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New Paradigms for the Treatment of Cancer: The Role of Anti-Angiogenesis Agents

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Angiogenesis, the sprouting of new blood vessels, plays a role in diverse disease states including cancer, diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, psoriasis, atherosclerosis, and restenosis. With regard to cancer, the clinical association of tumor vascularity with tumor aggressiveness has been clearly demonstrated in numerous tumor types. The observation of increased microvessel density in tumors not only serves as an independent prognostic indicator, but also suggests that anti-angiogenic therapy may be an important component of treatment regimens for cancer patients. The complexity of the angiogenic process, which involves both positive and negative regulators, provides a number of targets for therapy. Many positive

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regulators, including growth factor receptors, matrix metalloproteinases, and integrins, have been correlated with increased vascularity of tumors and poor prognosis for patient survival. Thus, these serve as ideal targets for anti-angiogenesis therapy. Many inhibitors of these targets are currently undergoing clinical evaluation as potential anticancer agents. In this article, we discuss the role of positive regulators in angiogenesis and tumor growth and describe the anti-angiogenic agents under development.

I. INTRODUCTION

In recent years, angiogenesis has become a rich area of research due to the role this process plays in a number of diseases as well as the potential for therapeutic intervention. Angiogenesis occurs during development and in normal adults during wound healing, pregnancy, and corpus luteum formation. Although angiogenesis is limited in normal adults, it is induced in many disease states including cancer, diabetic retinopathy, rheumatoid arthritis, psoriasis, atherosclerosis, and restenosis (reviewed in Folkman, 1995).

The requirement for angiogenesis in tumor growth was first hypothesized by Folkman in 1971. Ample evidence has since been collected to show that tumors require angiogenesis to grow beyond 1–2 mm^3 (Folkman, 1990). A switch to an angiogenic phenotype occurs when the balance of positive and negative regulators shifts, causing tumors to progress from hyperplasia to neoplasia (Hanahan and Folkman, 1996). The increased blood flow to the tumor allows for continued growth as well as metastasis because successful metastasis requires the presence of blood vessels to allow for the tumor cells to enter the circulation (Fig. 1, see color plate). The close interplay between angiogenesis and metastasis contributes to the poor prognosis seen in patients with highly angiogenic tumors. The main players in angiogenesis are the endothelial cells that line the blood vessels. Endothelial cells release proteases that degrade the basement membrane, which enables their migration through the membrane to form sprouts and their proliferation to extend the sprouts (Figs. 1A and B). The sprouts fuse to form loops, allowing blood to flow to the tissue (Fig. 1C). In metastasis, proteases released from the tumor cells also degrade the basement membrane as the tumor cells migrate from the tumor to a vessel (Fig. 1D) or from a vessel to a new site (Fig. 1E). Once the tumor cells are established in a new organ (Fig. 1F), the angiogenic process may begin again to allow growth of the metastases.

Tumor angiogenesis has been investigated in numerous studies in which archival tumor material has been analyzed by immunohistochemical staining of vasculature markers such as Factor VIII, CD31 or CD34. Increased tumor vessel density relative to normal tissue has been shown in many tumor types including brain (Wesseling *et al.*, 1998), colon (Takahashi *et al.*,

1995), and breast tumors (Weidner *et al.,* 1991). Furthermore, vessel density frequently correlates with progression and severity of disease. For example, astrocytomas and anaplastic astrocytomas have limited microvasculature, but higher grade glioblastoma multiforme have significantly increased microvasculature (Wesseling *et al.,* 1998).

In non-small-cell lung carcinoma (NSCLC), dysplasias and *in situ* carcinomas had higher vessel counts than less severe hyperplastic/metaplastic lesions (Fontanini *et al.,* 1996). Furthermore, 94 out of 253 NSCLC patients studied who developed metastases had higher vessel counts than the patients who did not develop metastases (Fontanini *et al.,* 1995). In two additional studies of NSCLC patients, microvessel count was found to be a better prognostic indicator than tumor size or lymph node status (Fontanini *et al.,* 1997; Matsuyama *et al.,* 1998).

Intratumoral vessel count was also found to be a prognostic indicator in gastric carcinoma (Tanigawa *et al.,* 1997). Metastatic colon cancers have been shown to have approximately 70% more vessels than nonmetastatic tumors (Takahashi *et al.,* 1995). In node-negative breast tumors, low microvessel density correlated with longer disease-free survival (Karaiossifidi *et al.,* 1996; Obermair *et al.,* 1997), whereas high vessel density associated with lymph node positivity and other metastasis (Karelia *et al.,* 1997; Fox *et al.,* 1997; Weidner *et al.,* 1991).

Clearly, neovascularization is required for solid tumor growth to supply the proliferating tumor cells with nutrients. Other tumor types such as Kaposi's sarcomas (KS) and hemangioblastomas originate from vessels and are characterized by abnormal, highly permeable vessels. KS lesions contain abundant and abnormally permeable vasculature (Masood *et al.,* 1997). The spindle-shaped cells in the lesions are of endothelial and macrophage origin (Ensoli and Sirianni, 1998). A virus, Kaposi's sarcoma-associated herpes virus (KSHV), is found in all KS lesions and carries an oncogene that encodes a G-protein-coupled receptor (Bais *et al.,* 1998). This receptor is constitutively activated and it couples to signaling pathways that lead to the expression of angiogenic factors. These factors stimulate proliferation of the endothelial cell-derived KS cells as well as mediating angiogenesis. Von Hippel–Lindau (VHL) disease is also characterized by vascular lesions. VHL is frequently manifested by hemangioblastomas of the retina and central nervous system (for review, see Maher and Kaelin, 1997; Wizigmann-Voos and Plate, 1996). These lesions consist of a capillary network made up of vascular endothelial cells, stromal cells, and pericytes. Angiogenic factors have also been implicated in development of and vessel permeability in these lesions.

The inhibition of angiogenesis through one of its positive regulators may serve as a valuable, novel therapy for solid tumors, KS, and VHL disease. The low incidence of angiogenesis in healthy adults would be expected to

minimize the potential for side effects by agents that specifically block the angiogenic process. Because angiogenesis is controlled by many positive and negative regulators, there are a number of potential targets for inhibition. Well-characterized inducers include growth factors, cytokines, and proteinases. Endogenous inhibitors of angiogenesis have also been described, including thrombospondin, platelet factor-4, tissue inhibitors of matrix metalloproteinase (TIMPs), transforming growth factor-β (TGF-β), interferon α, placental proliferin-related proteins, interleukin 12, angiostatin, and endostatin (reviewed in O'Reilly *et al.,* 1997; Folkman, 1995). Specific targets for anti-angiogenesis agents that have been investigated in clinical trials include inhibitors of growth factor receptor tyrosine kinases, matrix metalloproteinases, and integrins. These are reviewed in more detail here, and other important targets such as platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) and the plasminogen activator (PA) system are discussed.

II. GROWTH FACTORS AND RECEPTOR TYROSINE KINASES

Some of the most well-characterized regulators of angiogenesis are growth factors and receptor tyrosine kinases (RTKs) involved in the migration and proliferation of endothelial cells (Fig. 2, see color plate). More than 50 members of the RTK family are characterized by an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain that transfers phosphate from ATP to substrate proteins initiating a signaling cascade (reviewed in Shawver *et al.,* 1997). Of primary interest for angiogenesis are Flt-1 and Flk-1/KDR, the receptors for vascular endothelial growth factor (VEGF), as well as Tie1 and Tie2/Tek, the receptors for angiopoietins. These four receptors are expressed primarily on endothelial cells and play a direct role in angiogenesis. Additional RTKs with broader expression patterns implicated in angiogenesis are platelet-derived growth factor receptors (PDGFRs); fibroblast growth factor receptors (FGFRs); the hepatocyte growth factor/scatter factor (HGF/SF) receptor, Met; and epidermal growth factor receptors (EGFRs).

A. VEGF and Receptors

VEGF is a dimeric protein also known as vascular permeability factor because it acts on endothelial cells to regulate permeability as well as proliferation. These two activities are mediated through its tyrosine kinase recep-

tors, VEGFR1/Flt-1 and VEGFR2/Flk-1/KDR (KDR is the human homolog of Flk-1). VEGF and its receptors are expressed in angiogenic tissues during development, wound healing, and other situations when angiogenesis occurs. The temporal and spatial patterns of expression of VEGF and its receptors as well as the results of targeted mutagenesis support that they are required for angiogenesis during development. Similarly, the role of VEGF in tumor angiogenesis has been clearly demonstrated using tumor models in rodents (reviewed in Hanahan, 1997; Shawver *et al.,* 1997).

An extensive literature exists linking VEGF with human cancer. In human tumors, VEGF mRNA or protein has been identified by reverse transcriptase polymerase chain reaction, *in situ* hybridization, or immunohistochemistry in primary gliomas (Plate *et al.,* 1994), colon cancer (Takahashi *et al.,* 1995; Tokunaga *et al.,* 1998; Landriscina *et al.,* 1998), NSCLC (Fontanini *et al.,* 1999; Takahama *et al.,* 1998), pulmonary adenocarcinoma (Takanami *et al.,* 1997), renal cell tumors (Takahashi *et al.,* 1994), and KS (Cornali *et al.,* 1996). In pulmonary adenocarcinoma (Takanami *et al.,* 1997) and NSCLC (Fontanini *et al.,* 1999; Takahama *et al.,* 1998; Ohta *et al.,* 1996), survival of patients with VEGF-positive tumors was significantly less than patients with VEGF-negative tumors. For example, in one study of NSCLC, patients with low VEGF levels had a median survival time of 151 months, whereas those with high VEGF expression had a mean survival time of only 8 months (Ohta *et al.,* 1996). Likewise, poor prognosis, as well as increased microvessel counts were also found in patients with high VEGF expression at either the mRNA or protein level in many studies of both node-negative and node-positive breast cancer patients (Eppenberger *et al.,* 1998; Scott *et al.,* 1998; Anan *et al.,* 1998; Relf *et al.,* 1997; Brown *et al.,* 1995). The presence of VEGF in serum (Landriscina *et al.,* 1998; Fujisaki *et al.,* 1998; Kumar *et al.,* 1998) as well as in tumors (Landriscina *et al.,* 1998; Fujisaki *et al.,* 1998) was correlated with stage of disease in colon cancer patients, where liver metastasis (Tokunaga *et al.,* 1998) and poor prognosis correlated to VEGF levels (Tokunaga *et al.,* 1998; Ishigami *et al.,* 1998; Hyodo *et al.,* 1998).

In KS cells, VEGF expression is induced by the G-protein-coupled receptor oncogene expressed by KSHV. VEGF mediates angiogenesis in these lesions and also stimulates the growth of the spindle cells (Bais *et al.,* 1998). VEGF has also been detected in hemangiomas in VHL disease patients (Wizigmann-Voos *et al.,* 1995). This factor appears to be secreted from stromal cells and is likely to act through a paracrine mechanism to stimulate capillary formation and to increase vessel permeability in the lesions.

KDR and Flt-1 mRNA have also been detected in tumors such as gliomas (Plate *et al.,* 1994), neuroblastomas (Rossler *et al.,* 1999), colon cancer (Takahashi *et al.,* 1995), and adenocarcinomas (Takanami *et al.,* 1997). KDR was expressed at higher levels in malignant breast tissue than surrounding tissue (Brown *et al.,* 1995; Kranz *et al.,* 1999). In 51 cases of in-

testinal-type gastric cancer, vessel count correlated with high KDR expression on endothelium, and vessel count in turn correlated with progression of disease and liver metastasis (Takahashi *et al.,* 1996). High Flt-1 expression in pulmonary adenocarcinoma correlated with poor survival (Takanami *et al.,* 1997). In these tumors, as with the intestinal-type gastric tumors, the receptors were detected on the endothelial cells of the vessels and not the tumor cells. This strongly suggests a paracrine mechanism in which VEGF secreted from tumor cells stimulates migration and proliferation of endothelial cells.

In KS, KDR was found to be expressed on the tumor cells as well as on endothelial cells in stromal vessels (Brown *et al.,* 1996; Masood *et al.,* 1997). VEGF produced by tumor cells and squamous epithelium in these lesions may stimulate growth of the tumor itself as well as the vessels. This is supported by experiments with VEGF antisense oligonucleotides (Masood *et al.,* 1997) and antibodies (Nakamura *et al.,* 1997), both of which inhibited the growth of KS cells in culture. Recently, it was discovered that the Tat protein of human immunodeficiency virus can bind to and activate KDR (Ganju *et al.,* 1998), thus contributing to angiogenesis and the proliferation of spindle cells in lesions of AIDS-associated KS patients. In hemangiomas from VHL disease patients, KDR and Flt-1 were shown to be coexpressed on endothelial cells (Wizigmann-Voos *et al.,* 1995). Although VEGF and its receptors act by a paracrine mechanism in these lesions, it is unclear how they are regulated (Wizigmann-Voos and Plate, 1996).

VEGF and Flt-1 may function via an autocrine loop mechanism in hematopoietic neoplasms. Five of 12 human hematopoietic tumor cell lines were found to express mRNA for both VEGF and Flt-1, although there was no expression of KDR mRNA in these cell lines (Bellamy *et al.,* 1999). Although Flt-1 and KDR were not detected on normal stem cells, VEGF stimulated proliferation of hematopoietic cells from approximately 15% of chronic and acute myeloid leukemia (AML) patients studied (Ratajczak *et al.,* 1998). Leukemic blasts from AML patients were shown to express VEGF, Flt-1, and KDR transcripts in another study (Fiedler *et al.,* 1997). The role of angiogenesis in leukemias is an important area of further investigation.

B. PDGF and Receptors

PDGF is a pleiotropic factor that exists as a homo- or heterodimer of two polypeptides, the A and B chains, which interact with two receptor subtypes, the PDGF α receptor (PDGFRα) and PDGF β receptor (PDGFRβ). PDGF receptors have been found to be expressed in microvascular endothelium *in vivo* when endothelial cell activation and angiogenesis occur. However, the

mechanism whereby PDGF stimulates angiogenesis has been controversial. PDGF has been found to induce tube formation by endothelial cells (Battegay *et al.,* 1994) and an angiogenic response in the chick chorioallantoic membrane assay (Wilting *et al.,* 1992). In some studies, PDGF induced the proliferation of endothelial cells (Marx *et al.,* 1994; Koyama *et al.,* 1994) although not all endothelial cells respond mitogenically to PDGF (Sato *et al.,* 1993). Endothelial cell migration in response to the homodimer PDGF-BB has been shown using video time-lapse microscopy (Thommen *et al.,* 1997). PDGF also has been reported to upregulate other angiogenic factors such as VEGF (Enholm *et al.,* 1997) and thus it has been postulated that its activating role in angiogenesis is indirect. Another indirect mechanism was discovered by studies in PDGF-BB-deficient mice (Lindahl *et al.,* 1997). These mice were found to lack microvascular pericytes, small cells that form part of the capillary wall. It appears that neovessels were unable to attract PDGFRβ-expressing pericyte progenitor cells, leading to instability of the capillaries.

PDGF and its receptors have been detected in cancers such as gliomas, (Plate *et al.,* 1992; Hermanson *et al.,* 1992), lung carcinomas (Antoniades *et al.,* 1992), melanoma (Antoniades *et al.,* 1992), prostate carcinomas (Story, 1991), and esophageal carcinomas (Yoshida *et al.,* 1993). In breast cancer patients, circulating PDGF was found to be higher in patients with metastases (Ariad *et al.,* 1991). In another study, PDGF-A mRNA was detected in a higher number of tumor samples than nontumor breast tissue and vessel counts correlated with PDGF-A expression (Anan *et al.,* 1996). PDGFR has been shown to be expressed on vascular endothelial cells in breast tumors (Bhardwaj *et al.,* 1996). During tumor progression, PDGFRs on tumor neovasculature were upregulated (Plate *et al.,* 1992). In colorectal (Lindmark *et al.,* 1993) and breast carcinomas (Bhardwaj *et al.,* 1996), PDGF and PDGFRβ were detected on stroma. Because PDGF and its receptors are expressed in tumor cells, stroma, and pericytes, they may play both direct and indirect roles in angiogenesis.

C. Angiopoietin and Ties

Tie1 and Tie2 are expressed in the vascular endothelium during embryonic development. Tie2 (also known as TEK) knockout and transgenic mice suggest that Tie2 is important for vasculogenesis, while Tie1 may be important for maintaining vascular integrity (reviewed in Hanahan, 1997; Shawver *et al.,* 1997). Also, Tie2 and its ligand, angiopoietin-1 (ang-1), appear to be required for recruitment of pericytes and smooth muscle cells. Angiopoietin-2 (ang-2) can also bind to Tie2; interestingly ang-1 induces autophosphorylation of Tie2 while ang-2 does not. Instead, ang-2 competitively inhibits ang-1-induced Tie2 phosphorylation, thereby negatively reg-

ulating Tie2 (reviewed in Hanahan, 1997). Expression of ang-1 was downregulated by treatment of fibroblasts with serum or growth factors in parallel with upregulation of VEGF (Enholm *et al.,* 1997). This suggests that ang-1 and VEGF play different roles in the angiogenic process. Neither ang-1 or ang-2 bind to Tie1 and, in fact, the ligand for Tie1 is unknown at this time.

A recent investigation in a brain xenograft tumor model illustrated how the interplay between these positive (ang-1) and negative (ang-2) regulators, as well as VEGF, may influence tumor vasculature (Holash *et al.,* 1999). During early tumor growth, vessels were mature and similar to the surrounding brain vessels. Ang-1 and ang-2 were expressed, but minimal VEGF was detected. After 2 weeks, the expression of ang-2 increased and vessels began to regress, possibly because ang-2 blocked the stabilizing activities of ang-1. Two weeks later, neovasculature formed at the tumor margins in parallel with a large increase in VEGF expression. This model not only showed how the relative levels of ang-1, ang-2, and VEGF may influence tumor vasculature, but also demonstrated that tumor angiogenesis may not always be induced during early stages of tumor growth.

In human cancer, Tie1 mRNA has been shown to be highly expressed in capillaries and vessels of primary and metastatic melanoma, while low levels were observed in normal tissue surrounding the tumors (Kaipainen *et al.,* 1994). Tie1 has also been reported to be highly expressed on vessels of gliomas, meningiomas (Hatva *et al.,* 1995), and breast tumors (Salven *et al.,* 1996). In a recent study of breast tissue, the proportion of Tie2 positive vessels was significantly higher in tumors compared to either normal breast tissue or benign lesions (Peters *et al.,* 1998). More studies will be required to fully understand the role of Tie1 and Tie2 and their ligands in cancer, but based on the endothelial-specific nature of the expression of the receptors, it is likely that they may be significantly involved in tumor angiogenesis and may serve as a target for therapeutic intervention.

D. FGF and Receptors

FGF is a family of at least 13 structurally related heparin-binding growth factors (reviewed in Bikfalvi *et al.,* 1997). FGF-2, the prototype family member, is a potent mitogen of different cell types including vascular endothelial cells and fibroblasts. In addition to its mitogenic activity on endothelial cells, it has been associated with other activities involved in an angiogenic phenotype including migration, proteinase production and integrin expression (Figs. 1B, C, and D) (reviewed in Shawver *et al.,* 1997). Although FGF-2 knockout mice have no apparent defects related to impaired angiogenesis, FGF-2 is clearly an angiogenic factor *in vivo* (reviewed in Bikfalvi *et al.,* 1997). Transgenic mice overexpressing FGF-2 developed vascular cysts upon

injection of extracellular matrix, where the nontransgenic mice formed cysts without vessels (Fulgham *et al.,* 1999). FGF-2 also has been reported to be both synergistic with VEGF and to induce the expression of VEGF (Seghezzi *et al.,* 1998).

A number of studies have investigated the role of FGFs and their receptors in cancer. Many tumor cell lines express high levels of FGFs and/or receptors, including esophageal (Iida *et al.,* 1994), prostate (Nakamoto *et al.,* 1992), and renal cell carcinoma (Emoto *et al.,* 1997). A neutralizing antibody against FGF-2 inhibited the growth of two human glioblastoma cell lines in culture and in athymic mice (Takahashi *et al.,* 1991). Yet the role of FGFs in human cancer biopsies is less clear. In two studies of breast tumors, 45 cases of invasive cancer showed a positive correlation with microvessel density and FGF-2 expression (de Jong *et al.,* 1998), and FGF-2 expression was detected at higher levels in tumors than in surrounding breast tissue (Relf *et al.,* 1997). In contrast, in additional studies of breast cancer, no difference was seen between FGF-2 expression in normal breast tissue and tumors (Anan *et al.,* 1996), and no correlation was found between FGF-2 expression and patient survival (Eppenberger *et al.,* 1998). Furthermore, in colorectal cancer, FGF-2 was expressed at lower levels in the tumors than in the surrounding mucosa (Landriscina *et al.,* 1998). These patients had increased FGF-2 levels in mesenteric blood, possibly because FGF2 was released from tumor tissue.

Likewise, no clear expression pattern in tumors has been found with the FGF receptors. Amplification of two FGF receptor types was detected in approximately 12% of breast cancers, with a correlation to node positivity (Adnane *et al.,* 1991). In astrocytoma, FGFR type 1 increased with higher grade tumors, but FGFR type 2 expression decreased (Yamaguchi *et al.,* 1994). Because FGFRs are expressed by tumor cells as well as vascular endothelial cells, it is difficult to differentiate between their direct role in tumor cell growth and their role in angiogenesis.

E. HGF/SF and Met

HGF/SF and its receptor Met have many important roles in embryogenesis, but based on knockout data they do not appear to regulate vasculogenesis. On the other hand, cultured endothelial cells express Met and proliferate in response to HGF/SF. Furthermore, HGF/SF stimulates migration and tube formation by endothelial cells, as well as angiogenesis in Matrigel plug and cornea models. Additional experiments have demonstrated that VEGF and platelet-activating factor (PAF) are potent mediators of HGF-induced angiogenesis and therefore the role of HGF/SF in angiogenesis may partially occur though indirect effects (reviewed in Shawver *et al.,* 1997).

HGF/SF is overexpressed in many human cancers including non-small-cell lung tumors (Siegfried *et al.,* 1997), melanoma (Hendrix *et al.,* 1998), endothelial carcinoma (Wagastuma *et al.,* 1998), leiomyosarcoma (Jeffers *et al.,* 1996), and multiple myeloma (Börset *et al.,* 1996). In breast cancer, HGF/SF expression correlated with a marker of vascular endothelial cells and circulating HGF/SF was found to be higher in patients with breast cancer than healthy patients, with the highest levels in patients with positive nodes or liver metastases (Taniguchi *et al.,* 1995; Toi *et al.,* 1998). Furthermore, HGF/SF was found to be more highly expressed in breast tumors than in normal tissue, and HGF/SF and Met expression were both found to correlate with disease relapse (Nagy *et al.,* 1996). Met has been shown to play a role in tumor invasion and metastasis (Figure 1B-F) (Jeffers *et al.,* 1996) as well as being an independent prognostic indicator of survival (Wagastuma *et al.,* 1998). HGF/SF and Met appear to be involved in tumor angiogenesis although their effects may be indirect.

F. EGF and Receptors

The four members of the EGF receptor family are the EGF receptor, HER-2, HER-3, and HER-4 (the latter three are also known as erbB2, erbB3, and erbB4, respectively). As with the FGF and PDGF families, the ligands bind with differential affinity to the various receptors (reviewed in Shawver *et al.,* 1997).

The EGF receptor has been shown to be overexpressed in many tumor types including glioblastoma (Libermann *et al.,* 1985), cervical carcinoma (Skomedal *et al.,* 1999; Kersemaekers *et al.,* 1999), oral tumors (reviewed by Todd and Wong, 1999), bladder (Skriplakich *et al.,* 1999), and breast tumors (Harris *et al.,* 1994). Its overexpression correlates with shortened relapse-free survival in breast cancer (Harris *et al.,* 1994). HER-2 is also overexpressed in breast cancers and correlates to poor prognosis (Slamon *et al.,* 1987). Although neither of these receptors or their other family members have been convincingly linked to angiogenesis, it has been proposed that EGF may act indirectly by inducing VEGF (Tsai *et al.,* 1995). This suggestion is supported by an experiment in the A431 tumor model in athymic mice in which administration of a neutralizing antibody against the EGF receptor caused dose-dependent reduction in VEGF expression and vessel counts in the tumor (Viloria Petit *et al.,* 1997). In humans, one study of breast tumors showed that coexpression of the EGF receptor with one of its ligands, TGFα, correlated with microvessel density (de Jong *et al.,* 1998). However, the EGF receptor and HER-2 are likely to act predominantly to directly drive the growth of cancer cells rather than through angiogenesis.

G. RTK Inhibitors in Clinical Development

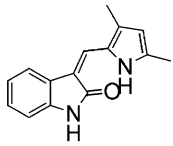
Receptor tyrosine kinases serve as excellent targets for therapeutic intervention in the treatment of cancer. Traditionally, tyrosine mimetics were used to block substrate binding to the kinase catalytic domain (reviewed in Burke *et al.,* 1997), but more recently, competitors of ATP binding have been developed (reviewed in Strawn and Shawver, 1998; McMahon *et al.,* 1998). Despite the utilization of ATP in many biochemical functions, specific inhibitors of RTKs have been developed (reviewed in McMahon *et al.,* 1998). Because several RTKs play a clear role in angiogenesis, blocking their kinase activity with specific inhibitors would be expected to inhibit tumor angiogenesis (reviewed in Shawver *et al.,* 1997).

1. Flk-1/KDR INHIBITORS

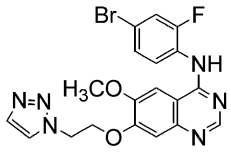
VEGF and its receptors serve as excellent targets for anti-angiogenesis therapy because KDR is an endothelial cell-specific VEGF receptor expressed primarily during the angiogenic process. The VEGF signaling cascade has been validated as a target for therapeutic intervention by several methods. Reduction of VEGF expression in C6 rat glioma (Saleh *et al.,* 1996) and human melanoma cells (Claffey *et al.,* 1996) with antisense constructs prevented their growth in mouse models. Also, a neutralizing antibody against VEGF inhibited growth of human rhabdomyosarcoma, glioblastoma, leiomyosarcoma (Kim *et al.,* 1993), and fibrosarcoma (Asano *et al.,* 1995) in mice. Furthermore, dominant-negative inhibition of Flk-1 by introduction of truncated Flk-1 significantly inhibited tumor growth in eight xenograft models (Millauer *et al.,* 1994, 1996). In these studies, the vessel density in the small tumors that did form was reduced compared to vehicle treated tumors, suggesting that the mechanism of tumor growth inhibition was through blocking of angiogenesis.

Several specific inhibitors of VEGF signaling are currently in clinical trials, including small molecules (Fig. 3), antibodies, and a ribozyme. SU5416 is a small molecule inhibitor of Flk-1/KDR under development by SUGEN. It is a potent, specific inhibitor of Flk-1/KDR kinase activity and VEGF-stimulated proliferation of human umbilical vein endothelial cells (HUVECs) in culture (Fong *et al.,* 1999). Eight out of 10 xenograft tumor models were significantly inhibited by the intraperitoneal administration of SU5416. Intravital multifluorescence videomicroscopy was used to help define the mechanism of SU5416 *in vivo* activity. This technique allows constant evaluation of tumor angiogenesis at the level of individual microvessels (Vajkoczy *et al.,* 1999). C6 rat glioma cells were implanted into the dorsal skinfold chamber of athymic mice and mice were treated daily with SU5416. The newly formed microvasculature within the fluorescently labeled tumor (intratumoral) and

Flk-1/KDR Inhibitors



SU5416



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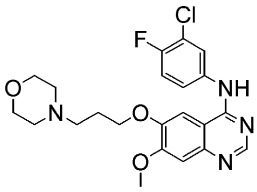
EGFR Inhibitors



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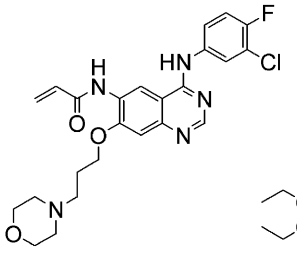

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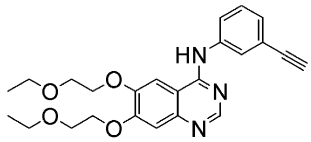
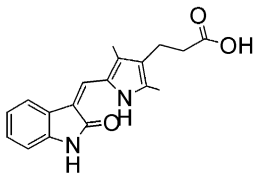


CP-358,774

Flk-1/KDR, FGFR and PDGFR Inhibitor



SU6668

Fig. 3 Small molecule receptor tyrosine kinase inhibitors in clinical development. The specific Flk-1/KDR inhibitor, SU5416, and Flk-1/PDGFR/FGFR kinase inhibitor, SU6668 (both from SUGEN), have indolinone core structures. The Flk-1/KDR inhibitor, ZD4190, and EGFR inhibitors, ZD1839 (both from AstraZeneca), PD183805 (from Parke-Davis) and CP-358,774 (from Pfizer) have quinazoline core structures.

at the periphery (peritumoral) were separately assessed. SU5416 showed a dramatic decrease in tumor growth (up to 92%) by day 22 postimplantation. In addition, SU5416 also caused a reduction both in the total and functional vascular density of the tumor microvasculature. As would be expected from a compound exhibiting anti-angiogenic properties, a greater reduction of the total vascular and functional density was observed in areas of higher angiogenic activity (60–70% in peritumoral; 30–40% in intratumoral areas). Additionally, SU5416 demonstrated a decrease in vascular leakage in this study.

In a phase I clinical trial with SU5416, stable disease was observed in many patients and objective responses were measured as well (Rosen *et al.,* 1999a). SU5416 therapy was well tolerated by patients, several of whom remained on treatment for over a year (Rosen *et al.,* 1999b). Currently, SU5416 is under phase III clinical evaluation for the treatment of NSCLC and colorectal cancer. It is also being evaluated in other diseases such as AIDS-KS and VHL disease.

ZD4190 (Fig. 3) is an inhibitor of Flk-1/KDR being developed by AstraZeneca. This compound inhibits VEGF receptor kinase activity, VEGF-stimulated HUVEC proliferation in culture, and the growth of many tumor cell lines in xenograft models when administered orally (Ogilvie *et al.,* 1999). Vascular permeability is also decreased by ZD4190 in tumor xenograft models, as determined by dynamic contrast medium-enhanced MRI (Wedge *et al.,* 1999).

CGP 79787 (structure not published) is another small molecule inhibitor of Flk-1/KDR. It has been reported to extend survival and reduce ascites in athymic mice implanted intraperitoneally with SKOV3 human ovarian tumor cells (Xu *et al.,* 1999). This compound is also currently in phase I clinical trials sponsored by Novartis.

Humanized neutralizing antibodies are also under clinical investigation as inhibitors of angiogenesis. An anti-VEGF antibody (rhuMAb VEGF) is under development by Genentech. As with the small molecule inhibitors of VEGF signaling, this antibody blocked tumor growth in a variety of xenograft models (Novotny *et al.,* 1999). In a phase I clinical trial, it was found to be well tolerated and no antibodies against rhuMAb VEGF were detected in treated patients. It was also tested in combination with carboplatin/paclitaxel, 5-FU/leucovorin, or doxorubicin with no adverse effects. This agent is currently in phase II clinical trials for the treatment of NSCLC, colorectal, prostate, and breast cancers (Novotny *et al.,* 1999). Another approach with inhibitory antibodies is being pursued by ImClone. They have generated an antibody against Flk-1/KDR that inhibits kinase activation and downstream signaling by blocking the binding of VEGF (Zhu *et al.,* 1999). This antibody induced apoptosis and also inhibited proliferation in endothelial cells (Bohlen *et al.,* 1999). The antibody also inhibited angiogene-

sis in a human skin/SCID mouse model (Bohlen *et al.,* 1999). This inhibitor has not yet entered clinical trials.

Another approach to inhibit VEGF signaling is via downregulation of its receptors. Ribozyme Pharmaceuticals is developing a catalytic RNA molecule, known as a ribozyme, that specifically degrades *flt-1* mRNA (Usman *et al.,* 1999). This agent has been investigated in two phase I clinical trials and was found to be well tolerated.

2. EGFR INHIBITORS

Although EGFR appears to play an indirect role in angiogenesis, therapy with anti-EGFR agents may affect angiogenesis as well as tumor cell growth. The reduction in VEGF expression and vessel counts in A431 tumors treated with anti-EGFR support this idea (Viloria Petit *et al.,* 1997). A number of EGFR inhibitors are in clinical trials for the treatment of cancer, including three anilinoquinazolines, ZD1839, PD183805, and CP-358,774 (Fig. 3).

AstraZeneca is developing ZD1839 (Fig. 3), a potent and specific EGFR inhibitor with a K_i = 2.1 n*M* (Averbuch, 1999). In preclinical experiments, ZD1839 was efficacious in several tumor models, including the EGFR-driven A431 model, when dosed orally (Fig. 4A). In phase I clinical trials, it was well tolerated (Hammond *et al.,* 1999) although it caused a rash at higher doses, consistent with a mechanism-based side effect (Averbuch, 1999).

PD183805 is similar in structure to ZD1839 but it contains an acrylamide group (Fig. 3) that becomes covalently linked to the EGFR, causing irreversible inhibition with low nanomolar IC_{50} values (Vincent *et al.,* 1999). This compound has been shown to reduce tyrosine phosphorylation on the EGFR in A431 tumor xenografts following oral administration. The growth of the xenografts was also significantly inhibited. PD183805 recently entered phase I clinical trials.

CP-358,774 is being developed by Pfizer. It has similar *in vitro* activity to ZD1839 and PD183805, and inhibited xenograft growth in athymic mice. It has been evaluated in phase I clinical trials and was found to be generally well tolerated with a rash and diarrhea being the major toxicities (Siu *et al.,* 1999; Karp *et al.,* 1999).

Similar to approaches taken to inhibit VEGF and its receptors, an inhibitory monoclonal antibody against EGFR is being explored. MAb 225, and its chimeric human/mouse form, C225, have been used in numerous experimental systems and found to block tumor cell growth in culture and in athymic mice (Fan *et al.,* 1993; Wu *et al.,* 1995; Viloria Petit *et al.,* 1997; Huang *et al.,* 1999). Interestingly, it causes tumor cells to enter apoptosis following accumulation in G_1 (Wu *et al.,* 1995; Huang *et al.,* 1999). C225 is in clinical trials sponsored by ImClone. In a phase II study in renal cell carcinoma, in which 85% of tumors overexpress EGFR, 54 patients have been treated (Gunnett *et*

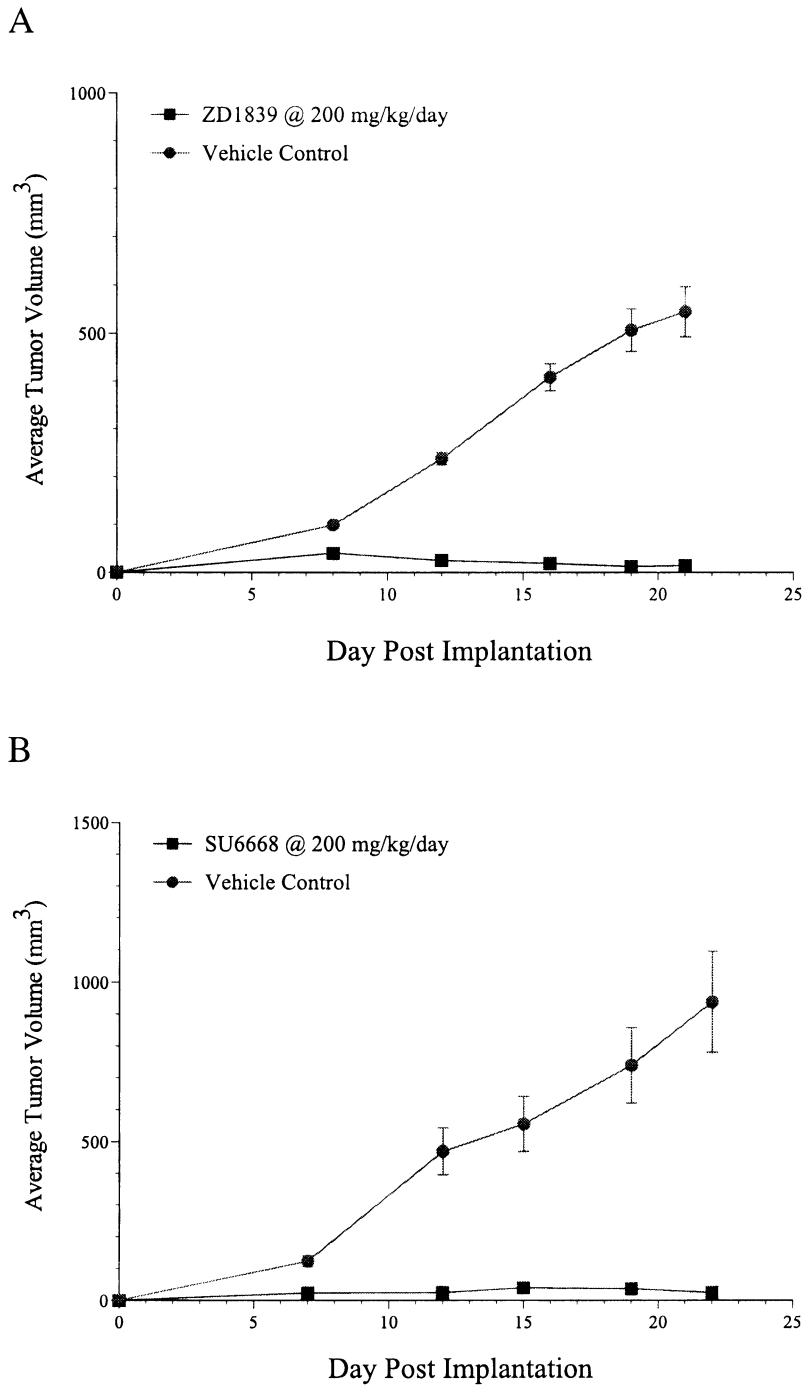


Fig. 4 Efficacy of RTK inhibitors in the A431 subcutaneous xenograft tumor model. Athymic mice ($n = 10–20$) were implanted with A431 cells (5×10^6 cells/mouse) on day 0. Daily dosing with ZD1839 (A) or SU6668 (B) in a Cremophor-based vehicle was initiated on day 1. Tumors were measured with vernier calipers twice weekly and volumes were calculated as the product of length × width × height.

al., 1999). Of those, one partial and two minor responses have been observed in patients with metastatic lung lesions. When seven patients with squamous cell carcinoma of the head and neck were treated with combinations of C225 and cisplatin, one complete response and three partial responses were achieved (Mendelsohn *et al.,* 1999). C225 also enhanced the effects of radiation treatment with 93% of patients with head and neck malignancies showing complete responses (Ezekiel *et al.,* 1999). As with the small molecule inhibitors of EGFR, the major toxicity caused by C225 was a rash. Phase II and III trials of C225 in combination with cisplatin and/or radiation are under way.

3. VEGFR/PDGFR/FGFR RTK INHIBITORS

Because RTKs play a variety of roles in tumor growth and angiogenesis, including tumor cell proliferation, endothelial cell growth and migration, and pericyte and stroma formation, agents that inhibit multiple receptors may be excellent therapeutics. One such compound, SU6668 (Fig. 3), is under clinical development by SUGEN. SU6668 is a potent inhibitor of Flk-1/KDR, PDGFR, and FGFR kinase activity and endothelial cell proliferation (Shawver *et al.,* 1999; Laird *et al.,* 1999). It is orally efficacious in all xenograft tumor models tested thus far, including those of glioma, lung, colon and ovarian origin. SU6668 also significantly inhibited the EGFR-driven A431 model similarly to the EGFR inhibitor, ZD1839 (Fig. 4B). Because SU6668 does not inhibit EGFR tyrosine kinase activity, the mechanism of tumor growth inhibition is likely to be through inhibition of angiogenesis. Phase I clinical trials of SU6668 are currently under way.

Small molecules, antibodies, and ribozymes that block signaling of angiogenic growth factors or their receptors are all currently under clinical investigation as anti-cancer agents. Reports to date indicate that these therapeutics are well tolerated by patients and positive responses have been observed in some studies. Because of the importance of these receptors in angiogenesis, many other companies also have programs to develop RTK inhibitors.

III. PLATELET-DERIVED ENDOTHELIAL CELL GROWTH FACTOR/THYMIDINE PHOSPHORYLASE

A. PD-ECGF/TP in Angiogenesis

PD-ECGF/TP was originally characterized as a factor produced in fibroblasts and other cultured cells that induced chemotaxis of endothelial cells and angiogenesis in model systems. Subsequently, this factor was shown to have thymidine phosphorylase (TP) activity (reviewed in Folkman, 1996).

Elimination of TP activity by mutagenesis blocked the angiogenic response. The enzymatic activity of TP produces 2-deoxy-D-ribose-1-phosphate from thymidine; subsequent hydrolysis results in 2-deoxy-D-ribose, which is the molecule that exerts the chemotactic and angiogenic effects (Fig. 5).

PD-ECGF/TP expression has been investigated in many tumor types. It was expressed at 260-fold higher levels in invasive bladder tumors than in normal bladder tissue (O'Brien *et al.,* 1995). In pancreatic (Fujimoto *et al.,* 1998) and colorectal cancers (Amaya *et al.,* 1997), TP protein levels detected by immunohistochemistry correlated significantly with increased microvessel density, and in the case of pancreatic cancer, to poor prognosis. A study of brain cancers revealed that in glioblastoma multiforme, but not other brain cancers, high amounts of PD-ECGF/TP protein were found in the macrophages around highly vascular regions in the stroma (Nakayama *et al.,* 1998). Similarly, in breast cancer, tumor-associated macrophages were associated with increased angiogenesis, apparently because tumor necrosis factor-α (TNF-α) produced by the macrophages induced PD-ECGF/TP in the tumor cells (Leek *et al.,* 1998). In this study, receptor-bound TNF-α on the tumor cells correlated with lymph node status. Another study of breast cancers showed that PD-ECGF/TP was present in tumor epithelial, stromal, endothelial, and inflammatory cells but was inversely correlated with tumor grade and size (Fox *et al.,* 1996). Medium-sized tumors were reported to have the highest PD-ECGF/TP expression in another study (Yonenaga *et al.,* 1998). Since PD-ECGF/TP exerts a chemotactic rather than mitogenic function, it may play a role in remodeling of existing vasculature, a function that is consistent with its association with smaller tumors.

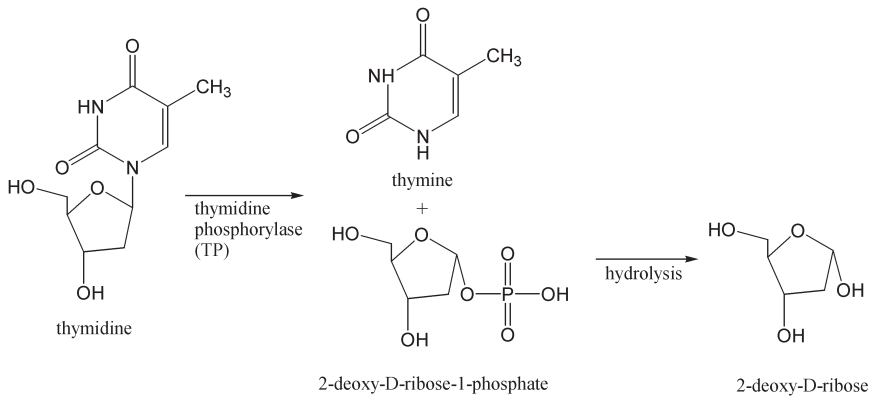


Fig. 5 Enzymatic activity of PD-ECGF/TP. Thymidine phosphorylase cleaves thymidine to thymine and 2-deoxy-D-ribose-1-phosphate, which is subsequently hydrolyzed to 2-deoxy-D-ribose. 2-Deoxy-D-ribose has chemotactic effects on endothelial cells and is the active angiogenic agent.

B. PD-ECGF/TP Inhibitors

Recently, two inhibitors of PD-ECGF/TP were described in the literature (Fig. 6). 7-Deazaxanthine (7DX) had an IC_{50} for thymidine phosphorylase activity of 40 μM (Balzarini *et al.,* 1998). 7DX also inhibited angiogenesis in the chorioallantoic membrane of chicken embryos. The second compound, 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride (TPI), inhibited the growth of KB tumor cells transfected with PD-ECGF/TP (KB/TP) in athymic mice (Matsushita *et al.,* 1999). Furthermore, TPI inhibited angiogenesis in a mouse model that involved the implantation of KB/TP cells in a chamber under the skin of the mice. Although inhibition of PD-ECGF/TP as a means of therapy for cancer has not yet been fully investigated, results with these inhibitors show promise.

IV. MATRIX METALLOPROTEINASES

A. MMPs in Angiogenesis

Matrix metalloproteinases (MMP), along with their endogenous inhibitors (TIMP), have been among the most thoroughly studied enzyme systems with respect to their role in the control of angiogenesis (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996; Benaud *et al.,* 1998; Moses, 1997; Yu *et al.,* 1997). One of the key elements for successful angiogenesis is degradation of the extracellular matrix (ECM), a function performed by MMPs. The MMPs are members of a multigene family of zinc-dependent enzymes. These proteases have been classified into four broad categories originally based on substrate specificity (Table I). With the exception of MT-MMPs and stromelysin-3, MMPs are secreted as proenzymes and then

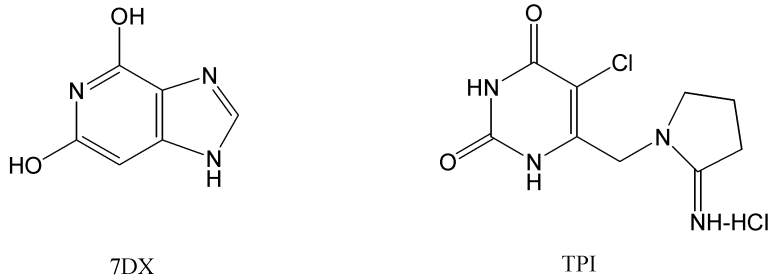


Fig. 6 Inhibitors of PD-ECGF/TP. 7DX (7-deazaxanthine) and TPI (5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride) inhibit angiogenesis in model systems.

Table I Matrix Metalloproteinase Family

Subfamily	Name	MMP #	Substrate
Interstitial collagenases	Fibroblast collagenase	MMP-1	Fibrillar collagen, IL1-β.IGF binding protein 3
	Neutrophil collagenase	MMP-8	Fibrillar collagen
	Collagenase-3	MMP-13	Fibrillar collagen
	Collagenase-4	MMP-18	Fibrillar collagen
Gelatinases	Gelatinase A	MMP-2	Gelatin, type IV collagen, fibronectin, elastic, IL-β, galectin 3
	Gelatinase B	MMP-9	Gelatin, collagen, IV, V, IL1-β, galectin 3
Stromelysins	Stromelysin 1	MMP-3	Laminin, fibronectin, non-helical, collagen, vitronectin, proteoglycans, elastin, α.2 macroglobulin, IL1-β
	Stromelysin 2	MMP-10	Same as above; α1 proteinase inhibitor,
	Stromelysin 3	MMP-11	Laminin, fibronectin, elastin, Entactin,
	Matrilysin (PUMP-1)	MMP-7	proteoglycan, collagen IV, uPa, pro-TNF-α
Elastases	Macrophage Metalloelastase	MMP-12	Elastin, collagen IV, gelatin, fibronectin, laminin, vitronectin, proteoglycan, myelin protein, pro-TNF-α, α1-antitrypsin
Membrane-type MMPs	MT1-MMP	MMP-14	Pro-gelatinese A, procollagenase 3
	MT2-MMP	MMP-15	Pro-gelatinase A
	MT3-MMP	MMP-16	
	MT4-MMP	MMP-17	Inflammatory cytokines?
Other MMPs	Enamelysin	MMP-19	Tooth enamel
		MMP-20	

activated extracellularly via cleavage by plasmin, MT-MMPs, and by autoproteolysis (Fig. 7). Recently, the crystal structure of MMP-2 was solved (Morgunova *et al.,* 1999). It was used to illustrate how cleavage of peptides within the propeptide exposes the catalytic site and activates the enzyme.

Type IV collagenase activity (carried out by MMP-2 and -9) is required for the early steps of endothelial cell morphogenesis/capillary formation while MMP-1 is required for angiogenesis *in vitro* and is responsible for degradation of interstitial collagen types I–III (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996; Benaud *et al.,* 1998; Moses, 1997; Yu *et al.,* 1997). Interestingly, VEGF induces MMP-1 expression in endothelial cells. MT-MMP-1 is also able to degrade collagen types I–III, as well as other parts of the ECM, plus it is able to activate pro-MMP-2. MT-MMP-1 is expressed on endothelial cells and may regulate angiogenesis by activating pro-MMP-2 and by cleaving collagens on the cell surface to allow for local invasion (Zucker *et al.,* 1995).

MMP expression can be upregulated by a number of factors including in-

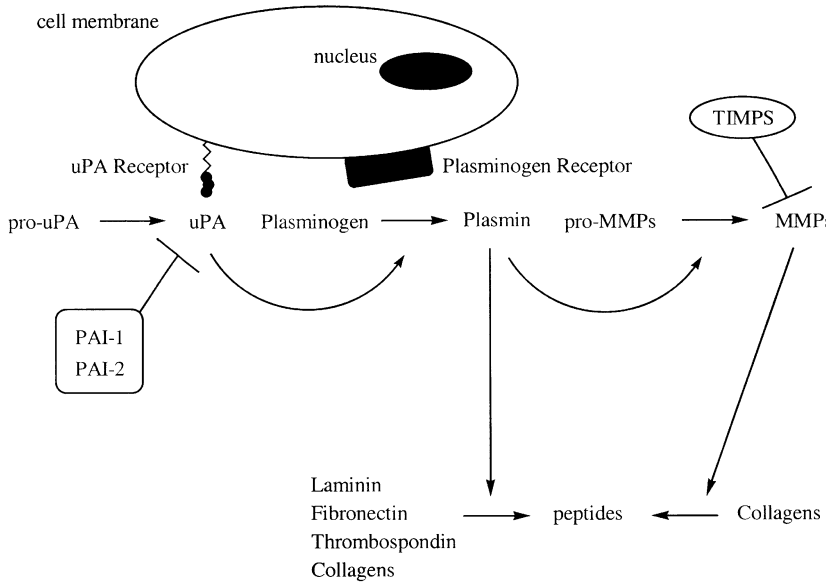


Fig. 7 Plasminogen/plasmin cascade. Pro-uPA binds to its receptor on the cell surface and is cleaved to uPA. Plasminogen is brought into proximity of uPA by binding to its receptor, and uPA cleaves plasminogen to plasmin. Pro-MMPs are in turn cleaved to MMPs by plasmin. Subsequently, MMPs cleave collagens in the ECM. Plasmin also cleaves other components of the ECM including laminin, fibronectin, thrombospondin, and collagens. Plasminogen activator inhibitors (PAI-1 and PAI-2) block the activity of uPA and tissue inhibitors of metalloproteinases (TIMPs) block the activity of MMPs.

terleukin-1, VEGF, EGF, PDGF, TGF-α and TNF-α (reviewed in Mignatti and Rifkin, 1996; Benaud *et al.,* 1998). Several of these factors have been implicated either directly or indirectly in the angiogenic process. On the other hand, factors such as TGF-β, interferon γ, and some steroid hormones, including glucocorticoids and retinoids, downregulate MMP expression (reviewed in Mignatti and Rifkin, 1996; Benaud *et al.,* 1998).

Inhibitors of the MMPs (TIMPs) have also been described. The TIMPs regulate ECM turnover and tissue remodeling by binding to MMPs and have been shown to inhibit neovascularization. Curiously, recent data have demonstrated that MMP-7, -9, and -12 are able to cleave plasminogen to release angiostatin, a potent inhibitor of angiogenesis (Dong *et al.,* 1997; Patterson and Sang, 1997). The significance of these results remains to be determined; however, it is clear that the MMPs and TIMPs play a critical role in the angiogenic process.

Studies of human tumors have shown that MMP-2 is increased in multiple tumor types, including breast cancer, colon cancer, thyroid cancer, and pulmonary adenocarcinomas (reviewed in Price *et al.,* 1997). In general, these enzymes are produced by stromal cells surrounding the lesion rather than by the tumor cells themselves (reviewed in Benaud *et al.,* 1998). Invasive carcinoma of the ovary showed the presence of MMP-2 protein, while benign ovarian cysts had none detectable. In addition, secreted MMP-2 activity has been demonstrated from ovarian carcinoma cells *in vitro* and from ovarian cancer patients. MMP-2 appears to be expressed very early in breast cancer but not in normal, resting breast tissue (Poulsom *et al.,* 1993), and thus may contribute to early events leading to tumor formation. Increased immunostaining of MMP-2 in hepatocellular carcinomas was associated with poor survival; increased serum MMP-2 levels in lung cancer patients was associated with the presence of distant metastases. Stromelysin-3 is considered a marker for highly aggressive tumors and late stages of primary breast tumor progression. In clinical studies, its expression has been shown to correlate with poor prognosis, tumor recurrence, and decreased survival (Chenard *et al.,* 1996).

Although expression information is useful, levels of activated enzyme may be more informative since MMPs are synthesized as proenzymes and the activated species are associated with the invasive phenotype. Addressing this issue, activated MMP-2 has been associated with the extent of tumor spread in one investigation of NSCLC, and MMP-9 production has been associated with lymph node metastasis (Iwata *et al.,* 1996). Also, an increase in activation of the proenzyme MMP-2 has been shown to be associated with more malignant tumors. A comparative study of normal breast tissue with benign and malignant tumors indicated that the proportion of the active enzyme (lower molecular weight) increased with the tumor grade, without a parallel increase in total production of MMP-2 (Davies *et al.,* 1993).

In addition to being detected in tumor cells and surrounding stroma, MMP-2 was also detected on the cell surface, with a similar pattern of expression during tumor progression as MT-MMP and TIMP-2 (Poulsom *et al.,* 1993; Polette *et al.,* 1994). This potentially sets up a complex interaction between MMP-2, the MT-MMPs, and TIMP-2, all of which are expressed in the same local vicinity.

The other members of the TIMP family, TIMP-1 (Yoshiji *et al.,* 1996) and TIMP-3 (Uria *et al.,* 1994; Byrne *et al.,* 1995), have been detected in breast carcinomas. In general, TIMP-1 expression has been correlated with low malignancy or early cancer stages (Polette *et al.,* 1993), yet other studies have shown its presence in malignant tissue samples (Yoshiji *et al.,* 1996). In stage I and II NSCLC patients, high expression of TIMP-1 correlated with poor disease-free survival (Tsao *et al.,* 1999) and in another study, both TIMP-1 and TIMP-2 were expressed at higher levels in later stage tumors (Brambilla, 1999). These results are unexpected in view of the inhibitory activity of TIMPs on MMPs. Their roles in tumor angiogenesis are not yet clear.

B. MMP INHIBITORS

Because MMPs play a role in tumor angiogenesis and invasiveness, they have been developed as targets for anti-cancer therapy. A number of MMP inhibitors are currently in clinical trials. Many are peptidomimetics designed to bind in the active site of MMPs. Such compounds have similar functional groups to substrates that hydrogen bond to amino acids and complex to zinc in the active site (Fig. 8). Inhibitors AG3340 (Prinomastat), marimastat, Bay 12-9566, and BMS-275291 are currently under clinical evaluation (for review, see Nelson, 1998).

AG3340 (Fig. 9) is a potent inhibitor of MMP-2, -9, -13, and -14. It was designed by Agouron Pharmaceuticals using models derived from crystal structures of MMPs. It significantly inhibited tumor growth in a number of xenograft models including colon, lung, breast, and glioma when administered orally (Shalinsky *et al.,* 1999a,b). Combinations with paclitaxel or carboplatin were more efficacious than either agent alone. It also decreased angiogenesis and increased apoptosis in the tumors. In phase I clinical studies alone or in combination with either paclitaxel and carboplatin (Olimpio *et al.,* 1999) or mitoxantrone and prednisone (Wilding *et al.,* 1999), AG3340 was well tolerated, although some patients complained of joint problems (Wilding *et al.,* 1999). This inhibitor is currently in phase III clinical trials in NSCLC patients in combination with paclitaxel and carboplatin or gemcitabine and cisplatin (Clendeninn *et al.,* 1999).

Marimastat (Fig. 9) is a synthetic peptidomimetic that potently inhibits MMP activity (Wojtowicz-Praga *et al.,* 1997). This compound is being de-

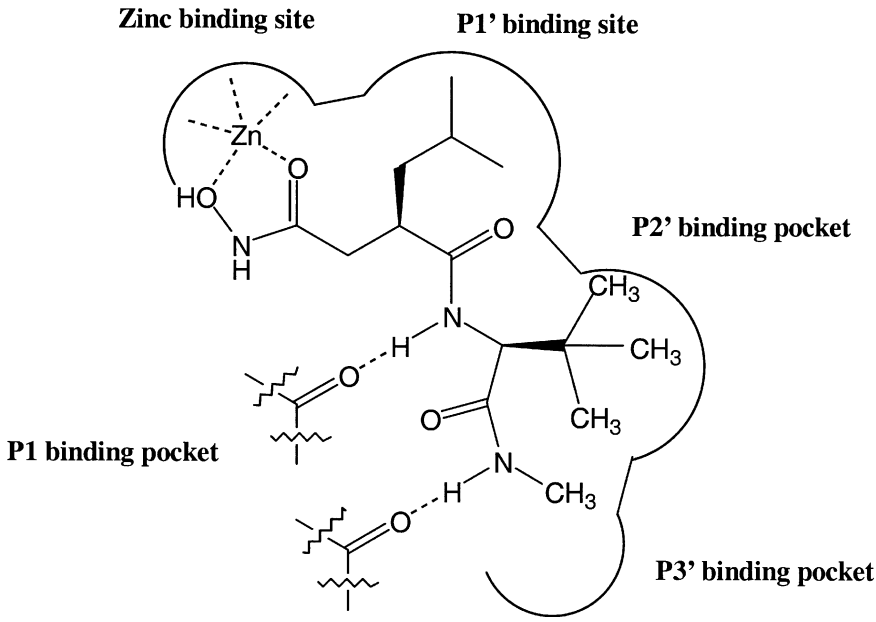


Fig. 8 Synthetic MMP inhibitors are peptidomimetics with a functional group that binds to zinc. Shown is an example of how marimastat may bind to the catalytic site of an MMP.

veloped by British Biotech. In preclinical studies, it was found to be orally bioavailable and to inhibit mammary tumor growth in rats and lung colonization of B16–BL6 melanoma in mice. In phase I/II clinical studies, marimastat was evaluated in combination with doxorubicin/cyclophosphamide in breast adenocarcinoma (Gradishar *et al.,* 1999), in combination with captopril/fragmin in a variety of tumors (Jones *et al.,* 1999), and in combination with paclitaxel/carboplatin in NSCLC (Anderson *et al.,* 1999). In the breast cancer study with 20 patients, 55% had stable disease and 45% had a partial response (Gradishar *et al.,* 1999), whereas in the NSCLC study of 22 patients, 18% had stable disease and 50% had a partial response (Anderson *et al.,* 1999). In all phase I/II studies, musculoskeletal toxicities were observed. Marimastat is also being evaluated in a phase II study in pancreatic cancer (Rosemurgy *et al.,* 1999) and in phase III clinical trials in a variety of solid tumors (Rasmussen *et al.,* 1999).

Bay 12-9566 (Fig. 9) is under clinical development by Bayer. This nonpeptidic small molecule is specific for MMP-2, -3, and -9 (Tolcher *et al.,* 1999) and has shown activity in angiogenesis and metastasis preclinical models (Humphrey *et al.,* 1999). In phase I clinical trials in tumors such as colorectal, ovarian, breast, and renal, Bay 12-9566 did not cause the mus-

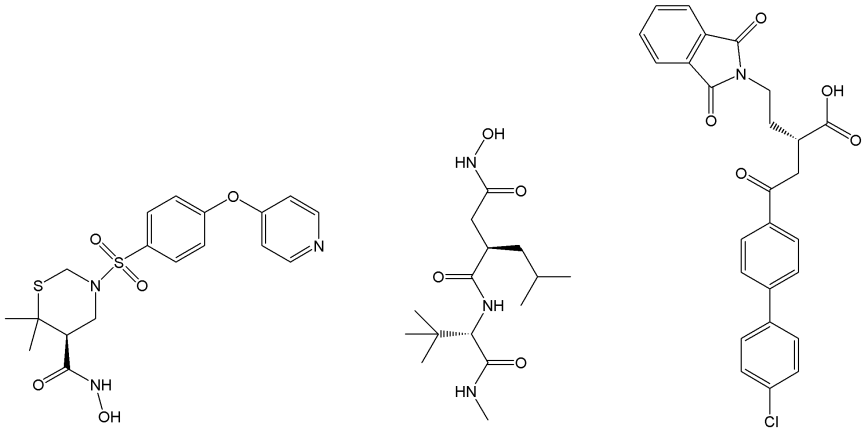


Fig. 9 Inhibitors of MMPs in clinical development. Marimastat is a peptidomimetic containing amide linkages.

culoskeletal toxicities observed with marimastat, and 18 of 29 patients had stable disease (Goel *et al.,* 1999). This compound was also well tolerated in combinations with paclitaxel and/or carboplatin, and was detected in plasma at concentrations much higher than those required for inhibition of MMP-2 and -9 enzymatic activity (Tolcher *et al.,* 1999). Bay 12-9566 is currently in phase III clinical trials in pancreatic, ovarian, NSCLC, and small cell lung cancer (Humphrey *et al.,* 1999).

Bristol-Myers Squibb also is evaluating an MMP inhibitor in phase I clinical trials. BMS-275291 is specific for MMP-2 and -9 (Humphrey, 1999). No musculoskeletal adverse events have been reported to date. Many other companies are also developing MMP inhibitors as anti-angiogenesis agents.

V. PLASMINOGEN ACTIVATOR/PLASMIN SYSTEM

The urokinase (uPA) and tissue (tPA) plasminogen activators are serine proteases that convert plasminogen to plasmin (Fig. 7). Plasmin is very broad acting, degrading fibronectin, laminin and gelatins but not collagen or elastin (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996). Furthermore, plasmin also activates MMPs (Fig. 7) and latent elastase. Because plasminogen is ubiquitously expressed at high levels, a small amount of PA can result in a high concentration of plasmin; therefore, PAs trigger a proteinase cas-

cade that results in the high local concentration of plasmin and MMPs. This increase in proteolytic activity results in three central events in angiogenesis: endothelial cell degradation and invasion of the vessel basement membrane, generation of ECM products that are chemotactic for endothelial cells, and activation and mobilization of growth factors from the ECM. Additionally, uPA modulates endothelial migration and proliferation independent of its proteolytic activities (Figs. 1B-F).

In vitro, endothelial cell expression of PAs and MMPs is modulated at the transcriptional level by a number of agents, including cytokines and growth factors that induce angiogenesis (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996). In addition, and again similar to the MMPs, PA activity is regulated extracellularly by several mechanisms, including control of proenzyme activation, interaction with binding sites on the cell membrane and ECM, and inhibition by specific tissue inhibitors. Endothelial cells express high levels of PA inhibitors (PAI) both *in vivo* and *in vitro*. These PAIs are members of the multigene family of serine protease inhibitors (serpins) (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996). As is the case for PAs, endothelial cell expression of PAI is also modulated by a variety of cytokines, some of which are angiogenic.

High levels of uPA have been observed in both human tumors and cell lines (Heiss and Allgayer, 1995). In bladder cancer, tumor progression and recurrence were associated with high uPA content (Hasui and Marutsuka, 1996). In addition, a greater number of cells expressed uPA in advanced, invasive lung carcinomas than in low-grade lung tumors (Oka and Ishida, 1991; Brambilla, 1999). Production of uPA in ovarian carcinoma cells was 17- to 38-fold higher than that found in normal ovarian epithelial cells (Moser and Young, 1994). The DU-145 human prostate cancer cell line has been shown to have five times more extracellular, secreted uPA activity than normal prostatic epithelial cells (Waghray and Webber, 1995). EGF stimulation of PC3 human prostate cancer cells increases their uPA expression and their invasive ability (Jarrard and Blitz, 1994). In addition to the presence of uPA in cancer cells, its receptor (uPAR) has also been detected in several different cancer types, including ovarian carcinoma, colon carcinoma, and glioma (Casslen and Gustavsson, 1991; Ossowski and Russo-Payne, 1991). Similar to uPA, expression of uPAR is up-regulated by a variety of growth factors and tumor promoters, including TGF-β, EGF, HGF/SF, and phorbol ester (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996). Patients with breast cancer expressing high uPA levels had an increased risk for early recurrence and had poor prognosis (Duffy and Reilly, 1990).

As was shown for the TIMPs, the PAIs have been shown to play a role in endothelial cell migration during angiogenesis (reviewed in Price *et al.,* 1997; Mignatti and Rifkin, 1996). The relationship between PAI levels and clinical outcome has been investigated in several studies, yet a consistent result

has not been achieved. In one study, cancer cells that overexpress PAI-1 were shown to have a decreased ability to degrade extracellular matrix and a reduction in their invasive potential (Cajot and Bamat, 1990). Consistent with this result, in another study, patients with PAI-1 negative gastric tumors were shown to have a significantly higher incidence of liver metastases and a poorer prognosis than those with PAI-1 positive tumors (Ito and Yonemura, 1996). However, in a third study, elevated antigen levels of uPA and of PAI-1 in tumor extracts of ovarian, breast, colon, and lung cancer correlated with increased incidence of relapse, shorter overall survival, and increased degree of invasion (Schmitt and Wilhelm, 1995). Higher levels of uPA and PAI-1 have also been reported in high-grade lung tumors compared to low-grade tumors (Brambilla, 1999).

Generally, higher levels of PAI-2 have been associated with better prognosis and less disseminated disease. Increased PAI-2 has been shown in malignant breast tumors compared with benign breast tumors (Foucre and Bouchet, 1991; Sumiyoshi and Serizawa, 1992). Also, PAI-2 concentrations were lower in breast carcinomas with lymph node involvement than those without. In this study however, PAI-1 levels were actually higher in breast cancers with involved lymph nodes (Bouchet and Spyratos, 1994; Foekens and Schmitt, 1994).

Although it may be a viable target for cancer therapy, no inhibitors of uPA have been described.

VI. INTEGRINS

A. Integrins in Angiogenesis

The invasion, migration, and proliferation of vascular endothelial and smooth muscle cells during angiogenesis are regulated by one or more of the integrin family of cell adhesion proteins as demonstrated by *in vivo* and *in vitro* models of angiogenesis (reviewed in Varner and Cheresh, 1996; Varner, 1997). The integrin family is composed of 15 alpha and 8 beta subunits that are expressed as different heterodimeric combinations on the cell surface (Fig. 10). Integrins bind to ECM or cell surface immunoglobulin family members through conserved short peptide sequences present in the ligands. Combinations of different integrins on cell surfaces allow cells to respond to a variety of different ECM proteins.

Several members of the integrin family of adhesion receptors are expressed on the surface of cultured smooth muscle and endothelial cells. Among these integrins is $\alpha_v\beta_3$, the endothelial cell receptor for von Willebrand factor, fibrinogen (fibrin), and fibronectin (reviewed in Brooks, 1996). This integrin

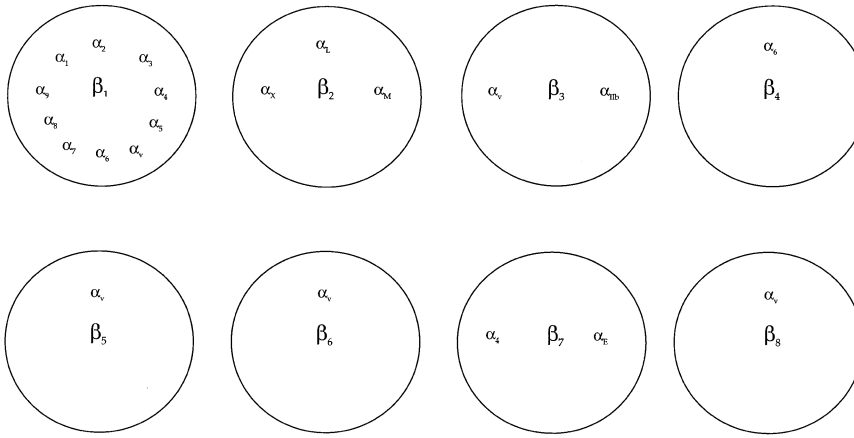


Fig. 10 Pairings of α- and β-integrins. Each integrin exists as a heterodimer of one α and one β subunit.

has also been shown to colocalize with MMP-2, together acting to induce endothelial cell migration (Brooks, 1996). $\alpha_v\beta_3$ is expressed at low levels on quiescent blood vessels but its expression is upregulated on tumor vessels and granulation tissue (Brooks *et al.,* 1995). FGF-2 and TNF-α both induce $\alpha_v\beta_3$ expression in endothelial cells (Brooks *et al.,* 1994; Friedlander *et al.,* 1995). In the chick chorioallantoic membrane angiogenesis model, an antibody against $\alpha_v\beta_3$ blocked angiogenesis induced by FGF-2 and TNF-α (Brooks *et al.,* 1994). Interestingly, it has been proposed that angiogenesis initiated by VEGF involves a separate integrin, $\alpha_v\beta_5$.

With regard to human cancers, $\alpha_v\beta_3$ is expressed on certain invasive tumors including late-stage glioblastomas and metastatic melanoma, and may play a role in regulation of tumor proliferation and metastasis (reviewed in Varner, 1997). It clearly plays an important role in angiogenesis and it will be interesting to determine the roles of other integrins in this process as well.

B. Inhibitors of Integrins

Because $\alpha_v\beta_3$ is not expressed on normal vessels but is expressed during angiogenesis, it is a good target for anti-angiogenic therapy. A humanized antibody against $\alpha_v\beta_3$, Vitaxin, and its mouse form, LM609, both inhibit angiogenesis in *in vivo* models (Brooks *et al.,* 1994; Gutheil *et al.,* 1998) and tumor growth in animals. LM609 and an antibody against $\alpha_v\beta_5$ both inhibited angiogenesis in a KS model in which the integrins are expressed in the KS cells (Burke and Leigh, 1999). Vitaxin has been evaluated in clinical tri-

als (Gutheil *et al.,* 1998). In a phase I clinical trial with 12 patients, six stable diseases and one partial response were observed (Gutheil *et al.,* 1998). Further results of clinical trials have not been reported.

