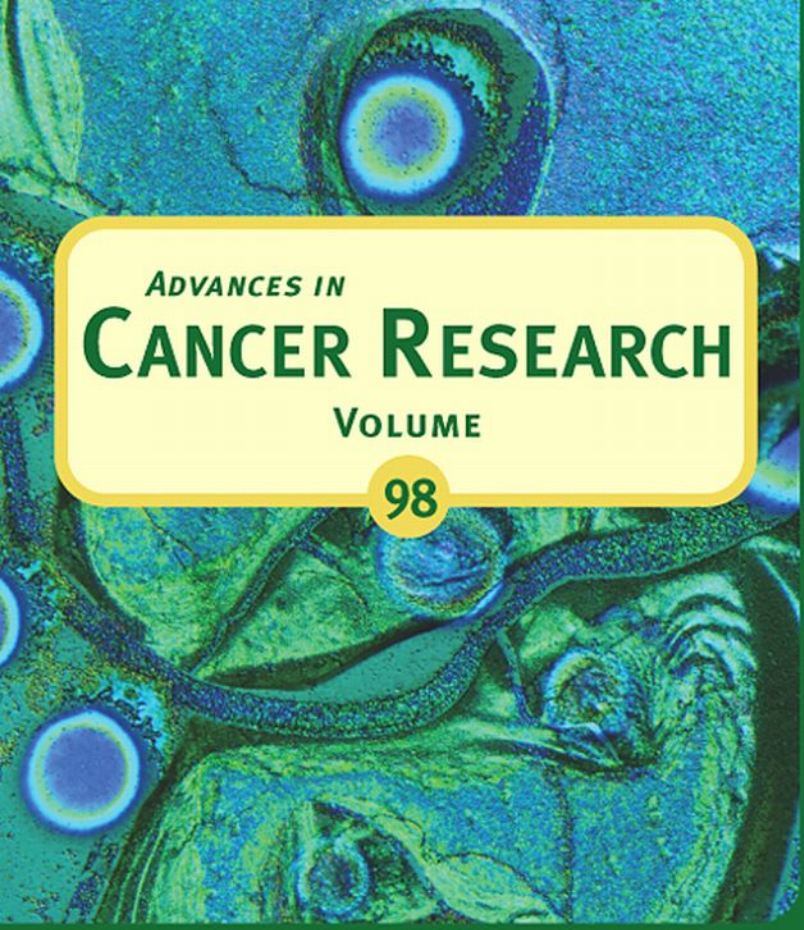

Advances in Cancer Research

Volume 98

Edited by

George F. Vande Woude
George Klein

Advances in CANCER RESEARCH

Volume 98

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Advances in CANCER RESEARCH

Volume 98

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FOUNDATIONS IN CANCER RESEARCH*

Why Do We Not All Die of Cancer at an Early Age?

George Klein, Stefan Imreh, and Eugene R. Zabarovsky

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I. Introduction
II. Immune Surveillance
III. Genetic Surveillance (DNA Repair)
IV. Intracellular Surveillance
V. Is There Epigenetic Surveillance?
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Traditionally, *surveillance* against cancer was thought of as mainly *immunological*. With the exception of tumors with a clear viral involvement, such as immunoblastomas (Epstein–Barr virus, EBV), cervical, anogenital, and skin carcinomas (HPV), and Kaposi's sarcoma (HHV-8) where the immune system is confronted with virally encoded, nonself targets, tumors with no viral involvement provide poor targets. Attempts to influence them by immunological means are akin to the breaking of tolerance. Robust *nonimmunological* surveillance mechanisms include *DNA repair*-based checkpoint functions, and the triggering of growth arrest and/or apoptosis pathways by DNA damage or by illegitimate oncogene activation (*intracellular surveillance*). There is emerging evidence for *epigenetic surveillance*, reflected in the stringency of imprinting. A fourth mechanism, *intercellular surveillance*, or microenvironmental control, is rapidly gaining momentum. It can be mediated by contactual controls or by differentiation-inducing signals. Somatic hybridization experiments have shown that *tumorigenicity is usually suppressed* in somatic hybrids between normal and malignant cells, as long as a fairly complete chromosome complement is maintained. Individual normal cell-derived chromosomes may have a similar suppressive effect. For example, genetic and molecular dissection of human 3p that shows frequent deletions in many human tumors has identified multiple tumor suppressor

*Drs. Klein and Vande Woude initiated the "Foundations in Cancer Research Series" in 1993 to provide a collection of views and perspectives of the extraordinary and historical developments in cancer research that will serve as an important resource for future generations.

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genes, which can inhibit both *in vitro* growth and *in vivo* tumorigenicity. In addition, five genes were found with an "asymmetric activity," capable of suppressing tumorigenicity, without affecting *in vitro* growth. These genes, *LTF*, *L1MD1*, *HYAL1*, *HYAL2*, and *VHL*, are of particular interest because they may be involved in microenvironmental control.

I. INTRODUCTION

Vitally important functions that can be exemplified by the protection of the mammalian fetus from rejection are safeguarded by multiple mechanisms. This is also true for protection against tumor development. The vulnerability of our somatic cells to potentially carcinogenic noxae and the plethora of genetic and epigenetic changes that can favor the microevolution of a cell clone toward malignancy would make us highly cancer prone in the absence of multiple defense systems.

Traditionally, protection against cancer has been mainly, if not exclusively, ascribed to the immune system. The basic idea has been already expressed by Paul Ehrlich in 1909 (Ehrlich, 1909). He wrote that the complicated fetal and postfetal development must generate a large number of "aberrante Keime," translatable to "mutated cells." Were it not for the defense mechanisms of the organism, Ehrlich continued, cancer would arise in an "enormous frequency."

II. IMMUNE SURVEILLANCE

The hypothesis of immune surveillance was formulated by two prominent immunologists, Lewis Thomas in 1959 (Thomas, 1959) and Mac Farlane Burnet in 1964 (Burnet, 1971). In Burnet's words: "In large long lived animals ... inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent steps toward malignancy. It is an evolutionary necessity that there should be some mechanism for elimination or inactivity of such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character."

The evolutionary necessity of cancer protection is certainly true, but the unique role attributed to the immune system ignores two salient facts: (1) tumor evolution involves the loss rather than the gain of many functions and (2) the cancer cell phenotype is easily malleable. This does not augur well for the immune recognition of tumors as "nonself" targets. Even if adventitious, immunologically recognizable mutations would occur, they may be readily circumvented by further mutations or phenotypic modulation. There is one important exception, however. Oncogenic proteins of DNA tumor viruses, such as SV40, polyoma, papilloma, and Epstein–Barr virus (EBV), are readily recognized by the immune system as nonself. They

are also relatively stable targets since their expression is a prerequisite for the proliferation of the virally transformed cell.

The strongest argument for immune surveillance is provided by the increased incidence of a given tumor in immunodefectives. Three types of human malignancies qualify by this criterion. All of them are virus related: EBV carrying immunoblastoma, papillomavirus-associated cervical, anogenital, and skin cancer, and HHV-8 carrying Kaposi's sarcoma.

The frequency of other tumors does not increase significantly in the immunosuppressed. This is in line with the notion, also supported by research on experimental animals, that spontaneous tumors with no viral involvement are regarded as "self" by the immune system (with skin melanomas as a possible exception). Immunological attempts to influence such tumors are therefore akin to breaking or circumventing tolerance. Current efforts toward that goal include the inhibition of regulatory T cells; the provision of appropriate costimulatory signals; the stimulation of proinflammatory cytokines such as IL-1, IL-6, or IL-12; the activation of dendritic cells, for example, by administering a ligand for a Toll-like receptor; and the attempts to stimulate natural immunity.

If the immune system cannot mount the robust immunity envisaged in the early statements of Ehrlich, Thomas, and Burnet, it is still true that we are strongly protected against cancer development. It is well established that the majority of tumor cells that disseminate during surgery do not give rise to metastasis. This is not necessarily an immune protection, however, as so frequently assumed. The well documented fact that dormant tumor cells can "wake up" years or decades later also speaks against immune killing.

What other mechanisms protect us against cancer? There is evidence for at least *four different types of nonimmune surveillance* against cancer. Two of them, *genetic* (DNA repair based and checkpoint control) and *intracellular* (largely apoptosis related) surveillance, are well established. Evidence for *epigenetic surveillance*, related to chromatin structure and particularly the stringency of imprinting, has only recently started to emerge. A fourth, already quite strong and rapidly increasing area, *intercellular surveillance*, points to the importance of the tumor microenvironment.

III. GENETIC SURVEILLANCE (DNA REPAIR)

Tumor risk is highly influenced by mutations in genes that control the fidelity of DNA replication, the efficacy of DNA repair, and the checkpoint controls of chromosome separation. Mutations in these genes, whether identified as point mutations, microsatellite instability (MSI), or loss of heterozygosis, are referred to as *mutator mutations*.

Xeroderma pigmentosum (XP) is the oldest known case of a specific DNA repair deficiency. It is due to recessive mutation in one of the essential components of the nucleotide excision repair (NER) system, the repairosome. The latter is composed of 30 different proteins, and its main function is to excise thymidine dimers from UV-exposed DNA in the skin epithelium.

XP patients must protect themselves from light all their lives, but they nevertheless develop multiple skin carcinomas. This points to the paramount importance of DNA repair as a first-line surveillance mechanism.

Hereditary nonpolyposis colon cancer (HNPCC) is due to a defect in one of several DNA mismatch repair (MMR) genes. Some of their products can splice out the mismatched region and insert new bases to fill the gap. MMR defects can be manifested as MSI and are associated with multiple cancers. *MLH1* is one of the frequently involved genes. *MLH1* mutation in the hereditary cases and epigenetic silencing by dense hypermethylation of the 5′ promoter region in sporadic cases can lead to the same MSI phenotype.

These and other examples have identified DNA repair as a robust protection mechanism against cancer.

IV. INTRACELLULAR SURVEILLANCE

Growth arrest and/or *programmed cell death* are best known. The former may end in apoptosis or other types of cell death. Apoptosis is the endpoint of multipathway, multistep programs that lead to the enzymatic breakdown of cellular DNA. It can be initiated either through the extrinsic death receptor-ligand or the intrinsic mitochondrial pathway (for a review see Klein, 2004). Most known programs converge toward the activation of caspases that cleave cellular substrates, leading to characteristic biochemical and morphological changes. There are also caspase-independent pathways of apoptosis, however. The recently described DNA damage response, capable of acting in very early precancerous lesions, is another case in point (reviewed in Höglund, 2006).

Many physiological growth control mechanisms that govern cell proliferation and tissue homeostasis are linked to apoptosis. It is therefore logical that a relative resistance of tumor cells to apoptosis is an essential feature of cancer cell development.

The cell death machinery can be disabled in cancer cells at many different levels. Examples include the overexpression of apoptosis inhibitors (e.g., *BCL2*), inactivating mutations of executioner caspases, downregulation and mutation of proapoptotic genes (like *BAX*, *APAF1*, *CD95*), alterations of the *PI3K/AKT* pathway, and others (Green and Evan, 2002; Hunt and Evan, 2001; Igney and Krammer, 2005; Lavrik *et al*., 2005; Viktorsson *et al*., 2005).

Similar to other tumor related genetic changes, like oncogene activation and tumor suppressor inactivation, many different genetic and epigenetic changes can bring about the same or similar phenotypic effects. Moreover, each pathway can be affected at different alternative levels.

Oncogenes and apoptosis. Proapoptotic oncogenes include *MYC, RAS, E2F1,* and *E1A*. They upregulate APAF-1 and procaspase 9, components of the apoptosome, associated with the mitochondrial pathway. A rise in the APAF-1 level increases the sensitivity of apoptosome activation. Oncoproteins activate the apoptosome at the point of holocytochrome C release due to mitochondrial outer membrane permeabilization. This is a sudden process that involves all mitochondria.

Integrin receptors. Proper attachment to the extracellular matrix, and especially to basal membrane, is mediated by integrin receptors. It allows the cells to gain proper polarity, assemble their cytoskeleton, and resist proapoptotic stimuli, like those mediated by TRAIL, Fas, and TNFα. The antiapoptotic state is correlated with cellular resistance to chemotherapeutic agents.

Integrin signaling molecules that promote cell survival are focal adhesion kinase (FAK), Shc, and ILK. Each of them may impinge on the AKT/PI3K pathway. Inactivation of the tumor suppressor gene *PTEN* constitutively activates both *ILK* and *AKT*.

Death by neglect. Normal cells require survival signals. Lack of such signals triggers apoptosis. Survival signals include growth factors, cytokines, hormones, and other stimuli. Some of them are mediated by adhesion molecules. They are transduced by the PI3K/AKT pathway.

Anoikis is a special case of "death by neglect." It is triggered by inadequate or inappropriate cell–matrix contacts. Anoikis maintains the correct cell number in epithelial tissues. The breakdown of anoikis contributes to neoplasia. It conveys selective advantage on precancerous epithelial cells. Resistance to anoikis may facilitate metastasis by allowing cells to survive following detachment from the matrix.

Attempts to decrease the apoptotic threshold of cancer cells. All cancer cells have an increased apoptotic threshold. Relative resistance against apoptosis is an equally essential part of the neoplastic evolution as the dysregulation of the cell cycle. Increase of the apoptotic threshold can occur in several steps and does not seem to reach an endpoint. Resistance to apoptosis is always relative. Even though tumors are more resistant than normal cells, many tumors can be cured by X-irradiation or by cytotoxic drugs that act by triggering residual apoptotic pathways. Treatment sensitive tumors may become resistant by a further rise of the apoptotic threshold. A completely apoptosis-resistant cell is not known.

In view of the multiple apoptotic pathways and the different levels where apoptosis can be triggered within each pathway, it may be asked whether

there is a hierarchy between the different pathways. According to current consensus, inactivation events may occur in a stochastic fashion and the apparent choice between different inactivation pathways is due to selection, depending on the cell type. A certain hierarchy among the pathways cannot be excluded, however, and it appears that inactivation of the Rb and p53 pathways could be a universal rule in neoplasia.

V. IS THERE EPIGENETIC SURVEILLANCE?

Recent findings speak for an affirmative answer. The normally inactivated maternal allele of the *IGF2* gene showed loss of imprinting (LOI) in about 10% of the normal human population (Cui *et al.*, 2003). This LOI was associated with a 3.5- to 5-fold increase in the risk of colorectal adenoma development. This surprising finding has been corroborated in a mouse model system (Sakatani *et al.*, 2005). Hybrid mice were generated by crossing two genetically engineered mouse strains. The females used for the cross were heterozygous for a deleted differentially methylated region (DMR). Inheritance of this deletion from the mother leads to the biallelic expression of *IGF2*—corresponding to LOI. The males entering the hybrid cross were of the Min strain that carries a mutation in the adenomatous polyposis coli (*APC*) gene. *APC* mutations provide a strong predisposition to familial colonic polyposis, a precancerous condition in humans and mice.

All hybrids derived from this cross carried the *APC* mutation, but only half of them inherited the imprinting defect. The frequency of intestinal adenomas was twice as high in the mice with the imprinting defect as in their littermate controls. Also, their intestinal crypts were longer and showed increased staining for proteins characteristic of intestinal-cell progenitors. Differentiation of the crypt cells to more specialized intestinal cell types was delayed.

These observations show that the impairment of normal parental imprinting may interfere with cellular differentiation and thereby increase the probability of cancerous development. Taken together with the data in the earlier paper (Cui *et al.*, 2003)—which showed person-to-person differences in the epigenetic silencing of *IGF2* that were related to a predisposition to colon tumors—the findings indicate that cancer susceptibility may be influenced by differences in the stringency of epigenetic control. This is consistent with earlier work showing that inbred mouse strains differ in the activity of enzymes involved in DNA methylation (Paz *et al.*, 2002).

In more general terms, the findings also show that mutation of a "cancer gene" (*APC*) and an epigenetically imposed delay of cell differentiation may drive tumor development synergistically. This inference is reminiscent of the

early concept of two-phase carcinogenesis, by "initiation" and "promotion," based on the finding that subliminal doses of initiating carcinogens that caused no tumors by themselves, became carcinogenic, following the application of tumor promoters, for example phorbol esters, that were noncarcinogenic by themselves (Berenblum, 1962).

Initiation may correspond to mutation or epigenetic change, whereas promotion probably occurs through epigenetic modification only. Impairment of differentiation was shown to be an essential part of tumor promotion.

Jaenisch and his coworkers have shown that the global LOI can lead to widespread tumorigenesis in adult mice (Holm *et al*., 2005). They generated imprint-free mouse embryonic stem cells (IF-ES cells) by transient demethylation. Embryonic fibroblasts derived from the IF-ES cells showed reduced p19 and p53 expression, as well as TGFβ resistance. They were immortalized and tumorigenic in SCID mice. They could also cooperate with H-ras in cellular transformation. Chimeric animals derived from the IF-ES cells developed multiple tumors, arising from the injected IF-ES cells. These findings directly proved that LOI can predispose cells to tumorigenesis. They also imply that imprinting plays an important role in tissue homeostasis by providing an essential tumor suppressor function in the adult.

VI. INTERCELLULAR SURVEILLANCE

Intercellular surveillance can also be referred to as *microenvironmental control*. A wide variety of observations fall into this category. The earliest experimental reports are based on the contactual interaction between tumor and normal cells. Authors like Michael Stoker, Leo Sachs, Harry Rubin, John Paul, and others have shown already in the 1960s that admixture of normal to tumor cell suspensions can dramatically decrease the focus or colony-forming efficiency of the tumor cells. Cell contact is required for the effect. Studies have identified some of the junctions involved (for review see Glick and Yuspa, 2005). Adherence junctions play an important role. E-cadherin, a major structural component of the adherence junctions, is downregulated in most epithelial tumors, usually by promoter DNA methylation. Structural constituents of the adherence junctions, like catenins or connexins, are frequently mutated. Reestablishment of cadherin expression by transfection can revert the tumor phenotype.

Other structural components of the tumor cell membrane may be involved in contactual control as well. β-Integrins are often abnormally expressed by tumor cells. Antibody targeting of a rearranged, tumor-associated β-integrin could inhibit tumor growth (Weaver *et al*., 1997).

Notch receptors and their ligands regulate differentiation and proliferation. Their deletion in the basal layer of mouse epidermis leads to epidermal hyperplasia and skin tumor. Notch signaling between normal and preneoplastic cells can contribute to the suppression of the neoplastic phenotype (Glick and Yuspa, 2005).

These and other *contactual controls* between normal and tumor cells may also explain the previously mentioned observation that many disseminated tumor cells never grow into metastatic tumors. In one experimental model (Naumov *et al.*, 2002), it was found that a significant fraction of injected mouse mammary tumor cells of either high or low metastatic potential persisted as solitary nondividing cells in the liver. Reinoculated to new hosts they were fully tumorigenic. Similar dormancy of solitary tumor cells has been observed with melanoma, squamous cell carcinoma, and prostate carcinoma cells. The "awakening" of the dormant tumor cells may be accelerated by disturbing the tissue equilibrium, for example, by phorbol esters.

The effects of the microenvironment on tumorigenicity are not restricted to contactual controls. Certain tumor cells can be *induced to differentiate* and loose tumorigenicity following their exposure to natural or nonnatural signals. The most spectacular experiment in this category is the demonstration by Beatrice Mintz that cells of a highly malignant but diploid teratoma could be "normalized" by implantation into early mouse embryos (Mintz and Illmensee, 1975).

Microenvironmental structure may also exert a profound influence. Tumorigenic two-dimensional (2D) cultures of mammary carcinoma cells could lose their tumorigenicity partly or completely, after they have been built into a 3D acinar structure *in vitro* (Nelson and Bissell, 2005).

The tumor environment can thus influence the propensity of neoplastic cells to proliferate *in vivo* in a number of different ways. Some act by direct contact between tumor and normal cells, while others may act in a more distal, signal-mediated fashion.

If there is microenvironmental control, it must be transmitted through appropriate receptors. Such receptors have not been identified. Our previous and current studies on somatic hybrids, generated by the fusion of malignant and normal cells, may give a first hint.

Somatic hybrid studies. Henry Harris and our group have shown in a series of collaborative studies during the 1970s that somatic hybrids generated by the fusion of normal with malignant cells loose their tumorigenicity, as long as they maintain a nearly full set of chromosomes from both parents. Subsequent to the loss of chromosomes from the normal parent, tumorigenicity may reappear (Harris *et al.*, 1969). Later, it was shown that single chromosomes including chromosome 3, 13, and 17 could suppress tumorigenicity by themselves.

Identification of normal parent-derived chromosomes that were regularly lost when the malignancy of normal tumor hybrids reappeared made it clear

that neither Rb nor p53, nor any other tumor suppressor gene known at the time were involved in the phenomenon.

Asymmetric suppression: in vivo *but not* in vitro. The design of our hybrid experiments involved the selection of permanently growing hybrid lines. Tumor suppressor genes acting by the induction of growth arrest or apoptosis were therefore automatically excluded.

What genes could be responsible for the suppression of tumor growth *in vivo*, without any inhibition of cell growth *in vitro*? There are at least three conceivable categories: genes encoding products required to respond to differentiation-inducing signals *in vivo*, products required for normal cellular responses to microenvironmental controls, and genes whose products inhibit angiogenesis.

Identification of genes on human 3p with tumor suppressor properties in vivo *but not* in vitro. Our tumor suppressor-related work was focused on the short arm of human chromosome 3 (for a review see Imreh *et al*., 2003). It was chosen because of the frequent occurrence of deletions in human tumors that can affect several regions on 3p with the 3p21.3 region as a particularly frequent target. Two different approaches were used.

The "elimination test," developed by Imreh *et al.* (1994), departed from our earlier cell fusion experiments. Instead of hybridizing normal with tumor cells, a single normal human chromosome 3 was introduced into murine or human tumor cells. The resulting "monochromosomal hybrids" were passaged repeatedly in SCID mice. The developing tumors were explanted, and the retention versus elimination of chromosome 3-derived sequences was assessed by FISH and by PCR. Following mouse passage, two commonly eliminated regions, designated CER1 and CER2, and one frequently eliminated region, designated FER, could be identified and their gene content determined (Imreh *et al*., 2003; Fig. 1).

The *lactoferrin* (*LF*) gene, also referred to as lactotransferrin (LTF), located near the centromeric border of the CER1 region, was the first case in point. To test whether the presence and expression of *LF* contributes to CER1 elimination, we have transfected a PAC that contained the entire gene with its own promoter, and an LF-cDNA into mouse fibrosarcoma cells. Fourteen SCID-derived tumors from two independent PACs and two independent cDNA transfectants were analyzed by real-time PCR at the DNA and RNA levels. After SCID tumor passage, LF expression decreased or was eclipsed in all tumors (Yang *et al*., 2003). In 2 tumors chosen for methylation analysis by bisulfite treatment and sequencing, 6 CpG sites out of 14 in the *LTF* promoter have become methylated, in contrast to the original *in vitro* propagated lines, where all CpG sites remained unmethylated (Fig. 2). Frequent epigenetic silencing of the *LF* gene has also been reported to occur in human tumors (Iijima *et al*., 2006). *LF* was also shown to have an antiangiogenic effect (Norrby *et al*., 2001; Shimamura *et al*., 2004).

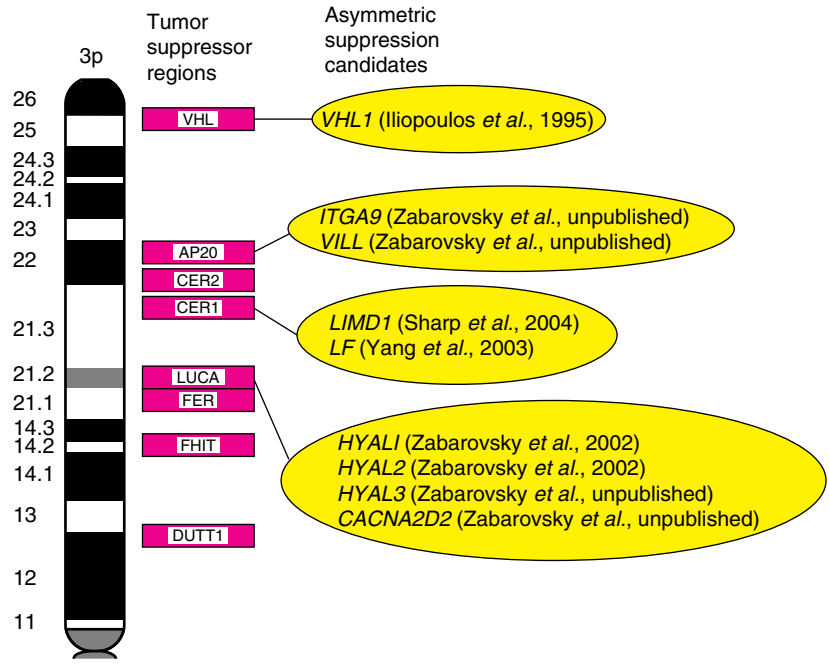

Fig. 1 Tumor suppressor regions on the short arm of chromosome 3 and candidate genes that may suppress tumorigenicity *in vivo* but not *in vitro*. References in parentheses. For megabase details see Imreh *et al.* (2003).

Another tumor suppressor candidate with *in vivo* but not *in vitro* inhibitory activity, *LIMD1* (LIM domain-containing gene 1), was discovered and cloned by us during the mapping and sequencing of CER1 (Kiss *et al.*, 1999). It belongs to the zyxin family of proteins, known to play a role in cell adhesion (Grünewald *et al.*, 2006).

LIMD1–PAC transfectants of a mouse fibrosarcoma line grew equally well *in vitro* as the nontransfectant controls, whereas their growth in SCID mice was inhibited and their ability to grow into metastatic nodules in the lungs after intravenous inoculation was reduced (Sharp *et al.*, 2004).

Zabarovsky *et al.* (2002) focused on two other 3p regions (LUCA and AP20) that are frequently subjected to homozygous deletions in human tumors. A selected set of genes were tested by introducing them within tetracycline-regulatable constructs into human and murine cancer cell lines. Cells carrying the construct were selected and tested for growth *in vitro* and *in vivo*, under conditions when the gene of interest was expressed or silenced.

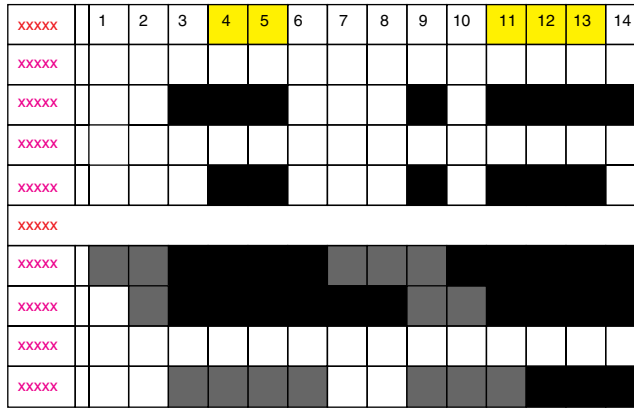

Fig. 2 Promoter methylation in lactoferrin (LF) transfectants and in lung tumor cells. The upper panel shows the methylation pattern of 14 CpG sites in LF promoter in LF–PAC transfectants *in vitro* and derived SCID tumors *in vivo*. White and black boxes indicate unmethylated and 'methylated CpG sites, respectively. Note that no methylation was found in transfectants *in vitro*. Common methylation sites: 4, 5, 9, 11, 12, and 13 in tumors (Yang *et al*., 2003). On the lower panel the same CpG site methylation status was investigated in SCLC line H1299 and NSCLC lines H2009, H1264, and control bronchial epithelial cell line BEAS-2B. White, gray, and black squares represent unmethylated, partially methylated, and completely methylated CpGs, respectively (Iijima *et al*., 2006). CpG sites 4, 5, 11, 12, and 13 overlap in both panels.

Using this approach, we have tested 16 genes from 3p21.3. Eight genes (*TCEA1, MLH1, RHOA, 3PK, PL6, 101F6, BLU, TGFBR*) did not show any effect in the tested cell lines. Six genes (*RBSP3, NPRL2/G21, RASSF1A, RASSF1C, SEMA3F, SEMA3F*) had strong inhibitory activity, both *in vitro* and *in vivo*. Two genes (*HYAL1* and *HYAL2*) showed strong inhibitory activity *in vivo* in SCID mice but not *in vitro*, as judged by colony formation inhibition and growth curve assays. A representative experiment with hyaluronoglucosaminidase 1 (HYAL1) is shown in Fig. 3.

The *HYAL1* gene is located in the LUCA region. It contains three exons coding for a 2.6-kb mRNA (436 aa). Its product is an extracellular protein that is well expressed in all analyzed normal human tissues including lung. It was not expressed in 18 out of 20 lung cancer cell lines (Zabarovsky *et al*., 2002).

The *HYAL2* (located in LUCA region) gene also contains three exons that encode a 2-kb mRNA (473 aa). It is well expressed in all analyzed human tissues including lung and majority of lung cancer cell lines. The protein is attached to the membrane by the glycosylphosphatidylinositol anchor (GPI-anchor).

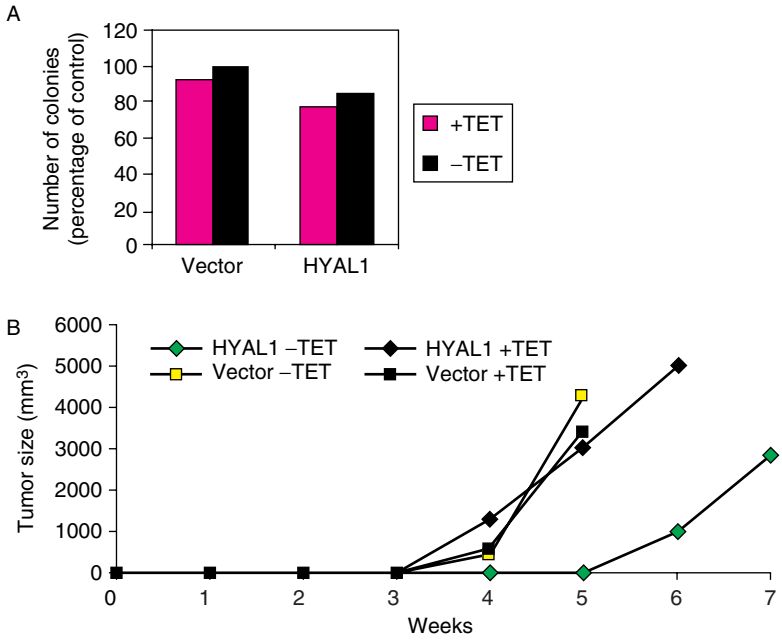

Fig. 3 (A) Colony formation efficiency of KRC/Y cells transfected with HYAL1 in a tetracycline-regulatable construct. No significant difference *in vitro*. (B) Tumor growth inhibition of KRC/Y cells by HYAL1 *in vivo* in SCID mice. Mice were drinking water with tetracycline (+TET, gene is OFF) or without tetracycline (–TET, gene is ON).

Hyaluronan (HA, a negatively charged, high-molecular-weight glycosaminoglycan) is claimed to affect tumor invasion and metastatic spread. The levels of HA in the immediate tumor cell environment is reportedly correlated with tumor aggressiveness and poor prognosis (Zhang *et al.*, 1995). Overproduction of HA enhances anchorage-independent tumor cell growth (Kosaki *et al.*, 1999; Liu *et al.*, 2001). Loss of hyaluronidase activity, permitting accumulation of HA, may be one of several steps required by cells in the multistep process of carcinogenesis (Csoka *et al.*, 2001). No frequent inactivating mutations were identified so far. It was shown, however, that HYAL1 is frequently inactivated in tumors by hypermethylation and illegitimate splicing (Csoka *et al.*, 2001; Frost *et al.*, 2000).

Hyaluronidases are thus known to play an important role for tumor–microenvironmental interactions. They may exemplify genes encoding interactive molecules that participate in microenvironmental growth control.

VHL1 (von Hippel–Lindau gene 1) is another tumor suppressor gene that inhibits tumor growth *in vivo* but not *in vitro*. Recent studies have shown

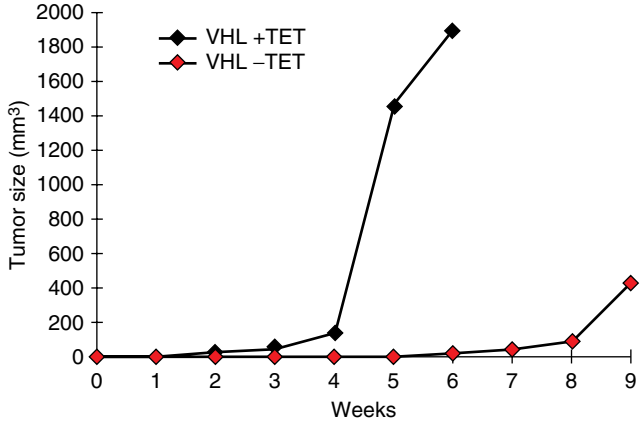

Fig. 4 Tumor growth inhibition of KRC/Y cells by VHL *in vivo* in SCID mice. Mice were drinking water with tetracycline (+TET, gene is OFF) or without tetracycline (–TET, gene is ON).

that pVHL serves as a substrate-recognition component of an E3 ubiquitin ligase complex that targets HIF, a transcription factor for polyubiquitination and subsequent degradation. Accordingly, tumor cells devoid of functional pVHL show an inappropriate accumulation of HIF, as well as downstream HIF-target genes, such as VEGF, a potent angiogenic factor (Maynard and Ohh, 2005).

In our colony formation experiments and growth curve assays, *VHL* had almost no or only a very modest inhibitory effect on the renal cell carcinoma cell line KRC/Y. On the other hand the tetracycline-controlled *VHL* gene significantly suppressed the growth of KRC/Y cells in SCID mice (Fig. 4). Consistent results were reported by Iliopoulos *et al.* (1995).

There may be many other, as yet unidentified, suppressor genes with similar asymmetric inhibitory properties. For instance, the *DEC1* gene (deleted in esophageal squamous cell carcinoma, ESCC) mapped to 9q32-q34 could also belong to this category. It strongly inhibited the tumorigenicity of the ESCC cell line in nude mice but had little inhibitory activity *in vitro* (Yang *et al.*, 2005).

A large number of LOHs that occur in the major human tumors and are not accounted for by known tumor suppressor genes, as well as the existence of "tumor suppressor gene clusters," as documented on human 3p (reviewed in Imreh *et al.*, 2003), raise the question of possible coregulation and point to a fertile area of investigation ahead.

VII. SUMMARY

A large number of genes are now known to influence cancer development, following their illegitimate activation or inactivation by genetic or epigenetic mechanisms. At least five types of surveillance functions have evolved to counteract the probability of cancer development. They can be classified as follows:

Immunological (mainly directed against virally induced tumors), *genetic* (DNA repair, checkpoint functions), *epigenetic* (imprinting, chromatin structure), *intracellular* (programmed cell death), and *intercellular* (microenvironmental). They reduce the otherwise high probability of cancer development. Their collective impact is a vastly magnified version of the multiple surveillance of the harbor of Cartagena, Colombia, against pirates. They relegate cancer development to a rare, low-probability event. Strengthening of the protective forces is desirable at all levels.

ACKNOWLEDGMENTS

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FOUNDATIONS IN CANCER RESEARCH*

The Early History of Plasma Cell Tumors in Mice, 1954–1976†

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I. Introduction
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XVII. Growing PCTs in Culture and Growth Factors
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Plasma cell tumors (PCTs) in mice became available at an exciting period in immunology when many scientists and laboratories were occupied with how to explain the genetic basis of antibody diversity as well as antibody structure itself. An unlimited source of PCTs in an inbred strain of mice became a useful adjunct in these efforts. A PCT was a greatly expanded monoclone and a source of a single molecular species of immunoglobulin (Ig) molecule. The PCTs provided not only the components of the Ig-producing cell but also potentially functional secreted products. Many of the monoclonal Igs produced by PCTs in the mouse and others found in humans were found to

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have specific antigen-binding activities. These became the prototypes of monoclonal antibodies. This chapter describes the origins of PCTs in mice and attempts to recapture some of the ambience of the day albeit from personal recollection. The great discovery of the hybridoma technology by Cesar Milstein and Georges Kohler in 1975 began a new direction in immunology.

I. INTRODUCTION

In the 1950s, a ferment of new theoretical ideas on the cellular and genetic basis of antibody formation electrified the field of immunology and began a new age in experimentation. Two problems dominated the thoughts of scientists during most of this decade—the cellular basis of antibody formation (how did it work?) and the genetic basis of antibody diversity (how could the genes in one individual generate antibodies for the thousands and thousands of antigens?). The period from 1954 to 1976 was one of the most exciting in the history of biology when an explosion of new information unfolded from many fields. This was paced by spectacular technological advances. This was the period when molecular biology emerged and the DNA-gene-messenger RNA–translated protein relationship was clarified, when proteins were first sequenced (Sanger, 1959), which led to the deciphering of the genetic code. New insights in the cell biology and genetics of tissue transplantation had a deep impact in the study of hematopoiesis. The use of inbred mice in immunological research greatly expanded. All of these developments in different fields supplied new ideas to each other. The pace of research was remarkable. One bridge between fields such as immunology, cancer research, protein chemistry, and molecular biology was the plasma cell, the antibody-secreting cell and its neoplastic derivative, the plasma cell tumor (PCT).

II. PLASMA CELLS BEFORE THE 1950s: DISCOVERY, UNCERTAIN ORIGINS

Plasma cells were first described in 1890 by the famous histologist Ramon y Cajal who found them in syphilitic condylomas (Cajal, 1906). Since they were unlike any other leukocyte, he thought they might be special embryonic cells [see Michels (1931) for an excellent review of the early plasma cell literature]. Cajal named them "cianophil cells." His descriptions were published in Spanish in "Manuel de Anatomia Patologica General," Barcelona, 1890. In 1891, Paul Unna (Unna, 1891), not aware of this work, independently described plasma cells in lupus vulgaris lesions caused by *Mycobacterium tuberculosis*. He thought they might have originated from fixed connective tissue elements and occurred only under pathological conditions, especially chronic inflammations. Marschalko (1895) found plasma cells in many kinds of normal tissues. Marschalko thought that plasma cells originated from emigrated hemic lymphocytes.

Now hematologists and histologists began debating about their cellular origin and two candidates were proposed—lymphocytes and fixed reticular cells. A voluminous literature was generated. Bloom (1938) who wrote the standard textbook of histology with Alexander Maximov defined reticular cells: "The other constituent of the stroma of the lymphatic tissue is the reticular cell." He went on to say, "There can be little doubt that lymphocytes may develop from fixed cells of lymphatic tissue." He could find transition stages between lymphocyte and plasma cell and stated rather emphatically, "The plasma cells are believed by all investigators to develop by individual hypertrophy from various sized lymphocytes." Others thought the plasma cell originated from a (fixed connective tissue cell) "reticular" cell (Marshall and White, 1950). In the ensuing years, many associations of plasma cells with antibody formation were made, and then Astrid Fagraeus in her elegant and seminal thesis produced evidence that convinced most workers that plasma cells were the source of antibodies: "The capacity of the red pulp (of the spleen) to form antibodies varied with the amount of plasma cells in the tissue and above all with the amount of immature plasma cells," but she too thought the plasma cell originated from a reticular cell (Fagraeus, 1948). This lack of consensus understanding about the origins of plasma cells in the 1930s carried into the 1950s (Marshall and White, 1950; Sundberg, 1955) and illustrated the lack of a clear understanding of the developmental history of these cells.

III. MULTIPLE MYELOMA

Multiple myeloma (MM) is a malignant tumor that grows primarily in the bone marrow cavities and spreads from one bone marrow cavity to another. The tumor cells can erode the overlying bone, causing terrible pain and pathological fractures. In 1901, James H. Wright made the important observation that the tumor cells were plasma cells. He obtained them from a patient with MM in which the neoplastic process protruded from the sternum:

> The cells making up the bulk of the tumors are very like or identical with 'plasma cells', which cells are a normal constituent of the red marrow. It therefore seems reasonable to think that the neoplasm, exclusive of its vessels and insignificant stroma, has arisen from an abnormal proliferation of these cells.
>
> (Wright, 1906)

IV. ABNORMAL PROTEIN

The feature of MM (mollities ossium or softening of the bones as it was called in 1845) that intrigued physicians beginning in 1845–1848 was its association with abnormalities in protein production. The first patient who was studied very intensively was a London grocer, Mr. Thomas McBean, who besides suffering from the agonies of his bone pain was found to excrete large amounts of protein in his urine [see Kyle (1985) for an exquisite history of MM]. His astute and concerned physicians recognized this was an unusual phenomenon and sent his specimens to Henry Bence Jones, a noted clinical chemist physician in London. Dr. Henry Bence Jones made a detailed analysis of this protein, demonstrating it could be distinguished from albumen (the common pathological urinary protein in renal disease) chiefly because this new protein when precipitated by nitric acid would go into solution when it was boiled (Jones, 1847).

During 1937–1941, serum protein abnormalities in MM were revealed by the analytical ultracentrifuge and moving boundary electrophoresis developed by Theodor Svedberg and Arne Tiselius (Putnam, 1993). These great technological advances paved the way to the analysis of serum proteins. It was discovered that the serum of MM patients contained extraordinary peaks or concentrations of proteins of β and γ electrophoretic mobility (Longsworth *et al.*, 1939). These abnormal proteins were called myeloma proteins or paraproteins, and many regarded them as pathological proteins, a stigma reinforced by Bence Jones proteins that caused renal disease (myeloma kidney). The pathological protein concept prevailed for many years until a better understanding of the genetics of antibody synthesis became known.

V. NEW IDEAS ABOUT THE CELLULAR BASIS OF ANTIBODY FORMATION IN THE 1950s

Niels Jerne (1955) reawakened the students of antibody formation with his natural selection theory. He proposed that an antigen combines with circulating natural antibodies and the complex is taken up by "a system of cells that can reproduce this antibody." This idea did not detail how this was done, but it contained fresh thoughts on a subject that had been preoccupied with instructionist theories that were gradually becoming less plausible as more was learned about proteins. Talmage (1957) extended and modified the natural selection idea by suggesting "... one of the multiplying units in the antibody response is the cell itself; according to this hypothesis only those cells are selected for multiplication whose synthesized product

had affinity for the antigen injected." In hindsight, Jerne was in part on the right track, as the "system of cells that can reproduce this antibody" could be the B lymphocyte itself that responds to an antigen-specific antibody complex. Later in 1957, Frank MacFarlane (Burnet, 1957) began proposing important specifics to the natural selection idea by harnessing the lymphocyte as a player in humoral immunity and linking it to antibody-secreting plasma cells. It is difficult to imagine in this era of sophisticated T and B cells that in the early 1950s the function of the lymphocyte was not firmly established. Burnet's hypothesis revealed a remarkable insight into the potential of the lymphocyte. He envisioned antibody formation in the context of a developing clone of cells. The "recognition of foreign patterns was ascribed to lymphocytic cells and not to circulating natural antibody" and, further, that the interaction with antigen caused the specific lymphocyte to proliferate and give rise to cellular progeny (plasma cells) that now secreted the recognition protein as antibody. Very critical to his theory was the brilliant idea that each lymphocyte was somehow limited to produce only one molecular species of antibody molecule. All this implied that the lymphocyte sensed the antigen through the antenna of the specific antibody molecule on its surface. This was a challenging hypothesis way ahead of the times.

During August to October 1957 when Burnet was formulating the ideas for the clonal selection hypothesis, Joshua Lederberg on a Fulbright fellowship paid him a visit during a trimester break from Madison. His original intent was to learn about recombination in influenza viruses, but Burnet had closed down his influenza work and turned his full attention to immunology. Lederberg also had been intrigued with the problems of the multiplicity of antigens and antibodies and there were many discussions about clonal selection [see Lederberg (1988) for an interesting historical insight on his interaction with Burnet].

Hearing these discussions, Gustav Nossal, a postdoc in Burnet's laboratory, proposed to Burnet and Lederberg an experiment that might support the Burnet hypothesis. He suggested isolating single antibody-producing cells from immunized rats, placing them in microdroplets, and challenging the cells with live motile *Salmonella* organisms. The specific antibody would stick to the flagella and stop their motion, all of which could be seen microscopically (Nossal, 1986) for a historical account of this experiment. Both Burnet and Lederberg were enthusiastic about Nossal's proposal. Nossal knew that Lederberg had experience with the incorporation of *Salmonella* bacteria in microdroplets using a micromanipulator, and they both dropped everything to carry out this experiment. Two motile flagellated species of *Salmonella* (*Salmonella adelaide* and *Salmonella typhi*) that differed from each other antigenically were chosen as the targets. Anti-*Salmonella adelaide* antibodies could not immobilize motile *Salmonella typhi* and vice versa.

Gustav Nossal and Joshua Lederberg performed this beautiful experiment by immunizing rats simultaneously with *Salmonella adelaide* and *Salmonella typhi*, then teasing out 1–6 cells per droplet from lymphoid tissues. They found 33 of the droplets contained antibodies that were active against *Salmonella adelaide* and 29 against *Salmonella typhi*, but none reacted with both (Nossal and Lederberg, 1958). This was the first evidence to support Burnet's "clonal individuation in antibody formation."

VI. FIRST PCTs IN MICE

Engelbreth-Holm (1942) in Copenhagen concluded in his book on "Leukemia in Animals" that "Only one form of human leukaemia, plasma cell leukaemia, and the related multiple myelomatosis has not yet been observed in lower animals." This proved to be an inspiration to Danish workers. Ragna Rask-Nielsen (RRN) along with Harald Gormsen in 1951 were studying a variety of tumors induced by carcinogenic aromatic polycyclic hydrocarbons in mice and recognized that some of the "leukemias" were, in fact, plasma cell leukemias (Rask-Nielsen and Gormsen, 1951).

"Leukemia" and "mouse leukemia" were jargon terms widely used to describe lymphomas, that is tumors involving lymph nodes, thymus, and spleen, as well as various kinds of hematopoietic leukemias.

The plasma cell leukemias, RRN found, developed in lymph nodes and histologically "showed all grades of differentiation ranging from practically immature cells to extremely immature plasma cells and reticulum cell-like elements side-by-side lending the picture an appearance resembling reticulosarcoma" (Rask-Nielsen and Gormsen, 1951). Another Danish worker, Jergen Bichel (1951), established the first transplantable PCT from a mass that had developed in the axilla of an Ak mouse. This tumor was carried for many generations but discontinued.

In 1954, Thelma B. Dunn (TBD), a renowned "mouse" pathologist at the National Cancer Institute (NCI) in Bethesda, MD, described PCTs in her classic monograph on tumors of reticular tissue in mice (Dunn, 1954). These PCTs (ileocecal plasmacytomas) appeared to arise in the intestinal wall at the ileocecal junction. At this site, there were frequently associated small mucosal ulcers. Underneath this epithelial erosion an extensive inflammatory tissue formed that contained both normal and atypical plasma cells.

Several years later, Pilgrim (1965) searching for these ulcerations in 168 C3H/Pi mice maintained in a colony in Salt Lake City found the associated inflammation and 27 ileocecal PCTs, some associated with reticulum cell sarcoma type B.

Thelma Dunn and RRN were good friends, and during RRN's yearly visits to the NCI, they had many discussions about PCTs—not agreeing about a true definition of PCTs. TBD contended (as best the author can recollect) that a tumor that contained lymphoid elements with primitive and intermediary morphological stages (as RRN's plasma cell leukemias) was not a true PCT. In hindsight they were both correct. Later, serological and transplantation studies revealed the plasma cell leukemias produced paraproteins in the early transfer generations, but eventually reverted to a more immature lymphocytic cell type that lost the ability to secrete serum protein. The ileocecal PCTs were more stable, morphologically and functionally.

VII. LLOYD LAW's SUGGESTION

In 1954, I came to Lloyd Law's (LWL's) laboratory at the NCI to learn how to do and publish experiments in experimental cancer. At first, he was very reluctant to give me an experiment to do but after some persuasion he acquiesced, and I helped him carry out an experiment on X-ray-induced thymic leukemia where mice were thymectomized, irradiated, and then grafted with unirradiated neonatal thymuses subcutaneously. The tumors developed in the grafts, and he was quite excited about the results. I learned many other procedures relating to the induction of "mouse leukemias," from him, including rotational skin painting with carcinogenic hydrocarbons. Mider and Morton (1939) had developed this method and discovered that by distributing the carcinogen to different sites in DBA/2 mice the incidence of skin tumors was reduced and the mice developed a high incidence of lymphomas. These lymphomas were quite heterogeneous—some originated in the thymus, but others arose in the peripheral lymphoid tissues. After 2 years of working on drug resistance, X-ray induced thymic leukemogenesis and attempting to isolate leukemia viruses (an effort that failed), LWL suggested an intriguing new line of experimentation to me. A long-standing interest of his was the variety of different hematopoietic tumors (leukemias) in mice. This fascination began with his association with Arthur Cloudman, a pathologist at the Jackson Laboratory, and his abiding global interest in mouse leukemia. LWL had achieved great success with the carcinogen-induced DBA/2 L1210 transplantable leukemia that was considered to be a model of acute lymphocytic leukemia (childhood leukemia). He thought there were other models to be discovered. His suggestion was very attractive, and I immediately pursued it by inducing leukemias in DBA2^{f}/Lw mice and establishing the various morphological types of leukemias from the Dunn classification in transplant. I then began a collaboration with Thelma

Dunn whose laboratory was two floors down to identify tumors and align them to her classification.

One of the first products of this collaboration was the DBA/2 carcinogen-induced mast cell tumor P815. This was particularly intriguing because it produced functional products serotonin, heparin, and histamine (Dunn and Potter, 1957). P815 was used by several laboratories at National Institutes of Health (NIH) to study the biosynthesis of these components. At first, the tumor cells contained beautiful granules characteristic of mast cells, but then during continuous passage they began to disappear to my horror. One day, Glenn Fischer from Yale visited our laboratory. He had just found ways to grow leukemias in tissue culture, so he took P815 home with him and successfully established a cell line. Years later, I began reading about P815, which turned out to be a highly sensitive target of T-cell cytotoxicity.

P388 was a lymphoblastic tumor cell that grew exponentially in mice, killing them in 5–7 days. It was widely used by radiologists because of its high sensitivity to radiation damage. Clyde Dawe spent a summer trying to put these lymphoblastic cells in culture, but to his dismay he repeatedly obtained cell lines with macrophage-like morphology (correspondence with Dawe). These were called P388D1 and, years later, were used as surrogates for macrophages (Koren *et al*., 1975). As tissue culture methods improved, Virginia Evans successfully grew out P388 in its lymphoblastic form. The relationship of P388 (lymphoblastic) to P388D1 was established many years later by Steve Bauer (Bauer *et al*., 1986), then a graduate student at NIH, who showed that both lines had the same IgG gene rearrangements. P388 is a B-cell tumor that can give rise to a macrophage-like variant, both of which produce tumors.

Although this collaboration was very productive, there was one type of tumor that was missing—the PCT. TBD had successfully transplanted a C3H tumor several years before, which she thought might be an ileocecal PCT called 70429 (Dunn, 1957). John Fahey and I could not find any evidence that it produced a myeloma protein. My quest for a myeloma protein-producing line was greatly charged by an inspiring lecture given in Wilson Hall at NIH by Frank Putnam in 1955. He had been characterizing a series of human Bence Jones proteins for sometime by various physical and chemical methods and found no two of them were alike (Putnam and Stelos, 1953). This raised intriguing questions. Why were they different? Was it due to polymorphisms of their structural genes in the human population? Or, were these mutant proteins? It would be so valuable then to propagate PCTs from a single inbred strain of mice to sort these questions out.

In the 1950s, the big yearly scientific meeting was held in Atlantic City and attended by 20,000 or more biomedical scientists from all over the United States. In 1955, I met Elliott Osserman here for the first time through our

mutual interest in MM. He was very proud of the fact that he had seen around 30 patients with MM. I told him flippantly, he would not be a success until he had seen 100 cases (and break the world record). This was a very large number as MM was considered a rare disease. I discussed my hope to find a PCT in mice that produced a myeloma protein. We compared notes on a yearly basis and within a few years he reached the 100 mark and became the leading expert in MM in New York City. A great increase in incidence of MM began about this time. Elliott Osserman became very interested in methods to detect myeloma proteins and went to Paris to work with Pierre Grabar and learn the exciting new technique of immunoelectrophoresis, which he kindly taught me how to do in 1958.

VIII. X5563 AND X5647

The major practical problem with TBD's ileocecal PCTs was their scarcity. It might be a year before she would find another one and establish a transplant line from it. Then one day in 1956, H. Ira Pilgrim, a graduate student of Dr. DeOme's laboratory in Berkeley, who had been studying mammary tumors in C3H mice, encountered two ancient mice with abdominal lymphomas. Realizing these might be interesting, he wrote to Dr. Dunn and sent her the histological sections for classification. She immediately recognized the tumors as ileocecal PCTs and wrote him back her findings with the query, by any chance had he transplanted these tumors? If so, would he be willing to send them to the NCI, as there was a young investigator there who was very interested in PCTs? The answers were, "yes" and "yes."

The mice bearing X5563 and X5647 subcutaneous tumors arrived the very day in May 1956 we had acquired our first paper electrophoresis apparatus. This had been purchased from Spinco/Beckmann for the large sum of around $300 so that I could screen all of the transplantable tumors for serological evidence of myeloma protein production and to study the 70429 tumor and its variants in more detail. Preliminary studies with John Fahey had not shown a myeloma band. Alvado Campbell and I loaded up the paper electrophoresis strips to fill out the overnight run and put in X5563 serum at the last minute. Then, to our surprise, the X5563 serum proved to be the unusual one. There was the familiar large albumen band, but a large amount of protein remained at the point where the sample had been applied. The first thought was that we had not carried out the procedure properly with this new instrument, but then when the finding was repeated, it was clear that the extra band near the origin was a massive myeloma protein. I ran across the hall and told LWL about it, announcing, "Gold has been struck."

In those days, I was preparing the bones from various mice using the very odiferous papain digestion method. I collected the skeletons to search for genetic anomalies of bone structure described in Hans Gruenberg's great treatise *The Genetics of the Mouse* (Martinus Nijhoff, The Hague, 1952). When the bones of mice carrying X5563 growing subcutaneously were examined, many of them had osteolytic lesions throughout the skeleton. In these early transfer generations, X5563 grew subcutaneously very slowly, and the bone lesions developed only in the mice that had carried these tumors for 90 days. Histological studies showed the bone lesions were due to metastases of the X5563 subcutaneous tumor to bone marrow cavities. It seemed then that X5563 was a model for human MM (Potter *et al.*, 1957). This was a premature, if not incorrect, conclusion primarily because the mouse X5563 PCT did not begin or arise in the bone marrow tissue. In later studies, we failed to find bone lesions associated with other primary or subcutaneously transplanted PCTs but were able to find them if the PCT cells were injected intravenously (Kobayashi *et al.*, 1962). However, even though much has been written about the origin of human MM, the fundamental issue of its histogenetic origin is not fully resolved. The possibility entertained by some workers is that the neoplastic transformation in MM could originate in extraosseous lymphocytes and then selectively grow out in bone marrow cavities as plasma cells where the neoplastic cells take on a clinical "life of its own."

Fahey *et al.* (1960) characterized the second pilgrim PCT X5647 and found that it produced a quite different myeloma protein with beta mobility with multiple peaks in the ultracentrifuge (characterisitic of an IgA myeloma protein). This was the beginning of a series of studies by John Fahey in the mouse that defined the classes of antibodies [or immunoglobulins (Igs) as they would soon be called].

IX. SPECIFIC INDUCTION OF PCTs IN MICE BY IMPLANTING MILLIPORE DIFFUSION CHAMBERS: RUTH MERWIN AND THELMA DUNN

One day in 1958, Thelma Dunn called me on the telephone. Something very exciting and very unusual relating to PCTs had happened most unexpectedly. Ruth Merwin had brought to her tissues from BALB/c mice that had been implanted intraperitoneally with Millipore diffusion chambers (MPDCs), and after 6 or 8 months the mice had developed ascites and their peritoneal cavities contained multiple tumors. When the histological sections became available, the tumors were found to be PCTs and fibrosarcomas. Fibrosarcomas formed around subcutaneously implanted foreign bodies, and

even one PCT had been previously described by Oppenheimer *et al.* (1955), but the consistent peritoneal PCTs that Ruth Merwin was finding had never been seen before in Thelma Dunn's or anyone else's experience. This was an extraordinary development because it provided for the first time a method for inducing PCTs in inbred mice. Some background on the MPDC experiments revealed the complexity of the problem for explaining why this notoriously rare type of tumor should suddenly be consistently found.

Ruth Merwin was completing a study she had begun with Glenn Algire over a year before. MPDCs had been developed by Richmond T. Prehn and Glenn Algire in 1954 (Prehn *et al.*, 1954), several years before at the NCI. The MPDCs essentially consisted of a plastic ring onto which Millipore membranes of various pore sizes had been glued. A small hole in the plastic ring permitted introduction of cell suspensions. Cells from one inbred strain could survive within the chambers implanted into another strain that differed genetically at the major histocompatibility (H-2) locus. The Millipore membranes would allow nutrients and large molecules such as antibodies to enter the chambers but exclude cells. A basic question that arose in discussions of the findings was, how long could foreign incompatible cells live under these conditions? To answer this, Merwin and Algire (1959) designed a long-term cell survival experiment. First, they chose to use a transplantable tumor as the source of cells because viability could be assayed by transplantation. Then they chose the most commonly available tumor—a mammary tumor. This led them to use a C3H ($H\text{-}2^k$) mammary tumor that carried the mammary tumor virus (MMTV), which was transmissible. They chose a pore size that would allow this virus to pass through. This now led to the most fortuitous choice of BALB/c Andervont (BALB/cAn) as the recipient, first because BALB/c had a different histocompatibility type ($H\text{-}2^d$) and second BALB/cAn mice did not carry the transmissible MMTV but were susceptible to the C3H MMTV virus, so the C3H mammary tumor in the chamber might induce mammary tumors in the BALB/c female host. Algire tragically died of periarteritis nodosa during the course of these experiments and never knew about the fascinating outcome. But Ruth Merwin then was presented with an exciting discovery and a new direction. She and Lena Redmon continued to search for the component of the MPDC that was responsible for the plasmacytoma and in 1963 showed that plastic disks or plastic shavings alone could induce PCTs and in fact were more effective than MPDCs (Merwin and Redmon, 1963).

Ruth Merwin kindly gave me several of her PCTs to carry in transplant. The *M*erwin PCT MPC11 tissue culture line is still in use. The MPC2 PCT produced a copious amount of protein in the urine that had the physicochemical properties of a Bence Jones protein and caused the renal pathology of "myeloma kidney" (Dunn *et al.*, 1960; Fahey and Potter, 1959; McIntire and

Potter, 1964). These new BALB/c and C3H tumors added up to eight, and John Fahey and I wrote up the description of them (Potter and Fahey, 1960). Each one produced a distinctive myeloma protein. Joshua Lederberg became interested in acquiring these tumors but was in the process of moving to Stanford. His letters included intriguing ideas and insights, one of which was a query about what might be the antigenic stimulus to which the PCTs were responding, suggesting the possibility of the gut flora.

X. MINERAL OIL AND THE HYPERIMMUNIZATION HYPOTHESIS

As Ruth Merwin was continuing to work on the MPDC system, I sought to find alternative ways to induce PCTs. The important clue from the Merwin–Algire experiment was that the BALB/cAn mouse, not C3H, appeared to be the inbred strain of choice to use. My working hypothesis on how the MPDC system worked was that the incompatible tissue in the chamber was continually leaking foreign antigen and chronically hyperstimulating the immune system of the BALB/c recipient. Implicit in this notion was that plasma cells would be activated and that during the proliferation of these cells errors (mutations) would occur that would lead to neoplasia (hyperimmunization hypothesis). In hindsight this was naïve, especially because I knew so very little about experimental immunology, but it did not deter me from exploring the idea. Several immunologists had moved into Building 10 at NIH around the corner from the laboratory, so I asked them what a good antigen (immunogen) might be, and they suggested horse serum. They told me stories about rabbits hyperimmunized with horse serum that developed massive plasma cell hyperplasias. This of course was all I needed to hear. Hastily giving weekly injections of horse serum to BALB/c mice produced a disastrous result: between the third and fourth weeks all of the mice had died of anaphylactic shock long before the time when they would possibly come down with PCTs.

A little reading led to enlightenment about agents that amplified immune responses—immunological adjuvants, the most famous being Freund's adjuvants (FA). Jules Freund had first described these adjuvants in 1937, a study that had a long history in tuberculosis research that searched for a method for improving immunity to *M. tuberculosis* (Freund *et al.*, 1937). Coating the organisms in paraffin oil was found to enhance immune responses. Numerous refinements were described leading to the development of water-in-oil emulsions that contained in one phase 8.5 parts of the mineral oil Bayol F, 1.5 parts of an emulsifying agent (Arlacel A) and heat-killed mycobacteria, and an equal volume of aqueous phase components containing antigen. After

vigorous emulsification the water-in-oil emulsions also had the property of slowly releasing antigen and were not associated with anaphylaxis.

Munoz (1957) at the Rocky Mountain Laboratory had injected FA (with antigen) into the peritoneal cavities (i.p.) of Swiss Webster mice and found that they developed an accumulation of peritoneal fluid (ascites) that contained substantial amounts of antibody.

Clarification of some terms used here and background is necessary. Most of the early studies of adjuvants were carried out in guinea pigs and rabbits and very few in mice. When water-in-oil emulsions were introduced into mice, the peritoneal cavity was the convenient site. This included complete FA (CFA) that contained a bacterial product (tubercle bacilli, staphylococci, and others) or incomplete FA (IFA) that had only the paraffin oil and emulsifying agent in the oil phase. All of these materials induced the formation of a chronic inflammatory process on peritoneal surfaces called the oil granuloma (OG). The growing OG obstructed the flow of lymph through the peritoneal space and this plus the inflammation caused the accumulation of fluid (ascites). The ascites contained inflammatory cells and in mice with PCTs, atypical plasma cells.

Tuberculoproteins had been injected into the peritoneal cavities of rabbits over 20 years before the studies in mice. These rabbits accumulated large populations of plasma cells in the omentum (Miller, 1931). I was not aware of this work in 1958. Studies by Robert White, Albert Coons, and Jeanne Connolly in 1950 (White *et al*., 1955) showed that the injection of water-in-oil emulsions with wax D from tubercle bacilli (a modified form of CFA) caused a local lesion to form at the site of injection. From the description, this was clearly an "oil granuloma" tissue rich in macrophages, fibroblasts, and neutrophils. In this subcutaneous site columns of plasma cells were found. Although antigen persisted there, antibody-forming cells were rare. The main cellular immune response occurred in the draining lymph nodes. The source of the antibodies in the peritoneum of the mouse then could be the draining lymph nodes (in the superior mediastinum), or from the plasma cell populations in the OG or from a distant lymphoid site.

By another turn of good fortune, there was an investigator in the National Institute of Allergy and Infectious Diseases (NIAID), Rose Lieberman (RL), who had modified FA by using staphylococci or *Salmonella* organisms instead of tubercle bacilli. When this adjuvant mixture was injected i.p. into mice of many different strains, it produced voluminous ascites that contained an abundance of antibodies. I asked RL if there were plasma cells associated with this response, but she had not studied the pathological response to her adjuvants in the peritoneal cavity. I struck a deal with her to study the pathology of the peritoneal reaction if she would inject a group of BALB/cAn mice for me with her adjuvant mixture. Abundant plasma cells

were seen in the reactive tissue that formed in response to the water-in-oil mixture (Lieberman *et al*., 1960). RL injected BALB/cAn mice in return. For good measure, Charlotte Robertson and I painted some of the mice with the carcinogen 3-methylcholanthrene. Six months later some of the mice developed ascites. The tissue sections showed these were due to PCTs that had developed in the OG, and transplant lines were initiated, of which only AdjPC5 exists today (Potter and Robertson, 1960). The PCTs occurred in noncarcinogen-painted mice as well as painted ones. The antigen was presumably the *Staphylococcus aureus* bacteria, but we did not test the mice for antibodies. This was a most exciting result, first because it was an easier way to induce PCTs and second it still implicated immunization in the pathogenetic scheme. The hyperimmunization hypothesis brightened but not for long.

Some paraffin oils had been known to contain carcinogens and this led to testing the components of the adjuvant mixture. IFA alone and the mineral oil were the easiest to test, and one of these alone yielded a quick solution to the problem. Mineral oil alone was highly effective (Potter and Boyce, 1962). This eliminated the need for antigen, skin painting with an aromatic polycyclic hydrocarbon carcinogen, or the heat-killed bacteria (tubercle bacilli or staphylococci). The results also seemed to eliminate my hyper-immunization hypothesis. At least it became much less tenable. The reason—paraffin oils were not thought to be antigenic nor directly immunogenic.

Pursuing the mineral oil story, we found that mineral oils which could be purchased at the local grocery store as well as highly refined ones used for medicinal purposes were just as effective as Bayol F, the component of IFA and CFA. Paul Anderson discovered from his reading that pristane (2,6,10,14-*te*tramethylpentadecane) was a typical component of mineral oils, and he tested this light white oil in BALB/c mice and found it was more effective than mineral oils in inducing PCTs (Anderson and Potter, 1969). The laboratory began using pristane exclusively, and the name of the new PCTs changed from MOPC to TEPC (the *te* was derived from the first two letters of the chemical formula). Pristane is of biogenic origin (Blumer *et al*., 1963) and found in the human food chain. Avigan *et al*. (1967) at the NIH found pristane in many human tissues. The carcinogen idea seemed to dissipate.

At age 66, Jules Freund (1890–1960) was asked to come to the NIH to head up the Laboratory of Immunology in NIAID, but very unfortunately he had been found to have MM and spent most of his time in bed in the clinical center. This did not deter him from editing papers, and he asked me to review a manuscript, so I visited him twice to discuss this. Unfortunately, we never talked about the induction of PCTs in mice with his adjuvants, although I sensed he knew about our PCT induction studies. Many investigators of his vintage (and long before, e.g., John Hunter) often injected themselves with their inventions or vaccines. I was of course keenly interested to know if Jules

Freund had given himself his own adjuvants but I dared not ask. One of his physicians (who knew about the mouse PCT work) told me much later he had asked Jules Freund if he had injected himself with adjuvants. The first answer was yes, but then on another occasion when I asked again he said he had not. I doubt he had because of subsequent experiences where large number of people had been given vaccines in FA, many of which had developed aggravating consequences and this should have been noted in the clinical record.

Intraperitoneal mineral oil (0.5 ml × 3 at 2-month intervals) evolved a similar oil granulomatous tissue that formed on mesenteric surfaces. Intriguingly, it contained isolated plasma cells and focal plasma cell proliferations of normal and atypical plasma cells. Ross MacCardle and I found the PCTs formed exclusively in this tissue (Potter and MacCardle, 1964). The relationship of these plasma cells to immune responses was not clear since no antigen had been intentionally introduced. In hindsight, the result implies that if there were a role played by antigen, it could have an endogenous origin, for example antigens resulting from tissue breakdown caused by the inflammation evoked by mineral oil or antigens generated by the microbial flora. The relationship of these local plasma cells in the OG to immune responses was going to be more subtle.

XI. DISCOVERIES ON ANTIBODY STRUCTURE USING HUMAN MYELOMA PROTEINS CHANGED THE COURSE OF IMMUNOLOGY, 1961–1965

The field of immunochemistry was continually advancing. Many recognized that progress would depend on a chemical definition of the antibody molecule. Rodney Porter was the first to seriously attack the difficult problem of solving the structure of antibody molecules (Porter, 1991). His first approach was to proteolytically cleave purified antibody molecules from rabbits into smaller fragments using enzymes such as papain. This yielded the widely used fragments, the Fab that contained the active antibody site and a second type of fragment, the Fc, that crystallized.

Rodney Porter was a revered scientist by all who knew and worked with him. He visited the NIH on several occasions to describe his latest findings. One sensed he was destined to win a Nobel Prize for his work.

Rodney Porter knew about myeloma proteins but was hesitant to use them as a model for structural studies because they were derived from tumors and had the "stigma of abnormality" about them. At meetings he was rather reserved. In several encounters, instead of talking about myeloma proteins

with him I finally found a topic of mutual interest—he loved to fish for trout in Scotland.

Two turning point landmarks in antibody structure set the field on a new course. The polypeptide chain structure held the key. This was the era of the one gene:one enzyme concept. Gerald Edelman perceived the problem and had no apparent reservations about using myeloma proteins as a model. Working with Henry Kunkel, he was no doubt familiar with the biologically active myeloma proteins (Kritzman *et al.*, 1961). This led him and his colleagues to try and dissociate the tightly knit myeloma protein molecule into separate chains. Using strenuous denaturing methods (6-M urea) and the reduction of disulfide bonds he succeeded in showing a human myeloma protein contained two components, one of molecular weight of 50,000 kDa and a smaller one of 25,000 kDa (Edelman and Poulik, 1961). The monomeric myeloma protein was made of four chains, two identical light (L) chains of 25,000 kDa each and two identical heavy (H) chains of 50,000 kDa each. He then showed that antibody molecules had a similar molecular weight and polypeptide chain structure. Then came the revelation that the Bence Jones proteins excreted in the urine were antibody light (L) chains (Edelman and Gally, 1962). The final structure of the antibody molecule using the Fab, Fc, and polypeptide chain structure was established (Fleischman *et al.*, 1962). These findings overcame a major roadblock in thinking by showing that myeloma proteins and antibodies had the same polypeptide chain structure. These molecules were then called Igs. The stigma of abnormality was beginning to fade away. Now the isotypes of the L (kappa and lambda) and the heavy (H) chains [M, D, G (four subclasses), E, and A] were defined. The one gene:one polypeptide chain relationship prevailed. But in1965 at Melvin Cohn's (MCs) greatest Antibody Workshop, this icon was destroyed by the amino acid sequence analysis of two human Bence Jones proteins carried out by Norbert Hilschmann who discovered that the two L chains shared an identical or common (C) sequence for the C-terminal half of the molecule chain but a strikingly variable one for the N-terminal half. Soon after Hilschmann and Craig (1965) published these findings and suggested they might be joined by a gene crossing-over mechanism. To explain this extraordinary phenomenon, Dreyer and Bennett (1965) proposed that the C and V (N-terminal segment) were controlled by separate genes and that the chain structure was determined by a rearrangement process that joined the two separate DNAs on the chromosome. The era of the two genes:one polypeptide chain prevailed (for a time).

XII. ENTER MEL COHN

MC brought the exciting emerging field of molecular biology into immunology. He founded the "Antibody Workshops" but this lasted just a short time because so many people wanted to attend, he would need a convention center not a close interactive group to conduct the meetings (Porter, 1986). He tried on many occasions to have molecular biologists become immunologists, but they would have no part in this. Instead, a new generation of immunologists trained in the techniques of molecular biology would accomplish this union and transform the field. He had a major impact on the mouse PCT field.

MC did his doctoral thesis under the inspiring mentor Alwin Pappenheimer, Jr. at New York University (NYU; 1946–1949) (Cohn, 1982). "The Department of Microbiology at NYU was the precursor (along with Delbruck) of the era of molecular biology. The head was Colin Macleod, coauthor of the paper with Avery, proving that DNA was the hereditary material. Mark Adams was a key member of the phage group; in fact he kept detailed notes on the meetings that became the basis for his handbook on "Phage Biochemistry and Genetics." Ephriam Racker was a biochemist of unrivaled expertise, studying mammalian viruses (polio) as well as intermediate metabolism. Pap and Alan Bernheimer were leaders in the study of bacterial toxins. Pap of course was an immunologist in the league of Heidelberger and Kabat. Heidelberger and Kabat were on the committee when I defended my Ph.D. thesis" (letter to author from MC, July 2, 2006). Pappenheimer was a great admirer and friend of Jacques Monod. During a visit to Pappenheimer's laboratory MC met Monod and this set the stage for MC to go to the Pasteur Institute for his postdoctoral work. In 1949, MC thought that immunology had become "boring and felt it was on the wrong track and needed the input from genetics, regulation and biology" (letter to author from MC, February 21, 2006). After returning from Paris, he began to introduce the excitement of molecular biology and the thinking of molecular biologists to the field of immunology. He joined the Department of Microbiology at Washington University in Saint Louis (1954–1958) and began working with phages and interacting with Max Delbruck's circle of phage geneticists. He met Ed Lennox in 1955 and with him developed an antibody phage neutralization assay that could be accomplished in microdroplets. In 1958, MC moved to Stanford University (Cohn, 1994). Gathered there in 1958–1959 was a newly assembled group of immunologists and molecular biologists: MC, Joshua Lederberg (in the apogee of his immunological period), Av Mitchison, Oli Makela, and Len Herzenberg. Together they taught a graduate course in immunology. About this time, MC and Joshua Lederberg became interested in the transplantable PCTs in mice as a model system. MC wrote me a letter in 1960, requesting the

eight known PCTs in our collection. While he was preparing his laboratory in Palo Alto to deal with mouse tumors and mice, he suddenly decided to return to Paris to complete several experiments. On returning to the United States in 1962, he joined the Salk Institute faculty and immediately began to establish a plasmacytoma laboratory and tackle the mouse problem. The famous mouse geneticist Leonell Strong who founded many of the inbred strains of mice was recruited in 1964 thus bringing a major "mouser" to La Jolla.

PCT induction was highly dependent on the proper inbred strain—the BALB/cAn subline. Realizing this, MC obtained 200 BALB/cAn mice from the NCI and began inducing PCTs. Some of these were "heavily immunized" before giving them mineral oil with the expectation of finding a PCT that would produce a myeloma protein that reacted with the immunogen. MC, of course, chose for this purpose bacteriophages (T_2, T_0, PLT22, and øX174) as the immunogens and developed an exquisitely sensitive plaque assay to detect activity. Many of the BALB/cAn mice in his induction study were immunized with phages, but none of the myeloma proteins produced by these PCTs reacted with the phages (Cohn, 1967).

XIII. PNEUMOCOCCAL TYPE C POLYSACCHARIDE (PnC) AND PHOSPHORYLCHOLINE

Matching a myeloma protein with an antigen would now depend on a screening procedure. Seymour Benzer convinced MC that this was an honorable, methodical, and probably necessary way to find an antigen-binding myeloma protein.

The first descriptions of biologically active M components in humans were made in 1957 by W. N. Christianson and J. V. Dacie (Christenson and Dacie, 1957) in the cold hemagglutinin syndrome. Kritzman *et al.* (1961) described a monoclonal IgM that reacted with human IgG. Waldenstrom, his associates, and others surveyed a number of myeloma sera and found several that reacted with streptolysin O (Seligmann *et al.*, 1968; Waldenstrom *et al.*, 1964). Streptolysin O is an antigen that is produced by beta hemolytic streptococci, a common pathogen in humans. Stone and Metzger (1967) described a human macroglobulin with anti IgG activity.

By 1967, MC had begun testing 123 myeloma transplant lines against a battery of available antigens. At the Cold Spring Harbor Meeting in May, MC and his colleague Gursaran Notani announced they had discovered an active myeloma protein, S63, that precipitated with the Pneumococcal type C polysaccharide (PnC) (Cohn, 1967; Cohn *et al.*, 1969). MC showed an impressive Ouchterlony precipitation band, but then cautioned everyone about the antigen, which was known to react with the acute phase non-Ig

C-reactive protein. Because of this admonition, I made no attempts to test for this antigen; we were busy assembling a screening panel and shipping samples to Herman Eisen (see below).

One afternoon in October 1967, Myron Leon (ML) from Cleveland was on his way to go behind the iron curtain to do some experiments with Jaroslav Sterzl's laboratory in Prague. He came a day early to conserve his resources and hopefully carry out a quickie experiment on the way. He planned to test IgM human myeloma proteins to a series of antigens and had arranged with John Fahey to utilize a number of samples that were stored in a freezer in a facility in Virginia. He had three antigenic cocktails, one containing a series of dextrans, another a series of levans, all obtained from Allene Jeanes. The third tube had a mixture of six highly purified Pneumococcal capsular polysaccharides from Michael Heidelberger's laboratory (where he once had been a student).

Petri dishes plated with a special agar that gave good visualization of the precipitation of an antigen with its specific antibody. The antigen and antibody were placed in separate wells and allowed to diffuse through the agar,

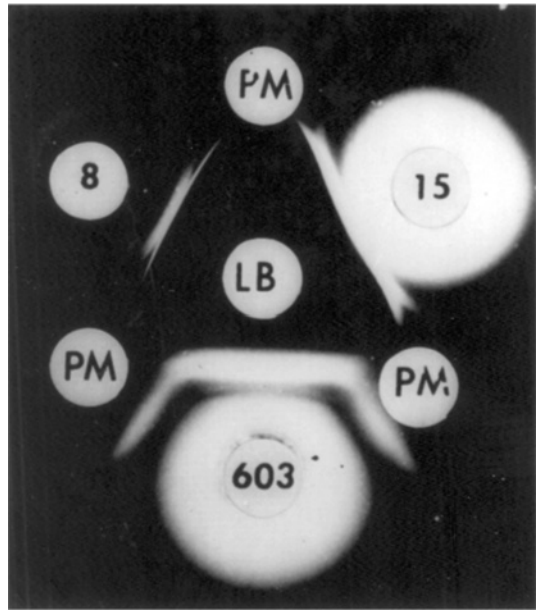

Fig. 1 Double diffusion in agar gel (Ouchterlony precipitin reaction) showing the precipitation bands with the myeloma proteins 8 (HOPC8), 15 (TEPC15), and 603 (McPC603) and extracts of four *Lactobacillus* spp. (LB) and *Proteus Morganii* (PM). Note 8 and 15 do not react with PM, while 603 reacts with both antigens.

and where and when the two diffusing waves met they reacted to each other and an opaque precipitin band formed (Fig. 1).

The day in Virginia proved frustrating because only three serum samples were found, and none reacted. Not wanting to call the day a total "bust," he called our laboratory, desiring to come over for a chat. He arrived around 4:00 PM and told me about the experiment in Virginia. I told him, "It is too bad you left all your antigens over in Virginia because I have 100 myeloma sera just waiting to be tested." "Oh, but I didn't," he replied, producing from his coat pocket the three vials of antigens. I opened the refrigerator door showing him ascites samples from 100 PCTs. My technician quickly cut a series of micro-Ouchterlony plates using the RL cutter. The plates were filled with reactants, and ML and I retired to a local restaurant. On our return 2 h later, we saw to our delight four bright precipitin bands, three in the Pneumococcal polysaccharide (PSS) plate (MOPC299, MOPC167, and McPC603) and one in the dextran plate (MOPC104E). What a memorable day! The Pneumococcal reactants were most exciting—sugarplums were dancing in my head. Could these be mouse myeloma proteins that were reacting with type-specific Pneumococcal antigens derived from the pathogens that caused Lobar pneumonia in humans? I was eager to sort out which PSS reacted with each of the myeloma proteins. But alas! ML would leave the next day for Prague, and the separate PSS were in Cleveland. But quickly, arrangements were made to send the proteins to Martin Young in Cleveland.