Other non-antibody inhibitors of the integrins are under development. The recognition sequence for $\alpha_v\beta_3$ in the proteins it binds is arginine-glycine-aspartic acid (RGD). Merck AG is evaluating cyclic peptides containing the RGD sequence. They have found peptides that specifically inhibit $\alpha_v\beta_3$ binding to vitronectin (Dechantsreiter *et al.,* 1999). Other peptidomimetics based on the RGD sequence have been prepared by Searle and screened for inhibition of vitronectin binding to $\alpha_v\beta_3$ (Carron *et al.,* 1998). One of these, SC-68448, inhibited the growth of endothelial cells, but not tumor cells, in culture and blocked FGF-2-induced angiogenesis in a corneal neovascularization model. Other peptidomimetics from Searle, S836 and S137, inhibited tumor xenograft growth (Westlin *et al.,* 1998, 1999; Nickols *et al.,* 1998) and lung metastasis in the Lewis lung model (Nickols *et al.,* 1998). Small molecule antagonists developed by DuPont, SM256 and SD983, also blocked angiogenesis in a mouse Matrigel model and inhibited tumor growth in a colon carcinoma model (Kerr *et al.,* 1999). Although these other integrin inhibitors are not yet in clinical trials, they appear to be promising for inhibiting tumor growth through blocking angiogenesis.

VII. OTHER

Growth factors, PD-ECGF, MMPs, plasminogen activators, and integrins are only a few of the positive regulators of angiogenesis. Although many other regulators are known, reports of development of agents to inhibit their actions are lacking. Such angiogenesis activators as interleukin-8 (IL-8) and pleiotrophin may serve as intriguing targets for future anti-cancer agents.

A. Cytokines

Many inflammatory disorders such as rheumatoid arthritis and psoriasis are also characterized by angiogenesis. Thus, investigations into possible angiogenic factors produced by inflammatory cells have been undertaken and IL-8 was identified as such a factor. IL-8 is chemotactic and mitogenic for endothelial cells (Koch *et al.,* 1992) and causes endothelial cells to assemble into tubes in Matrigel (Kumar *et al.,* 1996). Administration of IL-8 in numerous *in vivo* models (Koch *et al.,* 1992; Norrby, 1996) caused angiogenesis. Furthermore, transfection of nonmetastatic melanoma cells with the IL-8 gene, or induction of IL-8 with ultraviolet B light, increased tumor growth

and metastasis in nude mice (Bar-Eli, 1999). MMP-2 was upregulated by IL-8 and may have mediated its activity in this model.

In human tumors, IL-8 was detected in non-small cell lung tumors (Yatsunami *et al.,* 1997). It was also found in higher levels in breast tumors than in normal breast tissue, although there was no correlation to histological grade or lymph node status (Green *et al.,* 1997). Receptors for IL-8 have also been detected in breast tumors on both tumor cells and endothelial cells of the small vessels (Miller *et al.,* 1998). Although little research has been conducted to date on the role of IL-8 in cancer, there is some evidence that it may be involved in tumor angiogenesis.

B. Pleiotrophin

Pleiotrophin was initially isolated as a heparin-binding protein from a breast tumor cell line. It is a growth factor with many similar characteristics to FGF although it does not have sequence homology to members of the FGF family. Pleiotrophin and a related protein, midkine, may be involved in embryogenesis but are not highly expressed in normal adults (Kurtz *et al.,* 1995).

Pleiotrophin has transforming activity and stimulates proliferation of endothelial cells (Fang *et al.,* 1992) as well as angiogenesis in the rat cornea (Choudhuri *et al.,* 1997). Ribozyme inhibition of pleiotrophin inhibited tumor growth, angiogenesis, and the number of metastases to the lungs in *in vivo* models (Czubayko *et al.,* 1996).

In humans, increased pleiotrophin was detected in serum of patients with pancreatic and colon cancer; the levels decreased after tumor removal (Souttou *et al.,* 1998). In a study of 27 human breast tumors, 62% were found to express pleiotrophin while normal breast tissue did not express this protein (Fang *et al.,* 1992). A later study showed a correlation between pleiotrophin expression and FGF-1 expression in breast tumors, indicating that they may be coregulated (Relf *et al.,* 1997). As with IL-8, further investigation is required to determine whether pleiotrophin functions as an angiogenic factor in cancer.

VIII. CONCLUSIONS

The clinical association of tumor vascularity with tumor aggressiveness has been clearly demonstrated in numerous tumor types. The observation of microvessel density as an independent prognostic factor implies that anti-angiogenic therapy may be an important component of treatment regimens for

cancer patients. Microvessel density has been correlated with expression of many angiogenic factors; thus these factors have been developed as targets for therapeutic intervention. In this regard, several promising therapies are emerging that target many of the molecular events associated with angiogenesis. Inhibitors of RTKs and MMPs have progressed significantly in clinical trials, with several phase III studies under way. Because angiogenesis is a complex process that occurs during the growth of primary tumors and metastases, treatment with more than one agent may be beneficial. Recently, a study in a pancreatic islet cell carcinogenesis model demonstrated that different anti-angiogenesis agents were efficacious at different phases of tumor development (Bergers *et al.,* 1999). For example, an MMP inhibitor effectively blocked tumor growth when administered to the mice prior to tumor formation or when the tumors were very small, but not once the tumors had been established. In contrast, an inhibitor of endothelial cell proliferation had no effect when administered prior to tumor formation, but it caused regression of established tumors. Combinations of anti-angiogenesis agents that work through different mechanisms or of anti-angiogenesis agents and traditional cytostatic agents may have the best potential for cancer treatment. To this end, several clinical trials of anti-angiogenesis agents currently under way include combination strategies. Within the next few years, results of these trials, along with the trials of single agents, will demonstrate the most effective way in which to use this new and exciting class of inhibitors in the clinical management of cancers.

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The Hepatocyte Growth Factor/ Met Pathway in Development, Tumorigenesis, and B-Cell Differentiation

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I. INTRODUCTION

Hepatocyte growth factor/scatter factor (HGF), originally described as a strong mitogen for hepatocytes (Michalopoulos *et al.,* 1984; Nakamura *et al.,* 1984; Russell *et al.,* 1984a,b), is a multifunctional cytokine with a domain structure and a proteolytic mechanism of activation similar to that of the blood serine protease plasminogen. Unlike plasminogen, however, HGF is devoid of protease activity but has pleiotropic effects on target cells, including stimulation of growth, motility, and morphogenesis. All known biological effects of HGF are transduced via a single receptor, Met, the product of the *Met* protooncogene. The Met protein is a receptor tyrosine kinase and is the prototype of a distinct subfamily, also encompassing Ron and Sea.

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Upon ligand binding, Met interacts with several cytoplasmic target proteins, resulting in activation of a number of distinct signaling cascades including the Ras/MAP kinase and PI3-K/PKB pathways. In addition to binding Met, HGF has a high affinity for heparin and heparan sulfate. Heparan sulfate is present on the cell surface and in the extracellular matrix (ECM), in the form of heparan sulfate proteoglycans (HSPGs). By binding HGF, HSPGs function as coregulators of Met signaling.

Genetic studies in mice have indicated that HGF is indispensable for mammalian development, because mutations of the *HGF* or *Met* genes cause abnormal development of the liver and placenta, and disrupt the migration of myogenic precursors into the limb bud. Other studies have provided evidence for important roles of HGF in angiogenesis, and in the three-dimensional organization of kidney tubular cells and various glandular structures, for example, mammary glands. Apart from mediating these physiologic functions, the HGF/Met pathway is also believed to play a key role in tumor growth, invasion, and metastasis. For example, Met was originally isolated as the product of a human oncogene Tpr-Met, and Met and/or HGF overexpression have been reported in several human tumors. The tumorigenicity of HGF/Met signaling has been confirmed in transgenic mouse models, which develop tumors in many different tissues. In human hereditary papillary renal carcinomas, potentially activating Met mutations are found.

In this article, we discuss the structure, signal transduction, and physiologic functions of the HGF/Met pathway, as well as its role in tumorigenesis. Furthermore, we highlight recent studies that indicate a role for the HGF/Met pathway in antigen-specific B-cell differentiation and B-cell neoplasia.

II. STRUCTURE AND FUNCTION OF HGF AND Met

A. Structure of HGF and Met

1. STRUCTURE OF HGF

Hepatocyte growth factor/scatter factor was independently identified by groups working in two different fields of research. In 1984, a factor present in serum of partially hepatectomized rats and in rat platelet lysates was found to have a strong mitogenic effect on hepatocytes (Michalopoulos *et al.,* 1984; Nakamura *et al.,* 1984; Russell *et al.,* 1984a,b). Hence, this factor was designated hepatocyte growth factor (HGF). Almost simultaneously, Stoker and Perryman (1985) identified a molecule secreted by fibroblasts, which causes dissociation or "scattering" of epithelial cell colonies, and was thus named scatter factor (SF). Subsequent structural and functional studies showed

HGF and SF to be identical (Gherardi and Stoker, 1990; Weidner *et al.,* 1990, 1991; Furlong *et al.,* 1991; Konishi *et al.,* 1991; Naldini *et al.,* 1991c; Rubin *et al.,* 1991).

The mature HGF protein has a relative molecular mass of 90 kDa under nonreducing conditions and is a heterodimer composed of a 60-kDa α subunit and a 30-kDa β subunit linked by a disulfide interchain bridge (Nakamura *et al.,* 1987, 1989; Weidner *et al.,* 1990; Rubin *et al.,* 1991). Due to differential glycosylation, two β-chains, of ~34 kDa and ~32 kDa, respectively, can generally be detected. HGF is secreted as a biologically inactive monomer that is activated through proteolytic cleavage (Naka *et al.,* 1992). Several proteases have been shown to be able to activate HGF. These include urokinase-type (uPA) and tissue-type (tPA) plasminogen activator, proteases known to function in blood clotting and ECM breakdown, blood-coagulating Factor XII_a, and two new serine proteases, HGF activator and HGF converting enzyme (Naldini *et al.,* 1992; Mars *et al.,* 1993; Miyazawa *et al.,* 1993; Mizuno *et al.,* 1994; Shimomura *et al.,* 1995). Recently, a negative regulator of HGF activation was identified, underscoring the complexity of this activation process (Shimomura *et al.,* 1997).

The full-length human *HGF* cDNA encodes a protein of 728 amino acids (Fig. 1) (Nakamura *et al.,* 1989). Its amino acid sequence predicts translation as a precursor protein, which becomes activated by proteolytic cleavage at an Arg-Val cleavage site. This cleavage results in the above-mentioned α and β-chains. Furthermore, the cDNA sequence contains 4 putative N-linked glycosylation sites. Interestingly, significant homology was found between

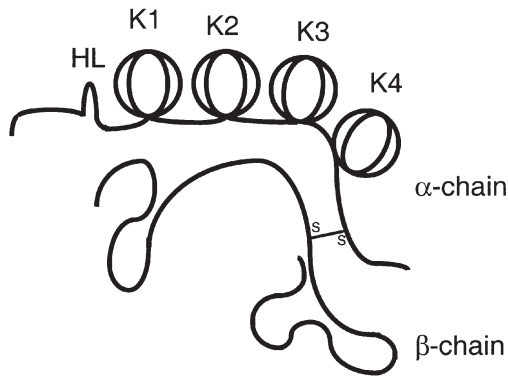

Fig. 1 Schematic representation of the HGF protein. HGF is a secreted glycoprotein composed of a 60-kDa α chain and a 30-kDa β chain linked by a disulfide bridge. The α chain contains an NH_2-terminal domain with a hairpin loop (HL), and four kringle domains (K1–K4). The β-chain is homologous to the protease domain of plasminogen, but has no catalytic activity due to the lack of several essential amino acids.

HGF and plasminogen. Like this serine protease, the α chain of HGF has four kringle domains, structures that play a role in protein-protein interaction. The β chain shows high homology with the catalytic domain of plasminogen, but, due to the lack of two crucial amino acids from the active site, HGF has no proteolytic activity.

Several structurally different HGF transcripts were shown to exist. For instance, in cultured human fibroblasts, Northern blotting revealed three HGF mRNAs of 6, 3, and 1.5 kb, respectively (Chan *et al.,* 1991; Miyazawa *et al.,* 1991b; Rubin *et al.,* 1991; Weidner *et al.,* 1991). Molecular cloning and Northern blotting indicated that the 6- and 3-kb messages emanated from differential polyadenylation (Weidner *et al.,* 1991). The 1.5-kb mRNA represents a splice variant encoding the N-terminal domain of HGF in combination with the first two kringle domains (Chan *et al.,* 1991; Miyazawa *et al.,* 1991b). This variant, NK2, behaves as an HGF antagonist (Chan *et al.,* 1991). The subsequently described one kringle domain variant, NK1, functions as a partial HGF agonist (Cioce *et al.,* 1996; Jakubczak *et al.,* 1998). In addition to these two variants, a putative splice variant containing a deletion of 15 nucleotides in the first kringle domain has been described (Rubin *et al.,* 1991; Weidner *et al.,* 1991). This deletion results in a change of the biological activity of HGF, presumably caused by a change in its tertiary structure (Shima *et al.,* 1994).

Genomic studies have revealed that human HGF is encoded by a single gene localized on the long arm of chromosome 7, band 21.1 (Weidner *et al.,* 1991; Saccone *et al.,* 1992). The gene spans about 70 kbp of DNA and contains 18 exons (Miyazawa *et al.,* 1991a). The promoter region contains a number of regulatory sequences, including a TATA-like element, an interleukin-6 (IL-6) responsive element, and a potential binding site for nuclear factor IL-6, a regulator of IL-6 expression. Also, wild-type, but not mutant, p53 was shown to activate the *HGF* promoter (Metcalfe *et al.,* 1997).

Han and colleagues (1991) identified a gene that shared about 50% sequence homology with HGF. The molecule was designated hepatocyte growth factor-like protein (HGFL), but was subsequently shown to be identical to macrophage stimulating protein (MSP) (Yoshimura *et al.,* 1993), a molecule involved in macrophage chemotaxis and in phagocytosis (Skeel *et al.,* 1991). Structural analysis suggests that HGF and MSP, together with plasminogen and apolipoprotein (a) have evolved from a common ancestral gene (Fig. 2) (Donate *et al.,* 1994).

2. STRUCTURE OF Met, THE HIGH-AFFINITY RECEPTOR FOR HGF

Met, the receptor for HGF, was originally identified as the product of an oncogene (Cooper *et al.,* 1984). This oncogene, *TPR-Met,* results from a chromosomal translocation, fusing the sequence encoding the intracellular

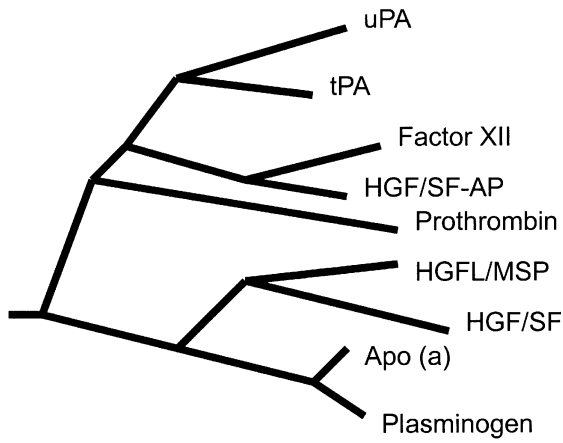

Fig. 2 Phylogenetic tree of plasminogen-related proteins, including HGF and HGFL/MSP. Evolution of the proteins was deduced from the structure of their serine protease domains. Apo (a), apolipoprotein (a); HGF-AP, HGF activator protein; HGFL/MSP, HGF-like protein/ macrophage stimulating protein; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator. (Adapted from Donate *et al.,* 1994.)

domain of Met to that of Tpr (Park *et al.,* 1986), a protein with unknown function. Tpr-Met functions as a constitutively active homodimer with a strong transforming capacity (Cooper *et al.,* 1984; Gonzatti-Haces *et al.,* 1988). Molecular cloning of the 8-kb *Met* protooncogene indicated that this molecule is a cell-surface tyrosine kinase receptor for growth factors (Dean *et al.,* 1985; Park *et al.,* 1987; Rodrigues *et al.,* 1991), whereas functional studies revealed that HGF is the ligand of Met (Bottaro *et al.,* 1991; Naldini *et al.,* 1991b; Rubin *et al.,* 1991).

The Met protein is synthesized as a single-chain 170-kDa precursor. After synthesis, the molecule is cleaved and rearranged into a 190-kDa heterodimer linked by a disulfide bridge (Fig. 3) (Giordano *et al.,* 1989a,b). Komada *et al.* (1993) demonstrated that Met can be cleaved by furin, but that endoproteolytic processing is not essential for HGF-induced signal transduction. The Met heterodimer is composed of a 50-kDa α-subunit and a 145-kDa β-subunit (Giordano *et al.,* 1989a). The cytoplasmic tail of the β-chain contains the tyrosine kinase domain and a "docking site," which interacts with multiple signaling molecules (Ponzetto *et al.,* 1994). Both functional domains are discussed in more detail in Section II,C.

The identification of Tpr-Met resulted in the assignment of the human *Met* gene to chromosome 7, band q31 (Cooper *et al.,* 1984; Dean *et al.,* 1985; Lin *et al.,* 1996). The gene spans more than 110 kbp and contains 21 exons (Duh *et al.,* 1997; Lin *et al.,* 1998; Liu, 1998). The sequence of the *Met* promoter region revealed a number of binding sites for regulatory elements, including AP1, AP2, NF-κB, and, like the *HGF* gene, an IL-6 responsive element (Liu,

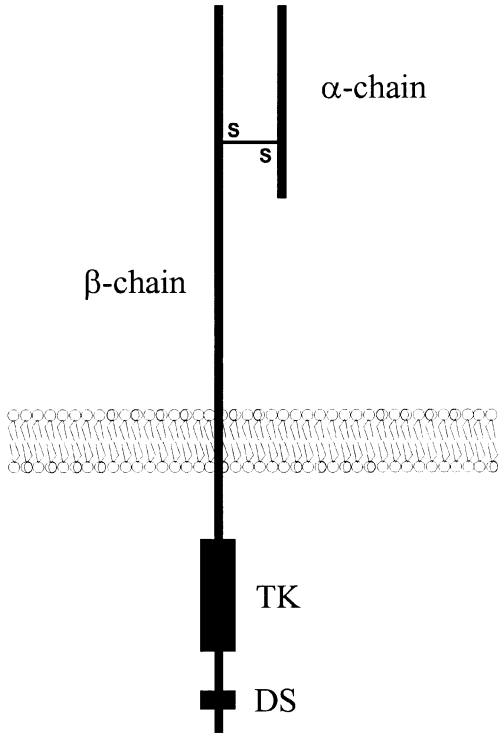

Fig. 3 Schematic representation of the receptor tyrosine kinase Met. The receptor is composed of two disulfide-linked chains: a 50-kDa α chain and a 145-kDa β chain. The β chain contains the tyrosine kinase domain (TK) and a "docking site" (DS), which interacts with signaling molecules.

1998). Recently, wild-type, but not mutant, p53 was shown to enhance the activity of the *Met* promoter (Seol *et al.,* 1999), as had been shown before for the *HGF* gene.

Two receptor tyrosine kinases related to Met, Sea (Huff *et al.,* 1993) and Ron (Ronsin *et al.,* 1993), have been identified. MSP was shown to be the ligand for Ron (Gaudino *et al.,* 1994; Wang *et al.,* 1994), whereas Sea remains, as yet, an orphan receptor. In addition, Met shows homology with the putative receptor tyrosine kinase stem cell-derived tyrosine kinase (STK) and with the SEX family of transmembrane proteins (Iwama *et al.,* 1994; Maestrini *et al.,* 1996).

3. LOW-AFFINITY RECEPTORS FOR HGF

Apart from binding to Met, HGF also binds to heparan sulfate proteoglycans. These interactions, which appear to play an important role in the regulation of HGF activity, are discussed in Section II,D.

B. Expression and Functions of HGF and Met

1. INTRODUCTION

The receptor tyrosine kinase Met is prominently expressed on a wide variety of epithelial cells, whereas its ligand, HGF, is expressed by stromal cells. This reciprocal expression pattern points to their important role in epithelial–mesenchymal interactions underlying branching morphogenesis and tubulogenesis during development of organs such as lungs, kidney, and mammary glands. During the past few years, it has become clear, however, that HGF and Met are also involved in a plethora of other biologic processes. In the next paragraphs we give an overview of the well-established expression pattern and functions of HGF and Met, as well as of those attributed more recently. It places HGF and Met in the center of developmental processes, leading to a proper organization not only of epithelial tissues, but also of muscle, endothelium, and the nervous and hematopoietic systems.

2. EXPRESSION PATTERN DURING AMPHIBIAN, AVIAN, AND MAMMALIAN DEVELOPMENT

During embryogenesis of the tadpole *Xenopus laevis*, Met is present as early as in the gastrula stage and remains expressed at high levels throughout neurulation (Aoki *et al.,* 1996). Sites of expression include the foregut region, tailbud mesenchyme, and, in neurulating embryos, neural tissues. HGF expression becomes apparent later, from the neurula stage onward. The spatiotemporal expression patterns of both *HGF* and *Met* point to multifarious roles in amphibian organogenesis. This has been shown more specifically by use of dominant-negative Met constructs, introduced into fertilized *Xenopus* eggs. Embryos thus treated fail to develop a normal liver, whereas organogenesis of the gut and early kidney are greatly impaired (Aoki *et al.,* 1997). Hence, in *Xenopus* embryos, a functional HGF/Met system is involved in early organogenesis, especially of organs derived from the primitive gut.

Interference with the HGF/Met system during early chick embryo development leads to abnormal axis formation, underscoring its determining role during avian development, especially in neural induction and limb bud elevation (Stern *et al.,* 1990). *HGF*, but not *Met*, is expressed in the mesoderm of the limb bud and in the central core region of mandibular arch and maxillary processes at stages 17 to 24 of development (Myokai *et al.,* 1995; Théry *et al.,* 1995). During limb bud extension, *HGF* is expressed in the mesenchyme and becomes later confined to the ventral and subapical mesenchyme of the limb bud, suggesting that HGF production in the limb bud is involved in the induction and maintenance of apical ectoderm during limb bud development (Myokai *et al.,* 1995).

During embryonic development of rodents, HGF is prominently expressed in a multitude of tissues, mainly at sites where epithelial/mesenchymal interactions determine organogenesis (Iyer *et al.,* 1990). In gastrulating mouse embryos, the expression of *HGF* and *Met* overlaps. Initially, the two genes are expressed in the endoderm and in the mesoderm along the rostro-intermediate part of the primitive streak and, later, in the node and in the notochord. Neither *HGF* nor *Met* is expressed in the ectodermal layer throughout gastrulation (Sonnenberg *et al.,* 1993; Andermarcher *et al.,* 1996). During early organogenesis, overlapping expression of *HGF* and *Met* is found in the heart, condensing somites, and neural crest cells. However, a second and distinct pattern of expression, characterized by the presence of the ligand in mesenchymal tissues and the receptor in the surrounding ectoderm, is seen in the bronchial arches and in the limb buds. At E13, only this second pattern of expression is observed in differentiated somites and several major organs, such as the lungs, the liver, and the gut (Andermarcher *et al.,* 1996). The expression of the *HGF* and *Met* genes throughout embryogenesis suggests a shift from an autocrine to a paracrine signaling system. Halfway through gestation, HGF is present in renal collecting tubes of the kidney, in the liver, in esophageal and skin squamous epithelium and in bronchial epithelium (Defrances *et al.,* 1992; Lee *et al.,* 1993). HGF is also detected in brain, somites, hematopoietic cells, and chondrocytes (Defrances *et al.,* 1992).

Similar patterns of *Met* and *HGF* expression are found along human embryonic development. From the 5th week of gestation onward, placental tissue highly expresses *HGF* and *Met*. HGF is secreted by amniotic epithelium, the placental villi and the villous core mesenchyme, whereas Met is present on the trophoblast and vascular endothelium (Kauma *et al.,* 1997; Somerset *et al.,* 1998; Wolf *et al.,* 1991). A human pathologic condition, known as intrauterine growth restriction, is associated with an underdeveloped placenta and could be linked to a decreased secretion of HGF by the villous stromal cells (Somerset *et al.,* 1998). The absolute dependence of placenta maturation on HGF has been unequivocally shown in *HGF* null mutant mouse embryos, whose placenta fail to develop properly and which die *in utero* (Schmidt *et al.,* 1995; Uehara *et al.,* 1995). From weeks 6–13 of gestation, when major organogenesis takes place, *HGF* and *Met* are coexpressed in liver, metanephric kidney, intestine, lung, gall bladder, and spleen (Kolatsi-Joannou *et al.,* 1997; Y. Wang *et al.,* 1994b). In the digestive tract of 7- to 8-week-old embryos, Met is localized in epithelia of the liver, pancreas, esophagus, stomach, the small and large intestine, and in smooth muscle layers, whereas HGF becomes concentrated in mesenchymal tissue and smooth muscle (Kermorgant *et al.,* 1997). Interestingly, *HGF* expression has also been shown in epithelial tissues in the interval from weeks 9–17 of gestation, particularly in the crypt region of the small intestine, keratinizing epithelium of the tongue, skin, and esophagus (Y. Wang *et al.,* 1994b).

In conclusion, HGF and Met are highly conserved molecules in a wide range of species, not only structurally (see Section II,A), but also with respect to their particular role during embryogenesis. In the next paragraphs some specific functions of HGF and Met are discussed. We focus on branching morphogenesis, muscle development, angiogenesis, and neuronal development.

3. MESENCHYMAL–EPITHELIAL INTERACTION AND BRANCHING MORPHOGENESIS

HGF induces scattering of epithelial cells *in vitro* (Stoker *et al.,* 1987; Uehara and Kitamura, 1992) through activation of Met (Weidner *et al.,* 1993) (Fig. 4). This effect can be mimicked by a constitutively active mutant of *Met* (Jeffers *et al.,* 1998a), suggesting that activation of Met is sufficient in this process. Once activated, Met can in turn activate PI3K and the Ras-MAPK pathway (Boccaccio *et al.,* 1998; Potempa and Ridley, 1998). Furthermore, enzymes involved in ECM proteolysis (e.g., uPA) are activated (Pepper *et al.,* 1992). Partial ECM proteolysis may increase cell motility by diminishing adhesion properties of epithelial cells toward matrix components. Although scattering *in vitro* can hardly be considered as its physiologic function, the phenomenon per se reflects the first phase of epithelial morphogenesis (by activation of MET) through mesenchymal induction (secretion of HGF), underlying the complex, but coordinated formation of branched organs, such as the lungs, the kidney, and mammary gland (Sonnenberg *et al.,* 1993).

Tubular differentiation can be induced under "ECM conditions," that is, when epithelial cells are cultured in a three-dimensional ECM-like environment. Thus, kidney epithelial cells, treated with HGF, form tubules resembling those emanating during kidney organogenesis in early embryonic development (Boccaccio *et al.,* 1998; Cantley *et al.,* 1994; Liu *et al.,* 1998a; Montesano *et al.,* 1991a,b; Sachs *et al.,* 1996). Ezrin, a member of the ERM family of membrane to cytoskeleton linkers (reviewed in Tsukita and Yonemura, 1997), and a substrate of MET, is involved in the cytoskeletal reorganization associated with tubulogenesis (Crepaldi *et al.,* 1997). Embryonic mesenchymal kidney cells undergo a mesenchymal to epithelial transition, which is accelerated by HGF (Karp *et al.,* 1994). This conversion mimics developmental processes in the metanephros *in vivo*, where mesenchymal specialization is induced by the ingrowth of a branching ureteric bud and is in accordance with expression patterns of Met and HGF during development (Santos *et al.,* 1994; Woolf *et al.,* 1995).

Surprisingly, kidney epithelial cells derived from *Met* null mutant mouse embryos, and hence unresponsive to HGF, were able to form tubular structures *in vitro* and to express epithelial-specific markers after treatment with epidermal growth factor (EGF) (Kjelsberg *et al.,* 1997). An intact HGF/Met

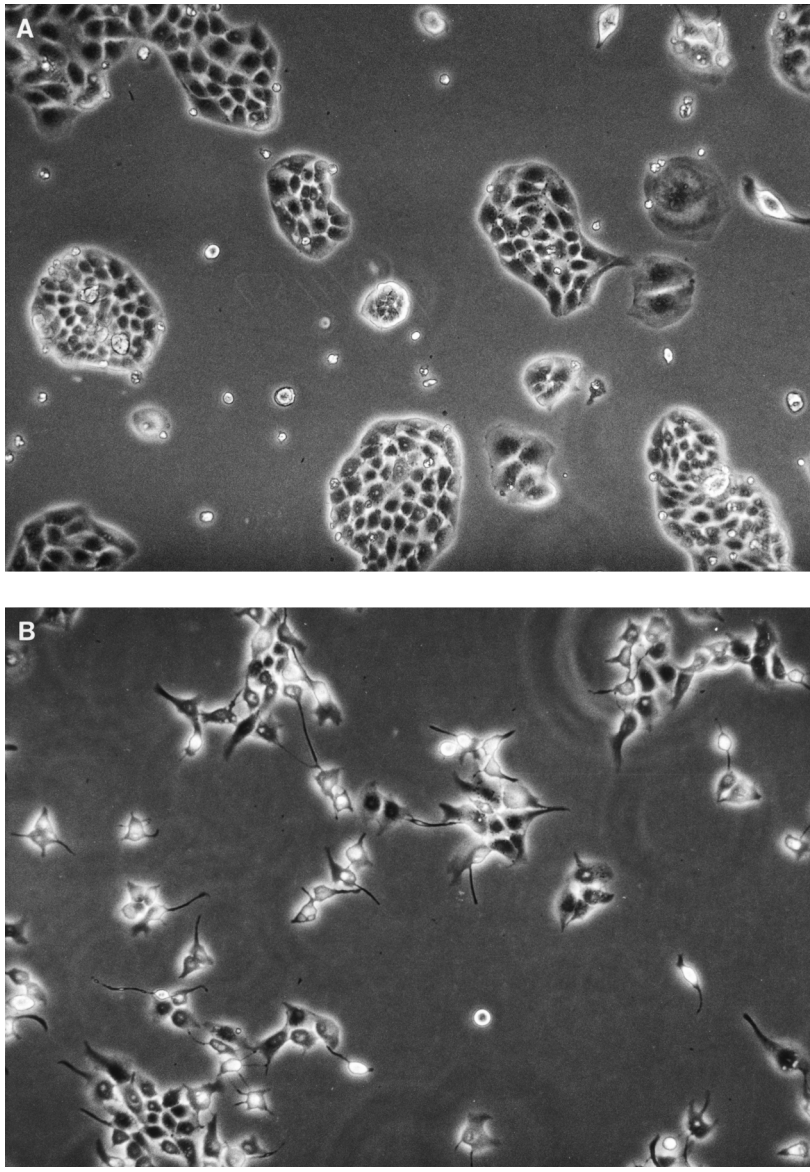

Fig. 4 Scattering of MDCK cells induced by HGF. (A) MDCK cells grown in the absence of HGF form islands. (B) HGF treatment leads to dissociation of the islands and to migration of the cells.

pathway may thus not be necessary for kidney development, although it can play an auxiliary role. This opens the possibility that HGF may be involved in kidney epithelial regeneration, rather than embryonic kidney development. Indeed, following renal injury, HGF expression is elevated (Horie *et al.,* 1994; Igawa *et al.,* 1993; Liu *et al.,* 1999). Moreover, transgenic mice, overexpressing HGF in the kidney, die of renal failure, associated with the stimulation of the HGF/Met autocrine pathway (Takayama *et al.,* 1997a). In these mice, kidney pathology is not apparent at birth, but rather develops progressively.

In epithelial cells derived from another branched, lumen-forming organ, the mammary gland, HGF treatment leads to the formation of branches and structures resembling mammary gland ducts when cultured in a three-dimensional matrix (Berdichevsky *et al.,* 1994; Brinkmann *et al.,* 1995; Niemann *et al.,* 1998; Soriano *et al.,* 1995; Yang *et al.,* 1995). In accordance with its role in mesenchymal-epithelial interaction in the mammary gland, *Met* expression is confined to the epithelial cells lining the mammary ducts, whereas HGF is produced by mammary gland fibroblasts (Niranjan *et al.,* 1995; Tsarfaty *et al.,* 1992; Y. Wang *et al.,* 1994a; Yang *et al.,* 1995). During pregnancy, *HGF* and *Met* transcripts are progressively reduced to background levels during lactation, and increase during the phase of involution to pre-pregnancy levels. The reduction in *HGF* and *Met* expression corresponds to periods in which functions other than tubulogenesis predominate in the mammary gland: alveolar budding and milk protein synthesis (Pepper *et al.,* 1995). Indeed, treating mammary gland cultures with the milk production inducing hormone prolactin, sharply reduces *Met* transcript levels (Pepper *et al.,* 1995).

In the developing lung, *HGF* is expressed in the mesenchyme and *Met* in the pulmonary epithelium (Ohmichi *et al.,* 1998). Alveolar type II cells, when cultured in the presence of HGF, are induced to proliferate (Mason *et al.,* 1994; Shiratori *et al.,* 1995), whereas tracheal epithelial cells are driven to differentiate into a polarized cell type (Shen *et al.,* 1997). HGF also proved to be a mitogen for bronchial epithelial cells (Singh-Kaw *et al.,* 1995) and furthermore to facilitate the organotypic rearrangement of cultured E15 mouse lung epithelial cells (Sato and Takahashi, 1997) and branching morphogenesis in organ cultures (Ohmichi *et al.,* 1998). Apart from the function in pulmonary development, HGF can act as growth factor *in vivo* for alveolar type II cells after lung injury and can thus add to the restoration of epithelial integrity (Panos *et al.,* 1996; Yanagita *et al.,* 1993). Its tissue distribution in the developing lung, together with its proliferation and differentiation-stimulating effects, renders HGF a paracrine growth factor in lung development and regeneration.

Pancreatic epithelial cells, as well as pancreas carcinoma cells, are induced to proliferate and differentiate by HGF, forming tubular structures com-

posed of a lumen, lined by polarized epithelial cells (Brinkmann *et al.,* 1995). These cells have characteristics of pancreas ductal epithelia, including apical microvilli (Jeffers *et al.,* 1996a) and the appearance of characteristic markers of normal ductal cells (Vila *et al.,* 1995). HGF further influences pancreatic islet formation and β-cell differentiation, leading to the secretion of insulin (Otonkoski *et al.,* 1994, 1996).

HGF has initially been described as a mitogenic factor for cultured hepatocytes (Michalopoulos *et al.,* 1984; Nakamura *et al.,* 1984) (see also Section II,A) and it has been implicated in embryonic hepatic development. In the liver, *HGF* is expressed in Ito cells, whereas *Met* transcripts are strongly expressed by hepatocytes (Hu *et al.,* 1993). After chemical or mechanical liver injury HGF levels sharply increase, leading to a strong hepatocyte proliferation (Horimoto *et al.,* 1995; Hu *et al.,* 1993). Livers from transgenic mice with liver-specific overexpression of HGF are twice the size of livers of control animals and they regenerate much faster after partial hepatectomy (Sakata *et al.,* 1996; Shiota *et al.,* 1994). Apart from their placental phenotype described earlier, *HGF* null mutant mouse embryos fail to develop a fully functional liver (Schmidt *et al.,* 1995), demonstrating that the presence of HGF is an absolute requirement during liver organogenesis. In rats with an experimental liver cirrhosis the administration of HGF through autologous gene transfer was shown to have a beneficial effect on overall survival (Ueki *et al.,* 1999). Thus, HGF acts as a paracrine factor for hepatocyte proliferation and differentiation, both during embryonic liver development, as well as during post-trauma regeneration.

HGF and Met are furthermore involved in the proliferation and migration of a wide variety of epithelial cells, and in the morphogenesis of epithelial tissue. In colon epithelial cells, a complete epithelial developmental program is enrolled upon treatment with HGF, including apical/basal polarization and the formation of crypt-like structures (Brinkmann *et al.,* 1995). Prostate stromal cells produce HGF *in vivo* (Kasai *et al.,* 1996) and prostate epithelial cells, grown in the presence of HGF, proliferate and develop tubular structures reminiscent of those found in the prostate (Brinkmann *et al.,* 1995). Other implications of HGF-Met include the development of bone (especially of cartilage) (Blanquaert *et al.,* 1999; Grumbles *et al.,* 1996; Takebayashi *et al.,* 1995), teeth (Tabata *et al.,* 1996), the (male and female) reproductive tract (Depuydt *et al.,* 1996; Naz *et al.,* 1994; Parrott and Skinner, 1998), thyroid (Schulte *et al.,* 1998; Trovato *et al.,* 1998), and the regulation of hair growth (Jindo *et al.,* 1994, 1998; Shimaoka *et al.,* 1995).

During epithelial wound healing, many cellular programs that play a role during embryonic development are reactivated. The HGF-Met axis has been implicated in epithelial wound healing of various epithelia, including gastric, intestinal and corneal epithelia (Nusrat *et al.,* 1994; Takahashi *et al.,* 1995a,b; Wilson *et al.,* 1999).

In conclusion, HGF and Met are involved in tissue-specific programs of differentiation in a wide variety of lumen-forming organs, leading to the formation of contiguous, polarized epithelial cell layers and, depending on the type of tissue, tubulogenesis and branching.

4. DEVELOPMENT OF THE NERVOUS SYSTEM

As described earlier, *HGF* and *Met* are already expressed in the developing central and peripheral nervous system (CNS and PNS, respectively), but they remain present during adulthood. Embryonic prospective chick neural plate explants, when treated with HGF, differentiate into cells with a neuronal morphology, and start to express neuronal markers (Streit *et al.,* 1995), whereas in transgenic mice that ectopically express HGF, cells of the neural crest lineage become inappropriately targeted (Takayama *et al.,* 1996). Thus, HGF is involved in neural induction, as well as in later stages of neuronal development, when neural cells adopt a migratory phenotype. In the mammalian CNS, *Met* is abundantly expressed in the neurons of the hippocampus, cerebral cortex, septum, amygdala, pons, olfactory bulb, medulla, and spinal cord (Achim *et al.,* 1997; Honda *et al.,* 1995; Jung *et al.,* 1994; Thewke and Seeds, 1999; Wong *et al.,* 1997). During embryogenesis, *HGF* expression seems to be confined to prospective target cells for the outgrowing neurites. HGF is therefore considered as a chemoattractant, for example, for spinal motoneurons. Furthermore, HGF has been identified as a survival factor for these neurons and is secreted by their target tissue, muscle, during later stages of development (Ebens *et al.,* 1996; Yamamoto *et al.,* 1997). HGF has been shown to act synergistically with another neurotrophic factor, ciliary neurotrophic factor (CNTF), in motoneuron survival (Wong *et al.,* 1997). During adulthood, *HGF* remains expressed in the CNS, where it is found, apart from its localization in neurons, in a/o central glial, ependymal cells, and cells lining the choroid plexus (Honda *et al.,* 1995; Jung *et al.,* 1994). In cultured sympathetic neurons, which express both *HGF* and *Met* throughout development, HGF acts as an autocrine axonal outgrowth-stimulating factor, and not as a survival factor (Maina *et al.,* 1998; Yang *et al.,* 1998). However, in the precursor cells of the sympathetic neurons, the sympathetic neuroblasts, HGF does have a stimulating effect on cellular survival, pointing to a shift in the dependence on HGF from a survival factor to an outgrowth-stimulating factor (Maina *et al.,* 1998). Transgenic mice expressing dominant-negative Met fail to develop a complete set of sensory innervating connections (Maina *et al.,* 1997). In cultured dorsal root ganglia of these mice, which contain predominantly sensory neurons, HGF acts synergistically with nerve growth factor (NGF) in axonal outgrowth (Maina *et al.,* 1997). Neurotrophic effects of HGF have also been reported in mesencephalic dopaminergic neurons from neocortical explants *in vitro*

(Hamanoue *et al.,* 1996). In these explants, HGF is mainly expressed in microglia, suggesting a role in CNS development.

Thus, depending on the spatiotemporal distribution pattern and the type of neurons involved, HGF may act as a neural inducer, a neuronal survival factor, or an axonal guidance factor.

5. ANGIOGENESIS

HGF is a potent *in vitro* motility-stimulating factor for endothelial cells under two-dimensional culture conditions (Rosen *et al.,* 1990), whereas in three-dimensional collagen matrices, endothelial cells can be induced by HGF to adapt an elongated phenotype or to even form tubular, vessel-like structures (Bussolino *et al.,* 1992; Grant *et al.,* 1993). Abundant and genuine angiogenesis *in vivo* is observed when rabbit cornea is treated with HGF (Bussolino *et al.,* 1992). Accordingly, after implantation into mice, tumor cells that express both *Met* and *HGF* expand much faster than cells that do not secrete HGF and they constitute larger tumors. This coincides with increased and abundant microvascularization of the HGF-secreting tumors (Lamszus *et al.,* 1997; Laterra *et al.,* 1997). Blood vessel endothelial cells express Met on their plasma membrane (Bussolino *et al.,* 1992), but it is not clear whether HGF-induced angiogenesis is a direct consequence of increased endothelial cell motility and proliferation. HGF can also enlarge the expression of vascular endothelial growth factor (VEGF) in gastric epithelial cells and could thus be responsible for neovascularization in gastric tumors (Takahashi *et al.,* 1997). On the other hand, VEGF induction has been described in endothelial smooth muscle cells after HGF treatment, where it may act synergistically with HGF in angiogenesis (Van Belle *et al.,* 1998). Finally, HGF has been described to induce platelet-activating factor in macrophages that are in the vicinity of the site of neovascularization (Camussi *et al.,* 1997). HGF thus increases proliferation and migration of endothelial cells and may engender angiogenesis directly, or indirectly via VEGF and platelet-activating factor.

6. MUSCLE DEVELOPMENT

During embryonic muscle development, HGF secreted by limb bud mesenchymal cells induces migration of Met expressing myogenic precursor cells from the somites (Bladt *et al.,* 1995; Yang *et al.,* 1996). Met signaling is essential for the detachment of the myogenic precursor cells and the subsequent migration into the limb bud and diaphragm (Brand-Saberi *et al.,* 1996). In *Met* null mutant mouse embryos, myogenic precursor cells remain in the dermomyotome, and consequently, the limb bud and diaphragm are not colonized, leading to the absence of skeletal muscles in the limb and di-

aphragm (Bladt *et al.,* 1995; Dietrich *et al.,* 1999). In contrast, development of the axial skeletal muscles proceeds in the absence of Met signaling. Ectopic HGF expression leads to aberrant muscle development as shown in chick embryos, where additional limb buds had been induced by the ectopic application of fibroblast growth factor (FGF). Here, myogenic precursor cells colonize this newly formed limb bud, through chemoattraction toward HGF (Heymann *et al.,* 1996), whereas in transgenic mice that inappropriately express HGF, ectopic muscle formation occurs (Takayama *et al.,* 1996).

Met and *HGF* mRNA are present in immature neonatal rat skeletal muscle, but in adult skeletal muscle their levels are below detection limits. After muscle damage, both *HGF* and *Met* expression are upregulated in the regenerating muscle (Jennische *et al.,* 1993; Anastasi *et al.,* 1997; Tatsumi *et al.,* 1998). In a cultured undifferentiated myoblast cell line both genes are also coexpressed, pointing to the existence of an autocrine pathway in the regulation of cell proliferation (Anastasi *et al.,* 1997). It appears that HGF expression is developmentally regulated in skeletal muscle and transiently reexpressed during muscle regeneration. The latter process may involve the concerted activation of quiescent satellite cells to proliferate (Allen *et al.,* 1995), while at the same time their differentiation is inhibited (Gal-Levi *et al.,* 1998).

HGF and Met are also expressed in progenitor cells of the cardiomyocytes and may play a role in cardiomyogenic differentiation and heart organogenesis (Rappolee *et al.,* 1996; Song *et al.,* 1999).

HGF is hence an inducer of myogenic migration during embryonic development and of satellite cell proliferation during muscle regeneration. Contrary to the mutually exclusive expression pattern generally found in mesenchymal/epithelial tissues, myoblast proliferation may be regulated by HGF/Met in an autocrine fashion.

7. HEMATOPOIESIS

The HGF/Met pathway has also been implicated in hematopoiesis. Both HGF and Met are expressed in the yolksac of the chicken embryo (Théry *et al.,* 1995), and in the human and rodent fetal liver, primordial sites of hematopoiesis (Selden *et al.,* 1990; Hu *et al.,* 1993). Within the adult hematopoietic microenvironment, the bone marrow, Met is expressed by a subset of haematopoietic precursor cells (HPC), whereas HGF is expressed by stromal cells, suggesting that HGF functions as a paracrine growth factor (Kmiecik *et al.,* 1992; Galimi *et al.,* 1994; Takai *et al.,* 1997; Weimar *et al.,* 1998). Indeed, it was shown that HGF promotes differentiation and proliferation of HPC induced by other hematopoietic growth factors. In the presence of IL-3, HGF stimulates the formation from $CD34^+$ progenitors of burst-forming units erythroid, as well as colony-forming units granulocyte

erythroid macrophage, but not of colony-forming units granulocyte monocyte (Galimi *et al.,* 1994; Takai *et al.,* 1997). In the presence of stem cell factor, an even stronger synergistic effect is obtained (Galimi *et al.,* 1994; Weimar *et al.,* 1998). Apart from effects on growth and differentiation, HGF stimulation of $CD34^+$ cells leads to integrin activation and adhesion to fibronectin. This adhesive interaction prolonged survival of hematopoietic cells in culture (Weimar *et al.,* 1998). Taken together, these data indicate that the HGF/Met pathway is involved in the regulation of the proliferation, differentiation, and survival of hematopoietic progenitors.

C. Signal Transduction by Met

1. INTRODUCTION

As with most other receptor tyrosine kinases, activation of the kinase domain of Met is believed to depend on receptor dimerization or oligomerization, resulting in intermolecular transphosphorylation. This process of di- or oligomerization may be facilitated by the action of HSPGs, as discussed in Section II,D. Upon stimulation by HGF, the C terminus of the β chain of Met is strongly tyrosine phosphorylated (Bottaro *et al.,* 1991; Naldini *et al.,* 1991a,b). The autophosphorylation of the tyrosine residues Y1349 and Y1356 of Met, as well as the equivalent residues Y482 and Y489 of the oncoprotein Tpr-Met, are critical for most biologic responses (Ponzetto *et al.,* 1994, 1996; Zhu *et al.,* 1994; Fixman *et al.,* 1995). These tyrosine residues serve as a multisubstrate docking site for several proteins, including Gab1, Grb2, phosphatidylinositol-3-kinase (PI3K), phospholipase C (PLCγ), Src, Shc, SHP-2, and STAT3 (Fig. 5). Except for Gab1, which has a unique Met-binding domain (Weidner *et al.,* 1996; see, however, discussion later), these proteins interact with Met via their SH2 domains: Grb2 specifically to Y1356, the other proteins to both Y1349 and Y1356 (Ponzetto *et al.,* 1993, 1994, 1996; Pelicci *et al.,* 1995; Nguyen *et al.,* 1997). Here we discuss the nature and function of the different Met-associating signaling molecules. Furthermore, the signaling pathways activating Met, and their biologic function, are discussed.

2. THE ROLE OF Grb2 AND SIGNAL TRANSDUCTION VIA Ras

One of the signaling molecules that associates directly with Met upon HGF stimulation is Grb2 (Ponzetto *et al.,* 1994). Grb2 is an adapter protein consisting of one SH2 and two SH3 domains. SH2 domains are involved in binding to phosphorylated tyrosine residues, whereas SH3 domains bind to proline-rich regions. By means of its SH3 domain, Grb2 is constitutively as-

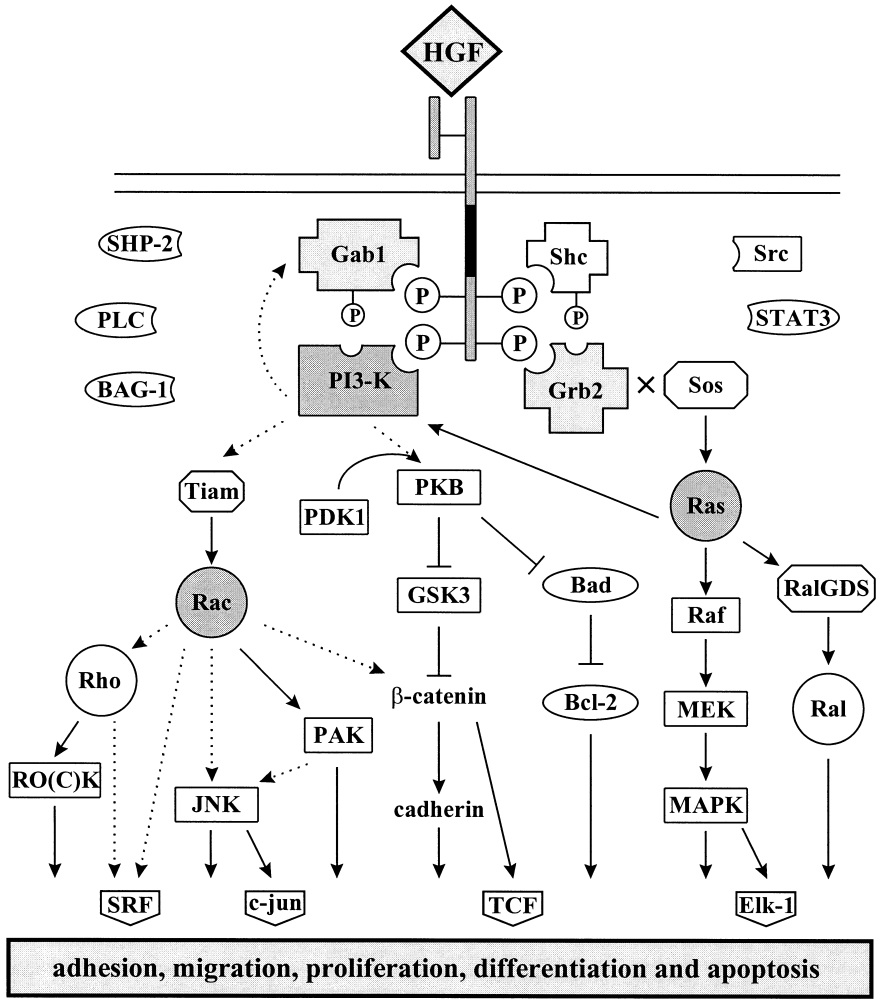

Fig. 5 A schematic representation of the most relevant signaling pathways activated by HGF. For reasons of clarity both relevant autophosphorylation sites of Met, that is, Y1349 and Y1356, are depicted twice. Furthermore, the Met-associating proteins, except for Grb2, are able to interact with either autophosphorylation site. Crossed shapes represent adapter or docking proteins without catalytic activity, squares represent kinases, circles represent GTPases, octagons represent guanine nucleotide dissociation stimulators, and pentagons represent transcription factors. The solid arrows indicate a direct activation, whereas the dotted arrows indicate activation via known or unknown intermediate proteins or phospholipid metabolites, and the blunted arrows indicate a direct inhibition. Although only Met is depicted, most signaling pathways also apply to the oncoprotein Tpr-Met. See text for further details.

sociated with Sos, an exchange factor for Ras. The Grb2–Sos complex is recruited by receptor tyrosine kinases, via their autophosphorylated tyrosine residues, to the plasma membrane where Ras is localized. As a consequence, Ras becomes activated (Fig. 5). After HGF-induced autophosphorylation, Met also associates with the Shc adapter protein (Pelicci *et al.,* 1995). Interestingly, upon phosphorylation, Shc is also able to associate with Grb2 (Pelicci *et al.,* 1995). Thus, HGF stimulation can trigger the Ras pathway by both direct and Shc-mediated association of the Grb2–Sos complex to Met (Fig. 5).

Activation of Ras has been implicated in a wide variety of cellular responses including cytoskeletal reorganization, adhesion, proliferation, differentiation, and apoptosis. The first identified and best characterized effector molecule for Ras is the serine/threonine kinase Raf1, which phosphorylates and activates MEK, resulting in the phosphorylation and activation of MAP kinase (Campbell *et al.,* 1998; Vojtek and Der, 1998) (Fig. 5). Among the substrates for MAP kinase are the transcription factors Elk-1 and Ets-2 involved in ternary complex formation at serum response elements. Upon activation, these transcription factors regulate expression of immediate early genes, such as *c-fos,* eventually leading to cell proliferation (Wasylyk *et al.,* 1998). Besides Raf, several additional effector molecules for Ras have been identified. These include PI3K (Rodriguez-Viciana *et al.,* 1994), which is discussed later, and RalGDS (Spaargaren and Bischoff, 1994), an exchange factor for Ral (Albright *et al.,* 1993; Feig *et al.,* 1996) (Fig. 5). Ral has been implicated in Ras-dependent proliferation, gene expression, phospholipase D activation, and transformation (Wolthuis and Bos, 1999), however, no studies have been conducted yet to investigate its involvement in Met signal transduction and functional responses.

In initial studies using mutants of Met, it was shown that Y1356, the Grb2 binding site, is required for scattering and branching tubulogenesis of MDCK cells, whereas the equivalent residue Y489 of Tpr-Met is required for cell proliferation and transformation (Zhu *et al.,* 1994; Fixman *et al.,* 1995). However, these mutations also reduced binding of other Met-associating proteins (Ponzetto *et al.,* 1993, 1994; Pelicci *et al.,* 1995). Using a more sophisticated mutant that selectively fails to bind Grb2 only, it was shown that Grb2 association by Met is required for HGF-induced branching tubulogenesis of MDCK cells, but not for scattering (Fournier *et al.,* 1996; Ponzetto *et al.,* 1996; Royal *et al.,* 1997). Similarly, whereas Grb2 binding by Tpr-Met is not required for motility, it is required for induction of transformation and invasion, *in vitro,* as well as metastasis and tumorigenicity, *in vivo* (Fixman *et al.,* 1996; Ponzetto *et al.,* 1996; Giordano *et al.,* 1997; Jeffers *et al.,* 1998b; Bardelli *et al.,* 1999). Intriguingly, however, Grb2 binding to Met is dispensable for transformation, metastasis, and tumorigenicity, when Met is activated by either a point mutation or by autocrine HGF stimulation (Jeffers *et al.,* 1998b).

Interestingly, it has been shown that expression of N17-Ras, a dominant-negative mutant of Ras, abolishes HGF-induced cell scattering (Hartmann *et al.,* 1994; Ridley *et al.,* 1995). Because scattering does not require a Grb2 binding site, this suggests that HGF/Met activates Ras by a Grb2-independent mechanism. Indeed, a study by Tulasne *et al.* (1999) shows that a mutant of Met, which lacks four major autophosphorylation tyrosine residues (including Y1349 and 1356, which constitute the multisubstrate docking site), despite its loss of Grb2 binding ability, is still able to induce Ras activation. Moreover, the scattering response, which was clearly not affected by these mutations, was abolished upon treatment with the specific pharmacologic inhibitor PD98059 of the Ras–MAP kinase pathway intermediate MEK (Tulasne *et al.,* 1999). Using either this MEK-inhibitor or constitutively active or dominant negative mutants of Ras or MAP kinase, it was concluded that the activation of the Ras–MAPK pathway is required but not sufficient for HGF-induced scattering (Ridley *et al.,* 1995; Potempa and Ridley 1998; Herrera, 1998; Khwaja *et al.,* 1998; Tanimura *et al.,* 1998; Tulasne *et al.,* 1999), and for tubulogenesis of MDCK cells (Khwaja *et al.,* 1998). In conclusion, Grb2 and the Ras–MAP kinase pathway appear to play an important regulatory role in a variety of responses elicited by HGF/Met, including mitogenesis, motogenesis, and morphogenesis, as well as in Tpr-Met-induced transformation, invasion, metastasis, and tumorigenicity.

3. THE ROLE OF Gab1 AND SIGNAL TRANSDUCTION VIA PI3K

Another important substrate for Met is the docking protein Gab1 (Weidner *et al.,* 1996). *In vitro*, Gab1 interacts directly with Met via a proline- rich binding domain (Weidner *et al.,* 1996), but it has been concluded that the interaction of Gab1 with Met and Tpr-Met *in vivo* is mediated by Grb2 (Nguyen *et al.,* 1997; Bardelli *et al.,* 1997; Fixman *et al.,* 1997). Gab1, which contains a PH domain as well as a proline-rich region, and can become heavily tyrosine phosphorylated, has the ability to directly associate with several signaling molecules such as Grb2, PI3K, PLCγ, and SHP2 (Holgado-Madruga *et al.,* 1996).

Overexpression of Gab1 partially mimics the action of HGF, because it results in tubulogenesis of mammary epithelial cells (Niemann *et al.,* 1998), as well as in enhanced MAP kinase activity, cell scattering and tubulogenesis of MDCK cells (Weidner *et al.,* 1996). The HGF responses in MDCK cells could be abrogated by overexpressing the Met binding domain of Gab1 (Weidner *et al.,* 1996). In NIH 3T3 fibroblasts, however, Holgado-Madruga *et al.* (1996) did not observe enhanced MAP kinase activity or activation upon overexpression of Gab1. Moreover, a recent study shows that Met with mutations of the multisubstrate docking site, which abolish recruitment of

Gab1, as well as Grb2, Shc, and PI3K, although indeed impaired in the induction of morphogenesis, is still able to activate the Ras–MAP kinase pathway and to induce MEK-dependent scattering (Tulasne *et al.,* 1999). Finally, the transforming potential of Tpr-Met mutants correlates with their ability to induce tyrosine phosphorylation of Gab1 (Bardelli *et al.,* 1997; Fixman *et al.,* 1997). Taken together, these findings convincingly demonstrate the involvement of Gab1 in Met-induced morphogenesis (Weidner *et al.,* 1996; Nguyen *et al.,* 1997; Niemann *et al.,* 1998; Maroun *et al.,* 1999; Tulasne *et al.,* 1999) and suggest a role for Gab1 in transformation by Tpr-Met.

One of the first molecules that was shown to become associated with Met upon HGF stimulation was PI3K (Graziani *et al.,* 1991; Ponzetto *et al.,* 1993). This interaction of PI3K with Met may enhance PI3K activity and/or localize PI3K in the proximity of its substrate (Ponzetto *et al.,* 1993). PI3K is composed of a p85 adapter subunit, which contains the Met interacting SH2 domain, and a p110 catalytic subunit. PI3K is able to phosphorylate PIP2 in order to produce PIP3. PIP3 in its turn can bind to the PH domain of target proteins, resulting in their translocation, membrane localization, and, indirectly, in their activation. Among the PH-domain-containing effector molecules of PI3K is the kinase Akt/PKB (Burgering and Coffer, 1995), which, upon membrane localization, is phosphorylated and activated by PDK1 (Stokoe *et al.,* 1997; Stephens *et al.,* 1998) (Fig. 5). Downstream effector molecules for the PI3K-regulated kinase PKB include the Bcl-2 family member Bad, which can exert pro-apoptotic activity by interacting with Bcl-2 (Datta *et al.,* 1997; del Peso *et al.,* 1997); glycogen synthase kinase 3 (GSK3), involved in regulation of glycogen synthesis and, as discussed below, in phosphorylation of β-catenin (Cross *et al.,* 1995); p70S6K, involved in regulation of protein synthesis and gene expression (Proud, 1996); and the forkhead transcription factor AFX (Kops *et al.,* 1999). The function of these effector molecules in Met signal transduction has not yet been investigated.

Besides the ability of PI3K to interact directly with Met, two additional mechanisms may account for Met-induced PI3K activation (Fig. 5). First, the p85 subunit of PI3K was also found to associate with Gab1 (Holgado-Madruga *et al.,* 1996), and, at least in cells overexpressing both Met and Gab1, more PI3K activity is associated with Gab1 than with Met (Maroun *et al.,* 1999). Interestingly, besides being able to associate with PI3K, Gab1 requires PI3K activity and an intact PH domain for proper localization and induction of morphogenesis (Maroun *et al.,* 1999). Secondly, PI3K has been identified as an effector molecule for Ras, because Ras has the ability to directly interact with the p110 catalytic subunit of PI3K (Rodriguez-Viciana *et al.,* 1994). To what extent these three different mechanisms contribute to HGF-induced PI3K activation remains to be established. However, Ras-mediated PI3K activation has been implicated in HGF-induced adherens junction disassembly in MDCK cells (Potempa and Ridley, 1998).

By means of either specific pharmacologic inhibitors such as Wortmannin and LY294002, or by expression of dominant-negative or constitutively active mutants of PI3K, its function in Met signaling has been extensively studied. These studies revealed a prominent regulatory role for PI3K in Met-induced mitogenesis, motility, and morphogenesis. (Royal and Park, 1995; Rahimi *et al.,* 1996; Royal *et al.,* 1997; Potempa and Ridley, 1998; Khwaja *et al.,* 1998). Activation of PI3K has been reported to be required and sufficient for tubulogenesis and required for scattering (Royal and Park, 1995; Khwaja *et al.,* 1998; Potempa and Ridley, 1998). Interestingly, however, mutation of the multisubstrate docking site of Met, which results in the loss of PI3K and Gab1 association with Met upon HGF stimulation, does not abrogate HGF-induced scattering or Ras activation (Tulasne *et al.,* 1999). This indicates that Ras-mediated, rather than direct Met-induced or Gab1-mediated, activation of PI3K is required for HGF-induced scattering.

Whether PI3K activation alone is also sufficient for HGF-induced scattering of MDCK cells is still a matter of debate. On the one hand, Potempa and Ridley (1998) reported that neither expression of an active mutant of PI3K, nor the combined expression of PI3K with active Raf or MEK, was sufficient for adherens junction disassembly, a prerequisite for scattering. On the other hand, Khwaja *et al.* (1998) reported that expression of an active mutant of PI3K is sufficient to induce scattering, provided a basal level of MAP kinase activity is present, however, expression of active Rac or PKB is not sufficient to induce scattering. Based on these observations, both studies suggested the requirement of an additional (novel) motogenic pathway for HGF-induced scattering, either downstream of Ras, other than PI3K or Raf (Potempa and Ridley, 1998), or downstream of PI3K, other than PKB or Rac (Khwaja *et al.,* 1998). Noteworthy, a recent study also suggested the existence of an additional mitogenic signaling pathway, because NK2, the truncated HGF isoform described in Section II,A, despite its ability to induce both PI3K and MAP kinase activation, as well as a motogenic response, is unable to induce a mitogenic response in breast epithelial cells (Day *et al.,* 1999). In agreement with the data from Khwaja *et al.* (1998), it has been reported that expression of active PI3K disrupts the polarized tubular growth of well-differentiated mammary epithelial cells, resulting in enhanced motility and invasion (Keely *et al.,* 1997). Taken together, PI3K activation is required, and may also be sufficient, for HGF-induced scattering.

PI3K has also been implicated in the responses elicited by Tpr-Met. A mutant of Tpr-Met, which preferentially binds PI3K over Grb2, although still able to elicit cell motility, is unable to induce transformation, invasion, and metastasis (Bardelli *et al.,* 1999). However, a mutant of Tpr-Met that selectively binds Grb2 only, is also impaired in its ability to induce invasion and metastasis (Giordano *et al.,* 1997). This could, however, be overcome by expression of constitutively active PI3K (Bardelli *et al.,* 1999). Thus, these data

indicate that simultaneous activation of the Ras and PI3K pathway is required and sufficient for full invasive and metastatic activity of Tpr-Met. In conclusion, the PI3K pathway is an important regulatory pathway in HGF/Met-induced mitogenesis, motogenesis, and morphogenesis, as well as in Tpr-Met-induced motility, invasion, and metastasis.

4. ROLE OF SIGNAL TRANSDUCTION VIA Rho-FAMILY GTPases AND β-CATENIN

Members of the Rho subfamily of Ras-related GTPases, as well as Ras itself, have been implicated in HGF-induced cytoskeletal reorganization, cell scattering, and tubulogenesis. Initially, mainly based on studies in fibroblasts, cdc42, Rac, and Rho were shown to regulate the formation of filopodia, lamellipodia, and stress fibers, respectively (Hall, 1998; van Aelst and D'Souza-Schorey, 1997). Evidence was presented indicating that cdc42 may function upstream from Rac, which in turn may function upstream from Rho (Zigmond, 1996). Furthermore, it was shown that Ras can induce PI3K-dependent activation of Rac (Rodriguez-Viciana *et al.,* 1997), which may be mediated by the PH domain-containing exchange factor for Rac, Tiam (Michiels *et al.,* 1995). More recently, these Rho-family GTPases were shown to be involved in the regulation of a wide variety of cellular functions, including regulation of membrane trafficking, transcriptional activation, and cell growth control (Hall, 1998; van Aelst and D'Souza-Schorey, 1997). Several effector molecules for Rho-family GTPases have been identified, including the Rho effectors ROK and ROCK, involved in stress fiber formation, and the Rac effector PAK, involved in JNK activation and cytoskeletal organization (van Aelst and D'Souza-Schorey, 1997).

With respect to HGF/Met signaling, it has been reported that the activation of Rho is required for HGF-induced membrane ruffling and cell motility in keratinocytes (Takaishi *et al.,* 1994; Nishiyama *et al.,* 1994). Furthermore, HGF-induced actin reorganization, membrane ruffling, spreading, and scattering of MDCK cells was reported to require activation of Ras and Rac (Hartmann *et al.,* 1994, Ridley *et al.,* 1995, Potempa and Ridley, 1998), but not of Rho (Ridley *et al.,* 1995). Interestingly, recent studies revealed that Rac and Rho are involved in intercellular E-cadherin-mediated adhesions in epithelial cells. In MDCK cells and keratinocytes, the basal cadherin-mediated cell–cell adhesion was inhibited by the dominant-negative mutant N17-Rac and by inhibition of Rho (Braga *et al.,* 1997; Takaishi *et al.,* 1997). Furthermore, expression of constitutively active V12-Rac enhanced cadherin-mediated cell–cell adhesion in MDCK cells (Takaishi *et al.,* 1997).

In agreement with the stimulatory effect of V12-Rac on cell–cell adhesion, overexpression of either V12-Rac or Tiam1 inhibits HGF-induced scattering of MDCK cells (Hordijk *et al.,* 1997). Furthermore, V12-Rac and Tiam1

suppressed the scattered appearance and invasion of fibroblast-like Ras-transformed MDCKf3 cells. This was shown to be due to restoration of E-cadherin-mediated cell–cell adhesion, as a consequence of enhanced levels of β-catenin and E-cadherin at intercellular junctions (Hordijk *et al.,* 1997) (Fig. 5). In contrast, however, in well-differentiated mammary epithelial cells, expression of active mutants of Rac, cdc42, or PI3K disrupts their polarized tubular growth and instead promotes their motility and invasion (Keely *et al.,* 1997). Most likely, these apparent contradictory results reflect the delicate balance between the Rac-dependent regulation of cell–cell adhesion, cell–matrix adhesion, and matrix-dependent cell migration (Sander *et al.,*1998). Finally, expression of N17-Rac in MDCK cells prevents HGF-induced and Ras-mediated dispersal of β-catenin and E-cadherin, adherens junction disassembly, and, as mentioned before, scattering (Potempa and Ridley, 1998). Because tyrosine phosphorylation of β-catenin has been implicated in the dissociation of the cadherin/β-catenin complex from the actin cytoskeleton, it is noteworthy that, although this was not observed in MDCK cells (Potempa and Ridley, 1998), β-catenin as well as plakoglobin (γ-catenin) become phosphorylated on tyrosine residues upon HGF stimulation of HT29 colon adenocarcinoma cells (Shibamoto *et al.,* 1994). In conclusion, Rac, Rho, and β-catenin play an important regulatory role in cell–cell adhesion and HGF-induced cytoskeletal organization and motogenesis.

Besides its involvement in cell–cell adhesion, β-catenin has also been implicated in the regulation of gene transcription. β-Catenin can form mutually exclusive complexes with either cadherins or with APC, the tumor suppressor gene product that is mutated in colon carcinoma (Rubinfeld *et al.,* 1993). GSK3, which can be phosphorylated and inactivated by the PI3K-dependent PKB, can in turn phosphorylate APC and β-catenin on serine residues (Rubinfeld *et al.,* 1996; Cross *et al.,* 1995) (Fig. 5). As a consequence of this phosphorylation, β-catenin is degraded (Morin *et al.,*1997). However, upon phosphorylation and thus inhibition of GSK3, free β-catenin will accumulate, translocate to the nucleus, and interact with the transcriptional regulator T-cell factor/lymphocyte enhancer-binding factor (TCF/LEF-1), thereby inducing expression of TCF/LEF-1 target genes (Behrens *et al.,* 1996; Molenaar *et al.,* 1996) (Fig. 5). Interestingly, HGF stimulation of mouse mammary cells was reported to result in a decrease in GSK3 activity, the nuclear accumulation of β-catenin, and the activation of TCF/LEF-1 (Papkoff and Aikawa, 1998). Thus, via direct or indirect phosphorylation of β-catenin on either tyrosine or serine residues, Met appears to be able to regulate cell–cell adhesion as well as gene expression.

Expression of N17-Rac has also been shown to inhibit both JNK activation and transformation by Tpr-Met (Rodrigues *et al.,* 1997). Based on this observation it was suggested that activation of the JNK pathway, which is mediated by the sequential activation of PI3K and Rac (Coso *et al.,* 1995;

Minden *et al.,* 1995) (Fig. 5), is essential for transformation by the Tpr-Met oncoprotein (Rodrigues *et al.,* 1997). JNK in turn is able to phosphorylate a number of transcription factors, including the immediate early gene *c-jun.* Noteworthy, both GTPases Rac and Rho have also been implicated in regulation of the transcriptional activity of the serum response factor (SRF) (Hill *et al.,* 1995). In conclusion, Rac, Rho, and β-catenin play an important regulatory role in HGF/Met-induced mitogenesis and motogenesis, whereas Rac is also involved in Tpr-Met-induced transformation.

5. ROLE OF ADDITIONAL SIGNALING MOLECULES

An additional Met associating protein is PLCγ (Ponzetto *et al.,* 1994). PLCγ mediates the production of IP3, which results in enhanced calcium release from intracellular stores, and diacylglycerol, which activates PKC. Indeed, PKC has been implicated in Met signaling in a variety of cell types (Santos *et al.,* 1993; Adachi *et al.,* 1996; Dunsmore *et al.,* 1996; Laping *et al.,* 1998; Machide *et al.,* 1998). Furthermore, both PKC and calcium have also been implicated in the negative regulation of Met signaling, by phosphorylation of residue S985 of Met, resulting in decreased kinase activity (Gandino *et al.,* 1990, 1991, 1994). Using a pharmacologic inhibitor, PLCγ has been implicated in the chemotactic response elicited by stimulation of a chimeric PDGF-Met receptor molecule expressed in renal epithelial cells (Derman *et al.,* 1996).