Weeks went by, then the letter came from Prague with the puzzling news that each of the three proteins reacted with all six of the individual PSSs. Remembering MC's findings with S63, I sought to obtain a pure PnC preparation to see if this was the antigen. By good fortune Emil Gottschlich, who had been working on the structure of PnC, was over at Walter Reed, and I asked him if he would test our proteins with his purified polysaccharide, which he did using a capillary tube precipitation reaction and quickly (in a few minutes) found the myeloma proteins precipitated the purified PnC. When I told ML this result, he was horrified because it meant that Heidelberger's revered capsular polysaccharide preparations (at least the ones he had) were contaminated with PnC, but he came to Bethesda and we published our findings (Potter and Leon, 1968). We began testing every available ascites sample in the laboratory and added MOPC511 and then TEPC15 and HOPC8 (7-n-*h*exyl*o*ctadecane PCT), both of which came from PCTs that Paul Anderson had induced. This brought the total of R36 PnC reactors to eight, six from our laboratory and two from MC's.

Despite the disappointment about not finding type specific anti-Pneumococcal capsular polysaccharide activity, we were nonetheless excited about finding six different myeloma samples that reacted with the same antigen. A collaboration with RL and Sheldon Dray was well underway at

the time. RL had developed a number of allotypic antisera, that is antibodies raised in mice that would react specifically with antibodies (Igs) from other inbred strains. Using myeloma proteins as immunogens, we also found that antisera could easily be prepared that recognized the individuality and antigenicity (idiotype) of a myeloma protein (Potter and Lieberman, 1967; Potter *et al*., 1966). These antisera possessed extraordinary specificity. Myeloma proteins of the same H chain class could be distinguished from each other by an idiotypic antiserum. We immediately began to develop individual idiotypic antisera to the R36A PnC-binding myeloma proteins and obtained a wonderful result. The anti-idiotypic serum to TEPC15 (T15) gave strong reactions of identity with MOPC299, HOPC8, and with MC's S63 and S107, which he had kindly sent us but did not bind the McPC603 and MOPC167 proteins (Potter and Lieberman, 1970). The seemingly endless variety of myeloma proteins had encountered for the first time substantial evidence that five of them at least were identical. RL then discovered that there were antibody molecules in the normal serum of mice that carried the T15 idiotope and that they were greatly increased when the mice were immunized with R36a (rough) Pneumococci (Lieberman *et al*., 1974). All this evidence collectively showed that some myeloma proteins were in fact natural antibodies.

Greatly influenced by the monumental work of Karl Landsteiner, efforts then focused on finding the specific chemical group on the polysaccharide macromolecule to which these anti-PnC myeloma proteins reacted. The structure of the PnC was being worked out by Brundish and Baddiley (1968) who found it contained repeating units composed of ribitol phosphate, *N*-acetyl-D-galactosamine, D-glucose, a diamino sugar, and choline. MC and Notani tested each of these and found only weak inhibition with choline (Cohn *et al*., 1969). Then ML and Martin Young in 1970–1971 made the critical discovery that the dominant hapten or reactive group was phosphorylcholine (PC) and that by the addition of the phosphate group, the inhibitory power of the choline hapten was increased four-hundred times (Leon and Young, 1971). PC side groups were a component of many macromolecules that could be found in prokaryotes and eukaryotes, including bacteria, fungi, plants, nematodes, and humans. We had no trouble in encountering some of these structures (antigens) in the microbial environment of the mouse. Cosenza and Kohler (1972) advanced a new insight into the significance of the TEPC15 idiotope by finding it was also found on specific B lymphocytes where it acted as an antigen receptor for normal cells participating in immune responses to PC.

XIV. HERMAN EISEN, DNP, AND MOPC315

Herman Eisen, in 1967, was one of the most respected immunochemists in the United States, well known for his work on antibodies to the dinitrophenyl (DNP) groups found on conjugated proteins (Eisen, 1964). He became interested in obtaining monoclonal antibodies to DNP and began screening human myeloma proteins for this activity. At the Cold Spring Harbor Meeting in 1967, he presented his findings on the human myeloma protein BRY that weakly bound DNP (Eisen *et al.*, 1967). To my surprise and delight, he tapped me on the shoulder in the line going to lunch and said he would like to collaborate with me to find a mouse myeloma protein that bound DNP. I had not envisioned this possibility might be achieved, but I eagerly agreed to start sending him ascites from our collection of transplantable PCTs that were all maintained by serial transplantation by Alvado Campbell. Herman Eisen would spend the summer at Woods Hole, but we were to ship the samples to St. Louis to Ernie Simms. Soon, numerous handwritten letters from Eisen arrived, describing the results of various immunochemical tests. Some of the proteins were active, but I was not able to evaluate these interesting results. To try and understand this, we set up an Ouchterlony plate with all the suspects that Herman Eisen had found using a DNP-conjugated human-gamma-globulin and let it react overnight. Coming to work the next day, I was greeted by Betty Mushinski, who did all of the immunological work in the laboratory, she excitedly announced, "We've got one!" The PCT ascites was from an IgA producing PCT MOPC315, and on the plate was a bright yellow precipitin band. MOPC315 was just another transplantable PCT maintained in the laboratory. We hastened down to the mouse colony to see if the mice carrying this tumor were alive and well, which they were. I immediately called Herman Eisen who cautioned me about extrapolating Ouchterlony and binding affinities. However, MOPC315 was high on his list from the immunochemical data, and he showed that MOPC315 had a binding affinity of 10^{-7} M/L for DNP, not quite the intensity of $>10^{-(10–12)}$ M/L for induced antibodies to DNP, but respectable enough that Herman Eisen began an intensive and careful study of MOPC315 and two other IgA myeloma proteins, MOPC460 and XRPC25 (Eisen *et al.*, 1968).

Herman agreed that I could present some of our findings at an American Association for Cancer Research (AACR) meeting, and when I announced that Herman Eisen said MOPC315 was a strong candidate to be a monoclonal antibody to DNP, one person got up and walked to the back. It was David Pressman, who immediately called Herman Eisen in St. Louis for confirmation. When the talk was over, Pressman was anxious to get MOPC315. Eisen said that we should not distribute the tumor until the paper was published, and I

agreed to do this. Several months later when we were both at another meeting, there was a presentation by a British group showing electron photomicrographs of MOPC315-binding DNP proteins. I had kept my word but was nonetheless embarrassed about how this had happened, but I had a vague recollection that a colleague of mine at NIH might have done this. Fortunately, he was at the meeting, and I asked him if he had any clues. Of course, he said everyone knows you give these tumors to anyone. There was a visitor in his laboratory from England, in fact on her way back that afternoon, and my friend arranged to give her the MOPC315 tumor, which then was distributed widely in Britain.

Herman Eisen and his group pursued MOPC315 in an extraordinarily productive and thorough way, discovering first its unique and previously unknown lambda-2 L chain, then finding he could produce anti-idiotypic antibodies in BALB/c mice to M315 protein, and then in exciting studies with Richard Lynch that mice immunized with MOPC315 cells could be protected from developing transplant outgrowths when they were challenged with live tumor cells (Rohrer and Lynch, 1977). This, of course, implied that M315 cells presented the M315 protein on its membrane where it could be the target of immune destruction. M315 tumor was composed of two cell types, one a lymphocyte expressing the cell surface M315 and the other a plasmacyte.

XV. IRRELEVANT AND RELEVANT ANTIGENS

We began by broadening our antigen screen first by collecting a variety of antigens from other laboratories. A key acquisition came from Otto Luderitz who graciously sent me lipopolysaccharides (LPSs or O antigens) that he had prepared from 40 different *Salmonella* species. This had been arranged by ML. When these were tested, we identified new antigen-binding activities to exotic salmonella antigens from all over the world. I soon found a local source and began "growing my own" and isolating a variety of antigens (Potter, 1971). The remarkable MOPC467 myeloma protein strongly precipitated all the *Salmonella* LPSs from different O antigen serogroups but not *Salmonella typhi*. But these were not relevant to the environment of the mouse unless of course the mice became infected. And so, we began isolating antigens from bacteria cultured from mouse fecal pellets (Potter, 1971). Strong reactions were obtained with the MOPC467 and heat extracts from a *Proteus mirabilis* species and then from several other bacteria. Mason Smith took up the problem of characterizing this ubiquitous antigen and showed it could be a flagellin, a heat stable protein (Smith and Potter, 1975). [MOPC467 myeloma protein

has been used commercially for the rapid detection of contaminant *Salmonella* antigens in food (Robison *et al*., 1983).]

PC groups are found on different kinds of biological macromolecules. PC is a component of cell wall teichoic acid of R36A *Streptococcus pneumoniae* (Pneumococcus). This organism is not a common natural pathogen in mice. It was also found in the LPSs of a *Proteus* (*Morganella*) *morganii* (PM) species isolated from the GI tract of a BALB/c mouse in our colony (Potter, 1970). In this LPS, the PC group is linked in a chemically different way to the underlying LPS and this is sensed by the myeloma protein. All this may be seen in the Ouchterlony reaction where the McPC603 IgA myeloma protein that binds to both PM LPS and R36A PnC and where the IgA TEPC15 binds only to R36A PnC (Fig. 1). The Ig genes that form these two proteins have structural differences, the same H chains but different L chains. Their binding sites differ in the ability to recognize the context of the choline group. The McPC603 IgA protein can bind the PC on the LPS, the TEPC15 cannot. Lathe Claflin and Katie Williams began a series of studies on immune responses to PM LPS and discovered that the antibodies to PM that strongly bound the LPS all contained characteristic somatic hypermutations that were required for this reactivity to the LPS. The M167 protein may have even yet another phosphorylcholine specificity that is associated with binding to lipoproteins (Leon and Young, 1971).

Using the T15 myeloma protein as a reagent, PC antigens have been found on a variety of species of bacteria and fungi and even many allergens (Baldo *et al*., 1979). Brown and Crandall (1976) discovered it on the parasite *Ascaris suum*, and others have since found on many nematode species (Lochnit *et al*., 2000). T15 also reacts with the antigenic determinant, 1-palmitoyl-2-(5-oxovaleroyl-α-glycero-3-phosphorylcholine)-oxidized LDL (so called bad cholesterol) in atherosclerotic lesions and apoptotic cells (Binder *et al*., 2005; Shaw *et al*., 2003).

Together, the PC-binding myeloma proteins represent germ line-encoded natural antibodies responsive to ubiquitous stimuli that are virtually "constitutive" and are components of a first line of humoral defense, but they may also react with autoantigens generated by cell death and inflammation (Binder *et al*., 2005), and here they may play a role in antigen clearance. Normal lymphocytes and plasma cells, which produce the T15 idiotype, have been shown to be peritoneal B1 cells, a B-cell subset known to be associated with natural antibody production (Masmoudi *et al*., 1990). The natural history of neoplastic development in plasma cells producing natural antibodies is probably quite different from the antigen-hyperstimulated clones generated in artificial immunizations.

XVI. THE ANTIDEXTRANS, ANTILEVANS, AND ANTIGALACTANS

The 1967 Cold Spring Harbor Meeting was a turning point in the search for an antigen-binding myeloma protein, driven primarily by the exciting presentations of MC and Herman Eisen. In 1968, numerous other antigens were screened in La Jolla and Bethesda. Many polysaccharides were used primarily because of their multivalency, which lent itself to good precipitin bands.

ML, K. Robert McIntire, and Martin Young described the B1355 dextran-binding myeloma protein MOPC104E in 1970. MOPC104E was an unusual myeloma protein in many ways. First it was found to have a macroglobulin type H chain (IgM) and a lambda L chain, and further it was produced by a PCT not a more lymphoid cell (McIntire *et al*., 1965). MOPC104E opened up the study of myeloma proteins with anti-polysaccharide activity. Leon *et al*. (1970) tested a large series of dextrans isolated by Allene Jeanes and found that B1355S1,3 which have the highest alpha 1,3 linkages and B1234 which contained no alpha 1-3 linkages but was rich in alpha 1,2, 1,3, and 1,6 were the most effective antigens. The reactions were most effectively inhibited by nigerose but also to a lesser degree by oligosaccharides with other linkages. Lundblad *et al*. (1972) while looking for antidextrans and antilevans found W3129, an antidextran that was inhibited by oligosaccharides with alpha 1-6 linkages (isomaltose). The first antilevan was J606 an IgG3 myeloma protein (Grey *et al*., 1971).

All this commotion brought the legendary Elvin A. Kabat (EAK) into the mouse myeloma business. He had long-standing interests in immunochemistry and anti-polysaccharide immunology, including antibodies to blood groups for which he was famous and importantly a large library of oligosaccharides. He and John Cisar (Cisar *et al*., 1974; Lundblad *et al*., 1972) began characterizing the available myeloma proteins with antidextran activity.

EAK was the true dean of immunochemists, author of a major (and huge) book on "Experimental Immunochemistry," and the recipient of honorary degrees and the National Medal of Science in 1991. After completing his college degree, EAK began his immunological career with Michael Heidelberger in 1932 as an overqualified laboratory assistant cleaning glassware. This was during the depression when jobs were very scarce. These duties soon gave way to participation in experiments, and Heidelberger must have quickly recognized his talents. EAK's early experiments dealt with the purification of antibodies. Michael Heidelberger had spent two summers in Uppsala with Arne Tiselius, who then became very interested in the analysis of serum proteins and antibodies. An arrangement was made by Tiselius and Heidelberger to send EAK. At age 23, he embarked for Sweden but not before carefully arranging with his benefactors to provide the $50 per month for his parents.

Table I Characteristics of Plasma Cell Tumors That Produced Myeloma Proteins with Antigen-Binding Activities

Irelevant antigen	B1355 S1,3 dextran	B512 dextran	Inulin Levans	Bacterial levans	Gum ghatti	R36A PnC	*Proteus morganii* (morganella) LPS	DNP protein conjugate
				Levans	β-1,6-D-Galactopyranoside protein conjugate			
Potential natural antigen	Dietary source?	Dietary source?	Dietary source?	Gut flora?	Hardwood bedding dietary components	*Lactobacillus*4 (gut flora mouse)	*Proteus morganii* (morganella) LPS (gut flora mouse)	Menadione?
Hapten-inhibiting ligand	α-1,3-D-glucan	α-1,6-Glucan	β-2,1-Fructans	β-2,6-Fructans	β-1,6-D-galactan	Phosphorylcholine	Phosphorylcholine	Dinitrophenyl Trinitrophenyl
Plasma cell tumor	MOPC104E **J558*** UPC102	**W3129*** **W3434*** QUPC52	**J606*** **W3082*** UPC61 ABPC4 EPC109 TEPC957 ABPC47N TEPC803 MOPC702	ABPC48 UPC10 **Y5476***	**J539*** XRPC24 JPC1 SAPC10 XRPC44 TEPC191 TEPC601 CBPC4	**S63*** **S107*** TEPC15 HOPC8 MOPC299 McPC603 MOPC167	McPC603	MOPC 315 MOPC460

The principal antigen-binding PCTs, many of which are referred to in the text, are listed according to their antigen-binding properties. Many of these were discovered by an interaction (usually in an Ouchterlony double diffusion reaction) of the myeloma protein with an "irrelevant" antigen that was available in the laboratory. Subsequently a source for that antigen could be found in the environment of the mouse, that is, a potential natural antigen. The haptenic or molecular specificity of the antigenic macromolecule to which the monoclonal Ig bound was often identified (hapten-inhibiting ligand). Igs produced in different tumors that bound the same hapten were selected out from screening M components for antigen-binding activities that shared common IgVL and IgVH genes (shaded). *, Originated in the Salk Institute PCT library; MOPC, mineral oil (induced) plasma cell tumor; TEPC, tetramethylpentadecane-induced PCT; UPC, QUPC, XRPC, EPC, and JPC were paraffin oil-induced PCTs that were named for a specific experiment at the time and have no pathogenetic significance. ABPC, PCT induced by paraffin oil and Abelson virus; McPC, PCT induced by K. Robert McIntire; CBPC, CB-20 PCT induced by paraffin oil, HOPC, 7N-hexyloctadecane-induced PCT. SAPC, PCT induced in a BALB/c germ-free mouse that had been monocontaminated with *Salmonella tel aviv* organisms by Richard Asofsky; R36A, rough strain of *Streptococcus pneumoniae* that lacks a capsule. The **W** and **J** PCTs originated at the Salk Institute and were initials of investigators who collected and typed the M components.

This was the true measure of this fine man. In 1937, EAK went to Uppsala armed with purified antibodies and showed that they migrated with the gamma globulins [see Kabat (1983) for a detailed account]. EAK had worked extensively on the antigenicity of dextrans, which were candidates as plasma expanders in World War II. He had also immunized himself with dextrans and followed his own responses over a period of many years. During numerous lunches in NIH cafeterias he told me about his unusual serology, including stories about monoclonal bands, but I never saw first hand any of these electrophoretic patterns. EAK's later work on complementarity regions, that is the parts of the polypeptide chain that interacted with antigen, revealed his great plasticity and insight in immunochemistry.

Alan Sher found the J539 protein reacted with proteins conjugated with B1-6 D-galactopyranoside (Sher and Tarikas, 1971) and then Neil Glaudemans, Betty Mushinski, Stuart Rudikoff, and I found a series of proteins that reacted with galactan polysaccharides, which contained β D-1,6 galactose linkage including antigens in the mouse diet and bedding (Potter *et al.*, 1972; Rudikoff *et al.*, 1973). The field of anti-polysaccharide myeloma proteins was expanding (Table I).

In 1969 Henry Metzger, Bill Terry, and I organized the first workshop on homogeneous antibodies, a series that continued until 1979 (Fig. 2). From 1971 onward, Martin Weigert and I organized this meeting on a yearly basis. These discussions and the contributions of the many participants continually produced exciting new insights on the structural and genetic basis of antibody diversity. They provided the experimental system that the molecular geneticists would subsequently use to define the mechanisms in antibody diversity.

The two collections of myeloma proteins in Bethesda and La Jolla began to create groups of proteins with the same antigen- and hapten-binding activities (Table I). When the light-chain variable domain (VL) or VH polypeptides within these groups were sequenced from Igs that bound the same hapten, they were found to have the same prototypic L and H chain amino acid sequences with minor variations (Rudikoff *et al.*, 1973). All this supported the multiple gene basis of diversity, suggesting that during the evolution of the mouse specific sequences had been conserved because binding activities to the respective antigens were useful for the survival of the species, specifically for the groups of functional proteins (Table I). Martin Weigert began comparing lambda L chains in the mouse. These were intriguing because lambda L chains were infrequently expressed and one could now focus on them as if they were all derived from a single gene. He established the first molecular evidence that sequence variants of this chain were generated by somatic mutations (Weigert *et al.*, 1970).

Fig. 2 Group Picture Homogeneous Immunoglobulin Workshop I, 1969. Front row: Kris Hannestad, M Potter, Ernest Simms, Paul Anderson, Mason Smith, K. Robert McIntire, William Terry, Frank Richards, U. I., Henry Metzger, Allen Kaplan, Lawrence Levine, Elvin Kabat, Tom Waldmann, William Konigsberg, Lee Hood, Henry Azar. Second row: Robert Ashman, J. Frederic Mushinski, Richard Asofsky, Herbert Rapp, Edward Goetzl, Herman Eisen, Philip Periman, Elliott Osserman, Allen Grossberg, Mathew Scharff, Richard Krause, Myron Leon, Oliver Roholt, Tibor Borsos, Michael Mage, Henry Kunkel, Melvin Cohn, Howard Grey.Third row: Normal Talal (between Rapp and Smith), John Fahey (between Krause and Kaplan), Martin Weigert (behind Leon), David Davies (between Kunkel and Konigsberg), U. I. (unidentified).

On the basis of various studies of the antigen-binding myeloma proteins in mice, probably less than 5% were found to bind an antigen and not all of these interactions involved relevant antigens.

In general, none had the extraordinary high affinity for their respective antigen that is characteristic of a T-cell-dependent immune response and many reacted as might be expected of a natural antibody. The antigens to which the other 95% of myeloma proteins react are unknown as yet. But possibly when more systematic approaches are taken many more matches will be found. This information may become very useful in understanding the population of B cells that get caught up in neoplastic transformation in both humans and mice.

XVII. GROWING PCTs IN CULTURE AND GROWTH FACTORS

When PCTs became more available, many workers attempted to establish them in tissue culture. The story was always the same: "We have put the PCT cells in culture, they are doing fine, growing vigorously, will call you back next week to report the progress"—but the calls never came because the cells failed to sustain their initial growth and died. George Sorenson and Olive Pettengill (Pettengill *et al.*, 1966), after a painstaking effort, finally established X5563 in culture. In 1964, MC, now well established at the Salk Institute, requested a series of established BALB/c transplant lines (AdjPC5, MOPC21, MOPC46, AdjPC6A, and so on). He was gearing up to generate his own library of tumors. He designated the Bethesda transplant lines, P1 (AdjPC5), P2, (?) P3 (MOPC21), and so on, for convenience. Leo Sachs from the Weizman Institute was visiting at the Salk Institute for several months. He casually asked MC if there was anything he would like for him to do and MC answered by saying, "Oh, yes, could you put some of these plasma cell tumors in culture?" "No problem," said Sachs. He planted the PCT cells in culture with the usual story; however, when his cultures seemed to begin failing, he injected them back in mice, and after several such mouse-culture cycles, he had selected a successful cell line from the P3 (MOPC21) tumor. This turned out to be an ideal line, as the cells produced an abundance of IgG1 myeloma protein. A beautiful study of this vigorous myeloma protein-producing line was written up and sent to Science for publication, and I was asked to review the paper. I told the editors of Science that this was a landmark paper and the authors should be prepared to receive many requests for the cell line. This was duly transmitted to Ed Lennox and Kengo Horibata who had frozen the line. They tried to revive it, but alas! it was not recoverable. They withdrew the paper from Science. Later Horibata and Harris

(1970) repeated the work and published it. MC's laboratory established many lines in culture and became a source of tissue culture lines to the scientific community.

However, the mystery of why PCTs were so difficult to establish in culture was not solved by Sach's method. Great insight into the problem unfolded later from a beautiful study by Yuziro Namba and Masao Hanaoka in Kyoto (Namba and Hanaoka, 1972), who showed that the MOPC104E PCT could be established in culture so long as supporting stromal cells were present. When these stromal cells failed to survive, as they routinely did, the PCTs began their denouement. When macrophages were added back the PCT cells revived. Namba and Hanaoka (1974) then went on to isolate the factor from the stromal cells, they designated Phagocytic Cell Factor. The estimated molecular weight of this factor was 50 kDa.

Later, Richard Nordan (Nordan and Potter, 1986) at the NIH found another PCT (TEPC1165), which required a factor produced by macrophages isolated from the peritoneal cavities of BALB/c mice injected with pristine. This new factor, Plasmacytoma Growth Factor, was soon renamed IL-6. Its molecular weight was 20–30 kDa, suggesting it was not the same as the Namba–Hanaoka factor (Phagocytic Cell Factor), but its biological behavior was the same. I still think Phagocytic Cell Factor was IL-6, but if I am wrong there is another factor waiting to be discovered. With the availability of IL-6, it is now possible to establish many (but not all) PCTs in tissue culture.

One recipient of P3 was Cesar Milstein at Cambridge, whose abiding interest was in the mutagenesis of Ig genes. He used P3 as a target for mutagenesis experiments and produced a fascinating number of P3 mutants with deletions of domain segments of the H chain, but this was not quite what he wanted. What he sought was a monoclonal Ig with a known binding activity. His laboratory then turned to fusing PCT cells. He first made 8-azaguanine- and 5-bromodeoxyuridine (BrdU)-resistant lines so that the rare hybrids could be selected. He made hybrids between P1 (AdjPC5) and P3 (MOPC21) and even fused a rat PCT with a mouse PCT. The colleague who did these experiments left the laboratory and Cesar Milstein went back to his original model P3 mutation experiments. He entertained the thought of using MOPC315 because it had a known antigen-binding activity but was not able to establish it in tissue culture (Wade, 1982). Then in 1975, George Kohler arrived as a postdoctoral fellow and he asked him to screen P3 to find an antigen-binding activity, but George Kohler was very reluctant to do this. Instead he proposed to use P3 as a fusion partner to normal plasma cells from a mouse immunized to a known antigen. Cesar Milstein agreed, and Georges Kohler immunized mice with sheep red blood cells to produce the first antibody-producing hybridomas. Many laboratories had attempted and hoped to do this and failed. From the moment of the Kohler–

Milstein success the world of antibodies changed forever. The age of hybridomas and monoclonal antibodies rendered the antigen-binding myeloma proteins as prehistoric. After hearing Milstein report of these findings at a conference at the Rockefeller Institute in 1976 (Milstein and Kohler, 1977), I walked back to the hotel with EAK. I told Elvin on the street corner, "If these guys are right, they have put me out of business," to which he replied, "Don't be hasty—let's see if this is reproducible."

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FOUNDATIONS IN CANCER RESEARCH*

Mouse Mammary Tumor Biology: A Short History

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For over a century, mouse mammary tumor biology and the associated *Mouse mammary tumor virus* (MMTV) have served as the foundation for experimental cancer research, in general, and, in particular, experimental breast cancer research. Spontaneous mouse mammary tumors were the basis for studies of the natural history of neoplasia, oncogenic viruses, host responses, endocrinology, and neoplastic progression. However, lacking formal proof of a human mammary tumor virus, the preeminence of the mouse model faded in the 1980s. Since the late 1980s, genetically engineered mice (GEM) have proven extremely useful for studying breast cancer and have become the animal model for human breast cancer. Hundreds of mouse models of human breast cancer have been developed since the first demonstration, in 1984, that the mouse mammary gland could be molecularly targeted and used to test the oncogenicity of candidate human genes. Now, very few scientists can avoid using a mouse model to test the biology of their favorite gene. The GEM have attracted a new generation of molecular and cellular biologists eager to apply their skills to these surrogates of the human disease. Newcomers often enter the field without an appreciation of the origins of mouse mammary tumor biology and the basis for many of the prevailing concepts. Our purpose in writing this short history of mouse mammary tumor biology is to provide a historical perspective for the benefit of the newcomers. If Einstein was correct in that "we stand on the shoulders of giants," the neophytes should meet their giants.

ABBREVIATIONS

CRGL, Cancer Research Genetics Laboratory; DBA, dilute brown agouti; DCIS, ductal carcinoma *in situ*; ER-alpha, estrogen receptor alpha; GEM, genetically engineered mice: HAN, hyperplastic alveolar nodule; HRE, hormone response element; LTR, long terminal repeat; MIN, mammary intraepithelial neoplasm; MMTV, mouse mammary tumor virus; NCI, National Cancer Institute; NKI, Netherlands Cancer Institute; PyV, Polyomavirus; RSV, *Rous sarcoma virus*.

I. INTRODUCTION

One hundred years ago, Hugo Apolant published his definitive description of spontaneous mouse mammary tumors (Apolant, 1906). Although the intervening century has brought numerous advances, the seeds for the current concepts of mammary tumor biology were carefully planted by our predecessors. Many of the fundamental principles developed by these pioneers are as sound today as they were 100 years ago. They are simply now confirmed, verified, and expanded by modern technology. The story has been

documented in various historical accounts (Dunn, 1945, 1958). However, the advent of genetically engineered mice (GEM), starting in 1984 (Stewart *et al*., 1984), has attracted a cadre of new investigators into the field. Most of these newcomers come from backgrounds in genetics and molecular and cellular biology. Although the field has been enriched by investigators with diverse backgrounds, this does not always include basic knowledge about the origins of mouse mammary tumor biology. This naiveté has sometimes resulted in the publication of "new" observations that, in fact, were previously described but not found on PubMed or other electronic media accessible to the current generation of scholars. The purpose of this chapter is to provide a concise review of the early history of mouse mammary tumor biology from the perspective of modern day research.

We will strive to cover the major concepts, trends, and directions in mouse mammary tumor biology. In so doing, we will try to provide the interested reader with access to more pertinent literature that might fit their specific interests. We may inadvertently slight some of our colleagues by omitting their work or by not giving sufficient detail to accurately portray their contributions. We believe that the strengths of the overview will outweigh its inevitable shortcomings.

Neither of us could thoroughly interpret the French and German texts of the early 1900s. We have made a valiant effort to interpret, or have had someone else interpret, key sections found in these papers. We did review the illustrations, when available. All of the papers quoted have been read or, at least, viewed by at least one of the authors. We are indebted to the many earlier reviews of mouse mammary tumor biology and of carcinogenesis in general. We are particularly indebted to Dr. Thelma B. Dunn who provided the scientific community a great service by her detailed accounts of the early German and French literature. Without her interpretations these articles would not have been as accessible to us (Dunn, 1945, 1958).

Our chapter is divided into early and more recent events. Each epoch is marked by a critical conceptual or technical advance and is based on the work of the previous period. Our goal is to convey the continuity and interdependence of these advances in our knowledge and ideas concerning pathogenesis of breast cancer.

The initial studies of mouse mammary tumors were based on mice obtained from "breeders." Some investigators maintained records of "families" (Bashford, 1911a; Haaland, 1911; Murray, 1911). The conceptual and technical barriers, however, were limiting, particularly in the efforts to transplant tumors between animals. The biology of transplantation was unknown and, with the sole exception of the Jensen lines (Jensen, 1903), all attempts at serial tumor transplantation were stymied until the development of inbred, genetically identical laboratory mice by Little and Tyzzer (1916) and Strong (1935, 1936). The inbred mouse strains also facilitated development of the new field of endocrinology with an emphasis on the effects of ovarian and

pituitary hormones on mammary tissues. These effects as well as chemical carcinogenesis and tumor biochemistry were explored (Moulton, 1945).

The search for the cause of cancer in high mammary tumor strains led to the discovery and verification of the extrachromosomal "milk agent" at the Jackson Laboratory and in the Netherlands. This discovery heralded a new epoch featuring the "cause" of familial breast cancer, namely the *Mouse mammary tumor virus* (MMTV) (Laboratory, 1933). Although comparable viruses have not been identified in human or other species, each generation, armed with new technologies, has renewed the search.

With the understanding of the major histocompatibility that governs transplant rejection (Snell, 1948), came the ability to study neoplastic progression using transplantation of preneoplastic breast tissue into inbred, syngeneic mice (De Ome *et al.*, 1959; Dunham and Stewart, 1953). The focus now turned to neoplastic progression and viral infection (Shimkin, 1979a). The 1970s were sparked by the newly emerging techniques of molecular biology and molecular genetics (Shimkin, 1977a). Dutch investigators discovered a germ line oncogenic MMTV before the viral oncogene hypothesis was developed (Bentvelzen and Daams, 1969). The era focused on the ramifications of the oncogenes and unraveling the role of MMTV. After the realization that some oncogenic viruses, like *Avian leukosis virus*, transduced a protooncogene, others, like MMTV, were found to inserts their DNA into the host genome thereby insertionally activating oncogenes (Hayward *et al.*, 1981; Nusse and Varmus, 1982; Nusse *et al.*, 1984).

MMTV itself became secondary to the molecular mechanisms of oncogenesis but, because of its long terminal repeat (LTR), the virus became the basis for considerable research on hormone regulation (Yamamoto *et al.*, 1983). The promotion–activation characteristics of the MMTV-LTR served as the foundation for the most recent period of research in GEM and mouse mammary tumor biology in which the LTR was used to promote the myc transgene, thus triggering a new era of mouse mammary tumor research (Stewart *et al.*, 1984).

Genetic engineering of mice and, specifically, by targeting mouse mammary glands introduced a new era in the history of mouse mammary tumor biology (Paigen, 1995). The new technology created tumors that were unknown to the early biologists, opening a new approach to breast cancer research. The mouse is now used to test the biological properties of every potential human mammary oncogene. The challenge for the modern investigators is to understand how we arrived at this point so that their research will advance the biology and not rediscover which is already known. The basic themes voiced at the turn of the twentieth century are the same as those studied by current investigators: What is the role of the three factors: genes, hormones, and etiology?

We will relate mouse mammary tumor biology to our current molecular-based understanding of breast diseases. The focus will be on mammary neoplasms and will only briefly mention adjunct fields, such as endocrinology and immunology, which have contributed to our understanding and have blossomed into separate fields worthy of separate historical reviews.

II. THE DAWNING OF EXPERIMENTAL CANCER RESEARCH

A. The Origins of the Laboratory Mouse

The house mouse has lived with mankind for as many years as man cultivated grains and probably before. Most authorities trace the origins of the laboratory mouse to Asian mouse fanciers where descriptions of fancy mice appear in ancient Chinese texts (Keeler, 1931; Morse, 1978, 1981).[1] The first written record appears in the Chinese lexicon "EhYah." In 1100 BC, the book recorded a dominant spotting mouse. The Egyptians from the same era have pictographs of colored mice. The dancing mouse also known as the waltzing mouse that became the basis for the first inbred strain (DBA, i.e., dilute brown agouti) was recorded in the annals of the Han Dynasty in 80 BC. The interest in colored mice increased during the subsequent centuries with over 30 separate descriptions. By the 1700s, the increasing interest in Japan led to a booklet on "Breeding Of Curious Varieties Of The Mouse" published in 1787. By the early 1800s, the fancy mouse had made its way to Europe and the United States. By 1895, formal mouse fancier clubs were established. These groups became the source of mice recorded in the scientific literature from 1890 to 1910 as "from a breeder."

In Western antiquity, the house mouse received considerable attention. The Pontic mice were the most famous in ancient western literature. They were venerated because they ate the leather armor straps of the Greek's enemy, leaving the Pontians vulnerable to attack. As a result, a shrine was built on the Pontic isle of Tenedos where the mice were kept at public expense. A cult developed around these mice and spread throughout the Grecian Mediterranean. Mutations apparently appeared in this population as Aristotle wrote of the white mice of Pontis (Keeler, 1931). White mice were also described by a number of subsequent authors.

Keeler notes that mice were generally considered pests in the west (Keeler, 1931; Morse, 1978, 1981). Perhaps, our distain originated with the ancient

[1] Excellent reviews of the ancient literature appear in Keeler's 1931 monograph on the Origins of the Laboratory Mouse and Morse's volumes on the Originof the Laboratory Mouse.

Egyptians who deified cats because they ate the mice who ate the grain. Thus, cat-like sculptures appear in Egyptian shrines. In spite of the distain for the mouse, the early western literature has mention of the mouse being used for "auguries" (fortune telling). They also showed up as part of medieval pharmaceutical potions. Consequently, mice were obviously kept by some groups of people and not considered completely useless. In fact, as Morse reminds us, mice were used as experimental animals and appear in published investigations including Priestly's (1775), who found, when placing mice in oxygen free chambers, that "... a mouse lived perfectly well ... but died the moment it was put into the other part ...." (Morse, 1981).

Therefore, we owe a debt of gratitude to the Asian culture which actively cultivated mice. The Chinese and Japanese kept "fancy mice" such as the famous waltzing mouse. The opening of trade between the orient and the west brought the fancy mice to the west. Breeders became fascinated with these creatures and domesticated them to form the foundation for the development of the modern laboratory mouse.

B. The First Mouse Mammary Tumors

> *6. Malignant Tumor on the Pectoral Muscle of a Mouse (M. musculus).*
> The animal, caught in a trap, was of the usual size, and upon the right pectoral muscle was a hard, scirrhus-like tumor, the size of a large nut. It presented, when microscopically examined, many of the appearances supposed to be characteristic of scirrhus.
> (Crisp, 1854, 4th April)

Although the early mouse breeders recognized that certain families of mice developed tumors, the first formal description of a mouse mammary tumor in the scientific literature is credited to Crisp in 1854 (Crisp, 1854; Dunn, 1945). He offered the opinion that the tumor was a "scirrhus-like tumor." The Crisp description is a short five-line paragraph appearing in an issue of Pathology Transactions. The first line is noteworthy in that the mouse in question was "caught in a trap." One should also recognize that the Crisp description was written before Virchow's cellular basis of disease and Waldeyer's description of the origin of epithelial cancers (Rather, 1978).[2] Classifications of epithelial tumors were not yet developed and many investigators, including Virchow himself, believed that all tumors arose from the connective tissues. This was a reasonable hypothesis given the dense stroma around many cancers and the primitive microscopes available to the scientists of that time.

By the 1890s, a number of European scientists were using mice obtained from "breeders" for experimental cancer research. Foremost laboratories

[2] The authors relied extensively on L. J. Rather's excellent book "The Genesis of Cancer" to provide the historical perspective of ideas in cancer research.

were in France under Moreau and Germany under Ehrlich. Moreau, in 1894, described a spontaneous subcutaneous tumor in the mouse as an adenocarcinoma (Dunn, 1945). Livingood, prompted by a suggestion of Dr. Welsh of Johns Hopkins, examined mammary tumors from five mice, providing convincing descriptions and drawings. His descriptions were illustrated by drawings showing pulmonary metastases and distinguishing them from pulmonary adenomas (Livingood, 1896). Because of its informal style and personal pronouns, Livingood's publication appears to be a transcript of a talk he delivered at Johns Hopkins.

The next microscopic descriptions of mouse mammary tumors were controversial and misleading as Eberth and Spude in 1898 concluded that, without obvious connections to the rest of the mammary tree, the tumors were endotheliomas (Eberth and Spude, 1898; Woglom, 1913). This conclusion is not completely unexpected since science was still under the influence of Virchow and Connheim, both of whom hypothesized that all cancer originated in the connective tissue either from blastema or from embryonic rests (Rather, 1978). Eberth and Spude interpreted the tubules and glands as endothelial. Further, the apparent lack of connection between the tumors and the mammary epithelium was a direct violation of Waldeyer's principle of direct continuity with normal epithelium (Rather, 1978; Waldeyer, 1867). The endothelial dispute persisted through 1910 with v. Hannesmann arguing that these tumors were endothelial (Woglom, 1913).

C. Spontaneous and Transplanted Tumors 1890–1911

> The most convincing of the reports was Jensen's and his classical paper published in 1903 marks the beginning of modern experimental cancer research.
>
> (Foulds, 1959, p. 8)

A century ago, in 1906, Apolant published his landmark paper that provided clear descriptions and classification of the epithelial origin of mouse mammary tumors (Apolant, 1906). His classification of the tumors was the first systematic organization based on a broad experience and has withstood the test of time. As Thelma Dunn later commented, there was little to add to Apolant's descriptions and classification (Dunn, 1945, 1958). This paper was followed by the work of Haaland, who in 1911 published the first comprehensive description of mouse mammary tumors in English (Haaland, 1911). Since Haaland, Bashford, Murray, Apolant, Borrel, and Moreau all published papers together at one time or another, quite clearly the serious scientists of the age knew each other's work and collaborated. Haaland, a Norwegian, also published papers in English, German, and French and spent time in the laboratories of Erhlich, Borrel, and Bashford until 1911 when he became the first director of the Norwegian Cancer Institute (Shimkin, 1977b).

The malignant potential of these tumors was vigorously debated in the early years and has remained topical to the current time. Livingood's (1896) paper describes and illustrates intravascular pulmonary metastases (Livingood, 1896). Borrel, in 1903, describes and illustrates, in color, intravascular pulmonary metastases (Borrel, 1903). Illustrations in Haaland (1911), Woglam (1913), and, finally, in Dunn (1945, 1958), all illustrate metastases. Nonetheless, when Jensen was awarded the Walker Prize given by the Royal College of Surgeons for his transplantable mammary tumor cell line, he was vigorously attacked by Williams who contested the malignant potential of mouse mammary tumors (Williams, 1906). This argument rages on with each generation because many of the mouse mammary tumors do not appear to be malignant by the histological criteria used for human breast cancer (Cardiff *et al*., 2000a; Foulds, 1959). Even the histologically "benign" mouse tumors, however, metastasize to the lungs making the arguments based on the histology of the primary tumor moot. The biology of the tumors provides unequivocal evidence.