HGF also activates STAT3 (Schaper *et al.,* 1997), and stimulates recruitment of STAT3 to the autophosphorylated Y1356 of Met (Boccaccio *et al.,* 1998). Upon phosphorylation, the STAT proteins can dimerize and translocate to the nucleus, where they act as transcription factors controlling the promoter activity of target genes. Inhibition of STAT-mediated transcription prevents HGF-induced tubulogenesis, whereas scattering and proliferation were unaffected (Boccaccio *et al.,* 1998).

Furthermore, the Src tyrosine kinase was shown to directly associate with Met (Ponzetto *et al.,* 1994). This association with Met and activation of Src was shown to play a critical role in carcinoma cell motility (Rahimi *et al.,* 1998), and in HGF-induced phosphorylation of FAK (Chen *et al.,* 1998). With respect to another Met-associating protein, the tyrosine phosphatase SHP2 (Nguyen *et al.,* 1997), no functional data are available yet. Recent data indicate that SHP2 can also be indirectly recruited to Met via Gab1 (Maroun *et al.,* 1999).

In addition, BAG-1, a cell death suppressor gene product that binds the anti-apoptotic protooncogene product Bcl-2 in a cooperative fashion (Takayama *et al.,* 1995), interacts with Met. This interaction was independent of phosphorylation of either Y1349 or Y1356 of Met (Bardelli *et al.,* 1996) and may very well be mediated by the molecular chaperone Hsp70 (Takayama

et al., 1997c). Overexpression of BAG-1 enhances the anti-apoptotic effect of HGF on liver progenitor cells (Bardelli *et al.,* 1996).

Finally, it is noteworthy that ezrin, a member of the ERM protein family involved in membrane–cytoskeleton interactions, is a substrate for Met *in vitro* and also becomes phosphorylated on tyrosine residues *in vivo*. Both a truncated and a tyrosine mutated variant of ezrin impair the motogenic and morphogenic response of epithelial kidney cells to HGF (Crepaldi *et al.,* 1997). Furthermore, as discussed in more detail in the next section, the observations that the HSPG CD44 can directly interact with ezrin (Tsukita *et al.,* 1994), and can bind and present HGF to Met (van der Voort *et al.,* 1999; Taher *et al.,* 1999), adds an extra dimension to the role of ezrin in Met signaling.

D. Heparan Sulfate Proteoglycans and HGF/Met Function

Besides Met, HSPGs have been identified as a second class of HGF-binding sites on various cell types. These binding sites have a lower affinity than the Met receptor, but they are considerably more numerous (10- to 1000-fold) (Higuchi and Nakamura, 1991; Tajima *et al.,* 1992).

HSPGs are proteins that carry one or more covalently linked heparan sulfate chains. They are widespread throughout mammalian tissues both as cell surface molecules, (e.g., the syndecans, glypicans, and CD44-HS) and as ECM components, (e.g., perlecan). HSPGs have been implicated in several important biologic processes including cell adhesion and migration, angiogenesis, tissue morphogenesis, and regulation of blood coagulation. In these processes, they are believed to function as scaffold structures, designed to accommodate proteins through noncovalent binding to their heparan sulfate (HS) chains (reviewed in Schlessinger *et al.,* 1995; Lindahl *et al.,* 1998). The ligand-binding sites reside within discrete sulfated domains formed by complex, cell-specific modifications to the HS disaccharide repeat. Binding of proteins, including many growth factors and cytokines, e.g., FGFs, VEGF, HB-EGF, IL-3, IL-7, GM-CSF, and certain chemokines, to HS chains may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of their biologic function. This functional importance is illustrated by fibroblast growth factor 2 (FGF-2), whose binding to its signal-transducing receptor and consequent biologic effects is critically dependent on its interaction with cell-surface HSPGs (Rapraeger *et al.,* 1991; Yayon *et al.,* 1991; Schlessinger *et al.,* 1995). Furthermore, a number of cell biologic and genetic studies have recently provided compelling evidence for an *in vivo* role of cell-surface HSPGs in growth control and morphogenesis in *Drosophila,* mice, and humans (reviewed by Selleck, 1998).

The modular structure of HGF has facilitated the identification of the domains responsible for binding to Met and heparin/HS. By using deletion mutants of *HGF* and examining their binding ability to immobilized heparin, Mizuno *et al.* (1994) identified the hairpin loop of the amino-terminal domain and the second kringle domain as sites essential for heparin binding. The same domains are also critical for Met binding and signaling (Matsumoto *et al.,* 1991; Hartmann *et al.,* 1992; Lokker *et al.,* 1992; Okigaki *et al.,* 1992). To dissect the binding sites for Met and heparin/HS in HGF, the groups of Gherardi and Blundell generated three-dimensional models of the individual HGF domains to help to design specific mutants (see Donate *et al.,* 1994). Based on the X-ray structures of antithrombin– (Carrell *et al.,* 1994) and FGF–heparin complexes (Faham *et al.,* 1996), they predicted the heparin-binding sites to contain clusters of positively charged residues that make electrostatic contact with negatively charged groups in HS chains. Indeed, three such clusters were identified on the surface of HGF, two in the hairpin loop and one in the kringle 2 domain (Donate *et al.,* 1994). By introducing specific mutations at these sites, it was confirmed that these residues play a key role in heparin binding (Hartmann *et al.,* 1998). A study by Chirgadze *et al.* (1999) has recently reported the crystal structure of NK1, a natural splice variant of HGF with agonistic activity, consisting of the N and first kringle domains (see Section II,A). It was shown that NK1 assembles as an asymmetric homodimer in which the N domain of one partner interacts with the kringle domain of the other. Short heparin fragments (14-mer) effectively dimerized NK1 in solution, suggesting that heparan sulfate chains expressed on cells or in the ECM may stabilize the NK1 dimers *in vivo*.

Although HS chains are composed of a linear array of disaccharide units consisting of alternating hexuronic acid [L-iduronic acid (IdoA) or D-glucuronic acid) and D-glucosamine, there is evidence that they are capable of highly specific protein binding. Variations in O-sulfation pattern, hexuronate composition, and length of the sulfated segments determine this specificity (Lindahl *et al.*, 1998). The first specific binding domain identified was a pentasaccharide that binds with high affinity to antithrombin III (AT III), a serine protease inhibitor. This sequence induces a conformational change in AT III and accelerates its binding to Factor X_a and thrombin, and in this way promotes the anticoagulant action of AT III (Lindahl *et al.,* 1984). Lyon *et al.* (1994) and Ashikari *et al.* (1995) have analyzed the structural basis of the interaction between HGF and HSPGs. Both studies indicate that high-affinity HGF binding requires oligosaccharides with a minimum length of 8–12 units containing 6-*O*-sulfated $GlcNSO_3$ residues, which may be flanked by IdoA(SO_3) units (Ashikari *et al.,* 1995). Interestingly, this structural specificity for binding to HS differs radically from that of FGF-2 (Maccarana *et al.,* 1993), illustrating the importance of structural diversity of the HS chain in selective growth factor binding.

Whereas HSPGs have been shown to be crucial for FGF interaction with its receptor, and thus for FGF functioning, their role in HGF/Met interaction is, as yet, less well defined. Binding to heparin/HS does not appear to affect the affinity of full-length HGF for the Met receptor, but it increases receptor phosphorylation and mitogenicity on rat hepatocytes (Zioncheck *et al.,* 1995; Schwall *et al.,* 1996). In contrast, Met binding and mitogenicity of NK1 has been reported to require HSPGs (Schwall *et al.,* 1996; Sakata *et al.,* 1997). Recently, we have shown that HGF binds to a HSPG splice variant of CD44 (CD44-HS) expressed on B cells (van der Voort *et al.,* 1999). This binding strongly promotes the HGF-induced tyrosine phosphorylation of Met as well as phosphorylation of several substrates (Fig. 6, see color plate) (see also Section III,C). Taken together, these *in vitro* studies indicate that HSPGs may play an important regulatory role in HGF/Met signaling.

Interaction of HGF with HSPG could modulate Met signaling via several mechanisms (Fig. 6). First, as already mentioned, HSPGs may promote dimerization of HGF, thereby promoting receptor cross-linking and tyrosine kinase activity (Chirgadze *et al.,* 1999). Second, by inducing a conformational change, HSPGs may influence the affinity of HGF for Met, as has been demonstrated for the NK1 splice variant (Sakata *et al.,* 1997). Third, HGF may mediate colocalization of HSPGs and Met, which may bring relevant intracellular signaling molecules in proximity with each other. For example, we have shown that the cytoplasmic tail of CD44 interacts physically and functionally with Src-family protein tyrosine kinases (Taher *et al.,* 1996), which have also been implicated in Met signaling (Ponzetto *et al.,* 1994). The above-mentioned processes may involve the formation of a ternary complex between HGF, Met, and a HSPG. In the case of ternary complex formation between CD44-HS, HGF, and Met, Src kinases associated with the cytoplasmic tail of CD44 might be recruited into the complex (Taher *et al.,* 1999). This may facilitate their activation by Met. Similarly, the ERM family member ezrin, which is also associated with CD44 and a downstream target for Met, may also be assembled into the complex. Ezrin acts as a linker between the intracellular domain of CD44 and the actin-based cytoskeleton (Tsukita *et al.,* 1994) and has been shown to be involved in HGF-induced cell migration (Crepaldi *et al.,* 1997). By recruiting ezrin, CD44-HS might thus contribute to the regulation of cell adhesion and migration.

The preceding data suggest an important physiologic role for HSPG in the regulation of HGF function. To directly address this issue, Hartmann *et al.* (1998) performed *in vivo* studies comparing wild-type HGF with HGF mutants with a strongly (50-fold) reduced affinity for heparin. Mutant HGF showed a delayed clearance from the blood and, interestingly, induced a higher DNA synthesis in normal mouse liver. Based on these findings, the authors suggest a role for HSPGs in promoting the internalization and degradation of HGF *in vivo*. Although no further *in vivo* data are as yet available,

it is tempting to speculate on other physiologic roles for HSPGs. HSPGs may help to localize HGF to specific cells or ECM components within the microenvironment and may be required for the establishment of a chemotactic gradient. Examples are the migration of myogenic precursor cells to the limb bud during embryogenesis, which is critically dependent on HGF and Met and, possibly, the migration of B cells within the germinal center. Furthermore, membrane and matrix HSPG may also protect HGF from proteolytic degradation (Säkselä *et al.,* 1988). Further studies are needed to explore these possible roles of HSPG.

E. HGF/Met Pathway in Tumor Growth, Invasion, and Metastasis

In Section II,A,2, we briefly discussed the oncogenic potential of the Tpr-Met chimera. In this chimera, the intracellular domain of Met is fused to Tpr, resulting in a constitutively active homodimer with transforming capacity (Cooper *et al.,* 1984; Park *et al.,* 1986; Gonzatti-Haces *et al.,* 1988). A vast body of clinical and experimental data show that, apart from Tpr-Met, the Met protooncogene and HGF also play a pivotal role in tumorigenesis. For instance, overexpression and high levels of autophosphorylation of Met have been found in human tumor cell lines (Fig. 7, see color plate) (Tempest *et al.,* 1988; Giordano *et al.,* 1989a; Ponzetto *et al.,* 1991; Kuniyasu *et al.,* 1992). Often this overexpression is caused by gene amplification (Giordano *et al.,* 1989a; Ponzetto *et al.,* 1991; Kuniyasu *et al.,* 1992). Interestingly, however, transfection of tumor cells with activated *ras* and *ret* oncogenes also causes Met overexpression and enhances HGF-dependent invasion (Ivan *et al.,* 1997; Webb *et al.,* 1998). Furthermore, Met overexpression can be induced by HGF itself, as well as by a number of other cytokines, including EGF, IL-1, and IL-6 (Chen *et al.,* 1997). These data indicate that both oncogenes and cytokines present in the tumor microenvironment can induce Met expression and thereby promote tumorigenesis.

In addition to Met overexpression, overproduction of HGF can also occur in tumors. For instance, it was demonstrated that tumor cells can release factors, for example, IL-1, FGF-2, or PDGF, that stimulate neighboring fibroblasts to secrete HGF (Fig. 7) (Rosen *et al.,* 1994a,b; Nakamura *et al.,* 1997). Alternatively, some tumor cell lines were shown to express both HGF and Met, suggesting the presence of an autocrine loop (Fig. 7) (Naidu *et al.,* 1994; Moriyama *et al.,* 1995; Tuck *et al.,* 1996; Trusolino *et al.,* 1998). Aberrant expression and activation of the HGF/Met pathway are not only present in tumor cell lines, but also in many native human tumors. Overexpression of Met was detected in carcinomas of the stomach, liver, colon, pancreas, lung, and thyroid gland (Di Renzo *et al.,* 1991, 1992, 1995a,b; Prat *et al.,* 1991;

Kuniyasu *et al.,* 1992; Liu *et al.,* 1992; Boix *et al.,* 1994; Ueki *et al.,* 1997). For colorectal, liver, thyroid, and brain cancer, it was shown to be correlated with disease progression (Di Renzo *et al.,* 1995a; Belfiore *et al.,* 1997; Koochekpour *et al.,* 1997; Ueki *et al.,* 1997). Similarly, overexpression of HGF was also reported in human cancer, for example, in tumors of the pancreas and in gliomas (Furukawa *et al.,* 1995; Koochekpour *et al.,* 1997; Lamszus *et al.,* 1998). Importantly, in patients with breast or non-small-cell lung cancer, expression of HGF was shown to be a strong and independent predictor of recurrence and tumor-related death (Yamashita *et al.,* 1994; Siegfried *et al.,* 1997).

Weidner and colleagues (1990) demonstrated that HGF induces invasion of carcinoma cell lines into collagen gels. Similar effects of HGF were subsequently reported for many other tumor cell lines, including mammary, colon and squamous cell carcinoma, and melanoma lines (Jiang *et al.,* 1993; Matsumoto *et al.,* 1994; Rosen *et al.,* 1994b; Hendrix *et al.,* 1998). In accordance with these findings, Date *et al.* (1998) showed that a four-kringle-containing HGF antagonist (NK4) inhibits HGF-induced tumor invasion. Interestingly, Jiang *et al.* (1993) reported that, while colon carcinoma cells became more motile in the presence of HGF, their growth was inhibited. Similar observations were made by Giordano *et al.* (1993) in NIH 3T3 fibroblasts. A recent report suggests that HGF-induced growth suppression can be caused by the induction of oxidative stress (Arakaki *et al.,* 1999).

At least two mechanisms may be involved in the promotion of invasiveness by the HGF/Met pathway. First, HGF may induce invasion and metastasis by causing cytoskeletal rearrangement and by activating adhesion molecules (Fig. 7). For example, HGF was shown to induce tyrosine phosphorylation of molecules involved in the assembly of focal adhesions, resulting in cell spreading and migration (Matsumoto *et al.,* 1994) (see also Sections II,B and C). In addition, we and others have shown that HGF induces activation of integrins and consequent adhesion and migration (van der Voort *et al.,* 1997; Weimar *et al.,*1997; Trusolino *et al.,* 1998). Secondly, activation of Met may lead to an enhanced degradation of the ECM by invasive cells (Fig. 7). Met activation by HGF increases the expression of urokinase-type plasminogen (uPA) and its receptor (Pepper *et al.,* 1992; Rosen *et al.,* 1994b; Jeffers *et al.,* 1996b), molecules known to play a role in ECM proteolysis.

Studies using *in vivo* models confirm the involvement of HGF and Met in tumorigenesis. Autocrine stimulation of Met transfected NIH 3T3 cells with HGF enhanced the tumorigenic and metastatic capacity of these cells in nude mice (Rong *et al.,* 1992, 1994). Similar findings were reported for SK-LMS-1 human leiomyosarcoma cells and for mouse mammary tumor cells (Rosen *et al.,* 1994b; Jeffers *et al.,* 1996b; Lamszus *et al.,* 1997). In the leiomyosarcoma model, HGF/Met signaling was shown to increase the expression of

both uPA and its receptor uPAR, suggesting a role for this proteolysis network in promoting invasiveness and metastasis. In the mouse mammary tumor model as well as in a glioma model, stimulation of angiogenesis by HGF appeared to play a key role (Fig. 7) (Lamszus *et al.,* 1997; Laterra *et al.,* 1997).

Studies in transgenic mice corroborate the role of HGF and Met in tumorigenesis. HGF transgenic mice were shown to develop a broad array of primary tumors and metastases of mesenchymal as well as epithelial origin, including malignant melanoma, fibrosarcoma, and mammary carcinoma (Takayama *et al.,* 1997b; Otsuka *et al.,* 1998). Many of these tumors arose from abnormally developed tissues, suggesting a functional link between HGF-dependent morphogenesis and tumorigenesis. Because most neoplasms, melanomas in particular, demonstrated overexpression and enhanced activation of Met, autocrine signaling via Met was thought to be a major cause of tumorigenesis.

Recently, Met has been implicated in the genesis of hereditary papillary renal carcinomas (HPRC). Schmidt *et al.* (1997) showed that missense mutations in the *Met* gene are present in the germline of affected members of HPRC families. Remarkably, affected individuals often have a duplication of the chromosome bearing the mutated *Met* allele (Fischer *et al.,* 1998; Zhuang *et al.,* 1998). Similar mutations were also found in a subset of sporadic papillary renal carcinomas and of childhood hepatocellular carcinomas (Zhuang *et al.,* 1998; Park *et al.,* 1999). Importantly, NIH 3T3 fibroblasts transfected with Met constructs containing the mutations detected in HPRC are transforming *in vitro* and tumorigenic *in vivo* (Jeffers *et al.,* 1997). Taken together, these data strongly suggest that the Met mutants expressed in HPRC initiate tumorigenesis.

III. HGF/MET IN B-CELL DEVELOPMENT AND NEOPLASIA

A. HGF/Met in Antigen-Specific B-Cell Differentiation

Interestingly, recent studies from our laboratory have provided evidence for a role of the HGF/Met pathway in the immune system, that is, in the regulation of antigen-specific B-cell differentiation (van der Voort *et al.,* 1997, 1999; Pals *et al.,* 1998; Taher *et al.,* 1999). During this process, naive B cells develop into memory cells or plasma cells. This requires multiple interactions of B cells with other cells, such as T cells and follicular dendritic cells (FDC), and with the ECM, which take place within distinct microenvironmental compartments of the lymphoid tissues (Fig. 8, see color plate) (MacLennan,

1994; Nossal, 1994; Thorbecke *et al.,* 1994; Liu *et al.,* 1996a; Rajewsky, 1996; Lindhout *et al.,* 1997). After their initial activation in the extrafollicular T-cell (paracortical) area, germinal center (GC) founder cells migrate into B-cell follicles where they initiate the formation of GCs (Liu *et al.,* 1991; Jacob *et al.,* 1991a). Once in the GC, the B cells first pass the dark zone where they undergo rapid clonal expansion and somatic hypermutation in their IgV genes (Berek *et al.,* 1991; Jacob *et al.,* 1991b; Küppers *et al.,* 1993; McHeyzer-Williams *et al.,* 1993; Pascual *et al.,* 1994). Mutated B cells then progress to centrocytes and move to the basal light zone of the GC. Here they reencounter antigen, presented as low levels of immune complexes on FDC, and undergo affinity selection (Tew *et al.,* 1990; Hardie *et al.,* 1993; MacLennan, 1994). Whereas low affinity mutants and autoreactive mutants die by apoptosis, high-affinity mutants internalize antigen and process it on their migration pathway to the apical light and outer zones of the GC. In these areas, the affinity selected B cells present antigen to antigen-specific GC T cells (Fuller *et al.,* 1993; Casamayor-Palleja *et al.,* 1995; Zheng *et al.,* 1996). Cognate T–B interaction results in expansion and Ig-isotype switching of high-affinity B cells (Kraal *et al.,* 1982; Liu *et al.,* 1996b) that mature into memory B cells or plasma cells and receive signals mediating their export from the lymphoid organ (MacLennan, 1994).

We observed that stimulation of human tonsillar B cells by phorbol ester and, more importantly, by concurrent CD40 and B-cell receptor (BCR) ligation, leads to a rapid transient Met induction (van der Voort *et al.,* 1997). Presumably, BCR- and CD40-mediated signals are also instrumental in the physiologic induction of Met, because Met is expressed *in vivo* on tonsillar centroblasts ($CD38^+CD77^+$), which are the offspring of B cells that have recently been activated at extrafollicular sites by antigen plus T-cells signal (MacLennan, 1994). These activating signals critically involve CD40/CD40L interactions: Patients with the X-linked hyper-IgM syndrome (due to mutated and consequently defective CD40L) do not develop GC, and blocking of the CD40/CD40L pathway in mice leads to complete inhibition of GC reactions (Banchereau *et al.,* 1994; Foy *et al.,* 1994; Han *et al.,* 1995; Kawabe *et al.,* 1994; Facchetti *et al.,* 1995). Our findings thus link Met induction to the initiation of the B-cell immune response.

Adhesion regulation, particularly regulation of lymphocyte integrin function, is believed to be fundamental to the control of cell migration and microenvironmental homing during B-cell differentiation (Koopman and Pals, 1992; Butcher and Picker, 1996). In functional studies, we observed that HGF augments adhesion of Met positive B-cell lines to VCAM-1 and fibronectin by activating the integrin $\alpha 4\beta 1$ (van der Voort *et al.,* 1997). Similar effects of HGF on integrin-mediated adhesion of B cells to fibronectin were also reported by Weimar *et al.* (1997). The physiologic relevance of these findings is strongly supported by our observation that HGF is produced

by stromal cells and FDC (van der Voort *et al.,* 1997; and unpublished observations). During B-cell differentiation the integrin α4β1 mediates B-cell adhesion to FDC (Freedman *et al.,* 1990; Koopman *et al.,* 1991, 1994), an interaction that regulates the formation of the microenvironment required for the affinity selection of GC B cells. Apart from establishing physical contact between B cells and FDC, α4βb1 presumably contributes directly to the B-cell selection process itself, because signaling through the α4β1/VCAM-1 pathway costimulates rescue of GC B cells from apoptosis (Koopman *et al.,* 1994, 1997). Furthermore, α4β1 also regulates cell adhesion to fibronectin (Wayner *et al.,* 1989), an important substrate for cell migration. Interestingly, Weimar *et al.* (1997) reported that HGF indeed stimulates B-cell migration on fibronectin.

In view of the pleiotropic effects of HGF on many cell types, it is possible that HGF may have other, as yet unknown, roles in antigen-specific B-cell differentiation in addition to adhesion regulation. For example, as discussed in Section III,B, Met signaling might promote B-cell proliferation and survival.

Preliminary results from *in vivo* studies support the involvement of the HGF/Met pathway in antigen-specific B-cell differentiation. We explored this role by a molecular genetic approach using *Met* knockout mice. Because homozygous *Met* knockout mice die *in utero* at around day E15.5 (Bladt *et al.,* 1995), the immune function of these mice cannot be studied directly. To circumvent this problem, we reconstituted *RAG-2*$^{-/-}$IL-2gR$^{-/-}$ mice with fetal liver cells from *MET*$^{-/-}$ mice or control littermates. After intravenous injection, the hematopoietic stem cells present in the fetal liver migrate to the bone marrow and regenerate B- and T-cell populations. Thus far, analysis of the B- and T-cell compartments, of the organization of the lymphoid organs, and of the baseline levels of immunoglobulin demonstrated no significant difference between *MET*$^{-/-}$ and control mice. Interestingly, however, the immune response against the T-cell-dependent antigen TNP-KLH was reduced in the *MET*$^{-/-}$ mice. Furthermore, in the spleens of immunized *MET*$^{-/-}$ mice, we observed a reduction in the number of plasma cells, the Ig-secreting population of B cells.

B. Met Signaling in B Cells

Although most data on Met signaling have been obtained in epithelial cells and, hence, do not necessarily apply to B cells, we have recently been able to demonstrate HGF/Met-induced phosphorylation and/or activation in B cells of at least several key signaling molecules such as Ras, MAP kinase, PI3K, PKB, and Gab1 (Fig. 5) (our unpublished observations). Here we outline the putative roles of these molecules in B-cell development.

As discussed earlier, the HGF/Met pathway is implicated in integrin regulation in B cells (van der Voort *et al.,* 1997; Weimar *et al.,* 1997). Several different signaling pathways have been implicated in inside-out signaling to integrins: Key regulatory proteins in these pathways appear to be PI3K and different Ras-like GTPases (Howe *et al.,* 1998; Kolanus and Seed, 1997; Hughes and Pfaff, 1998). PI3K is involved in the activation of integrins in leukocytes (Shimizu *et al.,* 1995), T cells (Zell *et al.,* 1998), and platelets (Zhang *et al.,* 1996a), whereas activated R-ras increases integrin activity in myeloid cells (Zhang *et al.,* 1996b) and epithelial cells (Keely *et al.,* 1999). Because R-ras is able to bind the same effector molecules as Ras, including PI3K (Spaargaren *et al.,* 1994; Spaargaren and Bischoff, 1994; Marte *et al.,* 1997), integrin activation by R-ras may involve the activation of PI3K.

In contrast to the stimulatory effect of R-ras, activated H-ras and Raf were found to inhibit activity of platelet integrins expressed in CHO cells (Hughes *et al.,* 1997). Interestingly, R-ras appears to function as an antagonist of Ras-suppressed integrin activity in this cell system (Sethi *et al.,* 1999). However, unlike its inhibitory effects on the activation of platelet integrins, active Ras promotes TCR-triggered integrin-mediated T-cell adhesion to ICAM-1 (O'Rourke *et al.,* 1998). These data imply that, dependent on the specific cell system studied, Ras may either inhibit or promote integrin activity. In addition to Ras and R-ras, the GTPase Rho has also been implicated in integrin activation, specifically in chemoattractant-induced integrin activation in lymphocytes and neutrophils (Laudanna *et al.,* 1996). Because HGF stimulation of Met in B cells results in activation of PI3K as well as the Ras-MAP kinase pathway (van der Voort *et al.,* 1999; our unpublished observations), and Rho has been implicated in Met signaling in epithelial cells (Takaishi *et al.,* 1994; Nishiyama *et al.,* 1994), it is likely that these proteins also play an important role in the regulation of integrin activity by HGF in B cells.

Recent studies have revealed several points of convergence between B-cell antigen receptor (BCR) and HGF/Met signaling. First, the prominent Met substrate Gab1 was recently shown to become phosphorylated, and associated with Grb2, PI3K, Shc, and SHP2, upon BCR triggering (Ingham *et al.,* 1998; Nishida *et al.,* 1999; our unpublished observations). Second, PI3K, which is activated upon stimulation of B cells by HGF, has a prominent role in BCR signaling and B-cell development: Targeted disruption of the gene encoding the p85 subunit of PI3K arrests B-cell development at the pro-B-cell stage, resulting in decreased immunoglobulin production (Fruman *et al.,* 1999; Suzuki *et al.,* 1999), similar to that in X-linked hypoglobulinemia. Third, STAT3, which becomes phosphorylated and translocates to the nucleus upon stimulation by HGF (Boccaccio *et al.,* 1998), also plays a role in BCR-mediated signaling (Karras *et al.,* 1997). Finally, we observed that HGF activates the Ras-MAPK pathway in B cells. This presumably constitutes an important transcription regulatory and proliferative signal for Met ex-

pressing GC B cells, which are in the process of undergoing rapid clonal expansion and selection (Lindhout *et al.,* 1997; Tarlinton, 1998b). Ras is also involved in BCR signaling and B-cell development. Expression of dominant-negative Ras arrests development at a very early stage, prior to formation of the pre-B-cell receptor (Iritani *et al.,* 1997). Furthermore, activated Ras causes progression of RAG1-deficient pro-B cells to pre-B cells and to cells with characteristics of the more mature GC B cells (Shaw *et al.,* 1999).

Successful B-cell selection in the GC requires tight regulation of cell survival. Interestingly, several studies indicate that the HGF/Met pathway may generate survival signals. HGF can rescue MDCK cells from apoptosis (Frisch and Francis, 1994) and inhibits apoptosis induced by staurosporin or DNA damaging agents of liver progenitor and carcinoma cells (Bardelli *et al.,* 1996; Fan *et al.,* 1998; Liu *et al.,* 1998b). In addition, overexpression of an active Met mutant renders hepatocytes resistant to anoikis and staurosporin-induced apoptosis (Amicone *et al.,* 1997). Given the ability of Met to interact with the anti-apoptotic BAG-1 upon HGF stimulation (Bardelli *et al.,* 1996), it is interesting to note that BAG-1 has been reported to play a role in survival and proliferation of the IL-3-dependent B-cell line Ba/F3 (Clevenger *et al.,* 1997). Furthermore, we have found that Met can activate PKB, in a PI3K-dependent fashion, in B cells (our unpublished observation). PKB is able to phosphorylate Bad, a Bcl-2 antagonist expressed in GC B cells (Ghia *et al.,* 1998), and thereby may suppresses the pro-apoptotic activity of Bad (Datta *et al.,* 1997; Mok *et al.,* 1999) (Fig. 5). Taken together, these data suggest that Met may play an important role in the regulation of apoptosis in the GC B cells, and thus in the process of affinity selection, which is critical for antigen-specific B-cell differentiation.

Finally, regulation of GC B-cell migration is important for the antigen-specific B-cell differentiation (Tarlinton, 1998a; Pals *et al.,* 1998). Several chemokines have been shown to be involved in this process, including SDF-1 , which is produced by reticulum cells surrounding the GC and acts via the G-protein-coupled receptor CXCR4 (Bleul *et al.,* 1996, 1998). A recent study has reported the ability of the BCR to arrest SDF-1a-induced migration (Bleul *et al.,* 1998). Interestingly, it was shown that this arrest was caused by PKC-mediated CXCR4 downregulation (Guinamard *et al.,* 1999). Because HGF stimulation also results in activation of PKC, this mechanism may provide a means to arrest migration of Met expressing B cells in the GC.

C. Heparan Sulfate Proteoglycans on B Cells Promote Met Signaling

We have recently obtained evidence that HSPGs expressed on the cell surface of specific B-cell subsets may play an important role in regulating Met

signaling. Several human B-cell subpopulations, including plasma cells and memory B cells, express HSPGs (van der Voort *et al.,* 2000). Interestingly, this HSPG expression was greatly enhanced by activation of B cells with the phorbol ester PMA and, more importantly, by ligating the costimulatory molecule CD40. An even stronger induction of HSPG was obtained after simultaneous ligation of CD40 and the BCR, signals which also induce expression of Met (van der Voort *et al.,* 1997). Because CD40 and BCR play key roles during T-cell-dependent B-cell differentiation (see Section III,A), these data suggest that Met and HSPGs act in concert during this biologic process. Indeed, we observed that, upon activation, B cells acquire the capacity to bind large amounts of HGF via HS moieties. CD44 isoforms carrying HS chains (CD44-HS) are the major proteoglycan core proteins on these activated B cells, which did not express the core proteins of syndecan-1, -2, -4, or glypican. However, others have shown that human as well as murine plasma cells express syndecan-1 (Sanderson *et al.,* 1989), whereas syndecan-4 was recently shown to be expressed by murine immature and mature B cells (Yamashita *et al.,* 1999).

By using Burkitt's lymphoma cells transfected with either CD44-HS or a CD44 isoform lacking HS (CD44s), we demonstrated that CD44-HS strongly promotes signal transduction via Met, including Met phosphorylation, phosphorylation of Gab-1, activation of the MAP kinases ERK1/2, and phosphorylation of PKB. Taken together, our results identify HSPGs, specifically CD44-HS, as functional coreceptors for HGF promoting signal transduction through Met (Fig. 6). We hypothesize that, via concentration and presentation of HGF, HSPGs regulate the biologic activity of the HGF/Met pathway in B cells.

D. HGF/Met Pathway in B-Cell Neoplasia

The HGF/Met pathway presumably is not only involved in normal B-cell differentiation, but also in the development and progression of B-cell neoplasia. Met is constitutively expressed by several Burkitt's lymphoma cell lines, including Raji, BJAB, and EB4B (Jücker *et al.,* 1994; van der Voort *et al.,* 1997; Weimar *et al.,* 1997), as well as by a subset of native Burkitt's lymphomas (Weimar *et al.,* 1997; our unpublished observations). On these tumor cells, which represent the malignant counterparts of GC centroblasts, HGF induces Met phosphorylation, as well as activation of downstream signaling molecules including MAP kinases and PKB (van der Voort *et al.,* 1997, 1999; Taher *et al.,* 1999; our unpublished observations) (Section III,B). Furthermore, HGF stimulation of Met positive Burkitt's lymphoma cells enhances $\alpha 4\beta 1$ and $\alpha 5\beta 1$-mediated adhesion to fibronectin, collagen, and VCAM-1, and promotes their invasion into fibroblast monolayers (van der

Voort *et al.,* 1997; Weimar *et al.,* 1997). Because HGF is produced by follicular dendritic cells and lymphoid stromal cells (van der Voort *et al.,* 1997; our unpublished observation), paracrine stimulation of Burkitt's lymphoma cells by HGF most likely takes place within the lymphoid microenvironment, promoting tumor growth and/or survival. HGF/Met signaling may stimulate survival via at least two distinct routes. As a direct consequence of HGF stimulation, Met may become associated with the anti-apoptotic protein BAG-1 (Bardelli *et al.,* 1996) (see also Section III,B). At the same time, HGF stimulation may downmodulate the activity of the Bcl-2 antagonist Bad (Datta *et al.,* 1997). Alternatively, activation of integrins by HGF/Met signaling may prevent tumor cell death by anoikis. This scenario is supported by our previous observation that integrin mediated adhesion to FDC presents a strong anti-apoptotic signal for GC B cells (Koopman *et al.,* 1994, 1997).

Although precise data concerning the expression of Met and HGF in different subtypes of malignant lymphoma are not available at present, a study by Weimar and colleagues (1997) indicates that Met expression is not confined to Burkitt's lymphoma. In 8 out of 11 follicle center cell lymphomas, and in some cases of large B-cell lymphoma, Met expression was observed. Furthermore, in approximately half of the cases of Hodgkin's disease Met expression was found in Hodgkin's/Reed-Sternberg (RS) cells, which presumably represent "crippled" GC B cells (Braeuninger *et al.,* 1997). Interestingly, Met expression in RS cells was strongly correlated with the presence of Epstein–Barr virus (EBV), suggesting a role for EBV in Met regulation (Weimar *et al.,* 1997). Although recent studies from our laboratory do not confirm the high percentage of Met positive cases among follicle center cell lymphomas, we did find Met expression in large B-cell lymphomas (our unpublished observations).

HGF has also been identified as a potential growth factor for multiple myeloma (MM), a neoplasm of terminally differentiated B cells (i.e., plasma cells). By screening myeloma supernatants for their ability to inhibit the activity of transforming growth factor-β (TGF-β), Borset and colleagues (1996c) isolated an antagonist. This protein was identified as HGF, and was produced by all five myeloma cell lines tested. Interestingly, in four of these cell lines Met was also expressed, suggesting the existence of an autocrine HGF/Met loop. Indeed, in the human myeloma cell line JJN-3, Met was found to be constitutively phosphorylated and could be dephosphorylated by anti-HGF antibodies (Borset *et al.,* 1996b). These findings were further extended by analyzing MM cells freshly isolated from patients. In all seven cases studied, coexpression of HGF and Met was observed on MM cells isolated from the bone marrow (Borset *et al.,* 1996a). Recently, the Nordic Myeloma Study Group reported the HGF serum levels of more than 400 MM patients. In approximately half of these patients, elevated HGF levels

were present; these patients had an unfavorable prognosis and poor response to melphalan/prednisone treatment (Seidel *et al.,* 1998).

In conclusion, while its precise role needs to be elucidated, these data suggest that deregulated HGF/Met signaling may contribute to the development and progression of specific subtypes of B-cell neoplasia, including Burkitt's lymphoma, large B-cell lymphoma, and multiple myeloma.

IV. SUMMARY

This article summarizes the structure, signal transduction and physiologic functions of the HGF/Met pathway, as well as its role in tumor growth, invasion, and metastasis. Moreover, it highlights recent studies indicating a role for the HGF/Met pathway in antigen-specific B-cell development and B-cell neoplasia.

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Clinical Targets for Anti-Metastasis Therapy

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Metastasis is responsible for most cancer deaths. Therapeutic strategies to prevent development of metastases thus have potential to impact on cancer mortality. Development of these therapies requires a better understanding of the biology and molecular events of the metastatic process. Metastasis is usually defined, clinically and experimentally, by evidence of the endpoint of the process, that is, the presence of metastatic tumors. Endpoint assays are suitable for determining if a therapeutic approach is effective, but can provide little information on how a treatment works *in vivo* and what steps in metastasis are affected. We describe here two methodological advances in the study of metastasis as a process: *in vivo* videomicroscopy, which permits direct observation of steps in metastasis, and a "cell accounting" technique that permits quantification of the fate of

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cells over time. These procedures have provided new and unexpected insights into the biology of the metastatic process. Based on these insights, we consider which steps in the metastatic process are biologically and clinically most appropriate as therapeutic targets for development of anti-metastasis therapies. We conclude that the most promising stage of the metastasis process for therapeutic targeting is the growth phase, after cancer cells have arrested in the microcirculation in secondary sites and have completed extravasation. Earlier phases in the process are either biologically inappropriate or clinically inaccessible, except in specific cases (e.g., chemoprevention strategies). The role of "seed" and "soil" in determining organ-specific metastasis is also discussed. The metastatic growth phase fortunately is a clinically broad target, and any treatment that limits growth of metastases prior to their causing irreversible harm to the patient has the potential to be clinically useful. A variety of therapeutic approaches to target this phase are under active development, including inhibition of angiogenesis or signal transduction pathways needed to support the growth of metastatic cells.

I. INTRODUCTION

In 1999, it is estimated that well over half a million Americans will die from cancer (Landis *et al.*, 1999). For the most part, these deaths will be due to the physiologic consequences of the growth of metastatic tumors, rather than to the effects of the primary tumor. Patients whose primary tumors have been detected before metastatic cells have been seeded to distant sites can generally be cured. However, these patients often cannot be readily identified. Two major difficulties in cancer treatment are, first, determining whether the tumor has indeed seeded metastatic cells at the time of detection, and second, how best to treat patients in which the metastatic process has (or is likely to have) already begun. In some cases, overt metastases are detected at the time of presentation. In others, however, no metastases can be detected, and the decision of whether metastasis has already been initiated in a given patient is based on probabilities, derived from a variety of prognostic indicators that have been shown, in other patients with similar tumors, to predict for future development of metastases.

There continues to be a great need to refine the predictive process for development of metastases, in order to more precisely assign patients to groups that likely require, or can be spared, further treatment following surgical removal or other treatment of the primary tumor. Furthermore, when a patient is identified by prognostic indicators as someone in whom metastasis is likely to occur, the treatment options to prevent metastases from developing are in many cases less than optimal. Similarly, the treatment options for patients in which overt metastases are present are often limited or less effective than one would like. For these reasons, metastases continue to be the primary source of cancer mortality. There thus remains a huge need for improved ways to treat existing metastases, in order to prevent their physiologic consequences to the patient, and to treat undetected metastases

believed to be present, to prevent them from growing and causing harm to the patient.

To achieve the goal of developing effective treatments to prevent mortality and morbidity due to metastasis, a better understanding of both the biology and the molecular biology of the metastatic process are needed. The steps in the process, and their timing over the course of the natural history of the disease, must be clarified. Similarly, molecules that contribute to the process of metastasis, and the steps at which each plays an important role, must be understood. This information is necessary for the development and appropriate clinical use of new anti-metastatic therapeutics.

II. METASTASIS: CLINICAL AND EXPERIMENTAL CONSIDERATIONS

Metastasis is defined clinically by the "endpoint" of the process, the detection of secondary tumors at a site distant from the primary tumor. These tumors can be detected either on the basis of the effects that they cause to the patient (e.g., bone pain, in breast cancer that has spread to and grown in bone), or by detection by various medical imaging procedures (e.g., radiologic detection of metastases in internal organs), whether or not they are causing symptoms to the patient. The general steps in the process can be logically inferred, and include growth and vascularization of the primary tumor, escape of cells from the primary into the blood or lymphatic circulations (intravasation), transport of cells to distant organs, extravasation (escape from the circulation into the new tissue), and growth and vascularization of the new metastatic tumor. However, the details of these steps cannot be deduced from detection of this endpoint (i.e., presence or absence, and numbers, of metastatic tumors).

Experimentally, most commonly used assays are similarly designed to detect the endpoint of detectable tumors in distant organs (reviewed by Welch, 1997). In *spontaneous metastasis assays,* a primary tumor is produced by injecting cancer cells into an appropriate experimental animal (e.g., mice syngeneic to a murine cancer cell line, or immune deficient nude or SCID mice for various human cancer cell lines or immunogenic murine lines). Cells can be injected subcutaneously (i.e., an ectopic site for most tumor types) or, better, orthotopically (e.g., mammary fat pad injections for breast tumor cells) (reviewed by Fidler, 1991; Hoffman, 1994; Price, 1994; Killion *et al.,* 1999). Various transgenic tumor-prone mice also can be used, in which primary tumors spontaneously arise, and metastasis from these tumors can be assessed (reviewed by Christofori and Hanahan, 1994; Eccles *et al.,* 1994; Dankort and Muller, 1996). Metastatic tumors at sites distant from the implanted or spontaneously arising primary are then detected, at a single point in time, generally by dissection of the organs and observation and counting of metastases. Cells are assumed to have left the primary tumor and traveled to sec-

ondary organs, via the blood or lymphatic circulations, and to have grown to form detectable metastases in these sites. In contrast, the *experimental metastasis assay* is designed to model the latter half of this process by injecting cancer cells directly into the circulation of experimental animals and again detecting the endpoint of the presence and numbers of metastatic tumors in various internal organs such as lung or liver.

Both of these assays are very well suited for determining the effect of genetic, molecular, or therapeutic manipulations on the endpoint being quantified. Compounds or genetic manipulations of cells that give rise to decreased or increased numbers of metastatic tumors are clearly involved in the metastatic process, and this information is extremely valuable in defining factors that affect metastasis and may thus be targets for inhibition. Much progress has been made in recent years, using such endpoint assays, in identifying molecular and genetic factors that contribute to metastasis. However, metastasis assays that detect the endpoint of metastasis are by their very design poorly suited to clarifying the nature of metastasis as a *process*. The functional contributions of molecules identified as affecting the endpoint of metastasis cannot be directly assessed by such assays. Thus the roles these molecules play in the process have often been logically inferred rather than assessed experimentally.

For example, many studies had clearly implicated various classes of proteolytic enzymes in metastasis. A reasonable inference was that a major functional contribution of these enzymes to the process was by facilitating extravasation, the escape of cancer cells from the circulation in secondary organs, because the basement membrane lining the vessels was considered to be a significant physical barrier to the passage of cells. This assumption (based on logical deduction of what these enzymes must do *in vivo*) turned out to be a simplification of the much more complex role these enzymes play in the process, when this assumption was tested using procedures that allow observation of metastasis as an ongoing process (reviewed by Chambers and Matrisian, 1997). When murine melanoma cells were transfected to express the matrix metalloproteinase (MMP) inhibitor TIMP-1, both their metastatic ability *in vivo* and invasiveness *in vitro* were significantly reduced (Khokha *et al.,* 1992a,b; Khokha, 1994). Thus we expected to see a reduced ability of the cells to extravasate *in vivo*. However, the TIMP-expressing cells extravasated in the same proportions and with the same kinetics as did the parental cell line, when directly observed using *in vivo* videomicroscopy (Koop *et al.,* 1994, 1995). By direct observation of metastasis as a process, we were able to determine that this molecular intervention resulted in altered growth properties of the cells, after they had completed extravasation. A similar conclusion was reached when the anti-MMP agent batimistat was used to treat mice injected with melanoma cells: No alteration in extravasation was observed, whereas the metastases that

formed were smaller, associated with a reduction in angiogenesis in the metastases (Wylie *et al.,* 1999).

Thus, the ability to observe metastasis as a process, rather than just as an endpoint, provides insights into how molecular and therapeutic interventions contribute to inhibition of metastasis. By relying on endpoint assays alone, one obtains a static view of metastasis. *In vivo* videomicroscopy, discussed in the next section, provides the opportunity to observe metastasis as a process.

A complete understanding of metastasis as a process also requires the ability to quantitatively follow the fate of cells during the process. Observations of cells at any point in the process need to be related to numbers of cells that began the process. To address this need, we have developed a new approach of "cell accounting," through which we can monitor and quantify the fate of a population of cells over time following their injection into an experimental animal, by providing a fixed reference point to the number of cells originally injected. Together, the procedures of *in vivo* videomicroscopy, coupled with a quantitative monitoring of the fate of cells over time afforded by the "cell accounting" approach, have offered new insights into the steps involved in metastasis, their relative ease or difficulty, and their relationship to the overall malignant phenotype of the cells. These methodological approaches, and results achieved using them, are discussed in the following sections.

III. NEW TOOLS FOR STUDYING THE METASTATIC PROCESS

A. I*n Vivo* Videomicroscopy

Direct observations of experimental metastasis as it occurs over time may be carried out in a number of different organs and tissues by means of *in vivo* videomicroscopy (Fig. 1). Cancer cells can be fluorescently labeled *in vitro,* then injected into an animal and viewed at later times *in vivo* in thin tissues or superficial ($\leq$50 μm) regions of thick tissues by both fluorescence and oblique transillumination. Fluorescence provides for positive identification of cancer cells and speeds the initial process of finding them within a field of view. Oblique transillumination then permits individual cells to be viewed at high magnification in relation to their immediate environment. Because detailed descriptions of the methodology have been provided elsewhere (Chambers *et al.,* 1995; MacDonald *et al.,* 1998), only a brief summary is given here.

To locate cancer cells in tissues by *in vivo* videomicroscopy, high levels of cellular fluorescence are needed. Exogenous cytoplasmic markers such as Cal-

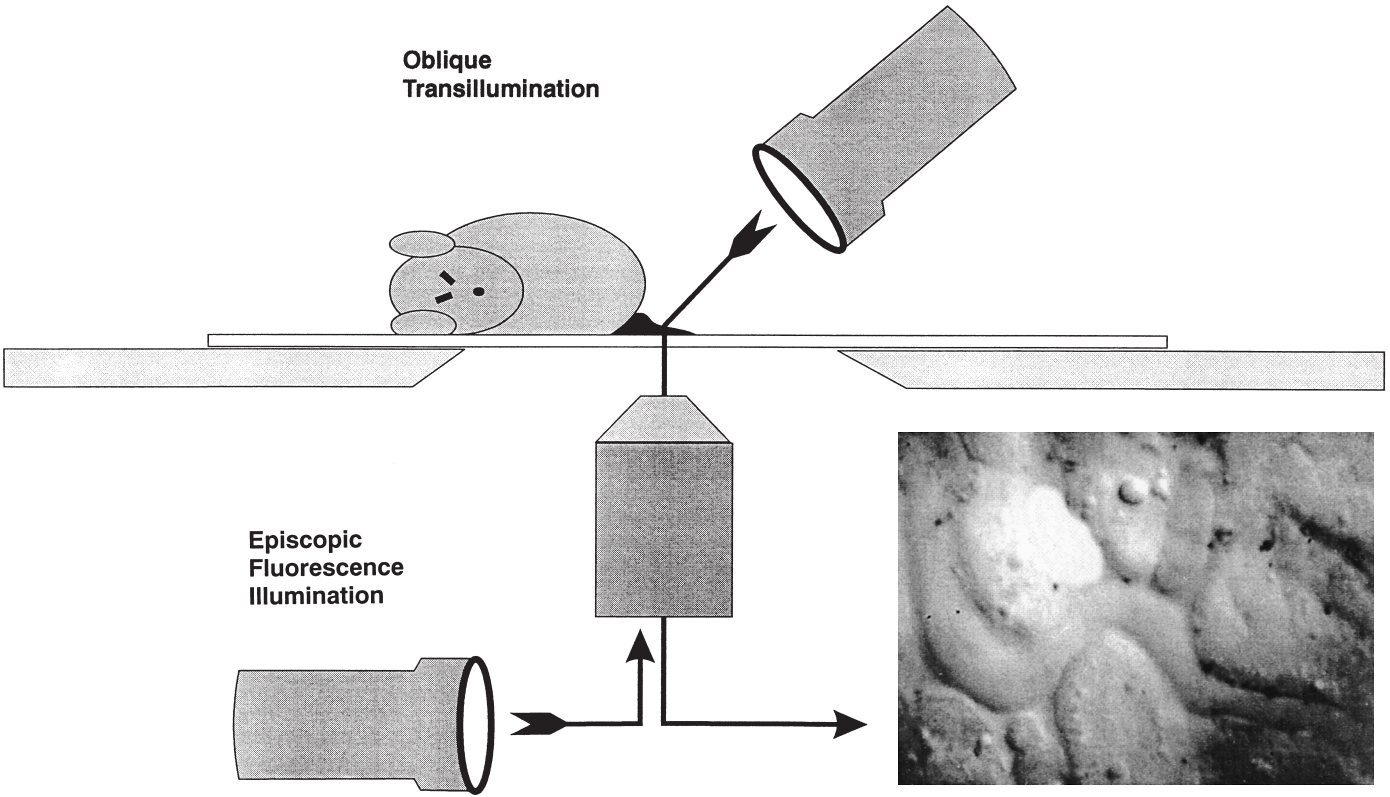


Fig. 1 Schematic diagram of *in vivo* videomicroscopy method. The mouse is placed on the platform of an inverted microscope, the exposed intact organ (e.g., liver) being visualized through a glass coverslip using 10–100× objective lenses. Oblique transillumination via a fiber optic light guide and/or episcopic fluorescence illumination with appropriate filter blocks is used to observe fluorescently labeled cells within the organ. Images are viewed using a videocamera and monitor, and recorded on SVHS tapes and/or sent to a computer image capture system for further analysis.

cein-AM or fluorescent "nanospheres" 0.05–0.07 μm in diameter, which are internalized by the cells, are effective for this purpose (Morris *et al.,* 1994). Both markers leave cellular membrane integrity and growth potential unimpaired. [We found that use of nuclear fluorescent markers such as acridine orange is toxic to cells and results in membrane disruption and cell lysis (Morris *et al.,* 1993).] Calcein-AM gives a clear outline of the cell profile (MacDonald *et al.,* 1992), but is gradually lost from the cells and useful only for periods of up to 24 hr. Fluorescent nanospheres do not fade with time but become diluted at each successive cell division, such that after two or three divisions the marker can no longer be detected; however, undivided (i.e., dormant) cells can remain brightly fluorescent several weeks later. Recently, transfection of cells to express green fluorescent protein (GFP) has provided a heritable endogenous cytoplasmic marker that yields excellent views of cells and their pseudopodia (see Fig. 3 later), and makes long-term studies of metastatic growth possible (Chishima *et al.,* 1997; Naumov *et al.,* 1999).

After labeling, cells are injected intravascularly so as to target the organ being studied, for example, mesenteric vein for liver, chorioallantoic membrane (CAM) vein for chick embryo CAM. [Other organs such as lung (Cameron *et al.,* 2000), gut, spleen, and pancreas may also be studied, as described by Chambers *et al.* (1995).] At selected times later, a superficial layer of the partially exteriorized organ with its blood flow intact is examined microscopically *in vivo* (Fig. 1). For this purpose, the animal is placed on the stage of an inverted microscope with the organ resting on a glass coverslip above the objective lenses (10–100×, dry). In this way, the lower surface of the organ remains stationary and within the plane of focus, in spite of any respiratory motion to which the rest of the tissue is subjected. Epifluorescence illumination is used to locate cancer cells, followed by or in conjunction with transillumination to obtain high-resolution images and see cells clearly in relation to their immediate surroundings.

Transillumination is best carried out using obliquely incident light via a fiber optic guide. This arrangement results in more light being refracted from one side of the cells than the other and produces a shadowing effect, greatly enhancing the contrast and imparting a three-dimensional quality to the image. Cells are then seen by virtue of their shape rather than their optical density. In an organ such as the liver, the large volume of blood in the path of the incident light gives rise to a monochromatic red image that is extremely difficult to view. However, a Newvicon tube video camera with extended red sensitivity (Panasonic WV1550; Hamamatsu C2400) attached to the microscope yields high-contrast, nongrainy black-and-white images that can be viewed comfortably on the monitor and recorded on SVHS tapes, or captured by computer, for later analysis. By focusing up and down, "optical slicing" of the tissue may be carried out, due to the shallow depth of focus at high magnification (~1 μm for a 100× objective).

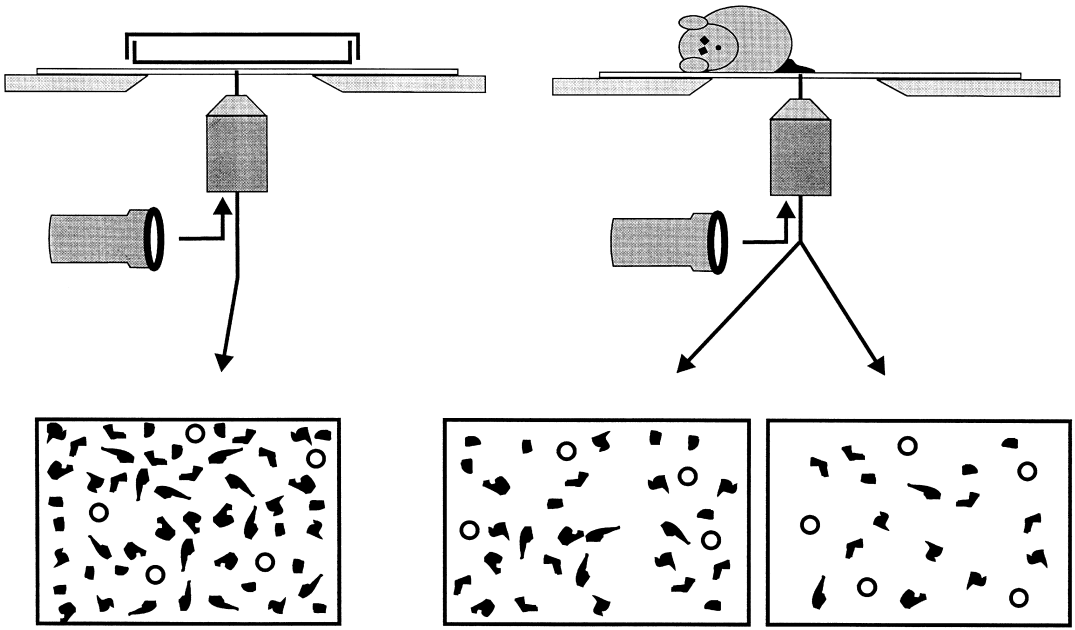
A limitation of *in vivo* videomicroscopy is that observations are restricted to a superficial layer of tissue 50–75 μm in thickness. For chick CAM this presents no problem since the total thickness of the tissue is less than this value. In mouse liver, studies using thick sections through the whole organ show that there is good reason to focus on this superficial layer of tissue, for it is at the surface of the organ, specifically, that metastases from various cell lines develop (Luzzi *et al.,* 1998; Naumov *et al.,* 1999). By *in vivo* microscopy one can study the metastatic process as it occurs over time, as well as the effects of molecular interventions on specific steps in metastasis. Using the same technique it is also possible to study the events of lymphogenous metastasis, as we have recently begun to do (Trites *et al.,* 2000).

B. Cancer "Cell Accounting" in Tissues

We have devised a new experimental procedure to quantify accurately the survival of injected cancer cells in tissue, at successive early steps in hematogenous metastasis (Koop *et al.,* 1994; Luzzi *et al.,* 1998). To determine survival at any particular instant, the number of cancer cells actually observed in a sampled volume of tissue must be expressed relative to the number of cells that originally entered that volume. Our approach, based on the standard method for measuring distribution of blood flow (Rudolph and Heymann, 1967), is to include in the cell suspension injected, inert plastic ~10-μm microspheres at a known ratio, for example, 1 microsphere per 10 cells. This cell-to-microsphere ratio was chosen to minimize the effects of capillary blockage. After injection, the microspheres become trapped by size restriction in blood capillaries (or liver sinusoids) and remain in the tissue indefinitely, since they do not extravasate or deform to pass through the microcirculation. Thus, the total number of microspheres present in any particular region of tissue provides a reference marker for the total number of cancer cells which originally entered that region. On this basis it may be seen that the percentage of cells surviving in the tissue at a particular time after injection (Fig. 2) is given by the ratio of cells to microspheres present in the tissue, divided by the corresponding ratio present in the syringe immediately prior to injection, ×100%.

This method may be used in conjunction with *in vivo* videomicroscopy to

Fig. 2 "Cell accounting" procedure for quantifying survival *in vivo*. The cell-to-microsphere ratio in the suspension is determined before injection and in the tissue of the target organ at various times after injection. The microspheres remain trapped in the tissue indefinitely, providing a reference marker for the number of cells delivered to that tissue. From the cell-to-microsphere ratios at later times, the percentages of surviving cells can be determined (see example provided).



before injection

10 cells/μsphere = 100%

in vivo

day 3

7 cells/μsphere = 70% survival

day 13

3 cells/μsphere = 30% survival

sample superficial regions of the tissue, or after fixation the tissue may be sampled throughout its entire thickness by counting cells and microspheres in 30- to 50-μm-thick sections of tissue (Luzzi *et al.,* 1998). Because metastases have been shown to be clonal in origin (Talmadge *et al.,* 1982; Chambers and Wilson, 1988), the same method may be used to quantify them as well. When used together, *in vivo* videomicroscopy and our cell accounting procedure provide, for the first time, a means to determine at sequential times after injection the proportions of injected cancer cells that extravasate and survive in the tissue, remain as solitary dormant cells, form micrometastases, or develop into tumors. Such information, in turn, opens the way to dissect out the separate contributions to metastatic inefficiency from each of the sequential steps in the metastatic process (Luzzi *et al.,* 1998).

C. Significance of the Procedures Described

What has been lacking in experimental metastasis methodology until now is the means to directly watch steps in the metastatic process as they occur *in vivo* over time, and to quantify the percentages of injected cancer cells surviving after each of these steps. The *in vivo* videomicroscopy and cell accounting procedures described in the preceding sections, coupled with fluorescence labeling for unambiguous identification of cancer cells *in vivo* or in thick sections of fixed tissue, provide new tools for studying early steps in the metastatic process. The use of these experimental approaches in combination with standard histologic and immunohistochemical procedures has led to a new conceptual understanding of early steps in metastasis and the contributions of individual steps to metastatic inefficiency. These approaches can now be used to determine which steps in metastasis are affected by a particular treatment or genetic manipulation, to test molecular mechanisms of metastasis, and to identify potential therapeutic targets.

IV. NEW INSIGHTS INTO THE METASTATIC PROCESS

A. Arrest and Survival of Cancer Cells in the Microcirculation

Using *in vivo* videomicroscopy, it is now possible to watch directly the arrival of blood-borne cancer cells in an organ and determine the way in which they become arrested within the microvasculature (Chambers *et al.,* 1992; MacDonald *et al.,* 1992; Morris *et al.,* 1993). It has become clear that in the organs and tissues we have studied, the arrest of cells from solid tumors oc-

curs by quite different means from those by which leukocytes are arrested. Thus, leukocytes pass through blood capillaries or liver sinusoids and are arrested by adhesion to walls of venules much larger than the cell diameter, rolling along the endothelium before forming shear-resistant bonds. In contrast to this, cancer cells from solid tumors are much larger than leukocytes and are less able to pass through vessels of capillary dimensions to reach the venules.

Our direct observations show that cancer cells become arrested by size restriction in small vessels at the input side of the microcirculation in mouse liver and muscle (Fig. 3a, see color insert) and in chick CAM (Morris *et al.,* 1993, 1994; Koop *et al.,* 1994, 1996). Immediately after arrest, the cells undergo deformation due to hemodynamic pressures and conform to the boundary restrictions imposed by the vessel (Fig. 3a, arrow). The degree of deformation is modest in relatively low-pressure circulations such as CAM and the portal circulation of the liver, but under the higher hemodynamic pressures found in muscle the cells reach length-to-width ratios of up to 8:1. However, in spite of such severe deformations the cells do not become lysed but retain their membrane integrity, as shown by exclusion of ethidium bromide (Morris *et al.,* 1993). In CAM and liver we have studied these cells over extended periods of time and found that the vast majority of cells successfully extravasate into surrounding tissue within 1–2 days, which further attests to the continued viability of cancer cells in spite of hemodynamic forces and arrest in the microcirculation (Chambers *et al.,* 1995; MacDonald *et al.,* 1998).

Based on extrapolation from *in vitro* studies, it has been believed that arrest of blood-borne cancer cells in an organ or tissue is determined solely by specific adhesive interactions between cells and the endothelium. This concept has appeared attractive because it would provide a rational basis for organ specificity of metastasis. However, our observations from *in vivo* videomicroscopy have demonstrated that for the systems we have studied this view is no longer tenable. These findings lead us to propose that cancer cells which are blood-borne, after release from solid tumors, will become arrested initially on the basis of size restriction in the microcirculation of whatever organ or tissue they enter. This view accords with findings in mesentery by Thorlacius *et al.* (1997). It is interesting that whereas Scherbarth and Orr (1997) confirmed our findings in normal mouse liver, they also discovered that in mice pretreated with interleukin 1α (IL-1α) the cancer cells could become arrested by adhesion to walls of presinusoidal vessels twice the cell diameter. This suggests the possibility that release of cytokines into the bloodstream could bring about arrest of some cancer cells in portal venules, any cells that escape such arrest becoming trapped by size restriction in periportal sinusoids.

Metastatic inefficiency has generally been considered the result of a massive destruction of cancer cells within the circulation, due to the immune system and/or hemodynamic forces. Studies with ^{125}IUdR-labeled cells in-

jected intravenously to target the lung showed a >100-fold reduction of radioactivity in the organ after 24 hr, suggesting that >99% of cells may have been destroyed (Fidler, 1970). Based on *in vitro* studies it was later proposed that such loss could arise if cells were arrested and deformed in blood capillaries, followed by stretching of their plasma membranes to the point of rupture (Weiss, 1987). Our own videomicroscopic studies, based on direct observation of cancer cells *in vivo,* following labeling *in vitro* with nontoxic fluorescent markers and injection into the circulation, demonstrate that the cells are indeed arrested and undergo deformation within capillaries or liver sinusoids (Fig. 3a). However, the vast majority of cells survive arrest and deformation, and go on to extravasate (Morris *et al.,* 1994; Koop *et al.,* 1994, 1995, 1996; Luzzi *et al.,* 1998; Wylie *et al.,* 1999). Similar results have been obtained for several different types of cells, including melanoma, mammary carcinoma, rhabdomyosarcoma, and normal and oncogene-transformed fibroblasts (Morris *et al.,* 1993, 1994, 1995; Hangan *et al.,* 1996; Luzzi *et al.,* 1998).

If these results from *in vivo* videomicroscopy can be translated to other vascular beds and cell types, as well as to the clinical situation, our findings suggest the new concept that most cells which escape from a solid tumor into the bloodstream (and go wherever the blood flow takes them) may not undergo rapid destruction within the circulation, but survive.

B. Extravasation of Cancer Cells into the Tissues

Divergent views are to be found in the literature regarding the way in which cancer cell extravasation occurs. These views are derived from histologic and ultrastructural examination of tissues after cancer cell injection into the circulation, rather than from direct microscopic observations *in vivo*. The first view is that arrested cells replicate within vessels and, after proteolytic destruction of adjacent vascular basement membranes, extravasate *en masse* (Chew *et al.,* 1976; Crissman *et al.,* 1985, 1988). In contrast, another view is that cancer cells extravasate on an individual cell basis, similar to leukocytes, and with minimal disruption of the vessel wall. This is followed by replication within the tissue (Dingeman and Roos, 1982). It has been generally believed that the process of extravasation constitutes a barrier that few cancer cells successfully overcome.

We have used *in vivo* videomicroscopy in chick CAM and mouse liver to observe directly cancer cells in the process of extravasation (Chambers *et al.,* 1992, 1995; MacDonald *et al.,* 1992, 1998; Koop *et al.,* 1994, 1995; Morris *et al.,* 1994, 1995, 1997). No evidence for intravascular replication has been found and, invariably, cells extravasated singly without observable disruption of the microvasculature. For several hours after initial arrest in liv-

er sinusoids, cancer cells completely obstruct the blood flow through the vessel segments concerned (Fig. 3a). Thereafter, cells gradually move away from one side of each vessel and extend themselves along the opposite wall, making possible a progressive resumption of blood flow (Morris *et al.,* 1994, 1995). This stage of the extravasation process is obviously dependent on the formation of shear-resistant bonds between cell and vessel wall, indicating that adhesion molecules (identity unknown) are playing a critical role. Each cell then forms pseudopodial projections, which extend out through the vessel wall and between the surrounding hepatocytes (Morris *et al.,* 1994). Finally, migration of the cell body occurs into the extravascular tissue, displacing hepatocytes and often wrapping around the abluminal surface of the sinusoid (Fig. 4a). In chick CAM the extravasated cells wrap around arterioles in the mesenchyme (Fig. 4c).

The first extravasated cells may be seen, in both CAM and liver, at between 2 and 3 hr after initial cell arrest in the microcirculation. However, for an entire population of cells to complete the process of extravasation takes considerably longer, since not all cells begin to extravasate at the same time. For each animal there is roughly a 3- to 5-hr time period during which observations by *in vivo* videomicroscopy can be carried out, and this permits the locations of large numbers of cancer cells to be assessed. Cells are classified as intra- or extravascular, or in the process of extravasation, and the percentages in each category are then determined. By studying a series of animals at successive times postinjection and combining the data, the time course of extravasation for an entire population of cancer cells can be obtained (Koop *et al.,* 1994, 1995). By 2–4 days after initial arrest in the liver microcirculation, virtually all of the injected cells are found to have extravasated (Morris *et al.,* 1994; Luzzi *et al.,* 1998; Wylie *et al.,* 1999).

The process of extravasation has been widely regarded as an important rate-limiting step in metastasis. This belief stems from (1) the recognition that degradation of vascular basement membrane and extracellular matrix is necessary for cancer cells to extravasate, taken together with (2) experimental results obtained from cell invasion assays through Matrigel *in vitro.* Based on differences in the extent of physical barriers in CAM capillaries (continuous endothelial lining and complete basement membrane by day 11, when cells were injected) versus liver sinusoids (fenestrated endothelium and incomplete basement membrane), one would predict that cells would extravasate with a shorter time course in liver than in CAM. However, the reverse is true, because it took ~18 hr in liver versus 7 hr in CAM for 40% of injected B16F1 cells to extravasate (Chambers *et al.,* 1995; MacDonald *et al.,* 1998). Clearly, the *in vivo* situation is more complex than that predicted from *in vitro* assays, and other factors such as differences in signaling molecules present in CAM versus liver must also play a major role in cancer cell extravasation.

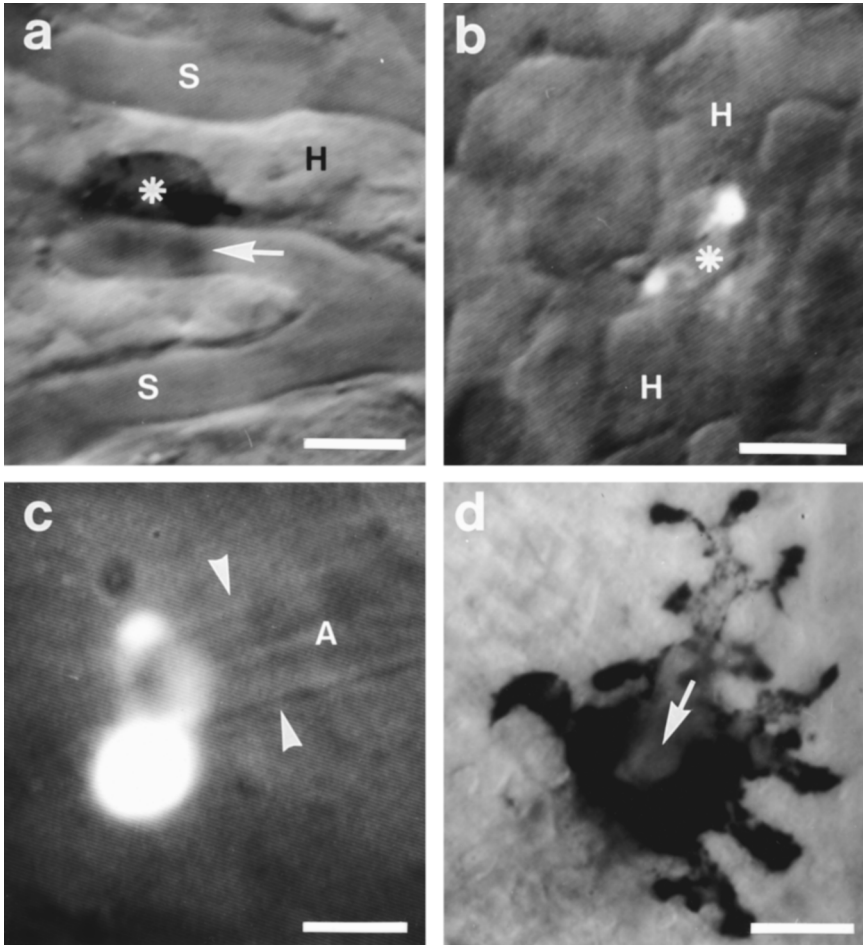


Fig. 4 Views of cancer cells *in vivo* by videomicroscopy. All marker bars = 20 μm. (a) Melanoma cell (*) in mouse liver has completely extravasated and displaces hepatocytes (H), at 24 hr after injection. At a deeper plane of focus the cell wraps around a sinusoid (arrow, blurred in this image) in which rapid flow has resumed. S, sinusoids. (b) Melanoma cell (*) that has extravasated and migrated to the hepatocyte layer in the subcapsular region of mouse liver, by day 2. Note outlines of hepatocytes (H). (The fluorescent nanosphere labeling gives the cell a spotty appearance, and several pseudopodia are visible.) (c) Extravasated melanoma cell wrapping projections around an arteriole (A) in chick embryo chorioallantoic membrane, ~4 hr after injection. Arrowheads mark the arteriolar wall. (Photograph courtesy of Sahadia Koop.) (d) Micrometastasis, highly melanotic, has formed by 3 days after injection in chick chorioallantoic membrane. It is growing around a terminal arteriole (arrow).

Based on the view that extravasation is a major rate-limiting step in metastasis, it has been inferred that highly metastatic cells will extravasate more readily than cells of lower metastatic potential. However, our findings from

in vivo videomicroscopy do not support this view. We compared the time course of extravasation in mouse liver for two mammary carcinoma cell lines, from separate tumors both derived from D2 hyperplastic alveolar nodules. The first cell line (D2A1) is highly invasive in Matrigel invasion assays *in vitro* and highly metastatic in both chick CAM and mouse liver, whereas the second (D2.OR) is almost entirely noninvasive *in vitro* and poorly metastatic. To our surprise, we found that both cell lines extravasated with the same time course (Morris *et al.,* 1994). When similar experiments were carried out in chick CAM (Koop *et al.,* 1996), comparing the extravasation of (1) highly metastatic *ras*-transformed NIH 3T3 cells, (2) control nontumorigenic nontransformed NIH 3T3 cells, and (3) primary mouse embryo fibroblasts, we found that all three cell lines extravasated with the same kinetics (Fig. 5). By 24 hr after injection and arrest in the microcirculation, more than 89% of observed cells had completed the process of extravasation.

Thus, a new key concept from direct observations of cancer cell extravasation *in vivo* is that highly and poorly metastatic cells, or even nonmetastatic cells, may extravasate equally well. Our evidence from both chick CAM and mouse liver supports the view that most blood-borne cancer cells, whether metastatic or not, can successfully extravasate into surrounding tissue after arrest in the microcirculation. If these results can be translated to other vascular beds and cell types, our findings suggest that, contrary to previous belief, the process of extravasation does not represent a major barrier for cancer cells. Therefore, extravasation can no longer be considered a rate-limiting step in metastasis.

Proteinases and integrins are known to play important roles in the metastatic process, based on endpoint analyses such as number and size of metastases. However, which particular steps in metastasis are affected most by these molecules has remained unknown. Using *in vivo* videomicroscopy together with our recently developed cell accounting assay, we have found (Koop *et al.,* 1994, 1995) that overexpression of metalloproteinase inhibitor in B16F10 cells does not affect extravasation but reduces tumor growth in chick CAM. At 24 hr after injection, more than 80% of injected cells of both cell lines survived and had successfully extravasated. Similar results have been obtained for B16F1 cells in mouse liver, using the synthetic metalloproteinase inhibitor batimastat (Wylie *et al.,* 1999). These studies indicated that MMP inhibitors, both endogenous and exogenous, produced a reduction in growth of metastases that was caused by effects on postextravasation events. In the case of batimastat, this reduction in growth occurred in conjunction with an inhibition of tumor angiogenesis (Wylie *et al.,* 1999). Other *in vivo* microscopy experiments that focused on the role of integrins in metastasis, also showed tumor inhibition because of effects on postextravasation cell migration and growth, with little or no effect on the process of extravasation itself (Morris *et al.,* 1995; Hangan *et al.,* 1996).

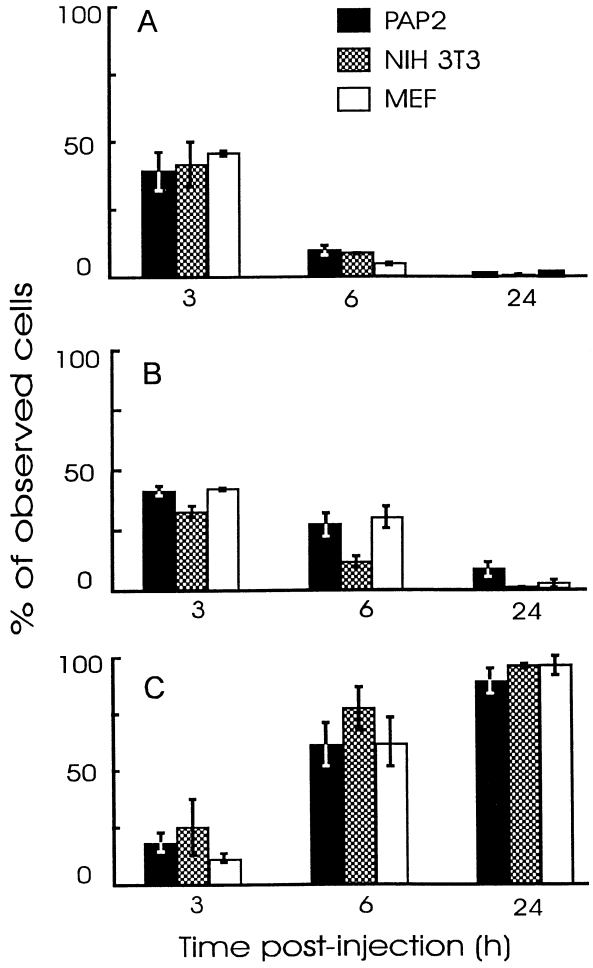


Fig. 5 Kinetics of extravasation of highly metastatic, *ras*-transformed NIH 3T3 cells (PAP2), control nontransformed NIH 3T3 cells, and primary mouse embryo fibroblasts (MEF) from the chick embryo CAM microcirculation. Percentages of cells that were intravascular (A), in the process of extravasating (B), or had extravasated (C) are shown at 3, 6, and 24 hr after injection. Different sets of experiments (involving different embryos) were performed at the stated time points. The data showed no significant differences among the three cell types, at any of the time points. By 24 hr after injection, >89% of observed cells of all three cell types had completed the process of extravasation. Bars represent means ± SE. (Republished from Koop *et al.*, 1996.)

Taken as a whole, the experiments reported in this and the previous section provide a strong indication that neither cancer cell arrest and survival in the microcirculation nor the process of extravasation into adjacent tissues

constitutes rate-limiting steps in the metastatic process. The reasons for metastatic inefficiency must therefore be sought at the level of postextravasation cell migration and growth.

C. Postextravasation Migration of Cancer Cells

Extravasated cancer cells need to be able to grow at their new locations, and *in vivo* videomicroscopy has revealed that the cells may migrate short distances to preferred sites in host tissue prior to replication. The site of replication can differ depending on the tissue in which the cell has extravasated. In chick CAM, most of the extravasated cancer cells migrate through the mesenchyme and attach to arterioles, rather than to venules or lymphatics (Koop *et al.,* 1994, 1996). We have observed that the individual extravasated cells extend pseudopodial projections that wrap around the abluminal surfaces of vessels (Fig. 4c), where cells may later develop into tumors (Fig. 4d). The reason for this specific cell migration is currently unknown, but the same behavior was seen in chick CAM or mouse liver for every cell type we studied (melanoma and mammary carcinoma cell lines, *ras*-transformed fibroblasts) and was independent of metastatic potential or transformed status of the cells (Morris *et al.,* 1994, 1995; Koop *et al.,* 1994, 1996). These results suggest that directed migration might be of widespread significance. The migration toward the arterioles is not simply explained by a requirement for oxygen since the CAM is a respiratory organ, and thus the arterioles contain deoxygenated blood. Also no differences between the levels of fibronectin, laminin, or collagen have been detected in venules versus arterioles of the CAM (Ausprunk *et al.,* 1991). Thus, the molecular basis for directed migration remains to be determined. In the process of angiogenesis new blood vessels grow from host tissues into small tumors; however, our observations in the CAM show a complementary situation, in which extravasated cancer cells preferentially migrate to the vicinity of preexisting vessels (Koop *et al.,* 1994, 1996). We found that the cells did not begin to divide until after they had reached the arterioles (Fig. 4c) and tumors encircling the vessels were later found at these locations (Fig. 4d).

Postextravasation cell migration is also observed in mouse liver. After extravasation from sinusoids, the cells wrap around the abluminal surface of these vessels, in a manner similar to that seen in the CAM (Figs. 4a and 4c). However, cells that have extravasated from the most superficial sinusoids may send pseudopodial projections 20–30 μm long up to the subcapsular region of the liver (Fig. 3b), which, in the mouse, is an avascular region approximately 10 μm in thickness. Thereafter, migration of the cell body to this location occurs (Fig. 4b). Cells from both poorly and highly metastatic cell lines may be seen to have squeezed between hepatocytes at the liver surface, and in mouse

this is the primary location where hepatic metastases from many cancer cell lines develop. Pseudopodial projections similar to those exhibited by migrating cells *in vivo* (Fig. 3b) have also been observed during cell movement *in vitro* and found to contain membrane-bound proteinases, which facilitate cell movement by localized degradation of the matrix components (Kelly *et al.,* 1994; Monsky *et al.,* 1994). In addition, these projections contain β_1 integrins, which may also play a role in this cell movement (Nakahara *et al.,* 1996).

Recent studies have shown that integrin $\alpha_2\beta_1$ mediates postextravasation migration of human rhabdomyosarcoma and erythroleukemia cells in mouse liver but has no effect on their extravasation (Hangan *et al.,* 1996; Ho *et al.,* 1997). This result is in agreement with a model of diapedesis of leukocytes, which proposes that different integrins are involved in the different steps of extravasation and postextravasation cell movement (Weber and Springer, 1998). In this model the integrins $\alpha_4\beta_1$ and $\alpha_L\beta_2$ are involved in the transendothelial migration of the leukocytes and their movement through interendothelial cell junction, respectively, while $\alpha_5\beta_1$ is involved in the spreading and migration of the extravasated cells on the extracellular matrix (ECM) components. Thus cell migration *in vivo* is likely to be a very complex process that depends on the interaction of different molecules.

From our *in vivo* study of metastasis in chick CAM and mouse liver the following key concepts concerning postextravasation cell migration have emerged: (1) Following extravasation cancer cells do not appear to replicate until after they have migrated to specific sites in tissue. It is possible that these sites may contain substances that attract cells and presumably enhance tumor growth. (2) Extravasation and postextravasation cell movement appear to be distinctly different migratory processes, because in the cases we have examined each process depends on a separate molecular basis. (3) During cell movement *in vivo,* cells can undergo considerable deformation, stretching out long extensions (20–30 μm) through the tissue. These extensions could potentially allow cells to sample their environment and move through the tissue matrix to preferred sites for growth. Thus, the possibility exists that tumor metastasis could be suppressed by blocking the postextravasation migration of cancer cells.

D. Postextravasation Cancer Cell Growth

Only a small proportion of cancer cells that enter the circulation from a primary tumor go on to form metastases, either clinically or in experimental animals (Liotta *et al.,* 1974; Butler and Gullino, 1975). Thus the metastatic process is "inefficient" (Weiss, 1983, 1990). Knowing where in the metastatic process this inefficiency occurs is of prime importance in devising strategies for preventing metastasis. In the organs we have examined, name-

ly, chick CAM and mouse liver, and for the cancer cell lines we have utilized, the vast majority of injected cells (generally >80%) are able to survive in the microcirculation and have successfully extravasated by 1–3 days later. Therefore, the fact that the number of tumors produced is so small must mean that very few extravasated cells ever succeed in growing into tumors. The fate of extravasated cells is thus of primary importance in understanding metastatic inefficiency.

In the mouse liver model, we quantified the proportions of injected melanoma cells (B16F1) remaining at successive stages of the metastatic process (Luzzi *et al.,* 1998). The key findings are summarized in Fig. 6. The vast majority (>81%) of the injected cells remained as solitary extravasated cells at day 3; only 2% of the injected cells had started to divide and form micrometastases of 4–16 cells. (Our recent use of GFP-transfected cells now allows such micrometastases to be visualized with remarkable clarity by *in vivo* videomicroscopy; Figs. 3c and 3d). No larger colonies of cells were observed at this time. By day 13, only 1 in 100 of these micrometastases had gone on

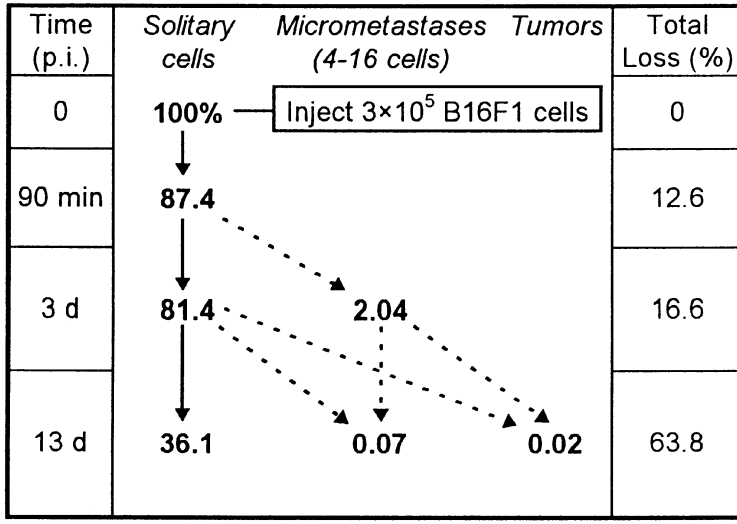


Fig. 6 Flowchart summarizing survival data for B16F1 cells in mouse liver, showing the multistep nature of metastatic inefficiency. Percentages of injected cells remaining as solitary cells, or forming micrometastases or macroscopic tumors, at different times after injection (postinjection: p.i.) are shown. (At 90 min, >85% of injected cells were intravascular, whereas by 3 days >80% had completed extravasation.) Note the slow loss of solitary cells with time. Dotted arrows indicate possible origins of micrometastases and tumors. Two distinct steps after extravasation were principal determinants of metastatic inefficiency: the failure of solitary cells to initiate growth, and the failure of micrometastases to continue growth into macroscopic tumors. (Republished with permission from Luzzi *et al.,* 1998.)

to form tumors (similar to those seen in Fig. 3e) and the rest had disappeared; more than 36% of the injected cells still remained as solitary cells. This observation on solitary cells could result from a balance of proliferation and death resulting in a steady-state situation, or it could mean that these solitary cells are simply remaining dormant. We addressed this question using immunohistochemistry. The cells were stained in adjacent serial sections for markers for apoptosis (TUNEL) and proliferation (Ki-67). The identification of the melanoma cells was made using immunostaining for S100. Only 5% of the solitary cells were either undergoing proliferation or apoptosis. By comparison, 91% of the tumor cells in metastases were proliferating and 6% were undergoing apoptosis. These findings indicate that the solitary cells were in fact dormant. If these results also apply clinically, then these solitary, dormant cancer cells potentially pose a double threat to cancer patients. Dormant cells are not effectively treated by conventional cancer therapies that target only actively proliferating cancer cells. In addition, dormant cells could potentially be activated at a future time and start dividing to form metastases.

Our observations of extravasated cancer cells in mouse liver suggested that the main sources of metastatic inefficiency are found at two stages of tumor development. Only a very small fraction of solitary, extravasated cells (1 in 40 by day 3) began to divide to form micrometastases (4–16 cells). In addition, only a tiny fraction of these micrometastases (1 in 100) continued dividing to form tumors; most of the rest disappeared. These results suggest that initiation and maintenance of growth of micrometastases, as well as the activation of dormant, solitary cancer cells, could be important targets for future therapeutic strategies.

These concepts arise from our work on chick CAM and mouse liver concerning postextravasation cell growth: (1) A high proportion of extravasated cells can remain in the tissue for extended periods as solitary, dormant cells. (2) Metastatic inefficiency occurs as a two-step process with only a small proportion of extravasated cancer cells dividing to form micrometastases (4–16 cells). Only a tiny fraction of the micrometastases then go on to form tumors, while most of the rest disappear. Our results indicate that failure of extravasated cells to initiate growth in the target organ and failure of micrometastases to grow into macroscopic tumors may be two major rate-limiting steps in metastasis.

V. TARGETS FOR ANTI-METASTASIS THERAPY: CLINICAL AND BIOLOGICAL CONSIDERATIONS

The goal for development of anti-metastatic therapeutics should be to prevent the negative physiologic consequences of growth of metastases to the patient. This goal can be met theoretically by a variety of strategies, target-

ed to specific steps in the process, from the prevention of the initial seeding of metastatic cells up to and including control of growth of established metastases (reviewed in Chambers, 1999; Fidler, 1999). This is a broader goal for cancer therapy than that taken by conventional cytotoxic treatment strategies (reviewed in Kohn and Liotta, 1995; Schipper *et al.,* 1996). Instead of requiring that all cancer cells be killed, this approach requires that the effects of the growth of the cells be minimized to the patient, by any strategy that achieves this end. Approaches to combating the physiologic effects of metastases are thus considered here in this context.

Three key questions must be addressed in developing new therapeutic approaches to combat the detrimental consequences of metastatic growth. First, what steps in metastasis offer appropriate targets, in terms of what is known about the *biology* of the metastatic process? Second, is there a *clinical* opportunity to apply a therapy designed to attack the chosen step? Third, are there *molecular* therapeutic approaches that can be developed to target the chosen step? Much of current research into anti-metastatic therapies is focused on this final question, with studies designed to determine, for example, how metastatic and nonmetastatic cells differ at the molecular level. Less attention is often paid to the first two questions, although answers to these questions are necessary in order to put molecular information about metastasis into a biological and clinical context. Careful consideration of biological and clinical factors that contribute to metastasis is required to determine how to use the molecular information that is rapidly accumulating about metastasis, as well as to prevent unnecessary effort being applied to inappropriate therapeutic targets. Here we focus our attention on lessons learned from biological studies on the metastatic process, as outlined earlier, coupled with clinical considerations as to the accessibility of biologically promising therapeutic targets in the natural history of cancer and metastasis in patients.

A. Clinical and Biological Relevance of Specific Steps in Metastasis as Therapeutic Targets

A list of steps that occur during the metastatic process is shown in Fig. 7, beginning with growth of the primary tumor and culminating with growth of the metastatic lesions. For each step in the process, two questions need to be answered: *Is this step a promising therapeutic target, based on what is known about the biology of the process?* and *Is this step a promising therapeutic target, based on the clinical opportunity for applying a therapy targeted to that step?* Only then can one profitably ask whether a drug or other therapeutic approach is available, or can be developed, to target the step in question.

Ideally, one would like to target and prevent the earliest steps possible in the metastatic process, by preventing the *growth of the primary tumor* (see

Fig. 7). Biologically, this step is clearly an appropriate target, and would essentially short circuit the whole metastatic process. Clinically, however, the opportunity to target the growth of the primary tumor is relatively limited, once a patient has been identified as having cancer. The goal for many cancers is to remove the primary tumor as promptly as possible, and this can often be achieved successfully with surgery. The time window for targeting growth of the primary tumor with the aim of preventing metastasis is thus relatively limited. In addition, by the time a tumor has been detected clinically, it may already have released metastatic cells that have progressed through several of the subsequent steps. In this situation, there might be little value in trying to prevent further growth of the primary tumor. In the future, however, this early phase of tumor progression may well be amenable to chemoprevention strategies, especially in patients deemed to be at higher risk for developing subsequent cancer. An example of this strategy is the recent Tamoxifen prevention trial for women at high risk for developing breast cancer (Fisher *et al.*, 1998). Similarly, dietary and lifestyle interventions designed to prevent cancer development in people who are not identified as already having cancer (reviewed by Schatzkin, 1997) would ultimately, if suc-

Steps in metastasis	Good Therapeutic Target: Clinically?	Biologically?
• Growth of 1° tumor	• Limited opportunity, after diagnosis of 1° tumor • Possibly useful in chemoprevention setting?	• Good biological target
• Intravasation	• Limited opportunity, after diagnosis of 1° tumor	• Insufficient evidence
• Survival in circulation	• Unlikely	• Unlikely
• Arrest in new organ	• Unlikely	• Unlikely
• Extravasation	• Unlikely	• Unlikely
• Initiation of growth/ dormant cells	• Promising clinical target	• Promising biological target
• Persistence of growth/ angiogenesis	• Promising clinical target	• Promising biological target

Fig. 7 Steps in the metastatic process and assessment of whether each step offers a good therapeutic target, based on biological and clinical considerations.

cessful, have the effect of preventing metastasis at the very earliest phase of the process.

Relatively little is known biologically or clinically about the *intravasation* process (the escape of cancer cells from the primary tumor to the blood or lymphatic circulation), making it difficult to know if this stage is an appropriate one for intervention (Fig. 7). Few models have been designed for studying the intravasation process by direct experimentation (e.g., see Kim *et al.,* 1998). Clinically, little can be known about the timing or extent of intravasation, for a given tumor. The consequences of intravasation are used clinically in, for example, detection of breast cancer cells in a patient's lymph nodes, as a marker of cells having begun to be shed from the tumor, and the presence of these cells is an indicator of poor prognosis in breast cancer. However, considerably more needs to be learned about the intravasation process, both biologically and clinically, before it can be determined if this step in metastasis offers a promising therapeutic target.

The steps in the metastatic process that include cancer cell *survival in the circulation, arrest in a new organ,* and *extravasation* from the circulation into the tissue of the new organ may be less than optimal steps for the development of anti-metastatic therapies (Fig. 7), for several reasons. First, our biological studies have indicated that cancer cells do not appear to be particularly vulnerable to attack at these stages, and that cells can successfully complete these steps in large numbers, as discussed earlier. In addition, these steps appear to be relatively independent of the malignant phenotype of the cells, with highly and poorly metastatic cells being similarly able to complete these steps. Furthermore, these steps appear to be completed quite rapidly, thus providing a very restricted temporal target. We have found, for a variety of cancer cell types injected to target to several different organs, that the vast majority of a population of circulating cells has completed the process of lodging in an organ and fully extravasating within 1–3 days of being injected into the circulation. For an individual cancer cell, the process of arrest and extravasation appears to require only hours to complete. Thus, these steps appear to be targets of limited utility in terms of the biology of the process.

Similarly, these steps (survival in the circulation, arrest and extravasation) may not be ideal targets in terms of the clinical course of the disease (Fig. 7). In a patient who has just been diagnosed with cancer, there would be a limited temporal window for treatment to prevent these steps in metastasis. If the primary tumor has progressed to the point of having shed cells into the circulation, the majority of those would already have arrested and extravasated in secondary sites, and only cells shed from the primary during the relatively brief time from diagnosis to initial treatment would be vulnerable to treatment of these steps. It is also possible that there may be a window of therapeutic opportunity at the time of primary surgery for cancer, if

appreciable numbers of cancer cells are shed at the time of surgery (discussed in Chambers, 1999). In general, however, this stage of the process seems to be of limited promise as a target, based on both biological and clinical considerations.

A much broader time window is offered by the final steps in the metastatic process, the *growth of metastases in secondary sites* (Fig. 7). Included in this process are the initiation of growth of extravasated cells (or the maintenance of dormancy in a population of single cells), the persistence of growth of pre-angiogenic metastases, and the attraction of new vasculature to support continued growth of metastases. Biologically, this phase of metastasis appears to offer a very promising and multifaceted therapeutic target. Our *in vivo* studies described earlier have consistently pointed to the growth phase, after cancer cells have successfully extravasated in secondary sites, as being key in the regulation of metastasis.

Clinically, the growth phase of the metastatic process also appears to offer an excellent target, since the restriction of growth at any point prior to the metastases causing irreversible physiologic harm to the patient has the potential to be clinically useful. During the clinical course of the disease, this phase occupies a much larger proportion of time than do the steps of arrest in the circulation and extravasation into the tissue.

B. Therapeutic Approaches for Restricting Growth of Metastases

Based on both biological and clinical considerations, it thus appears that the restriction of growth of metastases in secondary sites may offer an especially promising target for development of therapeutic strategies to combat the consequences of metastasis to the patient (Fig. 7). There are a number of approaches by which this could be achieved, and focused attention of research into this area seems warranted. Current cytotoxic chemotherapy is indeed directed at this general target, but is based primarily on the idea that cells populating the metastases need to be dividing rapidly in order to be killed. As is well known, the side effects of these therapies to the patient are based on the nonspecificity of this requirement, and many normal tissues are equally or more sensitive to the cytotoxic effects of this approach. Alternate strategies, based more on growth pathways or other aspects of growth with greater specificity for the cancer cells, are needed (Kohn and Liotta, 1995; Schipper *et al.,* 1996). A number of such strategies are under active development, and an understanding of biological and clinical factors in metastasis will be important for appropriate use of these approaches. Although details of these strategies are beyond the scope of this review, some promising means to achieve this end are mentioned here.

One approach that might lead to growth inhibition of metastases is through *inhibition of signal transduction pathways* used by metastatic tumors to support their growth. Considerable effort is devoted to identifying unique, or preferential, signaling pathways used by malignant cells, and to the development of inhibitors of these pathways. One example of this approach is the development of the cytostatic drug carboxyamidotriazole (CAI), an inhibitor of calcium-mediated signal transduction pathways (Kohn *et al.,* 1992). Interestingly, CAI has been shown to affect growth of tumor cells directly (Kohn *et al.,* 1994) as well as indirectly via an inhibition of metastasis-specific angiogenesis (Luzzi *et al.,* 1999). Another example of the strategy to limit growth of metastases via inhibition of signaling pathways active in malignant cells is the use of anti-HER2/neu monoclonal antibody in women with HER2/neu-positive breast tumors (M. D. Pegram *et al.,* 1998, 1999; Norton, 1999).

Another attractive approach to limiting growth of metastasis is through the inhibition of *angiogenesis,* the development of new blood vessels which are required for persistence of growth of tumors. By limiting the development of new vasculature, metastases would be restricted to small sizes, which are generally of minimal clinical consequences to the patient. This approach is particularly promising in that it is both biologically sound and there would be clinical opportunity for applying this strategy (both for restricting the growth of metastases, whether clinically detected or occult, as well as inoperable primary tumors). This strategy is the subject of considerable attention and research (reviewed by Folkman, 1995, 1996; Pluda, 1997; Zetter, 1998).

C. "Seed" and "Soil" Revisited: Organ-Specific Growth as an Anti-Metastasis Therapeutic Target

It has long been observed that certain tumors show an organ-specific pattern of metastasis. Breast cancer, for example, preferentially metastasizes to bone, liver, brain, and lung. In 1889, Stephen Paget published an article in the *Lancet* entitled "The Distribution of Secondary Growths in Cancer of the Breast" (see also Poste and Paruch, 1989). In his paper, Paget conceptually addressed the question "What is it that decides what organs shall suffer in a case of disseminated cancer?" Paget's conclusion can be summarized by the following quote from his article: "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil." Where metastases finally form is a relatively straightforward question, which can be addressed by looking at the "endpoint" of metastasis, that is, the presence or absence of detectable metastases in specific organs. The question of whether cells from a primary tumor are carried "everywhere" in the body is a much more difficult question to ask.

The concept that certain tumors "go" to specific target organs thus should more properly be replaced with the concept that certain tumors "grow" in specific target organs, since the endpoint of detectable tumors gives evidence only of successful growth, and no indication of whether cells arrived in the organ but failed to grow there. Our experimental *in vivo* studies, described earlier, have suggested that the majority of cells injected to target them to a specific organ in fact arrest and successfully extravasate in that organ, with the initial arrest in the microcirculation being determined primarily by size considerations. Cells from most solid tumors are large, relative to the sizes of most capillaries, although there are size differences both between cancer cell types and between capillaries in different organs. A small proportion of cells may pass through the first microcirculatory bed encountered and on to the next. Consideration of blood flow patterns predict to which organs cells shed from tumor growing in specific organs will be carried first (see Chambers *et al.,* 1995, Fig. 3 therein). For example, cancer cells shed from a primary tumor in a splanchnic organ such as the gastrointestinal tract or pancreas will first be taken by the hepatic portal circulatory system to the liver. Cells shed from a lung tumor would be taken via the arterial system to all parts of the body. Cells from tumors in other organs would be transported via the venous system first to lung capillaries. Patterns of lymphatic drainage of specific organs also are important, and cancer cells can be spread in this fashion as well.

A detailed study of autopsy data, coupled with considerations of relative blood flow to specific organs, was conducted by Weiss (1992). In that study, the presence of metastases detected at autopsy of cancer patients was used to determine the pattern of metastatic spread. Arterial blood flow to specific organs (in ml/min) was used to determine exposure of the organ to circulating cancer cells; arterial flow only was considered, and metastases to liver and lung were excluded. Based on this analysis, Weiss concluded that metastasis to most organs can be accounted for primarily by the relative volumes of blood delivered to the organ. However, some organs were deemed to be "hostile" or "friendly" in their support of growth of cancer cells delivered to the organ. For example, breast and prostate cancers showed preferential growth in bone, above that which would be expected on the basis of volume of blood flow to the bone. This study thus suggests that much of organ-specific metastasis may be due to the volume of blood passing through various target organs. However, several factors could not be assessed with this sort of approach, including numbers of cells shed from different primary tumors, patterns of blood flow between primary and secondary sites, and, importantly, numbers of cells carried to secondary sites. The relationship between numbers of cancer cells that arrest (most of which likely survive and extravasate, as discussed earlier) in an organ, and the formation of detectable metastases in that organ (i.e., organ-specific metastatic inefficiency), is important for a full understanding of organ-specific metastasis.

Based on the considerations mentioned, the important factors in determining organ specificity of metastasis can be summarized as in Fig. 8. This topic has been recently reviewed by Radinsky (1995) and Fidler (1995). Two factors determine the distribution for specific tumor types: the number of cells that arrest in an organ, and the ability of the environment of that organ to support growth of the cells. Metastasis clearly involves interactions between cancer cells (the "seeds"), and specific organs and microenvironments within organs (the "soil"). An improved understanding of the factors that contribute to organ-specific growth promotion or inhibition will be important for the development of anti-metastasis therapies.

Two features of organ-specific metastatic growth are important: First, what factors influence the preferential growth of certain types of cancer cells in specific organs? (That is, why do breast cancer cells preferentially grow in bone?) Second, what factors influence the selective growth of a small subset of a cancer cell population that arrives in an organ? (That is, why do only subsets of breast cancer cells begin and sustain growth in bone, while other

Organ-Specific Metastasis	
① Cancer cells are 'seeded' wherever the circulation takes them	• Circulatory patterns from the primary tumor • Size of cancer cells relative to capillaries in different organs
② Cancer cells grow only where they find a supportive growth environment	• 'Seed' (cancer cell) specific factors *(e.g. oncogenes, receptors, signal transduction pathways that are active, etc.)* • 'Soil' (organ) specific factors *(e.g. growth factors, extracellular matrix components, etc.)*

Fig. 8 Organ-specific metastasis. Two steps in organ-specific metastasis are listed, along with factors that contribute to each of these steps.

cells from the same breast tumor fail to grow? Are there differences within subpopulations of cancer cells, differences in microenvironment where the cells find themselves by chance, or a combination of both?) Clarification of these questions will add to our ability to target growth of metastases as an anti-metastasis therapeutic strategy.

VI. CONCLUSIONS

Metastasis is responsible for the majority of deaths due to cancer. Therapeutic strategies that effectively prevent the development and growth of metastases thus have the potential to impact on cancer mortality. Outlined in this article are biological considerations of the metastatic process, with a focus on information learned from *in vivo* videomicroscopy studies of the metastatic process, coupled with techniques to quantify the loss of cells at various steps in the process. These studies have led to the conclusion that the most promising targets for development of anti-metastasis therapies lie in the growth phase of the process, after cancer cells have arrived in secondary sites and extravasated there. This phase of the metastatic process is also attractive clinically, providing a broad time window for treatment, because clinical utility is possible at any point prior to growth of metastases causing irreversible clinical consequences to the patient. Earlier phases of the metastatic process, such as survival of cells in the circulation, arrest in secondary sites, and extravasation, appear to be less appropriate as therapeutic targets, based both on biological and clinical considerations. Approaches being developed to target the growth phase of metastasis include inhibition of angiogenesis of metastatic tumors, and inhibition of signaling pathways required by metastases for their growth.

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Animal Models of Melanoma: Recent Advances and Future Prospects

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I. INTRODUCTION

The rapid advances in understanding the molecular basis of cancer attained during the past 30 years have placed us at the threshold of translating this information to the cancer patient. As such, a critical need has developed for biological systems to mechanistically dissect and clinically validate

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emerging therapeutic advances. Cell culture-based systems have permitted high-throughput screening assays to identify and evaluate the cytostatic/cytolytic activity and specificity of novel compounds (Weinstein *et al.,* 1997), but such systems are limited to the assessment of drugs that impact on the growth and survival of cancer cells in a cell-autonomous manner. Traditionally, animal tumor models have been employed as a complement to *in vitro* systems in therapeutic studies. However, these models are genetically undefined. Furthermore, in most cases, the tumors do not evolve through stepwise progressive stages in their natural microenvironment and do not arise in an immune competent host, hence their fidelity to human malignancies is unclear. The evolutionary conservation of genes and gene function has provided the basis for more recent attempts to create faithful animal models for specific human cancers. New technologies of genetic manipulation, particularly in the mouse, have permitted the generation of cancer-prone animals that harbor genetic lesions homologous to those found in human cancers (Cohen, 1999; McClatchey and Jacks, 1998). Not only can they serve as *in vivo* systems to evaluate efficacy of drugs identified by other assays, these engineered animal models can provide a tractable system for the elucidation of tumorigenic mechanisms. Such understanding obtained through detailed molecular and genetic characterization will permit correlation of specific mutations with cancer genesis and progression, identification of potential markers for diagnosis and staging, and ultimately facilitate the development and design of therapeutics directed against novel targets.

This article reviews the current knowledge of the genetic basis of human melanoma and describes how such information is being employed to construct and refine *in vivo* animal model systems for this disease. Following this treatise on the human disease, focus is placed on the mouse and how genetics and transgenic technology are being used to generate an accurate melanoma model.

II. CLINICAL ASPECTS OF MELANOMA

A. Malignant Progression

The incidence of malignant melanoma increased steadily during the last half of the 20th century. In the United States the lifetime risk is approximately 1 in 90 and the mortality is 1 in 400 (Parker *et al.,* 1997). The high metastatic potential and therapeutic resistance of melanoma present great challenges to the management of this disease (Balch *et al.,* 1997). Melanoma arises from the melanocyte, pigment-producing cell of the skin, eyes, and inner ears (Bennett, 1993). The pathogenesis of this disease involves a

multistep process in which there is phenotypic progression from benign melanocytic precursor lesions, through a number of increasingly dysplastic intermediates eventually culminating in a highly invasive and metastatic tumor (Meier *et al.,* 1998; Newton Bishop, 1997). Although the earliest stages of melanoma genesis and progression are not well defined, the first transition appears to be the conversion of a normal melanocyte into a dysplastic nevus (atypical mole). These precursor lesions progress to *in situ* melanoma, which remains confined to the epidermis, growing laterally and hence is defined as the radial growth phase (RGP). RGP melanomas can be effectively treated by surgery with very low risk for recurrence or metastasis (Herlyn, 1993). Further progression to the vertical growth phase (VGP) is typified by invasion of the melanoma cells into the dermis, an event that presages the acquisition of metastatic potential and poor clinical outcome. VGP melanoma cells show numerous cytogenetic abnormalities suggesting that they are genomically unstable. Patients with VGP melanoma have an 8-year survival rate of 71% (Herlyn, 1993). Metastatic melanomas are highly aggressive and have the propensity to spread to any organ including the brain and heart (Balch *et al.,* 1997). Although there are rare reports of remissions (both with and without therapy), there is no effective treatment for metastatic melanoma and patients with advanced disease have a dismal prognosis (mean survival 6 months) (Balch *et al.,* 1997). Tumor staging systems based on histologic parameters have been devised to assess the degree of melanoma progression. These useful diagnostic systems are hampered by the absence of genetic and biochemical markers that unambiguously distinguish disease stages (Greene, 1997). The identification of specific molecular markers linked to stages of phenotypic progression would be expected to yield a revised staging system with increased reproducibility and improved predictive power (for a review of potential markers, see Meier *et al.,* 1998).

B. Epidemiology

Exposure to ultraviolet (UV) radiation and fair skin complexion are important risk factors for melanoma (Goldstein and Tucker, 1993; Langley and Sober, 1997). The incidence in fair-skinned individuals is related to place of residence with increased risk associated with more sun-exposed environments. The number of nevi (moles) is another risk factor and may itself be influenced by sun exposure (Rigel *et al.,* 1989). The recent rise in melanoma incidence can be explained in part by altered patterns of sun exposure, perhaps due to increased popularity of sun tanning as a leisure activity and the relative ease of global travel or migration of fair-skinned individuals to more sun-intensive environments (Gilchrest *et al.,* 1999). Further evidence for the role of UV radiation in melanoma induction derives from the risk conferred

by multiple episodes of acute sunburn during childhood and the increased susceptibility of individuals with inherited defects in DNA repair mechanisms, such as in xeroderma pigmentosum and ataxia telangiectasia patients (for review, see Halpern and Altman, 1999). The mode of exposure associated with increased risk of melanoma appears to differ from other skin cancers (Gilchrest *et al.,* 1999). Whereas squamous-cell and basal-cell carcinomas arise in areas of the body receiving maximal dosages of solar radiation, melanoma usually occurs in regions that have intermittent exposure. In addition, unlike the other types of skin cancer, melanoma risk is dependent on episodes of strong intermittent exposure rather than total cumulative exposure to the sun. In contrast, melanoma in darker skinned individuals occurs at lower frequency, predominantly in concealed areas such as the sole of the foot, and thus is not correlated with sun exposure (Armstrong and Kricker, 1993).

Other genetic factors not directly related to increased susceptibility to UV radiation are predisposing to melanoma (reviewed in Haluska and Hodi, 1998). For example, there exist melanoma-prone families whose susceptibility is linked to germline defects in the INK4a/ARF locus (see below) (Kamb *et al.,* 1994).

C. Melanocyte Biology and Melanoma

The epidemiologic profile of melanoma may be explained by consideration of several of the unique biological features of melanocytes. Human epidermal melanocytes play an important role in the protection of epidermal keratinocytes, a cell type that is highly sensitive to UV radiation-induced apoptosis. In response to UV light, melanocytes become increasingly dendritic, induce synthesis of the photoprotective pigment, melanin, resulting in tanning of the exposed skin, and enter the cell cycle (Eller *et al.,* 1996; Gilchrest *et al.,* 1996). Melanin is synthesized in membrane-bound organelles known as melanosomes that are transported to cover the nuclei of adjacent keratinocytes, thereby protecting the DNA of these cells (Bennett, 1993; Sturm, 1998). The activation of melanocytes is dependent on synergistic paracrine stimulation by adjacent keratinocytes. A number of keratinocyte-derived, melanocyte-stimulatory factors have been identified, including endothelin-1, alpha-melanocyte stimulating hormone (alpha-MSH), and basic fibroblast growth factor (Gilchrest *et al.,* 1996; Imokawa *et al.,* 1992; Tada *et al.,* 1998). In contrast to the highly proliferative and UV-sensitive nature of keratinocytes, melanocytes rarely divide in normal skin, survive well on UV exposure, and possess a robust DNA damage repair response (Hatton *et al.,* 1995). Based on these fundamental differences between keratinocytes and melanocytes, Gilchrest *et al.* (1999) have proposed a model

to rationalize the epidemiologic features of either type of skin cancer. Chronic moderate exposure to radiation predisposes to basal cell carcinoma, but not melanoma, since keratinocytes are less able to repair damaged DNA. The higher level of DNA damage associated with episodes of intense exposure is likely to result in apoptosis of keratinocytes, whereas under such conditions melanocytes survive and permit propagation of oncogenic mutations. The mechanism for survival may involve the inhibition of some aspects of p53 signaling (Tada *et al.,* 1998) and the constitutive expression of the anti-apoptotic factor, Bcl-2 in melanocytes (Plettenberg *et al.,* 1995; Selzer *et al.,* 1998). Irradiated melanocytes are able to induce p53 accumulation and transactivation of target genes such as p21 (Medrano *et al.,* 1995; Tada *et al.,* 1998). In response to this signaling pathway, melanocytes in culture undergo G_1 arrest. The administration of keratinocyte-derived factors such as alpha-MSH and ET-1, however, leads to a bypass of G_1 arrest in melanocytes despite the accumulation of functional p53 (Im *et al.,* 1998; Tada *et al.,* 1998). These cells also maintain RB in its growth inhibitory hypophosphorylated state and thus it appears that the survival and proliferative factors must be acting downstream of p53 and RB (see later discussion of the RB and p53 pathways). Members of the CREB (cyclic AMP response element binding protein)/ATF (activating transcription factor) family have been implicated in conferring radiation resistance to these cells (Jean *et al.,* 1998; Ronai *et al.,* 1998). Elucidating the basis for survival and enhanced DNA repair may help to explain the ability of melanoma cells to resist chemo- and radiotherapy.

III. MOLECULAR BASIS FOR MELANOMA

A. Genetics of Familial Melanoma

Familial cancer syndromes, despite their rarity relative to sporadic cancers, continue to provide substantive insights into the disturbed molecular circuitry present in a broad spectrum of malignancies (Fearon, 1997). The availability of extensive tumor-prone pedigrees in conjunction with evolving methodologies of genetic mapping has permitted the isolation of a number of important cancer susceptibility genes. Indeed, genetic studies of the familial form of melanoma provided a basis for the identification of a melanoma tumor suppressor (reviewed in Haluska and Hodi, 1998). Early epidemiologic studies recognized an inherited component to melanoma susceptibility that is now estimated to account for 8–12% of malignant melanoma cases (Fountain *et al.,* 1990). Cytogenetic and loss of heterozygosity (LOH) studies pointed to chromosomes 1p and 9p as potential loca-

tions for key melanoma susceptibility genes (for review, see Welch and Goldberg, 1997). Genetic linkage analyses of the chromosome 1p melanoma locus have produced conflicting results that may relate to confounding factors of differential diagnosis and inadvertent inclusion of sporadic melanomas. In contrast, linkage analysis of a subset of melanoma-prone families using chromosome 9p markers proved more definitive with subsequent molecular analysis of tumor specimens leading to the assignment of the INK4a gene as the familial melanoma gene. This gene resides at a locus that sustains homozygous deletions in a wide range of cancer types (Kamb *et al.*, 1994). Although the product of the INK4a gene, the cell cycle inhibitor $p16^{INK4a}$, has been convincingly shown to be a melanoma tumor suppressor in humans, the frequent occurrence of large homozygous deletions and the capacity of this locus to encode two additional regulators of growth and survival have raised questions regarding whether $p16^{INK4a}$ is the singular 9p21 tumor suppressor (Ruas and Peters, 1998).

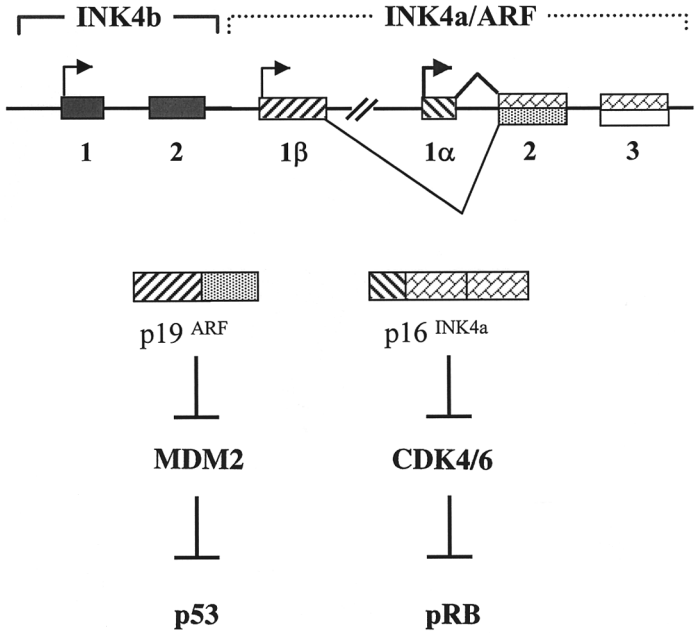

Fig. 1 The genomic organization of the 9p21 locus. $p16^{INK4a}$ and $p19^{ARF}$ share exons 2 of the INK4a/ARF gene. The products of this locus regulate both the p53 and RB pathways. $p16^{INK4a}/p15^{INK4b}$ cause cell cycle arrest by inhibiting the ability of CDK4/6-cyclin D complexes to phosphorylate RB. $p19^{ARF}$ physically interacts with MDM2, thereby preventing MDM2-mediated degradation of p53.

The INK4a gene and its neighbors have highly unusual genomic organization (for review, see Kamb, 1995; Haber, 1997). There are two upstream exons (1α and 1β) driven by separate promoters and the resulting alternative transcripts are spliced into two shared downstream exons (designated exons 2 and 3) (see Fig. 1). Although the different first exons splice into a common acceptor site in the second exon, the AUG-initiated open reading frames of each first exon continue in distinct open reading frames in the second exon. Thus, despite shared downstream nucleotide sequences, each transcript encodes a completely distinct protein product. Transcript initiating from the proximal promoter (1α) encodes $p16^{INK4a}$, a cyclin-dependent kinase inhibitor (INK4) that binds to cyclin-dependent kinases 4 and 6 (CDK4/6) and inhibits CDK4/6 phosphorylation of RB (for review, see Sherr, 1996; Weinberg, 1995). Loss of function mutations in all INK4a exons have been found to segregate with malignancy in some melanoma-prone kindreds, demonstrating that indeed this gene encodes a melanoma tumor suppressor gene (for review, see Haluska and Hodi, 1998). The second transcript, designated $p19^{ARF}$ (or $p14^{ARF}$ in humans) (Alternative Reading Frame), also encodes a negative regulator of cell growth (Quelle *et al.,* 1995) that inhibits MDM2, thereby stabilizing p53 (Kamijo *et al.,* 1998; Pomerantz *et al.,* 1998; Stott *et al.,* 1998; Zhang *et al.,* 1998). A final notable feature of this locus is the immediate proximity of another highly related INK4 family gene encoding a CDK4 inhibitor, $p15^{INK4b}$. Hence, this region of chromosome 9p21 is remarkable in that it harbors a cluster of genes that impinges on the two major growth/survival pathways, RB and p53 (see Fig. 1). The evolutionary advantage of such a genomic organization, which is conserved in man, rats, rodents, and opossum (Stone *et al.,* 1995; Mao *et al.,* 1995; Duro *et al.,* 1995; Swafford *et al.,* 1997; Quelle *et al.,* 1995; Sherburn *et al.,* 1998), is not immediately evident. One conjecture is that this gene organization exists to permit coordinate regulation of both INK4a and ARF, perhaps at the level of chromatin structure. In support of this notion, Bmi-1 regulates cell growth and senescence through the INK4a/ARF locus (Jacobs *et al.,* 1999). The *bmi-1* gene is a member of the polycomb group, a family of proteins that maintains stable repression of target genes by packaging DNA into higher order chromatin structures (Bell and Felsenfeld, 1999). *bmi-1*-deficient mice suffer neurologic and lymphoid defects, their MEFs are impaired in S phase progression and undergo premature senescence in conjunction with elevated $p16^{INK4a}$ and $p19^{ARF}$ levels (Jacobs *et al.,* 1999). Loss of INK4a/ARF function in *bmi-1*-deficient background dramatically rescues both the neurologic and lymphoid defects in mice as well as the premature senescence phenotype in MEFs, indicating that Bmi-1 functions upstream of $p16^{INK4a}$ and $p19^{ARF}$ *in vivo,* thus linking the INK4a/ARF locus to chromatin regulation.

B. The INK4a Tumor Suppressor Gene

$p16^{INK4a}$ is the founding member of the INK4 family of proteins, which includes $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, all sharing the capacity to control the RB-regulated G_1/S transition. By inhibiting the phosphorylation of RB by CDK4/cyclin D, the INK4 proteins ensure that RB remains in a complex with the E2F transcription factor (for review, see Sherr and Roberts, 1999; Weinberg, 1995). These RB/E2F complexes recruit histone deacetylase to promoter and repress transcription of target genes, leading to G_1 arrest (for review, see Dyson, 1998; Brehm and Kouzarides, 1999). In the absence of inhibition by the INK4, CDK4/cyclin D catalyzes an initial phosphorylation of RB. CDK2/cyclin E subsequently phosphorylates RB at other sites, thereby releasing E2F, which proceeds to activate genes necessary for progression into S phase. Recent evidence has also indicated that CDK4 can indirectly regulate CDK2/cyclin E by sequestering Cip/Kip proteins (p21 and p27), inhibitors of cyclin E- and A-dependent kinases. The binding of INK4 to CDK4/cyclin D not only inhibits the CDK4 kinase activity but also displaces Cip/Kip, which can proceed to inhibit CDK2/cyclin E function (reviewed in Sherr and Roberts, 1999).

Many of the $p16^{INK4a}$ mutations associated with familial melanoma have been tested in functional assays and are deficient in their ability to bind CDK4/6 and/or inhibit its catalytic activity (Ruas and Peters, 1998). Consistent with these data, the crystal structure of $p16^{INK4a}$ complexed to CDK6 reveals that these $p16^{INK4a}$ mutations map to residues in direct contact with CDK6 (Russo *et al.,* 1998). Tumors with loss of $p16^{INK4a}$ function have excess CDK4 activity resulting in a shift toward hyperphosphorylated RB, facilitating the G_1 exit. Interestingly, in a subset of familial melanomas not showing linkage to chromosome 9p and in some sporadic melanomas, there are mutations in CDK4 that prevent $p16^{INK4a}$ binding, rendering this kinase constitutively active (Tsao *et al.,* 1998a). CDK4 mutations thus appear to be epistatic to INK4a loss with respect to melanoma genesis, suggesting that the critical function of $p16^{INK4a}$ is to regulate CDK4/6 activity. Furthermore, the occurrence of RB and INK4a mutations in cancers is inversely correlated, again supporting the notion that these genes are functioning in the same pathway (Ruas and Peters, 1998). Moreover, recent epidemiologic studies have observed a greatly increased incidence of melanoma in survivors of hereditary, but not sporadic, retinoblastoma (Draper *et al.,* 1986; Moll *et al.,* 1996; Traboulsi *et al.,* 1988).

The incidence of INK4a mutation has been compiled for a wide range of cancers (Quelle *et al.,* 1995; Pomerantz *et al.,* 1998; for review, see Ruas and Peters, 1998; Sharpless and DePinho, 1999). In the case of melanoma, the incidence of INK4a mutations is much higher in familial versus sporadic cases, approaching 50% when stringent criteria are used to define a familial

setting. When families with linkage to 9p21 were analyzed, the frequency of INK4a mutation may be greater than 85% (Haluska and Hodi, 1998). The discovery of a mutation (G-34T) in the INK4a 5′ untranslated region (5′ UTR) in a number of melanoma-prone families indicated that the search for noncoding mutations may reveal still higher incidence of INK4a involvement in familial melanoma (Liu *et al.,* 1999). The G-34T mutation creates an aberrant initiation codon that is out of frame with $p16^{INK4a}$ and produces a truncated peptide. The mutation is likely to behave as a null allele since the novel AUG codon appears to inhibit translational initiation from the wild-type start site.

The frequency of INK4a mutations in sporadic melanomas has been the subject of ongoing study. Mutational profiles of melanoma cell lines have revealed INK4a alteration (mainly by homozygous deletions) in virtually all cases, however, it is now apparent that primary melanomas are much less likely to harbor defects in this gene (Walker *et al.,* 1998). Recent studies of primary melanomas suggest that the frequency of INK4a mutation in early-stage tumors is approximately 15%, although the relative occurrence of homozygous deletions versus point mutations remains unclear (Fujimoto *et al.,* 1999; Gruis *et al.,* 1995). The transcriptional silencing by methylation of the promoter appears to be a mechanism of disrupting INK4a in many tumor types (Herman *et al.,* 1995), although this does not appear to be common in melanoma (Fujimoto *et al.,* 1999). INK4a is also important in the pathogenesis of a number of other tumors, most notably sporadic pancreatic cancers, more than 50% of which show alterations at this locus (Rozenblum *et al.,* 1997).

The deletion of INK4b in conjunction with loss of INK4a in a subset of tumors and the absence of INK4a mutation in some tumors showing LOH at 9p21 has fueled speculation that INK4b itself may function as an additional 9p21 tumor suppressor (Wagner *et al.,* 1998). However, in humans, tumor-associated mutations that exclusively target INK4b have yet to be identified. Similarly the highly related INK4 members, INK4c and INK4d, are rarely mutated in human malignancies (for review, see Ruas and Peters, 1998). In the mouse, however, INK4c has been shown to be involved in the suppression of pituitary tumors (Franklin *et al.,* 1998).

$p16^{INK4a}$ is very frequently lost in the establishment of culture cell lines, a phenomenon that is likely explained by the prominent role of this protein in cellular senescence (reviewed in Huschtscha and Reddel, 1999). Primary cells in culture have a genetically determined replicative life span and eventually undergo permanent cell cycle arrest (reviewed in Reddel, 1998). Continuous propagation in culture (immortalization) requires the occurrence of genetic changes that abrogate pathways leading to senescence. There are several lines of emerging evidence that $p16^{INK4a}$ loss is a potent immortalizing event. Primary cells undergoing senescence accumulate $p16^{INK4a}$ and, conversely,

immortalized cells frequently lose $p16^{INK4a}$ function (Hara *et al.,* 1996; Noble *et al.,* 1996). Forced expression of $p16^{INK4a}$ induces premature senescence indicating that it plays a direct role in this process (Serrano *et al.,* 1997; Vogt *et al.,* 1998; J. Zhu *et al.,* 1998). Correspondingly, the loss of $p16^{INK4a}$ is perhaps the most common lesion in the establishment of cultured cell lines (for review, see Ruas and Peters, 1998).

C. The $p19^{ARF}$ Gene

Mounting evidence has supported a role for the alternative product of the INK4a/ARF locus, $p19^{ARF}$, as a suppressor of mouse and human cancers. Homozygous deletions or point mutations that affect both INK4a and ARF are common in tumors, however mutations that specifically target ARF are relatively infrequent and no inherited tumorigenic mutations have been identified (reviewed in Ruas and Peters, 1998). T-cell acute lymphoblastic leukemia (T-ALL) provides the strongest evidence that in a human malignancy $p19^{ARF}$ possesses inherent tumor suppressor activity independent of $p16^{INK4a}$ (Gardie *et al.,* 1998). Rearrangement of INK4a/ARF is characteristic of T-ALL. The ARF open reading frame is disrupted or deleted by these rearrangements, whereas INK4a remains unaffected in a subset of cases. Like INK4a, ARF is subject to silencing by promoter methylation, hence some tumors may potentially lose ARF function in the absence of intragenic mutations (Robertson and Jones, 1998).

Despite the relative paucity of data showing specific involvement of ARF in human tumorigenesis, cell culture experiments and mouse genetic studies have confirmed a tumor suppressor role for this protein. Although there are highly conserved domains, most notably in the amino terminus, mouse and human ARF share only modest homology (53% for exon 1 and 63% for exon 2) (compiled in Sharpless and DePinho, 1999). Alignment of the predicted amino acid sequence of $p19^{ARF}$ revealed no substantial homology to other known proteins and thus provided few clues to its function. However, $p19^{ARF}$ level is significantly high in p53 null cell lines and ectopic expression of $p19^{ARF}$ induces potent G_1 and G_2 phase arrest (Quelle *et al.,* 1995). This provocative result, coupled with two subsequent lines of genetic evidence, led to the first proposals that $p19^{ARF}$ functions in the same pathway as the p53 tumor suppressor gene, a central regulator of neoplastic processes (Chin *et al.,* 1997; Kamijo *et al.,* 1997). Analyses of melanomas arisen in $INK4a^{\Delta 2/3}$ knockout (KO) mice (null for both $p16^{INK4a}$ and $p19^{ARF}$) revealed that, in all cases, p53 remained in wild-type configuration, suggesting that in the absence of ARF, there was little selective pressure to mutate p53 (Chin *et al.,* 1997). This reciprocal mutation relationship, reminiscent of that described for $p16^{INK4a}$ and RB (Ruas and Peters, 1998), raised the possibility that

$p19^{ARF}$ and p53 functions in the same pathway. Similarly, immortalization of ARF+/− MEF in cultures involved loss of either $p19^{ARF}$ or p53 in a mutually exclusive manner, indicating that p53 and ARF inactivation might represent an alternative mechanism for bypassing the senescence block (Kamijo *et al.,* 1997). Moreover, ARF-null mice are highly cancer prone, displaying a tumor spectrum that is overlapping with that of p53 null (Kamijo *et al.,* 1997, 1999). Together, these *in vitro* and *in vivo* genetic data built a strong case for a common pathway on which $p19^{ARF}$ and p53 reside.

Consistent with the genetic data suggesting a functional link between $p19^{ARF}$ and p53 proteins, subsequent biochemical experiments demonstrated that $p19^{ARF}$ indeed stabilize and enhance p53 level (Kamijo *et al.,* 1998; Pomerantz *et al.,* 1998; Stott *et al.,* 1998; Zhang *et al.,* 1998). This stabilization is mediated by the physical interaction of $p19^{ARF}$ and MDM2. p53 level is low under normal conditions but can be transiently induced to high level in response to a wide range of stimuli such as DNA damage, oncogene activation, and hypoxia (reviewed in Giaccia and Kastan, 1998). This increase in p53 is accompanied by an induction of its ability to directly activate transcription of target genes, resulting in either cell cycle arrest or apoptosis depending on the cellular context (reviewed in Levine, 1997). The level of p53 is stringently regulated on multiple levels, most notably via a negative feedback loop with MDM2 (reviewed in Prives, 1998). MDM2 can bind to p53, repressing transcriptional activation of its targets (Haupt *et al.,* 1997; Kubbutat *et al.,* 1997). Moreover, MDM2 binding of p53 catalyzes the proteosomal degradation of p53 through the ubiquitin ligase pathway (Fuchs *et al.,* 1998; Thut *et al.,* 1997). The necessity of this strict regulation of p53 by MDM2 is best illustrated by the embryonic lethality resulted from MDM2 deficiency, which is completely rescued in the p53 null state (Montes de Oca Luna *et al.,* 1995; Jones *et al.,* 1995). $p19^{ARF}$ can bind to MDM2 and inhibit MDM2-mediated ubiquitination of p53, likely by blocking the ubiquitin ligase activity of MDM2 (Honda and Yasuda, 1999; Pomerantz *et al.,* 1998). Moreover, $p19^{ARF}$ can colocalize with MDM2 in the nucleolus (Tao and Levine, 1999; Weber *et al.,* 1999; Zhang and Xiong, 1999). It has been postulated that nucleolar localization of $p19^{ARF}$ serves to sequester MDM2 from the nucleoplasm where p53 is, thus freeing p53 to act on its target genes (Weber *et al.,* 1999). Alternatively, nucleolar compartment may represent part of the export machinery that can efficiently transport p53 from the nucleoplasm to the cytoplasm where proteosomal degradation occurs (Tao and Levine, 1999). In the mouse, it has been shown that the Δ26-37 mutant of ARF has lost nucleolar localization as well as its ability to arrest cell cycle and stabilize p53 (Weber *et al.,* 1999). In humans, however, the finding is less clear, but disruption of this nucleolar localization in some tumor-associated ARF mutants appears to partially reduce ARF's ability to stabilize p53 (Zhang and Xiong, 1999). Together, these data strongly suggest that there is

an important nucleolar-associated process, although the precise mechanism or biological consequences of which have yet to be identified.

The experiments discussed here firmly place $p19^{ARF}$ in a pathway with p53. However, because p53 is activated by diverse stimuli and triggers two distinct effector pathways (cell cycle arrest and apoptosis), it is important to delineate specific signals that induce $p19^{ARF}$ and the physiologic consequences of this induction. Evidence for a role of $p19^{ARF}$ in signaling inappropriate proliferation and inducing p53-dependent apoptosis was first demonstrated in the setting of RB deficiency (Pomerantz *et al.,* 1998). In the murine lens, RB deficiency alone is sufficient to cause abnormal proliferation of normally postmitotic lens fiber cells. This aberrant proliferation activates an apoptotic response that is dependent on functional p53 (Morgenbesser *et al.,* 1994). The observation that this apoptosis is significantly suppressed in lens that are doubly null for RB and INK4a provided *in vivo* evidence that $p19^{ARF}$ loss partially reversed p53-dependent apoptosis in the setting of RB deficiency, linking $p19^{ARF}$ to p53-dependent apoptosis (Pomerantz *et al.,* 1998). Subsequently, studies from several groups have convincingly established ARF as the mediator that signals inappropriate proliferation to p53 as a result of oncogenic stimulation by E1a (de Stanchina *et al.,* 1998), myc (Zindy *et al.,* 1998), v-abl (Radfar *et al.,* 1998), RAS (Palmero *et al.,* 1998), and E2F (Bates *et al.,* 1998; Robertson and Jones, 1998). On the other hand, ARF null MEFs were shown to able to transiently induce p53 and undergo G_1 arrest upon γ irradiation (Kamijo *et al.,* 1997). Moreover, human ARF-null tumor cell lines such as MCF7 and A375 retained the ability to upregulate p53 on treatment with actinomycin D (Stott *et al.,* 1998). These data would suggest that ARF does not signal DNA damage due to γ radiation or genotoxic drugs to p53.

D. Other Mutations

A notable aspect of the mutational profile of human and mouse melanomas is the very low frequency of p53 mutations in contrast to its prevalence in other adult malignancies (Gruis *et al.,* 1995; Sparrow *et al.,* 1995). These tumors are also unusual in that advanced-stage tumors express elevated amounts of wild-type p53 (Hartmann *et al.,* 1996). Mutations characteristic of UV mutagenesis (i.e., C-T or CC-TT transitions) do occur in p53 in some melanomas but are not always involved in melanoma genesis (i.e., mutations may be in the primary tumor but not in the metastases) (Zerp *et al.,* 1999). Thus, it appears likely that minimal selective pressure exists to lose p53 function for the genesis and progression of melanoma, perhaps reflecting the epistatic relation to ARF or the deregulation of signaling pathways downstream of p53 (see earlier discussion).

Recurrent structural abnormalities and LOH at chromosome 10q indicate the existence of a melanoma tumor suppressor gene in this region. The PTEN (phosphatase and tensin homolog deleted on chromosome 10)/MMAC (mutated in multiple advanced cancers) gene, mapping to chromosome 10q24, is an attractive candidate (Li *et al.,* 1997; Steck *et al.,* 1997; Li and Sun, 1997). This gene encodes a negative regulator of the phosphoinositide-3-kinase (PI3K)–PKB/Akt signaling pathway that mediates cell proliferation and survival (Stambolic *et al.,* 1998; reviewed in Cantley and Neel, 1999). PTEN mutations are found in a number of other tumor types including advanced stage glial and prostate tumors, endometrial tumors, and melanomas (see later discussion) (reviewed in Cantley and Neel, 1999). Germline PTEN mutations are associated with three clinically related, inherited cancer syndromes: Cowden disease, Lhermitte–Duclos disease, and Bannayan–Zonana syndrome (Liaw *et al.,* 1997; Nelen *et al.,* 1997; Marsh *et al.,* 1997; for review, see Cantley and Neel, 1999). In the mouse, heterozygous PTEN mutations confer a cancer-prone phenotype, although a variable tumor spectrum is observed depending on the specific PTEN mutation and/or the genetic background of the mouse (Di Cristofano *et al.,* 1998; Suzuki *et al.,* 1998; Podsypanina *et al.,* 1999). Melanomas have not been reported in these animals. In human, allelic loss or mutations of PTEN have been described in uncultured melanoma specimens, metastasis as well as established melanoma cell lines (Guldberg *et al.,* 1997; Teng *et al.,* 1997; Tsao *et al.,* 1998b). A mutation/deletion rate of 5–15% in primary tumors has been reported (Guldberg *et al.,* 1997; Tsao *et al.,* 1998b). *In vitro* chromosome transfer assays provide functional evidence that genes on chromosome 10q23 can mediate melanoma growth suppression (Robertson *et al.,* 1998). The LKB1/STK11 gene, mutation of which is associated with Peutz–Jeghers syndrome, encodes a serine/threonine kinase speculated to function as a PTEN regulator (Cantley and Neel, 1999; Jenne *et al.,* 1998). There is overlap of certain clinical features between Cowden disease and Peutz–Jeghers syndrome such as multiple hamartomas and predisposition to certain malignancy. Interestingly about 5% of primary melanomas have inactivating mutations of LKB/STK11 (Guldberg *et al.,* 1999; Rowan *et al.,* 1999).

IV. ROLE OF RECEPTOR TYROSINE KINASES

Receptor tyrosine kinases (RTKs) play crucial roles in coordinated development and proliferation of normal melanocytes and may contribute to uncontrolled growth and progression of melanomas. During development, melanoblasts originate from the neural crest and migrate along a dorsal-lateral path to the epidermis where they differentiate into mature pigment-

producing melanocytes of the integument, choroid, and ear (Bennett, 1993; Goding and Fisher, 1997). The spatiotemporal activation of various RTKs in the developing melanocyte precursors is thought to regulate the migration and terminal differentiation of melanocytes. The essential roles of RTKs in normal melanocyte biology have been demonstrated through the analyses of a variety of pigmentation defects, particularly piebaldism (see later discussion). In view of the dependence of melanocytes on complex RTK signaling for proper orchestration of basic cellular functions essential for development and homeostasis, it would not be surprising that RTK or their downstream signaling pathways would represent candidate targets during melanocyte transformation. In the sections that follow, we discuss specifically several RTKs that have been implicated in melanoma genesis or progression, either through correlative expression analyses in human melanoma cell lines or by genetic experiments in animal models.

A. bFGF/FGFR

Growth factor independence is a characteristic of transformed melanocytes. Extensive expression analyses of genes encoding for various growth factors and their RTKs in primary, metastatic melanomas and derivative cell lines have collectively implicated the acquisition of autocrine signaling loops as an important step in the transformation and progression of melanomas (Chin *et al.,* 1998, and references therein), particularly the autocrine loop involving basic fibroblast growth factor (bFGF) and its cognate receptors (FGFR). Aberrant expression of bFGF induces not only autonomous growth but also downregulation of melanocytic functions, thus may play an important role in melanocyte dedifferentiation. In culture, normal melanocytes require exogenous bFGF for their proliferation, whereas transformed melanocytes uniformly can produce a high level of bFGF, thus acquiring growth factor independence (Becker *et al.,* 1989, 1992). Moreover, abnormal expression of bFGF has been detected in atypical nevi *in situ,* and bFGF/FGFR autocrine loop has been shown to be essential for melanoma growth *in vivo* (Ahmed *et al.,* 1997; Wang *et al.,* 1996). Finally, the central role of bFGF/FGFR signaling in melanoma growth is demonstrated by induction of growth arrest of melanoma cells by interference with the biological activity of bFGF alone (Wang and Becker, 1997; Yayon *et al.,* 1997; Becker *et al.,* 1992).

B. c-Met/HGF/SF

In contrast to the bFGF/FGFR autocrine loop just described, overexpression of another RTK, c-met, has been observed in melanoma tissue and cells

without the accompaniment of its ligand, hepatocyte growth factor/scatter factor (HGF/SF) (Rusciano *et al.,* 1995). HGF/SF is produced in cells of mesenchymal origin, whereas c-met is expressed in adult and embryonic epithelium, indicating that HGF/SF functions almost exclusively as a paracrine regulator under normal physiologic conditions. HGF/SF can stimulate cellular movement or scatter, extracellular matrix degradation, and angiogenesis, any of which could contribute to tumor cell invasion and metastasis (Matsumoto and Nakamura, 1996; Vande Woude *et al.,* 1997). Transformed cells expressing both HGF/SF and c-met show metastatic activity *in vivo* (Jeffers *et al.,* 1998; Rusciano *et al.,* 1995). The autocrine signaling loop of HGF/SF/met is associated with tumorigenesis of a variety of tumors, however, the production of HGF/SF has not been demonstrated in melanoma cells (Halaban *et al.,* 1992; Saitoh *et al.,* 1994). However, in human uveal melanomas, a high level of c-met expression has been correlated with high mitotic response, and preferential dissemination to the liver, where high levels of HGF/SF are normally present (Hendrix *et al.,* 1998).

A transgenic mouse model in which HGF/SF is constitutively overexpressed induces the stepwise development of cutaneous melanoma with 20% of the tumor-bearing mice ultimately developing distant metastasis (Otsuka *et al.,* 1998). It is postulated that, in this model, constitutive expression of HGF/SF exerts genetic pressure and selection for melanocytes that have activated their c-met receptors, thus creating an autocrine loop in the transformed cells. The long latency period of melanoma and low frequency of metastastic disease in HGF/SF transgenic mice indicates that additional mutations, such as INK4a/ARF loss of function, are necessary for melanoma onset and progression.

C. EGFR/Xmrk

Several lines of evidence have pointed to the epidermal growth factor receptor (EGFR) as another candidate RTK important for melanoma development. The human EGFR gene resides on chromosome 7p12-13, a region that is frequently amplified in late-stage melanoma (Koprowski *et al.,* 1985). The amplification of this region corresponds to increased levels of EGFR in melanoma cells (Ellis *et al.,* 1992). Furthermore, analysis of a large series of human cutaneous melanoma showed that increased EGFR expression correlates with disease progression and that EGFR expression is significantly elevated in metastatic melanoma (de Wit *et al.,* 1992). The *Xiphophorus* fish model of melanoma provides additional support for EGFR involvement in melanoma genesis and progression. The aberrant expression of the activated Xmrk gene, a novel EGFR-related RTK, induces melanoma formation with high metastatic behavior (Malitschek *et al.,* 1995).

D. c-Kit/SCF

The importance of RTKs in murine melanocyte biology has been demonstrated by the pigmentation defects described in some mouse strains carrying spontaneous coat color mutations mapping to either the dominant white spotting (*W*) locus on chromosome 5 or the steel (*Sl*) locus on chromosome 10 (Halaban, 1992; Wehrle-Haller and Weston, 1997). Mice homozygous for these mutations suffer from anemia, sterility, and extensive white spotting (piebaldism) (Geissler *et al.,* 1981). Piebaldism is a condition characterized by patches of vitiliginous (white) skin and hair wherein there is a complete absence of melanocytes. This occurs as a result of aberrant migration and survival of melanoblasts during development. The coat color gene at the *W* loci was shown to be c-kit, a protooncogene encoding a RTK, whereas the *Sl* locus was determined to code for the ligand of c-kit, the steel or mast/stem cell growth factor (SCF). The c-kit/SCF signaling has been shown to be required for the melanocyte precursors' initial dispersal and migration on the dorsal-lateral pathway as well as their differentiation and subsequent survival (Morrison-Graham and Weston, 1993; Reid *et al.,* 1995). Mutations of the c-Kit have also been identified in human piebald patients, suggesting this signaling pathway is also involved in normal human melanocyte development (Fleischman *et al.,* 1991; Giebel and Spritz, 1991).

Interestingly, in contrast to the high level of expression in normal melanocytes, the majority of human melanoma cell lines have no detectable level of c-Kit expression. Furthermore, the lack of c-kit expression in melanoma has been associated with higher metastatic potential in nude mice (Gutman *et al.,* 1994; Luca and Bar-Eli, 1998). In addition, the c-Kit ligand, SCF, promotes the growth of normal melanocytes but inhibits the growth of c-Kit-expressing melanoma cells (Lassam and Bickford, 1992; Zakut *et al.,* 1993). Thus, it is postulated that the c-Kit/SCF signaling pathway is necessary in normal melanocytes to maintain its terminally differentiated state and its loss may be necessary in the steps of melanoma genesis (Gutman *et al.,* 1994; Zakut *et al.,* 1993).