The primary experimental evidence used from the very beginning was transplantation of the tumors. The initial assumption was that tumors that grew on transplantation could be regarded, by definition, as malignant. Bashford calculated that accumulative mass of transplanted Jensen tumors exceeded the mass of the single donor (Bashford, 1911a). Moreau, in 1891 and in subsequent publications, described attempts to transplant tumors autochthonously and into other mice with variable success (Dunn, 1945; Morau, 1894). The autochthonous transplants "took" but tumor transplants between unrelated animals frequently failed (Bashford, 1911a; Haaland, 1911; Murray, 1911; Woglom, 1913). The major focus in the 1890s, however, appeared to be on using transplantation to prove the existence of an infectious agent which was not found. Nevertheless, an interest in natural history and transplantation emerged from these early studies. The criterion of transplantation was later used to determine whether the tumors were malignant in inbred mice.

By 1903, Jensen, who worked with the Borrel laboratory, developed a transplantable mammary tumor line that could be passed between several unrelated mice (Jensen, 1903). He shared the line with numerous investigators and, thus, provided the resource that became the origins of modern cancer research (Dunn, 1958; Foulds, 1959). As documented above, the origin and biology of the Jensen line were controversial (Williams, 1906). The controversy was largely laid to rest by the pathological analyses of Apolant, Bashford, and others (Woglom, 1913). The Jensen line was apparently very angiogenic and described as a "hemorrhagic" carcinoma by Haaland (1911). The accompanying illustrations leave little doubt as to the epithelial nature of the tumor. Others, including Borrel, found pulmonary metastases in mice receiving transplants of the Jensen tumor.

n the early 1900s, Apolant and Erhlich also published several experiments describing transplantation of mouse mammary tumors. Morau and Borrel

continued transplantation experiments; Ernest Tyzzer was actively studying tumor transplants in the United States; and Bashford, Murray, and Haaland were doing the same in England during the early 1900s (Woglom, 1913).

Woglom's (1913) treatise provides an extensive and detailed literature up to that point (Dunn, 1945; Woglom, 1913). He provides amusing pro and con arguments as to whether the mouse mammary tumors were epithelial or endothelial, even suggesting that the German pathologists did not know the anatomy of the mouse mammary glands (10, not 2, glands) or the location of the sebaceous glands (limited to the footpads). He also records early proof of metastases and, thus, malignancy with camera Lucida drawings of tumor emboli. Judging from the attribution to an illustrator, J. R. Ford, these illustrations are from the Bashford monographs.

Woglom also discusses the cumulative evidence supporting a family distribution of breast disease and covers the concepts of etiology of breast cancer that were current in 1913 (Woglom, 1913). Familial breast cancer in mice was recognized by Haaland, Bashford, Murray, Tyzzer, and others (Bashford, 1911a; Dunn, 1945; Murray, 1911; Tyzzer, 1909; Woglom, 1913). Tyzzer, initiating transplantation experiments in the United States, recognized the importance of inheritance and is credited with the introduction of the phrase "somatic mutation" into tumor biology (Paigen, 2003). Tyzzer was also the senior mentor of C. C. Little and coauthored his 1916 paper announcing inbred mice. The project was begun by Little and Tyzzer in 1909 with the brother–sister mating of two "Japanese waltzing mice" (Little and Tyzzer, 1916). The 1916 paper was the first paper using inbred mice and featured serial transplants of the same tumor (J.w.A.) from a Japanese waltzing mouse into various genetic crosses. The "winner" was a dilute agouti mouse. Their conclusion was that transplantation susceptibility is a complex genetic trait. Ironically, the concept of the inbred mouse is not emphasized in the text.

The etiology of cancer was another topic of speculation in these early papers. Chronic irritation was the dominant theory but Erhlich's "side chain" theory and various bacterial and parasitic infections also were postulated and discussed as possible causes of cancer (Woglom, 1913). The leading hypothesis during the early 1900s was that cancer was caused by inflammation. The publications of Haaland and Murray contain illustrations of nematodes in the mammary vessels, a finding that was quite common in the mice obtained from mouse breeders (Haaland, 1911). Experiments were designed to prove the infectious or inflammatory origin of cancer in mice but provided no convincing evidence. Improved cage conditions and better hygiene cleared the colonies of nematodes but the mammary tumors persisted. Although the term "immunity" is invoked and a paper on the role of the lymphocytes in mouse mammary tumorigenesis appears, immunity as understood in the current era was unknown during the early 1900s.

Interest in inflammation, nematodes and bacteria as etiological agents of cancer, however, led to many fascinating observations. Strangely, none of these early mammary tumor biologists used bacterial filters in their attempts to isolate and characterize an infectious viral agent. The experiments of Ellerman and Bang and Rous were published during the first decade of the twentieth century (Ellerman and Bang, 1908; Rous, 1910). Borrel presented evidence that he interpreted as supporting a "cancer virus" (Borrel, 1910). However, in light of modern knowledge, Borrel's line of investigation, based largely on distribution of the disease in co-caged populations, is not at all convincing (Dunn, 1945). Ewing's book and Woglom's monograph both comment on the ongoing experiments with avian leukosis and sarcomata in chickens but this work had no apparent impact on the investigators of mouse biology (Ewing, 1919; Woglom, 1913). This is not to say that they were ignoring other possible infectious agents. The influence of such studies on Castles, Little, or Tyzzer's thinking is not clear from their writings. However, Little and Tyzzer had Jensen's cell lines and were familiar with the European literature (Little and Tyzzer, 1916).

As discussed, these early papers covered transplantation, infectious disease, inflammation, etiology, progression, and the rudiments of what we now call immunology. By 1919, Ewing in his treatise, "Neoplastic Diseases," discusses these papers on mouse mammary tumors and states that they laid the "foundations for experimental cancer research" (Ewing, 1919). Dunn credits Jensen with beginning experimental research by widely sharing his line (Dunn, 1945, 1958; Foulds, 1959). Foulds later states that Jensen's work "... marks the beginning of modern experimental cancer research." (Dunn, 1945, 1958; Foulds, 1959).

The lasting contributions from this era were the detailed descriptions of the epithelial origin of the mammary cancers by Apolant and Haaland (Haaland, 1911). Their papers were written from the perspective of the natural history of disease. As a result, they had it right and, in some ways, we have only added the details. Apolant described "adenomas" that might be precancerous (Apolant, 1906). Haaland discussed, in some detail, the role of the hyperplastic nodule, pointing out the early appearance of the nodules and their association with more aggressive cancers and direct connections with the mammary tree, leaving little doubt that these lesions were the immediate precursors of cancer (Haaland, 1911).

In view of modern concerns, it is noteworthy that, from the earliest times, investigators were also interested in the comparative pathology of human and mouse mammary tumors. Bashford initiated what has since been a century long discussion of the mouse as a model of human breast cancer (Bashford, 1911b). In retrospect, one can now be certain that the tumors examined by these early investigators were, indeed, induced by the MMTV, which explained their familial occurrence, maternal lineage, and the limited number of tumor phenotypes. We now know that *wnt*, *fgf*, *notch*, and other oncogenes are activated by MMTV provirus insertion (Jhappan *et al.*, 1992; Nusse and

Varmus, 1982, 1992; Peters *et al.*, 1983). Ironically, neither the virus nor these genes play a major role in initiating human breast carcinogenesis. These genes are more aligned with other human tumors including cutaneous neoplasms.

By 1913, the field had been preempted by the dramatic development and application of Mendelian Genetics to the mouse. Although the early experimental work was summarized by Woglom (1913) and in shorter form by Ewing (1919), we are all indebted to the more accessible summaries written in 1945 and 1954 by Dr. Thelma Dunn (Dunn, 1945, 1958). Her detailed summations are invaluable to the modern reader. Dr. Thelma Blumfield Dunn was a pathologist trained at George Washington who was in Harold "Red" Stewart's National Cancer Institute (NCI) Division of Pathology. Her publications record spans a period from 1945 to 1994. Much of her work involved the description and classification of murine tumors in a variety of organs, including the mammary gland. Each document remains useful today because each was concise, well organized, and clear. In particular, she eschewed the use of unfounded speculation and used simple terms.

III. MENDELIAN MOUSE GENETICS: 1909–1920

> Mendel, who had begun his research breeding mice in cages he kept in his two-room quarters, had to turn to his garden when his bishop insisted that it was not appropriate for a monk to share his living quarters with creatures that had sex and copulated!. . . .
> (Paigen, 2003, p. 1)

In 1900, de Vries, Correns, and von Tschermark rediscovered the work of Gregor Mendel (Paigen, 2003). Mendel apparently began his landmark studies using mice but was forbidden to continue because his Bishop, Anton Ernst Schaffgotsch, considered the mouse a lascivious animal whose sexual behavior was improper for study by a monk. As a result, Mendel is remembered for his study of sweet peas and not mice (Paigen, 2003). Cuenot is credited with the first demonstration of Mendelian principles in a 1903 publication on mouse coat colors (Paigen, 2003).

The Harvard group led by Castles was at the forefront of the new Mendelian genetics. One of his graduate students, Clarence Cook Little, took on the task of discovering the genetic basis of cancer (Snell, 1992). When accepted this undertaking, Little was in a scientific environment that already was studying familial distribution of mammary tumors in mice. His work with Ernest Tyzzer whose wide range of interests in murine pathology led to the identification of numerous infectious diseases as well as insight into cancer biology. Tyzzer, in fact, is credited with recognizing and naming somatic mutation as a cause of cancer (Wunderlich, 2002). Tyzzer was also experimenting with transplantation of mammary tumors (Tyzzer, 1909). Clearly, Little had the advantage of a very stimulating environment.

Other investigators, including Leo Loeb and the mouse fancier Ms. Abbie E. C. Lathrop, had shown that certain "races" of the waltzing mouse developed mammary tumors that could be transplanted to all strains of the waltzing mouse (Shimkin, 1975). Tyzzer and Little used the waltzing mouse as a test recipient for a complex series of tumor transplantations. Starting with a domesticated mouse with a long history among mouse fanciers gave them an obvious advantage. Through the series of transplantations of one mouse mammary tumor from a Japanese waltzing mouse (J.w.A.), Tyzzer and Little began to suspect they had found a complex Mendelian inheritance that might explain susceptibility to transplant tumors. Little realized that his goals would be best accomplished with genetically identical, inbred mice. Little started in 1909 with a brother–sister mating and after 20 such serial matings established the dilute brown nonagouti (Dba) which became the first inbred mouse strain, the DBA (Little and Tyzzer, 1916). The idea was accepted and extended by many of Little's colleagues with many of their initial papers appearing between 1920 and 1935 (Strong, 1935, 1936).

The foundations for the inbred strains had been laid by the mouse fanciers. The best known is the Granby (Massachusetts) Mouse Farm of Abbie Lathrop. Her career is well chronicled in several reviews (Morse, 1981; Shimkin, 1975). Ms. Lathrop was a retired school teacher and mouse fancier who had sufficient knowledge of her mice and their tumors that she not only pointed scientists in the right direction but she became a coauthor on eight of their papers. One of her papers with Leo Loeb suggests the possibility of an extrachromosomal factor (Lathrop and Loeb, 1916). In this case, they used castration as the operational tool to identify an "internal secretion." Sadly, Ms. Lathrop died at 50 years of age with pernicious anemia caused by vitamin B12 deficiency, a controllable disease in modern times (Shimkin, 1975).

The development of the DBA and other inbred strains has been thoroughly reviewed elsewhere (Morse, 1978). The inbred mouse opened the door to the next major chapter in experimental cancer research that has yet to be closed. With a few exceptions, subsequent studies have been largely based on the inbred laboratory mouse which has become a powerful surrogate for human disease (Paigen, 1995).

IV. THE INBRED MOUSE IN MOUSE MAMMARY TUMORIGENESIS: 1920–1930[3]

> I have often wondered what the world of genetics would be like if Little had studied guinea pigs and if Wright had studied mice.
>
> (Crow, 2002)

[3] The most comprehensive account of the development of the inbred mouse to date can be found in Karin Rader's extensively documented book "Making Mice" (Rader, 2004).

The introduction of inbred mice was a major advance in experimental cancer research. The 1920s are not particularly remembered for major breakthroughs. A review of the literature from the era, however, shows that the period was far from inactive (Moulton, 1945). In retrospect, the 1920s was a decade of consolidation as the scientific community began to evaluate and exploit the inbred mouse. A thorough review of this era can be found in the 1945 NCI monograph edited by Moulton (1945).

Most experimental cancer researchers of the era used mice in their investigations. During this period, the fundamental rules of reproductive endocrinology were written by Leo Loeb. Detailed descriptions of the anatomy and histology of the mouse organs and tissues were written. Chemical carcinogens were applied to the mouse. Transplantation of tumors went forward briskly. The mouse was increasingly used as the new ideas of biochemistry were tested. For example, mouse mammary tumors played a prominent role in Otto Warburg's hypothesis that all cancers are glycolytic, favoring anaerobic glycolysis for their energy supply (Greenstein, 1945).

However, the scientific world seemed to be waiting for the next two major advents in mouse mammary tumorigenesis, namely, the discovery of the "extrachromosomal factor" that became the MMTV and the discovery of the major histocompatibility genes (MHC).

V. THE EXTRACHROMOSOMAL FACTOR: 1933–1940

> John J. Bittner always emphasized that he took on the study of the Mother's milk because nobody else wanted it.
>
> (Personal communication from Dr. Franz Halberg, a long time collaborator and University of Minnesota fellow faculty member, 2006)

In 1933, the staff of the Jackson Laboratory, led by C. C. Little, announced in *Science* the discovery of an extra chromosomal milk factor that was the cause of mammary tumors in inbred mice (Laboratory, 1933). These observations led the way to a revolution in mammary tumor biology. Although the initial announcement was suitably understated, the 1933 paper triggered decades of research leading to contributions in retrovirology, such as insertion activation of protooncogenes, steroid response elements, and mammary gland-specific promoters. The discovery of MMTV also led to the extensive but, thus far, futile searches for a human breast cancer virus.

Like all of us who "stand on the shoulders of giants," a precedent was set by the former school teacher, Abbie Lathrop, and Leo Leob when they described hybrid crosses in which the mammary tumors sorted with the mother's tumor phenotype (Lathrop and Loeb, 1918, 1919; Shimkin, 1975). However, the mice were not inbred and other explanations were possible.

The landmark paper from the Jackson Laboratory staff came 15 years later (Laboratory, 1933). The 1933 paper describes reciprocal crosses between high incidence mice, identified only as "D, A, and Z," and low incidence "C57 blk," "I," or *Mus bactrianus* mice. The paper reported that mammary tumors always depended on the mother's risk (Laboratory, 1933). Korteweg, a Dutchman from the Netherlands Cancer Institute (NKI), used "Murray-Little dilute brown (DBA)" and "C57 black Little" strains he obtained from Jackson Laboratories in 1931 to reproduce the experiments and obtained the same results that included a backcross of F_1 to each parental strain (Korteweg, 1934, 1936). Although the effect was obviously not through the chromosomes, cytoplasmic inheritance and an "intrauterine factor" were offered as alternatives to a milk factor. Korteweg, in what appears to be a more extensive English version of his 1934 paper written in Dutch, particularly favored a cytoplasmic inheritance hypothesis (Korteweg, 1936).

In retrospect, the scientific community is fortunate that the GR/A or the DDD strains had not yet been developed and were not used in these experiments because the tumorigenic MMTV2 is located in the germ line DNA of these strains. Thus, the GR males transmit the "factor" to their progeny. Consider the confusion if the early investigators had access to GR/A or other strains such as DDD. Since the GR strain was primarily developed at the NKI, Korteweg was the most at risk of missing the importance of MMTV in milk. However, the GR strain was not developed until after World War II when the NKI Director Otto Muhlbock obtained the mice from a Swiss investigator (Hilgers and Sluyser, 1981).

John J. Bittner drew the assignment to explore the nongenetic alternative, the milk factor. His research was so compelling that the agent eventually became widely known as the "Bittner Virus" (Gross, 1970). Several aspects need consideration here. First, Little, along with his arch nemesis, Maud Slye, was certain that the heredity of mammary cancer could be understood through these mice (Crow, 2002). After the 1933 paper, Little chose to concentrate on the genetics and apparently assigned the young Dr. Bittner to pursue the infectious etiology. Bittner himself is quoted as saying that he took the study of mother's milk because no one else wanted it.[4] When Bittner left the Jackson Laboratory for the University of Minnesota in 1942, primary interest in "Bittner's virus" left with Bittner.

At that time in history, viruses were just becoming understood. However, the failure of earlier investigators in the 1890s and 1900s to find an infectious factor associated with mouse mammary tumors somewhat colored the field. Although tumor-associated viruses had been identified as early as

[4] Personal communication from Dr. Franz Halberg, Bittner's University of Minnesota colleague and collaborator.

1908 by Ellerman and Bang (1908) and 1911 by Rous (Rous, 1910), the scientific community resisted the concept of a comparable mammalian tumor virus. Borrel, in 1910, had suggested that mouse mammary tumors might be due to a virus (Borrel, 1910). Nonetheless, we cannot find any evidence that the early mammary tumor biologists made any effort to find a virus and the prevailing "wisdom" was that tumor viruses were limited to chickens. The identification of the mouse leukemia viruses by Gross in 1951 was still 18 years away (Gross, 1951). Thus, Bittner had almost no precedent and a potentially hostile environment to lead the way. Further, he had no handy biological assay to confirm his work, making each experiment excruciatingly lengthy. The emergence of tumors was the only proof of viral activity. He needed to wait for 1–2 years for his endpoint.

Bittner's first really informative experiment was the *foster* nursing of pups born of low-strain mothers by high-strain mothers and vice versa (Bittner, 1936). These studies published in 1936 elevated the extrachromosomal factor to the "milk factor" and clearly suggested an infectious agent. These observations were quickly confirmed and extended (Andervont, 1945). Various techniques used to prepare the milk proved that the agent was rather fragile. This led to the formulation of Bittner's three influences for the etiology of cancer: (1) a genetic factor of susceptibility, (2) a hormonal factor, and (3) a milk-borne agent with the general properties of a virus (Foulds, 1975).

VI. THE MILK AGENT 1936–1970

> Interesting as these factors were, however, they could not fully explain the development of breast cancer in mice. Thus, for many years, the etiology of mouse mammary carcinoma remained obscure.
>
> (Gross, 1970, p. 239)

A. Filterable Agent

Again, our task is to give a broad overview of the experimental activities that involve mouse mammary tumors. In the late 1940s to the late 1950s, new technological advancements made the examination of an old problem possible, that is detection of the "milk agent." The milk agent is what we now know as the MMTV. Bittner and colleagues, in 1936, suggested that a virus was contained in mammae and that the rates of viral duplication increased in midlactating mammae (Bittner, 1936). The critical experiment was the demonstration that the agent would pass through bacterial filters and through the Seitz or Berkfield filters (Andervont, 1945, 1957; Bittner, 1942). Thus,

the agent was smaller than a bacterium but particulate. Bittner then moved to the University of Minnesota Medical School. Again, the major problem with Bittner's observations over the years was the inability to detect the "agent" from fractionated lactating mammary glands. Excluding that minor limitation, his discovery was extraordinary in that, for the very first time, a "true" carcinoma caused by a filterable agent (virus) was detected in a mammal.

Bittner made several additional observations: the importance of the host in the final assault by the virus, including the significance of the genetic background, the hormonal makeup in the formation of the mammary tumor, and the age of the host. In some respects, the modern tumor biologist shares Bittner's concerns but now addressed with new words: (1) host factors (genetic), (2) initiating oncogenes (virus), and (3) promoter context (hormonal factor). By 1945, Andervont was emboldened to speculate that the Bittner agent was a virus (Andervont, 1945). A detailed account of Bittner's and Andervont's experimental observations has been provided in Ludwig Gross's monograph (Gross, 1970).

B. Electron Microscopy

The next set of discoveries concerning the "milk agent" was just as provocative. By the late 1940s, the particulate nature of what was increasingly called the Bittner agent had been established by several groups, including the new NCI. The first practical electron microscope was built in 1938 by Burton in Toronto, Canada (Burton *et al.*, 1939, 1940). After World War II, biologists started realizing the potential of magnifications of as great as 2,000,000×. The logical step was to use the Electron Microscopy (EM) to study the structure of submicroscopic particles. Starting with Porter and Thompson in 1948, investigators reported that abundant particulate "entities" could be observed *in vitro* from C3H mammary epithelial tumor cells (Porter and Thompson, 1948). The initial study did not use thin sections.

Leon Dmochowski gets credit for setting the record straight on the milk agent. An outgoing personality and M.D. Anderson electron microscopist, he usually kept his oral presentation slides stuffed in a trench coat. This was no easy task because, in those days, EM presentation slides were 3×5 in.^{2} double glass plate "lantern slides." He presented the most convincing evidence that the EM particles were the Bittner agent by simply using identical mouse strains, one noninfected with the "milk agent" and the other uninfected. Dmochowski showed that the agent could be observed in the intracellular spaces and cytoplasm and that the agent was composed of an internal dense center with an outer, paler zone, surrounded by a bilamellar

membrane (Passey *et al.*, 1950a,b). Later, Bernhard, a strict renaissance man somewhat resistant to change in scientific experimentation, further defined the A and B particles of the "agent" and confirmed the presence of virus-like particles (VLP) in other mouse strains (Bernhard *et al.*, 1955). Bernhard's nomenclature, although mostly in the French literature, was eventually reviewed and published in English (Bernhard, 1958). His criteria are still maintained to distinguish between oncogenic VLP.

Bernhard would be interested in N. Sarkar's observation of similar particles in the milk of high-risk Parsi women (Moore *et al.*, 1969; Sarkar and Moore, 1972). Unfortunately, Sarkar later showed that the EM "particles" were an artifact of negative staining. As provocative as this may all seem, intact viral particles have yet to be detected by EM in biopsies of human breast tissue or milk, an observation recognized some 40 years ago (Roy-Burman *et al.*, 1973). Dan Moore, in 1955 (Dmochowski *et al.*, 1955), published a report also examining by EM the "agent" in RIII and C57Bl mouse mammary tumor cultures, but only sporadically visible in thin EM sections of the primary tumors.

A bit surprising is the fact that both Moore and Dmochowski collaborated sometime before 1955 to examine EM sections of both normal and tumor epithelia in high and low tumor-bearing mice (Dmochowski *et al.*, 1955). Although not published, Moore also experimented during this time with Coon's fluorescent antibody technique in an attempt to determine the location of the "agent" but without success. The presence or absence of VLP was sometimes confusing as electron microscopists observed classical A and B particles in low-incidence strains of mice such as the C3Hf (Pitelka *et al.*, 1964). Either the virus existed in different functional states or the host was playing a major role in its control. EM was informative but not biologically revealing.

C. Infectivity Assays

As indicated above, the standard tumorigenesis assays were very cumbersome and time consuming limiting their usefulness. With EM showing VLP in both high and low cancer mouse strains, the mere presence or absence of particles was clearly not a surrogate breast tumorigenicity assay. Progress was accelerated somewhat as Nandi and De Ome introduced the "nodulogenesis assay" that became a surrogate assay for infectivity (Nandi, 1963a,b). The assay used 3- to 5-week-old nulliparous female BALB/c mice that were injected intraperitoneally with the tissue, milk, or blood extract and stimulated with either subrenal capsule or subcutaneous transplants of pituitary glands to produce a pseudo-lactation. With removal of the pituitary transplants, the mammary glands regressed leaving the hyperplastic alveolar

nodules (HAN). The HAN presence became the "marker" for MMTV infection. The process shortened detection assays from years to 4 months. Now, one could test for viral infectivity in a relatively short period.

Led by Etienne "Steve" Lassfargue, Moore's group explored tissue culture methods that had become popular with experiments using transforming agents such as *Rous sarcoma virus* (RSV), Polyomavirus (PyV), and other small DNA tumor viruses. However, the milk agent proved difficult to culture and even more difficult to detect. Even though Andervont had proven that transmission of the virus could be prevented by immunization using tissue extracts of tumors, by the 1960s, many investigators had concluded that the virus was not immunogenic and were not using immunodetection. Further, almost no morphological or cytological differences could be observed between mammary tumor cells and normal cells in tissue culture. MMTV-infected mouse mammary tumor cells are contact-inhibited *in vitro* and do not form foci, colonies, or plaques as observed in cultures with the transforming RNA and DNA tumor viruses (Vacquier and Cardiff, 1979). Therefore, the simple tissue culture technologies popularized by the new breed of cellular oncologist did not prove applicable to the mammary tumors and their viruses.

The immunology of the virus was soon worked out by Phyllis Blair and others using, at first, the Ouchterlony agar diffusion plates and then increasingly sophisticated immunological techniques (Blair, 1968). These confirmed Andervont's earlier observations of tumor immunogenicity (Andervont and Bryan, 1944) and led the way to other quantitative immunoassays and opened the way to immunodetection and quantification of the virus (Cardiff *et al*., 1968).

Experiments at the Cancer Research Genetics Laboratory (CRGL) of the University of California, Berkeley showed that the virus could be produced in large quantities from mammary tumor cells grown at high density and maintained as dome cultures (McGrath, 1971; McGrath *et al*., 1972; Young *et al*., 1976). Eventually, cell lines with sufficient virus production (Parks *et al*., 1974b; Yagi *et al*., 1978) were established and became a valuable source of MMTV. Several groups developed immunofluorescence for semiquantitative *in vitro* infectivity assays but even these proved too cumbersome for extensive use (Vacheron *et al*., 1997; Vacquier and Cardiff, 1979).

With the development of quantitative radioimmunoassays and reverse transcription assays, the virus could be detected readily (Cardiff, 1973; Howk *et al*., 1973; Lo Gerfo *et al*., 1974; Parks *et al*., 1974a; Ritzi *et al*., 1976; Sheffield *et al*., 1977; Verstraeten *et al*., 1973, 1975). These assays provided rapid detection methods but to this day, the field has no effective *in vitro* assays for the virus.

Through all of this period, the traditionalists resisted the viral concept. In 1968, the eminent virologist Harry Rubin taught his University of

California, Berkeley tumor virology classes that MMTV was not a real virus or, at least, was not tumorigenic.[5] Rubin's experience was based on the plaque assays he used so effectively for the study of RSV. The Duesberg and Blair paper (Duesberg and Blair, 1966) proving that MMTV, like the mouse and avian leukemia and sarcoma viruses, had an RNA genome was required to convince a skeptical Dr. Rubin. A further nail was put into that coffin with Duesberg's description of MMTV's bimolecular genome, a feature common to all RNA tumor viruses (Duesberg and Cardiff, 1968). However, not everyone got the message. As late as 1978 papers describing *in vitro* assays for MMTV were being rejected by eminent virologists because MMTV was "not a transforming virus and probably just a helper virus."[5] Much of the uncertainty could have been resolved with the development of a decent bioassay for MMTV.

D. The Natural History of Virus Infections

> If I had called it a virus, my grant applications would automatically have been put into the category of 'unrespectable proposals.' As long as I used the term 'factor,' it was respectable genetics.
>
> (J. J. Bittner quoted in "Making Mice," p. 201; Rader, 2004)

In the late 1930s and early 1940s, even before the milk agent was legitimatized as the MMTV, investigators were studying the natural history of the agent. Once improved assay systems became available, more detailed studies were performed. Ironically, the major impetus came not from the virological community but from immunology. Now, a great deal about the complete life cycle of the virus is known, how MMTV is adsorbed in the intestine, processed by lymphocytes, and transported to the mammary gland (Acha-Orbea *et al*., 1999). A brief summary follows.[6] Exogenous MMTV (and replication competent endogenous MMTV) is transmitted primarily from infected dams to nursing pups through the milk. Virus initially infects gut-associated M cells and dendritic cells, then replicates in B cells of Peyer's patches. Infected B cells express viral LTR-encoded superantigen (SAg) in the context of MHC class II proteins on T cells, which results in stimulation and proliferation of T cells through recognition by specific Vβ T-cell receptors. Activated T cells release lymphokines that further stimulate proliferation of bystander B cells. Dividing B cells are requisite for retrovirus infection, thereby amplifying virus-infected B lymphocytes, which subsequently transport MMTV to mammary tissue. The role of SAg is critical in MMTV

[5] Personal recollection by the first author.

[6] This section on endogenous viruses was adapted from a chapter by S. W. Barthold (Percy and Barthold, 2006).

biology, as more of the virus is produced, the greater the efficiency of transmission in the milk. Once virus enters the mammary gland via infected lymphocytes, it further replicates extensively within mammary tissue. Mammary tropism is favored by promoters and enhancers in the LTR region, thereby amplifying virus and favoring transmission in the milk. High titers of the virus within mammary tissue increase the chance of proviral integration near a site of a cellular proto-oncogene, with subsequent oncogenesis. There is a high correlation between virus titer in milk and mammary tumor incidence within a mouse strain. MMTV also utilizes other mechanisms that favor its replication by circumventing the host innate immune response.

An inheritable infectious MMTV was described in the GR/A mouse strain in the late 1960s. This sets the stage for the discovery of endogenous MMTV sequences. Liquid phase molecular hybridization to mouse DNA revealed numerous endogenous copies of MuLV and MMTV. Restriction mapping and Southern blots showed numerous endogenous retroviral sequences that differed from the exogenous infectious viruses. These observations caused excitement and confusion because they suggested testable hypotheses but seemed to conflict with accepted knowledge. Did the virus possess the "*Mam*" gene? Was the apparent amplification of viral DNA sufficient to explain tumorigenesis? Did the endogenous MMTV sequences explain the electronic microscopic observations of B particles in mice thought to be "virus free"?

Like the endogenous, genetically transmitted MuLVs that have been extensively studied, endogenous MMTV DNA sequences have been discovered in the early 1970s. In contrast to endogenous MuLV, the endogenous MMTV sequences appear to have a less prominent role in mouse mammary cancer. The exogenous MMTV-S (standard) has been subsequently eliminated from most breeding stocks via caesarian section or foster nursing reducing the prevalence of early onset "spontaneous" mammary tumors. The endogenous MMTC sequences are largely replication deficient and contribute relatively little to mammary tumorigenesis.

However, the endogenous MMTV provirus contributes to modern GEM tumorigenesis in unexpected ways. Mice that express SAg from an endogenous MMTV develop selective deletion of the corresponding Vβ T-cell subset. These mice resist infection with exogenous MMTV with the same *Sag* specificity, since the matching exogenous virus cannot effectively amplify itself in the Peyer's patches without the cytokine drive. Thus, Vβ6 T-cell depletion in mice with endogenous *Mtv-7* cannot be infected or superinfected with *Mtv-7*. Unexpectedly, *Mtv-7* is also a major factor for PyV-induced neoplasia susceptibility in C3H/Bi, C58, CBA, AKR, and RF mice. Mice that are resistant to polyoma neoplasia, such as C57BR, C3H/He, and CBA, lack *Mtv-7*, but possess other *Mtv*s with different SAg

specificity. Lack of the appropriate Vβ6 T-cell subset, induced by *Mtv-7* SAg, precludes host T-cell immunity against PyV-induced tumors. The immunity appears to be specifically related to the major PyV oncogene, the middle T protein. Thus, it may play a role in susceptibility and resistance in some GEM experiments.

However, the endogenous MMTV plays no detectable role in mammary growth and development or the overall growth of the animal. Several clusters of feral mice have been found which do not have any endogenous MMTV sequences (Faulkin *et al*., 1984). When developed as an inbred colony, the mice lacking endogenous MMTC sequences are perfectly healthy. Thus, in the wild, MMTV does not interfere with sexual reproduction. Some feral mice do not even carry endogenous provirus indicating that the viral DNA is not required for the growth and development of the species (Faulkin *et al*., 1984). On the other hand, MMTV occurs in around 50% of feral mice, yet, on aging, these mice only develop a low incidence of mammary tumors (20% at 24 months) (Gardner, 1994a). The high incidence of mammary cancers in certain families of inbred strains is, thus, clearly an artifact of domestication and inbreeding.

The observations that even the most susceptible mouse strains become less susceptible once the female ovulates and goes through estrus are of particular interest (Blakely *et al*., 2005; Dux and Muhlbock, 1966; Russo *et al*., 1982). The mammary gland typically undergoes a tremendous spurt of growth and differentiation during this period and extends through the entire fat pad. Empirically, viral infection after 6 weeks of age is less readily achieved in mice. As a result, mice exposed to the virus after the onset of estrus do not readily develop mammary tumors (Dux and Muhlbock, 1966).

Subsequently, animals were shown to remain susceptible until after their first litter and the nursing of their pups. These observations were also extended to chemical carcinogenesis in rats and mice. The concept was most clearly articulated by Russo who named the period between the onset of puberty and the first pregnancy "The Susceptibility Window" (Russo *et al*., 1982). This important observation has been confirmed in every mammal studied including GEM (Blakely *et al*., 2005). The concept applies to the age distribution and natural history of human breast cancer (Russo and Russo, 1995).

VII. MMTV AND THE RISE OF TUMOR IMMUNOLOGY

From the very beginnings of experimental cancer research, various investigators have suspected that cancer is related to altered host responses (Ewing, 1919; Woglom, 1913). Andervont and Bryan first established that naïve mice could be immunized against virus infection with extracts of

tumors (Andervont and Bryan, 1944). However, the results could not be replicated by other investigators (Shimkin, 1977a). Many of the early negative observations could be written off by the newly described transplantation histocompatibility antigens. The subject was more or less dropped in the 1950s and some authorities came to the conclusion that the virus was not immunogenic, echoing Woglom's conclusion in 1929 that tumor immunology was not a promising field (Shimkin, 1977a; Woglom, 1929, 1947). However, Prehn and Morton are credited with reviving the field of tumor immunology. Prehn used carcinogen-induced sarcomas and Morton used MMTV-infected C3H mice for his experiments (Shimkin, 1979b). However, it has been difficult to determine whether the immune system suppresses or stimulates tumorigenesis, or does both (Heppner, 1972; Prehn, 2006).

Importantly, note that with advances in immunological techniques, serological detection of anti-MMTV antibodies was first found with Ouchterlony immunodiffusion plates and then with radioimmune precipitation techniques. Cellular immunity has been an equally vexing problem that has yielded to advances in experimental techniques. This area of research has been the subject of various reviews and will not be recounted here (Acha-Orbea *et al.*, 1999; Stewart and Heppner, 1997).

The MMTV-LTR also became noteworthy because it contained the MMTV-related superantigen (Acha-Orbea *et al.*, 1999; Marrack *et al.*, 1991; Pullen *et al.*, 1992). In 1981, Clive Dickson and Gordon Peters described an open-reading frame in the MMTV-LTR that encoded proteins (Dickson and Peters, 1981; Dickson *et al.*, 1981). However, this observation languished until the discovery in 1991 that MMTV acted as a SAg (Marrack *et al.*, 1991; Pullen *et al.*, 1992). Since then, the viral superantigen (vSAG) has been identified as a glycosylated type II integral membrane antigen that interacts with specific subsets of T-cell receptor Vβ to eliminate that clone which binds to the specific type of the virus (Acha-Orbea *et al.*, 1999). These observations have provided an interesting and powerful tool to study tumor-related immune responses such as immune surveillance, immune tolerance, and immune stimulation (Acha-Orbea *et al.*, 1999). Clearly, the virus has developed a molecular strategy to evade the host immune system.

VIII. HORMONES AND THE EMERGENCE OF ENDOCRINOLOGY

"The more simplistic the more complex." A quote John Coltrane was noted as saying when asked about the variety of his "riffs" on the jazz set. Such can be said about the influence of hormones on mammary tumor development.

The mouse mammary tumor biologists were instrumental from the very beginning of experimental cancer research in pursuing the hormonal influences on the mammary gland and its tumors. However, the significance of hormones in mouse mammary tumorigenesis has been overshadowed by the detection of the hormone response elements (HREs) in the MMTV-LTR (Yamamoto *et al*., 1983). Since the HRE involve the hormonal regulation both of virus production and of activated oncogenes, most experiments with "spontaneous" mammary tumors are difficult to interpret because it is impossible to separate primary and secondary effects of hormones. Since the hormones influence both virus and abnormal growth, many of the historical studies of endocrine regulation of mouse mammary gland development and tumorigenesis are open to question. How does one distinguish between effects that are primarily on the host cells and effects that are mediated through the presence of the virus?

The initial focus was dictated by clinical observations. Remarkably, the first observation was made in 1895 and published in 1896 by Beatson who detected the regression and then recurrence of a breast lump in a 33-year-old Glasgow woman following removal of her ovaries. The temporary effect led Beatson to opine that "We must look in the female to the ovaries as the seat of the exciting cause of carcinoma, certainly of the mamma, ..." (Beatson, 1896). Some 40 years after the Beatson report, clinicians were administering either androgens or estrogens and/or removing the adrenals or pituitary in women with breast cancer (Haddow *et al*., 1944; Huggins and Bergenstal, 1952; Loeser, 1938; Ulrich, 1939).

Loeb and Lathrop performed the same experiment as Beatson 20 years later in mice (Lathrop and Loeb, 1916). Surgical ablation became the *modus operandi* for studying the hormonal effects of various endocrine organs. Loeb continued performing such "physiological experiments" without the advantage of isolated chemical hormones and without inbred mouse strains (Loeb and Kirtz, 1939; Shimkin, 1945). Like the human, the mouse required ovaries for the development of breast cancer but in contrast to human, mouse mammary tumorigenesis was driven by pregnancy (Lacassagne, 1936).

The early insights on the spontaneous mammary cancer in mice also contributed significantly to understanding estrogen and its secretion from the ovary. Once removed, the incidence of tumors was reduced, while the grafting of syngeneic ovarian tissue or, when synthetic hormones became available, the injection of estrogen into male mice which normally do not form mammary tumors induced tumorigenesis (Lacassagne, 1932; Lathrop and Loeb, 1916; Murray, 1928).

With the development of chemically pure estrogens, numerous investigators led by Lacassagne, starting in 1932, verified that the ovarian function could be replaced by estrogens (Lacassagne, 1932). However, administration

of estrogens seemed to accelerate tumorigenesis in susceptible strains but, in itself, was not carcinogenic. When the milk factor was elucidated, tumors induced by estrogen were found to be dependent on the presence of the milk agent. In modern terms, the MMTV-LTR promoter element has a number of HREs that drive MMTV expression and promote or enhance/activate upstream or downstream oncogenes.

The MMTV-HRE also created the endocrine paradox between human and mice. Early pregnancy and lactation protects the human from breast cancer but increases the risk of breast cancer in susceptible strains of mice (Nandi *et al*., 1995). Further, a higher percentage of breast cancers in humans are estrogen receptor alpha (ER-alpha) positive and estrogen responsive while the majority of "spontaneous" MMTV-induced mouse tumors are ER-alpha negative and hormone independent (Cardiff, 2001). The use of the MMTV-LTR as an organ-specific promoter to drive oncogenic transgenes has continued to produce hormone-independent mammary tumors (Cardiff *et al*., 2000a). The utilization of whey acidic protein and various "knockins" behind native promoters may encourage the development of ER-alpha positive, hormone-dependent tumors in GEM (Lin *et al*., 2004).

During the turbulent years leading up to and following World War II, the major question was whether only ovarian hormones such as progesterone and estrogen were the inducers and controllers of normal breast growth or were there other elements? Lyons, in 1958, clearly defined the minimal hormonal growth requirements for normal mammary development in mice as being estrogen, adrenocortical steroids, growth hormone, and cortisol (Lyons, 1958; Lyons *et al*., 1958). Muhlbock in the Netherlands was particularly interested in the role of the pituitary in mammary gland physiology (Dux and Muhlbock, 1969a,b; Muhlbock, 1956).

Foulds found the pregnancy-dependent plaque in his hybrid BR mice in 1947, which he used to describe the basic rules of neoplastic progression (Foulds, 1949, 1954, 1958). His observations of the hormone-dependent tumors were confirmed in the GR strain by Muhlbock in the Netherlands and in RIII mice by Squartini in Italy (Foulds, 1975). Again, the initiating oncogene appears to be activated with a germ line insertion of the MMTV (Morris *et al*., 1990).