E. RTK and Metastasis/Progression

In addition to the autocrine growth promoting effects described, a number of the RTK-activating factors characteristically produced by melanoma cells can also exert profound influence on the immediate microenvironment through paracrine signaling mechanisms. These mechanisms include promotion of angiogenesis, extracellular matrix degradation, adhesive interactions, suppression of immunologic response, and antiproteolytic activity. These biological endpoints of RTK signaling have been proposed to account

for the apparent association of RTK activation with metastatic progression. The relevance of the RTK signaling pathway in melanoma development is further strengthened by the demonstrated role of downstream signaling components, including RAS, PI3K, and PTEN, in processes of cellular transformation (Fig. 2).

V. MODEL SYSTEMS FOR MELANOMA

A. Nonmurine Models

Melanomas occur spontaneously or can be induced in a number of organisms. These experimental systems vary in their fidelity to the human dis-

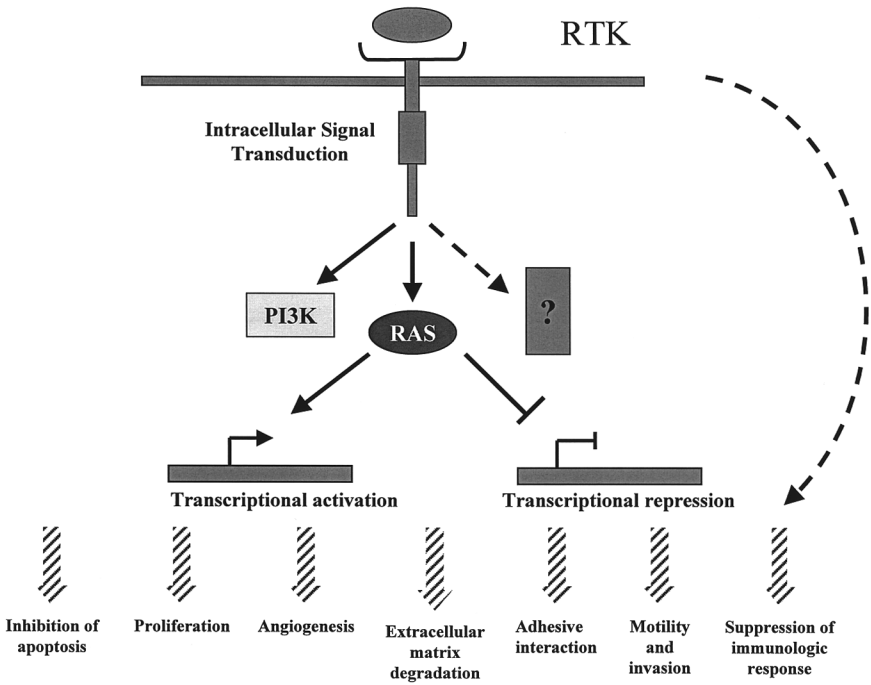

Fig. 2 The role of RTK in melanoma genesis and progression. Activation of RTK by its cognate ligand triggers the initiation of the intracellular transduction pathway through a series of adaptor molecules that may ultimately activate RAS, PI3K, or other effectors. These effectors, in turn, mediate signaling pathways regulating various cell growth and survival responses. The signaling pathways may be independent of transcription or may involve transcriptional activation/repression of gene targets.

ease and in the ease with which they can be manipulated. Because the primary focus of this review is on mouse models, we briefly mention some of the nonmurine model systems. Readers may refer to other comprehensive reviews for detailed discussion on these models (for review, see Kusewitt and Ley, 1996).

The freshwater fish genus *Xiphophorus* is susceptible to both spontaneous and UV-induced melanomas (Kusewitt and Ley, 1996). Hybrid crosses of different species of *Xiphophorus* have revealed the presence of a dominant allele for melanoma induction (Tu) as well as a series of recessive resistance allele (R) which oppose tumorigenesis. Depending on the number of R alleles present, Tu causes black spotting, benign melanoma, or malignant melanoma. The gene at the Tu locus, designated Xmrk, encodes a membrane-bound tyrosine kinase related to the epidermal growth factor receptor (see earlier section). An Xmrk protooncogene is found in all *Xiphophorus* species but the tumor-prone species harbors an extra, mutated copy that encodes a constitutively active oncogenic protein. A *Xiphophorus* homolog of INK4a maps to the R locus and thus it appears that there is conservation of the genetic basis for melanoma susceptibility from teleosts to humans (Kazianis *et al.,* 1998; Nairn *et al.,* 1996). *Xiphophorus* melanoma shows graded progression from benign, hyperpigmented lesions to metastatic tumors. The applicability of the *Xiphophorus* model to human melanoma may ultimately be limited however, due to the evolutionary divergence between organisms. Indeed, the tumor histology and type of melanocytic precursor cell differ substantially from the human disease (Kusewitt and Ley, 1996).

The South American opossum (*Monodelphis domestica*) has been used extensively in studies of UV-induced mutagenesis since the DNA repair pathways of this organism can be regulated experimentally (Kusewitt and Ley, 1996). UV radiation alone can induce melanomas in these animals in the absence of other mutagens or other tumor promoters. Unfortunately, few genetic studies have been done on this organism and there are currently no known inherited alleles conferring melanoma susceptibility. The *Monodelphis* ARF and INK4a genes have been cloned (Sherburn *et al.,* 1998), although melanoma-associated mutations in these genes have not yet been described (Robinson *et al.,* 1998; Sherburn *et al.,* 1998). In contrast to the epidermal origin of most human melanomas, the precursor cell type for *Monodelphis* melanoma originates from the dermis.

The Syrian hamster is susceptible to both spontaneous and chemically induced melanomas (Kusewitt and Ley, 1996). These tumors are characterized by progression from benign nevi to malignant tumors. Both the spontaneous and chemically induced melanomas show metastasis although the former is more aggressive. These animals appear to be resistant to UV-induced melanomas. As in the opossum, melanoma in the Syrian hamster arises from dermal melanocytes, although there has been suggestion that some of the

spontaneous melanomas in Syrian hamster originated from amelanotic melanocytes at the dermal–epidermal junction (Ghadially, 1982).

The systems described have successfully mimicked various aspects of human melanoma genesis, however, methodologies for the precise genetic manipulation of these animals are not yet well developed. Hence, it is not possible to refine these systems as new discoveries are made in the field of melanoma genetics.

B. Mouse Model Systems

The incidence of spontaneous melanomas is extremely low in the mouse. Melanomas are inducible by topical carcinogen treatment, although in the absence of a tumor promoter metastases are rare. Transgenic mouse strain expressing the SV40 early region (both large and small T antigens) under the control of the melanocyte-specific tyrosinase promoter developed spontaneous melanomas predominantly in the ocular site (Bradl *et al.,* 1991). Cutaneous melanomas were not observed presumably due to early fatality resulting from metastatic ocular melanomas. However, in a low susceptibility founder line that does not succumb to ocular melanoma, about 20% of the animals can be induced by UV radiation to develop cutaneous melanoma with metastatic potential (Kelsall and Mintz, 1998). This transgenic model system is of considerable value for studies of melanoma *in vivo* although its utility in identifying collaborating lesions may be somewhat limited since the T antigens modulate diverse growth control pathways (Butel and Lednicky, 1999).

Using the same tyrosinase promoter, others have generated a transgenic mouse strain that expresses activated H-RAS-^{V12G} in melanocytes (Powell *et al.,* 1995). These mice developed hyperpigmentation and melanocytic hyperplasia without melanocytic transformation. However, when induced with DMBA, these mice succumb to cutaneous and metastatic melanomas (Gause *et al.,* 1997). Cytogenetic analyses of derivative cell lines revealed homozygous and partial allelic deletions on chromosome 4, a locus syntenic to human 9p21 where INK4a/ARF locus resides, with resultant absence or reduced expression of p16^{INK4a} gene, as well as p15^{INK4b} and p19^{ARF} in some cases (Gause *et al.,* 1997).

C. Ras and I*nk4a* in a Mouse Model of Melanoma

The mapping of the *Ink4a* gene to the familial melanoma susceptibility locus at 9p21 (Kamb *et al.,* 1994) provided the initiative to generate a mouse model of melanoma mirroring the genetic lesion found in human. Because

the majority of 9p21-associated deletions in human tumors cluster around the exon 2 region of the *Ink4a* gene, conventional targeting strategy was utilized to generate mice with targeted deletion of exons 2/3 of the mouse *Ink4a* gene. Mice homozygous for this deletion ($INK4a^{\Delta 2/3}$) are functionally null for $p16^{INK4a}$ and $p19^{ARF}$ (Serrano *et al.,* 1996; R. DePinho, unpublished data) and they develop a cancer-prone phenotype. In contrast to the individuals with germline mutation of the *Ink4a* gene, these $INK4a^{\Delta 2/3}$ heterozygous and homozygous animals do not develop melanoma; instead they develop primarily B-cell lymphomas and fibrosarcomas (Serrano *et al.,* 1996).

The lack of concurrent phenotypes in mice and humans harboring mutations in homologous tumor suppressor genes is not unusual (Ghebranious and Donehower, 1998; McClatchey and Jacks, 1998). Rather than reflecting fundamental differences in gene function, this phenomenon is likely due to more subtle differences in physiology and in factors that permit collaborating lesions, such as target cell population and microenvironment (dermal versus epidermal melanocytes; see later discussion), life span, and environmental exposure (such as UV radiation). A factor that may contribute to the low incidence of melanomas in both wild-type and $INK4a^{\Delta 2/3}$ mice may relate to differences in the melanocyte microenvironment in mice and humans. In humans, most melanomas arise within the epidermal microenvironment as *in situ* lesions in radial growth phase. Progression toward the vertical growth phase (downward invasion into the dermis) is thought to require additional genetic alternations that may confer a survival advantage in the less "supportive" dermal milieu. This supposition is based on the observation that epidermal melanoma cells do not readily survive and proliferate when transplanted into a dermal microenvironment (Nesbit *et al.,* 1999). Because melanocytes of the adult mice reside in the dermis, it is, therefore, reasonable to assume that multiple prosurvival/growth stimulatory signals are needed in order to reach a critical transformation threshold in the mouse, providing a basis for observed resistance of rodents to melanoma. With this hypothesis in mind, we speculated that the addition of oncogenic stimuli in an $INK4a^{\Delta 2/3}$ null setting might promote the transformation of these murine dermal melanocytes.

As discussed earlier, activation of the RTK–RAS signaling pathway has been implicated in the pathogenesis of melanomas (Herlyn and Satyamoorthy, 1996). In addition, potent cooperative interaction between activated $H\text{-}RAS^{V12G}$ and *ink4a/arf* deficiency in cultured fibroblasts (Serrano *et al.,* 1997), and the presence of activating N- and H-RAS mutations in human nodular melanoma (Jafari *et al.,* 1995), provided the mechanistic basis for choosing activating $H\text{-}RAS^{V12G}$ as the cooperative genetic alteration on the $INK4a^{\Delta 2/3}$ KO background.

With the aide of the tyrosinase promoter and a newly identified upstream

enhancer element (Ganss *et al.,* 1994), transgenic mouse strain with melanocyte-specific expression of activated H-RAS^{V12G} was generated on INK4a$^{\Delta 2/3}$ null background (Chin *et al.,* 1997). These mice showed spontaneous development of multiple melanomas with high penetrance (60% incidence at 6 months of age). The tumors predominantly appeared as amelanotic dermal nodules. The melanocytic origin of the tumors was confirmed by the expression of melanocyte-specific markers, anti-TRP1. Against the wild-type or heterozygous INK4a$^{\Delta 2/3}$ background, activated RAS expression in melanocytes only rarely gave rise to melanomas and the resultant tumors invariably sustained deletion of the *ink4a* gene (Chin *et al.,* 1997). The weak oncogenic potential of RAS in the malignant transformation of melanocytes *in vivo* possessing a functional *ink4a/arf* locus may be due to the activation of senescence pathways as is observed in RAS-expressing primary fibroblasts. In these cells, the mitogenic and oncogenic actions of H-RAS were dominated by more potent G_1 arrest and cellular senescence response (Lin *et al.,* 1998; Serrano *et al.,* 1997). Thus, for RAS to induce malignant transformation in this system it appears that an associated immortalizing event such as INK4a or p53 loss is a prerequisite.

The genetic tractability of the mouse now permits the investigation of a number of other questions regarding the role of specific genes in melanoma development, genes such as *Ink4b* and p53 among others. For example, co-deletion of *Ink4a* and the adjacent *Ink4b* gene is common in human melanomas (Walker *et al.,* 1998). To distinguish whether *ink4b* loss is a necessary event in tumorigenesis rather than being merely a consequence of *ink4a* loss, the integrity of the locus was analyzed in tumors derived from tyr-RAS/INK4a$^{\Delta 2/3}$−/− animals. Since *ink4b* remained wild type in these tumors, it is reasonable to conclude that *ink4b* loss represents a bystander effect as a result of its proximity to *ink4a.* These data, however, cannot rigorously exclude the possibility that Ink4b may play a role in melanoma genesis in other genetic settings. To definitively address that issue, melanoma susceptability of *ink4b*-deficient mice will need to be examined directly.

Another curious feature of human melanoma is the rarity of p53 mutations (see earlier section). Similarly, mouse melanomas arisen in INK4a$^{\Delta 2/3}$ null background retained wild-type p53 (Chin *et al.,* 1997). The lack of p53 mutations in these *ink4a/arf* null tumors have led to the initial hypothesis that loss of ARF obviates the need for p53 mutation in tumorigenesis and that p19^{ARF} is mechanistically linked to p53 function, as was subsequently reported by a number of groups (Kamijo *et al.,* 1998; Pomerantz *et al.,* 1998; Stott *et al.,* 1998; Zhang *et al.,* 1998).

The above mouse model system generated a melanoma phenotype whose genetic and histologic features are conserved in relation to the human disease. The system did not, however, accurately model the temporal series of genetic lesions expected to arise during the graded progression of human

melanomas. Most dominant oncogenic mutations in cancers are acquired somatically, occurring after immunologic tolerance to self-antigen has been established. Because the tyrosinase promoter becomes active before the maturation of the immune system (Ganss *et al.,* 1994), it is likely that host response to the tumor antigens differs in comparison to adult human cancers. To overcome this deficiency, two transgenic mouse lines were engineered, harboring either a transgene for melanocyte-specific expression of the tetracycline-inducible activator (rtTA) or one for activated RAS expression driven by the tet-operon (Chin *et al.,* 1999) (see Fig 3A). This system permitted the tight regulation of H-RAS^{V12G} expression in double transgenic mice by administration of a tetracycline analog, doxycycline, to the drinking water. When crossed onto the INK4a$^{\Delta 2/3}$ KO background, double transgenic mice (e.g., Tyr/Tet-*RAS ink4a*$^{\Delta 2/3}$$-/-$) developed melanomas with short latency (25% incidence at 60 days) after doxycycline induction, whereas neither treated Tet-*RAS ink4a*$-/-$ or untreated Tyr/Tet-*RAS ink4a*$^{\Delta 2/3}$ $-/-$ animals were afflicted. Clinically and histologically, these doxycycline-induced tumors showed identical histologic and immunohistochemical characteristics to those occurring in the constitutive, tyrosinase-driven RAS model.

The ability to regulate RAS expression *in vivo* provided a unique opportunity to assess the requirement of continued RAS activation in tumor maintenance. Previous studies have suggested that activated RAS expression was necessary to sustain tumorigenicity of cultured tumor cells (Finney and Bishop, 1993; Shirasawa *et al.,* 1993; Ohta *et al.,* 1996). However, these studies could not address whether activated RAS expression still plays a role in *maintenance* of an established tumor *in vivo*. The inducible nature of this conditional transgenic model creates an *in vivo* system through which one can specifically evaluate the role of activated RAS expression in maintenance of primary established melanomas. Withdrawal of doxycycline led to a rapid and sustained regression of the established primary tumors. Tumors of greater than 1 cm^3 shrank to undetectable nodules in less than 2 weeks, although histologically, scattered foci of tumor cells still remained. Consistent with existence of residual disease, subsequent readministration of doxycycline resulted in the recurrence of a tumor at the original site (see Fig. 3B). As such, this *in vivo* system may find use in modeling minimal residual disease for design and testing of potential new therapeutic options. One of the major hurdles to overcome in the next generation of anti-oncologics is eradication of residual disease (Dwenger *et al.,* 1996; Negrin, 1998), an effort that will be greatly facilitated by availability of a useful animal model such as this.

As discussed, RAS induces pleiotropic effects, involving diverse signaling pathways including those that impact on cell cycle progression, inhibition of apoptosis, and cytoskeletal structure (for review, see Campbell *et al.,* 1998;

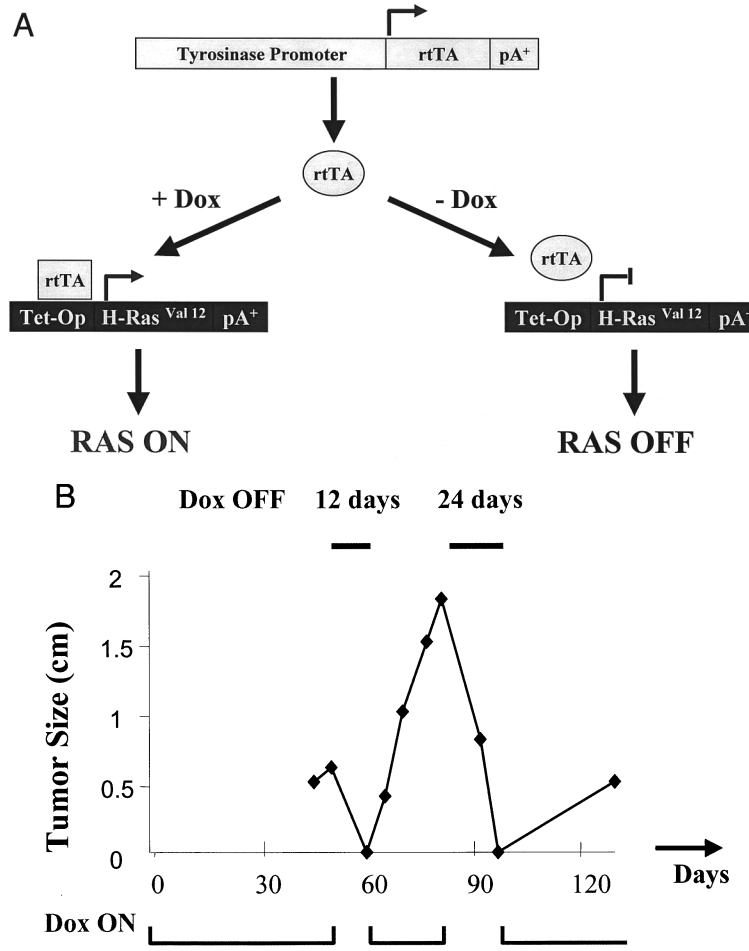

Fig. 3 An inducible mouse model for melanoma. (A) The tetracycline inducible system permits temporal regulation of oncogenic RAS expression in the melanocytes. The tyrosinase enhancer/promoter directed expression of the tetracycline transactivator (rtTA) to the melanocytes. In the presence of tetracycline or doxycycline, rtTA can transactivate the tet-operon (Tet-Op), which drives expression of oncogenic RAS. (B) tumors arisen in the inducible Tyr/Tet-RAS system can be regulated *in vivo*. Withdrawal of doxycycline *in vivo* causes regression of primary melanomas. Readministration of doxycycline results in growth of a new tumor at the site of the original lesion.

Vojtek and Der, 1998). As a preliminary step in obtaining evidence regarding the mechanism by which RAS sustains tumorigenicity, melanoma cell lines from the Tyr/Tet-*RAS ink4a*−/− tumors were established. In contrast to what is observed in the primary tumors, these cells do not require doxy-

cycline for their growth in culture. Indeed the growth of these cells was not substantially affected by doxycycline under a number of assay conditions and in several independently derived cell lines. In particular, neither the subconfluent growth rates nor the proliferative capacity in low serum was affected by doxycycline administration although growth at high cell density was slightly enhanced. The RAS-independent growth of the melanoma cells *in vitro* suggested that a component of the maintenance of the primary tumors might have involved non-cell-autonomous tumor–host interactions mediated by RAS expression. Subcutaneous injection of these cell lines into SCID mice produced tumors in a strictly doxycycline-dependent fashion and withdrawal of doxycycline lead to rapid tumor regression, similar to what is observed for the regression of primary tumors. This finding in the immunodeficient SCID mice suggested that the early phase of regression is not principally dependent on immune system function.

Histologic and immunohistochemical studies suggested that the mechanisms for tumor regression might involve interactions at the level of angiogenesis. The tumors showed dramatic morphologic changes and marked increases in apoptosis in the first 24–36 hr following doxycycline withdrawal. A significant proportion of the cells undergoing apoptotic death appeared to be closely associated with components of the blood vessel wall, perhaps endothelial cells, supporting the notion that vascular collapse was an early event in tumor regression.

The vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis and endothelial cell survival, emerged as a prime candidate for a RAS-regulated factor playing an essential role in tumor maintenance. This assumption was based on previous experiments showing that oncogenic RAS can activate the expression of VEGF (Grugel *et al.,* 1995; Larcher *et al.,* 1996). Although the melanoma cell lines in culture displayed the expected decline in VEGF levels following doxycycline withdrawal consistent with regulation by RAS, the SCID explant tumors showed a transient increase in VEGF before a gradual decrease was observed. The increase in VEGF was coincident with morphologic changes of tumor regression and likely reflects a response to hypoxia, a strong physiologic inducer of VEGF (Plate *et al.,* 1992; Shweiki *et al.,* 1992). These observations suggested that the basis for tumor regression and endothelial collapse is complex and extends beyond the regulation of VEGF gene expression. In support of this view, VEGF-transduced cells formed robust tumors on doxycycline exposure but still regressed following doxycycline withdrawal, showing extensive apoptosis in both the tumor cells and the host endothelial cells. These data demonstrate that VEGF is not sufficient for tumor maintenance, in accord with previous studies, and that oncogenic RAS regulates additional genes and processes essential for tumor growth and vascular integrity. This *in vivo* tumor model system will be of considerable value in the further dissection of the complex

host–tumor interactions. Experiments can be aimed at identifying paracrine signaling pathways, cell–cell adhesion changes, and angiogenic mechanisms. Ultimately this model may provide a testing ground for novel therapeutic strategies directed to the host compartment of the tumor.

D. Other Models

Chen and colleagues have established several transgenic founder lines expressing a genomic fragment that has been shown to induce adipocyte differentiation in transfection experiment *in vitro*. Of these lines, one spontaneously develops heritable metastatic melanomas (Chen *et al.,* 1996; H. Zhu *et al.,* 1998). It is hypothesized that random integration of this unrelated genomic fragment has disrupted the activity/function of a gene important in melanoma genesis. The highly penetrant melanoma phenotype in this transgenic model may be the result of perturbed regulation of a potential oncogene or of loss of function allele of a potential tumor suppressor gene. In either case, this model can serve as a system with which to identify this candidate melanoma gene.

The *Ret* protooncogene encodes a RTK that is expressed in cells and tumors of neuroectodermal origin, such as neuroblastoma, pheochromocytoma, and thyroid medullary carcinoma; its expression in normal pigment cells or transformed melanocytes has not been documented. However, a transgenic mouse strain engineered to express the *ret* oncogene under the control of a metallothionein (MT) promoter unexpectedly develops aberrant melanogenesis and melanocytic tumors, as well as progression toward malignant transformation in founder line 304/B6 (Iwamoto *et al.,* 1991; Kato *et al.,* 1998). In view of its prominent involvement in tumors of neural crest origin, it has been hypothesized that, although not expressed in differentiated melanocytes or melanomas, Ret may play a role in melanocytic development during embryogenesis. Alternatively, disturbance in activity of the *Ret* RTK may perturb the orchestrated network of RTK activation during development. As discussed earlier, coordinated activation of RTKs is likely to play a critical role in melanocyte biology.

E. Future Directions

The recent advances in identifying genetic changes in human melanoma are directing efforts to produce a more faithful melanoma model. In the RAS/INK4a$^{\Delta 2/3}$ melanoma model, the nonmetastatic phenotype is somewhat surprising given extensive evidence that RAS activation confers invasive and metastatic behavior in cell culture based systems. These observations suggest

that RAS activation may be necessary but not sufficient for the metastatic ability of melanoma. Because RTK activation has been implicated in melanoma metastasis (in particular activated Met and EGFR; see earlier section) it will be of interest to generate compound transgenic mice harboring these alleles. In addition, the loss of the PTEN tumor suppressor gene and consequent activation of the PI3K pathway may be a key genetic lesion necessary to generate a metastatic phenotype. PTEN is deleted in a subset of melanomas, and *in vitro* assays have shown that the Met metastatic potential requires simultaneous activation of RAS and PI3K pathways (Bardelli *et al.,* 1999).

The intriguing issue of the relative contributions of the roles of the p53 and RB pathways in melanoma pathogenesis can be addressed by crossing mouse strains harboring the melanocyte-specific *RAS* transgene with animals that have germline deletion of these tumor suppressor genes. As discussed, an unusual aspect of melanoma genetics is the rarity of p53 mutations in these tumors. Melanocyte-specific expression of *RAS* in p53 null animals will determine whether this gene behaves as a melanoma tumor suppressor. Similar studies can elucidate a role for the *ink4b* gene in melanoma genesis, that is, can elimination of this gene substitute for loss of the highly homologous *ink4a* gene in inducing melanoma? Finally, with respect to the *ink4a/arf* locus itself, what are the relative roles for either gene in melanoma pathogenesis? There is ample evidence that INK4a mutations are predisposing to melanoma. An equivalent role for *Arf* has not yet been established, although the frequent concomitant loss of this gene in association with *Ink4a* loss may reflect an important collaborative role for these genes and their pathways. In addition, the nearly equivalent cancer-prone phenotypes of the ARF$-/-$ and the INK4a$^{\Delta 2/3}-/-$ animals argue for an important role for p19^{ARF} in tumor suppression. Comparison of susceptibility to melanoma of knockout mouse strains specific for either of these tumor suppressor genes in the presence of melanocyte directed expression of activated RAS promises to provide key insights into the genetic basis for melanoma.

The field of cancer genetics has been successful in the identification of oncogenes, which exhibit recurrent activating mutations or chromosomal translocations, and in identifying tumor suppressor genes, which are lost during cancer development. There are likely to be classes of genes important for tumor biology that do not show such changes and instead show alterations in their level of expression during oncogenesis. Identification of the specific involvement of such genes remains elusive and constitutes a key future challenge in understanding the molecular basis of cancer. This task is obscured by widespread expression changes manifested by malignant cells, only a fraction of which are likely to be relevant in tumorigenesis. The identification of genes whose expression (or loss of expression) is contingent for tumor growth and survival may be facilitated using a binary system such as our inducible melanoma model. The switch for tumor regression (i.e., ex-

tinction of RAS expression) is likely to alter the expression of only a relatively small fraction of genes. Identification of this restricted group of genes using the emerging gene expression analysis methodologies may permit the isolation of effectors necessary for tumor survival (Johnston, 1998). The melanoma model system can also be used for the identification of genetic modifiers of tumor susceptibility. Modifier genes determine the penetrance and expressivity associated with the inheritance of a separate variant allele (de la Chapelle and Peltomaki, 1998). There are large differences in melanoma incidence when the Tyr-tTA/*Ras* and $ink4a^{\Delta 2/3}-/-$ alleles are bred onto different mouse strains, suggesting the existence of modifier gene(s) for melanoma susceptibility. Newly evolving methods of genetic mapping promise to facilitate the heretofore difficult task of isolating such modifier genes.

VI. CONCLUSIONS

We are now aware of a large body of genes whose alterations are associated with the development or progression of cancer but it remains a considerable challenge to dissect the biochemical processes that go awry as a consequence of these genetic lesions. Furthermore, the means by which such defective processes culminate in cancer as a disease of the organism continues to be an elusive problem. An emerging theme of cancer biology is the crucial importance of tumor–host interactions. Studies of angiogenesis and tumor immunology have highlighted the complex interplay required for tumor survival. This work emphasizes the requirement to study cancer in an *in vivo* setting. Mouse tumor model systems, employing advances in transgenic technology and cancer genetics, have been generated with varying fidelity; most fall short of being fully faithful versions of the human cancer on the histologic and genetic levels. Compound mutant mice, harboring mutations in multiple cancer-associated genes, moved us toward improved models. The development of inducible and conditional knockout strains may provide for a more physiologic approach enabling the stepwise acquisition of genetic lesions that is a hallmark of human malignancy. With these tools in hand we can strive to make inroads into the ultimate goals of cancer research: improvement in the diagnosis, treatment, and prevention of cancer.

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The Indispensable Role of Microenvironment in the Natural History of Low-Grade B-Cell Neoplasms

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Follicular lymphoma (FL) and B-cell chronic lymphocytic leukemia (B-CLL) are paradigmatic examples of lymphoid malignancies in which the relevant biological mechanisms are alterations in the control of apoptosis rather than an exaggerated proliferation. This explains why low-grade B-cell neoplasms still fail to be cured with current approaches. It is becoming increasingly clear that the defective apoptosis of FL and B-CLL has to be ascribed not only to intrinsic defects of the neoplastic cells, but also to extrinsic factors that influence their behavior. Malignant B cells retain the capacity to respond to microenvironmental signals, but have devised a monothematic responsiveness. They have a specific sensitivity to anti-apoptotic signals that favor their survival, whereas they seem to have become insensitive to pro-apoptotic signals. Bystander, nontumoral cells play a fundamental (though not sufficient) role both in the onset and in the progression of these diseases. The survival of leukemic cells appears to be dependent on direct cell–cell contacts. The localization of malignant B cells in bone marrow or neoplastic follicles is not a passive adhesion phenomenon but a crucial step for their survival. Bidirectional malignant lymphocyte–nontumoral cell interactions may lead to the amplification of a microenvironment able to inhibit the apoptosis of neoplastic B cells. The pressure of antigenic selection and the role of the tumor necrosis factor receptor family through the functional survival signal provided by CD40 together with the crippled death signal exerted by CD95 are new prominent characters on the stage.

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I. INTRODUCTION

Several successes have been obtained in the treatment of aggressive B-cell malignancies characterized by high proliferative activity like high-grade lymphomas and acute lymphoblastic leukemias, with long-term remissions and significant percentages of cure rate. In striking contrast, we are entering the new millennium with the heavy burden of low-grade B-cell neoplasms still hampering our efforts to find a cure.

Among these tumors, follicular lymphoma (FL) and chronic lymphocytic leukemia of B-cell type (B-CLL) can be taken as paradigmatic examples of lymphoid malignancies that are not, strictly speaking, life threatening, but still are capable of crippling patients' lives for several years and despite any treatment lead in most cases to an ineluctable death. FL is a tumor of mature peripheral B cells that operates within the germinal centers (GC) of secondary lymphoid follicles (Harris *et al.,* 1994) and accounts for approximately 50% of the non-Hodgkin's lymphomas (NHL). B-CLL is the most frequent leukemia in the Western World and is characterized by the accumulation of long-lived $CD5^+$ B cells, which show the morphology of small mature lymphocytes with a phenotype closely resembling that of lymphocytes detectable in the mantle zone (MZ) of lymphoid follicles (Caligaris-Cappio and Hamblin, 1999).

Both malignancies have a prolonged and substantially indolent course that allows delay of the onset of treatment until symptoms demand systemic intervention. Unfortunately, no treatment modality has so far significantly prolonged overall survival. FL and B-CLL malignant clones show an exquisite sensibility to nearly every treatment and all current therapeutic approaches are capable of inducing a response in a large proportion of patients. However, both diseases eventually always relapse, often in sites that seem to be privileged.

The clinical behavior of FL and B-CLL mirrors a slow but inexorable accumulation of neoplastic cells. This is the hallmark of malignant diseases where alterations in the control of apoptosis rather than an exaggerated proliferation are likely to be the relevant biological mechanism and explain why achieving disease-free survival is as yet an unattained therapeutic goal in FL and B-CLL.

II. MORE CHARACTERS ARE COMING ON THE STAGE

FL and B-CLL have emerged as the prototypes of malignancies where defective apoptosis leads to the progressive and relentless accumulation of neoplastic cells. The defective apoptosis of FL has been ascribed to the t(14;18)

translocation which juxtaposes the Ig heavy chain locus with the Bcl-2 gene and causes the overexpression of bcl-2 gene product (Tsujimoto *et al.,* 1985). Bcl-2 is overexpressed also in B-CLL cells even if the t(14;18) translocation is exceedingly rare and the mechanism involved is currently unclear (Schena *et al.,* 1992).

Irrespective of the underlying mechanism, the assumption that FL and B-CLL are malignancies primarily related to defects in the induction of apoptosis appears to be substantially true, judging from the clinical course and the *in vivo* data. However, when cultured *in vitro* both FL and B-CLL cells die quickly by apoptosis. This observation means that B-CLL and FL cells are not capable of self-maintaining and do lack the proper humoral and/or cellular factor(s) that allow their survival *in vivo*. Many scientific evidences are now pointing in the direction of a fundamental (even if not sufficient) role of bystander, nontumoral cells both in the onset and in the progression of these diseases. It is not yet clear how much of defective apoptosis has to be referred to intrinsic defects of the neoplastic cells and how much to extrinsic factors that influence their behavior. The implication is that, besides the malignant cells and their genetic alterations, other characters are coming on the stage that need to be properly analyzed in order to complete and hopefully clarify the whole picture of low-grade B-cell neoplasms.

Here, we review the most recent *in vitro* findings that elucidate which *other* cellular components play a relevant role in the natural history of FL and B-CLL. These findings will be critically discussed in the light of classical clinical observations to underline the interesting similarities and the differences between the two B-cell malignancies.

A. The Microenvironment

Long-standing clinical and histologic investigations strongly suggest an indispensable role for the microenvironment *nontumoral* cells in the onset and progression of FL and B-CLL. Accordingly, several *in vitro* data show how different nontumoral cells can prevent apoptosis and support the extended survival of malignant clones.

The presence of follicular dendritic cells (FDCs) is a highly characteristic feature of FL pathologic specimens irrespective of their anatomic localization. Because FDCs are normally present only in the germinal center (GC) of lymphoid follicles, their presence amidst malignant FL B cells in different sites may be taken to suggest that FL cells are trying to recreate their proper microenvironment wherever they are.

Immunocytochemical studies suggest a continuous stimulation of lymphoma lymphocytes by FDCs, and *in vitro* FDCs are capable of delaying apoptosis, while inducing activation and proliferation of the malignant clone (Petrasch *et al.,* 1992). Such a situation is reminiscent of the pivotal role of

FDC in the rescue of GC B lymphocytes from apoptosis (Lindhout *et al.,* 1993; Stein *et al.,* 1982). A physical interaction mediated by the LFA-1-α,β/ICAM-1, the VLA-4/VCAM-1, and the ICAM-1/C3bi receptor ligand on FDCs and GC B lymphocytes is able to rescue the latter cells from apoptotic cell death. The same interactions appear to take place between FDCs and neoplastic B cells (Petrasch *et al.,* 1992).

In addition, a common site of FL relapse is the bone marrow (BM), where it is usually difficult if not impossible to eradicate the disease. Such a clinical observation is matched by the recent experimental finding that an outgrowth of lymphoma cells has been observed *in vitro* in association with adherent stromal cells, obtained from patients' BM (Weekes *et al.,* 1998). This finding strengthens the notion that BM stromal cells play an important role in the maintenance of occult lymphoma by inhibiting apoptosis.

The BM milieu is a preferred environment for malignant B-CLL cells and BM infiltration is regularly present in all stages of the disease. In the early nodular phase of BM involvement, B-CLL cells are closely associated with FDCs, which are otherwise absent from normal BM (Chilosi *et al.,* 1985). Further, BM stromal cells seem to have a role in extending the leukemic cell survival (Lagneaux *et al.,* 1998; Panayiotidis *et al.,* 1996). BM-derived endothelial cells (BMECs), as well as human umbilical vein endothelial cells (HUVECs) or endothelial cell hybrids, have shown the capacity to protect B-CLL cells from apoptosis (Jewell and Yong, 1997), by means of physical interactions based on integrins expressed on the surface of malignant B lymphocytes (Long *et al.,* 1995).

Several *in vitro* studies have suggested that numerous cytokines might be important in a paracrine or autocrine fashion for the survival and/or proliferation of B-CLL cells (Meinhardt *et al.,* 1999). More recent data seem to indicate that humoral factors per se might not be responsible for the extended survival of the leukemic cells *in vivo*. Lagneaux *et al.* (1998) clearly show that direct physical contact between leukemic and stromal cells is essential for inhibition of apoptosis. In contrast, separation of leukemic cells from stromal cells by microporous membrane increased spontaneous apoptosis (Lagneaux *et al.,* 1998). Strikingly, normal B cells could not be rescued from apoptosis in the same co-culture system. It has also been shown that the viability of B-CLL cells cultured on fibronectin is consistently higher than the viability of control cultures (de la Fuente *et al.,* 1999). This finding confirms previous data indicating that B-CLL cells are able to bind to secondary lymphoid organs via the pair integrin α4β1/VCAM-1 (Behr *et al.,* 1998; Vincent *et al.,* 1996). These data led to the idea that direct cell–cell contact and/or matrix or membrane-bound cytokines rather than soluble factors may be important also for the *in vivo* survival of B-CLL cells.

Taken together, these observations provide evidence that the localization of malignant cells in BM or neoplastic follicles is not a passive adhesion phe-

nomenon but a crucial step for their survival. Bidirectional malignant lymphocyte–microenvironment interactions may lead to the amplification of a microenvironment able to inhibit the apoptosis of malignant B cells. In turn, the extended cell survival may create an intracellular milieu permissive for genetic instability and for the accumulation of gene mutations that favor the disease progression through disobeyance to cell cycle checkpoints and resistance to cytotoxic drugs and radiations.

As a corollary, such a scenario also provides a conceptual framework to tentatively explain some puzzling aspects of the heterogeneous clinical response observed in patients. It is not unreasonable to suggest that the different chemosensitivity shown by FL and B-CLL cells invading different anatomic sites might be due to modulation exerted by the microenvironment they infiltrate.

B. Tumor Necrosis Factor Receptor Family

Neoplastic B cells retain the capacity to respond to microenvironmental signals, and in many respects still behave as normal B cells. Interestingly, malignant clones have devised a monothematic responsiveness toward the microenvironment, which results in a specific sensitivity to "useful" (i.e., anti-apoptotic) signals that favor their survival while they seem to have become insensitive to negative (i.e., pro-apoptotic) signals.

One example is given by two members of the tumor necrosis factor (TNF) receptor family, CD40 and CD95 (Fas/APO-1). In physiologic conditions, both molecules are expressed on the surface of normal B cells where they have a dual role. Ligation of these molecules can result either in cell expansion or in apoptosis. A model has been proposed (Rathmell *et al.,* 1996) that explains this apparent paradox, based on signaling through the B-cell receptor (BCR). If the B cell has received the proper signal through BCR (i.e., it has recognized its cognate antigen -Ag-) it becomes resistant to Fas-mediated apoptosis. However, if the B cell has received an inappropriate signal through BCR, it undergoes apoptosis through the Fas-FasL pathway (Vogel and Noelle, 1998). In accordance with that, CD40 cross-linking upregulates the expression of CD95 on normal B cells and induces susceptibility to CD95-mediated apoptosis of tolerant B cells or of B cells in the absence of BCR stimulation (Garrone *et al.,* 1995; Lagresle *et al.,* 1995; Schattner *et al.,* 1995). In contrast CD40-activated B cells become resistant to CD95-based apoptosis if the BCR is engaged (Lagresle *et al.,* 1996; Rothstein *et al.,* 1995) and they undergo differentiation and proliferation. Thus, the concerted action of BCR, CD40, and CD95 results in the survival and expansion of Ag-reactive B cells and the elimination of self-reactive or tolerant B cells.

Several studies show how the fine-tuning between these molecules is some-

how disrupted in both FL and B-CLL. These findings corroborate the growing evidence that multiple counterregulatory mechanisms operate in B-cell malignancies to preserve cell survival and ensure their "safety."

1. A FUNCTIONAL SURVIVAL SIGNAL: CD40

The CD40 receptor is a 47- to 50-kDa glycoprotein that, within the immune system, is expressed on dendritic cells, monocytes, and B cells. The engagement of CD40 by its natural ligand, CD40 ligand (CD40L/CD154), which is expressed by activated T cells, induces B-cell growth, differentiation, and rescue from apoptosis (Grewal and Flavell, 1998).

Much *in vitro* evidence suggests a possible role of CD40/CD40L interactions in the pathogenesis of FL. Despite constitutive high levels of bcl-2 because of the t(14;18) translocation, fresh FL cells undergo spontaneous apoptosis when cultured *in vitro*. *In vitro* activation of malignant cells by the soluble form of CD40L results in protection of apoptosis (Ghia *et al.,* 1998), likely due to an upregulation of bcl-x_L. In this study, no correlation with the levels of bcl-2 protein has been shown in the spontaneous onset of apoptosis nor in the CD40-mediated rescue. Also, in contrast to previous work (Johnson *et al.,* 1993), no proliferation of malignant cells could be detected after CD40 stimulation. In accordance with these data, a recent study (Pound *et al.,* 1999) shows that CD40-dependent rescue of human B cells from apoptosis requires minimal cross-linking, whereas the requirements for promoting cell cycle progression and homotypic adhesion are more stringent. Therefore, the requirements for suppressing apoptosis via CD40 are less rigid than those necessary for inducing CD-40 mediated proliferation (Pound *et al.,* 1999).

To further underscore the possible role of such an interaction in the natural history of FL, evidences have also been reported that CD40 ligation can confer resistance to cytotoxic therapy, inhibiting both apoptosis and the antiproliferative effect induced by doxorubicin (Voorzanger-Rousselot *et al.,* 1998). This could support the idea of a mechanism of drug resistance promoted by nontumoral cells in the microenvironment of privileged sites.

In accordance with the *in vitro* evidence that points to a possible role for CD40L bearing nontumoral cells in FL, a striking predominance of polyclonal activated T cells, predominantly of the T helper type (Carbone *et al.,* 1995; Ghia *et al.,* 1998), is observed in involved lymph nodes. Moreover, the malignant clone appears to proliferate in response to T-cell-derived cytokines (e.g., IL4) that behave as paracrine factors (Schmitter *et al.,* 1997). In conclusion, FL cells are not only capable of responding to physiologic signals but also have availability of such signals within their neoplastic lesions.

The role of CD40 ligation in B-CLL seems to be quantitatively different.

The triggering of CD40 with monoclonal antibodies (mAb) as well as with the soluble ligand was not able to reduce the percentage of spontaneous apoptosis shown by B-CLL cells cultured *in vitro* (Buske *et al.,* 1997; Laytragoon-Lewin *et al.,* 1998; Romano *et al.,* 1998; Wang *et al.,* 1997). The strong cross-linking due to CDw32 transfected fibroblasts, instead, could enhance B-CLL viability *in vitro* (Buske *et al.,* 1997) and rescue B-CLL cells from spontaneous apoptosis (Younes *et al.,* 1998).

Moreover, as in FL, the CD40 stimulation is able to counteract *in vivo* the therapeutic effect of apoptogenetic drugs (Romano *et al.,* 1998), namely, fludarabine, and to upregulate adhesion and costimulatory molecules (Buhmann *et al.,* 1999) to levels high enough to turn the malignant cells into efficient Ag-presenting cells (Van den Hove *et al.,* 1997). These results are supported by the observation that the capacity of patients' autologous plasma to rescue B-CLL cells from spontaneous apoptosis can be reverted by anti-CD40L Ab. In fact, high levels of CD40L were shown to be present in the plasmas examined (Younes *et al.,* 1998). As in FL, the experimental evidence of a possible role for $CD40L^+$ nontumoral cells in B-CLL appears to be justified by the presence of significant proportions of $CD4^+$ helper T cells in involved BM and lymph nodes (Pizzolo *et al.,* 1983).

In addition, the presence of CD40 on the surface of leukemic B-CLL cells can provide a potential mechanism of immune evasion that protects the neoplastic clone from a cytolytic immune response. The CD40 molecules on the surface of malignant B cells are capable of inducing downmodulation and endocytosis of CD40L expressed on the membrane of activated T cells (Cantwell *et al.,* 1997). This seems in contrast with a hypothesis of CD40L-induced malignant cell survival. However, a situation of acquired CD40L deficiency would occur when the proportion of $CD4^+$ T cells relative to that of leukemic cells declines below a critical level. In B-CLL patients such proportions are generally noted in the later stages of disease, probably when the CD40-mediated mechanism of survival is no longer critical for the maintanence of the disease. Still, the question remains open as to whether the CD40-mediated anti-apoptotic pathway is part of the pathogenetic mechanism of the disease and can contribute to the prolonged life span and accumulation of the leukemic cells.

Fortunately such an efficacious mean of survival for FL and B-CLL may be a double-edged sword, as demonstrated by the full therapeutic potential of the soluble ligand *in vitro* (Hirano *et al.,* 1999) and of anti-CD40 antibodies *in vivo* (French *et al.,* 1999). The antibody treatment results in a cytotoxic T-cell response that is able to eradicate syngeneic lymphoma by passing T cell help. Moreover, the treatment provides protection against tumor rechallenge, indicating that an immunization process against syngeneic tumors has occurred (French *et al.,* 1999).

2. A CRIPPLED DEATH SIGNAL: CD95

CD95/Fas is a 40- to 50-kDa cell surface glycoprotein belonging to the TNF receptor superfamily (Krammer, 1999). Triggering through CD95 induces apoptosis of activated T, B, and NK cells (Robertson and Ritz, 1995). The natural ligand of Fas, Fas ligand (FasL), belongs to the TNF family and is expressed by activated T cells. Fas receptor and FasL are directly implicated in the regulation of apoptosis and seem to play a key role in eliminating activated lymphocytes and maintaining immune homeostasis (Van Parijs and Abbas, 1998). However, Fas expression is necessary but not sufficient to induce Fas-mediated apoptosis. Various cell populations and cell lines that express equivalent surface amounts of Fas have differential sensitivity or even resistance to Fas-mediated apoptosis.

FL is the NHL subtype that more frequently expresses high levels of CD95 (Nguyen *et al.,* 1996; Plumas *et al.,* 1998). Notwithstanding that, malignant FL cells are resistant to the apoptosis mediated by cross-linking CD95 either by specific mAb or by CD95L expressed on cytotoxic T cells (Plumas *et al.,* 1998). Even if Fas mutations have been detected in rare cases of FL (Gronbaek *et al.,* 1998), somatic disruption of Fas appears to play a limited role in the pathogenesis of the disease. Similar to normal B cells, CD40 ligation can strongly increase CD95 expression on FL cells (Plumas *et al.,* 1998; Wang *et al.,* 1997; Xerri *et al.,* 1998). Despite several studies, discrepancies still exist on the activity of CD95 after such a stimulation. In some instances it has been reported that CD40 activation increases sensitivity to Fas-induced apoptosis to a moderate and variable degree (Wang *et al.,* 1997; Xerri *et al.,* 1998), while in others it appears to poorly restore responsiveness to CD95-mediated apoptosis (Plumas *et al.,* 1998).

There is also disagreement on the explanation of the natural resistance of FL cells to CD95-induced apoptosis. Some groups referred it to the constitutive high levels of the anti-apoptotic protein bcl-2 (Nguyen *et al.,* 1996), while others failed to obtain a correlation with the level of bcl-2 expression (Wang *et al.,* 1997; Xerri *et al.,* 1998), suggesting that factors other than the levels of CD95 and bcl-2 may determine the susceptibility of malignant B cells to apoptosis.

Leukemic cells from B-CLL patients are either negative or only weakly positive for Fas expression (Kamihira *et al.,* 1997; Laytragoon-Lewin *et al.,* 1998; Mainou-Fowler *et al.,* 1995; Mapara *et al.,* 1993; Panayiotidis *et al.,* 1995; Tinhofer *et al.,* 1998; Tsuruda *et al.,* 1999; Wang *et al.,* 1997). As expected, malignant B-CLL cells do not exhibit significantly increased apoptosis after CD95 ligation (Laytragoon-Lewin *et al.,* 1998; Wang *et al.,* 1997). It has been recently shown that both full-length and alternatively spliced truncated forms of Fas mRNA can be detected in all B-CLL samples examined, and that the message is functional (Kamihira *et al.,* 1997; Tsu-

ruda *et al.,* 1999). Despite the fact that cellular activation [with SAC + IL2 (Malpara *et al.,* 1993), α or γ IFN (Panayiotidis *et al.,* 1995) or through CD40 ligation (Wang *et al.,* 1997)] upregulates the expression of Fas, B-CLL cells remain generally resistant to anti-Fas mAb-induced apoptosis (Panayiotidis *et al.,* 1995; Wang *et al.,* 1997). This resistance is not due to mutated FAS protein (Panayiotidis *et al.,* 1995). In contrast to such a remarkable resistance, it has recently been observed that after short-term *in vitro* culture, leukemic B cells show an increase in Fas density and a likewise increased susceptibility to Fas-mediated apoptosis (Tsuruda *et al.,* 1999) that enhance the spontaneous death rate in culture (Younes *et al.,* 1998). These new findings lend support to previous works where stimulated (Mapara *et al.,* 1993; Wang *et al.,* 1997) or unstimulated (Mainou-Fowler *et al.,* 1995) B-CLL cells appeared in some cases to be sensitive to anti-Fas-mediated apoptosis.

As in FL, discrepancies can be found on the role of bcl-2 in this death pathway. Some works suggested that, when present, CD95-induced apoptosis is independent of bcl-2 expression (Mainou-Fowler *et al.,* 1995; Wang *et al.,* 1997), while others indicated the opposite (Mapara *et al.,* 1993).

In summary, despite few discrepancies, there is a general agreement on the existence of a relative resistance to Fas-mediated apoptosis in both FL and B-CLL (Fig. 1). One can then speculate that such a resistance would allow tumor cells to escape, *in vivo,* from Fas/FasL-mediated regulatory control of

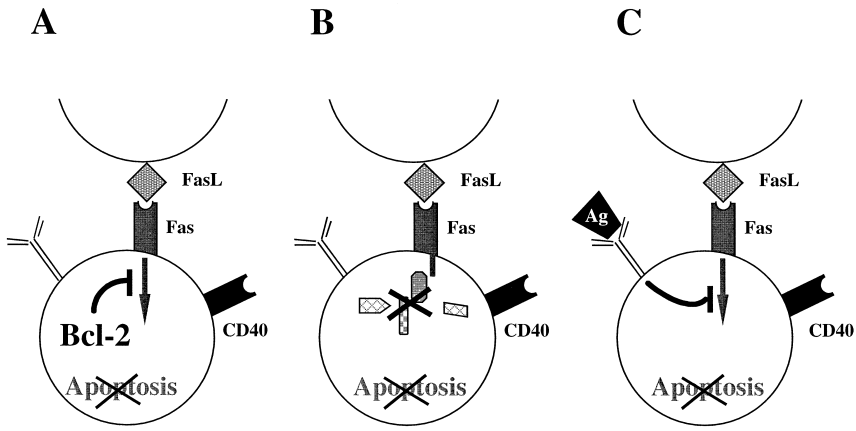

Fig. 1 Potential mechanisms responsible for the relative resistance to Fas-mediated apoptosis in FL and B-CLL. (A) Overexpressione of bcl-2 protein due to the t(14;18) translocation in FL and to so far unknown reasons in B-CLL. (B) Dysregulation of any of the molecules involved in Fas signaling pathway or overexpression of physiologic inhibitory proteins. (C) Stimulation through BCR by a cognate Ag (Rathmell *et al.,* 1996) as suggested by the presence of somatic mutations in FL and in a subset of B-CLL (see text).

$CD4^+$ lymphocytes and from anti-tumor cytotoxicity of $CD8^+$ cells. In agreement with this hypothesis, the CD95-deficient (*lpr*) mice develop lethal B-cell lymphoma only when they are deficient in T cells (Peng *et al.,* 1996). Therefore, resistance to CD95-mediated apoptosis together with functional abnormalities of T lymphocytes (e.g., anergy) could be of prime importance in facilitating lymphoma development or progression.

The mechanisms that counteract the CD95 apoptotic process in FL and B-CLL are still poorly understood. A number of signaling pathways are known to be activated on triggering of CD95 in sensitive cells. The salient feature of such signaling is the formation of a multimolecular complex of proteins, called the death-inducing signaling complex (DISC) (Kischkel *et al.,* 1995), constituted by several molecules (e.g., FADD and FLICE) (Peter and Krammer, 1998). Further studies are required to determine if expression of any of these molecules involved in CD95 signaling could be defective in CD95-sensitive versus CD95-resistant B cell tumors.

Interestingly, several molecules have also been described as specific and physiologic inhibitors of this cascade [e.g., sentrin (Okura *et al.,* 1996); FLIPs (FLICE inhibitory proteins) (Thome *et al.,* 1997)] while other molecules like phosphatidylinositide-3-kinase (PI3K) (Hausler *et al.,* 1998) and the Bruton's tyrosine kinase (BTK) (Vassilev *et al.,* 1999) have just recently been suggested to act as key players in the negative regulation of this death pathway. A dysregulation of any of these proteins could participate in the pathologic resistance to apoptosis shown by FL and B-CLL.

Beside the role of intrinsic defects, one alternative and attractive explanation can be hypothesized to explain the mechanism of resistance to CD95 apoptosis in malignant B cells. As previously mentioned, when a normal B cell is stimulated through BCR by a cognate Ag, it becomes resistant to Fas-mediated apoptosis (Lagresle *et al.,* 1995; Rathmell *et al.,* 1996; Rothstein *et al.,* 1995). This possibility does not seem so bizarre anymore for neoplastic B lymphocytes also (Fig. 1C).

C. Possible Role of Antigen Selection

1. FOLLICULAR LYMPHOMA

A potential influence on the development of lymphoid malignancies is exerted by the Ag encounter that occurs in GC of secondary lymphoid follicles and leads to the process of Ig somatic mutation during T-cell-dependent Ab response (Spencer and Dunn-Walters, 1999). Point mutations are introduced into V gene sequences and result in the production of Ab of increased affinity. The existence of an Ag recognized by tumoral BCR has rarely been described in NHL. However, the demonstration of Ig somatic hypermutations

with intraclonal diversity in FL suggests that antigenic stimulation and selection are involved in the evolution of the malignant clone (Friedman *et al.,* 1991; Zelenetz *et al.,* 1992). More recent and independent works (Matolcsy *et al.,* 1999; Ottensmeier *et al.,* 1998) strengthen and extend the initial claim. The high degree of intraclonal diversification with highly divergent $V_HD_HJ_H$ gene sequences has been confirmed in more cases. Matolcsy *et al.* have studied the transformation from low-grade FL into high-grade diffuse large B-cell lymphomas (DLBLs), an event that occurs in 30% of the FL (Cullen *et al.,* 1979). The Ig sequence observed in these DLBLs appears to be clonally related to the sequence detected in the low-grade neoplasms from which they have evolved; still it cannot be detected in the original tumor and carries somatic mutations that are distinct from those of the original tumor (Matolcsy *et al.,* 1999). The pattern of somatic mutations supports the hypothesis that a single, Ag-selected FL subclone can give rise to the transformed phenotype that will reveal as DLBL and suggests once more that not only the onset but also the progression of the disease might be associated with antigenic pressure (Matolcsy *et al.,* 1999).

The process of ongoing mutation in FL appears to occur according to the same mechanisms that operate within normal follicles. Somatically mutated follicular B cells constantly travel from the follicle to the interfollicular region, where they acquire the distinct phenotype of memory B cells (Liu *et al.,* 1995). They then return to the follicle where they undergo more rounds of somatic mutations. In FL, the presence of a separate interfollicular neoplastic B-cell population that diffusely infiltrates the areas between the follicles has been observed for a long time (Harris *et al.,* 1984). A recent finding shows that neoplastic follicles contain a clonally linked but phenotypically distinct population (Dogan *et al.,* 1998), which resembles the normal subpopulation of postfollicular (memory) B cells. The two populations (follicular and postfollicular), though phenotypically distinct, are clonally related, showing similar frequency of intraclonal Ig gene sequence variations in both compartments. This evidence points in the direction of an active tumor cell traffic between the two compartments of malignant cells with repetitive reentry in the follicle. The existence of these two distinct cellular fractions can therefore account for the progressive accumulation of somatic mutations shown by FL cells, as well as for different intracellular sensitivity to chemotherapy and radiotherapy (Dogan *et al.,* 1998; Longo, 1993).

All of these data together strengthen the notion of a scenario where a B/T cognate interaction has occurred through CD40/CD40L interactions, at a level sufficient to induce rescue from apoptosis of activated B cells. A concomitant Ag stimulation through Ig receptor of the malignant or premalignant clone would have a direct effect in protecting against Fas sensitivity induced upon CD40 ligation (Fig. 1C).

2. B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

In contrast to FL, it has been assumed for a long time that B-CLL cells would accumulate little, if any, somatic mutations. On these bases, B-CLL cells were considered to be mature, naive, Ag inexperienced lymphocytes. Studies challenging that assumption, however, have been accumulating.

Several groups during the last 5 years have accumulated data on the presence of somatic mutations in B-CLL cells (Hashimoto *et al.,* 1995b; Schroeder and Dighiero, 1994) and have provided compelling evidence that B-CLL is heterogeneous. All of these studies were recapitulated and confirmed by Fais *et al.* (1998), who showed in a series of 88 patients from different centers and different ethnic groups that about 50% of IgM^+ B-CLL and about 75% of the non IgM^+ B-CLL exhibit somatic mutations (Fais *et al.,* 1998).

Recently these experimental data have been correlated with the clinical course and the response to therapy, taking into account the clinical heterogeneity of the disease with some patients never requiring any treatment and others that need intensive therapy. An initial study performed in 18 cases showed different rates of V_H gene somatic hypermutation among subsets of B-CLL characterized by chromosomal abnormalities (Oscier *et al.,* 1997). The cases carrying trisomy 12, which are usually associated with advanced disease and a less favorable prognosis, showed a minimal level of mutation. In marked contrast, cases with 13q14 abnormality, which usually have a better prognosis, showed significant levels of somatic diversification (Oscier *et al.,* 1997). More recently, two studies have extended this preliminary observation. In a large series of 84 B-CLL patients, Hamblin *et al.* (1999) clearly showed that the germline configuration of Ig H genes is significantly associated with a more aggressive form of the disease. These data are mirrored by a smaller series of patients (Damle *et al.,* 1999), which adds the further information of potentially relevant diagnostic significance that patients experiencing a worse clinical outcome not only lack somatic mutations but also show higher proportions of CD38-positive leukemic cells.

These data elegantly show how the structure of the clonal Ig can help in discriminating distinct entities within B-CLL and are also relevant for a better understanding of the natural history of the disease (Fig. 2). The presence of somatic mutations and the localization of replacement changes and the sequential and nonrandom pattern of the V_H mutations are all consistent with the notion that, at least in a proportion of cases, Ag selection has occurred and suggests that Ag stimulation may be a promoting factor in the evolution of some B-CLL clones (Hashimoto *et al.,* 1995b). This possibility is also supported by the BCR features of B-CLL cell. BCR is a multimeric complex formed by the sIg and the Ig_α/Ig_β (CD79a/CD79b) heterodimer that translates the Ig stimulation into the B-cell response. The extracellular Ig-like domain of CD79b is lacking in most B-CLL patients (Zomas *et al.,*

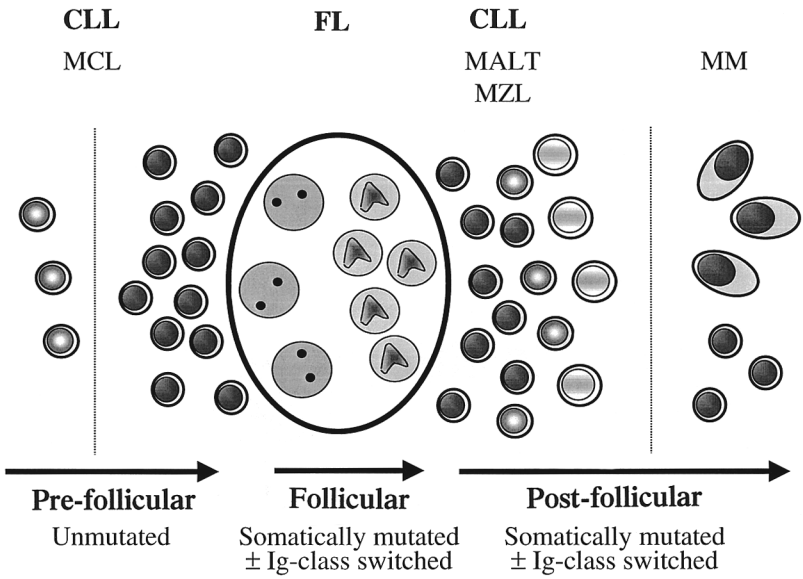

Fig. 2 A (tentative) model of origin of different B-cell neoplasms. B-CLL and MCL (mantle cell lymphoma) are thought to derive from prefollicular B lymphocytes, according to the absence of somatic mutations in their Ig gene sequences. FL is thought to derive from centrocytic or centroblastic follicular cells. Ig genes are somatically mutated and can be class switched. MALT (mucosa associated lymphoid tissue) lymphoma, MZL (marginal zone lymphoma), and MM (multiple myeloma) are thought to originate from postfollicular B cells carrying somatically mutated and, in some cases, class switched Ig genes. According to the new finding of somatic mutations in more than half of the B-CLL patients, we propose that a large subset of B-CLL can also be derived from postfollicular B cells (see text).

1996). A CD79b truncated form arises by alternative splicing of CD79b gene and lacks exon 3 that encodes the extracellular domain (Hashimoto *et al.*, 1995a; Koyama *et al.*, 1995). This spliced variant has been found in a variety of human B cells and B-cell lines (Hashimoto *et al.*, 1995a; Koyama *et al.*, 1995) and has been detected in all B-CLL cases analyzed (Alfarano *et al.*, 1999). Also, the spliced variant levels are higher in activated normal B cells as compared to resting B cells, suggesting that the process of B-cell activation per se favors the post-transcriptional negative regulation of CD79b. Accordingly, lymphoid tissue sections stained with CD79b mAb reveal a strong staining of mantle zone cells and a barely detectable staining of GC (Mason *et al.*, 1992), indicating that *in vivo* activated GC B cells are essentially CD79b negative. Therefore, it is conceivable that B cells physiologically use alternative splicing to downregulate BCR expression on activation. This would allow an undisturbed B-cell proliferation and differentiation after the encounter with an individual triggering Ag. On these bases, it becomes con-

sequent to conclude that the lack of CD79b in B-CLL likely reflects the state of activation of the B cell where the malignant transformation has occurred.

III. CONCLUSIONS

Several findings indicate that the defective apoptosis of FL and B-CLL is not adequately explained by intrinsic defects of the neoplastic cells. Rather, a scrupulous investigation of extrinsic factors reveals the fundamental (though not sufficient) role of bystander, nontumoral cells both in the onset and in the progression of these diseases. Therefore, the real turnaround in the central issue of which mechanisms lead to the relentless accumulation of monoclonal B cells in low-grade B-cell neoplasms and how they dictate the clinical behavior now seems at hand. In these diseases a defective apoptosis underlies both the pathogenesis and the drug resistance. It follows that understanding the cross-talk between malignant and nontumoral cells within different microenvironments is of major importance in order to revitalize the treatment of FL and B-CLL.

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Epstein–Barr Virus Latency: LMP2, A Regulator or Means for Epstein–Barr Virus Persistence?

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Like other herpesviruses, Epstein–Barr virus (EBV) persists in its host through an ability to establish a latent infection that periodically reactivates, producing infectious virus that infects naïve hosts. Disease syndromes in humans caused by EBV reflect the cell types that EBV infects, being primarily of lymphoid or epithelial origin. The most notable lymphoid disease, infectious mononucleosis, is a self-limiting lymphoproliferative disease that occurs in normal adolescents on primary infection. Children are normally able to resolve primary EBV infection with few or no symptoms. By the age of 25 most individuals are EBV seropositive. EBV is associated with a variety of hematopoietic cancers such as African Burkitt's lymphoma, Hodgkin's, and adult T-cell leukemia. EBV-associated lymphoproliferative disease occurs in individuals with congenital or acquired cellular immune deficiencies. The two notable epithelial diseases associated with EBV infection are nasopharyngeal cancer, a malignancy endemic to southern China, and oral hairy leukoplakia, an epithelial hyperplasia of the lingual squamous epithelium in AIDS patients. Latent membrane protein 2 (LMP2) is expressed both in normal EBV latency and EBV-associated pathologies. LMP2 may regulate reactivation from latency by interfering with normal B-cell signal transduction processes and in doing so may also provide a survival signal that could be important for viral persistence. Current knowledge about the function of LMP2 is described, defining a new class of regulators of herpesvirus latency.

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I. INTRODUCTION

Epstein–Barr virus (EBV) is one of eight human herpesviruses that routinely establishes latent infections in human hosts following initial infection (Roizman, 1993). The eight human herpesviruses are organized into three families (α, β, γ) depending on biological characteristics and evolutionary relatedness. The three human α-herpesviruses, herpes simplex virus 1 (HSV-1), HSV-2, and varicella zoster virus (VZV), are characterized by their rapid reproductive cycle and capacity to establish latent infections in sensory ganglia. The three human β-herpesviruses, human cytomegalovirus (HCMV), human herpesvirus 6 (HHV6), and HHV7, typically have a longer lytic reproductive cycle and may have the ability to remain latent in more than one cell type. The two human γ-herpesviruses, EBV and KSHV (or HHV8), are distinguished by their latent infection of transformed lymphocyte cell lines in culture and their link with human proliferative disorders (Chang *et al.,* 1994; Longnecker, 1998; Rickinson and Kieff, 1996).

Considerable interest has focused on EBV since its discovery and its link with Burkitt's lymphoma in the early 1960s (D. Burkitt, 1962; D. P. Burkitt, 1983; Epstein *et al.,* 1964). Along with HHV8, EBV is the only herpesvirus with a known role in human malignancies. EBV infection is linked with endemic Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) (Rickinson and Kieff, 1996). The virus has also been recognized as an important pathogen in individuals lacking cellular immunity either from genetic defects, immune suppression for organ transplantation, or loss of immune function due to HIV infection (Liebowitz, 1998; Longnecker, 1998; Rickinson and Kieff, 1996). In immunosuppressed patients, EBV causes a variety of proliferative disorders including oral hairy leukoplakia in AIDS patients, immunoblastic lymphomas, and an unusual tumor of muscle origin in children with AIDS or those who are under immune suppression after liver transplantation (Liebowitz, 1998; Longnecker, 1998; Rickinson and Kieff, 1996). EBV may also be a factor in a variety of other human malignancies including some T-cell lymphomas, gastric carcinomas, and Hodgkin's disease (Liebowitz, 1998; Longnecker, 1998; Rickinson and Kieff, 1996).

Infection with EBV usually occurs early in childhood, resulting in an asymptomatic infection. If primary infection occurs later in life, proliferation of B lymphocytes infected with EBV and the resulting immune response can result in infectious mononucleosis. After primary infection, most individuals will harbor the virus for the remainder of their life, and carriers develop cellular immunity against a variety of both lytic and latency associated proteins (Liebowitz, 1998; Longnecker, 1998; Rickinson and Kieff, 1996). Periodically, virus is shed from latently infected individuals by the induction of lytic replication in B lymphocytes. The true site of latent infection has not been determined, but the virus likely resides in B lymphocytes. Recent studies have

shown that EBV can be detected in circulating peripheral blood lymphocytes in carriers of EBV latent infections by PCR both for viral DNA and viral mRNA (Chen *et al.,* 1995; Decker *et al.,* 1996; Khan *et al.,* 1996; Miyashita *et al.,* 1995; Qu and Rowe, 1992; Tierney *et al.,* 1994).

The virus can also be isolated by culturing peripheral lymphocytes (Yao *et al.,* 1985). It has not been determined if this is the true site of latency. Other potential sites of EBV latency include bone marrow, lymph nodes, or other lymphoid organs. Further evidence of the hematopoetic site of EBV latency comes from engraftment of bone marrow cells, which can result in the loss of the resident virus or the appearance of a new virus strain from donor lymphocytes (Gratama *et al.,* 1988, 1989). Lytic replication is presumed to occur when EBV-infected B lymphocytes traffic through oral epithelium. This resulting infectious virus provides a source for infection of other individuals. There is considerable disagreement about whether lytic replication occurs in epithelia cells and if this replication is important for the transmission. Recent studies using samples from patients with acute infectious mononucleosis indicate no detectable lytic replication in oral epithelial cells despite abundant lytic replication in lymphocytes which have trafficked to the epithelium (Karajannis *et al.,* 1997). Studies from AIDS patients with the unusual epithelial hyperplasia of the tongue indicate that the virus can gain access to epithelial cells and undergo lytic replication (Liebowitz, 1998; Longnecker, 1998; Rickinson and Kieff, 1996). Whether this is a pathological consequence from the underlying immune suppression found in AIDS patients needs to be resolved. EBV is also found in NPC, an epithelial malignancy found predominately in Chinese populations (Liebowitz, 1998; Rickinson and Kieff, 1996).

II. EBV LATENCY

B lymphocytes are readily infected with EBV *in vitro* and provide a tissue culture model of EBV growth transformation and latency. Latently infected B lymphocytes grown *in vitro* are called lymphoblastoid cell lines (LCLs) and express nine proteins and two small RNAs. The proteins are expressed either in the nucleus (EBNA, Epstein–Barr nuclear antigen) or plasma membrane (LMP, latent membrane protein). The EBNAs are responsible for maintaining the viral episome and regulating viral gene expression (Kieff, 1996; Longnecker, 1998). LMP1, a tumor necrosis factor family receptor mimic, is an inducer of cell proliferation (Kieff, 1996; Longnecker, 1998). The LMP2 genes are the topic of this article.

The genes expressed in EBV immortalized B-cell cells grown in tissue culture are not strictly conserved in any other human herpesvirus, but in the hu-

man γ-herpesvirus KSHV there are hints of functional homologs. In the case of LMP2, two proteins have been identified that share structural as well as sequence homology with the EBV LMP2 proteins. The KSHV K1 contains a functional immunoreceptor activation motif (ITAM), but it appears to be expressed only in lytic infection and has a single transmembrane domain (Lagunoff *et al.,* 1999; Lee *et al.,* 1998; Russo *et al.,* 1996). Also in KSHV, K15 or LAMP is a gene composed of multiply spliced mRNAs expressed during latent infection. Predicted sequence from the group of transcripts indicates a maximum of 12 transmembrane domains with predicted products of fewer transmembrane domains as well (Glenn *et al.,* 1999; Poole *et al.,* 1999). The predicted proteins have a short amino terminus with a larger carboxyl terminus containing motifs reminiscent of both LMP1 and LMP2A of EBV (Glenn *et al.,* 1999; Poole *et al.,* 1999). In addition, homologs to many of the EBV latent genes expressed during *in vitro* latent infection have been identified in the EBV-related herpesviruses that infect monkeys (Franken *et al.,* 1995, 1996; Ling *et al.,* 1993; Rivailler *et al.,* 1999; Yates *et al.,* 1996).

Of the nine viral proteins expressed in latent infection, only EBNA1, LMP1, and LMP2A are consistently detected in NPC tumor biopsies, and other EBV-related proliferative disorders and only LMP2A and EBNA1 have been detected by polymerase chain reaction (PCR) analysis from B lymphocytes purified from healthy individuals harboring EBV latent infections (Chen *et al.,* 1995; Miyashita *et al.,* 1995; Qu and Rowe, 1992; Tierney *et al.,* 1994). The detection of EBNA1 is not surprising since it is known to be required for EBV genome persistence in infected cells (Yates *et al.,* 1985). Because of the uniform detection of LMP2A messages *in vivo*, it is likely to be important for viral replication, persistence, and EBV-related diseases in the human host. Because the full complement of viral genes expressed *in vitro* is not found in normal EBV latent infection in human hosts, the use of *in vitro* tissue culture models of EBV latent infection should be regarded with caution. This review concentrates on our analysis of LMP2A and LMP2B function in our models of *in vitro* and *in vivo* latency.

III. LATENT MEMBRANE PROTEIN 2

A. The LMP2 Genes

The two LMP2 genes (LMP2A and LMP2B) are transcribed into two mRNAs across the circularized viral genome from two spatially distinct promoter elements (Laux *et al.,* 1988, 1989; Sample *et al.,* 1989). The LMP2A start site is approximately 5 kb from the bidirectional promoter responsible for the expression of LMP1 and LMP2B. The two transcripts are mul-

tiply spliced, and the resultant mRNAs differ only in their first exons. The LMP2A first exon contains an initiator of translation that results in a 119-hydrophilic-amino-acid amino terminus. The LMP2B first exon lacks a methionine codon, and translation of LMP2B initiates at a methionine near at the beginning of the second exon, which is common to both mRNAs. This latter methionine codon immediately precedes the first of 12 transmembrane domains, each of which traverses the B-cell plasma membrane, and a 27-amino-acid cytoplasmic carboxyl-terminal tail both of which are common to LMP2A and LMP2B (Fig. 1).

LMP2A and LMP2B localize to numerous small patches in the plasma membrane of latently infected B lymphocytes (Longnecker *et al.,* 1991; Longnecker and Kieff, 1990). LMP2A patches colocalize with LMP1 (Longnecker *et al.,* 1991; Longnecker and Kieff, 1990) and LMP2A associates with LMP1 in immunoprecipitates (Longnecker *et al.,* 1991). However, LMP2A and LMP1 each independently have the ability to aggregate in patches in plasma membrane of B lymphoblasts (Liebowitz *et al.,* 1986; Longnecker *et al.,* 1991). Truncation of LMP2A after the fifth or seventh transmembrane domain results in diffuse plasma membrane localization, confirming the importance of the multiple membrane domains in the fully aggregated phenotype (Liebowitz *et al.,* 1986; Longnecker *et al.,* 1991, 1993a). Most of the phosphotyrosine reactivity in latently infected B lymphocytes is associated

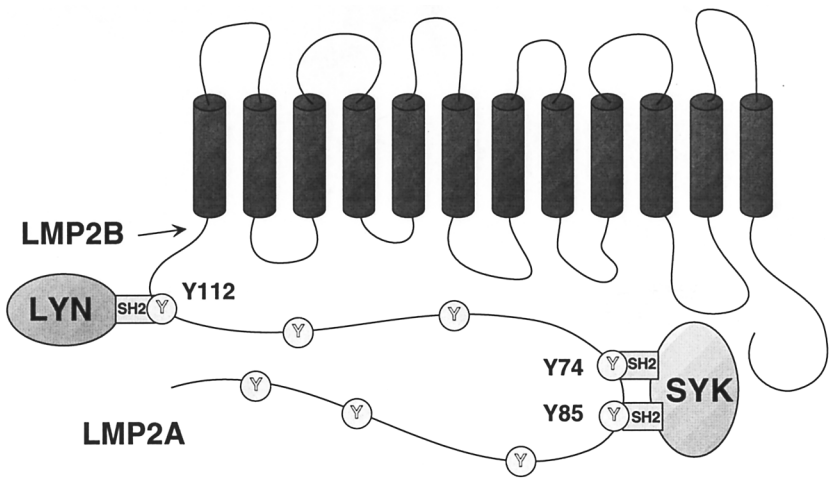


Fig. 1 Predicted structure of LMP2A and LMP2B. The 12 transmembrane domains of LMP2A and LMP2B are shown traversing the plasma membrane. The large amino terminal and small cytoplasmic domain, both found within the cytoplasm, are also indicated. The SH2–phosphotyrosine interactions of LMP2A with the Lyn and Syk PTK at specific LMP2A tyrosine residues are indicated. An arrow indicates the LMP2B initiator methionine.

with LMP2A patches (Longnecker *et al.,* 1991). LMP2A is tyrosine phosphorylated in transfected B lymphoma cells and in EBV-transformed B lymphocytes (Burkhardt *et al.,* 1992; Longnecker *et al.,* 1991). Both LMP2A and LMP2B have been shown to be dispensable for EBV-mediated transformation of primary B lymphocytes (Kim and Yates, 1993; Longnecker *et al.,* 1992, 1993a,b; Speck *et al.,* 1999).

B. Tyrosine Motifs

The LMP2A 119-amino-acid amino-terminal domain includes eight tyrosine residues, some of which are phosphorylated (Longnecker *et al.,* 1991). Each of these phosphorylated tyrosine residues provides a potential site for binding with cellular proteins containing Src homology 2 (SH2) domains (Fig. 1 and Table I). SH2 domains are noncatalytic domains conserved among cytoplasmic signaling proteins that bind tyrosine phosphorylated proteins (Pawson and Scott, 1997). Comparison of the LMP2A tyrosine motifs with tyrosine motifs known or predicted to bind SH2 domains contained within specific cellular proteins reveals homology to many cellular proteins involved in signal transduction (Table I) (Songyang *et al.,* 1993, 1994). Different SH2 domains have differing preferences for hydrophobic or hydrophilic residues immediately carboxyl prime to the tyrosine residue (Songyang *et al.,* 1993, 1994). Comparison of the eight tyrosine residues and their surrounding motifs reveals homology to several motifs that predict optimal binding to identified proteins involved in signal transduction (Table I). The Y31 motif (YPSA) has homology with group III members defined by Y-hydrophobic-X-hydrophobic (Songyang *et al.,* 1993, 1994). This phosphotyrosine motif optimally binds the SH2 domain of proteins such as the 85-kDa subunit adapter protein of PI3 kinase, PLCγ2, and Shc. Y60 (YEDP) contains the general motif Y-hydrophilic-hydrophilic-I/P or the group IB optimal motif. Of the group IB proteins, Y60 has the best homology with Crk (YDHP) and Nck (YDEP) motifs. LMP2A tyrosine residues, Y74 and Y85, form an immunoreceptor tyrosine-based activation motif (ITAM) (Cambier, 1995; Reth, 1989). ITAMs consist of paired tyrosine and leucine residues and have been shown to play a central role in signal transduction of the B-cell receptor (BCR) and the T-cell receptor (TCR) (Benschop and Cambier, 1999; Cambier *et al.,* 1994; Weiss and Littman, 1994). Interesting, Y85 bears some resemblance to an immunoreceptor tyrosine-based inhibitory motif (ITIM). It has been demonstrated that the phosphatase SHP-1 binds to the ITIM within the cytoplasmic domain of the Fc receptor. This interaction is important in the negative regulation of the BCR (Benschop and Cambier, 1999; Cambier *et al.,* 1994; Weiss and Littman, 1994). The consensus ITIM binding motif for SHP-1 is T/S-X-X-Y-X-X-L, which is very similar to

Table I. LMP2A Tyrosine and Serine Motifs

LMP2A tyrosine	Tyrosine motif	SH2 domain site homology[a]	Conservation of tyrosine motif: EBV clinical[b]	Conservation of tyrosine motif: *h. papio*[c]
Y23	YDGG	None?	No	—
Y31	YPSA	Shc, PI3-K, PLCγ2	Yes	YPPS
Y60	YEDP	Abl, Crk, Nck	Yes	YDAP
Y64	YWGN	None?	No[d]	YGGS
Y74	YQPL	Syk	Yes	YATL
Y85	YLGL	Syk	Yes	YAGL
Y101	YSPR	Csk	Yes	YSPR
Y112	YEEA	Lyn, Src family	Yes	YEEP

LMP2A serine	Serine motif	Ser/Thr kinase site homology[e]	Conservation of serine motif: EBV clinical[b]	Conservation of serine motif: *h. papio*[c]
S15	AGPPSPGGD	MAPK	Yes	GGPRSHGGP
S52	EERESNEEP	Casein kinase II	Yes	—
S102	PPPYSPRDD	MAPK, PKC	Yes	PPPYSPRQG

[a]Phosphopeptide sequences (one-letter code) within the LMP2A amino-terminal domain that are predicted to bind specific SH2 domains or are recognized by specific serine-threonine kinases. Proteins with an SH2 domain that are predicted to bind to LMP2A from Songyang *et al.* (1993, 1994).

[b]LMP2A sequences from EBV clinical samples were previously reported (Berger *et al.*, 1999; Busson *et al.*, 1995).

[c]LMP2A sequences from *H. papio* were previously reported (Franken *et al.*, 1995).

[d]Only 43% (Busson *et al.*, 1995) or 17% (Berger *et al.*, 1999) of the LMP2A clinical sequences contained Y64.

[e]Recognition motifs for specific serine-threonine kinases as reported by Songyang *et al.* (1996).

the Y85 motif (SLYLGL). Y101 (YSPR) contains an unusual arginine residue at +3. Peptides highly selected by the Csk SH2 domain contained T, A, or S at position +1 and M, I, V, or R at position +3 (Songyang *et al.*, 1993, 1994). Y101 contains a S at +1 and an R at +3. The Y112 (YEEA) phosphotyrosine motif, the site of Lyn binding to LMP2A, is clearly homologous to the Src family preferred SH2 binding motif (YEEI) (Songyang *et al.*, 1993, 1994). Y23 and Y64 contain phosphotyrosine motifs that have no predicted specificity to any identified SH2 domains.

Additional evidence for the putative importance of the LMP2A tyrosines is obtained from the comparison of LMP2A sequences from clinical EBV isolates. In the first study, in which 28 EBV isolates were analyzed, there was absolute conservation of Y31, Y60, Y74, Y85, Y101, and Y112 in the 28 sequences analyzed (Table I) (Busson *et al.*, 1995). Y23 was not found in any

of the isolates whereas Y64 was contained in only 43% of the sequenced isolates. A more recent study, which analyzed the LMP2A sequence in patients with EBV-associated reactive and malignant lymphoproliferations as well as normal healthy EBV carriers, found that Y74 and Y85 was strictly conserved in 76 cases (Berger *et al.,* 1999). In 54 cases, Y60 and Y64 were analyzed. Y64 was changed to asparagine in 45 cases, whereas Y60 was strictly conserved. There was no correlation with the changes in the LMP2A amino acid sequence with any disease state. Seven of the eight LMP2A tyrosine residues are homologous and conserved between LMP2A and the amino acid sequence of LMP2A homologs in nonhuman γ-herpesviruses that infect primates (Fig. 2 and Table I) (Franken *et al.,* 1995). Only tyrosine 23 is not found in the nonhuman LMP2A-like protein sequence (Fig. 2 and Table I).

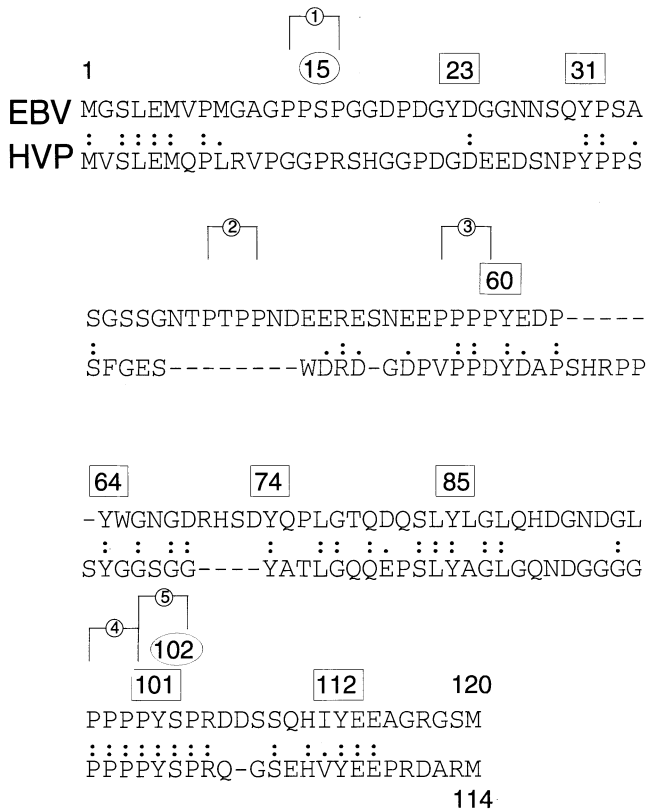


Fig. 2 Sequence conservation of the LMP2A genes from EBV and *H. papio*. EBV serines (circle) or tyrosines (box) are indicated. Proline pairs, which may bind SH3 domains, are indicated by brackets. Y60 and Y101 may form PY motifs and be sites of binding with proteins containing WW domains. Sequence data are adapted from Franken *et al.* (1995).

C. Serine, Threonine, and Proline Motifs

In addition to the LMP2A phosphotyrosine interactions with cellular proteins, the amino-terminal domain of LMP2A also includes multiple serine and threonine residues, as well as proline-rich regions, many of which are conserved in related γ-herpesviruses (Fig. 2). LMP2A is serine and threonine phosphorylated (Longnecker *et al.,* 1991), so at least a subset of serine residues and threonine residues in the LMP2A amino-terminal domain provides potential sites recognized by serine and threonine kinases. Two LMP2A serine residues, S15 and S102, are sites of *in vitro* mitogen-activated protein kinase (MAPK) phosphorylation of a purified LMP2A fusion protein (Panousis and Rowe, 1997). MAPK bound both the LMP2A fusion protein and LMP2A in wild-type LCLs, presumably at either or both of these serine residues (Panousis and Rowe, 1997). The functional significance of the MAPK interaction with LMP2A is not known.

LMP2A also contains proline-rich regions that are conserved in both the LMP2A sequence from the different EBV clinical isolates and *Herpesvirus papio* (Fig. 2). These proline-rich regions may recruit Src homology 3 (SH3) domain-containing proteins (Pawson and Gish, 1992) or proteins with WW domains (Sudol, 1996). SH3 domains are noncatalytic conserved domains that are found in a number of cellular kinases and adapter proteins that recognize proline-rich regions of proteins and are essential in many signal transduction pathways linking cellular receptors to downstream signaling events. WW domains consist of paired tryptophan residues separated by 20–22 amino acids and have been shown to specifically bind to the motif XPPXY (Sudol, 1996), of which LMP2A has two (Fig. 2) and recently been shown to bind to ubiquitin ligases (Ikeda *et al.,* 2000). WW domains are contained in a diverse group of proteins also implicated in the regulation of cellular growth.

IV. B-CELL SIGNAL TRANSDUCTION AND LMP2A

In primary B lymphocytes and EBV- B-cell lines, activation of the BCR leads to an intricate signal cascade. Initially, there is recruitment and activation of the Src family protein tyrosine kinases (PTKs) followed by the recruitment and activation of the Syk PTK. The B-cell linker protein (BLNK or SLP-65) then interfaces the Syk PTK with PLCγ, the Vav guanine nucleotide exchange factor, and the Grb2 and Nck adapter proteins (Fu *et al.,* 1998; Ishiai *et al.,* 1999; Wienands *et al.,* 1998). These initial interactions lead to the mobilization of calcium and activation of the Ras pathway and protein kinase C (PKC), ultimately leading to the expression of BCR signal-specific genes (Fig. 3) (Benschop and Cambier, 1999; Cambier *et al.,* 1994; Weiss and Littman, 1994).

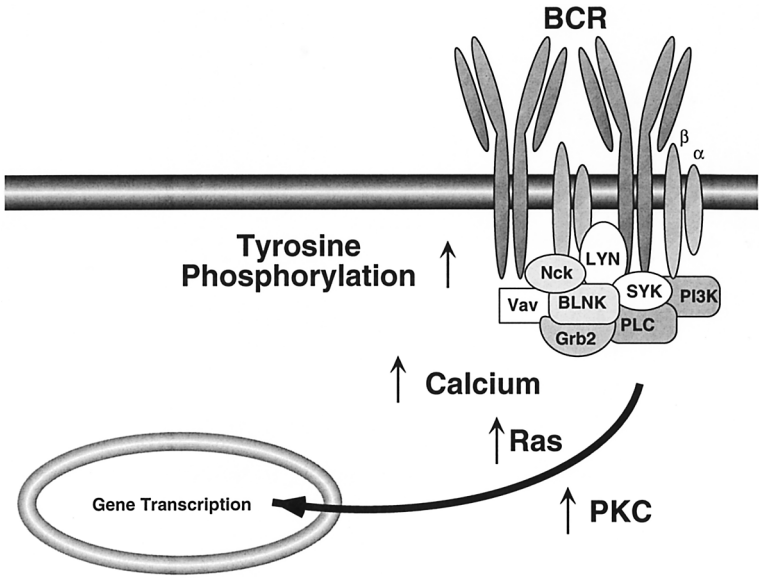


Fig. 3 B-cell signal transduction. Ligation of the BCR results in the activation of the Lyn and Syk PTKs, resulting in the tyrosine phosphorylation, recruitment, and activation of various cell proteins important for BCR-mediated signal transduction. These initial interactions and activation result in the activation of PKC, the Ras pathway, and the release of intracellular calcium, which results in the activation of nuclear gene transcription.

The first indication that LMP2A might be involved in BCR signal transduction was transfection studies utilizing the EBV- B-cell line BJAB. In BJAB cells expressing LMP2A, calcium mobilization was blocked following BCR cross-linking (Miller *et al.,* 1993). Further studies using LCLs infected with wild-type EBV indicated that normal B-cell signal transduction was also blocked as measured by induction of tyrosine phosphorylation, calcium mobilization, and activation of nuclear gene transcription following BCR cross-linking (Miller *et al.,* 1994a,b, 1995). In these wild-type infected LCLs, the amino-terminal domain of LMP2A was tyrosine phosphorylated and was associated with the Src family PTKs and the Syk PTK (Burkhardt *et al.,* 1992; Miller *et al.,* 1995). BCR cross-linking failed to activate Lyn, Syk, PI3-kinase, PLCγ2, Vav, MAPK, and Shc in these same LCLs (Miller *et al.,* 1995). Syk, PI3-kinase, PLCγ2, and Vav were constitutively tyrosine phosphorylated, and the tyrosine phosphorylation remained stable after BCR cross-linking (Miller *et al.,* 1995). In contrast, cross-linking the BCR on LCLs with null mutations in LMP2A resulted in normal BCR signal transduction as measured by induction of tyrosine phosphorylation, calcium mobilization, and switch to lytic replication (Miller *et al.,*1994b, 1995). These results in-

dicated that LMP2A is sufficient for the block in B-cell signal transduction observed in wild-type EBV-infected LCLs (Fig. 4).

V. LMP2A SITE-SPECIFIC MUTANT LCLs

To further understand the LMP2A-mediated block on BCR signal transduction, a number of site-specific mutations changing tyrosines into phenylalanines (Y to F) and three deletion mutations have been engineered into the LMP2A gene and then incorporated into the viral genome to generate LMP2A recombinant LCLs (Fruehling *et al.,* 1996, 1998; Fruehling and Longnecker, 1997; Swart *et al.,* 1999). These mutations are described next. Site-specific mutations at the proline-rich regions, serine, and threonine residues are currently under investigation. These studies will be vital in iden-

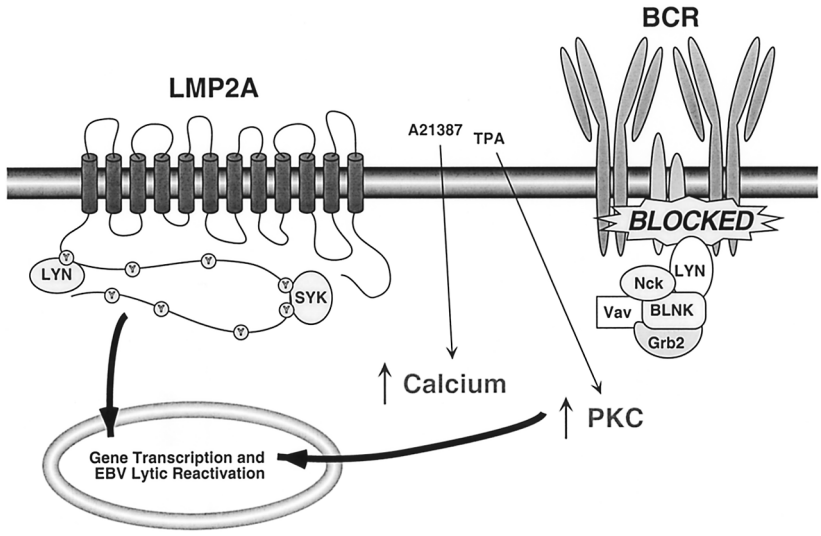


Fig. 4 B-cell signal transduction in LMP2A expressing cells. In EBV latently infected B cells, LMP2A is expressed and the multiple hydrophobic domains of LMP2A mediate aggregation in the plasma membrane, where the amino-terminal domains of LMP2A resemble cross-linked receptor tails and become tyrosine phosphorylated. The Src family PTKs and the Syk PTK bind. Other SH2-containing proteins may also bind. This complex then blocks signal transduction through the BCR, preventing activation of lytic replication following BCR activation. This LMP2A-mediated block can be bypassed by chemical inducers of gene transcription, such as phorbol esters (PMA) or calcium ionophores (A23187). Activation to EBV lytic replication may be mediated by an as yet unidentified pathway such as an interleukin cytokine pathway (IL-x and IL-xR) that is not blocked by LMP2A.

tifying regions of LMP2A that may be important for LMP2A function and provide insight into the involvement of yet identified proteins in LMP2A regulation of EBV latency.

A. Tyrosine Point Mutant LCLs: Y112

Initial studies using transient transfection of LMP2A cDNA mutants into BJAB cells identified tyrosine 112 (Y112) as the site of Lyn binding to LMP2A (Fruehling *et al.,* 1998). Unlike wild-type EBV-infected LCLs, LCLs with the LMP2AY112F mutation behaved as LMP2A null mutations. They were not blocked in BCR signal transduction, calcium mobilization, induction of tyrosine phosphorylation, or activation of lytic replication (Fruehling *et al.,* 1998). Interestingly, LMP2A in the Y112F mutant LCLs was not tyrosine phosphorylated before or after BCR cross-linking (Fig. 5) (Fruehling *et al.,* 1998). In addition, two cellular proteins of 60 and 57 kDa were constitutively phosphorylated in LMP2AY112F mutant infected LCLs. These proteins may represent an important early step required for LMP2A func-

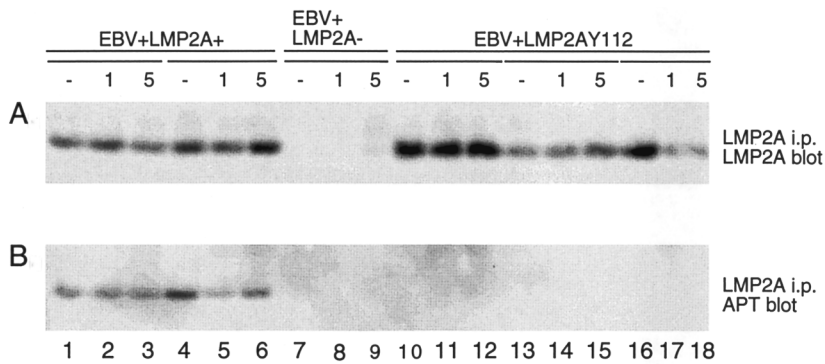


Fig. 5 LMP2A phosphorylation in EBV+LMP2AY112F LCLs. The BCR was untreated (−) or activated for the indicated time (1 or 5 min) in wild-type or mutant infected LCLs. The cells were then lysed and LMP2A was immunoprecipitated and separated by duplicate SDS–PAGE. Separated proteins were transferred to immobilon. Parallel immunoblots were probed with an anti-LMP2A antibodies (A) or anti-phosphotyrosine antibodies (B). (A) LMP2A expression in two EBV+LMP2A+ LCLs (A, lanes 1–6) and three EBV+LMP2AY112F point mutant LCLs (A, lanes 10–18) was similar before (−) and after (1 or 5 min) BCR activation. No LMP2A was detected in the EBV+LMP2A- LCL included as a negative control (A, lanes 7–9). (B) LMP2A tyrosine phosphorylation before and after BCR activation. In two EBV+LMP2A+ LCLs, LMP2A was constitutitvely phosphorylated, which did not change with BCR activation (B, lanes 1–6). LMP2A was not phosphorylated in the three EBV+LMP2AY112F point mutant LCLs even after BCR activation (C, lanes 10–18). (Reprinted with permission from Fruehling *et al.,* 1998)

tion. These EBV recombinant LCLs infected with the LMP2AY112F mutation have defined tyrosine Y112 as essential for LMP2A function and the binding of the Src family PTKs to LMP2A.

B. Tyrosine Point Mutant LCLs: Y74 AND Y85

To further test the importance of the LMP2A ITAM in LMP2 function, LCLs were derived containing either a tyrosine to phenylalanine point mutation at LMP2A tyrosine 74 (Y74F) or tyrosine 85 (Y85F). In LMP2AY74F and LMP2AY85F LCLs, B-cell signal transduction was similar to LMP2A null LCLs as assessed by mobilization of intracellular calcium, activation of tyrosine phosphorylation, and the induction of lytic viral replication after BCR cross-linking (Fruehling and Longnecker, 1997). In addition, Syk was co-immunoprecipitated with LMP2A in wild-type LCLs, but not in either the LMP2AY74F or LMP2AY85F LCLs despite LMP2A being tyrosine phosphorylated (Fig. 6) (Fruehling and Longnecker, 1997). Finally, like LMP2A null LCLs, Syk was not phosphorylated in the absence of BCR cross-linking, but rapidly became phosphorylated with a concomitant increase in kinase activity following BCR cross-linking, unlike wild-type LCLs in which Syk phosphorylation and activity remained unchanged after BCR cross-linking (Fruehling and Longnecker, 1997). These data demonstrated that each of the two tyrosine residues of the LMP2A ITAM was essential for the LMP2A-mediated block of BCR signal transduction and binding of the Syk PTK to LMP2A.

C. Deletion Mutant and Tyrosine Point Mutant LCLs: Y60, Y64, and Y101

In addition to the Y74, Y85, and Y112 tyrosine point mutations, the five remaining tyrosines have been mutated by either point or deletion mutation. Analysis of EBV-transformed LCLs infected with a tyrosine to phenylalanine mutation at tyrosine 60, 64, or 101 indicated that the expression, subcellular localization, or phosphorylation of LMP2A was unaffected by these mutations (Swart *et al.,* 1999). In addition, normal BCR signal transduction was similarly blocked when compared to wild-type EBV-infected LCLs as measured by calcium mobilization, tyrosine phosphorylation, and the induction of BZLF1. Although somewhat surprising, it is apparent that tyrosines 60, 64, and 101 of LMP2A are not required for LMP2A function *in vitro*. Previously, it was hypothesized that specific SH2 domain containing proteins may bind LMP2A on phosphorylation of the LMP2A tyrosines (Longnecker and Miller, 1996). The potential Y60 SH2 binding motif has the best ho-

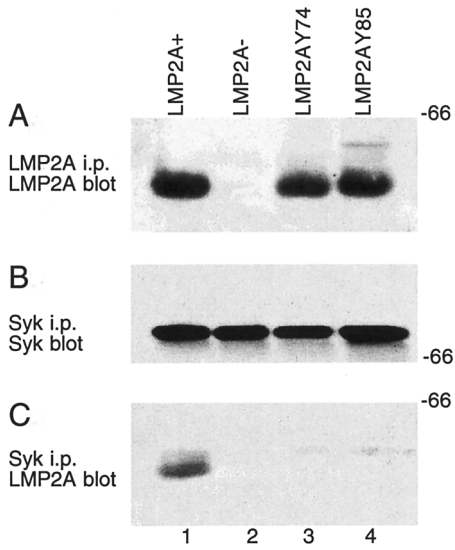


Fig. 6 LMP2A co-immunoprecipitates with the Syk PTK in EBV+LMP2A+ LCLs but not in LMP2A ITAM mutant LCLs. (A) Expression of LMP2A is detected in an EBV+LMP2A+ LCL (A, lane 1), an EBV+LMP2AY74 LCL (A, lane 3), and an EBV+LMP2AY85 LCL (A, lane 4), whereas no LMP2A was detected in an EBV+LMP2A- LCL (A, lane 2). (B) Equivalent levels of the Syk PTK are immunoprecipitated from all four of the LCLs: EBV+LMP2A+ LCL (B, lane 1), EBV+LMP2A- LCL (B, lane 2), EBV+LMP2AY74 LCL (B, lane 3), and EBV+LMP2AY85 LCL (B, lane 4). (C) LMP2A co-immunoprecipitates with Syk in an EBV+LMP2A+ LCL (C, lane 1), but not in an EBV+LMP2A- LCL (C, lane 2), an EBV+LMP2AY74 LCL (C, lane 3), and an EBV+LMP2AY85 LCL (C, lane 4). Unstimulated LCLs were lysed and immunoprecipitated in parallel with anti-LMP2A or anti-Syk antibodies. Following immunoprecipitation, proteins were separated by SDS–6% PAGE, and transferred to an immobilon. LCL clone numbers are indicated above each lane and protein standards are indicated in kilodaltons. (Reprinted with permission from Fruehling and Longnecker, 1997.)

mology for phosphotyrosines, which bind the SH2 domain of the adapter proteins Crk or Nck (Table I). These adapter molecules once recruited to LMP2A complexes might recruit additional proteins important in B-cell signal transduction and LMP2A function in B lymphocytes. The tyrosine 101 motif contains an arginine at the +3 position that is characteristic of the Csk SH2 preferred motif (Songyang *et al.*, 1994). Csk downregulates the activity of Src family PTKs when they are recruited to the BCR (Howell and Cooper, 1994). The interaction of LMP2A with Csk would provide a convenient model for the LMP2A-mediated affect on Lyn activity seen in wild-type infected LCLs.

Previous studies had indicated a potential important functional role for tyrosines 60 and 64 of LMP2A. Deletion of amino acids 21–64 but not amino acids 21–36 resulted in an LMP2A protein with only partial function in

EBV-transformed LCLs grown *in vitro* (Fruehling *et al.,* 1996). In particular, there was little constitutive phosphorylation of cellular proteins in the 21–64 deletion mutant when compared to wild-type infected cells in the absence of BCR activation. In addition, LMP2A was only phosphorylated following BCR activation in contrast to wild-type infected LCLs in which LMP2A is highly tyrosine phosphorylated. Finally, calcium mobilization was blocked as in wild-type infected LCLs, whereas lytic viral replication was readily induced following BCR activation similar to LMP2A null infected LCLs. Because mutation of tyrosines 60 and 64 resulted in none of the phenotypes associated with the 21–64 deletion mutant, it is apparent that other LMP2A sequences within the deletion must be considered. Two proline-rich sequences were deleted, one a potential binding site for SH3 domains, and the other a PY motif that may be important for the binding of proteins containing a WW domain.

In summary, tyrosines 23, 31, 60, 64, and 101 are nonessential for LMP2A function *in vitro* (Fruehling *et al.,* 1996; Swart *et al.,* 1999). The importance of tyrosines 74, 85, and 112 in binding cellular PTKs was previously shown to be essential for LMP2A *in vitro* function. Although tyrosines 23, 31, 60, 64, and 101 are not necessary for the LMP2A-mediated block on BCR activation of protein tyrosine phosphorylation, calcium mobilization, and lytic replication, these tyrosines may be important in other LMP2A functions related to the persistence of EBV in the human host. A recent *in vivo* study, described later, using LMP2A transgenic mice indicates that LMP2A provides both B-cell development and survival signals (Caldwell *et al.,* 1998). Studies using a similar transgenic approach using these same mutations may reveal that these tyrosines are required for promoting B-cell development and/or survival *in vivo*.

VI. MODEL OF LMP2A AND LMP2B FUNCTION *IN VITRO*

From our studies of the LMP2A mutations in LCLs, we propose the model of LMP2A and LMP2B function depicted in Fig. 7. LMP2A is expressed and forms complexes by interaction of the multiple hydrophobic membrane-spanning domains in the plasma membrane of EBV-infected B cells. By forming constitutive membrane complexes, LMP2A is able to mimic an activated BCR receptor. Initially, the Src family PTKs, such as Lyn, are recruited to the LMP2A complexes possibly through the interaction of the Lyn SH3 domain with a LMP2A proline-rich region (Fig. 7A). Alternatively, Lyn may be recruited to the DQSL sequence and is similar to the DCSM sequence, which has been shown to be important for recruitment of Lyn to the BCR associated pro-

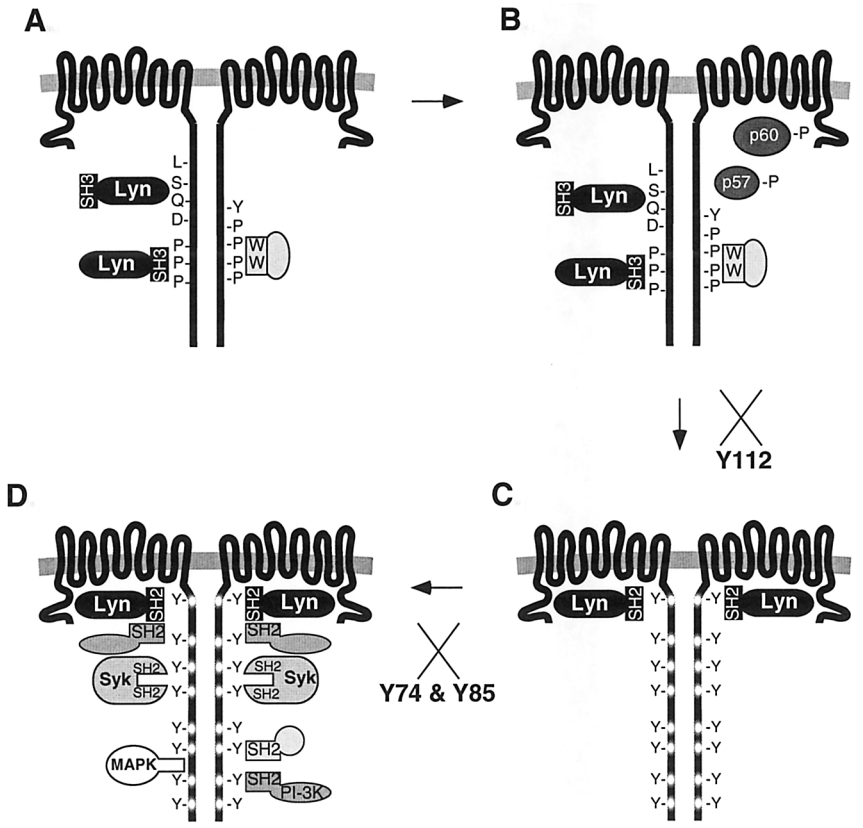


Fig. 7 Model for LMP2A function. (A) The Src family PTK Lyn is recruited to LMP2A possibly by an interaction of the Lyn SH3 domain with a LMP2A proline-rich region or interaction of the Lyn unique region with the LMP2A DQSL sequence. WW domain-containing proteins may also be recruited to a nonphosphorylated PPPPY motif. (B) LMP2A is phosphorylated at Y112, by the Lyn PTK. Phosphorylation of the unidenitified cellular proteins, p57 and p60, is induced in both wild-type EBV+LMP2A- and EBV+LMP2AY112F LCLs prior to BCR cross-linking. (C) Once Y112 is phosphorylated, the Lyn SH2 domain binds. Subsequent to the binding of Lyn, the remaining LMP2A tyrosines are phosphorylated including the LMP2A ITAM. Binding of Lyn to Y112 and the subsequent phosphorylation of LMP2A is blocked in the Y112F mutant. (D) The Syk PTK, other SH2 containing proteins, and MAPK bind to phosphorylated LMP2A. Once bound to LMP2A their activities are reduced and they are no longer able to participate in BCR signal transduction. Binding of Syk is blocked in LMP2A ITAM (Y74F and Y85F) mutants, but not LMP2A phosphorylation.

tein Igα (Pleiman *et al.*, 1994). Upon recruitment of Lyn to LMP2A, a 60-kDa protein and 57-kDa protein become phosphorylated (Fig. 7B). This is followed by the phosphorylation of LMP2A and the binding of Lyn via its SH2 domain to Y112 of LMP2A (Fig. 7C). Once bound, Lyn phosphorylates the remaining LMP2A tyrosines. In the Y112F mutation, the initial phosphorylation of

LMP2A at Y112 is blocked, thereby preventing interaction of the Lyn SH2 domain with Y112 and subsequent phosphorylation of LMP2A.

Following phosphorylation of LMP2A, other SH2 containing proteins are recruited to LMP2A complexes. Specifically, the Syk PTK binds to the phosphorylated LMP2A ITAM (Fig. 7D). Binding of Syk to LMP2A is blocked when either of the tyrosines within the LMP2A ITAM is mutated, although LMP2A is tyrosine phosphorylated in both ITAM mutants (Fruehling and Longnecker, 1997). Other unidentified SH2 domain-containing proteins may also be recruited to LMP2A phosphotyrosines, such as PI(3)-kinase or candidate proteins shown in Table I. LMP2A associates with at least eight unidentified proteins (Burkhardt *et al.,* 1992). If activated, PI(3)-kinase may prevent apoptosis of EBV infected cells by activation of the serine-threonine kinase Akt resulting in the phosphorylation of BAD, which is an important regulator of apoptosis (Datta *et al.,* 1997; del Peso *et al.,* 1997; Downward, 1997). Other potential targets of PI(3)-kinase and the Akt pathway include Caspase 9 (Cardone *et al.,* 1998), or the transcription factor forkhead (Brunet *et al.,* 1999; Kops *et al.,* 1999). The function of other proteins bound to LMP2A may also be altered. Proteins such as MAPK, WW domain-containing proteins, or the SH2 domain-containing tyrosine phosphatase 1 (SHP-1) may also be recruited to LMP2A patches (Figs. 7A and 7D). SHP-1, when recruited to the BCR complex by the Fc receptor, results in down-modulation of BCR signal transduction (D'Ambrosio *et al.,* 1995). Ongoing studies are focused on determining the intriguing mechanism of LMP2A regulation of BCR signal transduction.

VII. *IN VIVO* MODELS OF LMP2A FUNCTION

Because EBV is host specific, animal models need to be developed to understand LMP2A and LMP2B function *in vivo*. Recent studies using transgenic mice have proven informative for dissecting a function for LMP2A *in vivo* (Caldwell *et al.,* 1998). Transgenic mice were constructed that express the LMP2A in B lineage cells (Caldwell *et al.,* 1998). LMP2A expression resulted in the bypass of normal B-lymphocyte developmental checkpoints, allowing immunoglobulin negative cells to colonize peripheral lymphoid organs, indicating that LMP2A possesses a constitutive signaling activity (Caldwell *et al.,* 1998). B lymphocytes from mice bearing targeted deletions of recombinase activating gene 1 (RAG-1), RAG-2, or the immunoglobulin heavy chain are unable to make functional immunoglobulin and as a result have a compete absence of mature B cells in peripheral lymphoid organs (Kitamura *et al.,* 1991; Mombaerts *et al.,* 1992; Shinkai *et al.,* 1992). In animals lacking peripheral immunoglobulin gene expression by means of inducible Cre-*loxp*-mediated gene targeting, receptorless B lymphocytes are

eliminated by apoptosis (Lam *et al.,* 1997). These experiments illustrate the necessity for competent BCR signaling in all stages of B-cell development, from early B-cell ontogeny to peripheral B-cell maintenance. Surprisingly, B cells lacking BCR expression are prominent in the bone marrow and periphery of the LMP2A transgenic mice. Although these B cells exhibit many of the hallmarks of appropriately developing B lymphocytes; including downmodulation of CD43 expression, IL7 responsiveness, and colonization of splenic environments, they are unable to rearrange functional immunoglobulin genes and subsequently do not express a BCR.

As well as providing a developmental signal that bypasses the requirement for immunoglobulin gene expression in bone marrow, LMP2A provides a survival signal to progenitor and peripheral B cells as evidenced by the increase in CD19+BCR cells in spleens of LMP2A transgenic mice. Most striking is that the LMP2A transgene allows receptorless B cells to mature into CD43− cells in the bone marrow that are able to colonize the spleen in a RAG-1−/− background. LMP2A transgenic animals were bred to RAG-1−/− mice and subsequent TgE+RAG-1−/− animals were identified by PCR analysis of genomic tail DNA. Flow cytometric analysis of splenic cells from TgE+RAG-1−/− animals identified significant numbers of CD19+ cells, a population absent in spleens of nontransgenic RAG-1−/− animals (Fig. 8A). Similarly, bone marrow of TgE+RAG-1−/− animals exhibited a dramatic increase in CD19+ cells when compared to the nontransgenic RAG-1−/− littermates (Fig. 8B). Examination of CD43 expression in these bone marrow samples revealed that unlike nontransgenic RAG-1−/− littermates, TgE+RAG-1−/− bone marrow contained a large percentage of CD19+ cells that did not express CD43 (Fig. 8C).

VIII. LMP2 FUNCTION IN EPITHELIAL CELLS

LMP2 transcripts are readily detected in NPC biopsies, but little is known about the role of the LMP2 genes in NPC and other epithelial proliferative disorders. LMP2A has been shown to coprecipitate with PTKs and was phosphorylated in *in vitro* kinase assays from epithelial cell lines expressing LMP2A (Scholle *et al.,* 1999). The use of LMP2A deletion mutants indicated that multiple LMP2A tyrosines were important for phosphorylation of LMP2A (Scholle *et al.,* 1999). The phosphorylation of LMP2A was induced on adhesion to extracellular matrix proteins and the PTK Csk was shown to be important for LMP2A phosphorylation (Scholle *et al.,* 1999). The phosphorylation of LMP2A in epithelial cells is particularly tantalizing because it provides evidence that LMP2A may alter normal epithelial biology in a fashion similar to the alteration of normal B-cell function by LMP2A. Ep-

ithelial cells are also rich in proteins that regulate signal transduction. The generation of transgenic models with targeted LMP2A expression to epithelial cells may provide answers.

IX. DISCUSSION

From the *in vitro* studies and initial studies with LMP2A transgenic mice, several roles for LMP2A function *in vivo* can be surmised. The LMP2A transgenic studies suggest LMP2A may resemble a BCR thus providing an inappropriate developmental and survival signal to B cells in latently infected human hosts. This observation may have importance in EBV-associated

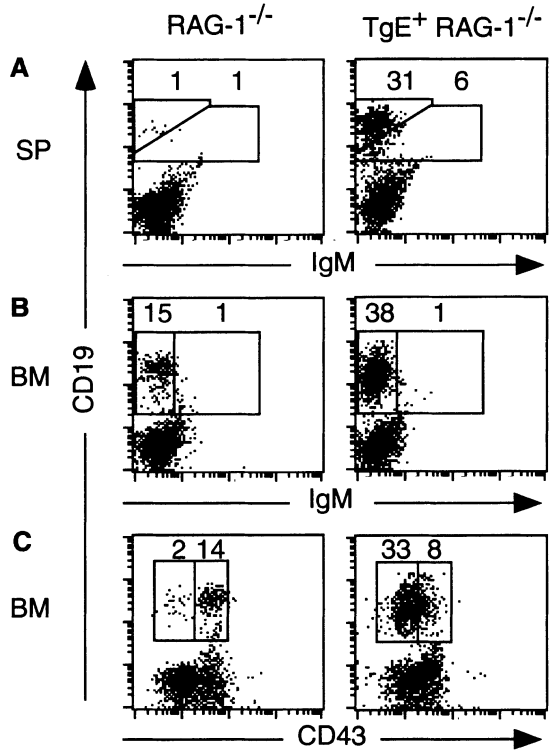
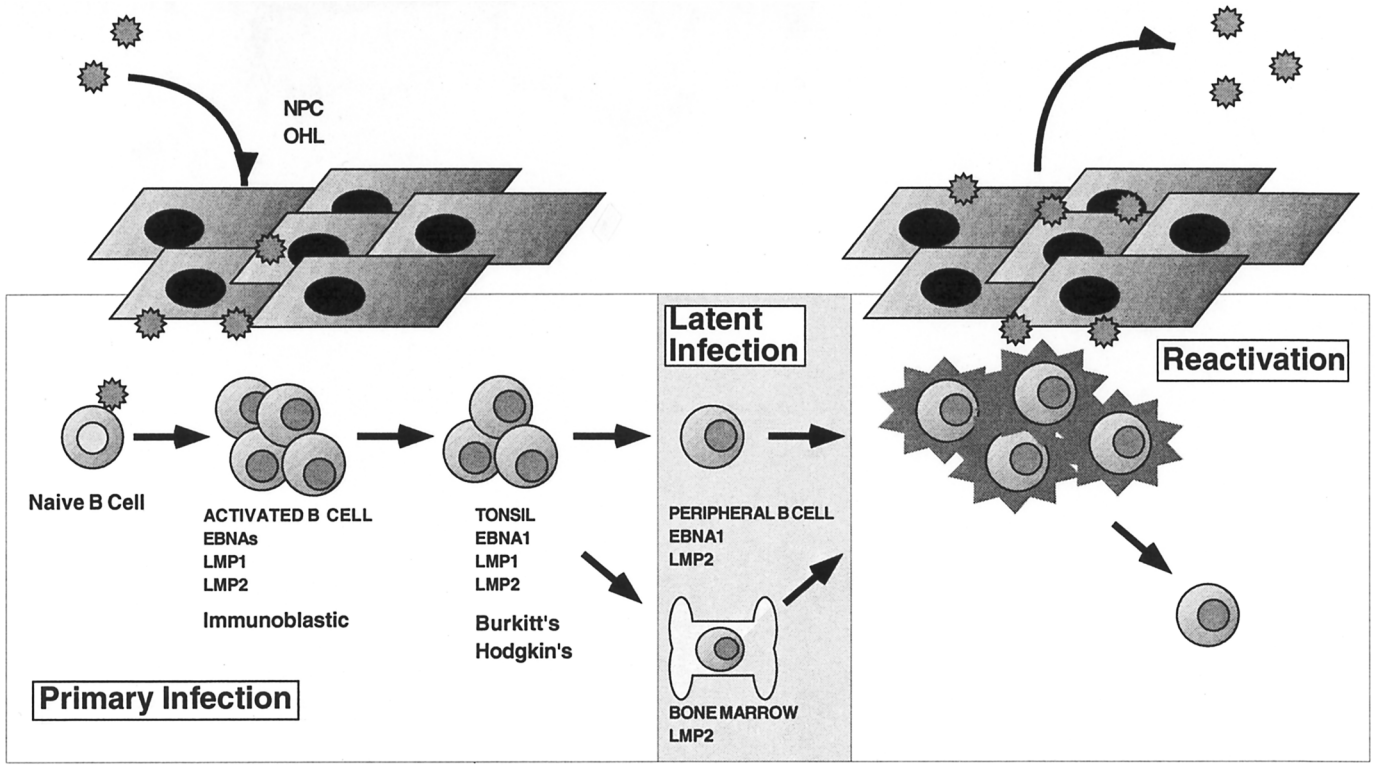


Fig. 8 Analysis of B lymphocytes in TgE x RAG-1−/− animals. Single cell suspension of bone marrow and spleen cells from nontransgenic RAG-1−/− and TgE+RAG-1−/− littermates were stained with the indicated antibodies and analyzed by flow cytometry. Plots show representative data from two independent experiments.

malignancies. Although Reed-Sternberg cells in Hodgkin's disease have been characterized as germinal center derived B lineage cells, some of these cells contain somatic mutations resulting in the absence of immunoglobulin expression, thereby indicating that these cells do not require BCR signaling for survival (Braeuninger *et al.,* 1997; Kanzler *et al.,* 1996). By mimicking signals derived from the BCR, LMP2A may provide a signal that would maintain these cells in the absence of a competent BCR. This may be an important first step in the development of HD.

The role of LMP2A in EBV latency has been the subject of much conjecture as the message for LMP2A is readily detected in immune competent hosts with EBV latent infections (Miyashita *et al.,* 1995; Qu and Rowe, 1992; Tierney *et al.,* 1994). Our studies discussed earlier indicate LMP2A blocks normal BCR signal transduction in EBV latently infected LCLs grown in culture. This block in BCR signal transduction prevents switch from latent to lytic replication following BCR activation (Miller *et al.,* 1994b, 1995). These *in vitro* observations suggest that the *in vivo* role of LMP2A in latent EBV infection was to prevent activation of lytic EBV replication by BCR-mediated signal transduction (Longnecker and Miller, 1996; Miller *et al.,* 1994b, 1995). This LMP2A function would be important in preventing lytic replication in latently infected lymphocytes as they circulate in the peripheral blood, bone marrow, or lymphatic tissues where they might encounter antigens, super antigens, or other ligands that could engage B-cell receptors and activate EBV lytic replication (Fig. 9).

Fig. 9 EBV infection and latency in the human host. Primary infection: Infectious virus is spread by saliva where it encounters a circulating B lymphocyte trafficking in the oral epithelium. EBV can infect oral epithelial cells, which can result in NPC and OHL. Once the B cell is infected, it becomes activated and is driven to proliferate by expression of all of the latency associated gene products (EBNAs, LMP1, and LMP2s). This activated phenotype is similar to immunoblastic lymphomas seen in patients with immune suppression. The infected cells may then traffic to other lymphoid organs where progenitor B cells may become infected. These may be important for the development of EBV-related malignancies, such as Hodgkin's or Burkitt's or latency. Upon the activation of a vigorous cytotoxic T-cell response, the proliferating infected B lymphocytes are killed. Latent infection: EBV is found in about 1 in 10^7 or 10^9 peripheral B cells depending on the human donor (Khan *et al.,* 1996; Yao *et al.,* 1985). This level remains relatively stable throughout the lifetime of the given human host. There is limited viral gene expression of LMP2 and EBNA1 (Chen *et al.,* 1995; Decker *et al.,* 1996; Khan *et al.,* 1996; Miyashita *et al.,* 1995; Qu and Rowe, 1992; Tierney *et al.,* 1994; Yao *et al.,* 1985). EBV may also gain access to the bone marrow where it may infect a B lineage stem cell. Reactivation: Virus is spread to uninfected individuals when a latently infected cell traffics to the oral epithelium where it is induced to undergo lytic replication by an as yet undefined mechanism. LMP2A may have roles in each of the phases of the establishment and persistence of EBV latent infections. In the proliferation stage, LMP2A may provide a growth-stimulating signal, whereas in the latent stage, it may provide a signal that is important for the survival of the latently infected cell. In the reactivation stage, LMP2A may be important in blocking the activation of lytic infection, allowing it to occur only when an EBV-infected B cell traffics to the oral epithelium.



LMP2A Function

Proliferation **Survival** **Inhibition**

More recent results from our transgenic model of EBV latency suggest another potential function for LMP2A *in vivo*. This function of LMP2A would be to provide a constitutive survival signal in mature B cells. This LMP2A derived signal would obviate the necessity for BCR-induced survival signals in EBV latently infected peripheral B cells, allowing persistence of virally infected B cells in the infected host. The importance of signal transduction through the BCR for B-cell survival has been demonstrated in murine conditional knockouts of the BCR receptor (Lam *et al.,* 1997) and in *in vitro* studies showing that activation of the BCR prevents apoptosis (Rathmell l*et al.,* 1996; Rothstein *et al.,* 1995). Finally, LMP2A may be important in maintaining EBV-infected lymphocytes in an inactivated state, thereby preventing killing by cytotoxic T cells specific for LMP2A.

Studies to determine the site of EBV latency in immune competent human hosts have shown that in peripheral blood EBV resides in memory B lymphocytes which are CD19+, CD23−, B7−, sIg+, and sIgD− (Babcock *et al.,* 1998; Lam *et al.,* 1994; Miyashita *et al.,* 1995, 1997) and express EBNA1 and LMP2A (Babcock *et al.,* 1998; Kahn *et al.,* 1996; Miyashita *et al.,* 1995, 1997; Qu and Rowe, 1992; Tierney *et al.,* 1994). Other potential sites of EBV latency may include bone marrow, lymph nodes, or other lymphoid organs because EBV can be isolated from virtually any lymphoid tissue (Rickinson and Kieff, 1996). Our recent results with our transgenic model of EBV latency (Caldwell *et al.,* 1998) and the observation that bone marrow cells can harbor EBV (Gratama *et al.,* 1988, 1989), have led us to speculate that bone marrow may serve as the site of EBV latency. In this model of EBV latency, progenitor B cells may be infected by EBV when circulating B cells containing the virus traffic to bone marrow. Progeny of latently infected bone marrow cells could generate the relatively stable number of EBV-infected lymphocytes observed in peripheral blood of latently infected individuals (Lam *et al.,* 1991, 1994; Lewin *et al.,*1987; Miyashita *et al.,* 1995; Yao *et al.,* 1985, 1991). Theoretically, once latently infected bone marrow cells enter the periphery, constitutive signaling from LMP2A could provide a survival signal that would maintain these cells in the absence of a competent BCR or the requirement for BCR signal transduction.

Whatever the site of EBV latency in the human host, peripheral B cells harboring the virus may undergo lytic replication when trafficking to the oral epithelium, thereby allowing shedding of virus to naive hosts. In infected hosts, latent infection enables the virus to escape immune destruction, while periodic reactivation transfers virus back to epithelial tissue where the virus replicates. However, the signals *in vivo* that may induce lytic replication are unknown. But it is likely that under specific conditions, such as the interaction of an EBV-infected lymphocyte with epithelial-produced cytokines or with epithelial surfaces, lytic replication may be induced. Both the *in vitro* and *in vivo* studies discussed will allow for the determination of the validi-

ty of the current ideas of LMP2 function in EBV latent infection and elucidate the mechanisms underlying the persistence of EBV in latently infected humans. The understanding of the unique pathologies associated with EBV latent infections may allow the development of novel therapeutics to treat EBV infections and its associated diseases.

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Biochemistry and Pathological Importance of Mucin-Associated Antigens in Gastrointestinal Neoplasia

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I. INTRODUCTION

Interest in mucin-associated carbohydrate and peptide antigens has increased in different fields of cancer research during the last years. Numerous studies have elucidated the structures of the antigens applying various techniques in order to analyze the involved genes, enzymes, and carbohydrates. Their distribution in human tissues and sera was investigated in detail and the diagnostic and prognostic relevance of a certain number of these anti-

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gens in cancer patients was reported. Some *in vitro* and *in vivo* studies shed light on mechanisms of cell adhesion and metastasis, which may explain the importance of these antigens for the course of tumor disease. Additionally, immunotherapeutic approaches were initiated in animal models and in clinical trials, which could indicate novel strategies in anticancer therapy. This review concentrates on the mechanisms of alterations of mucin-associated antigens in gastrointestinal neoplasia and their implications in clinical diagnostic and therapeutic oncology.

II. BIOCHEMISTRY AND MOLECULAR BIOLOGY OF MUCINS

A. What Is a "Mucin"?

Before we get into the body of this article, we should define what is meant with the term *mucin*. The meaning of this designation has changed during the last three to four decades. During the 1960s researchers in the field used *mucin* to refer to the major glycoprotein components in secreted mucus lining the surfaces of glandular epithelia in the gastrointestinal, the bronchial, and the urogenital tracts, and the best characterized species, the mucins from submaxillary glands of sheep and cow, served as structural models of this class of macromolecules. Meanwhile, knowledge about the structural aspects of mucins has increased tremendously with respect to both their protein cores and their glycan moieties, and the term *mucin* has a more precise meaning on the one hand, whereas on the other, its discrimination from members of the membrane bound "mucin-like" glycoproteins has become more difficult.

Initially, a mucin was thought to consist of a serine- and threonine-rich peptide core that served primarily as a scaffold for the addition of uniform, simple, and mainly acidic oligosaccharides. The submaxillary mucins from cows and sheep, for example, are predominantly glycosylated with *O*-linked di- and trisaccharides of the structures NeuAc(Gc)α2–6GalNAc and NeuAc-(Gc)α2–6(Galβ1–3)GalNAc. This simple structural model was in accordance with the proposed function of mucins, which was regarded to lie in the formation of a viscoelastic gel serving to provide physicochemical protection of epithelial surfaces. Up to now all "mucineers" agree with respect to several characteristic features of mucins: (1) their high carbohydrate content, which makes up between 50 and 80% by weight; (2) their correspondingly high buoyant density, which is generally in the range of 1.4–1.5 g/ml; and (3) their preferential glycosylation with *O*-linked chains, which often occurs in clusters.

After the evolution of sophisticated analytical instrumentation, such as high field mass spectrometry (FABMS) and 500-MHz NMR, and the development of hybridoma technology in the 1970s, we learned during the following decade that mucins are much more complexly glycosylated than expected (Lamblin *et al*., 1984; Hounsell *et al.,* 1985, 1989; Hanisch *et al*., 1985, 1986; Mutsaers *et al*., 1986). Moreover, we realized that mucins are secreted or shed from tumor cells and can be detected as circulating antigens using carbohydrate-specific antibodies (Magnani *et al*., 1983).

These two important observations have made the mucins more and more attractive as a research topic, and it was at the end of the 1980s that several groups were able to sequence the first human mucin species on the DNA level (Gendler *et al*., 1990; Lan *et al*., 1990; Ligtenberg *et al*., 1990; Wreschner *et al*., 1990). Designated as MUC1, this mucin and a series of others (MUC2–MUC8) were shown to exhibit large domains of tandemly repeated peptides as a structural characteristic of the "real" mucins (Gum *et al*., 1994; van Klinken *et al*., 1997; Porchet *et al*., 1991; Nollet *et al*., 1998; Meerzaman *et al*., 1994; Toribara *et al*., 1993; Bobek *et al*., 1993; Shankar *et al*., 1994).

More recently a functionally based definition of mucins has been introduced that refers to members of the "mucin-like" glycoproteins such as GlyCAM1 and MadCAM1 serving roles in cell adhesion (Shimizu and Shaw, 1993). The present article focuses on the real mucins, which are now described in more detail.

B. The MUCs: Two Classes of Real Mucins

Up to now nine genes have been identified that code for distinct mucin species (Table I). The major portion of the peptide cores of these mucins is characterized by large domains of tandemly repeated peptide sequences. Each of these units has a specific length and sequence (Table I), and in several mucin species its repetition varies individually according to a genetic polymorphism. Exceptionally, MUC5B (11 repeats) and MUC7 (6 repeats) have constant numbers of peptide repeats (Meerzaman *et al*., 1994; Bobek *et al*., 1993). Interestingly, the currently known MUC species can be subdivided into two groups dependent on further structural aspects characterizing domains outside the "variable number of tandem repeats" (VNTR) domain. While membrane-bound mucins exhibit hydrophobic sequences or "transmembrane domains" responsible for their becoming anchored in the lipid bilayer, the secretory mucins possess one or several D domains, cystein-rich peptides that play roles in the oligomerization of mucin monomers and in the packaging into secretory vesicles. Again there is one exception, MUC7, which is devoid of D domains (Bobek *et al*., 1993). Three members of the membrane-bound

Table I. MUC Series of Human Mucins

MUC species	Secretory (s) and/or membraneous (m)	D domains	Chromosomal localization	Tandem repeats	
				Number	Sequence
MUC1	m,s	−	1q21	pm[a]	AHGVTSAPDTRPAPGSTAPP
MUC2	s	+	11p15.5	pm	PTTTPITTTTTVTPTPPTPTGTQT
				16	PPTTTPSPPTTTTTTP
MUC3	m,s	−	7q22	pm	HSTPSFTSSOTTTETTS
MUC4	m,s	−	3q29	pm	TSSASTGHATPLPVTD
MUC5AC	s	+	11p15.5	pm	TTSAPTTS
MUC5B	s	+	11p15.5	11	irregular repeats of 29 aa
MUC6	s	+	11p15.5	pm	169 aa repeat
MUC7	a	−	4q13–21	6	TTAAPPTPSATTPAPPSSSAPPE
MUC8	?	?	12q24.3	?	TSCPRPLQEGTRV and TSCPRPLQEGTPGSRAAHALSRRG HRVHELPTSSPGGDTGF

[a]pm, polymorphic.

mucins, MUC1 (Gendler *et al*., 1990), MUC3 (Gum *et al*., 1997), and MUC4 (Nollet *et al*., 1998), have been identified so far, whereas MUC2 (Gum *et al*., 1994), MUC5AC (Guyonnet Duperat *et al*., 1995), MUC6 (Toribara *et al*., 1993), and MUC7 (Bobek *et al*., 1993) belong to the group of secretory mucins (Table I). Because not all MUC genes have been sequenced completely, a classification of MUC8 is currently not possible. The MUC genes were localized in good accordance with this classification to distinct chromosomal sites (Table I): chromosome 1q21 (MUC1) (Gendler *et al*., 1990), chromosome 7q22 (MUC3) (Fox *et al*., 1992), chromosome 3q29 (MUC4) (Gross *et al*., 1992), and chromosome 11p15.5 (MUC2, MUC5AC, MUC5B, MUC6) (Pigny *et al*., 1996). MUC7 was localized to chromosome 4q13–21 (Bobek *et al*., 1996) and MUC8 to chromosome 12q24.3 (Shankar *et al*., 1994).

We should mention at this point, however, that a clear-cut distinction between membrane-bound and secretory mucins cannot be made, because the former are also found as secreted mucins. One member of each class, MUC1 and MUC2, should serve to describe the structural aspects of membrane-bound and secretory mucins in more detail.

C. Structural Aspects of Mucins: MUC1 and MUC2 as Models

Four research groups were able to clone and sequence the full-length cDNA of MUC1 from different human carcinoma cell lines (Gendler *et al*., 1990; Lan *et al*., 1990; Ligtenberg *et al*., 1990; Wreschner *et al*., 1990). From these studies the organization of exon and intron sequences was deduced as shown in Fig. 1. Among the seven exon sequences, the second contains the entire repeat region. On the protein level this domain can make up about 50–80% of the molecule dependent on the number of peptide repeats (Fig. 1). The remainder of the protein consists of 480 amino acids (aa). The highly conserved VNTR domain in the center of MUC1 is flanked by short regions containing several degenerate repeat peptides that exhibit a high degree of sequence variation. The short N-terminal domain contains a signal peptide, whereas the more extended C-terminal portions outside the VNTR domain comprise a transmembrane (31 aa) and an intracellular domain (69 aa).

Between VNTR and transmembrane domains a proteolytic cleavage site is located that is used during processing of the mucin protein in the Golgi and results in the formation of a noncovalent membrane anchored heterodimer (Ligtenberg *et al*., 1992). The peptide backbone of about 120–225 kDa is heavily glycosylated within, but also outside the VNTR domain. The peptide stretch between VNTR and transmembrane domains contains five potential N-glycosylation sites, which are substituted with complex-type glycans (Hilkens and Buijs, 1988). Five potential *O*-glycosylation sites are

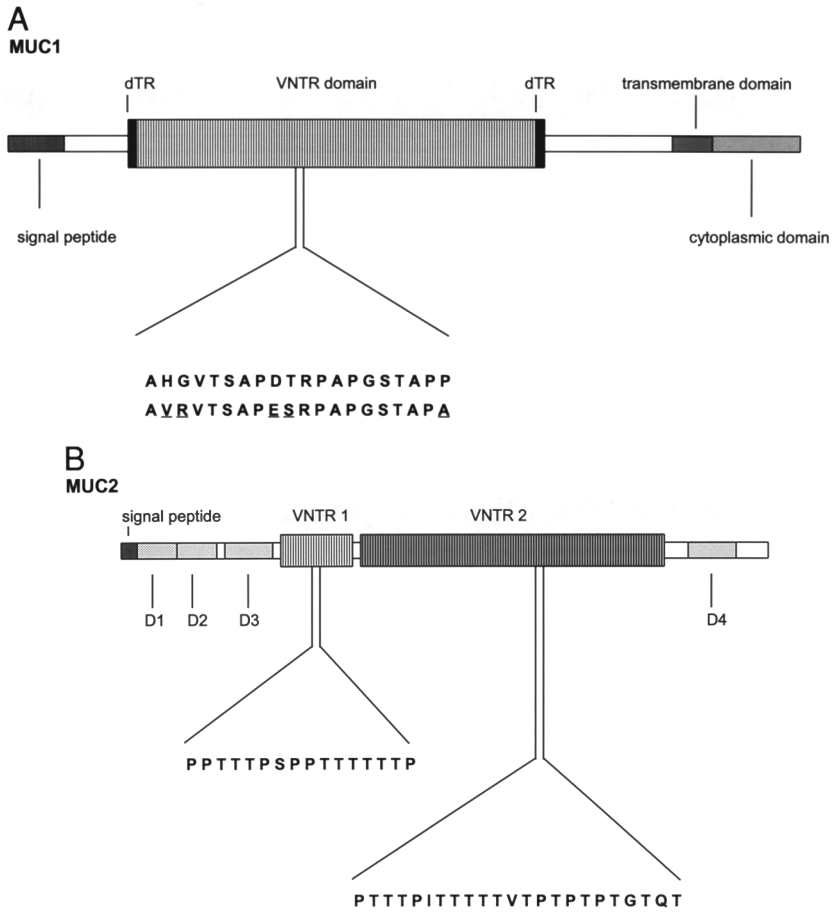

Fig. 1 Primary structures of human mucins: MUC1 and MUC2 as models. (A) The primary structure of MUC1 is shown as deduced from DNA sequence data (Gendler *et al*., 1990). Regions flanking the VNTR domain (variable number of tandem repeats domain) and designated as dTR correspond to degenerate tandem repeats with a low degree of homology to the "conserved" repeats within the VNTR domain. (B) The primary structure of MUC2 is shown as deduced from DNA sequence data (Gum *et al*., 1994). The regions designated D1 to D4 correspond to the Cys-rich D domains, which are responsible for vesicular packaging, secretion, and oligomerization of the mucin. VNTR1 refers to the 16 repeat domain (see Table I) and VNTR2 to the polymorphic domain (see Table I).

found per repeat peptide that carry a complex pattern of mucin-type oligosaccharides at varying chain lengths and densities (see below).

Since the repetitive peptide of the VNTR domain had been sequenced (Fig. 1), it was regarded to be highly conserved. Only low levels of mutational alterations were reported. Surprisingly, a recent study of the VNTR core pro-

tein revealed that the established sequence of the repeat peptide may represent only one of several alternative icosapeptides (Fig. 1). Three positions within the AHG 20 sequence were reported to be replaced at high incidence: Pro9 → Ala, Asp18 → Glu, and Thr19 → Ser (Müller *et al*., 1999). The latter two replacements, which were found in 50% of the repeats, always occur in concert. Originally reported for a breast cancer cell line (T47D), the same peptide variants were also detected in other human carcinoma cell lines on the DNA level (Siddiqui *et al*., 1988; F.-G. Hanisch *et al*., unpublished). The obvious discrepancy to earlier investigations is explained by considering that the entire VNTR domain had never been sequenced in the previous analyses.

One of the most complex and largest mucins is MUC2, the major component of intestinal mucus. The polypeptide backbone of the monomeric protein contains more than 4500 aa and exhibits two different tandem repeat domains (Fig. 1). As a characteristic feature of secretory mucins MUC2 is composed of four repetitive cysteine-rich D domains (~350 aa each) homologous to those of von Willebrand factor (Gum *et al*., 1994). Three of these domains are on the N-terminal side of the repeat domains. Small intervening sequences, but no potential transmembrane sequence, were found (Gum *et al*., 1994). Hybridization experiments revealed sequence and length polymorphisms and transcript heterogeneity. The more C-terminally located Thr-rich repeat domain is composed of a repetitive 23-aa peptide (Fig. 1) and is polymorphic in size (100–115 units). The second, more N-terminal repeat domain is characterized by a Ser/Thr-rich 16-mer exhibiting a lower degree of sequence conservation, but no length polymorphism. This domain is interupted by 7- to 8-mers that show some sequence homology. The four D domains are involved in the disulfide-mediated oligomerization of MUC2, which is a prerequisite for its gel-forming capacity (Gum *et al*., 1994).

D. Biosynthesis of Mucins: MUC1 and MUC2 as Models

The biosynthesis of MUC1 has been studied in detail by the group of John Hilkens (Hilkens and Buijs, 1988). Three precursors were identified by pulse chase and immunoprecipitation experiments: two early precursors appearing within 4 min, which are related by proteolytic cleavage and differ in apparent molecular mass by 20–30 kDa, and two late precursors detectable after 30 min at considerably higher apparent masses. Processing by glycosylation is terminated within ~3 hr. N-glycosylation by cotranslational transfer of high-mannose glycans occurs in the endoplasmic reticulum (ER) as the initial step. After proteolytic cleavage the processing of N-linked chains to complex-type glycans and the initiation/elongation of O-linked glycans take place in the *cis* to *trans* Golgi. The latter process contributes mainly to the increase in apparent molecular mass of MUC1, which generally exceeds 400

kDa. The product after 30 min of processing represents, however, an incompletely glycosylated product, since sialic acid is added initially only to a fraction of the glycans. The fully mature form of the mucin which is detectable after hours on the cell surface, is formed by constitutive reinternalization of membrane exposed molecules and their recycling through the *trans* Golgi network where sialyltransferases are localized (Litvinov and Hilkens, 1993). Several rounds of recycling (10 times on average) are neccessary to fully sialylate the mucin.