The preneoplastic HAN is also regulated by hormones (Nandi *et al*., 1960a). Early experiments related the HAN to pregnancy and when used for infectivity assays, either pituitary implants or hormone injections induced the HAN. However, the HAN does not regress after withdrawal of hormones but, in contrast to the normal mammary gland, persists in the presence of the constitutive levels of native hormones. Bern and Nandi provided a detailed analysis of the endocrinology of the HAN (Bern and Nandi, 1961; Nandi *et al*., 1960a,b). The more subtle effects of MMTV on normal mammary gland required morphometric analysis to demonstrate

that the presence of the virus is associated with alveologenesis (Squartini, 1962; Squartini and Bistocchi, 1977; Squartini *et al*., 1963, 1981, 1983).

On researching this chapter, we have discovered a researcher, the aforementioned Leo Loeb, who devoted his life to mammary biology and thus is probably the first true mammary biologist. Dr. Loeb spent some 60 years studying the mammary glands of mice. Loeb began his career in 1895 with Ribbert in Zurich and participated in the early transplantation experiments but immigrated to Chicago in 1896. He had far reaching interests that included some of the first attempts at tissue culture of tumor cells on blood clots (Loeb, 1958; Witkowski, 1983). In 1903, Loeb manipulated adult and embryonic mice to understand the attributes of tumor growth and sustainability. Along with Lathrop, he discovered ovarian influence on the development of mouse mammary tumors (Lathrop and Loeb, 1916). Twenty-eight years later, he furthered his observations by demonstrating that pituitary transplantation drives mammary tumor incidence and that variability in MMTV-induced tumor growth potentials varies in different mouse strains. Loeb also made major contributions to tissue culture and genetics of individuality (Loeb, 1953). Although rarely mentioned or referenced in today's literature, this field is deeply indebted to Leon Loeb's triumphant years of scientific observations.

Significant credit also must be given to Jull who, in early 1954, observed the effects of ovarian secretion on carcinogen-induced mouse mammary tumors (Jull, 1954), and later along with Bonser and Dossett discovered that forced breeding and pregnancies may also enhance the growth of spontaneous mammary tumors (Bonser *et al*., 1961). These observations have been very insightful for those studying mammary tumors in today's multiparous transgenic mice.

In the 1960s, a period of transition occurred from the mouse to the rat as an experimental model for endocrinology and chemical carcinogens (Shimkin, 1979b). Many of the observations on chemical-induced, hormone-dependent or hormone-independent mammary tumors occurred in the rat systems during this period. For the sake of this chapter, we will only mention that in the rat model, a single intragastric or intravenous injection of DMBA induces estrogen-dependent adenocarcinomas 60–100 days postinjection. This tumor model developed by Huggins in 1959 was aptly named after him (Huggins *et al*., 1959).

Russo used the rat model to develop the "window of susceptibility" concept which postulates that the mammary gland is highly sensitive to carcinogens between puberty and the first pregnancy (Russo and Russo, 1995; Russo *et al*., 1982). This model has been confirmed in mouse strains free of MMTV expression (Dandekar *et al*., 1986; Gardner *et al*., 1985). Almost parenthetically, specific mutations of the *Ras* gene have been observed in both the rat and the mouse models (Dandekar *et al*., 1986). In modern

terms, the DMBA-induced tumors have activation of the *Wnt-1* pathway (Currier *et al.*, 2005).

IX. THE NATIONAL CANCER INSTITUTE AND THE BIRTH OF MOLECULAR BIOLOGY: 1970–1980

A. Schools of Mouse Mammary Tumor Biology

The modern mammary tumor biologist has been well served by the NCI and other funding agencies organized to support cancer research. We must remember the extraordinary dedication of the pioneers in this field. Dr. and Mrs. Leonel Strong spent their honeymoon caretaking in the church rectory with Lionel's priceless mice stored under the church pews (Morse, 1978; Strong, 1978). Descriptions of the early days at the Jackson Laboratory recount the community vegetable gardens used to feed staff and mice (Crow, 2002). The Dutch recall that their mouse colonies were taken home and kept during most of the German occupation because the Netherlands Cancer Institute (NKI) did not have heat, electricity, or food.[7] Otto Muhlbock, a German national, had to serve as a medical officer during the occupation caring for the German nurses while sheltering and adopting a Jewish boy.[7] CRGL of University of California, Berkeley was initially housed in one of the temporary buildings in Strawberry Canyon just east of the football stadium. At times, people who came looking for him were "knocked for a loop" when they found "Dr. De Ome" identifying himself as the person mopping the animal room floor. But all of this was soon to change.[8]

In 1937, the US NCI was founded (Shimkin, 1977a). C. C. Little had become the managing director of the American Society for the Control of Cancer (founded in 1913) in 1929, which became the American Cancer Society in 1945, a calling he embraced with his usual enthusiasm and energy. In 1955, Little, a dedicated pipe-smoker, also became the first director of the Tobacco Research Council because he wanted to study the influence of genetics on susceptibility to smoking in humans (Crow, 2002).

In 1938, the Jackson Laboratory received the first extramural grant from the newly formed US NCI. These events reflected the increasing cancer awareness of the American public and marked the beginning of large-scale federal and private support for cancer research. The newly formed NCI took the lead and published the proceedings of a symposium on mouse mammary tumors in 1945 (Moulton, 1945). After the war, the funding, tools, and concepts of scientific research underwent major changes that led to the current era.

[7] Personal communications from R. van Nie and P. Hageman.

[8] Personal communication from L. J. T. Young.

The post-World War II infusion of cancer research funds encouraged the development and rise of mammary gland biology-focused centers located on the campuses of University of California, Berkeley, NCI, NIH, M.D. Anderson, Texas, NKI, and the University of Minnesota. Each of these centers assessed and contributed to transplantation assays, virus detection techniques, and hormonal influences on mammary gland development.

In the United States, the west coast contingent was led by Kenneth B. De Ome who had moved from Purdue to the University of California, Berkeley and persuaded Chancellor Robert Sproul to support a CRGL, incorporating a west coast production facility for inbred mice (Cardiff *et al.*, 2002). The CRGL faculty included endocrinology (Howard Bern), EM (Dorothy Pitelka), and De Ome who is best known for his work with the HAN. Later, other scientists such as Satyabrata "Ranu" Nandi (endocrinology) and immunologists David Weiss and Phyllis Blair joined the CRGL faculty. On the east coast, Dan Moore at the Rockefeller Institute in New York was working on the biophysical isolation and characterization of the virus. In Texas, Leon Dmochowski, the flamboyant Polish émigré, held sway with his emphasis on EM and viral structure. In the Netherlands, Otto Muhlbock was the director of the NKI. Muhlbock, a gynecologist, was particularly interested in the pituitary. The NKI developed many investigators whose major contributions are recorded in the monograph edited by Hilgers and Sluyser. Of course, the NCI had Heston, Andervont, and Bryant leading the effort in Bethesda. Francisco Squartini led the Italian mouse mammary biologists. Oddly, with Bittner's return to the Midwest in 1942, the Jackson Laboratory chose to concentrate on mouse genetics and MHC, thus abdicating its leadership in the field of mammary tumorigenesis.

In retrospect, the "schools" that developed in the 1950s and became prominent in the 1960s were led by the last of the natural historians of disease. De Ome and Muhlbock began holding informal "MTV Meetings" starting in 1955. By 1964 the group became international and met in California (Fig. 1). The group has maintained its international orientation and has become the International Association for Breast Cancer Research which is holding its twenty-fifth congress this year (2006) in Montreal, Canada. The group in Fig. 1 is counted as the "Third Congress." These leaders viewed mouse mammary tumor biology as a subject worthy of study in and of itself. They were disease oriented and understandably unprepared for the impact of molecular biology on scientific thinking and progress.

B. The Emergence of Molecular Biology

Molecular biology proved to be a powerful approach to fundamental biological problems. The structure of DNA was now known by 1953 (Watson and Crick, 1974). The Central Dogma had been pronounced by Crick (1958),

MTV Conference, Inverness, California, 1964

Back row, left to right: O. Mühlbock, K. B. De Ome, W. S. Murray, H. B. Andervont, A. Dux, M. Lyons, J. J. Elias, F. Squartini, L. J. Faulkin, Jr., S. Nandi, W. E. Heston, D. W. Weiss, W. Feller, G. Miroff.

Front row, left to right: L. Dmochowski, D. H. Moore, E. Y. Lasfargues, B. Fairchild, C. C. Little, D. Pitelka, H. A. Bern, P. Blair, J. Sykes.

Fig. 1 Photograph obtained from a published photo album from CRGL, University of California, Berkeley showing the participants at a 1964 meeting of investigators in Inverness California. The meeting was supported by the Lillian Babbitt Hyde Foundation and organized by Drs. Kenneth B. De Ome and Daniel Moore. The meeting also included leaders from Italy (Squartini), France (Fairchild), Netherlands (Muhlbock), and NCI (Andervont and Heston), MD Anderson (Dmochowski), and the Jackson Laboratory (Little). The photograph was taken in front of De Ome's house in Inverness.

suggesting a unidirectional flow of genetic information from DNA to RNA to protein (Crick, 1970). The John R. Platt paper on "Strong Inference," published in 1964, discussed the unique reductionist approach of molecular biology and inspired a generation of investigators (Davis, 2006; Platt, 1964; Watson, 1993) Gunther Stent declared in his essay "This was the molecular biology that was" that molecular biology had pretty much developed the solutions for all of biological problems and the only problem of interest was the organization of the central nervous system (Stent, 1968).

The successful molecular biologist sought models that could be used to test hypotheses. If a biological system was too complex or could not be used to test the hypothesis, other less complex systems were sought by the molecular biologists. As a result, the natural history of a single disease promulgated by the earlier mammary tumor biologists fell into some disfavor and the schools started by De Ome, Muhlbock, Moore, Dmochowski, and Squartini have faded into obscurity and with them, the institutional memory of mammary tumor biology.

In one sense, Moore's group was a harbinger of things to come because their emphasis on the biophysics and the structure of MMTV was regarded by many as "not real" tumor biologists. However, Moore's group brought new insights into mouse mammary tumor biology. His approach emphasized the physical separation and isolation of the virus. Trained in biophysics, Moore introduced a more quantitative approach to the field and a willingness to explore nonbiological aspects of the virology.

As is frequently the case in creative endeavors, impetus comes from outside the field. The *Drosophila* geneticist, Peter Bentvelzen, was hired by Muhlbock to study the complex MMTV genetics of the NKI GR/A mice (Hilgers and Bentvelzen, 1981). Bentvelzen confounded the mammary tumor community, in the late 1960s, by demonstrating germ line transmission of an infectious MMTV in this strain (Bentvelzen and Daams, 1969). He consulted with Monod, the phage geneticist, and adopted the idea of "infectious heredity" that was well documented in bacteriophages. Bentvelzen's papers suggested lysogeny as one of the potential mechanisms. He used the term "virogene" that foreshadowed the viral and cellular "oncogene." Bentvelzen's virogene hypothesis was clearly discussed before the Todaro and Huebner Viral Oncogene Hypothesis that was based on the inheritance of the *Murine leukemia viruses*' proviral DNA in AKR mice (Gardner, 1994a,b; Huebner and Todaro, 1969) in 1969. The Viral Oncogene paper does not reference Bentvelzen work with MMTV. But the stage was set for the discovery of reverse transcriptase.

Ironically, the conceptual hold of the Central Dogma on molecular biology was overturned with the 1971 discovery of reverse transcriptase in avian and murine leukemia viruses that then became known as retroviruses (Baltimore, 1970; Temin and Mizutani, 1970). MMTV was one of the retroviruses that contained reverse transcriptase which now provided the "milk agent" full

stature among the tumor virologists (Spiegelman *et al*., 1970). The oncogene hypothesis and reverse transcription became the basis for President Nixon's "War on Cancer," the National Cancer Act of 1971 which led to the Virus Cancer Program (Gardner, 1994b). The Virus Cancer Program brought together a large and diverse group of investigators and dictated much of the funding for mouse mammary tumor biology. The availability of funds during the 1970s targeted the oncogenic RNA viruses and supported most of the investigators primarily came from a molecular background and brought new perspectives to tumor virology (Shimkin, 1979b).

Almost immediately, the Virus Cancer Program came under attack by critics (Culliton, 1973, 1974; Wade, 1971). The VCP was, in essence, the first real programmatic venture into large scale, managed science. Many felt that such directed science hindered innovation while others argued that it provided the resources for innovative research (Gardner, 1994b). All through its existence (1968–1980), critics questioned the validity of the program. For example in 1974, the Zinder report from a panel of distinguished scientists found that the program supported research that was irrelevant to human cancer, citing Temin's discovery of reverse transcriptase as a prime example (Culliton, 1974).

C. The Search for a Human Breast Cancer Virus

One of the premises of Virus Cancer Program was that comparable oncogenic retroviruses and oncogenes would be found in humans (Gardner, 1994b). This rekindled the long-standing search for the elusive human breast cancer virus. This search deserves separate mention since the possibility has long fueled the imagination and provided a source of financial support. From the virological viewpoint, each technical advance in virology and molecular biology has led to reports of virus-like footprints that, when pursued to their logical end, have never led to a satisfactory conclusion.

Epidemiologists, virologists, and geneticists have long sought evidence for an "extrachromosomal factor" in humans and have failed (Korteweg, 1952). Evidence from family histories, first described by Brocca (1866), eventually led to the discovery of BrCa1 and BrCa2 but not an infectious agent (Ellisen and Haber, 1998; van de Vijver, 1999; Welcsh and King, 2001). As soon as the "extrachromosomal factor" was found in humans, the search went on in mice. When the factor was determined to be the Bittner agent or the MMTV, the virologists held sway and every new technique was applied to the hunt.

Electron microscopists have found VLP in human cells and in milk (Moore *et al*., 1969). These particles have never been validated as legitimate transmissible agents. With the improvement in immunological techniques, many investigators have found MMTV-related cross-reacting antibodies and

antigens in human sera (Black *et al.*, 1975, 1976; Bowen *et al.*, 1976; Feller, 1973; Goedert *et al.*, 2006; Heppner, 1972; Hollmann, 1973; Mehta *et al.*, 1977; Mesa-Tejada *et al.*, 1978; Muller and Grossman, 1972; Zotter *et al.*, 1978, 1981). Radioimmunoassays detected antibodies that proved in some cases to be nonspecific (Newgard *et al.*, 1976). Several groups have claimed to find MMTV-related antigens in human tumors and cancer cell lines (Keydar *et al.*, 1984; Mesa-Tejada *et al.*, 1978; Ohno *et al.*, 1979; Yang *et al.*, 1978). Some antigens have proven to be interesting shared carbohydrate moieties none of which have been proven to be viral. The advent of molecular biology brought reverse transcriptase assays and "simultaneous detection" hybridization techniques that have died a dignified death surrounded by negative results and many false alarms (Cardiff and Gardner, 1983; Roy-Burman *et al.*, 1973).

Improved tissue culture techniques in the 1970s brought claims of a human breast cancer virus that was proven by Walter Nelson-Rees to be the result of a worldwide cross contamination by HeLa cells containing the Mason-Pfizer Virus (Nelson-Rees *et al.*, 1981). Molecular hybridization, particularly under relaxed conditions, led to a series of suggestions of endogenous human sequences (May and Westley, 1989; Westley and May, 1984) that when sequenced were more related to primate viruses than MMTV (Callahan *et al.*, 1982). The PCR technology has spawned a new crop of investigators who claim detection of virus-like footprints in human breast cancer (Ford *et al.*, 2004; Holland and Pogo, 2004; Lawson *et al.*, 2001, 2006; Levine *et al.*, 2004; Wang *et al.*, 1998). This long record of futility does not mean that there are not viruses associated with human breast cancer. The search, however, has derailed the careers of some very bright people and perhaps the wiser of the lot quietly abandoned the effort when they realized that correlative proof was impossible to provide using other techniques. None the less, advocates still persist.

Since the premises of the Virus Cancer Program were that comparable retroviruses would be found in human cancers and to that time none had been found, the program was terminated in 1980. Overall, the program proved that laboratory and feral mice had oncogenic viruses but failed to provide definitive evidence of any oncogenic viruses related to human breast cancer. However, for the record, the program developed and supported a cadre of investigators who discovered most of the oncogenes and who have been instrumental in AIDS and HIV research (Gardner, 1994b).

D. MMTV and Molecular Oncology

One of the outcomes of molecular biology's use of viruses was the development of what we now regard as molecular oncology. Molecular oncology was foreshadowed by the landmark theoretical paper proposing the Viral

Oncogene Hypothesis by Huebner and Todaro (1969) and the emerging tools of molecular biology including reverse transcriptase (Baltimore, 1995). The tools were quickly applied to mammary tumor biology using MMTV as the major reagent. The discovery of reverse transcriptase in avian leukemia virus and *Murine leukemia virus* by Temin and Baltimore in 1971 was quickly followed by confirmation of a reverse transcriptase in MMTV by Spiegelman *et al.* (1970). The 1971 meeting of what is now the International Association for Breast Cancer Research was held in Cherry Hill and hosted by Dan Moore. The highlight of the meeting was Sol Spiegelman's announcement of the "simultaneous detection technique" for the detection of MMTV-related sequences in milk (Michalides *et al.*, 1975; Schlom and Spiegelman, 1971). He introduced his talk with the comment that no other laboratory represented in the audience could reproduce the hybridization technique. To a large extent he was correct since the specific hybridization technique proved to be nonspecific and unreliable (Roy-Burman *et al.*, 1973).

Peter Duesberg and Peter Vogt demonstrated that transforming viruses such as RSV carried a longer strand of DNA than the helper chicken leukemia viruses (Duesberg and Vogt, 1970). Varmus and Bishop showed that RSV carried a gene, the *src* gene, that came from a segment of the chicken chromosome, proving that the oncogene was of cellular rather than viral origin (Stehelin *et al.*, 1976). These findings provided the oncogene predicted in the Todaro-Huebner paper (Varmus, 1993). While RSV and src did not explain the other oncogenic viruses, RSV did inform the scientific community that they were looking for host genes (Varmus, 1993). When Varmus and Bishop demonstrated that the RSV sarcoma gene (*src*) was transduced from a gene normally found in chicken DNA, the race was on (Stehelin *et al.*, 1976). The current list of "oncogenes" is largely based on genes of rats, chickens, and cats that were first identified in transforming retroviruses or identified as sites of insertional mutagenesis by nontransforming animal leukemia viruses (Bishop, 1993). *Ras*, *Myc*, *ErbB*, *Fos*, *Myb*, and other oncogenes were all isolated from transforming viruses and then found to be part of the normal vertebrate genome.

With new technologies such as liquid phase hybridization, the molecular biology of mammary tumors began to be examined. First, like other murine retroviruses, MMTV-related sequences were encoded in the mouse genome. Intriguingly, numerous laboratories demonstrated that the MMTV DNA was amplified in tumors and in premalignant hyperplasias (Cardiff, 1984; Michalides *et al.*, 1982). Since MMTV was not a "transforming virus," the question became whether the amplification itself induced tumors (Altrock *et al.*, 1982).

The amplification hypothesis soon became moot with the description in 1981 that the *Avian leukosis virus* activated the *Myc* cellular oncogene

by insertion activation (Hayward *et al*., 1981). However, determining the genes activated by MMTV was not as easy since the virus was variably inserted some distance from the genes activated by the insertion. Nusse and Varmus were able to locate a common insertion site that activated a gene initially called *Int1* which was subsequently found to be homologous to a well-known developmental gene, *Wnt1* or wingless in *Drosophila* (Nusse and Varmus, 1982; Nusse *et al*., 1984). Later, Dickson and Peters isolated another site dubbed *Int2* for integration site number 2 which turned out to be *Fgf 3* (Dickson *et al*., 1984, 1990). A third common integration site was identified by Callahan as *int3* which was later determined to be *Notch* (Jhappan *et al*., 1992). The common theme here was that the insertion of MMTV activated genes that had already been identified in other species. *Notch* was another developmental gene previously identified in *Drosophila*. One of the scientists involved in these investigations was Roel Nusse who has devoted most of his subsequent career to studies in *Drosophila*. Ironically, his colleague at NKI, the *Drosophila* geneticist Peter Bentvelzen, had been recruited to study mouse mammary tumors. Little did he know, in describing the theoretical MMTV "*mam* gene" in GR mice, that the gene would turn out to be a homologue of a well-known *Drosophila* gene and a wonderful example of comparative biology (Hilgers and Bentvelzen, 1981).

With the identification of insertional activation of oncogenes as the oncogenic mechanism of many retroviruses, the focus of research in mammary tumor biology turned from the virus to the cellular genes. The virus remained of interest to tumor biologists only as a donator of the mammary-specific promoter from the MMTV-LTR. The LTR was also of interest to the endocrinologists as it contains various steroid response elements that were used to study the molecular basis for hormone responsiveness (Yamamoto *et al*., 1981, 1983).

X. NEOPLASTIC PROGRESSION: 1954

The ability to observe the growth of neoplasms in animals brought new concepts to experimental cancer research. From the very beginning of experimental cancer research, mice have been used to determine the origins of tumors and to watch how they evolve from benign (*in situ*) to invasive and from progress to metastatic. One key concept was that tumors do progress through stages. The term "progression" was first applied to virus-induced papillomas and carcinomas by Rous and Beard (Rous and Beard, 1935; Rubin, 1994). However, the seminal papers on the subject came in the 1950s from Leslie Foulds who used the mouse mammary tumor system for

his experimental model. He articulated and, in his own way, carefully defended six rules of neoplastic progression (Foulds, 1954):

Rule I: Progression occurs independently in different tumors in the same animal.
Rule II: Progression occurs independently in different characters in the same tumor.
Rule III: Progression is independent of growth. It occurs in latent tumor cells and in tumors whose growth is arrested.
Rule IV: Progression is continuous or discontinuous, by gradual change or by abrupt steps.
Rule V: Progression follows one of alternative paths of development.
Rule VI: Progression does not always reach an end point within the lifetime of the animal.

The reader should remember that the Papanicolaou and Trout monograph on cervical cytology to detect precancers of the cervix only appeared in 1943 (Papanicolaou and Traut, 1943) and the "Pap Smear" did not have widespread acceptance when Foulds' 1954 paper appeared (Shimkin, 1977a). However, the Pap Smear has saved countless lives from cervical cancer and became the basis for early cytological detection of cancers of many other origins. The theme of early detection is now the mantra for the American Cancer Society and NCI.

Foulds' ideas were primarily based on his observations of hormone-dependent mammary tumors in RIII × Black 6 mice that he named "BR" (Foulds, 1949, 1954, 1959). The tumors, Foulds observed, were initially hormone dependent (plaques) but frequently evolved into hormone-independent tumors. Foulds, a medical pathologist as well as a tumor biologist, developed these concepts and expanded them into a two volume treatise on "Neoplastic Development" (Foulds, 1959, 1975). His concepts were consistent with the morphological observations of the tumors in human and mouse. They complied with the ideas of initiation and promotion applied to carcinogen-induced skin tumors in the mouse (Berenblum and Shubik, 1954; Rous and Beard, 1935; Shubik, 1950; Shubik *et al.*, 1953). Interestingly, the terms *initiation* and *promotion* were also introduced by Rous (Foulds, 1954) in describing experiments with chemical carcinogens in the rabbit. These concepts were later applied to the mouse (Rous and Kidd, 1941).

Further, this idea fits well into the concepts of linear progression and clonal evolution currently in vogue in human colon and breast cancer (Burstein *et al.*, 2004; Fearon and Vogelstein, 1990). The observations of heterogeneity in induced MMTV tumors by Heppner and her group in Detroit during the 1980s also seem consistent with Foulds' observations (Heppner, 1972; Michalides *et al.*, 1982). Not all of Foulds' rules were

accepted by the scientific community. For example, Rubin, basing his criticism on spontaneous *in vitro* transformation of fibroblasts, contested Foulds' rules number III, IV, and V (Rubin, 1994).

Developed during an era in which the Willis "field effect" was the prevailing hypothesis for tumor origin, Foulds' diagrams of progression leave open the possibility of a unicellular origin, although one cannot find mention of the clonal origin of cancer in Foulds' own essays (Foulds, 1959; Willis, 1953).

XI. NEOPLASTIC PROGRESSION: 1959 THE HAN

A. The HAN

Although the term "progression" was first applied to neoplasia by Rous and Beard in a study of the evolution of rabbit papillomas to invasive cancers (Rous and Beard, 1935; Rubin, 1994), observation of the mouse mammary gland has long been favored to understand neoplastic progression. Foulds' hypothesis was built on the evolution of mouse mammary tumors. Other investigators were more interested in the origin of the tumor.

The early literature is interesting in that some investigators were correct and others were just confused. Breast cancer itself had been known since antiquity. However, the origin of breast cancer, or any other cancer, remained obscure with a variety of imaginative hypotheses extant. Remember that in 1850, the difference between true cancer and chronic inflammation was not completely understood. The light microscope added a new dimension. Even then, authorities such as Virchow maintained that all cancers arose from connective tissues (Rather, 1978). This idea was a reasonable hypothesis given the dense connective tissue stroma associated with most cancers.

The German pathologist Waldeyer first accurately described the origin of epithelial cancers although he was using primitive histological techniques and microscopes. He used microscopic slides of colon and breast for evidence that human cancers arose from preexisting epithelium (Rather, 1978; Waldeyer, 1867). He invoked the same line of reasoning used today. That is, that the breast cancer arose in direct continuity with preexisting mammary acini. The acini were themselves atypical. Although others such as Connheim continued to hypothesize embryonic rests, Waldeyer's observations were eventually confirmed and accepted by others. When Elberth and Spude examined mouse mammary tumors in 1898, they did not observe direct connections with the mammary epithelium and classified the mouse

tumors as "endotheliomas" (Eberth and Spude, 1898). This diagnosis was disputed by Apolant and Haaland but neither was able to demonstrate direct continuity with the normal mammary gland (Haaland, 1911), which was subsequently demonstrated by others (Woglom, 1913).

It is difficult to ascertain the scientific context during these years. Human breast cancer had long been associated with chronic mastitis. In 1913, James Ewing delivered an address to the New York Medical Society entitled "Precancerous Diseases and Precancerous Lesions: Particular in the Breast (Ewing, 1914). In this essay he credits his German mentor, Orth, for the term "precancerous diseases." Orth himself was a distinguished German pathologist who was Virchow's successor. The point here is that the term "precancer" was being used at the turn of the twentieth century. Medical scientists were thinking about precancers 100 years ago.

Apolant, working with Paul Ehrlich, also identified a smaller lesion he called adenomen or adenoma simplex (Apolant, 1906). He falls short of declaring that these lesions were precancers and did not show a clear relationship between these lesions and larger tumors. His colleague (Haaland, 1911), in 1911, not only redescribed Apolant's lesions but wrote a lengthy section on these hyperplastic nodules and their biological potential. Although he did not directly use the term, Haaland's discussion leaves no question that they were precancerous (Haaland, 1911).

Subsequent descriptions by Fekete and others in the late 1930s associated the hyperplastic nodules with the subsequent development of mammary cancers (Fekete, 1938). MMTV infection and hormones were associated with adenomas or adenomatous nodules by Pullinger (1947). While many investigators could relate the presence of HAN to the tumor incidence and the presence or absence of MMTV, others were not able to confirm the association because they found "nodules" in elderly, low-incident mice and in carcinogen-treated and irradiated mice (Dunn, 1945, 1958). It was also very clear that more HAN were present in each mouse than tumors. So, by 1959, the biological potential of the lesions was still a hotly disputed subject with many conflicting lines of evidence. Dr. Dunn, who had a knack for such terminology, cleverly entitled her section on this topic "Preneoplastic and early neoplastic changes (Dunn, 1958)."

The controversies over which lesions, if any, were precursors to cancer were subsequently eliminated by De Ome using his technique for transplantation into a gland-cleared fat pad. His studies provided definitive experimental proof that the HAN were in fact the precursor tissues to mouse mammary cancers (De Ome *et al.*, 1959). Interestingly, De Ome used the term "precancer" in his original 1959 paper. The first time "preneoplasia" appears in the De Ome HAN literature was in 1961 (De Ome *et al.*, 1961).

B. The Test-by-Transplantation

Although the earlier work clearly implied that the HAN was a precursor of mammary tumors, the hypothesis was by observation and inference (Haaland, 1911). No one had really tried to transplant these lesions. De Ome, using the classical transplantation approach to the biological problems in mammary tumor biology, developed a novel system of "clearing" the mammary fat pad by the surgical removal of the mammary bud at 3 weeks of age before the gland extended through the mammary fat pad. This simple maneuver left the number four mammary fat pad free of any host mammary gland and this location proved to be an ideal transplantation site (Faulkin and De Ome, 1958). De Ome was then able to transplant all types of mammary tissues into this site to observe their biological growth potential and properties. The key observation was that the HAN would grow into and fill the fat pad but would not grow when transplanted into subcutaneous tissue (Medina, 1973, 1975). Tumors will grow in any site, while the HAN will grow only in the fat pad. These observations demonstrated a key distinction between the HAN and mammary tumors and supported a sequential model of tumor progression which De Ome named "nodulogenesis" and "tumorigenesis."

Using the gland-cleared fat pad transplantation site, the Berkeley group demonstrated that transplants of normal tissue filled the mammary fat pad with normal mammary ductules. Transplants of HAN developed very atypical hyperplastic outgrowths. The normal epithelium did not progress to cancer while the transplants of HAN frequently have invasive tumors emerge from them, thus, defined the HAN as high-risk, precancerous tissues.

The numerous different types of "hyperplastic nodules" found in the mouse mammary gland are of historical interest since early investigators were not able to determine their biological properties. De Ome's experiments proved that hyperplastic nodules with alveolar differentiation were the only type of "hyperplastic nodule" that had a high risk of progressing to invasive tumor. After his experiments the precancers were identified as HAN. The other types of nodules, such as squamous nodules and inflammatory nodules, did not prove to be precancerous when tested by transplantation (Cardiff *et al.*, 2002).

Further studies demonstrated other growth differences between HAN, tumors, and normal epithelium. For example, transplants of normal mammary gland senesce, while the HAN are immortalized (Daniel *et al.*, 1968). Growth of the HAN and normal mammary gland is inhibited by adjacent gland while tumors are not. Overall, these experiments developed operational definitions of normal, precancerous, and cancerous mammary epithelium. The subsequent history of preneoplasia in mouse mammary tumorigenesis

has been extensively reviewed by Medina and others (Foulds, 1975; Medina, 1973, 1975, 1996, 2000, 2002).

Key observations have resolved some of the controversies about precancers by showing that the HAN from high tumor lines have different biological potential. Thus, a low-risk and a high-risk line can be isolated and serially transplanted from the same mouse (Cardiff *et al*., 1981, 1983; Pathak *et al*., 1987). Some low-tumor mouse strains produce low-tumor potential exogenous MMTV that induce nodules that rarely result in tumors (Young *et al*., 1984). Some types of focal nodules such as those with predominantly squamous epithelium and/or inflammation rarely develop tumors. Finally, other carcinogens, such as DMBA, result in mammary cancer but also produce preneoplastic focal atypias that lead to tumors (Medina, 1976). Thus, the test-by-transplantation was a handy surrogate assay for mammary precancers and has been applicable to preneoplasias in other organs (Cunha *et al*., 2001).

Study of the precancerous outgrowths initially using MMTV insertion sites and then protooncogene restriction mapping demonstrated their clonal origin. Different outgrowths from the same animal were clearly derived from different clones. Subdivisions of the same HAN gave rise to outgrowths with restriction patterns with the same signature fragments in each outgrowth but with unique patterns in each subdivision. Further, the tumors arising from the outgrowths had the same restriction patterns as the parent outgrowth. Thus, rearrangement of the oncogene or the viral DNA had no apparent role in the transition from MMTV-induced precancer to cancer or neoplastic progression. From a tumor virus standpoint, these observations were also difficult to reconcile with those of mouse mammary tumor heterogeneity (Calabresi *et al*., 1979; Dexter *et al*., 1978; Heppner *et al*., 1983). However, Foulds' rules No. II and III suggest that the different characteristics of tumors progress independently. Progression to invasive and metastatic tumors occurs independently of MMTV and probably involves the expression or silencing of other oncogenes and tumor suppressor genes.

C. Comparative Pathology of Preneoplasia

> Attempts have been made to correlate the histologic appearances in the mammary tissue of old female mice of high cancer strains, with supposedly precancerous conditions in women. Such attempts have been much hampered by the fact that there has been no clear agreement as to what constitutes a precancerous condition in either species. It is an attempt to check one unknown against another unknown.
>
> (Dunn, 1945, 1958)

Throughout the history of mouse mammary tumor biology, investigators have sought comparisons with human breast disease. As early as 1930 comparisons were appearing in the literature (Cheatle, 1934–1935;

Goormaghtigh and Amerlinck, 1930; Lacassagne, 1936; Taylor and Waltman, 1940). The comparative pathology was reviewed by Dunn (1945, 1958).

Cheatle and Cutler had performed serial sections of human breast cancers in 1928, the primary tool for observing these phenomena became the mammary gland whole mounts which allowed three-dimensional observation of the entire mammary fat pad. Students of De Ome encouraged similar approaches to study preneoplasia in other species such as dog, rats, and human (Beuving *et al.*, 1967; Cameron and Faulkin, 1971; De Ome and Young, 1970; Russo *et al.*, 1990).

The groundbreaking studies of human precancers by Wellings, an M.D. pathologist, using a variation of the whole mount technique to observe the human breast in "subgross" preparations are noteworthy in this regard because they exemplify the cross-fertilization between murine and human pathology (Wellings *et al.*, 1975). Wellings received his Ph.D. under De Ome. Wellings and Jensen observed some 36 types of focal abnormalities of the human breast that had previously been classified under "chronic mastitis" and confirmed Waldeyer's original observations that human breast cancer originates in the "acini" or terminal ductal lobular units. Like Waldeyer, they documented a morphological continuum between atypical foci, through *in situ* carcinoma to invasive cancers. To a large extent, the De Ome studies, followed by the Wellings studies, set the record straight and have led to our modern concepts of breast cancer. The Wellings and Jensen findings were widely disseminated and although initially resisted by the medical community, they were used by David Page in his landmark studies of precancers and breast cancer in a large cohort to demonstrate increased relative risk of the atypical hyperplasias (Page and Dupont, 1988; Page and Simpson, 2000; Page *et al.*, 2000). Most students of human breast cancer now agree that the separation of human breast cancer into ductal and lobular types is anatomically incorrect. Both originate in the terminal ductal lobular units.

D. Tumor Clonality

The concept of clonal origin of tumors had not been completely developed by the early 1950s. Boveri's proposal that chromosomal imbalance caused cancer was developed in 1913 but not published in English until 1927 (Balmain, 2001; Wunderlich, 2002). The Philadelphia Chromosome, discovered in 1960 (Nowell and Hungerford, 1960, 1961), led to the karyotypic recognition of tumor clonality. Monoclonal gammopathies lent credence to the clonal derivation of myelomas and, soon, other lymphocytic tumors were found to be monoclonal (Bernier and Putnam, 1964). In 1965,

the Linder and Gartler's study of uterine leiomyomas using x-linked recessive alleles provided another type of assay for demonstrating tumor clonality in certain populations (Linder and Gartler, 1965). This technology provided tools for examining solid tumors and was later extended to a large number of neoplasms in women (Alexander, 1985). It is now assumed, until proven otherwise, that most tumors are either mono- or oligoclonal. Clonality implied that most neoplasms originate from a single cell.

Although karyotyping of mouse mammary tumors was performed during this early period (Dofuku and Matsuzawa, 1983; Dofuku *et al*., 1979), the karyotypes were inconsistent and the mouse chromosomes were difficult to characterize. However, with the development of DNA restriction mapping, a number of laboratories reported clonal tumors based first on the appearance of additional and unique MMTV restriction fragments and later on the rearrangements in the *int* genes (Cardiff, 1984; Cardiff *et al*., 1981, 1983; Fanning *et al*., 1982, 1985; Michalides *et al*., 1982; Morris and Cardiff, 1987; Pathak *et al*., 1987). Although no one disputed the clonality, several groups reported oligoclonality based on MMTV integration patterns (Michalides *et al*., 1982). Serial transplantation of single tumors suggested that the restriction fragment patterns were remarkably stable. Thus, insertion or rearrangement of the MMTV or *int* genes could not explain any biological progression in the mouse mammary tumors.

E. Developmental Biology and Neoplastic Progression: Mammary Stem Cells

> Any tumor can serve as a convenient model of the normal process. One must always remember that negative results do not mean anything, and positive results must always be confirmed in the normal tissue. There is much to be learned by using tumors as models of tissue renewal.
>
> (Pierce, 1983; Arechaga, 1993)

The cancer stem cell hypothesis is now so popular that journals such as *Nature* have devoted cover pages and editorial reviews to the subject (Janzen and Scadden, 2006). It was, in many senses, foreshadowed by the "embryonic rest" hypothesis that Connheim and Virchow espoused in various forms in the late 1800s (Ewing, 1914; Rather, 1978). However, the modern hypothesis came from the perspective of developmental biology. Weiss, in 1933, described in classic fashion how vertebrate developmental structures are formed by certain cells that adapt, react, and create separate living systems (Weiss, 1933). Leighton, in 1967, some thirty years later, observed that cells within a tumor hold a structure or "niche" separate and distinct from the tumor as a "biological unit" (Leighton, 1967).

Following in their footsteps, the University of Colorado pathologist, Barry Pierce, who also moonlighted as an embryologist, identified mammary stem cells in mouse mammary tumors (Pierce, 1974, 1975, 1977, 1983; Pierce and Verney, 1961; Pierce *et al.*, 1977; Sell and Pierce, 1994). Pierce, an avid outdoorsman, began his stem cell research in the late 1950s with Frank Dixon (University of Pittsburgh) and with the help of Roy Stevens of the Jackson Laboratory, a colleague of C. C. Little. Before Pierce set sail into stem cell biology, he cultivated his research training in mouse embryogenesis and along the way utilized the peroxidase-labeled antibody techniques "Immunohistochemistry" and a new method for "Immunofluorescence" based on a variation of Coon's technique (Nakane and Pierce, 1967). Reflecting even further back, Pierce was exposed to mammary biology in the late 1940s when his work "The iron content of mammary epithelium" led to his Master's Thesis from the University of Alberta in 1950 (Pierce, 1950). As transitioned from clinical pathology to experimental pathology Pierce took some 17 years before rediscovering the mouse mammary gland and examining the location of stem cells in mouse mammary tumors (Pierce *et al.*, 1977).

Paralleling Leighton's tumor microenvironment observations, Pierce confirmed through EM and radioactive tritium labeling that "long-lived label-retaining cells" or undifferentiated mammary stem cell epithelium can be detected in mouse mammary tumors within distinct microenvironments (Pierce *et al.*, 1977). This observation stands firmly in history but is rarely cited in the current stem cell literature. The implications of these ideas will now be reviewed in the context of GEM. Recently, with the popularization of the embryonic stem cell research, the mouse has become a favorite tool for yet another branch of scientific research.

XII. GENETICALLY ENGINEERED MICE: 1984–2006

A. Genetically Engineered Mice

The modern era of mammary tumor research was initiated in 1984 by Stewart, Pattengale, and Leder's paper using the MMTV-LTR to promote *Myc* gene expression in the mouse mammary gland (Stewart *et al.*, 1984). This led to the rapid development of mammary tumors. The demonstration of organ-specific targeting of gene expression opened the door for using the mouse as the test bed for gene action. Since that time, the mouse has been used to test an exhaustive list of potential oncogenes and tumor suppressor genes.