MUC1 that is released from the cell corresponds primarily to the mature form of the mucin, while membrane-associated MUC1 comprises a mixture of premature and mature glycoforms, which are both constitutively recycled to achieve or maintain a high degree of sialylation. The outlined biosynthetic route may represent a general model for the processing of membrane-associated mucins, since similar mechanisms including proteolytic cleavage into a heterodimer and sialylation by recycling have also been reported for the rat homolog of MUC4, the ASGP1/ASGP2 complex (Sheng *et al.*, 1990). However, recycling seems not to be involved in the maturation of secreted gel-forming mucins.

The biosynthetic pathway of MUC2 may be similar to that of the von Willebrand factor with which MUC2 shares sequence similarities at its C and N termini by expressing D domains. A few major differences to the MUC1 processing are evident. First, MUC2 and other members of the mucin family clustered to chromosome 11p15.5 homodimerize within 30 min after initiation of their synthesis (Asker *et al.*, 1995). This process is mediated by formation of disulfide bonds between D domains of different molecules and has been shown to be retarded by inhibition of N-glycosylation (Asker *et al.*, 1998). The latter modification is a prerequisite for the transfer of the monomers and dimers to the Golgi apparatus. Hence, dimerization occurring in the ER always precedes *O*-glycosylation in the Golgi. After initial *O*-glycosylation started in the Golgi, MUC2 oligomerizes to larger complexes. There is evidence that at least some of the cross-links formed during this process are nonreducible intermolecular bonds (Axelsson *et al.*, 1998). MUC2, like MUC1, has been proposed to undergo a specific intracellular cleavage as part of its processing to a multimeric mucin.

E. Glycosylation of Mucins

Mucin-type *O*-glycosylation is characterized by a series of structural features, some of which are common to all *O*-linked chains, whereas others are related to organ- and differentiation-dependent fluctuations. All mucin-bound *O*-glycans are built up in a sequential step-by-step process in the Golgi apparatus starting with the addition of *N*-acetylgalactosamine (GalNAc)

to the hydroxyamino acids serine (Ser) and threonine (Thr). Two positions of the GalNAc hydroxyl groups, at C3 and C6, are the exclusive targets for the transfer of further monosaccharide residues, which are mainly galactose (Gal) and/or *N*-acetylglucosamine (GlcNAc). In this way a series of "core" structures is formed, core 1 to core 4, representing the most commonly found di- and trisaccharide structures linked to mucin proteins (Fig. 2). There are

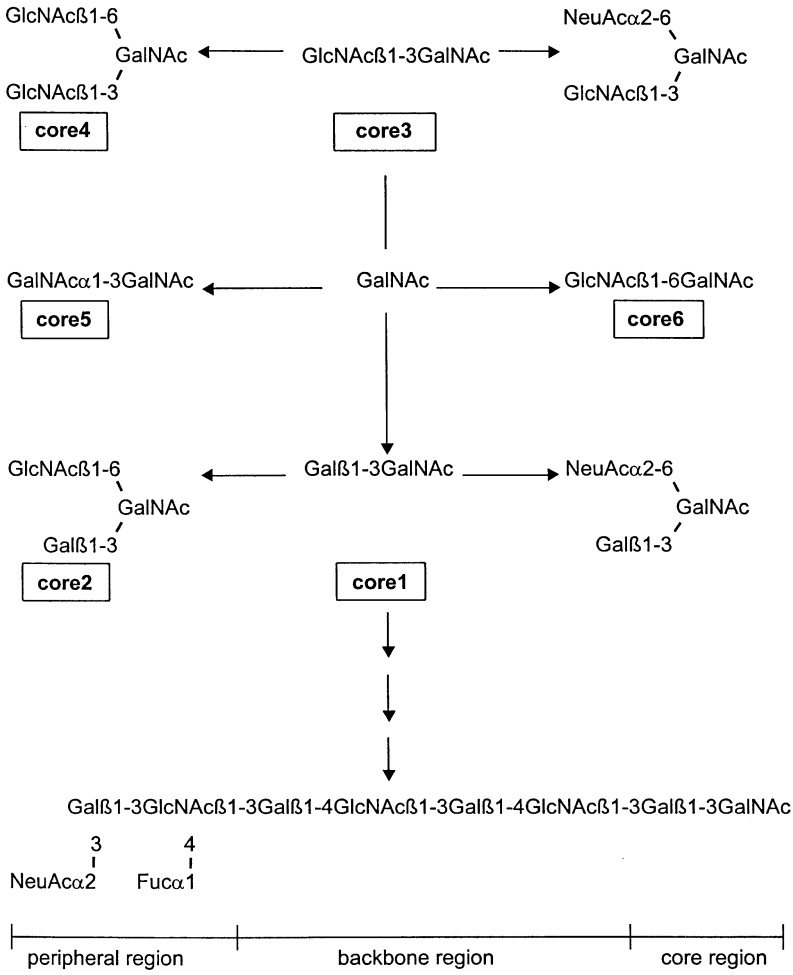

Fig. 2 Mucin-type *O*-glycosylation: structural aspects. Mucin-type *O*-glycans are built up by sequential addition of monosaccharides to the core GalNAc residue in linkage to Ser/Thr. In human mucins at least six core structures have been defined and in addition several sialic acid-containing derivatives. The polylactosamine-type sequence at the bottom is a representative model of complex mucin-type *O*-glycans expressing sialyl-Lewis[a] in the peripheral region.

also more rarely expressed cores, like core 5 and core 7, which contain *N*-acetylgalactosamine (GalNAc) instead of GlcNAc.

Considering also the existence of sialic acid-containing core structures it is evident that mucin-linked *O*-glycans exhibit a high degree of structural variation already on the level of the core chains. This compares to the structurally invariant pentasaccharide core of *N*-linked glycans. The two branches of the core structures can be elongated by sequential addition of *N*-acetyllactosamine units ending up in the formation of linear or branched polylactosamine-type backbone chains (Fig. 2). The peripheral regions of complex *O*-glycans are again polymorphic in structure, often contain α-anomeric monosaccharides like α-fucose (Fuc), α-sialic acids (NeuAc, NeuGc), α-galactose (Gal), or α-*N*-acetylgalactosamine (GalNAc), and express in this way blood group and blood group-related oligosaccharide epitopes like Lewis^a, Lewis^b, and the A, B, H blood group tetrasaccharides (Fig. 2).

O-glycan structures on MUC1 from different sources have been reported by several groups, but MUC2 glycosylation can be inferred only indirectly from the available studies on colonic mucins (Fig. 3). MUC1 from human milk has been characterized to express primarily long polylactosamine-type chains, which are built up by elongation of the C6 branch of core 2 (Hull *et al*., 1989; Hanisch *et al*., 1989, 1990, and unpublished). The linear and branched structures, which can comprise up to 16 monosaccharide units, are highly fucosylated and only up to 20% of the glycans contain sialic acid. The peripheral sequences have been shown to be derived from type 1 (Galβ1–3GlcNAc) or from type 2-lactosamine (Galβ1–4GlcNAc), and, hence, express the fucosylated Lewis^a and Lewis^x epitopes.

In breast cancer the chain lengths of the *O*-linked glycans on MUC1 are strongly reduced to the core-type level (Lloyd *et al*., 1996; Hanisch *et al*., 1996), acidic (sialylated) glycans predominate over neutral ones, and some cell lines do not express functional β-GlcNAc-transferases or α-fucosyltransferases. Accordingly, the most prominent glycans found on cancer-associated MUC1 were the trisaccharides NeuAcα2-3Galβ1-3GalNAc and Galβ1-3(NeuAcα2-6)GalNAc (Fig. 3) with a concomitant loss of blood group antigen expression. A characteristic feature of cancer-associated MUC1 is also the expression of the oncofetal Hanganutsiu-Deicher antigen (Hanisch *et al*., 1996), which is identical to an *N*-glycolylated sialic acid variant (NeuGc) that is not regularly found in tissues of adult humans.

With reference to MUC2, no carrier-specific information on the carbohydrate structures is available. However, the colonic mucin was analyzed *in toto* and demonstrated to carry mainly core 3-based oligosaccharides with extended backbone chains (Podolsky, 1985a,b) (Fig. 4).

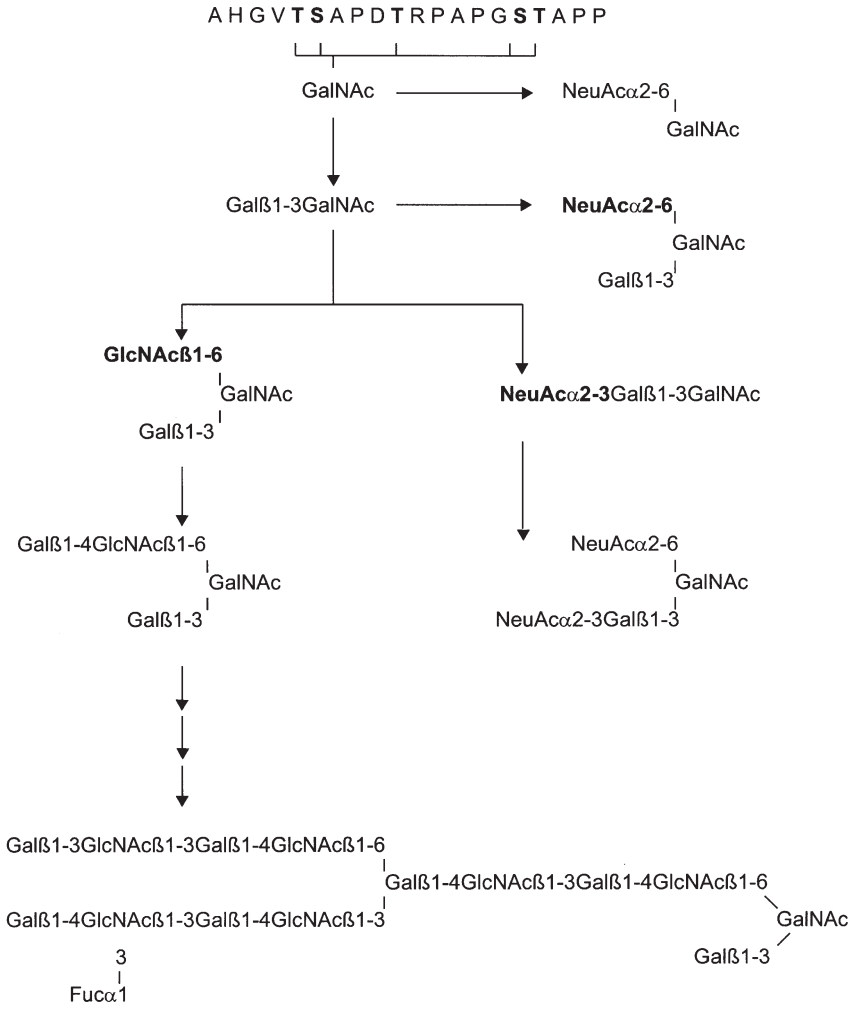

Fig. 3 *O*-Glycosylation of lactation- and cancer-associated MUC1 glycoforms. A characteristic feature of breast carcinoma cells is the reduced expression or lack of core 2-specific *N*-acetylglucosaminyltransferase and the concomitant increase of the α3/6-specific sialyltransferases. This results in the formation of short sialylated oligosaccharides instead of the lactation-associated polylactosamine-type chains synthesized by normal glandular cells.

F. Site Specificity and Biosynthetic Routes of *O*-Glycosylation

In contrast to *N*-glycosylation, initial GalNAc transfer to Ser/Thr by polypeptidyl *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts) is not ruled

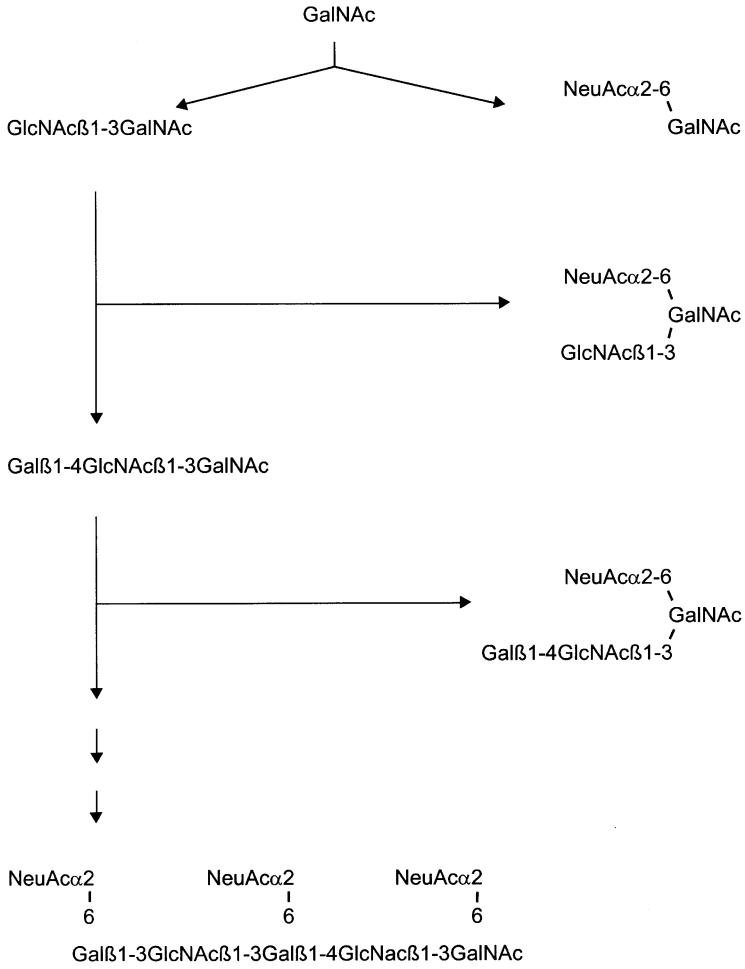

Fig. 4 *O*-Glycosylation of human colonic mucins. A few representative structures as defined by Podolsky (1985a,b) are shown in the context of their biosynthetic origins. Most glycans on normal colonic mucins contain core 3-based oligosaccharides with one or multiple NeuAc residues linked α6 to Gal and GalNAc.

by a consensus sequence of the peptide substrate. However, currently, much progress has been made with respect to our knowledge about the initial events regulating site-specific incorporation of *O*-linked glycans into mucin-type peptides. Up to seven recombinant GalNAc-Ts have been cloned and functionally expressed thus far and several of these were demonstrated to exhibit distinct, but partially overlapping, site specificities (Clausen and Bennett, 1996; Wandall *et al.*, 1997; Bennett *et al.*, 1998; Hanisch *et al.*, 1999).

While the rGalNAc-T1 and rGalNAc-T2 show nearly ubiquitous or broader expression, other enzyme variants, like rGalNAc-T3 and rGalNAc-T6, are much more restricted. Some cells or organ sites express only one particular ppGalNAc-T.

In the MUC1 model site-specific GalNAc-transfer to the five potential positions within the repeat peptide has been studied *in vitro* (Fig. 5) using rGalNAc-Ts. Whereas rGalNAc-T1 prefers the Thr within VTSA, rGalNAc-T2 transfers the sugar more rapidly to Thr within GSTA (Wandall *et al*., 1997; Hanisch *et al*., 1999). The latter enzyme is also responsible for initial glycosylation of Ser in GSTA. The remaining sites cannot be glycosylated by these two enzymes, but it has been shown that the fill-up reactions (glycosylation of Thr in DTR and of Ser in VTSA) can be catalyzed by rGalNAc-T4 (Bennett *et al*., 1998). This enzyme shows a strict dependency on previous glycosylation, since it does not act on the naked peptide substrate. While GalNAc in certain positions can enhance the initial glycosylation rates at other proximal sites, elongation of core-GalNAc to core 1 or core 2 di(tri)saccharides was shown to suppress initial GalNAc transfer to proximal sites (Hanisch *et al*., 1999). In this way, elongation and initiation are competitive processes and determine the density of mucin glycosylation (Fig. 5).

The biosynthetic routes of mucin *O*-glycosylation are ruled by the substrate specificities of the various glycosyltransferases involved and expressed in a cell (Brockhausen, 1997) (Fig. 6). There are biosynthetic "stop" signals, such as α6-linked sialic acid on core-GalNAc, that prevents further elongation at C3 of GalNAc, but also "go" signals, such as C3-glycosylation of core-GalNAc (with Gal or GlcNAc) that are prerequisites for elongation at C6 of GalNAc (the "3 before 6" rule). At the periphery similar rules are responsible for the ordered addition of Fuc and NeuAc in the biosynthesis of Lewis-related cancer epitopes sialyl-Lewis^a and sialyl-Lewis^x (sialylation precedes fucosylation). Blood group H-type 2 is a general precursor of blood group A and B tetrasaccharides, but also of the Lewis^y tetrasaccharide (2Gal substitution precedes 3/4GlcNAc substitution). This implies that the various fucosyltransferases exhibit distinct substrate specificities, some showing a preference for type 1 or type 2-based lactosamines (H gene coded α2-fucosyltransferase), others act after terminal sialylation of lactosamines only (X gene coded α3-fucosyltransferase), while the Se gene coded α2-fucosyltransferase and the Le gene coded α3/4 fucosyltransferase add their sugars to both type 1- and type 2-based lactosamines.

G. Antigenicity of Mucins

Mucins are heavily glycosylated proteins and, hence, it is not surprising that a target of B-cell responses is found in the carbohydrate portion of these

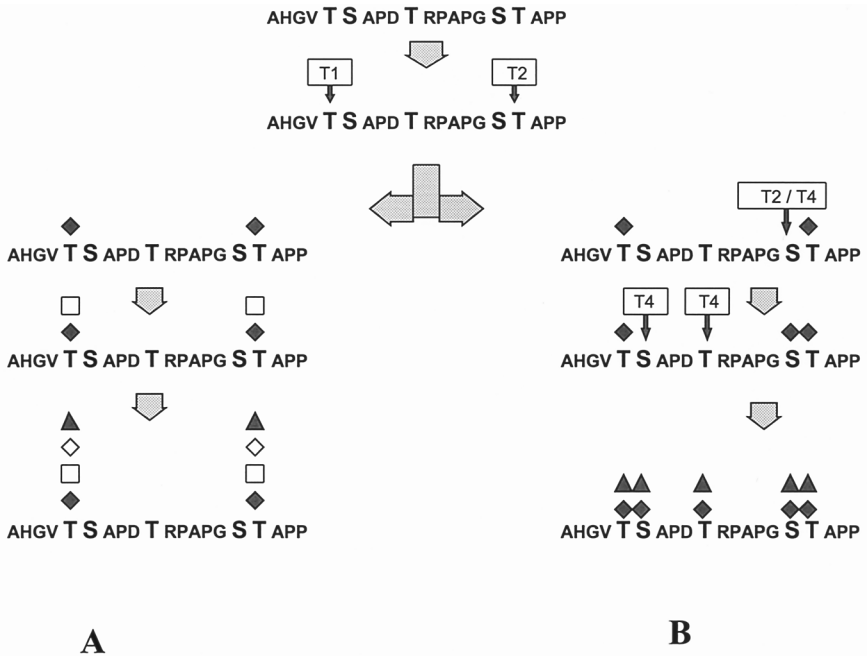

Fig. 5 Site-specific *O*-glycosylation of MUC1 tandem repeats by peptidyl GalNAc-transferases. The sequential order of GalNAc addition to the five potential sites is shown. T1 to T4 correspond to the recombinant enzymes acting in a site-specific and sequential manner. (A) Formation of core structures by addition of Gal or GlcNAc to the GalNAc prevents initial glycosylation at proximal sites and results in the low-density glycosylation characteristic for MUC1 in milk. (B) If there is no competition with core-specific enzymes, the peptide scaffold can be fully glycosylated at each putative site. By final addition of sialic acid the high-density glycoform of MUC1 is formed, which is characteristic for carcinoma cells.

molecules. However, in the case of MUC1 the protein core seems to elicit the stronger immune response in mice, since most hybridoma antibodies generated to this mucin bind to peptide epitopes within the VNTR domain (Price *et al.*, 1998). Reduced glycosylation of cancer-associated MUC1 has been assumed for a long time to permit the immune system access to the peptide core of the mucin (Burchell and Taylor-Papadimitriou, 1993). The preferred target for most peptide-specific antibodies generated to the tumor mucin is located at the DTR motif within the tandem repeat peptide (Price *et al.*, 1998). In humans, however, the natural response to MUC1 shows a second, even stronger immunodominant motif that comprises the STA sequence within the repeat (Petrarca *et al.*, 1996). The immunodominance of the DTR motif has been attributed to its knob-like secondary structure (Fontenot *et al.*, 1993) and to the finding that Thr within the motif represents a poor sub-

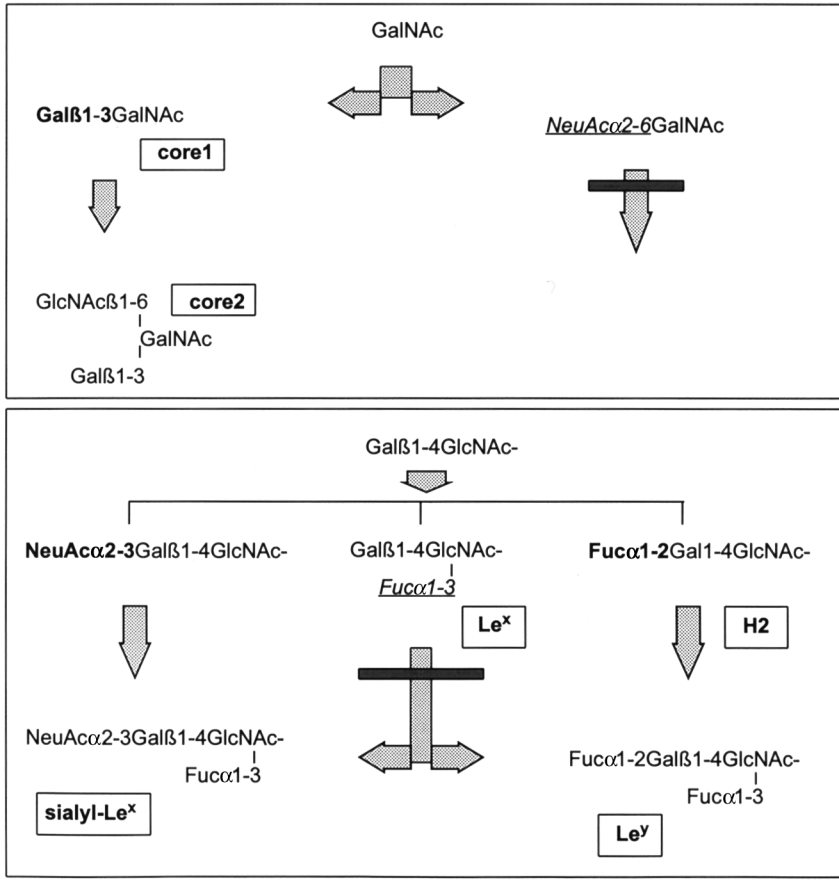

Fig. 6 Sequential order of *O*-glycan synthesis ruled by substrate specificities of glycosyltransferases. The addition of Gal to position C3 of GalNAc serves the function of a "go" signal for the β6-*N*-acetylglucosaminyltransferase and the formation of core 2. On the other hand, sialic acid in β6-linkage to GalNAc represents a "stop" signal and prevents elongation at C3 of GalNAc. Similar mechanisms rule the formation of peripheral sequences such as sialyl-Lewis^x or Lewis^y starting from type 2-lactosamine chains.

strate for ppGalNAc-Ts *in vitro* (Nishimori *et al*., 1994; Stadie *et al*., 1995).

In contrast, it has recently been shown that the DTR motif is a glycosylation target *in vivo* (Müller *et al*., 1997) and that cancer-associated glycoforms of the mucin are characterized by a higher density than normally processed mucin (Müller *et al*., 1999). Surprisingly, core-type glycosylation of the DTR motif was revealed to enhance its antigenicity for the majority of site-specific antibodies rather than to reduce it (Karsten *et al*., 1998).

Accordingly, glycans can stabilize particular peptide epitopes, in the same way as they can mask others by sterical hindrance or by exerting conformational changes. Besides "sequential" epitopes of the peptide or carbohydrate moieties, MUC1 directed antibodies have been demonstrated to recognize "conformational" epitopes, which are difficult to characterize. Also "mixed sequential" epitopes have been described that comprise both a carbohydrate and portions of the underlying peptide (Hanisch *et al.*, 1995). The described epitopes of mouse antibodies or human T-cell clones reflect the immunogenicity of MUC1 in the respective species, however, there are also approaches that circumvent the natural response to an antigen by selection of random combinatorial libraries of recombinant single-chain antibodies displayed on phages. By using this approach, less immunogenic peptide epitopes could be targeted with high-affinity single-chain antibodies to the PAP motif (Hendrikx *et al.*, 1998).

III. ALTERATIONS OF MUCIN PEPTIDE AND MUCIN GLYCOSYLATION DURING CARCINOGENESIS

Refering to cancer-associated changes as revealed by immunohistochemical staining of mucin epitopes, we need to distinguish between those related to altered expression of the protein core and those resulting from aberrant processing. Although glycosylation of proteins should be dependent on the rate of synthesis, and overexpression of mucin species in certain cancers is indeed associated with reduced glycosylation, there is no clear-cut evidence for a causal relationship between these events. Moreover, reduction in glycosylation can refer to both the chain lengths of the glycans and their density on the peptide scaffold. In breast carcinoma it has been shown that MUC1 expresses shorter chains of the core type, but these are found at much higher density than on the lactation-associated glycoform of the mucin (Lloyd *et al.*, 1996; Müller *et al.*, 1999). No such information is currently available on mucin glycosylation in gastrointestinal carcinoma. Data obtained with *in situ* hybridization indicate that the expression of MUC1 protein core increases in colon cancer at least 10-fold. In contrast, the expression of MUC2 and MUC3 mRNA can be higher or lower in colon cancer than in the corresponding normal tissue.

With respect to cancer-associated alterations of mucin glycosylation in the colorectum, there is only rare chemical evidence, since most studies are based on immunologic approaches. These, however, suffer from the inherent problem that they give evidence for epitope accessibility rather than for actual *de novo* synthesis or loss of a certain structure. One of the earliest structural reports has revealed that mucins from rectal adenocarcinoma are character-

ized by four major sialyloligosaccharides comprising about 50% of all *O*-linked glycans (Kurosaka *et al*., 1983):

NeuAcα2–6GalNAc
NeuAcα2–6(Galβ1–3)GalNAc
NeuAcα2–6(GlcNAcβ1–3)GalNAc
NeuAcα2–6(GalNAcα1–3)GalNAc

The glycosylation profile of rectal mucins from adenocarcinoma reveals a common motif, since all major glycans contain NeuAcα2–6GalNAc (sialyl-Tn antigen) as a partial structure. This common motif, however, is linked to different core disaccharides (core 1, core 3, core 5). These early findings are in partial agreement with enzymatic studies revealing evidence that the core 3-based glycosylation of normal colonic mucin should shift to core 2-based sequences (Fig. 7) according to a cancer-associated deficiency of UDP-GlcNAc/GalNAc β3-*N*-acetylglucosaminyltransferase and a concomitant increase of UDP/GalNAc β6-*N*-acetylglucosaminyltransferase (Corfield *et al*., 1995; Vavasseur *et al*., 1995; Yang *et al*., 1994; Brockhausen *et al*., 1991). The immunochemical detectability of Tn and sialyl-Tn antigens in 73–80% of colon carcinomas probably does not result from *de novo* synthesis of these antigens, but instead from a better accessibility of short-chain epitopes due to a general reduction of the glycan chain lengths. Sialyl-Tn expression correlates with the grade of malignant transformation in polyps indicating that incomplete glycosylation of mucins is an early alteration during carcinogenesis. The addition of sialic acid into C6 of GalNAc may contribute to the formation of shorter chains, since the acidic sugar in this position serves as a stop signal and prevents elongation of the core-GalNAc via C3 substitution (Fig. 7).

An alternative pathway in colonic cancer is found in the formation of core 1 (Thomsen-Friedenreich antigen) by addition of Gal to C3 of core-GalNAc (Fig. 7). This antigen has been detected by several groups in the mucin of malignant, but not of normal colonic mucosa (Boland *et al*., 1982a; Yuan *et al*., 1986; Itzkowitz *et al*., 1989; Baldus *et al*., 1998b). Conflicting results reported by another group (Orntoft *et al*., 1990) demonstrate that detectability of TF antigen is dependent on the antibody used. A more recent study has revealed carrier-specific TF expression on MUC1 from colonic cancer (Baldus *et al*., 1998b). Carrier-specificity of core-type glycosylation implies that the peptide sequence of the target protein influences the activity of the respective glycosyltransferase acting on GalNAcα-Ser/Thr. Evidence for a site-specific formation of core 1 (TF antigen) was reported (Granovsky *et al*., 1994).

Also with respect to the peripheral regions of complex mucin glycans a number of characteristic alterations were revealed. The most common peripheral regions shared by glycolipids and glycoproteins on erythrocytes and on epithelial cells are the antigenic determinants of the ABH and Lewis blood

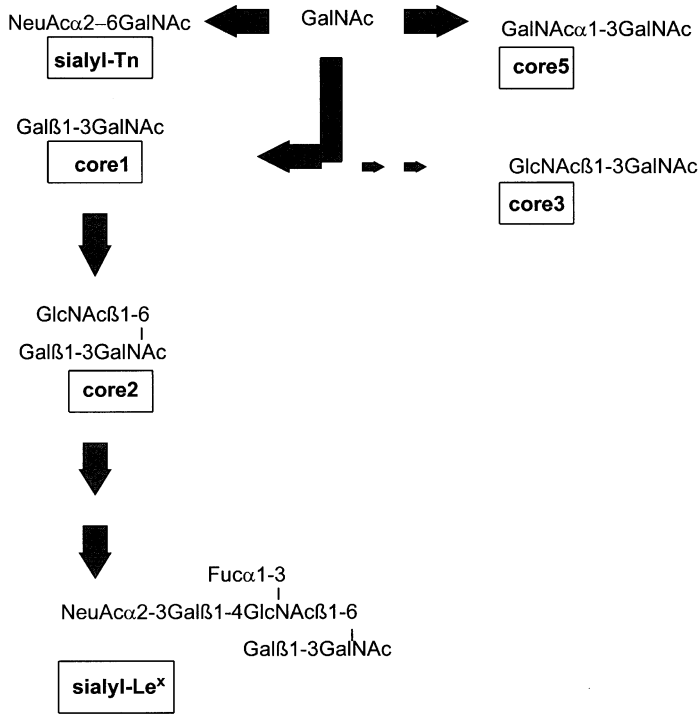

Fig. 7 Altered biosynthetic routes of O-glycosylation in colonic cancer. The major event in the context of colonic mucin glycosylation during carcinogenesis is the shift from core 3- to core 1- and core 2-based sequences. *De novo* synthesis of sialyl-Lewis^x is a characteristic feature of carcinoma-associated MUC1 and MUC2 (Hanski *et al.*, 1995).

groups. The expression of these antigens is determined by the tissue-specific distribution of at least four fucosyltransferases coded by Se and H as well as Le and X genes. While the products of the Se and Le genes are expressed in cells of ectomesodermal origin (including digestive and salivary epithelium), those of the H and X genes are restricted to mesodermal cells (including erythrocytes). The former fucosyltransferases use type 1 and type 2 chain substrates in contrast to the latter two, which are specific for type 2 chain substrates.

In the normal gastrointestinal mucosa type 1 chain is preferentially synthesized by the respective β3-galactosyltransferase (Holmes, 1989), and the derived type 1-based antigens predominate. In colonic carcinoma the activity of A and B transferases is reduced compared to normal mucosa (Kim *et al.*, 1974). In secretors the Se transferase is increased, while the Le transferase remains unchanged (Orntoft *et al.*, 1990). These alterations should result in the accumulation of H and Lewis^b antigens in Le gene positive secretors, and

in the accumulation of Lewis^a and type1 chains in nonsecretors. In accordance with this, a sequential loss of ABH expression in gastric carcinomas has been observed (Picard *et al.*, 1978), and similarly the majority of colonic carcinomas exhibited accumulation of AB blood group precursors like H-type 2 and Lewis^y antigens (Cooper *et al.*, 1991). Deletion of ABH antigens is not restricted to gastric carcinomas, because in the proximal colon H antigen also accumulates (Yuan *et al.*, 1985). Only the distal colon, where ABH antigens in adults are normally absent, does ectopic expression of A, B, H, Lewis^b, and Lewis^y take place (Yuan *et al.*, 1985; Kim *et al.*, 1986). Also blood group incompatible expression of antigens, like Lewis^b expression in nonsecretors, has been reported in colonic carcinomas (Kim *et al.*, 1986).

IV. EXPRESSION OF MUCIN-ASSOCIATED ANTIGENS IN GASTRIC TISSUES

A. Non-Neoplastic Gastric Tissues

1. PEPTIDE CORES (APOMUCINS)

In normal gastric tissues, some mucin peptide cores are typically expressed (as shown in Table II), whereas others do not or only faintly occur. A MUC1 immunoreactivity of gastric surface epithelium, mucous neck cells, and gastric glands of gastric fundus could be described applying monoclonal antibodies; antral mucosa showed similar results. A limitation of MUC1 immunoreactivity in the fundic glands to secretory canaliculi of parietal cells has also been described (Walker *et al.*, 1995). On the other hand, MUC2 was demonstrated by immunohistochemistry in superficial epithelium and neck cells, whereas, like MUC3, it was unreactive in other investigations. In mRNA analyses, MUC1, but not MUC2 or MUC3, RNA could be detected (Ho *et al.*, 1993; Cao *et al.*, 1997a). Additional extended studies showed an expression of MUC5AC, MUC5B, and MUC6 mRNA and an immunoreactivity of the proteins, whereas MUC4 was not present. Some MUC1-specific mabs reacted strongly with gastric surface epithelia of Le(a+b−) nonsecretors, whereas Le(a−b+) secretor tissues were positively stained only after pretreatment of the sections with α-fucosidase, indicating masking of the MUC1 epitopes by fucosylated saccharides (Bara *et al.*, 1993). MUC2 immunoreactivity was observed in all cases with intestinal metaplasia (Filipe *et al.*, 1996). Others described MUC1, MUC5AC, and MUC6 to be present in intestinal metaplasia without regard for its type, whereas MUC2 cores were only strongly expressed in types II and III (Reis *et al.*, 1999), thus allowing the identification of two "mucin patterns."

Table II. Expression of MUC Peptide Cores in Normal Gastric Tissue

MUC core	Expression	Ref.
MUC1	Surface epithelium, mucous neck glands, gastric glands	Bara *et al.* (1993), Ho *et al.* (1993, 1995), Gambús *et al.* (1993), Carrato *et al.* (1994), Baldus *et al.* (1998a)
MUC2	Superficial epithelium, mucous neck cells	Gambús *et al.* (1993), Carrato *et al.* (1994), Baldus *et al.* (1998a)
MUC2	Not expressed	Ho *et al.* (1993, 1995)
MUC3	Not expressed	Ho *et al.* (1993, 1995)
MUC4	Not expressed	Audie *et al.* (1993),
MUC5AC, 5B, 6	Surface epithelium, neck cells	Carrato *et al.* (1994), DeBolos *et al.* (1995), Ho *et al.* (1995), Sotozono *et al.* (1996), Reis *et al.* (1997)

2. CARBOHYDRATE ANTIGENS

In a number of studies, different lectins were used to characterize carbohydrate antigens in the normal stomach (Table III). However, the specificity of lectins often suffers from cross-reactivities with related carbohydrate structures. The first studies used Helix pomatia agglutinin (HPA) and Dolichos biflorus agglutinin (DBA), which bind to terminal GalNAcα residues. Their binding sites may at least in part represent Tn core antigen, but are also carried as terminal saccharides by blood group antigen A. They stained a variety of gastric cells including superficial epithelia, mucous neck, and parietal cells, whereas Tn-specific mabs exhibited only a weak positivity in gastric epithelium and glands. Reactivity of PNA, which most strongly binds to the TF core antigen, was shown in a number of studies in different cell types, especially in nonsecretors (Picard and Feizi, 1983; Macartney, 1986). Brunner's glands of the duodenum were also PNA reactive (Crescenzi *et al.*, 1988).

Interestingly, gastric tissues from some individuals contained PNA binding sites, whereas others expressed the α1–2 fucosylated TF epitope recognized by monoclonal antibody MBr1, indicating a α1–2 fucosylation of TF, which is regulated by the Se gene (Okada *et al.*, 1994). At least in part, α1–2 fucosylated TF antigen may be present on MUC1 peptide cores (Bara *et al.*, 1993). Most TF-specific monoclonal antibodies do not or only very inconsistently stain normal epithelium, whereas mab HB-T1 stained foveolar epithelial and neck cells of the antrum as well as mucous antral glands and the intracellular canaliculi of parietal cells (Table III). These conflicting results may be due to different fine specificities of the antibodies. On the other hand, sialyl-Tn antigen could be detected in parietal cells (especially within intracellular canaliculi) or goblet cells/intestinal metaplasia (Table III). In

Table III. Immunoreactivity of Carbohydrate Core Antigens in Normal Gastric Tissues

Antigen	Lectin/mab	Reactivity	Ref.
GalNAcα	HPA, DBA	Reactive	Fischer *et al.* (1984), Bur and Franklin(1985), Ito *et al.* (1985), Madrid *et al.* (1990), Kakeji *et al.* (1991)
Tn	mabs	Weakly reactive	Cao *et al.* (1996)
TF	PNA	Reactive	Picard and Feizi (1983), Fischer *et al.* (1984), Ito *et al.* (1985), Macartney (1986)
TF	mabs	Very inconsistently reactive	David *et al.* (1992), Yamashita *et al.* (1995) Cao *et al.* (1996)
TF	mab HB-T1	Epithelial, neck, and parietal cells reactive	Carneiro *et al.* (1994)
Sialyl-Tn	mabs	Reactive (especially parietal and goblet cells)	Ohuchi *et al.* (1986), Iwata *et al.* (1993), Kushima *et al.* (1993), Werther *et al.* (1994), Yamada *et al.* (1995), Yamashita *et al.* 1995), Cao *et al.* (1996), Victorzon *et al.* (1996)

conclusion, only sialyl-Tn antigen is consistently immunoreactive in normal gastric tissues, whereas Tn and TF antigens are possibly masked by oligosaccharide chain elongation in most instances.

Backbone antigens were rarely investigated in gastric tissues. Erythrina cristagalli and Erythrina corallodendron agglutinins, both recognizing type 2 backbones (Galβ1–4GlcNAc), stained superficial foveolar epithelium and fundic glands, especially the secretory canaliculi of parietal cells. In the pyloric mucosa, superficial and deep (mucous) glands were strongly positive (Vierbuchen *et al.*, 1988; Baldus *et al.*, 1996a). Furthermore, Ii blood group antigens were investigated in gastric tissues using anti-I and anti-i sera (Kapadia *et al.*, 1981; Picard and Feizi, 1983).

The expression of Lewis and ABH blood group antigens in the stomach underlies complex regulation mechanisms involving the Lewis (Le) and secretor (Se) genes. Besides other genes, they control the expression of certain α1–2 and α1–3 fucose transferases determining the individual antigen patterns. α1–2 fucosylation may be the result of H- or Se-gene-coded fucose transferases, whereas α1–3 fucosylation depends from Le- or X-gene-coded

fucose transferases. Se- and Le-coded enzymes accept type 1 as well as type 2 backbone structures, whereas H- and X-dependent transferases prefer type 2 backbones (Oriol *et al.*, 1986). Due to localization-dependent enzyme patterns, superficial gastric and small bowel epithelia of secretors (representing about 75% of European individuals) express Le^b, Le^y, and H-type 1. Instead of them, nonsecretors exhibit Le^a and Le^x antigens. However, in the deep mucous glands of the pylorus and Brunner's glands of the duodenum, Le^x and Le^y antigens are synthesized by H-gene-coded fucose transferases, since these tissues do not express type 1 chains and Se-coded fucose transferases (Mollicone *et al.*, 1985, 1986; Sakamoto *et al.*, 1989). Lectin histochemical studies applying Ulex europaeus agglutinin I (UEA I), Lotus tetragonolobus, and Anguilla anguilla agglutinin confirmed the findings (Bur and Franklin, 1985; Macartney, 1987; Baldus *et al.*, 1996b). These circumstances may also influence *Helicobacter pylori* infection since fucosylated blood group antigens Le^b and H-type 1 were shown to mediate its adherence to human gastric epithelial cells *in situ* (Borén *et al.*, 1993; Falk *et al.*, 1993, 1994). Le^b is bound by BabA adhesin of *H. pylori* and the binding phenotype is associated with the presence of cag pathogenicity island among clinical isolates of *H. pylori* (Ilver *et al.*, 1998).

B. Expression in Neoplastic Gastric Tissues

1. PEPTIDE CORE ANTIGENS

High levels of MUC1, MUC2, MUC3, and MUC4 mRNA as well as immunoreactive proteins were detected in gastric carcinomas, whereas the levels of MUC5 and MUC6 decreased (Ho *et al.*, 1995). Immunohistochemically, MUC1 antigens showed an expression in the majority of gastric cancers (Table IV), but MUC2 cores were only expressed in a minority of them (Ho *et al.*, 1995; Baldus *et al.*, 1998a). MUC1 was more strongly expressed in intestinal-type cancers with tubular or papillary differentiation and expansive growth patterns than in diffuse-type carcinomas, which often exhibit a signet-ring cell appearance (Sakamoto *et al.*, 1997; Baldus *et al.*, 1998a; Reis *et al.*, 1998). Additionally, MUC1 immunoreactivity increased with the pTNM stage (Baldus *et al.*, 1998a). However, MUC1 antigens may be masked by α1–2 fucosylation of TF antigen bound to the peptide core (Bara *et al.*, 1993). The investigation of MUC1 gene polymorphism led to the observation that the distribution of MUC1 alleles was significantly different in a population of patients suffering from gastric carcinoma and a control group of blood donors. Individuals with small MUC1 genotypes are at increased risk for carcinoma development according to these data (Carvalho *et al.*, 1997). Using different mabs, contradictory results were obtained regarding the distribution in histopathological subtypes: A significantly stronger staining of intestinal

Table IV. The most important mucin-associated antigens in gastric cancer

Antigen	Ref.
MUC1	Bara *et al.* (1993), Ho *et al.* (1995), Sakamoto et al. (1997), Baldus *et al.* (1998a), Reis *et al.* (1998), Utsunomiya *et al.* (1998), Nakagawa *et al.* (1999)
TF	Fischer *et al.* (1983), Kuhlmann *et al.* (1983) using PNA; David *et al.* (1992), Yamashita *et al.* (1995), Chung *et al.* (1996) using mabs
Sialyl-Tn	Ohuchi *et al.* (1986), Ma *et al.* (1993), Werther *et al.* (1994, 1996), Miles *et al.* (1995), Yamada *et al.* (1995), Yamashita *et al.* (1995), Victorzon *et al.* (1996), Baldus *et al.*, (1998c)
Sialyl-Le^a	Atkinson *et al.* (1982), Hirohashi *et al.* (1984), Sipponen and Lindgren (1986), Sakamoto *et al.* (1989), Sowa *et al.* (1989), Inagaki *et al.* (1990), Ikeda *et al.* (1991), Nakamori *et al.* (1997a), Baldus *et al.* (1998c)
Sialyl-Le^x	Fukushima *et al.* (1984), Sakamoto *et al.* (1989), Hanski *et al.* (1990), Nakamori *et al.* (1997a), Baldus *et al.* (1998c)

type than diffuse type cancer was observed (Baldus *et al.*, 1998a), whereas others described an inverse pattern (Sakamoto *et al.*, 1997). MUC5B and MUC5C were detected in gastric carcinomas (Carrato *et al.*, 1994) and MUC5AC was reported to be present in a greater percentage of diffuse compared to intestinal carcinomas and it was more frequently expressed in early than advanced gastric cancer (Reis *et al.*, 1997).

2. CARBOHYDRATE CORE ANTIGENS

A number of studies reported a heterogenous PNA reactivity of gastric adenocarcinomas (Table IV). Neuraminidase digestion enhanced PNA binding in intestinal-, but not diffuse-type, cancers (Bur and Franklin, 1985). Others did not observe a different reactivity of these histological subtypes (Sotozono *et al.*, 1994). Biochemical analyses detected the polymorphic epithelial mucin (MUC1) and a novel 24-kDa protein derived from KATO-III cell line as carriers of PNA binding site (Masuzawa *et al.*, 1992). Applying mabs, different percentages of gastric carcinomas were stained (Table IV). Differentiated or intestinal-type carcinomas, respectively, were more strongly positive (Chung *et al.*, 1996). After partial deglycosylation by periodate treatment, mab staining increased (Bara *et al.*, 1993).

Characteristically, gastric carcinomas show a strong expression of sialyl-Tn antigen, which could be demonstrated in a great number of studies (Table IV). Some authors observed an association with intestinal differentiation (Iwata *et al.*, 1993; Kushima *et al.*, 1993), others an association between lymphatic metastasis and sialyl-Tn expression in Borrmann type IV carcinomas (Kakeji *et al.*, 1995).

GalNAcα antigens were also expressed in gastric adenocarcinomas, however more often masked by sialic acid than in normal tissues (Bur and Franklin,

1985; Macartney, 1986). HPA binding site was expressed in the majority of the specimens without correlation with the histologic type (Kakeji *et al.*, 1991).

3. PERIPHERAL AND BACKBONE OLIGOSACCHARIDE ANTIGENS

Besides expression of incompatible ABH antigens (Denk *et al.*, 1974a), partial deletion of ABH blood group substances may be observed in gastric cancer. As a result of ABH deletion, type 1 backbones (Galβ1–3GlcNAc), I(Ma) antigen representing branched type 2 chains, and i antigen (repetitive linear type 2 backbones) become detectable (Kapadia *et al.,* 1981). Blood group i antigen, for example, could be recognized in about 40% of gastric carcinomas (Miyake *et al.*, 1989), and α1–3 monofucosylated polylactosaminoglycans in about 55% (Hanisch *et al.*, 1993a; Schwonzen *et al.*, 1993). Another carbohydrate structure, which gains reactivity, is GlcNAcβ1–3Galβ1–4GlcNAcβ1–6(GalNAc) as defined by mab 2B5 (Hanisch *et al.*, 1993b).

The Lewis blood group system is also involved in carcinoma-associated alterations. Anomalous Le^a antigen appeared in gastric dysplasia and intestinal metaplasia, but also in the majority of gastric cancers in patients with Le(a−b+) phenotype; others showed a Le^a deletion. At least in part, these modifications may be due to blocked synthesis of Le^b antigen, i.e., precursor accumulation (Sakamoto *et al.*, 1989; Torrado *et al.*, 1989, 1990). Le^a antigen revealed a tendency to be preferentially expressed in well or moderately differentiated and advanced carcinomas (Inagaki *et al.*, 1990). Another important mechanism, increase of sialylation, is represented by an increase of sialyl-Le^a as well as sialyl-Le^x antigens. Sialyl-Le^a and sialyl-Le^x were demonstrated in the majority of gastric carcinomas (Table IV). Neither showed correlations with histologic differentiation or tumor stage (Nakamori *et al.*, 1997a; Baldus *et al.*, 1998c). Difucosylated sialyl-Le^x-i antigen was preferentially expressed in differentiated cancers (Dohi *et al.*, 1989), whereas a monofucosylated polylactosamine sequence exhibited a stronger expression in undifferentiated carcinomas (Dohi *et al.*, 1990).

V. EXPRESSION IN COLORECTAL TISSUES

A. Non-Neoplastic Colorectal Tissues

1. PEPTIDE CORES (APOMUCINS)

MUC2, MUC3, and MUC4 (Table V) represent the main mucin peptide cores expressed by intestinal cells. Normal small intestinal and colorectal epithelia were frequently reactive with MUC2- and MUC3-specific anti-

Table V. Expression of MUC Peptide Cores in Normal Colorectal Tissue

MUC core	Expression	Ref.
MUC1	Inconsistent or absent	Zotter *et al.* (1987), Andrews *et al.* (1993), Ho *et al.* (1993), Nakamori *et al.* (1994), Ajioka *et al.* (1996)
MUC2	Frequently reactive	Tytgat *et al.* (1994), Blank *et al.* (1994), Chang *et al.* (1994), Toribara *et al.* (1991)
MUC3	Frequently reactive	Audie *et al.* (1993), Ho *et al.* (1993), Chang *et al.* (1994)
MUC4	Frequently reactive	Porchet *et al.* (1991), Ogata *et al.* (1992), Audie *et al.* (1993)

bodies, whereas MUC1 was only inconsistently observed or absent from normal tissues. In another study, MUC1 was observed in all specimens of normal colon (Carrato *et al.*, 1994). MUC1 reactivity could be enhanced by periodate oxidation, resulting in a staining of apical membranes within the crypt base (Ohe *et al.*, 1994). Additionally, there is some controversy in the literature regarding the tissue distribution of MUC2: It is either held to be goblet cell specific (Chang *et al.*, 1994) or to be also reactive in columnar cells (Blank *et al.*, 1994). MUC3 is expressed in the supra- and perinuclear cytoplasm of goblet cells, especially in the colorectum, and also in columnar cells of small intestine and large bowel, being more abundant in superficial epithelium compared with crypt bases (Ho *et al.*, 1993; Chang *et al.*, 1994). According to mRNA analysis, both MUC2 and MUC3 are strongly expressed throughout the intestine with a higher level of MUC3 mRNA in the small intestine compared with the colon (Ho *et al.*, 1993). MUC5C was not immunoreactive in colorectal tissue, whereas MUC5B showed binding (Carrato *et al.*, 1994), especially in the crypt bases (van Klinken *et al.*, 1998).

2. CARBOHYDRATE ANTIGENS

The expression of TF antigen in normal colorectal mucosa has led to controversial discussions in the past. PNA showed a reactivity with columnar or goblet cells in some studies, whereas others did not observe any or only minimal staining. The more specific monoclonal antibodies did not stain at all or only very faintly (Table VI). These conflicting data can be explained by the cross-reactivities of PNA and the fine specificities of the different mabs. However, biochemical data suggest that TF antigen can be detected after removal of sialic acid and fucose, indicating that it may be (almost completely) masked in normal colorectal tissue (Campbell *et al.*, 1995). GalNAcα residues—present on Tn and A blood group antigen—could be demon-

Table VI. Immunoreactivity of Carbohydrate Antigens in Normal Colorectal Tissues

Antigen	Lectin/mab	Reactivity	Ref.
TF	PNA	Columnar or goblet cells	Cooper (1982), Kellokumpu *et al.* (1986), Yuan *et al.* (1986), Campo *et al.* (1988)
TF	PNA	Minimal or absent	Klein et al. (1981), Boland *et al.* (1982a), Rhodes *et al.* (1986), Calderó *et al.* (1989), McGarrity *et al.* (1989)
TF	mabs	Minimal or absent	Yuan et al. (1986), Itzkowitz et al. (1989), Orntoft *et al.* (1990), Cao et al. (1995), Baldus et al. (1998b)
GalNAcα	DBA	Reactive	Boland *et al.* (1982a), Kellokumpu *et al.* (1986), Nakayama *et al.* (1987), Campo *et al.* (1988), Calderó *et al.* (1989), Jass *et al.* (1994), McMahon *et al.* (1994)
Tn	mab	Non-reactive	Cao *et al.* (1995)
Sialyl-Tn	mabs	Absent or faint	Itzkowitz et al. (1989), Wolf *et al.* (1989), Orntoft *et al.* (1990), Itzkowitz et al. (1992), Cao et al. (1995)
ABH, Le^b, Le^y	mabs	Absent from distal colon and rectum	Szulman (1966), Davidsohn *et al.* (1966), Szulman and Marcus (1973), Wiley *et al.* (1981), Yonezawa *et al.*, (1982), Ernst *et al.* (1984), Yuan *et al.* (1985), Bara *et al.* (1988)
H type 2, Le^y	mabs	Strongly reactive in proximal colon	Yonezawa *et al.*, (1982), Brown *et al.* (1984), Bara *et al.* (1988), Calderó *et al.* (1989)
Le^x	mabs	Weakly reactive throughout the colon	Shi *et al.* (1984), Gong et al., Gong *et al.* (1985), Cordon- Cardo *et al.* (1986), Itzkowitz *et al.* (1986)

strated using DBA, but Tn- and sialyl-Tn specific mabs showed absent or only faint expression in colorectal tissue (Table VI). However, these immunohistochemical results are obviously due to the *O*-acetylation of sialic acids in the normal colorectum. When *O*-acetylated sialic acids were con-

verted by saponification into non-*O*-acetylated sialic acids, normal goblet cells (mainly in the lower crypt area) became sialyl-Tn or (after subsequent neuraminidase digestion) Tn positive (Jass *et al.*, 1995).

Type 2 backbones were not detectable by the above-mentioned Erythrina lectins in the majority of specimens under study, showing a slight reactivity of columnar cell cytoplasm and goblet cell mucus in some of them (McMahon *et al.*, 1994; Baldus *et al.*, 1998a).

In the adult distal colon and rectum, $\alpha 1$–2 fucosylation and therefore expression of ABH, Le^b, and Le^y blood group antigens does not occur, but there is a strong immunoreactivity of H type 2 with UEA I and Le^y, with mabs (Table VI), especially in the proximal colon, since Se gene expression is restricted to the proximal colon after the fetal period. Additionally, the Le^a, antigen is not present in the entire colorectum in individuals who do not express the Le gene (Ernst *et al.*, 1984). Le^x, on the other hand, is (weakly) immunoreactive throughout the colon (Table VI). These type 2 chain antigens are mainly expressed by immature crypt base columnar cells. Sialyl-Le^a was only reactive in goblet cells of the upper crypts; sialyl-Le^x in a few cases at the crypt base. However, both antigens were strongly exhibited in normal colonic tissues after de-*O*-acetylation (Ogata *et al.*, 1995). These data indicate that they may be expressed, but masked by *O*-acteylated sialic acids in the normal colorectum (like sialyl-Tn antigen).

B. Colorectal Adenoma-Carcinoma Sequence

Like other tumor-associated antigens, some mucin-associated petides and carbohydrates show a significant decrease or increase during the evolution of colorectal neoplasms.

1. PEPTIDE CORE ANTIGENS

MUC1 peptide core, representing antigens formerly designated MAM-6 and DF3, is strongly expressed in colorectal cancer. In most studies, it was additionally detected in adenomas, especially in highly dysplastic areas (Table VII). The levels of mature (heavily glycosylated) MUC1 as detected by mab HMFG-2 were higher in advanced tumor stages and in metastasizing cancer (Nakamori *et al.*, 1994). MUC1 immunoreactivity also correlated with p53 protein expression and a relatively high apoptotic index in intramucosal carcinomas (Tanimoto *et al.*, 1999). MUC2 peptide could also be described in colorectal carcinomas, showing a frequent and strong expression in the mucinous carcinoma subtype (Table VII). Thereby, it revealed a binding pattern inverse to MUC1, which exhibited a higher staining score in tubular/papillary cancer (Baldus *et al.*, 1998b).

Table VII. Immunoreactivity of Mucin-Associated Core Antigens in the Colorectal Adenoma-Carcinoma Sequence

Antigen	Adenomas	Ref.	Carcinomas	Ref.
MUC1	Absent, increasing with dysplasia	Andrews *et al.* (1993), Zotter *et al.* (1987), Cao *et al.* (1997b) Baldus *et al.* (1998b)	Strong expression in tubular carcinomas	Zotter *et al.* (1987), Andrews *et al.* (1993), Ho *et al.* (1993), Baldus *et al.* (1998b)
MUC2	Decreasing with dysplasia	Ajioka *et al.* (1997), Baldus *et al.* (1998b)	Strong expression in mucinous carcinomas	Ho *et al.* (1993), Blank *et al.* (1994), Ajioka *et al.* (1996), Hanski *et al.* (1997), Baldus *et al.* (1998b)
MUC3	Decreasing with dysplasia	Cao *et al.* (1997b)		
TF	Increasing with dysplasia	Boland *et al.* (1982b), Cooper and Reuter (1983), McGarrity *et al.* (1989), Yuan *et al.* (1986), Cao *et al.* (1997b), Baldus *et al.* (1998b)	Strong expression	Klein *et al.* (1981), Boland *et al.* (1982a), Cooper (1982), Kellokumpu *et al.* (1986), Rhodes *et al.* (1986), Yuan *et al.* (1986), Campo *et al.* (1988), Calderó *et al.* (1989), Itzkowitz *et al.* (1989), Cao *et al.* (1995), Baldus *et al.* (1998b)
Tn	No correlation with dysplasia increasing with dysplasia	Itzkowitz *et al.* (1992), Cao *et al.* (1997b)	Strong expression	Itzkowitz *et al.* (1989), Orntoft *et al.* (1990), Cao *et al.* (1995, 1997b), Jass *et al.* (1995)
Sialyl-Tn	Increasing with dysplasia	Wolf *et al.* (1989), Itzkowitz *et al.* (1992), Cao *et al.* (1997b)	Strong expression	Stramignoni et al. (1983), Itzkowitz *et al.* (1989), Itzkowitz *et al.* (1990)

The relationship between MUC1 and MUC2 immunoreactivity in the adenoma-carcinoma sequence also revealed a divergent development: MUC1 positivity correlates with the development of high-grade dysplasia, whereas MUC2 and MUC3 diminish (Table VII). As recently reported (Bara *et al.*, 1998), MUC5AC is identical with the formerly described so-called M1 mucin, which was detected in normal mucosa adjacent to colon carcinomas (Bara *et al.*, 1984) as well as hyperplastic polyps and adenomas (Bara *et al.*, 1991). Summarized, MUC1 is a marker of a late stage of the adenoma-carcinoma sequence (high-grade dysplasia), whereas MUC2, MUC3, and MUC5AC are markers of the early stages.

2. CARBOHYDRATE CORE ANTIGENS

TF antigen represents the most extensively investigated carbohydrate core antigen in colorectal neoplasms. PNA as well as specific mabs exhibited a strong staining of large bowel cancer (Table VII) as reviewed earlier (Hanisch and Baldus, 1997). Additionally, PNA and mab reactivity correlated with increase of polyp size, histologic type, and dysplasia in adenomas (Table VII). TF antigen exhibits a similar staining pattern in tubular/papillary and mucinous carcinomas, but MUC1-bound TF antigen (BW835 epitope) is only faintly expressed in the latter subtype (Baldus *et al.*, 1998b). Furthermore, a stronger TF positivity in liver metastases compared with primary colorectal carcinomas was reported (Cao *et al.*, 1995). The expression of TF antigen in colonic adenocarcinomas could also be demonstrated biochemically by *O*-glycanase treatment (Campbell *et al.*, 1995). Correspondingly, the β3-galactosyltransferase was also increased in colorectal adenocarcinomas (Yang *et al.*, 1994) and in a cell line derived from a familial polyposis coli patient (Vavasseur *et al.*, 1994).

Tn antigen could be demonstrated in colonic cancer and adenomas by lectin (Vicia villosa agglutinin) and mab immunohistochemistry. It did not show a significant alteration or an increase with the different grades of adenoma dysplasia (Table VII).

Sialyl-Tn antigen was strongly reactive in colorectal adenomas and carcinomas. Its expression correlated with the grade of dysplasia, polyp size, and histologic type (Table VII). In addition to sialyl-Tn, the presence of the α2–6 sialylated binding site of Sambucus nigra agglutinin (SNA) in colorectal carcinomas was described (Sata *et al.*, 1991), which is not reactive in normal mucosa even after de-O-acetylation (Murayama *et al.*, 1997). It accompanied metastatic tumor growth (Vierbuchen *et al.*, 1995). According to biochemical data, the lack of immunoreactivity of TF, Tn, and sialyl-Tn in normal colorectal tissues is not due to a lack of expression, but a masking effect of other additional glycans (Dahiya *et al.*, 1992).

3. BACKBONE AND PERIPHERAL CARBOHYDRATE ANTIGENS

Type 2 chain backbone Galβ1–4GlcNAc as detected by Erythrina lectins showed a weak reactivity in less than 50% of hyperplastic polyps and (irrespective of the grade of dysplasia) adenomas. However, primary carcinomas and liver metastases were strongly positive (Baldus *et al.*, 1996a). The nonfucosylated blood group i antigen was observed in ~65% of the carcinomas (Miyake *et al.*, 1989). Type 2 chain dependent fucosylated antigens are generally enhanced in colorectal adenocarcinomas: Le^x antigen is expressed by ~85–95% of the carcinomas in the proximal as well as distal colon independently from the secretor status, whereas the difucosylated Le^y structure, on the other hand, showed a stronger reactivity in carcinomas of the distal colon (Table VIII). This expression is ectopic, since adult and fetal distal colorectum does not express Le^y. Their biosynthesis is obviously the result of an enhanced production of type 2 chains, since the β1–3 fucosyltransferase is also expressed in the normal colon (Holmes *et al.,* 1987). A number of epitopes consisting of elongated, linear type 2 chains bearing Le^x or Le^y antigenic determinants were also detected in 60–95% of the carcinomas, but rarely present in normal tissues (Itzkowitz *et al.*, 1986; Kim *et al.*, 1986; Baldus *et al.*, 1995). The FW6 epitope, an α1–3 monofucosylated polylactosaminoglycan, exhibited a significant association with the development of high-grade dysplasia in the adenoma-carcinoma sequence (Baldus *et al.*, 1995), as well as other elongated type 2 chain antigens with Le^x and Le^y determinants (Kim *et al.*, 1986; Yuan *et al.*, 1986).

ABH blood group antigens can exhibit a great variety of possible cancer-associated alterations (Table VIII): In some tumors, antigens incompatible with the patient's blood group may be expressed. A focal deletion of ABH antigens in the proximal colon may also be observed; a complete deletion is infrequent (Yuan *et al.*, 1985). Another typical alteration is the (oncofetal) reactivity of ABH and related α1–2 fucosylated antigens (Le^b, Le^y) in the distal colorectum, which could be documented in a large series of studies. In colorectal adenomas, expression of ABH antigens increased with the grade of dysplasia (Denk *et al.*, 1975). In carcinomas, blood group A antigens may be carried by type 1, 2, or 3 chains, thereby differing from the exclusive formation of A type 1 chains in the normal adult proximal colon (Dabelsteen *et al.*, 1988).

Another typical mechanism of modification of mucin-associated antigens is the increased reactivity of sialylated Lewis antigens. Different mabs detected sialyl-Le^x in colorectal carcinomas (Table VIII). Its expression is even more homogenous in liver metastases (Baldus *et al.*, 1995). Sialyl-Le^x is expressed in low-grade and high-grade adenomas as well, showing a stronger reactivity in the latter (Hanski *et al.*, 1990; Baldus *et al.*, 1995). The modified sialyl-Le^x-i antigen has similar characteristics, especially a correlation

Table VIII. Immunoreactivity of ABH and Lewis Blood Group Antigens in Colorectal Cancer

Antigen	Reactivity	Ref.
Le^x	Independent from secretor status, proximal/distal colon	Itzkowitz *et al.* (1989), Sakamoto *et al.* (1989), Baldus *et al.* (1995)
Le^y	Proximal colon	Brown *et al.* (1984), Bara *et al.* (1988)
ABH	Expression incompatible to patient's blood group	Denk *et al.* (1974a), Cooper *et al.* (1980), Yuan *et al.* (1985)
	Oncofetal (neo-)expression in distal colon	Denk *et al.* (1974b), Yonezawa *et al.* (1982), Ernst *et al.* (1984), Yuan *et al.* (1985), Cordon-Cardo *et al.* (1986), Bara *et al.* (1988)
Sialyl- Le^x	Increased reactivity	Fukushima *et al.* (1984), Hanski *et al.* (1990), Hanisch *et al.* (1992), Nakamori *et al.* (1993)

with dysplasia in adenomas (Itzkowitz *et al.*, 1986). Sialyl-Le^a is also expressed in colorectal carcinomas (Atkinson *et al.*, 1982), even more strongly in lymph node metastases (Nakayama *et al.*, 1995). The increase in sialyl-Le^x (Mann *et al.*, 1997) and sialyl-Le^a (Ogata *et al.*, 1995) immunoreactivity in carcinomas and metastases is at least in part due to a reduced *O*-acetylation of sialic acid. The gradual loss of sialic acid *O*-acetylation has been regarded as an early event in the adenoma-carcinoma sequence (Corfield *et al.*, 1995).

As outlined earlier, MUC2 has to be regarded as the predominating mucin of colonic goblet cells. On the other hand, MUC1, H type 2, Le^x, and Le^y are coexpressed in crypt base columnar cells (Brown *et al.*, 1984; Itzkowitz *et al.*, 1986; Yuan *et al.*, 1985). Obviously, colorectal cancer columnar cells exhibit a very similar antigen pattern, resulting in a colocalization, especially on luminal tumor cell membranes and within cancer secretions. Sialyl-Le^x and sialyl-Tn may be associated with both goblet and columnar cell lines (Ajioka *et al.*, 1996) and sialyl-Le^x is carried by MUC1 as well as MUC2 mucin peptide cores (Hanski *et al.*, 1993, 1995).

VI. PROGNOSTIC RELEVANCE IN GASTROINTESTINAL CANCER

During the last few years, carbohydrate antigens gained clinical importance as prognostic markers in a great variety of human carcinomas (Dabelsteen, 1996). Besides carbohydrate core as well as peripheral oligosaccharide antigens, peptide core antigens could also be characterized.

MUC1 and MUC2 peptide core antigens were investigated with regard to their relevance as markers of prognosis in gastrointestinal cancer. In three studies, MUC1 was described as a marker of a worse survival rate in gastric carcinoma patients (Sakamoto *et al.*, 1997; Baldus *et al.*, 1998a; Utsunomiya *et al.*, 1998). In another study, a significant correlation was not observed (Reis *et al.*, 1998). In colorectal cancer, MUC1 immunoreactivity also exhibited an association with a worse survival probability (Hiraga *et al.*, 1998). MUC2 immunoreactivity, on the other hand, correlated with a favorable prognosis of gastric cancer, especially intestinal-type gastric cancer, according to Laurén's classification (Baldus *et al.*, 1998a; Utsunomiya *et al.*, 1998).

Immunoreactivity of sialyl-Tn antigen was shown to exert a negative impact on survival probability in gastric cancer. If tumor stages were separately investigated, conflicting data resulted: A prognostic role of sialyl-Tn was emphasized in stage I (Victorzon *et al.*, 1996), stage III/IV (Ma *et al.*, 1993; Yamada *et al.*, 1995), or T3 and T4 (Werther *et al.*, 1994) gastric carcinomas. In an international multicenter study, a TNM-stage-dependent prognostic importance of sialyl-Tn was postulated (Werther *et al.*, 1996). Others did not observe such correlations with prognosis (Miles *et al.*, 1995; Baldus *et al.*, 1998c). In colorectal cancer patients, a worse prognosis of sialyl-Tn positive tumors with regard to 5-year survival as well as risk of disease recurrence could also be described (Itzkowitz *et al.*, 1990; Vierbuchen *et al.*, 1995).

Expression of TF antigen represents a marker of reduced survival probability in colorectal cancer irrespective of the tumor stage, but especially in stage I carcinomas and early gastric cancer (Baldus *et al.*, unpublished). On the other hand, another study reported a negative prognostic impact of TF antigen in advanced gastric cancer (Chung *et al.*, 1996).

Gastric cancer patients with a tumor reactivity of Helix pomatia agglutinin binding sites recognizing GalNAc, expressed on Tn and A antigen, and GlcNAc also had a lower survival rate, particularly if cancer cells were present at the serosal surface (Kakeji *et al.*, 1991).

Sialyl-Le^a antigen was reported to be a marker of worse prognosis in gastric (Nakamori *et al.*, 1997a; Baldus *et al.*, 1998c) as well as colorectal cancer (Nakayama *et al.*, 1995). In gastric cancer, this feature was generally independent from tumor stage, but pronounced in stage I cancer (Baldus *et al.*, 1998c). Sialyl-Le^x, on the other hand, had no prognostic importance according to one study (Nakamori *et al.*, 1997a). According to other results (Baldus *et al.*, 1998c) it represents a marker of bad prognosis only in early gastric cancer. In colorectal carcinomas, sialyl-Le^x immunoreactivity correlated with distant metastasis, the probability of recurrence and lower 5-year survival rate (Nakamori *et al.*, 1993, 1997b). Its non-sialylated variant, Le^x antigen, was found to be associated with advanced disease (UICC stage) and to be an independent parameter of poor prognosis in gastric carcinomas (Mayer *et al.*, 1996).

In summary, these partly conflicting results suggest that MUC1 peptide, TF and sialyl-Tn core carbohydrates as well as sialyl-Le^a and -Le^x may play a possible role as markers of an unfavorable course of gastrointestinal carcinomas. In this context, peptide core-specific glycosylation may be relevant, but it has not been investigated extensively up to now. Possible interactions of the antigens in the process of metastasis as well as tumor immunology are discussed later.

VII. SERUM TUMOR MARKERS (CIRCULATING ANTIGENS)

Several carbohydrate tumor markers have been thoroughly introduced in clinical tumor diagnostics for a couple of years (Orntoft and Bech, 1997). In gastric cancer, the sialyl-Tn based assay CA72–4 exhibited a sensitivity of about 30–40% for the detection of gastric cancer in most studies involving great numbers of patients (Gero *et al.*, 1989; Wobbes *et al.*, 1992; Guadagni *et al.*, 1992, 1993; Joypaul *et al.*, 1995), sometimes even higher percentages of positivity (Ohuchi *et al.*, 1989; Heptner *et al.*, 1989; Byrne *et al.*, 1990). The serum levels additionally correlated with stage or serosa infiltration (Byrne *et al.*, 1990; Kodama *et al.*, 1995; Gonzalez *et al.*, 1996) as well as presence of lymph node metastasis (Guadagni *et al.*, 1993; Spila *et al.*, 1996). Additionally, an association with grade of differentiation and high CA72–4 levels with a shorter survival was described (Gonzalez *et al.*, 1996). The CA19–9 and CA50 assay systems, which detect circulating sialyl-Lewis^a antigens, showed a lower than or comparable sensitivity to CA72–4, but in the case of CA19–9, most investigations showed a comparable specificity (Heptner *et al.*, 1989; Marechal *et al.*, 1990; Wobbes *et al.*, 1992; Guadagni *et al.*, 1993; Joypaul *et al.*, 1995; Kodama *et al.*, 1995; Spila *et al.*, 1996). Combination of CA72–4 and CA19–9 values, eventually also including carcinoembryonic antigen (CEA), increased their diagnostic potential (Heptner *et al.*, 1989; Ohuchi *et al.*, 1989; Guadagni *et al.*, 1992). CA125, on the other hand, exhibited only a very low sensitivity (Omar *et al.*, 1989).

In colorectal cancer, the same antigens were investigated.The CA242 assay (sialyl-Lewis^a) had a specificity of 100%, a positive predictive value of 100% and a negative predictive value of 60%. Additionally, the CA242 level strongly correlated with clinical course (Ward *et al.*, 1993). Serum levels of sialyl-Lewis^a as measured by another serum assay, CA50, strongly predicted cancer mortality within the first 2 years after surgery. The predictive value was independent of Duke's tumor stages (Stahle *et al.*, 1989a,b). The preoperative serum levels of CA19–9 were also higher in patients with cancer relapse (Gebauer and Müller-Ruchholtz, 1997). The sialyl-Tn epitope

bound by CA72.4 assay is an important early marker for colorectal cancer and/ or dysplastic colonic diseases (Guadagni *et al.*, 1996). Circulating anti-MUC1 antibodies could also be detected in about 48% of the patients, which may allow insights into the immunology of colorectal cancer, especially anti-MUC1 antibody-dependent cell-mediated cytotoxicity (Nakamura *et al.*, 1998).

VIII. FUNCTIONAL ASPECTS REGARDING INVASION AND METASTASIS

Mucin-associated peptide as well as carbohydrate antigens obviously play an important role in different aspects of proliferation, local invasion, and metastasis of cancer cells.

Regarding influences on proliferation, peanut agglutinin was revealed to represent a mitogen for normal human colorectal epithelium as well as human HT29 colorectal cancer cells, as measured by an increase in thymidine incorporation (Ryder *et al.*, 1992). Monoclonal antibodies directed against TF antigen also exhibited a proliferation-stimulating effect on colon cancer cells (Yu *et al.*, 1997). Such an effect is also exerted by dietary peanut ingestion of patients with normal colonic mucosa (Ryder *et al.*, 1998), whereas galactose-containing vegetable fibers would have an inhibitory effect by competing with lectin binding. How far PNA binding to mucin-associated TF antigens is involved in these effects remains to be investigated.

As indicated by several studies, MUC1 in particular may be involved in the local invasion of tumor cells. In breast cancer, MUC1 was reported to be a ligand for the intercellular adhesion molecule-1, ICAM-1 (Regimbald *et al.*, 1996). An inhibition of MUC1–ICAM-1 binding could be achieved by a 120-aa MUC1 peptide corresponding to six tandem repeats (Kam *et al.*, 1998). On the other hand, MUC1 overexpression inhibited the integrin-mediated carcinoma cell adhesion to extracellular matrix components like laminin and kalinin as well as the E-cadherin-mediated cell–cell interactions (Kemperman *et al.*, 1994; Wesseling *et al.*, 1995). In breast cancer cell lines, after reduction of MUC1 expression by using an antisense oligonucleotide, cell–cell and cell–surface adhesion of these cells *in vitro* was reduced. This alteration was explained by a functional suppression of E-cadherin due to a basolateral MUC1 expression, resuming its activity after suppression of MUC1 expression (Kondo *et al.*, 1998). An interaction of the cytoplasmic domain of MUC1 with β-catenin was decreased by phosphorylation of MUC1 by glycogen synthase kinase 3β. Thereby, E-cadherin–β-catenin complexes could be restored (Li *et al.*, 1998). MUC1 transfected gastric cancer cells MKN74 showed a more aggressive tumor growth in mouse *in vitro*

studies, invading the muscle layer after subcutaneous injection. Additionally, they showed a decreased binding to laminin, fibronectin, type I, and type IV collagen as well as an increased mobility. These effects could be abolished by inhibition of *O*-glycan synthesis (Suwa *et al.*, 1998). The accumulated data suggest an "anti-adhesive" effect of MUC1 on tumor cells, which also seems to be dependent on *O*-glycosylation of the peptide core.

The asialoglycoprotein receptor of hepatocytes has been described as a possible receptor for galactosylated antigens like TF (Ashwell and Morell, 1974). A similar asialoglycan-binding receptor was described on Kupffer cells (Kolb-Bachofen *et al.*, 1982). Their relevance was studied in animal models by Springer *et al.* (1983). After sorting of murine colon carcinoma cells by soybean agglutinin (SBA) into weakly and highly galactosylated subpopulations, liver metastasis caused by intrasplenic injection correlated with cell–surface galactose expression (Yeatman *et al.*, 1989). The potential relevance of the hepatic asialoglycoprotein receptors was further supported by studies reporting an (at least partial) inhibition of experimental liver metastasis by galactose or arabinogalactan infusions (Beuth *et al.*, 1988) or by the anti-TF mab A78-G/A7 (Shigeoka *et al.*, 1999). A reduction of hepatic metastasis and overall survival was additionally described in a clinical trial involving 76 colon adenocarcinoma patients perioperatively treated with D-galactose or D-glucose containing electrolyte solution (Warczynski *et al.*, 1997). An involvement of TF antigen and related galactosylated structures into liver metastasis, as suggested by these data, could help to explain the negative impact of TF immunoreactivity in human gastrointestinal cancer, as reviewed earlier.

The most extensively investigated adhesion mechanism involving mucin-associated antigens is, however, the interaction of selectins (E-, L-, and P-selectin) with sialylated Lewis antigens (sialyl-Le^x, sialyl-Le^a). This phenomenon was described for the first time by several groups in the early 1990s, demonstrating E-selectin (ELAM-1) binding to sialyl-Le^x, (Lowe *et al.*, 1990; Philipps *et al.*, 1990; Walz *et al.*, 1990; Tiemeyer *et al.*, 1991), as well as sialyl-Le^a (Takada *et al.*, 1991; Berg *et al.*, 1991; Tyrrell *et al.*, 1991). Both contributed to the adhesion of different malignant tumor cells including colonic cancer cells to vascular endothelia (Takada *et al.*, 1991, 1993). In a comparative study it was shown that L- and P-selectin interact primarily with mucin-bound sialylated Lewis antigens, whereas E-selectin binds to mucin- and non-mucin-substituted antigens as well (Mannori *et al.*, 1995). Additionally, the importance of E-selectin binding of breast and colon cancer cells to endothelial cell monolayers could also be demonstrated under flow conditions comparable to that found in lymphatics and postcapillary blood venules (Tözeren *et al.*, 1995). In nude mice, sialyl-Le^a (Yamada *et al.*, 1997) as well as sialyl-Le^x (Nakamori *et al.*, 1997b) expression correlated with a higher metastatic capacity of human colorectal carcinoma cells. In this con-

text, it is also well known that the expression of dimeric sialyl-Lewis^x antigen is increased in liver metastases compared to primary colorectal carcinomas (Hoff *et al.*, 1989; Hasegawa *et al.*, 1993). Antigen-selected carcinoma cells additionally showed an increased invasive capacity (Matsushita *et al.*, 1991).

IX. PERSPECTIVES IN TUMOR IMMUNOLOGY AND CANCER THERAPY

Mucin peptide cores, in addition to substituting carbohydrate structures, are obviously involved in different types of cellular and humoral immune response in cancer. Up to now, however, most immunotherapeutic studies focused on breast and ovarian cancer, therefore this survey considers only a selection of these works, especially those involving gastrointestinal carcinomas.

During the last few years, various aspects of MUC1 in tumor immunology have been emphasized. In this context, it was postulated that higher MUC1 levels induce T-cell anergy (Reddish *et al.*, 1996; MacLean *et al.*, 1997). Others reported an apoptose induction regarding activated T cells mediated by MUC1 (Gimmi *et al.*, 1996). Cancer-associated and synthetic MUC1 tandem repeats suppressed human T-cell proliferative responses, but this effect could be reversed by addition of exogenous interleukin-2 or anti-CD28 monoclonal antibodies to T-cell cultures (Agrawal *et al.*, 1998). The MUC1 effect seems to depend on the number of tandem repeats: Whereas native mucins exerted the above-described immunosuppressive effects, small peptide-based immunotherapy could be immunostimulatory (Ding *et al.*, 1992; Apostolopoulos *et al.*, 1995; Agrawal *et al.*, 1996). On the other hand, the presence or absence of anti-MUC1 antibodies in patients' sera did not correlate with the levels of circulating mucins or the stage of disease (Kotera *et al.*, 1994). However, MUC1 detection by the immune system is MHC independent; therefore, it would be a broad-spectrum target for immunotherapies (Finn *et al.*, 1995). As a consequence of these findings, several strategies have been developed to use MUC1 in cancer therapy, for example, recombinant MUC1 expression in vaccinia- or baculovirus and retroviral expression, respectively (Balloul *et al.*, 1994; Ciborowski and Finn, 1995; Henderson *et al.*, 1998).

Another approach was elected using autologous Epstein–Barr virus (EBV)-immortalized B cells as carriers of tumor-associated MUC1 in chimpanzees. The application of these costimulatory molecules resulted in an increase of cytotoxic T-cell precursors (Pecher and Finn, 1996) and an MHC-unrestricted T-cell receptor triggering (Magarian-Blander *et al.*, 1998). However,

passive transfer of MUC1-specific antibodies did not result in autoimmune responses against normal secretory epithelia, and also did not provide protection against the growth of syngeneic tumors expressing MUC1 in a mice model (Tempero *et al.*, 1999). Besides CD8+ cytotoxic T lymphocytes and the production of IgM antibodies, a CD4+ T-cell response can also be elicited by an adequate MUC1 peptide (Hiltbold *et al.*, 1998; Tempero *et al.*, 1998). In a clinical trial, 13 out of 25 patients with various adenocarcinomas developed IgG1 anti-MUC1, additionally T-cell proliferation and CTL responses were observed in some cases after immunization with MUC1 fusion proteins (Karanikas *et al.*, 1997). However, the role of MUC1 in tumor immunology as well as cancer immunotherapy needs to be investigated further, especially with regard to gastrointestinal cancer.

Mucin-associated carbohydrate antigens may also be involved in these complex interactions. For example, sialyl-Tn antigen was shown to inhibit natural killer (NK) cell activity against K562 target cells (Ogata *et al.*, 1992). In colorectal cancer, TF as well as sialyl-Tn conjugates with keyhole limpet hemacyanin (KLH) induced high IgG and IgM responses, but only the IgM antibodies showed a weak to moderate reactivity against the natural antigens (Adluri *et al.*, 1995). The postimmunization increase in anti-ovine submaxillary mucin (OSM, containing many sialyl-Tn epitopes) immunoglobulin IgM titer showed an independent association with longer survival of colorectal cancer patients (MacLean *et al.*, 1996). The level and specificity of the immune response after OSM-immunization was elevated after induction by rIL-1 and rIFN-γ. Therefore, these cytokines may be powerful adjuvants modulating the immune response against mucin-associated carbohydrate antigens (Piera *et al.*, 1993).

Some years ago, the different types of natural TF-reactive antibodies from human sera were characterized, revealing at least three subpopulations of TF antibodies with distinct specificities (Wolf *et al.*, 1987). Synthetic TF and Tn haptens stimulated delayed-type hypersensitivity (DLH) effector T cells, indicating an anticancer effect mediated by these antigens (Henningsson *et al.*, 1987). However, up to now, most immunotherapeutic investigations using TF antigen were performed on ovarian and breast cancers (MacLean *et al.*, 1992; Springer, 1997).

Bispecific monoclonal antibodies binding sialyl-Le^a antigen as well as CD3 T cells were also tested and resulted in a great augmentation of their cytotoxicity against gastrointestinal tumor cells (Ohta *et al.*, 1995). An additional *in vivo* effect against human colon cancer cells implanted in nude mice was also observed in the same study.

Extending these observations, further investigations are warranted in order to define the role of mucin-associated peptide and carbohydrate antigens in the immunotherapy of human cancer. Biosynthesized glycopeptides containing clustered saccharide domains (Sames *et al.*, 1997) as well as peptides

that mimic carbohydrates (Kieber-Emmons *et al.*, 1997) may also be applied in future studies on therapy of gastrointestinal carcinomas.

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Studies on Polyomavirus Persistence and Polyomavirus-Induced Tumor Development in Relation to the Immune System

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I. INTRODUCTION

Murine polyomavirus, a potentially oncogenic small DNA virus in the Papova virus group, was discovered independently by Gross and Stewart in 1953, as a contaminant in murine leukemia extracts (Gross, 1953; Stewart, 1953). The virus was subsequently found to induce tumors in different tissues of mice, hamsters, and rabbits, hence, the name *polyoma* (Stewart *et al.,* 1958; Eddy *et al.,* 1958a,b, 1959a,b). It was isolated and grown in tissue cul-

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ture (Stewart *et al.,* 1958), and its *in vitro* transforming capacity was demonstrated (Vogt and Dulbecco, 1960). Although polyomavirus was found to be common in populations of wild mice as well as in a number of laboratory animal colonies, the mice rarely developed polyomavirus-induced tumors (Rowe *et al.,* 1958, 1959a,b; Stewart and Eddy, 1958; Vogt and Dulbecco, 1960). Subsequently, a line of evidence suggested that this was due to a potent immunologic control of viral infection and tumor induction. These features indicated that the virus and its natural host were well adapted to each other and may have had a long common evolutionary history. Over the years polyomavirus has served as a useful tool for the studies of basic molecular events of cellular transformation, as well as for studies of virus–host interactions and immune recognition of virus-related tumors (for reviews, see Sjögren, 1964, 1965; Vandeputte and Datta, 1972; Allison, 1980; Griffin and Dilworth, 1983; Dalianis, 1990; Berke, 1997).

II. INITIAL STUDIES ON POLYOMAVIRUS INFECTION AND POLYOMA-SPECIFIC IMMUNE RESPONSES *IN VIVO* AND *IN VITRO*

The natural way of virus spread is through excretion of infectious particles, readily infecting mice in a colony (for review, see Shah, 1990). Although polyomavirus is potentially oncogenic, tumors are normally not observed under natural conditions. In polyomavirus-infected mouse colonies, passively transferred maternal antibodies protect newborn mice from infection for the first couple of weeks after birth when the murine immune system is generally immature (Stewart and Eddy, 1958; Law *et al.,* 1959). Primary infection normally occurs after the age of 3 to 4 weeks when the murine immune system is mature (Law *et al.,* 1959). At this time the mice are normally resistant to polyomavirus-induced tumor development (Law *et al.,* 1959; Law and Ting, 1965). Polyomavirus-induced tumors develop only in normal newborn mice that are not protected by passively transferred maternal antibodies and in adult immunocompromized mice, e.g., nude mice (Law *et al.,* 1959; Miller *et al.,* 1964; Vandeputte, 1968; Allison and Law, 1968; Allison, 1980). Furthermore, adult mice or hamsters immunized with polyomavirus are resistant to the outgrowth of a small polyoma tumor cell inoculum in contrast to nonimmunized controls (Sjögren *et al.,* 1961; Habel, 1961).

Subsequently, several studies have focused on the importance of the immune system in the prevention of polyomavirus-induced tumor development and polyoma tumor rejection. Neonatal thymectomy abrogating T-cell immunity increases the frequency of polyoma tumor development in neonates and renders adult mice susceptible to tumor development (Miller *et al.,* 1964;

Law and Ting, 1965; Allison, 1974). Treatment with anti-lymphocytic serum (ALS) has the same effect as neonatal thymectomy and when neonatal thymectomy is combined with ALS treatment an even higher tumor frequency is observed (Allison and Law, 1968; Vandeputte, 1968; Sjögren and Borum, 1971; Gaugas *et al.,* 1973; Allison, 1980). Similar to thymectomized mice, nude mice lacking functional T cells exhibit an arsenal of polyoma tumors on infection (Allison and Taylor, 1967; Vandeputte and Datta, 1972). However, transfer of lymphocytes from polyoma immune donors into thymectomized animals inhibits polyoma tumor development (Gaugas *et al.,* 1973; Vandeputte and Datta, 1972). The incidence of tumor induction can also be reduced in thymectomized and ALS treated mice if serum containing polyoma-specific antibodies is given within 24 hr but not 1 week after polyomavirus infection (Allison, 1980). Taken together, these results suggested that the cellular immune system, in particular T cells are important in preventing polyomavirus-induced tumor development. The humoral response also plays an important role, since it can prevent viral infection; however, it cannot prevent tumor development once viral spread has occurred. The cellular immune response has also been shown to be important for the rejection of polyoma tumors. Injection of a mixed population of polyoma tumor cells and lymphocytes from polyoma immunized mice resulted in decreased frequency of tumor outgrowth (Sjögren, 1964). Furthermore, both $CD4^+$ and $CD8^+$ T cells are necessary for the polyoma tumor rejection since inoculation of polyoma immunized mice with either anti-CD4 or anti-CD8 antibodies inhibited the ability of the animals to reject a polyoma tumor cell inoculum (Ljunggren *et al.,* 1994).

Several attempts have been made to characterize the cellular and humoral immune responses to polyomavirus-induced tumors *in vitro*. Nevertheless, the number of published reports is limited. Initial studies show that specific growth inhibition of polyoma tumor cells can be obtained in a colony inhibition assay with lymphocytes from rats immunized with polyomavirus or bearing polyomavirus-induced tumors (Datta and Vandeputte, 1971; Sjögren and Borum, 1971). Also antibody-dependent cellular cytotoxicity (ADCC) against syngeneic polyoma tumors is demonstrated by a microtoxicity assay using antisera from mice immunized with polyomavirus transformed cells (Walia *et al.,* 1980). In addition, polyoma-specific responses can be detected by macrophage migration inhibition assays with cell and membrane extracts from polyoma tumors, as well as with synthetic peptides derived from the sequences of middle T and small T antigens of polyomavirus (Szigeti *et al.,* 1982; Ramqvist *et al.,* 1986; Reinholdsson-Ljunggren *et al.,* 1989, 1992). Furthermore, although several attempts have been made, there are only two reports of *in vitro* cytotoxicity (CTL) directed against syngeneic polyoma tumor cells (Greene *et al.,* 1992; Lukacher *et al.,* 1995).

In summary, both *in vivo* and *in vitro* studies suggest that the cellular im-

mune system, in particular T cells, plays a major role in the prevention of polyoma tumor development and the rejection of polyomavirus-induced tumors. Irrespective of the mechanisms involved, polyomavirus-induced tumor development is observed several months after virus infection and, consequently, the virus has been suggested to persist. This was also supported by the data of McCance (1981) demonstrating the excretion of polyomavirus in the urine of mice infected as newborns. However, it has never been determined how the virus can persist without causing tumors. The present review gives a summary of recent data in the field dealing with the role of the immune system in polyomavirus persistence and polyomavirus-induced tumor development.

III. MOLECULAR CHARACTERISTICS OF POLYOMAVIRUS

The polyoma viral genome is divided into a noncoding region, and an early and late coding region (Griffin *et al.,* 1974). The latter division is made according to the stage during productive infection at which the encoded genes are expressed. The early region includes the genes for the three T antigens: large T antigen (LT), middle T antigen (MT), and small T antigen (ST). The early region of the genome is economically used and the genes for the three T antigens are partially overlapping. The three early mRNAs are generated through differential splicing from a precursor RNA (Treismann *et al.,* 1981). This results in a common N terminus for all three T antigens, but each T antigen has a unique C terminus due to frame shifts (Kamen and Shure, 1976; Ito, 1980). The early region gene products of polyomavirus have several functions in the lytic cycle, in transformation and regarding the host range of infection (for a review of T antigens, see Pipas, 1992). LT is expressed early during the lytic cycle, it promotes initiation of S phase in the cell cycle, and is necessary for viral replication (for review, see Cole, 1996). It binds to the noncoding region, initiates replication of viral DNA, represses early transcription, and promotes the production of viral capsid antigens (reviewed by Ito, 1980). The fact that LT pushes the cell into S phase can explain its immortalizing capacity (Land *et al.,* 1983). A function for MT in the lytic cycle has not been described so far; however, MT is important for the host range of the virus and it plays a pivotal role in cell transformation (Treisman *et al.,* 1981; Kiefer *et al.,* 1994). ST also influences the host range and promotes viral DNA synthesis and virus maturation (Nilsson and Magnusson, 1983; Martens *et al.,* 1989). Furthermore, ST elevates the transforming efficiency of MT (Cuzin, 1984). LT is located in the nucleus, MT is associated with cytoplasmic membranes, and ST is localized both in the nucleus and the cytoplasm (Dilworth *et al.,* 1986).

The late region of the virus genome encodes the three capsid proteins. These are also generated from a common pre-mRNA by alternative splicing; the reading frame for VP1 differs from that of VP2 and VP3 (Griffin *et al.,* 1980).

The early and late coding regions are separated by the noncoding region where the origin of replication and the enhancers and promoters are situated (for a review, see Griffin *et al.,* 1980; Ito, 1980). Mutations within this region influence host range, viral DNA replication, and the oncogenic capacity of the virus *in vivo*.

IV. POLYOMAVIRUS TUMOR-SPECIFIC TRANSPLANTATION ANTIGENS

The fact that polyomavirus-induced tumors are very rare under natural circumstances was suggested to be due to a potent T-cell-dependent immune response against polyoma tumor cells, as discussed above (Allison, 1980). The target of this specific immune recognition on tumor cells was functionally defined as the polyoma tumor-specific transplantation antigen (TSTA) (Habel, 1962, 1966; Sjögren, 1964). Initially, it was not clear if TSTA was of viral or cellular origin and the nature of TSTA was identified only later (for review, see Dalianis, 1990). TSTA was eventually correlated to expression of the early antigens, since TSTA was lost only when expression of all T antigens was abolished (Ramqvist *et al.,* 1988). Subsequently it was shown that recombinant polyoma MT and ST, although not expressed on the cell surface, could be used to immunize against polyoma tumors (Ramqvist *et al.,* 1988). Polyoma T antigens were therefore suggested to undergo intracellular processing and to be presented as peptide antigens on the tumor cell surface in complex with molecules of the major histocompatibility complex (MHC) (Ramqvist *et al.,* 1989). Functionally this seems to be the case, since short synthetic peptides derived from the sequence of LT and MT immunize efficiently against the outgrowth of a polyoma tumor cell inoculum (Reinholdsson-Ljunggren *et al.,* 1992). Furthermore, when MHC class I restricted peptides were eluted from a polyoma positive tumor cell line, one of the identified peptides corresponded to amino acids (aa) 578–585 of LT (Berke *et al.,* 1996b). Immunization with a synthetic peptide corresponding to this sequence protects against the outgrowth of the original polyoma positive tumor, and thus by definition this LT-derived peptide is a TSTA (Berke *et al.,* 1996b). In summary, today TSTA is regarded to consist of several T-antigen-derived peptide antigens. Consequently, it is an enigma how a virus with such a plethora of viral antigens can persist. Next, some factors influencing polyomavirus persistence are discussed.

V. FACTORS INFLUENCING POLYOMAVIRUS PERSISTENCE AND POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT

Several studies have focused on identifying viral and host factors that influence viral replication, persistence, and polyomavirus-induced tumor development.

A. Viral Factors

A number of different mutants of polyomavirus have been generated in order to dissect the mechanism of viral replication and persistence (for a review of polyoma mutants, see Griffin and Dilworth, 1983). These mutants can arbitrarily be grouped into structural mutants and nonstructural mutants, or a combination of the two. The tsa (temperature sensitive), hrt (host-range, nontransforming), and mlt (middle, large T antigens) mutants are all structural mutants of the early region, influencing viral replication and host range (for review, see Griffin and Dilworth, 1983). Tsa mutants with lesions in the LT coding region are defective in viral DNA replication at 37°C, replicate only at 32°C, and have an altered capacity of cellular transformation (Eckhart, 1975; Fried *et al.,* 1974). Hrt mutants contain lesions in the ST and MT coding regions, but not in LT and are nontransforming and nononcogenic (Benjamin, 1970; Staneloni *et al.,* 1977; Eckhart, 1977; Fluck *et al.,* 1977). Mlt mutants carry lesions in MT and LT and some of these mutants have a reduced capacity to replicate and persist in the kidneys of newborn infected mice (McCance, 1981). One particular mutant (1387T) carries a truncation only in MT. When injected into newborn mice it shows a reduced replication capacity, reduced organ distribution of persistent virus, and is defective in tumor induction (Freund *et al.,* 1992).

Two other mutant types of polyomavirus, PTA and RA, which have different mutations in the noncoding region as well as in the coding regions of both LT and VP1, have profoundly different capacities to induce tumors in newborn C3H/BiDa mice (Freund *et al.,* 1987, 1992; Dubensky *et al.,* 1991). PTA induces tumors at a very high frequency, 90–100%, in newborn infected C3H/BiDa mice, while tumor induction by RA is restricted to 15% in the same strain (Freund *et al.,* 1987, 1992). Furthermore, the tissue tropism of the two strains differs greatly. PTA-induced tumors are of varied origin, including epithelial and mesenchymal tumors, while RA-induced tumors are strictly of mesenchymal origin (Freund *et al.,* 1987). In addition, only the PTA but not the RA strain can establish a disseminated infection with extensive replication in the kidneys of newborn infected mice (Dubensky *et al.,* 1991).

Variations in the A and B enhancer regions of the virus genome also influ-

ence the level of viral replication and host range (Amalfitano *et al.,* 1992; Rochford *et al.,* 1990, 1992). Loss of the B enhancer in deletion mutants leads to a decreased viral replication *in vivo* (Rochford *et al.,* 1990). Loss of the glucocorticoid-like consensus sequence within the enhancer region reduces polyomavirus replication in the kidneys, thus restricting the organ specificity of the polyomavirus replication (Rochford *et al.,* 1990). On the other hand, loss of the c-fos, Ad5 E1A, or SV40 consensus sequences within the enhancer region expands the organ specificity of polyomavirus replication *in vivo* (Rochford *et al.,* 1990). Furthermore, the A and B domains have been suggested to control different phases (acute and persistent) of polyomavirus infection *in vivo* (Amalfitano *et al.,* 1992; Rochford *et al.,* 1992).

B. Host Factors

Different inbred laboratory mouse strains develop polyomavirus-induced tumors at a varying frequency and location. The inbred C3H/BiDa laboratory mouse strain shows an elevated sensitivity to polyomavirus-induced tumor development (Lukacher *et al.,* 1993). Subcutaneous injection of the wild-type polyoma strain A2 induces tumors at a 100% frequency in a wide variety of tissues in newborn C3H/BiDa mice, while the same injection in Balb/c mice induces a restricted tumor incidence of 30–50% (Berebbi *et al.,* 1988; Demengeot *et al.,* 1990). This enhanced sensitivity of C3H/BiDa mice was shown to be due to the presence of the dominant Pyv^s gene (Lukacher *et al.,* 1993). Pyv^s codes for the Mtv-7 superantigen and is suggested to be responsible for the elimination of T cells bearing Vβ6, which are considered important for the elimination of polyoma tumors (Lukacher *et al.,* 1995).

Likewise, different organs of the mouse vary in sensitivity to polyomavirus infection. According to the classification of Wirth *et al.* (1992), the organs of the mouse can be divided into two groups (class I and II) with regard to polyomavirus replication *in vivo*. In class I organs (mammary gland, skin, and bone) viral replication is high in both newborn and adult infected mice. In class I organs (kidney, liver, and lung) viral replication is high after infection of newborn mice and low after infection of adults (Wirth *et al.,* 1992; Amalfitano *et al.,* 1992). This discrepancy is most likely due to the developmental state of neonate and adult organs, involving different rates of cellular division and differentiation.

C. Route of Inoculation

Persistence of polyomavirus in newborn infected mice has also been shown to depend on the route of inoculation (Dubensky and Villarreal, 1984; Dubensky *et al.,* 1991). In newborn infected mice, persistent polyomavirus

DNA is observed in both the lungs and kidneys after intranasal infection, while after intraperitoneal inoculations the lungs are only transiently polyomavirus positive (Dubensky and Villarreal, 1984).

VI. STUDIES ON POLYOMAVIRUS PERSISTENCE AND POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT IN IMMUNOCOMPETENT AND IMMUNODEFICIENT MICE

A. Definition of a Persistent Polyomavirus Infection

Persistent polyomavirus infection is in this review defined as the presence of polyomavirus DNA in tissue samples from infected mice up to several months p.i. Studies on polyomavirus replication and persistence summarized below are based on a number of different techniques, such as hybridization of whole-mouse sections, Southern blotting or *in situ* hybridization, and polyomavirus-specific polymerase chain reaction (PCR) (Dubensky *et al.,* 1984, 1991; Dubensky and Villarreal, 1984; Berke and Dalianis, 1993; Wirth *et al.,* 1992, 1997). The sensitivity of the methods differs, but the results are comparable. Below, we review how the different arms of the immune response, in particular the presence or absence of T and B cells, influence the outcome of polyomavirus infection. The data 1–6 months p.i. for newborn infected mice are summarized in Table I and for adult infected mice in Table II. In Tables IIIA–D, the presence of polyoma DNA in different organs is followed 2 days to >1 month p.i. in strains of mice where a persistent infection is not necessarily established in all mice.

B. Studies in Normal Newborn and Adult Mice

1. POLYOMAVIRUS INFECTION AND PERSISTENCE IN NORMAL NEWBORN INFECTED MICE

a. General Findings

Polyomavirus infection in newborn mice causes extensive virus replication in several organs as observed by hybridization of whole-mouse sections, Southern blotting, *in situ* hybridization, or PCR and virus infection may persist (Dubensky and Villarreal, 1984; Dubensky *et al.,* 1984, 1991; Wirth *et al.,* 1992, 1997; Berke *et al.,* 1996a). Table I presents a summary of virus persistence, organ distribution, and virus-induced tumor development 1–6 months p.i. in different strains of newborn infected normal and immunoin-

Table I. Polyomavirus Persistence, Organ Distribution, and Virus-Induced Tumor Development 1–6 Months P.I. in Different Strains of Newborn Infected Normal and Immunoincompetent Mice

Immune state Mouse strain Organs	Normal CBA[a]	Normal C57BL/6[a]	Normal A/SN[a]	Normal Balb/c[a,b]	$CD4^{-/-}$ Balb/c[a] C57BL6[a]	$CD8^{-/-}$ Balb/c[a] C57BL/6[a]	$CD4^{-/-}8^{-/-}$ C57Bl/6[a]	XID CBA[a]	$IgM^{-/-}$ C57BL/6[a]
Bone	+	+	+[c]	+	+	+	+	+	+
Heart	+	+	+	+	+	−	+	+	+
Stomach	+	+	−[d]	nt[e]	+	−	+	+	+
Gonads	+	+	−	nt	−	−	+	+	+
Lung	+	−	−	nt	−	−	+	+	+
Parotid	+	+	−	nt	−	+	+	+	+
Skin	+	+	+	+	−	−	+	+	+
Brain	+	+	−	nt	−	−	+	+	+
Thymus	+	+	−	nt	−	−	+	+	+
Liver	+	+	−	nt	+	−	+	+	+
Spleen	+	+	+	+	−	−	+	+	+
Kidney	+	+	+	+	+	+	+	+	+
Lymph node	+	+	+	+	−	+	+	+	+
Mammary gland	+	nt	nt	+	nt	nt	nt	nt	nt
Salivary gland	nt	nt	nt	+	nt	nt	nt	nt	nt
Blood	+	nt	nt	nt	nt	nt	−	+	+
Persistence	95%	33–50%	33%	95–100%	28%	11%	41%	95%	100%
Tumor development	29%	0–4%	7%	10%	0–3%	5–28%	29% (early death)	35%	0%

[a]Polyomavirus DNA detected by PCR.
[b]Polyomavirus DNA detected by *in situ* hybridization.
[c]Polyomavirus DNA detected.
[d]No polyomavirus DNA detected.
[e]Not tested.

competent mice. In normal newborn mice lungs, kidneys, skin, bone, mammary gland, and salivary glands are primary targets for virus replication which peaks at 6–10 days p.i. (Dubensky *et al.,* 1984; Wirth *et al.,* 1992, 1997). Later, by 14–30 days p.i., viral replication decreases and viral DNA is detected in a more limited number of organs (Wirth *et al.,* 1992; Berke *et al.,* 1996a). A persistent polyomavirus infection is still observed 1 month p.i. most often in the kidneys and bone, but also in mammary glands, heart, skin, spleen, and lymph nodes, as summarized for different strains (CBA,C57BL/6 and A/Sn) of newborn mice in Table I (McCance, 1981; Wirth *et al.,* 1992, 1997; Berke *et al.,* 1996a). All mice with a persistent polyomavirus infection harbor virus DNA only in a limited number of organs, but individual mice may have a varied distribution of polyomavirus (McCance, 1981, Wirth *et al.,* 1992, 1997; Berke *et al.,* 1996a).

b. Strain Differences

Polyomavirus persistence varies in different inbred laboratory mouse strains (Lukacher *et al.,* 1993; Berke *et al.,* 1996a; Wirth *et al.,* 1997; Heidari *et al.,* 1999). When tissues of newborn infected mice are examined by PCR for the presence of polyoma DNA 1–6 months, p.i., 95–100% of Balb/c and CBA mice are positive as compared to 33–50% in C57BL/6 and A/Sn mice as summarized in Table I (Berke *et al.,* 1996a; Wirth *et al.,* 1997; Heidari *et al.,* 1999). Also the extent of virus dissemination differs between these mouse strains. The kidneys, bone, and mammary gland are the primary targets for viral persistence in all strains by all methods. However, persistent polyomavirus DNA is limited to only a few organs in A/Sn mice, while in C57BL/6 and CBA mice all organs are targets for virus persistence as demonstrated by PCR in Table I (Berke *et al.,* 1996a; Heidari *et al.,* 1999). Balb/c mice harbor persistent polyoma DNA in bone in one report (Demengeot *et al.,* 1990), while in bone, as well as in mammary gland, spleen, skin, salivary glands, and kidneys in another report as summarized in Table I (Wirth *et al.,* 1997). This discrepancy is most likely due to the different sensitivity of the techniques used. Hybridization of whole-mouse sections was used in the first study, while a polyoma-specific PCR was used in the second study (Demengeot *et al.,* 1990; Wirth *et al.,* 1997).

2. POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT IN NORMAL NEWBORN INFECTED MICE

a. General Findings

Polyomavirus can induce a wide variety of tumors in newborn infected mice of the C3H/BiDa, Balb/c, CBA, A/Sn, and C57BL/6 strains where the primary targets of polyomavirus-induced oncogenesis are bone, mammary glands, salivary glands, skin, and occasionally the kidneys (Dawe *et al.,*

1987; Berebbi *et al.,* 1988; Wirth *et al.,* 1992, 1997; Berke *et al.,* 1996a). Polyomavirus-induced tumors arise within a wide time range varying between 2 and 8 months p.i.

b. Strain Differences

Similar to the incidence of persistent infection, the incidence of tumor development varies between different strains. Among newborn infected mice polyoma tumors are observed in 90–100% of C3H/BiDa mice (data not shown), 29% of CBA, 10% of Balb/c, 7% of A/Sn mice, while in C57BL/6 mice tumors are observed only occasionally (0–4%) (Table I) (Dawe *et al.,* 1987; Miller *et al.,* 1964; Wirth *et al.,* 1992; Berke *et al.,* 1996a; Heidari *et al.,* 1999). Furthermore, the tumor profiles vary between different strains (Demengeot *et al.,* 1990; Wirth *et al.,* 1992, 1997; Berke *et al.,* 1996a; Berke and Heidari, unpublished observations). C3H/BiDa mice develop tumors of almost every tissue, while A/Sn, CBA, and Balb/c show a restricted tumor profile involving mainly bone, skin, salivary gland, and mammary gland (Dawe *et al.,* 1987; Wirth *et al.,* 1992; Lukacher *et al.,* 1993). C57BL/6 mice are more resistant to polyomavirus-induced tumor development and tumors are observed most often in the bone and parotid gland (Miller *et al.,* 1964, Z. Berke, unpublished observations).

3. POLYOMAVIRUS INFECTION IN NORMAL ADULT MICE

In normal adult polyomavirus-infected mice, polyomavirus DNA is cleared by 1 month p.i. (Berke and Dalianis, 1993; Berke *et al.,* 1996a, 1998). Table II presents a summary of virus persistence, organ distribution, and virus-induced tumor development 1–6 month p.i. in different strains of adult infected normal and immunoincompetent mice. Despite that infection is cleared by 1 month p.i. in normal adult infected mice, it is possible to detect polyomavirus DNA by PCR in a limited number of organs between 48 hr and 3 weeks p.i., as summarized for CBA, C57BL/6 and A/Sn mice in Table IIIA. The peak of infection is observed 1–2 weeks p.i. when bone, heart, lymph nodes, gonads, skin, and brain are positive for polyoma DNA (Berke and Dalianis, 1993; Berke *et al.,* 1996a, 1998). A variation in the organ distribution of virus DNA is observed between individual mice. Bone is polyomavirus DNA positive in almost all animals, heart is frequently positive, while virus DNA can be detected only occasionally in lymph nodes, gonads, skin and brain, and the kidneys are never polyoma positive as summarized in Table IIIA (Berke and Dalianis, 1993). The number of polyoma positive organs decreases with time and viral DNA is not detected by PCR later than 1 month p.i. as discussed earlier (Berke and Dalianis, 1993; Berke *et al.,* 1996a, 1998). Polyomavirus-induced tumors do not normally develop upon polyomavirus infection of immunocompetent adult mice as sum-

Table II. Polyomavirus Persistence, Organ Distribution, and Virus-Induced Tumor Development 1–6 Months P.I. in Different Strains of Adult Infected Normal and Immunoincompetent Mice

Immune state Mouse strain Organs	Normal CBA[a,b]C57BL/6[a] A/SN[a]Balb/c[b]	Nude Balb/c[b]	THXl A/SN[a]	CD4$^{-/-}$ Balb/c[a] C57BL6[a]	CD8$^{-/-}$ Balb/c[a] C57BL6[a]	CD4$^{-/-}$8$^{-/-}$ C57BL6[a]	XID CBA[a]	IgM$^{-/-}$ C57BL/6[a]	IgM$^{-/-}$CD8$^{-/-}$ C57BL/6[a]	SCID C.B–17
Bone	–[c]	+[d]	+	–	+	+	–	+	+	+
Heart	–	–	+	–	–	+	+	+	+	+
Stomach	–	nt[e]	–	–	–	+	–	+	+	+
Gonads	–	–	+	–	–	+	–	+	+	+
Lung	–	–	+	–	–	+	–	+	+	+
Parotid	–	+	+	–	–	+	+	+	–	+
Skin	–	+	+	–	–	+	+	+	–	+
Brain	–	–	+	–	–	+	+	–	+	+
Thymus	–	nt	+	–	–	+	–	–	+	+
Liver	–	–	+	–	–	+	–	+	–	+
Spleen	–	+	+	–	–	+	–	+	–	+
Kidney	–	–	+	–	–	+	–	+	–	+
Lymph nodes	–	nt	+	–	–	+	–	+	+	+
Mammary gland	–	+	nt	nt	nt	nt	nt	nt	nt	+
Salivary gland	–	+	nt	nt	nt	nt	nt	nt	nt	+
Blood	nt	nt	nt	nt	nt	–	–	+	+	+
Persistence	0%	100%	100%	0%	9%	75%	30–50%	100%	100%	100%
Tumor development	0%	30–60%	nt	0%	0%	56%	0%	0%	(early death)	(early death)

[a]Polyomavirus DNA detected by PCR.
[b]Polyomavirus DNA detected by *in situ* hybridization.
[c]No polyomavirus DNA detected.
[d]Polyomavirus DNA detected.
[e]Not tested.

marized in Table II for the CBA,C5/BL/6, A/Sn, and Balb/c strains of mice and reviewed by Allison (1980).

4. POLYOMAVIRUS PERSISTENCE AND TUMOR INDUCTION IS AGE- AND STRAIN-DEPENDENT IN NORMAL MICE

The above observations demonstrate that polyomavirus persists only in newborn infected mice, and that the incidence of long-term persistence is strain dependent (Berke and Dalianis, 1993; Berke *et al.,* 1996a, 1998; Heidari *et al.,* 2000). In contrast, persistent polyomavirus infection is normally not observed in adult infected immunocompetent mice independent of mouse strain (Berke and Dalianis, 1993; Berke *et al.,* 1996a, 1998; Heidari *et al.,* 2000). Consequently, it is not surprising that newborn infected normal mice may develop polyomavirus-induced tumors in a variety of organs later in life, while tumors are not observed in adult infected normal mice (compare Tables I and II). The differences observed in persistence between newborn and adult infected mice are suggested to be primarily due to the immature immune system of the newborn mouse (Berke and Dalianis, 1993). The variation between long-term persistence observed in different strains is most likely also dependent on the immune system. It is known, e.g., that C3H/BiDa mice that are

Table IIIA. Detection of Polyomavirus DNA in Organs of Normal CBA, C57BL/6 and A/Sn Adult Mice

	No. of polyomavirus DNA-positive mice by PCR at indicated time after infection		
Organ	**2–5 days**[a]	**1–4 weeks**[b]	**>1 month**[c]
Bone	2	19	0
Heart	1	10	0
Stomach	1	0	0
Gonads	2	5	0
Lung	0	0	0
Parotid	0	0	0
Skin	2	3	0
Brain	0	2	0
Thymus	0	0	0
Liver	0	0	0
Spleen	1	0	0
Kidney	0	0	0
Lymph nodes	1	4	0

[a] 8 mice were tested.
[b] 20 mice were tested.
[c] 10 mice were tested.

polyomavirus sensitive lack Vβ6 positive T cells as discussed above and reported by Lukacher *et al.* (1993). Furthermore, it has been described that the immune system of C57BL/6 mice is more mature than that of CBA at birth (Billingham and Brent, 1957). This fact could possibly explain why both virus persistence and tumor incidence in the latter strain is more frequent than in the former strain of newborn infected mice as summarized in Table I (Miller *et al.,* 1964; Dawe *et al.,* 1987; Heidari *et al.,* 2000).

Another difference between polyomavirus infected newborn and adult mice is the organ distribution (Berke and Dalianis, 1993). The kidneys and lungs described as primary targets for viral infection and persistence after infection of newborn mice are consistently polyoma DNA negative by PCR after infection of normal adult mice (compare Tables I and IIIA) (Berke and Dalianis, 1993). One explanation for the difference in susceptibility of the kidneys of newborn and adult mice to polyomavirus replication is that the newborn kidney is not fully differentiated. It has been shown that ongoing cellular replication and differentiation during the regeneration of the tubular epithelium as such is necessary for polyomavirus replication (Atencio *et al.,* 1993). The regeneration process can be evoked by mechanical (renal artery clamping), or chemical (glycerol, cisplatin, or methotrexate) damage in adult kidneys, which thus become susceptible to polyomavirus infection (Atencio *et al.,* 1993). Also, a persistent infection in the kidneys of newborn infected mice can be reactivated after such types of damage (Atencio *et al.,* 1993).

C. Studies in Nude and Thymectomized Mice

1. DESCRIPTION OF NUDE MICE AND THYMECTOMIZED MICE

Mice homozygous for the mutation *nude* have a malformed and dysfunctional thymus, which abrogates normal T-cell development, and thus these mice lack functional T cells (Pantelouris, 1968; Rygaard, 1969). Another type of mice that lacks complete T-cell function can be generated by neonatal or adult thymectomy. Newborn C57BL/KaLw mice are thymectomized at 3 days of age and infected with polyomavirus 24 hr later (Miller *et al.,* 1964). Alternatively, adult A/Sn mice are thymectomized at 4–6 weeks of age and 3 weeks later the mice are treated with cytosine-b-D-arabinofuranoside (Ara-C) and total body irradiation (TBI) prior to polyomavirus infection (Steel *et al.,* 1978).

2. POLYOMAVIRUS INFECTION AND PERSISTENCE IN NEWBORN NUDE MICE

Nude Balb/c mice infected as newborns show high levels of viral replication in all organs from 7 days p.i. and on as demonstrated by Southern blot-

ting and *in situ* hybridization (Wirth *et al.,* 1992). These mice succumb to a systemic infection by 7 weeks p.i. during which time period tumor development is not observed (Wirth *et al.,* 1992).

3. POLYOMAVIRUS INFECTION AND PERSISTENCE IN ADULT NUDE MICE

Nude Balb/c mice infected as adults harbor replicating polyomavirus in bone, mammary gland, spleen, salivary glands, lymph nodes, and skin during the systemic phase of infection, as shown by *in situ* hybridization and Southern blot (Demengeot *et al.,* 1990; Wirth *et al.,* 1992). Viral replication peaks 7–10 days p.i., thereafter the level of virus specific signals decreases, and by 5–10 weeks p.i. viral DNA can be detected most often in bone, skin, mammary glands, spleen, salivary glands, and lymph nodes as summarized in Table II (Wirth *et al.,* 1992). The tissues that are positive for polyomavirus shortly after infection are also the organs where later on a persistent infection is established (Demengeot *et al.,* 1990; Wirth *et al.,* 1992). Similar to normal adult mice, systemic viremia is not observed in nude adult mice and the kidneys, lungs, and liver are polyomavirus DNA negative as shown in Table II (Demengeot *et al.,* 1990; Wirth *et al.,* 1992).

4. POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT IN ADULT INFECTED NUDE MICE

Polyomavirus induces a wide variety of tumors in adult infected nude Balb/c and C3H mice observed (Vandeputte *et al.,* 1974; Demengeot *et al.,* 1990; Wirth *et al.,* 1992, 1997). Tumors of the bone, mammary gland, skin, kidneys, and thymus (osteosarcoma, adenocarcinoma, spindle cell carcinoma, hemangioma, and thymic epithelioma) are observed (Vandeputte *et al.,* 1974; Demengeot *et al.,* 1990; Wirth *et al.,* 1992, 1997). The latency period from infection until tumor formation is around 3–5 months; however, the tumor incidence varies in different mouse strains and depending on the age of the mice at infection (Vandeputte *et al.,* 1974; Demengeot *et al.,* 1990; Wirth *et al.,* 1992, 1997). In 3- to 6-week-old polyomavirus infected C3H nude mice, a 100% tumor incidence is observed, while in 10-week-old and 15-week-old polyomavirus infected C3H nude mice, the tumor incidence drops to 80% and 20%, respectively (Vandeputte *et al.,* 1974). Balb/c nude mice infected at 6 weeks of age exhibit a persistent infection (100%) and an overall tumor incidence of 30–60% as shown in Table II (Demengeot *et al.,* 1990; Wirth *et al.,* 1992). Note, however, that mammary adenocarcinomas are observed at a very high frequency (90%) in female Balb/c nude mice (Demengeot *et al.,* 1990).

5. POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT IN NEWBORN THYMECTOMIZED MICE

In the past extensive studies have been performed in newborn thymectomized mice with regard to tumor development. Nevertheless, polyomavirus persistence was not followed in these studies, most likely due to the fact that relevant methods had not been developed at the time. Thymectomy at 3 days of age followed by polyomavirus infection within 24 hr increases the susceptibility to polyomavirus-induced tumor development, as compared to normal newborn infected mice (Malmgren *et al.,* 1964; Miller *et al.,* 1964; Law and Ting, 1965; Law, 1965; Allison and Taylor, 1967). The effect of thymectomy is observed in all mouse strains studied and the frequency of polyomavirus-induced tumors is 73–90% in C57BL/6, 84% in C3H/Bi, and 30% in C3H/Law mice after a latency period of 2–9 months (Miller *et al.,* 1964; Law and Ting, 1965; Allison and Taylor, 1967). The primary targets of polyoma oncogenesis are the salivary glands (Miller *et al.,* 1964; Law and Ting, 1965; Allison and Taylor, 1967). Interestingly, similar to nude mice, the frequency of polyoma tumors is reduced as the time of infection is delayed after neonatal thymectomy (Law and Ting, 1965; Allison and Taylor, 1967). A combination of thymectomy and treatment with antilymphocytic serum (ALS) results in an elevated tumor frequency after polyomavirus infection in thymectomized mice (Vandeputte, 1968; Sjögren and Borum, 1971).

6. POLYOMAVIRUS INFECTION AND PERSISTENCE IN ADULT THYMECTOMIZED MICE

Polyomavirus persistence has been shown in normal adult A/Sn mice immunosuppressed by thymectomy (THX), Ara-C treatment, and total body irradiated (TBI) and infected with polyomavirus 3–4 weeks after thymectomy (Table II) (Berke *et al.,* 1995). During the peak of infection 1–2 weeks p.i. bone, heart, gonads, skin, liver, and lymph nodes are most frequently PCR positive for polyomavirus DNA (Berke *et al.,* 1995). Persistent polyomavirus DNA is still detected in all mice in most organs tested by PCR 1–2 months p.i. (Table II) (Berke *et al.,* 1995). However, polyomavirus-induced tumor development was not studied in these mice.

D. Studies in Mice with Specific T-Cell Deficiencies

1. DESCRIPTION OF $CD4^{-/-}$, or $CD8^{-/-}$ SINGLE-KNOCKOUT AND $CD4^{-/-}8^{-/-}$ DOUBLE-KNOCKOUT MICE

$CD4^{-/-}$ single-knockout C57BL/6 mice lack functional $CD4^{+}$ receptors, which results in a greatly reduced T-helper function (Rahemtulla *et al.,* 1991).

However, development of $CD8^+$ T cells is normal in these mice and virus specific cytotoxicity against lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (VV) is functional (Rahemtulla *et al.,* 1991). $CD8^{-/-}$ single-knockout C57BL/6 mice lack $CD8^+$ receptors and as a consequence thereof lack functional $CD8^+$ T cells (Fung-Leung *et al.,* 1991). MHC class I restricted cytotoxic response against alloantigens and viral antigens is not detected in these mice (Fung-Leung *et al.,* 1991). Nonetheless, $CD4^+$ T cells develop and function normally and respond to MHC class II alloantigens, as detected by proliferation tests (Fung-Leung *et al.,* 1991). $CD4^{-/-}8^{-/-}$ double-knockout C57BL/6 mice lack both $CD4^+$ and $CD8^+$ receptors and as a consequence thereof are unable to mount a T-helper or cytotoxic response against virus infected cells (Schilham *et al.,* 1993). Interestingly, in contrast to $CD8^{-/-}$ mice, recognition of alloantigens is observed, which is due to $CD4^{-/-}8^{-/-}$ double negative ab T cells (Schilham *et al.,* 1993).

2. POLYOMAVIRUS INFECTION AND PERSISTENCE IN NEWBORN INFECTED $CD4^{-/-}$ and $CD8^{-/-}$ SINGLE-KNOCKOUT AND $CD4^{-/-}8^{-/-}$ DOUBLE-KNOCKOUT MICE

$CD4^{-/-}$ and $CD8^{-/-}$ single-knockout Balb/c and C57BL/6 mice infected with polyomavirus at the age of 1–7 days harbor persistent polyoma DNA in the kidneys, bone, heart, skin, stomach, liver, and lymph node 2–6 months p.i., as detected by PCR (Table I) (Berke *et al.,* 1996a). Similar to that observed in normal newborn infected C57BL/6 mice and Balb/C mice, the organ distribution of polyomavirus becomes limited with time and persistent polyomavirus DNA is detected only in some of the organs of individual mice (Berke *et al.,* 1996a; Wirth *et al.,* 1992, 1997; Heidari, personal communication). The incidence of a persistent infection is around 11% in $CD8^{-/-}$ (C57BL/6 and Balb/C) mice and 28% in $CD4^{-/-}$ (C57BL/6 and Balb/C) mice as summarized in Table I (Berke *et al.,* 1996a; Heidari, personal communication).

$CD4^{-/-}8^{-/-}$ double-knockout C57BL/6 mice infected with polyomavirus at 1–7 days of age harbor persistent polyoma DNA in almost all organs tested 1–6 months p.i. (Table I) (Berke *et al.,* 1996a). Bone and brain are positive in all mice, and polyoma DNA is frequently observed also in the kidneys. One-third of the mice succumb to the infection 2–3 months after infection and half of these mice have signs of hind leg paralysis, which could be due to a persistent polyoma infection in the brain (Berke *et al.,* 1996a). The incidence of a persistent polyomavirus infection in the surviving mice is 41% (Table I) (Berke *et al.,* 1996a). In summary, when newborn $CD4^{-/-}8^{-/-}$ double-knockout mice are compared to newborn $CD4^{-/-}$ or $CD8^{-/-}$ single-knockout and normal mice they exhibit an increased frequency of polyomavirus persistence as well as a disseminated organ distribution of viral DNA (Table I) (Berke *et al.,* 1996a).

3. POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT IN NEWBORN INFECTED CD4$^{-/-}$ AND CD8$^{-/-}$ SINGLE-KNOCKOUT AND CD4$^{-/-}$8$^{-/-}$ DOUBLE-KNOCKOUT MICE

Polyomavirus induces a variety of tumors in CD4$^{-/-}$, CD8$^{-/-}$, and CD4$^{-/-}$8$^{-/-}$ knockout mice. The tumors observed are of the subcutaneous tissue, bone, salivary glands, and of the peritoneum (adenocarcinoma, fibrosarcoma, osteosarcoma) (Berke *et al.,* 1996a). The latency period from infection until tumor formation is 2–4 months in CD4$^{-/-}$8$^{-/-}$ mice and 3–6 months in CD4$^{-/-}$ and CD8$^{-/-}$ single-knockout mice (Berke *et al.,* 1996a). Tumors develop in 0–3% of CD4$^{-/-}$ C57BL/6 and Balb/c mice, and 5–28% of CD8$^{-/-}$ C57BL/6 and Balb/c mice and 29% of CD4$^{-/-}$8$^{-/-}$ C57BL/6 mice as summarized in Table I. Furthermore, as in polyomavirus infected newborn nude mice, a high rate of early deaths is also observed among the CD4$^{-/-}$8$^{-/-}$ mice. Of the infected mice without tumors, one-third died by 3 months p.i., as mentioned above, which should also be taken into account when comparing tumor incidence with other groups of mice (Table I) (Berke *et al.,* 1996a).

4. POLYOMAVIRUS INFECTION IN ADULT INFECTED CD4$^{-/-}$ AND CD8$^{-/-}$ SINGLE-KNOCKOUT MICE AND POLYOMAVIRUS PERSISTENCE IN CD4$^{-/-}$8$^{-/-}$ DOUBLE-KNOCKOUT MICE

After infection of adult CD4$^{-/-}$ and CD8$^{-/-}$ single-knockout Balb/c and C57BL/6 mice the organ distribution of polyomavirus DNA is limited and virus DNA is generally cleared by 1 month p.i. (Tables II and IIIB). Polyomavirus DNA is detected during the peak of infection in bone and heart in several, but not all animals and occasionally in stomach, gonads, spleen, parotid, and skin as shown in Table IIIB (Berke *et al.,* 1995). Furthermore, similar to that observed in normal adult mice virus DNA is never detected in the kidneys or lungs (Berke *et al.,* 1995). Persistent polyoma DNA is only rarely (1/20 mice) detected later than 1 month p.i. (Table IIIB) (Berke *et al.,* 1995). In summary, infection of mice deficient in one T-cell population (CD4$^{-/-}$ or CD8$^{-/-}$ single-knockout mice) resembles that observed in normal, immunocompetent adult mice (compare Tables II, IIIA, and IIIB) (Berke *et al.,* 1995).

Polyomavirus infection in adult CD4$^{-/-}$8$^{-/-}$ double-knockout C57BL/6 mice causes an extensive viral spread (Berke *et al.,* 1995). At the peak of infection, 1–3 weeks p.i., bone, heart, and skin are most frequently polyoma DNA positive, but most organs can be virus DNA positive at some time point (Table IIIC) (Berke *et al.,* 1995). The extensive spread of the virus and the positive viral signals in all organs can be due to viremia, since virus is de-

Table IIIB. Detection of Polyomavirus DNA in Organs of Normal $CD4^{-/-}$ and $CD8^{-/-}$ Single-Knockout C57BL/6 and Balb/c Adult Mice

	No. of polyomavirus DNA-positive mice by PCR at indicated time after infection		
Organ	**2–5 days[a]**	**1–4 weeks[b]**	**>1 month[c]**
Bone	0	6	1
Heart	0	6	0
Stomach	0	2	0
Gonads	0	2	0
Lung	0	0	0
Parotid	0	1	0
Skin	0	1	0
Brain	0	0	0
Thymus	0	0	0
Liver	0	0	0
Spleen	2	3	0
Kidney	0	0	0
Lymph nodes	1	0	0

[a]8 mice were tested. [b]20 mice were tested. [c]10 mice were tested.

Table IIIC. Detection of Polyomavirus DNA in Organs of Normal $CD4^{-/-}CD8^{-/-}$ Double-Knockout C57BL/6 Adult Mice

	No. of polyomavirus DNA-positive mice by PCR at indicated time after infection		
Organ	**2–5 days[a]**	**1–4 weeks[b]**	**>1 month[c]**
Bone	1	6	4
Heart	0	5	1
Stomach	0	1	0
Gonads	1	3	0
Lung	0	1	0
Parotid	0	1	0
Skin	2	5	2
Brain	0	0	0
Thymus	0	1	1
Liver	0	2	0
Spleen	2	3	0
Kidney	0	1	0
Lymph nodes	0	4	2
Blood	0	3	1

[a]8 mice were tested. [b]20 mice were tested. [c]10 mice were tested.

tected also in the blood during the observation period (Berke *et al.,* 1995). One to 2 months p.i., polyomavirus DNA still persists in bone, heart, skin, thymus, and lymph nodes, but the dissemination of virus DNA is not as extensive as during the first 4 weeks of infection as shown in Table IIIC and reported by Berke *et al.* (1995). Several months p.i. polyomavirus still persists in 75% of the tested mice (Table II) (Berke *et al.,* 1995).

5. POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT OBSERVED IN ADULT INFECTED $CD4^{-/-}8^{-/-}$ DOUBLE-KNOCKOUT MICE BUT NOT IN ADULT INFECTED $CD4^{-/-}$ AND $CD8^{-/-}$ SINGLE-KNOCKOUT MICE

Tumors of the subcutaneous tissue, thymus, and bone are observed in more than half of the adult infected $CD4^{-/-}8^{-/-}$ double-knockout C57BL/6 mice after a latency period of 4–6 months (Table II) (Z. Berke *et al.,* unpublished). As expected, polyomavirus-induced tumors do not develop in adult infected $CD4^{-/-}$ and $CD8^{-/-}$ single-knockout Balb/c or C57BL/6 mice, where the virus does not persist (Table II) (Berke and Heidari, unpublished).

E. Studies in Mice with Specific B-Cell Deficiencies

1. DESCRIPTION OF XID AND $IgM^{-/-}$ SINGLE-KNOCKOUT MICE

X-linked immunodeficiency (XID) CBA mice carry a missense mutation in the Bruton's agammaglobulinemia tyrosine kinase (btk) gene (Scher, 1982). XID mice have a decreased number of B cells and are unable to elicit a proliferative response upon surface Ig receptor cross-linking (Scher, 1982; Rawlings *et al.,* 1993). The immune response to protein antigens is unaffected, but the mice are unable to respond to polysaccharide antigens (Scher, 1982). $IgM^{-/-}$ single-knockout C57BL/6 mice have a disruption of one exon in the m-chain constant region gene (Kitamura *et al.,* 1991). The mice lack functional B cells, since B-cell differentiation is blocked at the pre-B-cell stage.

2. POLYOMAVIRUS PERSISTENCE IN NEWBORN INFECTED XID AND IgM$^{-/-}$ KNOCKOUT MICE

Polyomavirus infection leads to a disseminated virus infection and virus persistence in both newborn infected XID CBA and IgM$^{-/-}$ C57BL/6 mice (Heidari *et al.,* 1999). Polyomavirus DNA is detected in almost all mice and in all organs examined with PCR throughout the observation period of 2–10 months (Heidari *et al.,* 1999). Virus is also detected in the blood, suggesting viremia. Data available 1–6 months p.i. are summarized in Table I.

3. POLYOMAVIRUS-INDUCED TUMOR DEVELOPMENT IN NEWBORN INFECTED XID CBA AND IgM$^{-/-}$ KNOCKOUT C57BL/6 MICE

The incidence of tumor development in newborn infected XID CBA mice is around 35%, which is similar to the 29% observed in normal newborn infected CBA mice (Table I) (Heidari *et al.,* 2000). Similar to newborn infected normal C57BL/6 mice newborn infected IgM$^{-/-}$ C57BL/6 mice are resistant to polyomavirus-induced tumor development, although all IgM$^{-/-}$ mice harbor persistent polyomavirus DNA 1–6 months p.i. (Table I) (Heidari *et al.,* 2000).

4. POLYOMAVIRUS INFECTION AND PERSISTENCE IN ADULT INFECTED XID AND IgM$^{-/-}$ MICE

Polyomavirus infection of adult XID mice is generally limited with regard to the organ distribution of polyomavirus DNA. Between 4 days and 4 weeks p.i. the animals examined show detectable levels of polyoma DNA in some organs, most frequently in bone and heart and occasionally in skin, gonads, lymph nodes, and spleen as summarized in Table IIID (Berke *et al.,* 1998). However, 6–12 weeks p.i., around 30–50% of the mice are still polyoma DNA positive in heart, skin, lymph nodes, parotid, and brain (Table II) (Berke *et al.,* 1998; Heidari *et al.,* 1999). An extensive dissemination of polyomavirus is observed in polyomavirus infected adult IgM$^{-/-}$ single-knockout mice (Berke *et al.,* 1998). Virus DNA can be detected in most organs from 1 week p.i. and on and the positive signals do not decrease with time (Berke *et al.,* 1998). Virus DNA is still detected in most tested organs in 100% of the animals 2–6 months p.i., which indicates the establishment of an extensive, long-term polyomavirus persistence (Table II) (Berke *et al.,* 1998).

Polyomavirus-induced tumor development is not observed in any of the adult infected XID or IgM$^{-/-}$ mice around 6 months p.i., although all adult

Table IIID. Detection of Polyomavirus DNA in Organs of XID CBA Adult Mice

Organ	No. of polyomavirus DNA-positive mice by PCR at indicated time after infection		
	2–5 days[a]	1–4 weeks[b]	>1 month[c]
Bone	2	3	0
Heart	2	6	2
Stomach	0	0	0
Gonads	1	1	0
Lung	0	0	0
Parotid	0	0	1
Skin	1	1	1
Brain	0	0	1
Thymus	0	0	0
Liver	0	0	0
Spleen	1	0	0
Kidney	0	0	0
Lymph nodes	0	1	1
Blood	0	0	1

[a] 8 mice were tested.
[b] 20 mice were tested.
[c] 10 mice were tested.

$IgM^{-/-}$ mice and one-third to one-half of the XID mice are still polyomavirus DNA positive (Table II) (Heidari *et al.*, 2000).

F. Studies in Mice with Combined T- and B-Cell Deficiencies

1. DESCRIPTION OF $IgM^{-/-}$ $CD8^{-/-}$ DOUBLE-KNOCKOUT AND SCID MICE

$IgM^{-/-}$ and $CD8^{-/-}$ single-knockout mice were crossed and $IgM^{-/-}$ $CD8^{-/-}$ double-knockout C57BL/6 mice were bred at the animal facilities at the Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm. These mice lack CD8 receptors and have a disruption of one of the exons in the gene encoding the m-chain constant region gene, thus they do not have functional B cells or $CD8^+$ cytotoxic T cells (Fung-Leung *et al.*, 1991; Kitamura *et al.*, 1991).

Severe combined immune deficiency (SCID) C.B-17 and C57BL/6JSz mice are unable to repair double-stranded DNA breaks (Bosma *et al.*, 1983, 1989). They lack both functional B and T cells, since no functional rearrangement

of the immunoglobulin and T-cell receptor genes can occur. However, antigen presentation in the spleen is normal and functional macrophages and NK cells are present (Bosma *et al.,* 1983).

2. POLYOMAVIRUS INFECTION AND PERSISTENCE IN ADULT $IgM^{-/-}$ $CD8^{-/-}$ DOUBLE-KNOCKOUT MICE

All adult polyomavirus infected $IgM^{-/-}CD8^{-/-}$ double-knockout C57BL/6 mice included in a study by Berke *et al.* (1998) are polyomavirus DNA positive by PCR in several organs from 1 week p.i. and on (Table II). During the 8-week observation period polyoma DNA is detected most frequently in bone, heart, gonads, and skin and occasionally also in other organs at certain time points (Berke *et al.,* 1998). Polyomavirus DNA is also detected in the blood of several mice, indicating viremia (Berke *et al.,* 1998). The observation period was not extended beyond 8 weeks because the animals succumbed to the infection around 6–8 weeks p.i. (Table II) (Berke *et al.,* 1998). No tumor development was observed in any of the mice during this 8-week-long observation period.

3. POLYOMAVIRUS INFECTION AND PERSISTENCE IN ADULT INFECTED SCID MICE

In adult polyomavirus infected SCID C.B.17 mice, viral DNA is detected in all organs of all mice by 1–2 weeks p.i. and throughout the 2-month observation period (Table II) (Berke *et al.,* 1994). Extensive polyomavirus replication is shown in all tested organs from 1 week p.i. and on both in SCID C.B.17 and C57B1/6JSz mice (Berke *et al* ., 1994; Szomolanyi-Tsuda and Welsh, 1996). The mice studied by the latter group also developed acute myeloproliferative disease (AMD) at a 100% frequency at 14–16 days p.i., the time when high levels of viral replication is detected (Szomolanyi-Tsuda and Welsh, 1996). SCID mice succumb to a systemic polyomavirus infection with wasting syndrome 8–10 weeks p.i. (Table II) (Berke *et al.,* 1994; Szomolanyi-Tsuda and Welsh, 1996). Polyomavirus-induced tumor development is not observed, since the mice most likely succumb to the infection before tumor development could occur.

VII. CONCLUDING REMARKS

The present review attempts to summarize recent findings regarding polyomavirus infection *in vivo,* including data on immunologic control of viral persistence and tumor development.

Similar to previous reports the present literature indicates that tumor development is only observed after infection of normal newborn mice or adult immunodeficient mice with T-cell deficiencies (Tables I and II) (Wirth *et al.,* 1992, 1997; Berke and Dalianis, 1993; Berke *et al.,* 1996a). The present explanation for this discrepancy is that the immature immune system of the newborn mouse in contrast to that of the adult mouse cannot prevent virus persistence and subsequential tumor development (Wirth *et al.,* 1992, 1997; Berke and Dalianis, 1993; Berke *et al.,* 1996a). However, persistent infection does not necessarily lead to tumor development (Berke *et al.,* 1994, 1996a; Szomolanyi-Tsuda and Welsh, 1996; Heidari *et al.,* 1999). In fact, mice with very severe combined B- and T-cell immunodeficiencies succumb to polyomavirus infection before possible tumor development can occur (Berke *et al.,* 1994, 1996a; Szomolanyi-Tsuda and Welsh, 1996; Heidari *et al.,* (1999).

Recently however, studies on polyomavirus infection in newborn and adult mice with limited B- or T-cell deficiencies allow us to separate to some extent the immune mechanisms responsible for prevention of virus persistence and tumor development (Wirth *et al.,* 1992, 1997; Berke *et al.,* 1995, 1996a, 1998; Heidari *et al.,* 1999).

In mice with a limited T-cell deficiency, but with functional B cells, such as in adult $CD4^{-/-}$ or $CD8^{-/-}$ single-knockout mice, similar to normal adult mice, polyomavirus does not persist when assayed by PCR (Berke *et al.,* 1995). However, in mice with a full T-cell deficiency, such as in adult nude and $CD4^{-/-}8^{-/-}$ double-knockout mice, persistent polyoma is detected and tumor development is enhanced, also in the presence of functional B cells (Berke *et al.,* 1995; Berke, unpublished data; Wirth *et al.,* 1992).

The role of B cells and antibodies in prevention of polyomavirus persistence and tumor development was recently followed in XID and $IgM^{-/-}$ mice, the former with a more limited and the latter with a more severe B-cell deficiency (Heidari *et al.,* 1999; Scher, 1982; Rawlings *et al.,* 1993; Kitamura *et al.,* 1991). The XID mutation was on the CBA background, a strain susceptible to the induction of tumors by polyomavirus, whereas the $IgM^{-/-}$ mutation was on the more resistant C57BL/6 background (Heidari *et al.,* 1999). Newborn and adult infected XID and $IgM^{-/-}$ mice were found to harbor persistent polyomavirus in most organs (Heidari *et al.,* 1999). Nevertheless, tumors developed only in one-third of the newborn infected XID mice, a frequency close to that observed in newborn infected control CBA mice (Heidari *et al.,* 2000). Furthermore, similar to infected control mice, tumors were not observed in newborn and adult infected $IgM^{-/-}$ mice or adult infected XID mice (Heidari *et al.,* 2000). Taken together these results indicate that the absence of functional B cells does not influence tumor development (Heidari *et al.,* 2000).

Summarizing data from the early 1990s and on, our conclusion is that polyomavirus infection does not lead to persistence in normal adult mice

(Berke and Dalianis, 1993). Furthermore persistence is not always observed in all adult mice with a limited B- or T-cell deficiency such as XID mice or mice that lack only $CD4^+$ or $CD8^+$ T cells (Wirth *et al.,* 1992, 1997; Berke and Dalianis, 1993; Berke *et al.,* 1994, 1995, 1996a, 1998; Heidari *et al.,* 2000). However, B-cell function is definitely important for the prevention of polyomavirus persistence in both adult and newborn mice (Berke *et al.,* 1998; Heidari *et al.,* 2000). Likewise, virus persistence is also observed in adult mice that lack both T-cell arms (Berke *et al.,* 1995).

It is also obvious that persistent polyomavirus infection is necessary for the induction of polyoma tumors as demonstrated in newborn infected mice and in adult infected mice, e.g., nude mice and $CD4^{-/-}8^{-/-}$ mice (Wirth *et al.,* 1992, 1997; Berke and Dalianis, 1993; Berke *et al.,* 1995, 1996a; Berke, unpublished results). However, a persistent infection is not sufficient for the development of polyomavirus-induced tumors as indicated by the studies performed in XID and $IgM^{-/-}$ mice, as long as functional T cells are still present (Heidari *et al.,* 2000). In conclusion, B-cell-deficient mice differ from T-cell-deficient mice in that persistent infection does not significantly enhance the risk of tumor development (Heidari *et al.,* 2000).

VIII. FUTURE PROSPECTS

Polyomavirus, although oncogenic, does not induce tumors in immunocompetent mice that clear polyomavirus infection. Mice with a persistent polyomavirus infection are at risk of developing polyomavirus-induced tumors if their T-cell function is abrogated. Detailed studies on various ways of immunizing or preventing a persistent infection may create new paths of also inhibiting tumor development.

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