The irony of the current era is that the molecular proof of the genetic origins of breast cancer that C. C. Little, Maud Slye, and others so tenaciously sought in the early part of the twentieth century has been provided.

However, the proof now comes, in a dramatic twist, via reverse genetic engineering. Not so much that the mouse genes have revealed the secrets of breast cancer but the genes discovered in humans and other animals have been tested in mouse and found to cause or support breast cancer.

Although a definitive history of the current developments cannot be written, this closing section will be used to reflect on the significance of previous research and, perhaps, speculate about its implications.

As mentioned above, the first mice genetically engineered for the expressed intention of testing known oncogenes were the *Myc* mice promoted by the MMTV-LTR (Stewart *et al*., 1984). These mice were soon followed by the transgenic *ras* and *c-neu* mice [Tg(*ras*) and Tg(*neu*)] (Guy *et al*., 1992; Muller *et al*., 1988; Sinn *et al*., 1987). The experiments were so startlingly successful that they were immediately taken up by a wide variety of laboratories using what has become a long list of promoter systems and genes. The GEM technology attracted a large number of molecular and cellular biologists whose training is outside mouse mammary biology. Their scientific perspective has enriched and reinvigorated mouse mammary tumor biology. Their use of the fundamental reductionist approach of modern molecular and cellular biology has led to major advancements. However, their relative ignorance of the natural history of disease frequently has been problematic. Many of the remaining "old guard" regarded many of the interpretations as being simplistic, not good biology, and without regard to the previous advancements in mouse mammary tumor biology. Our hope is that this brief history will repair the gap between these two generations by reminding both camps of their heritage.

The first lesson of GEM is the very satisfying realization that when scientists recapitulated what MMTV had done in nature, that is, place the *Int* genes behind the MMTV-LTR promoter and the mice develop tumors that are morphologically identical to those described by Apolant and Haaland (Haaland, 1911) and classified by Dunn (Cardiff *et al*., 2000a; Dunn, 1958; Sass and Dunn, 1979). These two genes were subsequently identified as *Wnt* and *FGF* (van Leeuwen and Nusse, 1995). The complementary nature of the first two *Int* genes has also been demonstrated by infecting either Tg(*Wnt*) or Tg(*Fgf*) mice with MMTV. The resulting tumors showed that the virus insertional activation occurred in a *Fgf* locus in the Tg(*Wnt*) mice and in a *Wnt* locus in the Tg(*Fgf*) mice (Kwan *et al*., 1992; Lee *et al*., 1995; Shackleford *et al*., 1993).

Other types of GEM exhibit different histological patterns that rarely mimic the spontaneous MMTV-induced tumors (Cardiff *et al*., 2004). Many of the mammary tumors in these mice mimic morphological features that resemble human breast cancers (Cardiff and Wellings, 1999). Thus, these animals open a new chapter in mouse mammary tumorigenesis.

The natural history of neoplastic progression in these GEM tumors is very similar to that recorded over the past century for the spontaneous tumors. Although the initial descriptions recorded a "simultaneous" transformation of all mammary glands in some transgenic strains, many investigators have featured the presence of precancers in the animals with what one might consider an overemphasis on the morphological diagnosis of ductal carcinoma *in situ* (DCIS)-like lesions (Cardiff *et al*., 2000b). However, some investigators have utilized the De Ome test-by-transplantation criteria for precancers to demonstrate both high-risk (Cardiff *et al*., 1981, 1983, 2002; Fanning *et al*., 1982; Medina, 1975, 1976, 1996, 2000; Morris *et al*., 1990; Pathak *et al*., 1987) and low-risk hyperplastic growths (Jhappan *et al*., 1992; Lin *et al*., 1992). The Annapolis pathology workshop recommended that these lesions be designated mammary intraepithelial neoplasms (MINs) to distinguish them from the MMTV-induced HAN and the human DCIS (Cardiff *et al*., 2000a).

Studies of GEM mammary tumors have documented how some mammary tumors undergo an epithelial-to-mesenchymal transition (EMT) (Moody *et al*., 2005; White *et al*., 2001). The EMT tumor theme has been picked up in human cancers and an increasing number of papers have appeared showing that undifferentiated tumors often have sarcomatous characteristics and express one of several EMT-associated transcription factors (Yauch *et al*., 2005). The sarcomatous tumor types recorded in GEM were earlier described and illustrated and discussed in common lab mice by Apolant in 1906 (Apolant, 1906), Haaland (Haaland, 1911) and Bashford in 1911, and Woglom in 1913 and reviewed by Dunn in 1945 and 1954 (Dunn, 1945, 1958). The early investigators referred to these tumors as carcinosarcomas. Since these spindle cell tumors were frequently associated with transplanted tumors, some investigators considered them an artifact of transplantation. Bereft of immunohistochemistry, some early investigators speculated that these types of tumors could arise from a malignant stroma (Bashford, 1911a). The reader will detect the continuing shadow of Virchow in these suggestions. EMT tumors now can be identified by immunohistochemistry as dual staining for the intermediate filaments, cytokeratin, and vimentin and loss of E-cadherin (Moody *et al*., 2005; White *et al*., 2001). They are currently the object of intense scrutiny.

As recognized previously, most mouse mammary tumors metastasize to the lung. Livingood recorded pulmonary metastases in 1896 (Livingood, 1896). As recorded by Borrel (1903), Livingood (1896), Haaland (1911), and then Dunn (1945), many of the metastases appear as intravascular tumor emboli. Illustrations in Borrel's early paper and Livingood's Figure 3 clearly show intravascular tumor emboli. Illustrations of metastasis used by Woglom and Dunn have well-defined double layers of endothelium around the emboli

(Dunn, 1945). The same embolic metastases occur in the current crop of GEM (Oshima *et al*., 2004; Siegel *et al*., 2003) and are considered by some investigators a "new phenomenon" (Sugino *et al*., 2002). However, they are more an example of rediscovery of a previously described phenomenon that has been lost in with the loss of continuity of institutional memory.

Modern investigators are not limited to descriptions of the phenomenon but they have numerous GEM that can interrogate mechanism. The "knockout" mice from which the gene in question has been removed from the genome with genetic engineering is a good example (Cardiff and Wellings, 1999). The knockout mice demonstrate which genes control the type and rate of metastases (Man *et al*., 2003; Pollard, 2004; Siegel *et al*., 2003). The EMT tumors have been rediscovered in the context of transgenic mice the develop tumors that no longer express the initiating transgene or with recurrent tumors in doxycycline-inducible models (Moody *et al*., 2005; White *et al*., 2001). With control over the molecule, the modern investigator can directly demonstrate the mechanism and no longer needs to speculate.

The earliest investigators associated breast cancer in humans and mice with inflammation and other stromal events (Ewing, 1914, 1919; Haaland, 1911). These observations were correlative guilt-by-association that were somewhat ignored after the milk agent (MMTV) was understood. Most of the tumors in colonies with good husbandry had relatively little inflammation and contained less fibrosis than was characteristic for the human disease. However, modern investigators have began experiments that demonstrated the importance of stroma (Barcellos-Hoff and Medina, 2005; Cunha *et al*., 1997; Howlett and Bissell, 1993; Pollard, 2001). Now we have a growing list of stromal genes that appear to play a major role in epithelial tumorigenesis. Research by Pollard and Condeelis has riveted attention to the macrophage as a major inflammatory cell that stimulates the metastatic phenotype (Wyckoff *et al*., 2004).

B. Comparative Pathology of Breast Cancer

The initial studies of mouse mammary tumors questioned whether the mouse tumors were related in any way to human breast cancer. Livingood states that he undertook his studies in 1894 at the suggestion of Dr. Welsh, of Johns Hopkins fame, who told him that comparative pathology was a worthy pursuit (Livingood, 1896). In 1911, Bashford provided an extensive rationale for studying mouse mammary tumor biology (Bashford, 1911b). In 1945, Shimkin further defended the focus of NCI on mouse mammary tumorigenesis (Shimkin, 1945). As recorded herein, each development in either clinical breast cancer or in basic breast cancer research has led to research in the other field.

Originally, there was a question as to the malignant potential of the disease in mice. This was resolved in the affirmative by Bashford (1911b). Next, the role of genetics and family history became the new focus. With the discovery of the milk agent and the availability of new hormones, the role of hormones was studied in both species. The finding of an infectious agent that caused cancer in mice set off a search for a comparable agent in humans that continues to the present day.

The MMTV-induced tumors have always been recognized as being morphologically distinct from human breast cancers. Some authorities have speculated that the differences are due to the supposed large ductal origin of human breast cancer as compared to the acinar origin of mouse mammary tumors (Dunn, 1945, 1958). However, the detailed subgross studies of Wellings and his colleagues (Wellings and Jensen, 1973; Wellings *et al*., 1975, 1976) exploded the myth of the ductal origin of human breast cancer that originated with Foote (Foote and Stewart, 1941). The commonest site of origin of breast cancer in all species now appears to be in the acinar cells. However, the details of the histology of spontaneous mouse mammary cancers have always been very different from human breast cancers. In fact, when the NIH convened the Annapolis pathology workshop to assess the GEM models, several of the surgical pathologists without experience with mouse pathology were surprised to learn that the MMTV-induced tumors could be metastatic (Cardiff *et al*., 2000a).

The morphology of spontaneous, MMTV-induced tumors of mice does not resemble human breast cancers. However, many of the GEM-associated mammary tumors resemble human breast cancers in striking detail (Cardiff and Wellings, 1999). For example, Tg(*ErbB2/neu*) transgenics have microscopic fields that can be easily mistaken for human DCIS and Tg(*src*) GEM produce remarkably scirrhous tumors (Cardiff and Wellings, 1999). Numerous other examples can be provided and others will become apparent as the field of genetic engineering expands.

C. "Validation" of Mouse Models

In 2000, the Mouse Models of Human Cancers Consortium Steering Committee charged the Pathology and Laboratory Medicine Committee with the task of "validating" the new GEM models of human cancers (Cardiff *et al*., 2004). Several publications have discussed the validity of mouse models (Cardiff, 2001; Cardiff *et al*., 2004; Green *et al*., 2002). Validation has been defined as the matching of attributes of cancer in one species with those in another species (Cardiff *et al*., 2004). When considering the possibilities, the GEM mammary tumor model and human breast tumors express the same oncogene in the same organ and same cell set. In the mouse,

this results in the expression of many of the same genes observed in human breast cancer (Desai *et al*., 2002; Fargiano *et al*., 2003; Qiu *et al*., 2004). Overexpression of human genes, such as *erbB2* or *myc*, in the mouse mammary gland results in malignant neoplasms.

In summary, genes known to be associated with human breast cancer have been used to create breast cancer in mice. This, in one sense, fulfills what has become Koch's postulate of tumor biology (Cardiff *et al*., 2004). The gene is identified in human tumor, isolated, cloned, and inserted back into the mouse genome to produce the tumor. The mouse is used to recapitulate the biology of cancer. The same gene in the same organ results in the same disease.

Strikingly, most transgenes induce morphologically unique tumors that have genotype-specific phenotypes (Cardiff *et al*., 1991). Many of these new GEM mammary tumors have phenotypic characteristics indistinguishable from human (Cardiff and Wellings, 1999). As stated before, the morphology of spontaneous MMTV-induced tumors is recapitulated by the insertion of the protooncogenes, *Fgf3* or *Wnt1*, activated by the virus. Genetic engineering can accomplish what the infectious agent does at the molecular level.

As documented above, cancer precursors were recognized in the mouse mammary gland 100 years ago by Apolant and operationally proved in 1959 by De Ome's test-by-transplantation. Although the initial studies of mouse tumorigenesis in GEM were interpreted as a one-step transformation (Muller *et al*., 1988), many papers have identified focal atypias that are *in situ* (Cardiff *et al*., 2000b). However, the initial attempts at test-by-transplantation suggested that these lesions were hyperplasias with low risk of neoplastic progression (Jhappan *et al*., 1992; Lin *et al*., 1992). Two groups have developed transplantable lines from p53 knockout mice and PyV middle T mice that fulfill the criteria for precancerous, high-risk tissues (Maglione *et al*., 2001, 2004; Medina *et al*., 2002). These precancerous tissues, in addition to fulfilling the operational criteria, have many of the molecular, morphological, and biological characteristics of human DCIS (Namba *et al*., 2004).

The GEM, however, have not solved the problem of neoplastic progression, clonality, and heterogeneity. The precancers were initiated by the same oncogene and arose in the fat pads of the same mouse (Maglione *et al*., 2004; Namba *et al*., 2004). This could be envisioned as modeling Willis's Field Effect. However, each transplanted precursor has a separate, unique morphology, clinical course, and molecular biology. Karyotyping may demonstrate clonality but each outgrowth is unique. Further, the tumors arising from the premalignancies are members of the same clone. Thus, an animal which could model the field effect exhibits the evolution of diverse clonal variants.

The biology of the spontaneous mouse mammary tumors is significantly different than human breast cancers (Cardiff, 2001). This can be attributed to

the etiological agent in the mouse. MMTV is programmed to be hormone driven and transmitted via the mother's milk. Therefore, pregnancy-related hormones have driven the tumor biology in the mouse in a different manner distinct from the human. Except for the well-documented, pregnancy-dependent plaque in the GR/A, BR, and the DD mice, most mouse mammary tumors are not ovarian hormone dependent and have low levels of estrogen receptors (Foulds, 1959). We must note that, in this context, most GEM transgene models use the MMTV-LTR to promote transgene expression in the mouse mammary gland. Since the LTR has the HREs, one should not be surprised to find a similar tumor biology in the transgenic mice. However, with the development of knockin mice and other types of promoters, a new type of tumor biology may be expected.

Given the similarities with human cancers, one would expect that the metastases also would be similar. Although, metastatic tumors have been found in mouse lymph nodes and other organs, the vast majority of metastases are pulmonary and, typically, embolic. In contrast, human breast cancer metastases are typically to regional lymph nodes and the bone marrow. Thus, GEM have not changed the biology of metastasis in mice.

The GEM have provided the scientific community with tremendous resources. The neoplastic potential of any gene can now be tested in the specific organ of an immunologically intact mouse. Genes associated with the familial influence sought by Bashford, Murray, Tyzzer, and Little at the turn of the twentieth century are now identified and being tested in GEM. The chromosome factors postulated by Boveri have been tested. The influence of individual genes and combinations of genes controlled by different promoter systems are now being studied. The potential seems unlimited.

D. Biotechnology and the Commercialization of Science

The advent of molecular biology has been a dramatic inducement for the commercializing of science. An entire biotechnology industry has arisen from the molecular revolution. This is salutary in the sense that the industry is poised to quickly bring the discoveries in basic sciences to medical care. The commercial value of the technology is enormous. Unfortunately, the value is accompanied by the proprietary need to protect investments and frequently involves patents.

The Oncomouse patent, licensed by Harvard to DuPont, is one of the prime examples of commercialized science (McBride, 2004). Again, mouse mammary tumor biology has led the way. The patent, primarily based on the introduction of *myc*, *ras*, and *neu* into the mouse, in the view of the Licensee, DuPont, covers all mice developed for the purpose of creating

tumors. The issue has been debated in public forums and private meetings without complete resolution. The proponents believe that the patent protects the intellectual property of Harvard and DuPont, encouraging rapid development (McBride, 2004). However, others believe that the patent has been a major impediment to using the GEM in drug development (Check, 2002; Kondro, 2002). DuPont and Harvard maintain that licenses for nonprofit and academic institutions are readily obtainable (McBride, 2004). Others have still found significant impediments.

Whatever one's stance on the patent issue, the use of tumor-bearing GEM in preclinical treatment and prevention trials has been sparse (Green and Hudson, 2005). Recently the NCI has mandated that its mouse models of Human Cancers Consortium develop preclinical trials using the GEM models including mouse mammary tumors. These trials are starting to appear in the literature (Green and Hudson, 2005; Namba *et al*., 2005, 2006; Wislez *et al*., 2005). Since the trials are a recent development, the long-term advantage of GEM is still open to question.

Many of the questions about the host contribution voiced in Bittner's tripartite hypothesis can now be addressed in an extremely efficient manner. Knock out of numerous host genes has provided insights into the role of specific molecules that have never been available before. The examples already are too numerous to review or list here. However, the technology has permitted elucidation of macrophage function (Pollard, 2004), transcription factors (Man *et al*., 2003), adhesion molecules (White *et al*., 2004), cytokines (Siegel *et al*., 2003), and many other molecules involved in tumorigenesis.

Overall, the genetic modification techniques have meant that mice can be remodeled to make them exhibit mammary tumors and other diseases from which they would not normally suffer. As more genes that encode for more proteins are added or subtracted from their genomes, the more closely they mimic human cancers. Scientific advances should make tests in these mice more rather than less valuable. They offer new opportunities to understand human breast cancer more completely and to screen treatments before human trials can occur. But, will we solve the 100 year-old riddles?

XIII. EPILOG

> The first attempts to write the history of a scientific discipline often presage its imminent senescence.
>
> (Stent, 1968)

What started with Apolant comes now to the century mark (Apolant, 1906). Those who laid the foundation for experimental cancer research

posed questions have been refined and extended by powerful new technologies. The "giants" on whose shoulders "we stand" would surely be amazed and pleased if they could see the extent to which their mouse mammary tumor model and its modern day GEM equivalent has and will continue to contribute to cancer research. Many of the original questions, however, still resonate through the years: Is the mouse a good model for human disease? What is the role of host factors? What is role of hormones? What is the role of immunity? Does human breast cancer have an infectious etiology? Each question is as resoundingly familiar to modern readers as 100 years ago. Are we merely mindlessly repeating previous experiments? Have we learned, with all of our sophistication, more about less and less?

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FOUNDATIONS IN CANCER RESEARCH*

Ordered Heterogeneity and Its Decline in Cancer and Aging

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I. Introduction
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Ordered heterogeneity was introduced as a basic feature of the living state in the mid-1950s. It was later expanded to "order in the large over heterogeneity in the small" as the first principle of a theory of organisms. Several examples of ordered heterogeneity were given at the time to illustrate the principle, but many more have become apparent since then to confirm its generality. They include minimum size requirements for progressive embryological development, the errant behavior of cells liberated from tissue architecture, their sorting out to reconstitute tissues on reaggregation, and contact regulation of cell proliferation. There is increasing heterogeneity of cell growth with age, and marked heterogeneity of many characters among cells of solid epithelial tumors. Normal growth behavior is reintroduced in solitary, carcinogen-initiated epidermal cells by contact with an excess of normal epidermal cells. Contact normalization also occurs when solitary hepatocarcinoma cells are transplanted into the parenchyma of normal liver of young, but not of old, animals. The role of the plasma membrane and adhesion

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molecules in ordering heterogeneity is evaluated. Organizing the results in a conceptual structure helps to understand classical observations of tumor biology such as the lifetime quiescence of carcinogen-initiated epidermal cells and the marked increase of cancer incidence with age. The principle of order above heterogeneity thus provides a unifying framework for a variety of seemingly unrelated processes in normal and neoplastic development. Whereas contact between cells is required for these processes to occur, gap junctional communication is not required.

ABBREVIATIONS

CEA, carcinoembryonic antigen; ECM, extracellular materials; GJC, gap junction communication; LOH, losses of heterozygosity; PAH, polycyclic aromatic hydrocarbon.

I. INTRODUCTION

In 1955, a small group of distinguished biologists was convened to consider the perceived lag in conceptual maturation in biology in the face of rampant empirical development (Gerard, 1958). The most clearly defined concept to emerge from that meeting came from the pioneer molecular geneticist Rollin Hotchkiss in his definition of life as "the repetitive production of ordered heterogeneity." This concept was then elaborated by the prominent neuroembryologist Paul Weiss on the basis of his own systems analysis, and applied in diverse ways by the other participants to their experience. Weiss specified ordered heterogeneity as a hierarchy of levels starting from free molecules to their assembly in organelles, upward to cells, tissues, organisms, and their environment. Later he formulated the terms macrodeterminism and microdeterminism to characterize higher order states that cannot be generated simply as the sum of lower order elements (Weiss, 1973).

Perhaps because molecular biology and genetics were expanding in an explosive way following resolution of the structure of DNA, little notice was taken of the concept of ordered heterogeneity. A notable exception appeared in the biological writings of the eminent theoretical physicist Walter Elsasser who had independently forged a similar but more abstract approach in developing a theory of organisms (Elsasser, 1958, 1975). He later adopted Hotchkiss' terminology of ordered heterogeneity as the first principle of a formal biological theory in which he postulated that "there can be regularity in the large where there is heterogeneity in the small" or "order above heterogeneity" (Elsasser, 1998).

Heterogeneity of many cellular characteristics is a pervasive feature of malignant tumors (Heppner, 1984). Cellular growth rates in normal tissue also become heterogeneous in aging animals (Fry *et al.*, 1966). In both cases it would appear that there is a loosening of the ordering capacity associated with intact normal tissue. The purpose of this chapter is to review a variety

of situations in which the general principle of order above heterogeneity is manifest in order to better understand the circumstances of its loss in cancer.

II. THE ROLE OF TISSUE SIZE IN DEVELOPMENTAL BIOLOGY

Unbeknownst to Elsasser, there was already a considerable body of information about a minimal size requirement for progressive morphogenesis and differentiation in organisms. Fragments of various sizes cut from the head process stage of the mouse embryonic shield were cultivated for several days and implanted into the eye to determine the effect of reduction in size on the capacity to give rise to nervous tissue (Grobstein, 1952). Intact shields, even though precultured, yielded implants with nervous tissue. However, shields that had been cut into fragments prior to culturing in close clusters showed declining incidence of nervous tissue in the subsequent implants as the fragment sizes decreased from 1/2 to 1/16 of the intact shield sizes (Grobstein, 1955). At the 1/16 level, nervous tissue was virtually absent even though the total explant mass in the cluster was the same as that of the intact shield.

Similar experiments were done with fragments of the much larger primitive streak blastoderm of the chick embryo, which were transplanted on to the chorioallantoic membrane after cultivation *in vitro* (Grobstein and Zwilling, 1953). In decreasing the size of the fragments below 1/8 of the blastoderm, the graft weight and incidence of nervous tissue dropped off sharply. This result on a small part of the chick blastoderm repeats what was observed with the whole mouse embryonic shield and indicates that capacity to grow and differentiate is not the fractional value of the piece with regard to the whole but the absolute size of about 0.1 mm.

This size requirement recalled observations made much earlier in oviparous (egg laying) marine invertebrates (Berrill, 1941). Attempts were made to determine just how small a part is capable of forming a new organism out of its contained material. Fragments of compact tissue less than 0.1–0.2 mm in diameter fail to undergo regeneration to form a new individual, however simplified (Table I). It is notable that the order of size is the same as that of the eggs, which is representative of the minimum egg size of the vast majority of oviparous marine invertebrates. Both cases, the unicellular egg and the multicellular piece, represent protoplasmic masses that develop into an organized individual without recourse to material other than that initially apparent. It is evident that the minimal size for regeneration of these primitive organisms is of the same order as that required to carry on differentiation in the mouse and the chicken (Grobstein, 1952; Grobstein and Zwilling, 1953). Similar minimal dimensions have been found for reconstitution of

Table I Minimal Sizes of Tissues and Eggs of Oviparous Marine Invertebrates for Development

Animal	Minimal size (diameter) of pieces capable of complete reconstitution (mm)	Approximate diameter of eggs of same animal (mm)
Hydra (Coelenterata)	0.17	0.18
Lineus (Nemertinea)	0.15	0.12
Ephydatia (Porifera)	0.20	0.14
Clavelina (Tunicata)	0.30 (not compact tissue)	0.23

After Berrill (1941).

new individuals from aggregates for sponges and the hydroid, *Corymorpha* (Grobstein, 1955). The data suggests that the developmental capacity of tissues lies more in the pattern of cell interactions than autonomously in the cells themselves.

What might be a related phenomenon is known as the community effect first described in the differentiation of muscle in *Xenopus* blastula (Gurdon, 1988). Cells from the animal region of the blastula were placed in sandwiches between vegetal regions. The cells had to be in a three-dimensional aggregate, not in a monolayer, to differentiate into muscle. In later experiments using muscle progenitor cells from *Xenopus* mid-gastrula, more than 100 cells had to be in aggregates to activate early myogenic genes and later muscle-specific genes. Muscle gene activation did not require gap junction communication (GJC) among cells in the aggregate (Gurdon *et al.*, 1993). If the transfer of the muscle precursor cells is delayed until the late gastrula stage, even single cells differentiate into muscles, indicating they can complete their differentiation in the absence of their original neighbors (Kato and Gurdon, 1993). If the muscle progenitor cells from the mid-gastrula are exposed to dorsalizing molecules, only 10 cells are required to induce differentiation (Carnac and Gurdon, 1997). The reduction in cell number required for muscle differentiation as the embryo matures suggests that the dorsalizing treatment may have advanced the cells toward the late gastrula stage at which no community effect is required. Similar community effects are seen in muscle differentiation of mouse embryos (Cossu *et al.*, 1995) and of embryonal carcinoma cells of mice transfected with the MyoD gene, which is a regulatory factor for myogenesis (Skerjanc *et al.*, 1994). A complementary aspect of the community effect has been reported in zebra fish and *Drosophila* in which the transplantation of single cells of one fate of the early embryo to a region that has a different fate changes the fate of the single cells to that of the regional mass, but transplantation of cells in clumps allows them to retain their original identity (Ho, 1992; Stüttem and Campos-Ortega, 1991).

III. BEHAVIOR OF DISSOCIATED CELLS AND THEIR REASSOCIATION

Given the requirement for a minimal mass of tissue for its progressive development, it would not be surprising if dissociation of the tissue into its individual cells and their cultivation in a dispersed state on a solid substrate would result in their dedifferentiation. This is in fact the case for most tissues with the notable exception of fibroblasts which occur in connective tissue as isolated, mobile cells (Harris, 1964). Indeed, if they are maintained at very low density, even freshly explanted fibroblasts fail to multiply (Todaro and Green, 1963). Chick embryo fibroblasts increase three- to fourfold in average size, protein and RNA content, and become very heterogeneous in size within a day after explantation from the embryo into monolayer culture (Rubin and Hatié, 1968). However, if dissociated homophilic cells of the chicken or mouse embryo are rotated in suspension, they aggregate to form structures characteristic of the tissue of origin (Moscona and Moscona, 1952). The capacity of cells to aggregate is high when taken from 7-day-old chick embryos, but it declines as the embryo source ages, reaching close to zero in cells from 16-day-old embryos (Moscona, 1962). Growth of the cells in monolayer culture also results in a rapid decline in mutual cohesiveness of cells. Specialized markers are lost quickly in sparse monolayer cultures but are restored in reaggregated cultures (Linser and Moscona, 1979). If cells from different tissues are mixed, they initially associate with each other at random, but sort themselves out in the next few days into separate masses within a single structure (Moscona, 1960). The different tissues are reconstructed in definite positions in relation to one another, for example muscle is always rebuilt external to cartilage, never the other way around (Steinberg, 1963b). The position of a tissue in a mixture of two different cell types is determined by the strength of the homophilic adhesions, the one with the stronger adhesions forming the inner mass and the one with the weaker adhesions external to it.

In contrast to the complete separation of reaggregating cells from different tissues, cells of the same tissue from different species, such as chicken and mouse, randomly aggregate without discrimination into the same mass (Moscona, 1960). It was originally believed that viscous extracellular materials (ECM) released from the cells served as a structural matrix and binding agent for the reassembly of cells in definitive patterns. It was later shown however that tissue reconstruction from dissociated cells did not require the presence of the ECM (Auerbach and Grobstein, 1958). ECM proved to be a highly hydrated DNA gel containing proteins formed by chromosomes of disrupted cells (Steinberg, 1963a). These results indicated that the tissue-specific aggregates are formed by intimate contact between

the cell surfaces. This conclusion was reinforced by the failure to find electron microscopic evidence for intercellular "cement" substance in the 50 Å of separation between parenchymal cells of the intact liver (Coman, 1954).

IV. THE MOLECULAR BASIS OF CELL–CELL ADHESION

Given that there is a minimum size for the progressive development of tissue structures and that isolated cells lose their capacity for differentiated function, it is apparent that the mutual adhesion of homophilic cells plays an important role in guiding their function. This is particularly true in the case of epithelial tissues, such as epidermis, where there is a tight association between cells. Intercellular adhesion in epithelial tissue is accomplished in the epidermis by two types of intercellular junction, the adherens junction and the desmosome (Fuchs and Raghavan, 2002). One of the most important and ubiquitous types of adhesive interactions required for the maintenance of solid tissues is that mediated by the classic cadherin adhesion molecules (Gumbiner, 1996). Cadherins are transmembrane, Ca^{2+}-dependent homophilic adhesion receptors that play important roles in cell recognition and cell sorting during development (Takeichi, 1991). However, they continue to be expressed at high levels in virtually all solid tissues of adults (Gumbiner, 1996).

The cadherins form a superfamily with at least six subfamilies (Foty and Steinberg, 2004). Some of these subfamilies comprise classical or type-I cadherins and atypical or type-II cadherins of adherens junctions. Ten or more cadherins have been identified in the type-I and type-II subfamilies, and many more exist in the other subfamilies. Desmocollin and desmogleins are some of the adhesion proteins in desmosomes (Fuchs and Raghavan, 2002).

Both the adherens junction and the desmosomes are required for epithelial sheet formation. The core components of the adherens junction, its cadherin transmembrane anchor and its link to the actin cytoskeleton, are used in the first step of intercellular adhesion to draw epithelial cells together. The second step in the epidermis involves desmosomal cadherins and their indirect associations with the keratin intermediate filament cytoskeleton which are required to clamp the elements into place. The redirected actin polymerization then seals the membranes to make the adhering sheets of cells that form the epidermal barrier. The strength of intercellular adhesion is significantly increased by the clustering of cadherins as well as interactions of cadherin with the actin cytoskeleton (Adams and Nelson, 1998).

The role of cadherins in the sorting out of cells was demonstrated in L cells, which are an established cell culture line of neoplastic mouse fibroblasts (Steinberg and Takeichi, 1994). These cells initially express little or no cadherins and do not aggregate. They were transfected to express two substantially different levels of a cadherin. When the two cell populations were combined as intermixed single cells or as opposed aggregates, they sorted out with the more strongly adhesive population expressing the higher level of cadherin arranged in the center of the aggregates and the less adhesive population on the outside. This agreed with the "differential adhesion hypothesis" in which tissue movements in development are guided by cell adhesion-generated tissue surface tension (Steinberg, 1963b). Support for the hypothesis was obtained from measurements of surface tension of aggregates from five different tissues of the chick embryo, which predicted the mutual developmental behavior of different combinations of the tissues (Foty *et al.*, 1996).

In contrast to early concepts of adhesion in development, which postulated only a few types of adhesion molecules (Edelman, 1984) and that each cadherin subclass has specific binding affinity (Takeichi, 1988), the situation has become far more complicated. Not only are there large numbers of cadherins, but all type-I and type-II cadherins tested cross-adhere, in most cases with strengths close to those of their self-adhesions (Foty and Steinberg, 2004). The very same molecules that give rise to tissue compartmentalization during morphogenesis are involved in the elimination of these boundaries during invasion and metastasis by tumors. As an approach to understanding tumor behavior, it has been suggested that attention be focused on measuring differential tissue cohesive and adhesive relationships of living cells, and that the contribution of any single adhesion system not be overemphasized (Foty and Steinberg, 2004).

The reason for this caution can be illustrated by the case of E-cadherin that has been implicated as an important molecule in invasion and metastasis because its expression is in some cases inversely correlated with tumor aggressiveness (Mareel *et al.*, 1992). However, decrease in cadherin expression is not always predictive of invasiveness, probably because its expression does not necessarily reflect its function. There are many examples of highly invasive cell lines that express normal levels of E-cadherin but exhibit abnormalities of their cytoplasmic catenins, or in the ability of E-cadherin to form strong bonds (Foty and Steinberg, 2004). Mechanisms of metastasis appear to involve a complex array of genetic and epigenetic changes, many of which appear to be specific both for different types of tumors and different sites of metastases (Cairns *et al.*, 2003). Indeed there are cases in which increased expression of E-cadherins by neoplastic epithelial cells enables intraepithelial expansion of tumors, presumably by increasing cohesion of the tumor cells (Auersperg *et al.*, 1999; Bindels *et al.*, 2000).

The improved understanding of the expanded roles of the individual molecules involved has resulted in a mechanistic blurring of the previously described discrete stages of tumor invasion and metastasis. For example, the adhesion molecule known as carcinoembryonic antigen (CEA) shows surface *overexpression* in nearly all human colorectal carcinomas (Shuster *et al.*, 1980). It mediates homophilic aggregation of human colorectal carcinoma cells in culture and the sorting of a cell line transfected with the gene that encodes CEA (Benchimol *et al.*, 1989). Yet, all six human colorectal cancers tested directly after surgical removal of the tumors exhibited a reduction in adhesiveness as compared with the normal colorectal epithelium (McCutcheon *et al.*, 1948). As shown below, this functional loss of adhesiveness is typical of carcinoma cells (Coman, 1944; McCutcheon *et al.*, 1948). However, the increase in CEA adhesion molecules would have predicted the opposite. It may be argued that the small amount of CEA in normal colorectal cells appears mainly on the luminal surface of columnar epithelial cells lining the upper parts of the crypt (Benchimol *et al.*, 1989). In colorectal cancers, which no longer conform to the single-cell layer organization by bulging into the crypts and invading through the basement membrane in multicellular arrays, CEA is produced in large amounts along borders between cells. In the lateral position, CEA may replace other adhesion molecules and *reduce* intercellular adhesiveness. But this explanation seems unlikely to hold for the increase of the CEA adhesion molecules that occur in a high proportion of human tumors other than colorectal cancers (Chevinsky, 1991).

What is evident is that cancer involves a progressive loosening of ordering capacity of a tissue over the heterogeneous behavior of isolated cells (Rubin, 2006), and that loosened ordering capacity is associated with a reduction of the adhesive force between epithelial cells. This became patently clear in measurements made of homophilic adhesions among normal squamous epithelial cells, benign papilloma cells, and invasive squamous carcinoma cells (Coman, 1944). The method was to measure adhesiveness (in milligrams) by amplification of the bend produced in a microneedle when a pair of cells was pulled apart (Table II). It is apparent from the data that far more force was required to separate normal cells from the lip than from carcinomas of the lip, while papillomas of the skin had almost as strong adhesions as the normal cells of the lip. The difference in adhesion between normal cells of the cervix and cervical carcinoma cells was even greater than that between comparable pairs in the lip.

The method used to measure the adhesiveness of squamous carcinoma cells by pulling cells apart was not applicable to glandular carcinoma cells. Instead, a shaking device with graded increases in shaking intensities was used to determine its capacity to dislodge cells from tissues and to separate them from each other (McCutcheon *et al.*, 1948). Those cells less strongly

Table II Forces Required to Separate Pairs of Cells by Micromanipulation

Derivation of cells	Mean and standard error (mg)
Normal lip	1.42 ± 0.041
Carcinoma, lip	0.47 ± 0.051
Papilloma, skin	1.25 ± 0.032
Normal cervix	1.11 ± 0.039
Carcinoma, cervix	0.18 ± 0.022

Each figure is based on 50 pairs of cells and represents the mean (with its standard error) of the force in milligram required to separate the cells by micromanipulation. The cells in each group were obtained from 5 individuals, and from each specimen 10 pairs of cells were examined. Coman (1944).

bound together were separated, and those more firmly bound remained attached. In 20 out of 21 cases of different types of human adenocarcinoma, more cells were shaken out of cancerous than out of normal tissue as revealed by photographs of the tissues before and after shaking; counts of single cells separated from cancerous tissue were correspondingly higher. The results from the experiments with squamous and glandular cancers justify the conclusion that the mutual adhesion of human carcinoma cells is less than that of corresponding normal cells. The conclusion is consistent with the hypothesis that the invasive character of cancer is, at least in part, the result of decreased cellular adhesion. The combined results add to the impression that the loss of adhesion is an indication, and perhaps the cause of escape from the ordering capacity of the tissue by the cancer cells. They do not rule out the possibility that the apparently normal cells surrounding the tumors have lost some of their ordering capacity, as will be seen later (McCullough *et al.*, 1997; Strickland *et al.*, 1992).

The most extreme example of the loss of adhesiveness of cells is seen in the development of ascites tumor cells from solid tumors inoculated into the peritoneal fluid (Klein, 1951). The ascites tumor cells can maintain themselves and increase in number in the peritoneal fluid. Such cells exhibit an increasing negative charge as they show an increasing tendency to grow in the ascites form (Purdom and Ambrose, 1958). The increase in negative charge and ability to grow in the ascites form are correlated with the capacity to form metastases in the lung (Ringertz *et al.*, 1957). The increase in negative charge indicates either a change in the number of charged groups attached to high-molecular-weight material at the cell surface or a decreased capacity to absorb positive ions (Ambrose *et al.*, 1956). That the latter may indeed be the case is suggested by the decreased calcium content of tumor tissues in comparison with normal tissues (de Long *et al.*, 1950; Sanui and Rubin, 1982).

V. CONTACT RELATIONS AMONG HOMOPHILIC CELLS IN REGULATION OF GROWTH AND PROLIFERATION

The term contact inhibition was derived to signify that normal fibroblasts which attach to the surface of a culture dish move randomly over the surface until they encounter another like cell (Abercrombie and Heaysman, 1954). The movement of the cell is usually preceded by a ruffled membrane which forms a strong adhesion and stops ruffling when it touches any part of the peripheral membrane of another fibroblast (Abercrombie and Ambrose, 1958). The forward movement of the cell stops, giving rise to the term contact inhibition. It may break off the adhesive contact and move in another direction, but when a monolayer of cells becomes confluent and each cell is completely surrounded by other like cells, the ruffling of the membrane and independent movement stop.

The term contact inhibition was applied to cell proliferation because there is a marked decrease in proliferation rate in confluent monolayers, indicating a relationship between membrane movement and the rate of cell proliferation (Todaro *et al.*, 1965). Sarcoma cells are rounded in culture and do not form ruffled membranes, but have a very actively moving surface in the form of very fine, rather spiky processes all around the cell periphery (Abercrombie and Ambrose, 1958). There is little or no adhesion or change in either surface activity or locomotion when one sarcoma cell meets another, or meets a fibroblast; nor do these changes occur in a fibroblast when it meets a sarcoma cell. Epithelial cells also exhibit inhibition of motion and proliferation in sufficient contact with each other (Castor, 1968), but they neither inhibit nor are inhibited by fibroblasts (Eagle and Levine, 1967). The results indicate that homophilic cells inhibit their proliferation by forming adhesions between their surface membranes, which in turn reduces the physical activity of the membranes. Unlike fibroblasts, in which the intercellular adhesions are broken when one of the cells can move into open space, the adhesions between epithelial cells are stable and the cells do not move apart (Middleton, 1973). As a result, epithelial cells tend to move as a coherent sheet extending from an explant. These epithelial sheets consist of closely adherent sheets with ruffled membranes restricted to the cells at the free margin. The structure and locomotion of the sheet are thus in marked contrast to the loose network of cells formed by fibroblasts under appropriate culture conditions. Given that epithelial cells exhibit contact inhibition (Castor, 1968; Eagle and Levine, 1967), proliferation would be maximized at the free margin of a coherent sheet.

Paradoxically, primary cultures of fibroblasts require some limited contact with each other for optimal function. They proliferate poorly at very

low population density in fresh medium (Rubin, 1966; Todaro and Green, 1963). Seeding the cells in contact with a relatively large but nonconfluent population of x-irradiated, nonproliferating primary cells allows the living cells to proliferate at a maximal rate (Rein and Rubin, 1968). This rate is greatly reduced if the living cells on an inverted coverslip are separated from the irradiated cells by as small a distance as 0.2 mm. The results show a wide range of regulation, from optimal function with limited contact among themselves to inhibited proliferation when in confluent cultures.

VI. ROLE OF THE PLASMA MEMBRANE IN THE REGULATION OF CELL GROWTH

It has long been known that proteins in serum are required for the proliferation of animal cells in monolayer culture (Temin, 1966). It was widely assumed that the multiple protein growth factors of serum bound to receptors on the cell membrane to initiate their stimulatory effect, but it was not until individual growth factors that contribute to the effect were identified that it could be firmly established that entry into the cells was not required. The most significant of these proteins for establishing the site of action of growth factors was insulin, which could replace serum for the multiplication of chicken embryo fibroblasts (Temin, 1967). It was first reported that insulin could be attached to large polymers of Sepharose which were not taken into cells and still induce its classic metabolic response of increased glucose utilization and lipolysis (Cuatrecasas, 1969). The validity of such studies was questioned primarily because of the amount of soluble material that was released and the possibility that such material may be superactive (Carney and Cunningham, 1977). However, it was shown that trypsin immobilized on polystyrene beads initiated chicken embryo fibroblast division which could not be accounted for by its release into the medium or into the cells (Carney and Cunningham, 1977).

It was also shown that insoluble floccules of calcium pyrophosphate brought on the full coordinate response in an established line of mouse fibroblasts, whereas pyrophosphate in solution had no effect (Bowen-Pope and Rubin, 1983). The floccules were only effective if they attached to the cells; their removal from the surface at low pH blocked further action. In another regard, attachment to the culture dish of only a small fraction of the total surface area of a single cell from a line of mouse fibroblasts is sufficient to stimulate DNA synthesis without any increase in the total surface area of the cells (O'Neill *et al.*, 1986). This indicates that it is membrane activation and its intracellular consequences rather than change in cell shape (Ingber, 2005) that drives cell proliferation. Visual evidence of membrane perturbation

in growth stimulation is seen when insulin is added to serum-starved quiescent cells (Evans *et al.*, 1974). The serum-starved cells exhibit few microvilli at their surface, but within an hour of insulin addition microvilli appear at the surface in large numbers, along with subcellular organization characteristic of cells in exponential growth.

Contact-inhibited confluent cells are limited in their movement and in surface activity (Abercrombie and Ambrose, 1958). It had been proposed that contact inhibition of growth is mediated by the establishment of GJC and exchange of metabolites between the cells (Loewenstein and Penn, 1967). The alternative possibility is that the simple reduction of membrane activity in confluent cultures is sufficient to account for the inhibition. That the latter is indeed the case was strongly supported by demonstrations that the addition of surface membranes isolated from nontransformed cells to nonconfluent cells imitated the effects of contact between living cells in confluent cultures. A surface membrane-enriched fraction of mouse 3T3 fibroblasts added to a sparse living culture of the same cells inhibited the rate of DNA synthesis (Wittenberger and Glaser, 1977). It had no effect on transformed 3T3 cells. Glutaraldehyde-fixed plasma membranes of confluent human embryonal lung fibroblasts inhibited the growth of sparsely seeded lung fibroblasts (Wieser *et al.*, 1985). The inhibitory effects were abolished by those treatments of the membranes which inhibit the synthesis of the oligosaccharides of glycoproteins or enzymatically destroy them, indicating that cell growth is inhibited by specific cell–cell contact. Some of the molecular reactants in this process are membrane glycoproteins with asparagine-linked oligosaccharides (Wieser *et al.*, 1985).

Plasma membrane purified from adult rat liver inhibited growth-related activities of low-density monolayer cultures of mature rat hepatocytes (Nakamura *et al.*, 1983). The same preparations *stimulated* production of hepatocyte-specific enzymes characteristic of the differentiated state, just as confluence among live cells did. Specific differentiated functions and morphology of adult rat hepatocytes can be maintained up to 8 weeks *in vitro* only when they are cultured in the presence of rat liver epithelial cells, which are presumably derived from primitive biliary cells (Mesnil *et al.*, 1987). When the primary hepatocytes are cultured alone they lose the differentiated state in 2–3 days. Contact between the hepatocytes and the biliary epithelial cells of the liver is required to maintain the differentiated function of the former. Although there was increasing GJC with time among hepatocytes themselves in cocultures, and GJC was high throughout the experiment among liver biliary cells themselves in the cocultures, there was no GJC between the two cell types at any time during the culture period. The results indicate that the maintenance of hepatocyte-specific functions requires intercellular contact, but not GJC, of molecules up to 1000 Da.

It is possible, however, that GJC among the hepatocytes is necessary for transmission of the signal originating from the biliary cell membranes.

Normal cells in culture must attach to the surface of the dish in order to proliferate. They fail to multiply when suspended in semisolid medium or in liquid medium over a surface that does not allow attachment. If a narrow strip of cells is removed from a confluent, contact-inhibited culture, cells remaining on the edge migrate on the bared surface and proliferate rapidly until the gap is filled (Gurney, 1969). These results and others (O'Neill *et al.*, 1986) suggest that physical stress on the cell membrane in addition to the presence of serum growth factors is a component of proliferation. It has long been known that cells in certain tissues respond to mechanical signals. For example, mechanosensitive cells in bone, such as osteoblasts, osteoclasts, osteocytes, and cells of the vasculature are so placed in the tissue to react to strain, stress, and pressure (Rubin *et al.*, 2006). The sensations of touch and hearing are generated by mechanical stress, and other examples have been found in bacteria, worms, flies, mice, and humans (Kung, 2005). The proteins of mechanosensitive ion channels sense forces from the lipid bilayer which open and close the channels. Given the presence of such arrangements throughout the evolutionary scale of organisms, it is not unlikely they contribute to the necessity of cell attachment for growth of vertebrate cells in culture, and may well be involved in regulation of tissue function, as in the examples cited above.

VII. NORMALIZATION OF NEOPLASTIC CELLS BY CONTACT WITH NORMAL CELLS

The primary role of the cell membrane in regulation of proliferation, particularly with regard to contact inhibition, raises the question whether surrounding neoplastic cells with normal cells of the same type could reverse neoplastic behavior. A relevant consideration is that the mutual adhesiveness of papilloma cells and of carcinoma cells is slightly less and substantially less, respectively, than that of their normal counterparts (Table II; Coman, 1944). It has long been known that transformation of fibroblasts by infection with RNA and DNA tumor viruses could be prevented or reversed by surrounding single cells with contact-inhibited normal fibroblasts (Rubin, 1960; Stoker, 1964; Stoker *et al.*, 1966) although such reversal is only possible in the case of some RNA viruses in the presence of high concentrations of serum in the medium (Rubin, 1960). Related observations have been made for fibroblasts transformed spontaneously (Rubin, 1994) as well as by treatment with chemical carcinogens or ultraviolet light (Bertram, 1977; Herschman and Brankow, 1986).

A. Carcinogenic Initiation of Epidermal Cells and Its Suppression by Adjacent Cells

The possibility of suppressing neoplastic development in epithelial carcinogenesis took on special significance in view of the classic phenomena of initiation and promotion in development of chemically induced skin tumors in mice. Repeated painting of the skin with carcinogenic polycyclic aromatic hydrocarbons (PAHs) over a period of several months results in the appearance of multiple papillomas, some of which develop into carcinomas (Hieger, 1936; Rubin, 2001). A single application of the PAH however produces no tumors unless it is followed by repeated applications of an agent, which by itself produces no tumors (Berenblum and Shubik, 1947; Mottram, 1944). The single PAH treatment is known as initiation, and the repeated treatment with the noncarcinogenic agent is called promotion. The promotional treatment can be delayed for many months after initiation with no loss of capacity of the initiated epidermis for tumor production (Van Duuren *et al.*, 1975). The common interpretation of these results is that tumorigenic mutations are induced in the basal cells of the initiated skin, known as keratinocytes, but those mutations do not result in tumor formation until some physiological change is produced by the promoter. That change is thought to be induction of terminal differentiation in the main population of the keratinocytes and proliferation in a minority population, which includes the initiated cells (Yuspa *et al.*, 1982). Selective proliferation of the initiated cells results in papilloma formation.

Lines of initiated keratinocytes in culture multiply in the presence of calcium concentrations in which normal keratinocytes cease proliferation and terminally differentiate (Hennings *et al.*, 1990; Rubin, 2003). In a valid culture model for initiated epidermis, the clonal expansion of initiated cells would most likely be inhibited by immediately adjacent normal keratinocytes. When a small number of initiated keratinocytes from a selected line was mixed with a large excess of normal keratinocytes, which were replaced weekly, they completely suppressed the formation of colonies by the initiated cells (Table III). The addition of a promoting agent restored colony formation by many but not all of the initiated cells. Coculture of the initiated keratinocytes with an excess of dermal fibroblasts failed to inhibit either the number or the size of colonies by the initiated cells (Table III) even though the fibroblasts produced confluence in 1–2 days after plating. Conditioned medium from keratinocyte cultures also had no inhibitory effect on the growth of the initiated cells, suggesting that direct contact between the initiated cells and the large excess of normal keratinocytes is required to suppress colony formation by the former. There was no fluorescent dye transfer between the initiated cells and the normal keratinocytes in

Table III Modulation of Neoplastic Cell Growth in Presence of a Large Excess of Non-neoplastic Skin Cells

		Neoplastic cell growth	
Neoplastic cells	Non-neoplastic cells	Cell culture[a] Transformed colonies	Skin grafts[b] Tumor size
Papilloma	None	+++	+++
Papilloma	Normal keratinocytes	—	±
Papilloma	Normal keratinocytes plus TPA treatment	++	+
Papilloma	Dermal fibroblasts	+++	+++
Papilloma	Initiated, non-neoplastic keratinocytes	ND	+++
Carcinoma	None	ND	+++
Carcinoma	Normal keratinocytes	ND	+++

[a]Hennings *et al.* (1990).
[b]Strickland *et al.* (1992).
—, no neoplastic growth; ±, average about 90% reduction in tumor size; +, moderate reduction in neoplastic cell growth; ++, slight reduction in neoplastic cell growth; +++, maximum neoplastic cell growth.
ND, not done.

either direction (Hennings *et al.*, 1992). The lack of GJC between the cells suggests that direct contact of plasma membranes between confluent cultures of normal keratinocytes and initiated cells is sufficient to suppress the growth of the latter. A similar lack of GJC has been reported in the suppression of transformation in glial cells by normal glial cells (Alexander *et al.*, 2004), indicating cell contact is sufficient for the effect.

A variation of the above experiments was used in which the initiated cells derived from a line of papilloma cells were grafted to a prepared site in the skin of mice, where they proliferated to form a papilloma (Strickland *et al.*, 1992). Addition of an excess of dermal fibroblasts to the initiated keratinocytes actually doubled the size of the papillomas, so they were routinely added to improve growth of the tumors. Adding a large excess of normal keratinocytes to the mixture resulted in an average 90% reduction in size of the papillomas (Table III). A further large excess of dermal fibroblasts had no inhibitory effect on papilloma formation. A special line of initiated cells resistant to terminal differentiation in culture, which had a normal epidermal phenotype when grafted to the skin, failed to inhibit papilloma growth. Despite their production of normal epidermis on grafting, this line had lost the capacity to inhibit tumor formation by initiated papilloma cells (Table III). Furthermore, normal keratinocytes that did

suppress tumorigenesis by initiated papilloma cells failed to suppress tumor formation by a malignant variant of the papilloma cells. As was the case with colony formation *in vitro* by the initiated papilloma cells, there was no effect of conditioned medium from the keratinocytes. The combined results from the *in vitro* and *in vivo* experiments indicate that cells initiated by a single application of a carcinogenic PAH to the skin of a mouse behave like normal keratinocytes for the remainder of the lifetime of the mouse because they are held in check by contact with the surrounding normal keratinocytes, that is, the majority of normal cells exert an ordering effect on the potentially tumorigenic individual initiated cells. This ordering effect is overcome by promoter treatment, which selects for the growth of the initiated cells by terminally differentiating the normal keratinocytes.

It is of interest to note that the adhesiveness of papilloma cells to each other is almost as high as that between normal cells (Table II). This suggests that the normal cells exert their normalizing effect on the initiated cells by forming strong adhesions with their membranes and modulating membrane activity. It also suggests that the microenvironment of the initiated papilloma cells may be altered by repeated application of the PAH to the extent that the surrounding keratinocytes lose the capacity to suppress tumor growth. However, once there is progression to the malignant state with substantial loss of adhesiveness among the cells, there is also the apparent loss of adhesiveness with normal cells (McCutcheon *et al.*, 1948), which is likely to interfere with the capacity of the latter to normalize the malignant phenotype. If the progression is only to a low grade of epidermal malignancy, the proliferation of the cells can be suppressed with an excess of normal keratinocytes in three-dimensional organotypic cultures (Javaherian *et al.*, 1998). The formation of squamous cell carcinoma by lines of preneoplastic tracheal epithelial cells can be suppressed by normal tracheal epithelial cells (Gillett *et al.*, 1989). The sensitivity of the neoplastic cells to suppression appears to decrease with their progression to higher degrees of malignancy.

B. Suppression of Solitary Hepatocarcinoma Cells by Intact Liver

The above experiments involved mixing phenotypically normal cells with neoplastic cells *in vitro* before testing them for colony formation in culture or tumor development in skin grafts. A different protocol was used in which two lines of aneuploid rat hepatocarcinoma cells derived from a culture of diploid liver stemlike cells were transplanted directly or indirectly into normal liver, or into other body sites of different-aged rats, and their fate studied over extended periods of time (Coleman *et al.*, 1993; McCullough *et al.*, 1994, 1997, 1998;

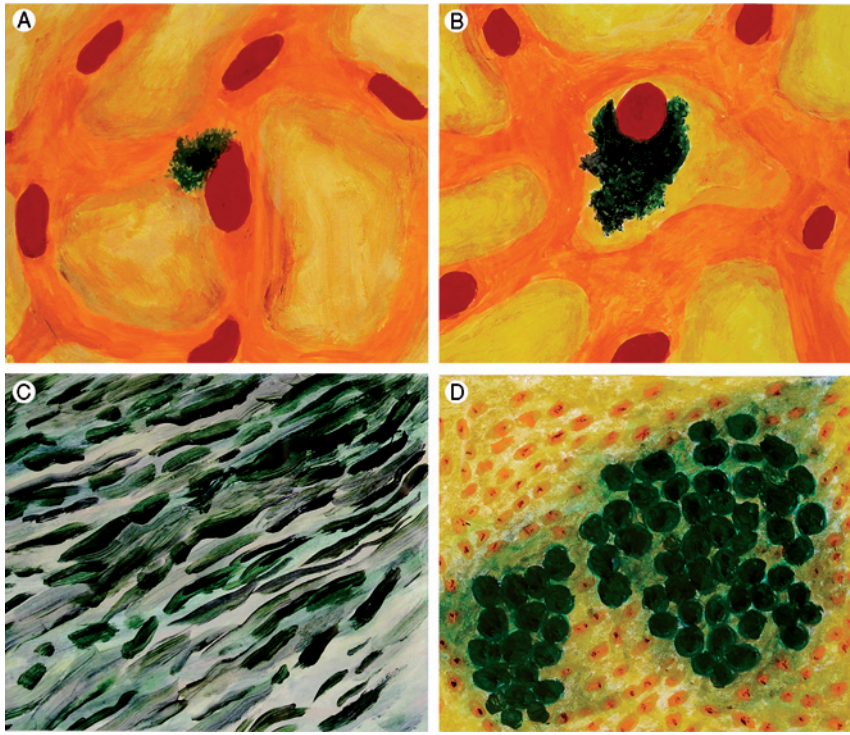

Fig. 1 Artistic representations of frozen sections of normal diploid or transformed aneuploid rat liver cells transplanted into the liver parenchyma or subcutaneous region of young rats. The transplanted cells carried copies of a chromosomally integrated lac Z reporter gene and are green while the nuclei of the liver parenchyma are red and cytoplasms are orange. (A) A normal diploid, stemlike liver cell 30 days after transplantation of many such cells into the liver. The cell had differentiated into a normal liver parenchymal cell. (B) One of many moderately aggressive transformed liver cells transplanted into the liver integrated into hepatic plates and morphologically differentiated with no tumor formation 87 days after transplantation. (C) Spindle cell tumor arising with short latency (17–21 days) after subcutaneous transplantation of malignant liver cells. Both moderately and highly aggressive transformed cells form tumors quickly when transplanted subcutaneously. (D) Tumor formed in liver after intrahepatic injection of highly aggressive transformed cells. The tumors exhibited a higher degree of differentiation than those observed at subcutaneous sites. (A) and (B) are at higher magnification than (C) and (D). The paintings were adapted by Dorothy M. Rubin, with permission, from photomicrographs in Figs. 2 and 4 of Coleman *et al.* (1993).

Smith *et al.*, 2000). The liver is an ideal site *in vivo* for homophilic transplantation of neoplastic cells because of its large size and overwhelming predominance of relatively uniform parenchymal epithelium. Following intrahepatic transplantation of the normal liver stem cells, they integrate into hepatic plates and differentiate into mature hepatocytes (Fig. 1A). Both of the neoplastically

transformed derivatives of the stem cells produce aggressively growing tumors when transplanted subcutaneously (Fig. 1C), but cells of the moderately aggressive line do not produce tumors of the liver when examined at 3 months. Instead they individually integrate into the hepatic plates and morphologically differentiate (Fig. 1B). Cells of the more aggressive line retain tumorigenicity in the liver but are more differentiated than are tumors at subcutaneous sites (Fig. 1D vs C). The results suggest that the hepatic parenchymal microenvironment regulates the differentiation of the neoplastically transformed hepatic stemlike cells, thereby eliminating or reducing their tumorigenic potential.

Since age is the most important risk factor in the development of many types of cancer (Newell *et al.*, 1989), an experiment was designed to determine the effect of age of the recipient rats on tumor formation by the transplanted liver cancer cells (McCullough *et al.*, 1994). The results in Table IV show that the moderately aggressive line produced no tumors at the site of transplantation over a 3-month period in 3- or 9-month-old rats, but the incidence of tumor formation increased with age beginning with transplantation at 12 months, and reaching 100% in those transplanted at 24 months. The latency time for the appearance of these tumors decreased with the age of transplantation. The overall results show a loss of capacity of the liver to regulate the growth of neoplastic liver cells as the rats aged.

Later studies showed that the transformed liver cells did produce tumors in both the young and old animals in the first 2 weeks after transplantation, but they disappeared from all the young animals at 85 days (Table V). In contrast, tumors remained at 85 days in 90% of the old rats. Tumors that arose at the early time points were roughly localized at the site of injection in both young and old animals and were indistinguishable from the undifferentiated spindle cell carcinomas that arose after subcutaneous transplantation (see Fig. 1C). The cells that had migrated into host hepatic plates acquired the appearance of differentiated hepatocytes (see Fig. 1B).

Table IV Tumorigenicity at Site of Injection into the Liver of Neoplastically Transformed Liver Cells in Rats of Increasing Age Examined 3 months After Transplantation

Host age (months)	Animals with tumors/ Animals transplanted	Latency (days)
3	0/8	–
9	0/8	–
12	2/11	70 ± 4
18	5/8	65 ± 6
24	8/8	40 ± 3

After McCullough *et al.* (1994).

Table V Tumorigenicity at Site of Injection into the Liver at Short and Long Intervals After Transplantation of Neoplastically Transformed Liver Cells into Young and Old Rats

	Animals with tumors/Animals transplanted	
Days after transplantation	Young rats (3–9 months)	Old rats (18–24 months)
7	9/9	5/5
14	10/10	5/6
85	0/18	17/19

After McCullough *et al.* (1997).

In the 85-day assay, peritoneal tumors were detected in the majority of young animals despite the absence of liver tumors. Such extrahepatic tumors developed as a result of leakage of tumor cells from the site of injection and showed that they were capable of colonizing and thriving at sites other than the liver. The results showed the liver of young, but not old, rats was able to dispose of tumors that had developed within the first 2 weeks after transplantation of the neoplastic liver cells. The peritoneal tumors that developed from leakage of the transplanted cells from the liver continued to grow without abatement. The observation that tumors do develop at the site of injection at 2 weeks, but not where solitary cells migrate into the hepatic plates recalls the effects of local numbers of ectopically transplanted cells on their developmental fate discussed earlier (Ho, 1992; Stüttem and Campos-Ortega, 1991).

It was of interest to determine the long-term fate of the solitary neoplastic liver cells that had migrated into the hepatic plates. To avoid any complications that might be induced by the formation of tumors at the site of injection into the liver, the neoplastic cells were transplanted into the spleen and carried by the circulation into the liver where they lodged as solitary neoplastic cells in the hepatic plates. In old rats these individual cells multiplied into multicellular foci that were present from 7 days through the 85 days that was as long as they were observed (Table VI). Only one of these foci developed into a tumor. In contrast, the cells transplanted into the liver of young rats via the spleen remained as single and small groups of cells that showed little or no multiplication for 85 days. When allowed to age naturally for 14–15 months however, 4 of the 5 rats from this group had developed multicellular foci. This indicates that the neoplastic cells that had maintained their normal appearance retained their capacity for multiplication when the normalizing capacity of the liver declined with age.

Progeny of the neoplastic cells that had resided with a normal phenotype in the livers of host rats for extended periods were reestablished in cell culture

Table VI Frequency of Hepatocyte Focus Formation in Young and Old Rats After Introduction into the Liver by Injection into the Spleen

Days	Young rats (3-month old)	Old rats (18-month old)
7	0/3	4/5
14	0/3	4/6
21	0/3	3/3
28	0/3	3/5
85	0/3	5/5
440	4/5	ND

ND, not done.
After McCullough *et al.* (1998).

from collagenase-dispersed liver cell preparations. Cell lines established from the transplant recipients exhibited a transformed morphology *in vitro* that was indistinguishable from the parental neoplastic line (McCullough *et al.*, 1998). All the lines gave rise to neoplastic colonies in agar and, most significantly, produced tumors with short latency periods in 100% of the rats that were transplanted subcutaneously or intraperitoneally. The tumors consisted of the same type of undifferentiated spindle cells seen in tumors produced by the parental line at the same sites. All the rats that received transplantations were males. The loss of capacity of the liver to restrain growth of the tumor cells that accompanied aging in males did not occur in females, which continued to suppress tumor formation into old age (Smith *et al.*, 2000). This continuing suppressive capacity might be related to the much lower incidence of liver cancer in women than in men, and the greater resistance of female than male rats to the chemical induction of liver cancer.

There has been a long-standing debate as to whether the marked increase in the incidence of cancer with age is due to some intrinsic property of the aging process or represents the duration of exposure to background and spontaneous carcinogenic stimuli (Ebbesen, 1974; Ebbesen and Kripke, 1982; Peto *et al.*, 1975). The results described here of the normalization of hepatocarcinoma cells after transplantation into the liver of young rats and their neoplastic growth in old male rats (McCullough *et al.*, 1994, 1997, 1998) add a new dimension to the debate. They show in an unequivocal manner that the intact organ of young animals can suppress the neoplastic phenotype of homophilic cells and that capacity is intrinsically lost in the aging liver. This result does not rule out a contribution from the duration of exposure to background and spontaneous carcinogenic stimuli. The carcinogenic effect of such stimuli is not limited to the potentially neoplastic cell, but includes a decline in the tumor suppression capacity of surrounding cells. A multiplicity of age-related changes in cells occurs naturally in animals (Rubin, 1997).

Of particular interest with regard to the theme of ordered heterogeneity is an increase in the variance of cell cycle times with age in the mouse small intestine (Fry *et al.*, 1966) and in gene expression of the mouse heart (Bahar *et al.*, 2006). Both results suggest a decline in the ordering capacity of tissues with age which is associated with a slowdown in cell cycle times in the case of the intestine (Lesher *et al.*, 1961).

One of the most important issues in clinical cancer is the dormancy of solitary metastatic cells in tissues such as the liver, and their emergence years later into a clinical occurrence. An experimental example of such dormancy is the occurrence in mice of solitary cells in the liver that originate from primary tumors of the mammary gland (Naumov *et al.*, 2002). These cells can be recovered from the liver months after their appearance and will produce mammary tumors after injection into the mammary fat pad. The metastatic mammary cells retain their neoplastic mammary morphology in the liver, in contrast to the transplanted hepatocarcinoma cells, which take on the morphology of normal, mature hepatocytes. It would be of great interest to determine whether the solitary mammary cancer cells start to proliferate as the mouse ages as the hepatocarcinoma cells do (McCullough *et al.*, 1998).

C. Regional Loss of Ordering Capacity by Carcinogenic Treatment

Such a decline in ordering capacity is also exhibited by carcinogen treatment of primary cultures of epidermal cells followed by selection for resistance to calcium-induced terminal differentiation (Strickland *et al.*, 1992). As described earlier, these cells have a normal skin phenotype when grafted to prepared sites in mouse skin but have lost the capacity to inhibit tumor formation when mixed in excess with papilloma cells (Table III). The possible significance of this observation can be appreciated by the fact that a single application of a carcinogenic PAH to the skin of a mouse results in the formation within 24 h of about 50,000 stable adducts to the DNA of every exposed epidermal cell. (Melendez-Colon *et al.*, 1999). Even if only 0.1% of those adducts resulted in a mutation there would be 50 mutations in every exposed cell and about 2 in the 5% of the mouse genome that codes for protein (Taft and Mattick, 2003), which could have the potential of reducing the ordering capacity of the treated area. Evidence that suggests it is reduced is that 5 weeks after a single application of a highly carcinogenic PAH to mouse skin, DNA synthesis in the entire cell population responds to a single promoter treatment sooner and involves more (up to 100%) of the cells in the PAH-treated area than in the untreated control (Frankfurt and Raitcheva, 1973).

This increased responsiveness is suggestive of a long-term loosening of the regulatory capacity of the entire basal cell population of the initiated region. Longer term exposure of initiated skin to promoters would be expected to produce widespread hyperplasia, a common precursor of neoplasia (Auerbach *et al.*, 1962; Chang, 1978; Setala *et al.*, 1959). Hyperplastic growth in cell culture markedly increases the selective multiplication of those initiated cells that have progressed furthest toward the neoplastic state (Rubin, 2005). When serially repeated, it leads to the emergence of frankly neoplastic cells capable of forming multilayered transformed foci in culture and tumors in animals. The extent of transformed focus formation is enhanced by the increased permissiveness of the entire population of nontransformed cells that surround the foci and proliferate to successively higher saturation densities with each round of selection. In that sense the cell culture system simulates the regional neoplasia so prominent in human carcinomas of the skin, uterine cervix, urinary bladder, and mammary gland that are evoked by long exposure to chemical carcinogens (Foulds, 1969).

D. Cellular Heterogeneity in Cancer

Not surprisingly, malignant tumors are paradigmatic examples of the loss of capacity to order heterogeneity. Although arising from single cells such tumors contain spatially distributed subpopulations of cells that differ in morphology, histology, karyotype, receptors, enzyme activities, immunological characteristics, metastatic ability, and sensitivity to therapeutic agents (Heppner, 1984). It is from such disordered populations that cells can be further selected for metastasis, drug resistance, and other harmful effects.

The heterogeneity of tumors is commonly attributed to genetic instability of the cells (Hill, 1990; Loeb, 2001; Loeb *et al.*, 2003). Others argue that the patterns of spatial diversity in tumors can be generated without a need to invoke increases in mutation rates (Gonzáles-Garcia *et al.*, 2002). Losses of heterozygosity (LOH) are the most common genetic alterations in human cancers, which may contain >10,000 regions of LOH per cell (Shih *et al.*, 2001; Stoler *et al.*, 1999). These are thought to result from chromosomal instability caused by mutations affecting DNA repair (Loeb *et al.*, 2003). It has however been found that brief exposure of stem cells to nontoxic levels of a variety of mutagens increases the frequencies of LOH by one to two orders of magnitude without inducing persistent instability (Donahue *et al.*, 2006). These results suggest that LOH contributes significantly to the carcinogenecity of a variety of mutagens and raises the possibility that genome-wide LOH observed in some cancers may reflect prior exposure to genotoxic agents rather than a state of chromosomal instability during

the carcinogenic process. Recent studies also show that epigenetic modulation of gene expression is sufficient to cause heterogeneity in tumor lineages (Gerdes *et al.*, 2006).

A final point to consider in generating the heterogeneity of cell behavior in human tumors is that genetic studies have been confined to protein-coding genes. These represent less than 2% of the human genome and only 5% of the mouse genome (Taft and Mattick, 2003). The noncoding portions of genomes were considered junk DNA but recent studies have revealed important regulatory roles for the RNA transcribed from these genes (Mattick, 2003). The noncoding DNA may also serve some functions such as loci for centromeres and sites for nuclear matrix attachment (Sternberg, 2002). Altogether therefore, mutations in the noncoding DNA genome are likely to contribute to the cellular heterogeneity of tumors, especially because the ordering function of size on the expression of mutations in normal tissues (Rubin, 2006) has apparently been reduced or disappeared in neoplastic tissues (Heppner, 1984).

VIII. CONCLUDING REMARKS

When ordered heterogeneity was first presented as a biological concept, it was illustrated by only a few empirical observations (Gerard, 1958) and was largely ignored in biological science. It is now evident that the concept serves as a framework for empirical observations in a wide variety of biological situations, which include the need for minimal size or cell number in embryological development (Berrill, 1941; Grobstein and Zwilling, 1953; Gurdon, 1988), cell heterogeneity in cultures of dissociated cells (Rubin and Hatié, 1968), the role of adhesion in the sorting out of cells (Moscona, 1960; Steinberg, 1963b), dedifferentiation and redifferentiation of cells on dissociation and reassociation (Linser and Moscona, 1979), and the normalization of the neoplastic phenotype by extensive contact with homophilic normal cells (Rubin, 2003, 2006). Where these phenomena have been properly tested as in the maintenance of hepatocyte-specific functions (Mesnil *et al.*, 1987), the community effect in development (Gurdon, 1988), the normalization of the neoplastic phenotype (Alexander *et al.*, 2004; Hennings *et al.*, 1992), and contact inhibition of cell proliferation (Nakamura *et al.*, 1983; Wieser *et al.*, 1985; Wittenberger and Glaser, 1977), contact between cells or between cells and their isolated plasma membranes is required, but not passage of molecules from cell to cell via GJC.

Elsasser introduced the term organismic function to designate phenomena not fully reducible to physics and chemistry because of their complexity (Elsasser, 1975). The organismic function was distinguished from mechanistic

functions such as the readout of DNA into protein which can be defined in strict physical and chemical terms, and are considered part of the mechanistic skeleton. The organismic function maintains or regenerates morphological features guided by the chemical relationships of the mechanistic skeleton. Relating the organismic function to the concept of order in the large over heterogeneity in the small, Elsasser suggested that microorganisms are a form of biological specialization in which the organismic function is very limited. In other words, microorganisms resemble a machine and can be considered an "automated" organism. It is quite remarkable that recent discoveries in molecular biology support this notion. DNA can be divided into that part of the code that is translated into proteins and that which is not. Much of the noncoding DNA, which was once dismissed as junk DNA, is transcribed into regulatory RNA, and is now related to the evolution of complexity. Nonprotein-coding DNA constitutes, on average, about 10% of bacterial genomes and 98.3% of human genome (Taft and Mattick, 2003). It seems apparent therefore that the principle of order in the large that presides over heterogeneity in the small anticipated the molecular evidence for a relation between nonprotein-coding DNA and evolutionary complexity.

Neoplastic tissue serves as a paradigm for the loss of the capacity to order cellular heterogeneity. This loss is usually assumed to arise from the intrinsic instability of the cells that constitute the tumor. However, the significance of tissue size in ordering heterogeneity raises the question whether the cells that surround the tumor have themselves lost their ordering capacity. Their greatest ordering effect is likely to operate in maintaining regulated behavior on initiated cells (Strickland *et al.*, 1992) or on solitary neoplastic cells lodged in normal tissue (McCullough *et al.*, 1998). In both cases, suppression of the neoplastic phenotype is effective only among homophilic cells, and in the case of liver the normalizing capacity is diminished as the organism ages (McCullough *et al.*, 1997). Mutations in the nuclear structural protein lamin A have been implicated in physiological aging (Scaffidi and Misteli, 2006). Cell populations that comprise normal tissues become heterogeneous in growth behavior with age (Fry *et al.*, 1966; Lesher *et al.*, 1961). Such heterogeneity suggests a loss of ordering capacity of tissues which would facilitate the selection and neoplastic development of the most disordered cells.

The mechanism of ordering the behavior of cells presumably depends on the interactions between them. The normalization of neoplastic cells by normal cells has been a particularly useful system for studying these interactions. It was first shown with fibroblasts transformed by polyomavirus that inhibition of their proliferation required direct contact with a confluent sheet of contact-inhibited normal fibroblasts (Stoker, 1964; Stoker *et al.*, 1966). Contact of initiated epidermal cells with their homophilic normal

counterparts is also required for their normalization (Hennings *et al.*, 1990; Strickland *et al.*, 1992). It was at first thought that the contact is necessary to establish GJC between the neoplastic and the normal cells (Mehta *et al.*, 1986) but more discriminating experiments showed that only contact is required (Alexander *et al.*, 2004; Hennings *et al.*, 1992). More recent experiments indicate that cadherin molecules play a significant role in forming adhesions between the two cell types that result in normalizing the behavior of the neoplastic cells (G. S. Goldberg, personal communication). There are, of course, many cadherins as well as other kinds of molecules involved in maintaining adhesions between cells, and it is difficult to obtain a full account of those that are required for the normalization process because they can substitute for one another to a considerable extent (Foty and Steinberg, 2004). It has in fact been suggested that only functional measurements of adhesiveness can give reliable estimates of the role of adhesion in maintaining the tissue integrity that is necessary for normal function (Foty and Steinberg, 2004). It may therefore be informative to reexamine earlier physical techniques for measuring adhesiveness between cells (Coman, 1944; Foty *et al.*, 1996) to better understand the role of mutual intercellular adhesion in maintaining normal cellular behavior.

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Reversal of Tumor Resistance to Apoptotic Stimuli by Alteration of Membrane Fluidity: Therapeutic Implications

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In recent years, significant development and improvement have been observed in the treatment of cancer; however, relapses and recurrences occur frequently and there have not been any current therapies to treat such cancers. Cancers resistant to conventional therapies develop several mechanisms to escape death-inducing stimuli. A poorly understood mechanism is the involvement of the cancer cell plasma membrane composition and architecture and their involvement in regulating drug-inducing stimuli leading to cell death. Although the basic structure of the biological membrane was established 80 years ago, study of the physical properties of lipid bilayers still provides significant information regarding membrane organization and dynamics. Membrane fluidity is probably the most important physicochemical property of cell membranes. Alterations of membrane fluidity can seriously affect functional properties of the cell and induction of apoptotic pathways resulting in cell death. The role of membrane fluidity in the apoptotic process is clearly exemplified as it is seriously disrupted as a result of cell injury. The molecular signaling pathways leading to apoptosis are currently promising areas of research investigation and lead to unravel the underlying molecular mechanisms of tumor cells resistance to apoptotic stimuli and hence the development of new effective therapeutic agents. Recent findings indicate that most anticancer agents induce apoptosis, directly or indirectly, through alterations of tumor cell membrane fluidity. The present chapter summarizes the relationship between alterations of tumor cell membrane fluidity and tumor cell response to apoptotic-inducing stimuli. Several potential therapeutic applications directed at tumor cell membrane fluidity are proposed.

I. INTRODUCTION

A. General

Current treatment of cancer consists of chemotherapy, radiation, hormonal therapy, or immunotherapy. The initial response rates are significant and in few instances curable. However, many patients experience tumor recurrences and relapses and such patients do not respond further to conventional therapies. The underlying mechanisms of resistance are not totally clear, although several mechanisms have been proposed (Hendrich and Michalak, 2003; Liscovitch and Lavie, 2002; Luqmani, 2005). Several studies have examined alterations in the cell membrane lipid bilayer in tumor cells as compared to normal cells (Hendrich and Michalak, 2003; van Blitterswijk, 1988). Such studies have suggested that differences in lipid membrane fluidity are found in cancer cells and, compared to normal tissues, such differences may account for tumor cell sensitivity or resistance to various cytotoxic therapeutics. The role of the membrane lipid composition and fluidity in the cell response to drug-induced apoptotic stimuli has not been clearly defined.

B. From "Structureless Bilayers" to Multicomponent Systems

Although the lipid bilayer was recognized as the universal basis of cell membrane architecture since 1925, the first proposed models treated this pattern as a rigid lipid structure, which provided the foundation to attach proteins in a "rather structureless manner" (Danielli and Davson, 1935; Gorter and Grendel, 1925). Almost 50 years later, the fluid-mosaic model introduced a certain degree of fluidity to the membrane, proposing that the membrane proteins are floating in a sea of excess lipid molecules organized in a lipid bilayer (Singer and Nicolson, 1972). Soon thereafter, new dynamic aspects of the cell membrane were recognized and their characterization in a quantitative manner became crucial in understanding its different functions. Fluidity is perhaps the most obvious physical feature of a membrane and is an essential requirement for its biological viability (Robertson and Thompson, 1977).

The strong relationship between the membrane fluidity and functional properties of both normal and tumor cells, including programmed cell death or apoptosis, is now well appreciated. The interaction with and the permeability of molecules as well as the activation of cell surface receptors by ligands will be influenced by the structure and fluidity of the cell membrane lipid bilayer. Most chemotherapeutic drugs and cytotoxic immune cells as well as certain microbial toxins mediate their cytotoxic action through the stimulation of apoptotic pathways leading to apoptosis in sensitive tumor cells (Cameron and Feuer, 2001). Resistance to apoptosis may be due in part to fluidity of the cell membrane. Therefore, a better understanding of the mechanisms involved in both chemo-induced apoptosis as a function of alterations in tumor cell membrane fluidity is essential. In the present chapter, we have attempted to provide an overview to correlate the pathways involved in drug- and immune-mediated apoptosis and how alterations of cell membrane fluidity regulate tumor cell sensitivity to various apoptotic stimuli. In addition, we describe present and future prospects for therapeutic intervention by modulation of the membrane fluidity.

II. MEMBRANE STRUCTURE AND DYNAMICS

A. Basic Structure

1. LIPID COMPOSITION

Despite their variable composition, all biological membranes contain a phospholipid bilayer as their basic structural unit. Phospholipids are amphipathic molecules and the primary physical forces for organizing biological

membranes are the hydrophobic interactions between their fatty acyl chains. There are three different classes of membrane lipid molecules, namely, phospholipids (PLs), cholesterol (CL), and glycolipids. Four major phospholipids predominate in the plasma membrane of most mammalian cells: phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylserine, and phosphatidylethanolamine (PE). They constitute more than half the mass of lipid in most membranes. Other phospholipids (i.e., inositol phospholipids) are present in smaller quantities but are functionally important in a variety of tasks such as cell interactions and signaling (Bretscher, 1973, 1985). Glycolipids are the result of a large variety of glycosylation patterns in membranes. They show remarkable and constant asymmetry in distribution since they are exclusively found in the noncytoplasmic domain of the lipid bilayer and may function as antigens, mediators of cell adhesion, and modulators of signal transduction (Hakomori, 1986, 2003; Hakomori and Handa, 2003). Finally, cholesterol has been shown to be the main determinant influencing membrane fluidity. Cholesterol intercalates among phospholipids, restricting the random movement of the part of the fatty acyl chains lying closest to the outer surface of the bilayer while, at the same time, separating and dispersing the tail of the fatty acyls, causing the inner region of the bilayer to become slightly more fluid (Chapman, 1975a,b, 1993; Yeagle, 1985) (Fig. 1A).

2. MEMBRANE PROTEINS AND FUNCTIONS

Although lipid bilayers provide the basic structure of biological membranes, most of their biological functions are carried out by carbohydrates, lipids, and proteins (glycoproteins and lipoproteins) (Branden *et al.*, 1991). Membrane proteins can be associated with lipid bilayers in various ways (Chow *et al.*, 1992) (Fig. 1B). Some have one region buried within the membrane and another protruding from it, while others extend through the lipid bilayer with part of their structure on either side. Like the lipid molecules, many membrane proteins are able to diffuse rapidly in the surface of the membrane; cells also have the ability to immobilize specific membrane molecules and confining both membrane proteins and lipids to particular domains of the lipid bilayer (Section II.C). Most plasma membrane proteins play a crucial role in membrane functions, participating as cell surface receptors, transport channels, enzymes, cell surface identity markers, cell adhesion molecules, or as molecules serving the plasma membrane anchoring to the cytoskeleton (Bennett and Gilligan, 1993; Bretscher, 1985). Furthermore, transmembrane proteins balance the passive cellular induction (influx) and active extraction (efflux) of chemical agents, thus controlling one of the primary pathways of cellular-induced drug resistance.

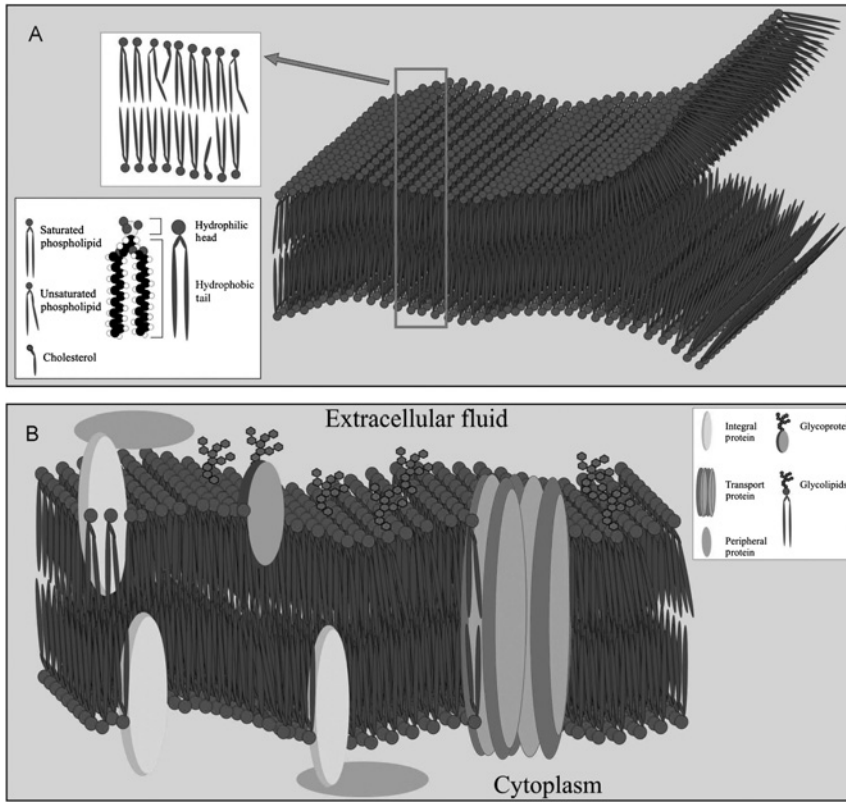


Fig. 1 (A) The phospholipid bilayer constitutes the basic structure of all biological membranes. There are three different classes of membrane lipids: phospholipids, cholesterol, and glycolipids. Four major phospholipids predominate in the plasma membrane of most mammalian cells: phosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine. The hydrophobic fatty acyl tails of the phospholipids form the middle of the bilayer, while the polar, hydrophilic heads of the phospholipids line both surfaces. Furthermore, cholesterol is abundant in the plasma membrane. (B) All biological membranes besides containing a substantial proportion of phospholipids also contain proteins. There are two major types of membrane proteins: integral membrane proteins and peripheral proteins. Integral proteins span or penetrate the lipid bilayer. Therefore, some of these proteins extend through the lipid bilayer with large parts of their structure found on either side of the membrane and are specifically referred to as transmembrane proteins. Moreover, other integral proteins are located in a large part on one side of the membrane, either in the cytoplasm or protruding from the cell surface, with only a small area extending into the bilayer, while others are almost completely buried within the hydrophobic region. Peripheral proteins usually bind to exposed regions of integral proteins and are associated with the surface of biological membranes. Transport proteins are membrane-spanning molecules that allow certain ions and polar molecules to pass through the lipid bilayer. Except lipids, oligosaccharides may also bind to membrane proteins forming glycoproteins.

B. Membrane Mobility

Lipid bilayers are asymmetrical with respect to the lipid composition of the inner and outer monolayers, and such asymmetry reflects the different functions of the two faces of the membrane. In addition, individual lipid molecules are able to diffuse freely within lipid bilayers (van Meer, 1989). Several motion patterns have been studied thoroughly in artificial spherical vesicles of lipid bilayers called liposomes. In such models, phospholipids are free to rotate around their long axis and diffuse laterally within the membrane leaflet, due to their natural thermal motion. Migration of lipid molecules from one membrane monolayer to the other (flip-flop) occurs very rarely in synthetic pure phospholipid bilayers since it is extremely energetically unfavorable. In natural models, though, rapid flip-flop of specific phospholipids is essential for the homeostasis of membranes and the process is catalyzed by a special class of membrane-bound enzymes called phospholipid translocators (Devaux, 1993; Yechiel and Edidin, 2002).

C. Advanced Membrane Formations

More complex liquid-ordered membrane microdomains with a unique protein and lipid composition have been identified within the plasma membrane of most, if not all, mammalian cells and have been described as lipid rafts (Zajchowski and Robbins, 2002). These rafts are dynamic assemblies of compactly packed proteins and lipids [enriched in cholesterol, sphingomyelins, and glycosphingolipids (GSLs)] that float freely within the cellular membrane bilayer or crowd together to form large ordered platforms. The tighter packing is a result of the saturated hydrocarbon chains in raft sphingolipids and phospholipids compared with the unsaturated fatty acids of phospholipids in the nonraft phase (Simons and Vaz, 2004). The high degree of organization observed in these structures, coupled with their dynamic nature, appears to be important in modulating and amplifying signals by acting to provide a signaling microenvironment adapted to produce specific biological responses. Changes in protein or lipid composition, size, structure, number, or membrane localization of lipid rafts could potentially affect the functional capabilities of these domains in signaling with important physiological consequences (Zajchowski and Robbins, 2002). Some of the membrane proteins are permanent raft residents, whereas others move in and out of rafts (Dimanche-Boitrel *et al.*, 2005).

Caveolae were first described in 1955 by Yamada as small flask-shaped noncoated plasma membrane invaginations observed by electron microscopy (Yamada, 1955). Later studies provided an additional broader definition of caveolae that included both morphologically identifiable flask-membrane

structures and flattened caveolin-rich membrane microdomains morphologically indistinguishable from the rest of the plasma membrane (Chang *et al.*, 1992; Rothberg *et al.*, 1990). Some researchers consider caveolae as a caveolin-enriched lipid raft, whereas others consider the two microdomains as completely separate entities. Caveolae have been implicated in many membrane functions, including the organization of signaling proteins, cholesterol transport, endocytosis, and phagocytosis (Sowa *et al.*, 2001).

III. INVOLVEMENT OF PHYSICOCHEMICAL PROPERTIES OF THE PLASMA MEMBRANE IN CELLULAR FUNCTIONS OF NORMAL AND TUMOR CELLS

Several studies have shown that membranes isolated from tumor cells present significant alterations in their composition, structural organization, and functional properties (Galeotti *et al.*, 1986). These alterations may include: (1) chemical parameters related to phospholipid/protein, cholesterol/protein, and cholesterol/phospholipid ratios, as well as in fatty acid composition of the phospholipids; (2) physical parameters such as the molecular order of the lipids (known as static membrane phase, which is closely related to the degree of membrane rigidity) and the fluidity status (representative of the dynamic membrane phase); and (3) functional properties such as the ability of the membranes to undergo superoxide-induced lipid peroxidation.

A. Membrane Fluidity

The extent of molecular disorder and molecular motion within a lipid bilayer is referred to as the fluidity of the membrane. The membrane fluidity is mainly determined by the membrane lipid composition (cholesterol, the degree of saturation and chain length of the fatty acids, and the proportion of lecithin and sphingomyelin) as well as protein–lipid interactions (Grunberger *et al.*, 1982). Various kinds of environmental events, such as temperature stress, osmotic stress, and chemical agents cause alterations in the membrane fluidity of living cells (Laroche *et al.*, 2001; Los and Murata, 2000; Murata and Los, 1997). In addition, modification in fluidity affects a number of cellular functions, including carrier-mediated transport, properties of certain membrane-bound enzymes and receptors, phagocytosis, endocytosis, depolarization-dependent exocytosis, immunological and chemotherapeutic cytotoxicity, prostaglandin production, and cell growth (Kinnunen, 1991; Spector and Yorek, 1985).

B. Monitoring Membrane Fluidity

Two methods are commonly referred to in the literature for monitoring membrane fluidity. (1) The most popular one involves measurements of the anisotropy of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). DPH is integrated into membranes in parallel to the acyl chains of membrane lipids and the fluorescence of DPH is only weakly depolarized when it interacts stably with rigidified membranes (Lentz, 1993). However, since DPH is not completely free to rotate within membranes, the extent of the interactions between DPH and membrane lipids is restricted. Thus, this method is not applicable for quantitative measurements of membrane fluidity. (2) Fourier transform infrared (FTIR) spectroscopy can be used for quantitative analysis of the physical state of membrane lipids (Szalontai *et al.*, 2000). FTIR spectroscopy allows the monitoring of acyl chain disorder and the interactions between lipids and membrane proteins in terms of the frequency of the symmetric CH_2-stretching mode near 2.81 cm^{-1}. This frequency decreases by approximately 2–5 cm^{-1} on the transition of the membrane lipids from an ordered to a disordered state. Thus, low and high frequencies of the CH_2-stretching mode correspond to the rigidified and fluid states of membrane lipids, respectively.

A different method was applied by Masuda *et al.* (1987) who measured the membrane fluidity of polymorphonuclear leukocytes (PMN) using the excimer formation of pyrenedecanoic acid with flow cytometry. The excimers are formed between excited pyrene molecules and ground-state pyrene molecules. The pyrene molecules are incorporated into the cell membrane, and the rate of the excimer formation depends on their translational diffusion rate. The fluorescence intensity ratio of excimer and monomer pyrenedecanoic acid (I_E/I_M) indicates the membrane fluidity: a high I_E/I_M ratio indicates high fluidity and vice versa (Masuda *et al.*, 1987). Similarly, Hu *et al.* (2003) used an excimer-forming lipid technique with pyrenedecanoic acid and flow cytometry to assay the membrane fluidity of fish blood cells. Another largely applied technique of investigating the biophysical basis of membrane formations is by using electron paramagnetic resonance (EPR) of spin-labeled lipids. Fatty acid spin-labels are used as lipid probes in the cell membrane. They are dissolved in lipid bilayers and their ordering and dynamics reflect the motion of the surrounding phospholipid hydrocarbon chains (Kusumi *et al.*, 1986; Subczynski *et al.*, 1992).

IV. MEMBRANE FLUIDITY AND APOPTOSIS

Apoptosis is an essential and complex process for balancing cell numbers during animal development and homeostasis. Typical morphological and biochemical changes are observed in cells undergoing apoptosis.

These include DNA fragmentation, changes in membrane symmetry, activation of caspases, and alteration in the cell death-related protein levels (Earnshaw, 1995; Fadeel *et al.*, 1999). Apoptosis can be induced by various extracellular factors, including ultraviolet (UV)-C irradiation, cytokines, and activation of death receptors expressed on the cell surface (Martin and Green, 1994; Riccardi *et al.*, 2000; Sellins and Cohen, 1987; Strasser *et al.*, 2000).

Various cellular functions are modulated by the physical properties of the cell membrane (Kinnunen, 1991), thus the perturbation of the lipid bilayer and the alteration of its fluidity may contribute toward setting the cell onto the pathway of apoptosis. In fact, several studies suggest that a number of drugs or lipid compounds promote apoptosis in many cell types, enhancing the membrane fluidity at a very early stage of apoptosis (Fujimoto *et al.*, 1999). The loss of membrane integrity may result in an alteration of the selective transport of ions and solutes across the plasma membrane and modification of protein expression and receptor exposure on the cell surface, thereby affecting the functional properties of cells, and inducing apoptosis or altering their susceptibility to apoptosis (Donner *et al.*, 1990; Gotz *et al.*, 1994; Iwagaki *et al.*, 1994; Martin *et al.*, 1994).

Furthermore, it has been postulated that alteration of lipid metabolism, specifically sphingolipid metabolism, may confer to the regulation of apoptotic pathways or may lead to enhanced resistance to apoptosis-stimulating agents. Sphingolipids comprise a class of lipids, found most predominantly in the plasma membrane, where they display a fundamental role in cell processes such as growth, differentiation, and adhesion mediated possibly by interaction with a variety of cellular proteins (Morales *et al.*, 2004; Radin, 2004). In addition to these functions, early studies demonstrate an emerging role of glycosphingolipid family members such as gangliosides in the stress response and cell death by acting as apoptosis regulators (Morales *et al.*, 2004). Their synthesis is dependent on the availability of ceramide generation, the carbon backbone of all sphingolipids. Ceramide synthesis could result *de novo* from condensation of L-serine and palmitoyl coenzyme A by the action of the enzyme serine palmitoyltransferase or ceramide synthetase (Weiss and Stoffel, 1997). A number of cytotoxic agents such as anthracyclines, vinca-alcaloids, or synthetic retinoids have been shown to activate *de novo* synthesis of ceramide in various malignant cell lines by different ways, inducing its intracellular elevation and apoptosis (Aouali *et al.*, 2005; Perry, 2000). Ceramide glycosylation, an essential process for glucosylceramide (GlcCer) synthesis, the metabolic precursor of GSLs, has been reported to influence tumor sensitivity to chemotherapeutic agents (Liu *et al.*, 2001). Apparent ceramide glycosylation has been observed in drug-resistant tumors compared to the drug-sensitive counterparts, preventing apoptosis induction (Lavie *et al.*, 1996). The increase in GlcCer level has been explained by a higher activity or overexpression of GlcCer synthase which may confer to chemoresistance (Liu *et al.*, 1999a). Inhibition of

ceramide glycosylation by GlcCer synthase inhibitors like tamoxifen has been shown to decrease the GlcCer levels and partially restores sensitivity to apoptotic agents in various chemoresistant tumor models (Aouali *et al.*, 2005; Lucci *et al.*, 1998).

In addition to the *de novo* synthesis of ceramide, the precursor of sphingolipids may also be generated from sphingomyelin hydrolysis by the action of sphingomyelinases (SMases) (Kolesnick and Kronke, 1998). This pathway may be of significance in promoting specific macrodomain formation in plasma membrane, allowing oligomerization of certain cell surface proteins such as ligated death receptors, as it will be discussed in the following sections (Cremesti *et al.*, 2002). Evidence suggests that ceramide, generated from a distinct pool of sphingomyelin by the activation of SMases induced by a variety of cytotoxic drugs, death ligands, or ionizing radiation, may be used by cells to propagate the initiation of apoptosis with various kinetics (Modrak, 2005; Modrak *et al.*, 2000, 2002, 2004; Morales *et al.*, 2004). In fact, the distribution of sphingomyelin on both inner and outer leaflets of the plasma membrane as well as the amount and the disposability of hydrosoluble sphingomyelin pool constitute additional candidates for regulating ceramide production and apoptosis (Linardic and Hannun, 1994).

Moreover, some researchers have emphasized the critical role of dihydroceramide (H_2Cer), the saturated precursor of ceramide, and the resulted lipid, sphingamine, in the cell membranes. Compared to ceramide, H_2Cer is characterized as inert or even an inhibitory structure in terms of efficient interaction with cellular regulatory proteins, possibly attributed to its slightly different physical properties, which may change the physical properties of critical ceramide-rich membranes and thus influence adjacent ceramide-induced protein activation (Gulbins and Kolesnick, 2003). The hypothesis that sphingolipid functions as coenzymes in anion transfer and cell death has been described by Radin (2004); it seems to be based on the presence of an allylic alcohol moiety in the sphingolipid structure, which may act as an anion transferring agent, forming transient esters for the synthesis or hydrolysis of phosphoproteins or proteins like cell cycle kinases. In addition to the above functions, the apoptosis-related function of some allylic sphingolipids on reactive oxygen species (ROS) generation could be inhibited by non-allylic sphingolipids, as those resulted by H_2Cer, which may possibly displace the active sphingolipids from their sites in the enzymes' active regions, or bind to their allosteric regions. This observation emphasizes the critical importance of cellular mechanisms able to control this competition of sphingolipids in terms of cell redox status and apoptosis induction. It is also obvious of the significance of allylic alcohol moieties in the design of many apoptosis-stimulating agents able to produce ROS or to affect protein activation.

Hence, cells react to alterations of physical aspects of their membrane, related to the composition and location of different ceramide metabolites, and such reactions can regulate the pathways leading to apoptosis. Newly discovered techniques have provided strong evidence underlining the feedback regulation of membrane fluidity and enabled a better understanding of such events (Hu *et al.*, 2003; Lentz, 1993; Masuda *et al.*, 1987). Noteworthy, we believe that the important issue is whether such newly accumulated knowledge regarding the role of membrane fluidity and membrane lipid composition in cell functions could be directly applied in the diagnostic and disease management practices.

V. MEMBRANE FLUIDITY AND CANCER

A. Membrane Fluidity in Normal and Cancer Cells

The main difference between normal and tumor cells in the biophysical parameters of their plasma membranes resides in the status of the membrane fluidity (Hendrich and Michalak, 2003; van Blitterswijk, 1988). However, no general trend has been discerned in tumors regarding the direction of alteration in membrane fluidity. For example, the physical state of hepatoma tumor cell line membranes, compared to normal membranes, appears to be characterized by increased rigidity (increased molecular order of the lipids and decreased fluidity), lower PL content and degree of fatty acid unsaturation, increased cholesterol/phospholipid ratio, and reduced oxyradical enzymatic defense mechanisms (Deliconstantinos, 1987; Galeotti *et al.*, 1986). This last observation demonstrates that tumor membranes are altered functionally and structurally, partly as the result of oxyradical-induced damage that takes place *in vivo* under conditions of increased oxygen toxicity.

Nevertheless, alteration in membrane fluidity of tumor cells compared to normal cells does not always result in membrane rigidization. Increased membrane fluidity has been observed in neural tumors (Campanella, 1992; Rodrigues *et al.*, 2002), lymphomas (Ben-Bassat *et al.*, 1977; Sherbet, 1989) and leukemias, where it has been found that fluidity is controlled by components of the blood serum (Inbar *et al.*, 1977), and in lung cancer (Sok *et al.*, 2002). Additionally, the membrane fluidity of hepatocellular carcinoma (HCC) cells has been found higher compared to non-HCC cells in patients who developed recurrences within 2 years and lower for those who did not develop recurrences for more than 2 years (Funaki *et al.*, 2004). The method used for membrane fluidity measurements in those living HCC cells was a modified approach of the classic fluorescence polarization method performed in two- and three-dimensional cultures.

The process of carcinogenesis is complex and results from altered patterns of cellular growth. In the resistant hepatocyte model for liver carcinogenesis in the rat, few persistent nodules developed into malignant tumors (Farber, 1991). In this experimental animal model, hepatocarcinogenesis has been associated with changes in lipid metabolism with decreases in the polyunsaturated fatty acid (PUFA) profile, especially the long-chain PUFA (C20 and C22 carbon chains, LC-PUFA) and the lipid peroxidative status accounting for alterations in membrane fluidity, activity of certain enzymes, and affinity of receptors to their ligands (Mahler *et al.*, 1988a,b). Moreover, fatty acids could act as signaling molecules involved in cell proliferation and/or apoptosis. A study using both resistant hepatocytes and regenerating liver rat models has demonstrated that the altered lipid parameters associated with hepatocyte nodules formation closely mimics cellular proliferation in regenerating liver (Abel *et al.*, 2001). In fact, fluorescence polarization indicated that the nodule membranes were more rigid than the respective controls at 1, 3, and 6 months after cancer promotion, in contrast to the increased fluidity in regenerating liver. However, at 9 months, the nodule membrane fluidity increased above the control, mimicking regenerating liver. These data suggested persistent altered lipid metabolism in the hepatocyte nodules at early and late time points after cancer promotion that could account for increased cellular proliferation, progression, and subsequent development of the lesions into neoplasia. Finally, these results may explain the different data obtained on membrane fluidity measurements in human tumors since this parameter could change with the progression of the cancer. Moreover, the membrane fluidity may also be influenced by the tumoral microenvironment, particularly its acidic pH (Shinitzky, 1984) which is a characteristic of some human tumors (Wike-Hooley *et al.*, 1985).

B. Membrane Fluidity and Cell Cycle

Since fluorescence polarization mesurements have shown significant differences in microviscosity between normal and cancer cells (Fuchs *et al.*, 1975; Shinitzky and Inbar, 1974), it has been suggested that the microviscosity of the membrane fluidity could be modulated during the cell cycle. In fact, De Laat and colleagues have demonstrated that the microviscosity, as measured by DPH-fluorescence polarization of synchronized neuroblastoma cells, changed markedly during the cell cycle, reaching a maximum in mitosis and a minimum during S phase (De Laat *et al.*, 1980). Moreover, cell differentiation has also been associated with a progressive increase in microviscosity of the cell membrane (Shinitzky, 1984). In this way, both the levels of cell proliferation and cell differentiation in cancer may modulate

the membrane fluidity and may also account for discrepancy in term of fluidization or rigidization observed in tumors.

C. Fluidity and Metastasis

The ability of tumor cells to infiltrate and metastasize seems to be also related to the degree of membrane lipid fluidity and its influence on the integral protein activity (Campanella, 1992; Funaki *et al.*, 2001; Hendrich and Michalak, 2003; Sherbet, 1989). The basic conformation or the quaternary structure of certain membrane transporters, receptors, and enzymes are likely sensitive to changes in the structure of their lipid microenvironment, leading to changes in activity. For example, melanoma and lymphoma tumor cells with a higher metastatic potential are characterized by higher lateral mobility of membrane receptor proteins in metastasis; however, the above observation does not constitute incontestable evidence that increase in fluidity might be relevant in the metastatic process. Additionally, the above membranes of the metastatic variants show reduced cholesterol/phospholipid ratio and higher unsaturated phospholipid content, but no significant differences in "bulk" fluidity (Sherbet, 1989).

In human gliomas, an increase in the malignancy is accompanied by a gradual accumulation of membrane molecules (lactosylceramide and gagliodide GD3), which is a valid marker of the malignancy grade. Furthermore, in the same setting, increased malignancy has been related with gradual elevation of PC/PE and PC/SM ratios, and alteration of membrane microviscosity, as well as a reduction of total lipids (all the principal classes of lipids), which is significantly associated with histological grading. The presence of more complex glycolipids in the glycolipidic composition of high degree tumor membranes has also been found to be an additional differentiation marker between high- and low-grade tumors (Campanella, 1992).

VI. MEMBRANE FLUIDITY IN CANCER THERAPY

A. Immune-Induced Cell Death

Altered fluidity in tumors compared to normal cells offers the tumor cells the possibility of escaping immune surveillance and immunotherapeutic approaches. According to data provided by Muller and Krueger (1986), it is strongly suggested that membrane lipid fluidity modulates the expression of membrane proteins by vertical phase separation. In the case of "syndromic" proteins, such as hormone receptors, H-2 antigens, and others, when the membrane fluidity is elevated their surface expression is also increased,

whereas it decreases when the membrane becomes more rigid. In contrast, the "antidromic" proteins, such as human blood group antigens, Thy 1.2, and other membrane proteins, display the opposite behavior with respect to the lipid fluidity (Muller and Krueger, 1986). It is possible that the tumor cell plasma membrane contains "cryptic antidromic" antigens (including tumor antigens) reduces the immune responses and the efficiency of antibody (Ab)-mediated immunotherapeutic approaches. By modulating the membrane lipid fluidity the above antigens may become exposed and targets for cell or Ab-mediated immune responses. Alternatively, overexpression of "syndromic" proteins induced by changes in fluidity may result in efficient ligand binding in the case of receptor proteins implicated in significant cellular functions such as the induction of apoptosis.

Data have shown an aggregation of death receptors in the lipid rafts and enhanced apoptosis after drug treatment, suggesting that these events could be mediated by changes in the physicochemical parameters of the plasma membrane resulting from accumulation of new ceramide molecules in the outer leaflet of the plasma membrane (Dimanche-Boitrel *et al.*, 2005; Lacour *et al.*, 2004). The sequence of the events which take place in death receptor-mediated apoptosis induced by changes in fluidity will be discussed below (Section VII.C).

Interferons (IFNs) have recently been demonstrated to partially mediate their action by causing membrane structural changes in the cell membrane including alterations in the cholesterol/phospholipid ratio and changes in fluidity. These changes result in enhanced expression of different surface proteins, including MHC class I and class II antigens and proteins related to apoptosis. Collectively, all the above may result either in IFN-induced growth arrest and apoptosis or enhanced cell-mediated cytotoxic response (Guha *et al.*, 1997).

B. Radiation-Induced Cell Death

Radiation-induced apoptosis usually occurs rapidly and appears to be a modulated process (Sellins and Cohen, 1987).The triggering events responsible for radiation-induced apoptosis are not well understood. One hypothesis is that radiation-induced cell death could be regulated by the biophysical state of the membrane (Benderitter *et al.*, 2000). Ionizing radiation alters the structure and function of biological membranes. Effects of ionizing radiation on biological membranes have been found to cause alterations in membrane proteins and peroxidation of unsaturated lipids accompanied by perturbations of lipid bilayer polarity (Berroud *et al.*, 1996). Alterations in membrane fluidity by the action of ionizing radiation and UV rays have been stated in several reports (Berroud *et al.*, 1996; Gaboriau *et al.*, 1993; Pandey and Mishra, 1999). These alterations could

account for Fas receptor clustering on the cell surface independently of FasL after UV light (Aragane *et al.*, 1998) or irradiation (Huang *et al.*, 2003a) and may sensitize cancer cells to immune-induced cell death.

The exact effect of ionizing radiation on membrane fluidity is not clear. Although most scientists agree that in synthetic lipid bilayers ionizing radiation results in a predictable dose-related effect, biological membranes respond in a rather unpredictable and cell-specific way. More explicitly, changes in both permeability and fluidity in the bilayer of liposomes have been observed, depending on the radiation dose, exhibiting fluidization after exposure to doses of up to 1 kGy, but rigidization of the bilayer at higher doses (Marathe and Mishra, 2002). Concerning biological membranes, Benderitter *et al.* (2000) report that alterations in membrane fluidity can occur since lipid peroxidation itself is known to induce membrane fluidization, while Bhosle and colleagues propose that rigidization of the bilayer membrane core after ionizing radiation exposure occurs due to peroxidation of the lipids in the bilayer (Bhosle *et al.*, 2005).

The mechanisms involved in membrane alterations induced by ionizing radiation are also unclear. According to Pandey and Mishra (1999), ionizing radiation causes peroxidative damage to unsaturated acyl residues of the phospholipid membrane in the presence of oxygen, resulting in the formation of hydrogen peroxide and acyl chain cross linkage. Oxidative damage in membrane phospholipids induced by ionizing radiation has also been found to cause significant alterations in membrane structure and function, possibly leading to apoptosis (Dainiak *et al.*, 1991; Ojeda *et al.*, 1994; Ramakrishnan *et al.*, 1993). Ionizing radiation in biological membranes and in pure lipid membranes results in malondialdehyde formation, indicating a lipid peroxidation process (Kolling *et al.*, 1994). Free radical damage initially induced by radiation can be propagated and magnified by lipid peroxidation chain reactions (Wolters *et al.*, 1987). Radiation has also been postulated to alter the expression of membrane-associated molecules, that is cytokines that are critical for cell survival (Dainiak and Tan, 1995; Dainiak *et al.*, 1991). Subsequently, membrane-bound reactions take place to restore the physiological membrane fluidity, causing imbalance of the second messenger systems (McClain *et al.*, 1995; Ojeda *et al.*, 1994; Ramakrishnan *et al.*, 1993). This disturbance triggers a chain reaction that results in an activation signal for endonucleases leading to the characteristic end point of programmed cell death (Benderitter *et al.*, 2000; Lockshin and Zakeri, 2001). Additionally, it has been proposed that the membrane's radiosensitivity is seriously affected by its lipid composition. In a study by Pandey and Mishra (1999), rigidity of the liposomal membrane was substantially enhanced with an irradiation dose but weaker dependence was noted in the presence of cholesterol, supporting the view that peroxidation caused by radiation was prevented in the presence of cholesterol. In contrast, Bhosle *et al.* (2005) by studying membrane fluidity among other effects

of fractioned radiotherapy in cervical carcinoma cells have reported absence of correlation between membrane fluidity and radiosensitization, suggesting that membrane fluidity could not be used as an indicator of the radioresponse in patients. However, a negative correlation between apoptosis and membrane fluidity was observed as well as rigidization of the membranes induced by peroxidation of the membrane lipids (Bhosle *et al.*, 2005).

Although a direct link between radiation-induced membrane damage and cell death has been well established, the exact mechanism of radiation-induced membrane alteration remains to be investigated. Moreover, the fact that membrane fluidity plays an important role in apoptosis induction and possibly in cellular radiosensitivity suggests a promising area in their application in the treatment of cancer.

C. Chemotherapeutic-Induced Cell Death

Other than DNA, the plasma membrane has been considered as the most important target of many anticancer therapies (Arancia and Donelli, 1991; Tritton and Hickman, 1990). Several antitumor agents induce cytotoxicity directly or indirectly through altering membrane fluidity of tumor cells (Fu *et al.*, 2002; Kabanov *et al.*, 2002; Lacour *et al.*, 2004; Schuldes *et al.*, 2001). Although most of the used chemotherapeutic drugs kill cells predominantly through induction of apoptosis, little is understood about the kind and the order of the plasma membrane events involved in chemotherapy-induced cell death, as well as the role of lipid rafts in drug-induced apoptosis (Nagane *et al.*, 2000; Nakamura *et al.*, 1995; Singh *et al.*, 2003). However, the importance of lipid bilayer as a medium in which diffusion of drugs takes place has been by far well established (Kaye and Merry, 1985). Considering membrane fluidity and its influence on integral protein activity, we can speculate the significance of the balance between the passive cellular influx and active efflux of the drug molecules. Here again, agents that can modulate membrane fluidity may sensitize resistant tumor cells to chemotherapy-induced cell death.

VII. MODULATION OF MULTIDRUG RESISTANCE BY ALTERATIONS OF MEMBRANE FLUIDITY

Numerous past and recent studies have shown that multidrug resistance (MDR), one of the main obstacles limiting the efficacy of chemotherapy treatment of tumors (Fu *et al.*, 2002; Kaye and Merry, 1985), is related, in large part, to membrane fluidity (Huang *et al.*, 2003b; Schuldes *et al.*, 2001)

and to quantitative and qualitative composition of phospholipids, cholesterol content, and lipid unsaturation index in plasma membranes (Chekhun *et al.*, 2003; Hendrich and Michalak, 2003). Resistant cells to anthracyclines, such as doxorubicin, compared to sensitive cells have been shown to present increased membrane fluidity, a lower unsaturation level of fatty acids in the membrane, and higher sphingomyelin and phosphatidylserine content which may suggest the activation of P-glycoprotein (P-gp)-mediated drug efflux (Chekhun *et al.*, 2003; Kaye and Merry, 1985). All of the above changes result in elevation of plasma membrane microviscosity. Additionally, a number of tumor cell lines resistant to DNA-damaging drugs, such as cisplatin (CDDP), show increased membrane potential (related to drug influx) resulting in increased fluidity (Huang *et al.*, 2003b; Liang *et al.*, 2004). However, it remains unclear whether the observed differences in the biophysical membrane status and/or fatty acid composition alone is the main reason for increased drug resistance, rather than the secondary effect of these differences on the structure or function of some transmembrane proteins.

A. Mechanisms of Drug Resistance Related to Membrane Events

Four mechanisms for MDR have been proposed, namely, inhibition of drug accumulation or active drug efflux, overexpression of the drug target, accelerated repair of chemotherapy-induced cellular damage, and inhibition of cell death pathways (Liscovitch and Lavie, 2002; Luqmani, 2005). All of the above mechanisms seem to be affected and related to host factors, tumor–host interactions, or cellular resistance. In examining resistance at the molecular level, many investigators conclude that the main root of the problem is often a matter of transport through the plasma membrane (Hendrich and Michalak, 2003; Kaye and Merry, 1985). The proposed hypothesis is that a cell surface receptor or another protein will recognize an incoming drug and, with the aid of an adenosine triphosphate (ATP) pump mechanism, pumps the drug out of the cell and make the tumor cell resistant to drug-induced apoptosis. If a drug is constantly pumped out of a cell, then it never gets a chance to carry out its desired course of action.

Researchers have focused on a surface molecule called P-gp, a member of the ATP-binding cassette (ABC) transporter superfamily, which seems to confer resistance in many type of cancers (Fu *et al.*, 2002; Higgins, 1992) by acting as a multidrug efflux (removal from the cell) pump. The overexpression of P-gp has been found to reduce intracellular drug accumulation due to enhanced active drug efflux triggered by P-gp (Gottesman and Pastan, 1993). This specific glycoprotein, which is produced in excess and is inherited, has been identified in the cell membrane of certain drug-resistant

solid tumors such as those of the liver, kidney, colon, adrenal, and pancreas (Kaye and Merry, 1985).

It has been noted that alterations in the membrane lipid environment modify the degree of resistance to chemotherapy. Several studies have shown that changes in MDR in different kinds of tumors and P-gp function are associated with alterations in the fluidity of the plasma membranes (Callaghan *et al.*, 1993, 1997; Drori *et al.*, 1995; Rothnie *et al.*, 2001; Sharom *et al.*, 1995). MDR tumor cells overexpressing P-gp demonstrate alterations in biophysical properties of the plasma membranes, specifically, an altered membrane lipid composition. P-gp affects membrane biophysical properties such as phospholipid packing, lipid translocation, bilayer fluidity, and overall permeability. In this way, P-gp has been shown to be an outward translocator of cholesterol (Liscovitch and Lavie, 2000). Furthermore, cholesterol modifies the "destabilizing" effects of P-gp on the bilayer and its interaction with drugs (Rothnie *et al.*, 2001).

The role of the cholesterol-binding protein caveolin-1 in the function of the P-gp, which is dependent on the cholesterol content in the membrane, is not clear in P-gp-related MDR tumors. By using cholesterol-depleting agents such as methyl-β-cyclodextrin (MβCD) and 2-hydroxypropyl-β-cyclodextrin (HβCD) as well as membrane packing density measurements, Cai *et al.* (2004) observed a decrease in fluidity and tighter packing density in drug-resistant breast cells due to the overexpression of caveolin-1. Therefore, alterations in the cholesterol levels and membrane fluidity status could be significantly related to reversal of MDR in cancer cells overexpressing P-gp. Moreover, the use of MβCD for decreasing the levels of cholesterol in Madin Darby canine kidney (MDCK) cells and lipid rafts showed shedding of the ABC transporter P-gp in both the intact cells and the rafts on treatment with MβCD (Kamau *et al.*, 2005). The highest decrease in cholesterol levels was observed on treatment with 100-mM MβCD for 2 h. However, cell viability and caveolin-1 levels were not found to be affected by MβCD. Caveolin-1 is regarded as a new prosurvival protein whose function needs to be clarified (Shatz, 2004).

Hence, due to studies indicating that drugs interact with P-gp via the lipid phase (Homolya *et al.*, 1993; Raviv *et al.*, 1990), any alterations in membrane biophysical properties may also influence the subcellular distribution of drugs and their access to P-gp. An altered membrane biophysical environment may also influence the MDR phenotype, affecting directly P-gp activity since it requires interaction with a defined lipid environment (Callaghan *et al.*, 1997; Rothnie *et al.*, 2001). However, it still remains unclear whether the alterations in membrane biophysical properties directly result from P-gp expression are caused by the activity of P-gp or are a consequence of prolonged exposure to chemotherapeutic agents in resistant cells (Rothnie *et al.*, 2001).

Although increased fluidity has been implicated in cisplatin resistance, Liang and his colleagues observed that an increase in membrane fluidity of the sensitive KB-3-1 epidermal carcinoma cell line did not result in higher resistance to cisplatin (Liang *et al.*, 2004). It was suggested that DNA-related effects and alterations of plasma membrane might not account for changes in resistance but rather facilitate a mechanism of resistance found only in the selected cell line. It is possible that increased fluidity amplifies the effect of other defects in the cell membrane rather than independently causing resistance. These defects seem most likely to be related to membrane trafficking. Decreased drug accumulation noted in cisplatin-resistant KB cells for many drugs was also associated with decreased expression on the cell membrane of additional transporters, carriers, and channels (Shen *et al.*, 1995). The reduced cisplatin accumulation was associated with an intracellular redistribution of some membrane proteins, including the increase of the protein in the cytoplasm. A defect in neutralization of the acidic pH of lysosomes suggested a general defect in the regulation of endocytosis and membrane vesicle recycling (Chauhan *et al.*, 2003). Furthermore, failure to properly anchor the membrane protein to the cytoskeleton results in mislocalization or failure to remain in the plasma membrane and accumulation of certain transporters in the cytoplasm. Therefore, alterations of the biophysical properties of the plasma membrane in cisplatin-resistant cells could facilitate the above defects in membrane trafficking that might underlie cisplatin resistance.

Despite the fundamental role of membrane transporters or other proteins in MDR, the development of drug resistance in tumor cells has additionally been associated with change in the composition and the properties of the membrane phospholipids, including microviscosity and fluidity. Many chemotherapeutic drugs exhibit membrane effects via weak hydrophobic interactions or via electrostatic binding to membrane phospholipids prior to entering the cytoplasm. Moreover, changes in the plasma membrane phospholipids have been observed during apoptosis of tumor cells treated with chemotherapeutic drugs. For example, Huang *et al.* (2003b) observed a higher ratio of saturated lipids and a lower ratio of cholesterol in the cisplatin-sensitive cell line A549, compared to the resistant cell line A549/DDP.

Several years ago, another mechanism of drug resistance related to membrane events has been called the confluence-dependent resistance independently of the P-gp expression (Pelletier *et al.*, 1990). A decrease of the drug passive diffusion in confluent cells has been correlated with a reduced membrane fluidity (Dimanche-Boitrel *et al.*, 1992) and a decrease in cell proliferation in relation with the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Dimanche-Boitrel *et al.*, 1998). This mechanism of resistance may also contribute to the low responsiveness to chemotherapy of most common

solid tumors such as digestive tract carcinomas (Dimanche-Boitrel *et al.*, 1993).

On the basis of the above findings, there seems to be a relationship between drug-resistant phenotypes and changes in the components and properties of the plasma membrane, directly depending on the cell type of the tumor, the proliferation rate, and the type of chemotherapeutic drug used. Further, studies may define optimal conditions of alterations of membrane lipid composition for optimal response to therapeutic drugs in selected subsets of cancer patients.

B. Reversal of MDR by Membrane Fluidity Modulators

Noted effects are exerted on membranes by different kinds of MDR modulators (chemosensitizers). Agents modifying the biophysical membrane status can either induce direct cell death or they can cooperate with other sensitizing approaches and trigger the cells to drug-, radiation-, or immune-mediated apoptosis. MDR modulators for the treatment of P-gp-mediated MDR cancers, including bile salt, R-verapamil, and tetrandrine, are already in clinical use. Bile salt and R-verapamil have been shown *in vitro* to reduce the membrane fluidity in the drug-resistant ovarian tumor cell line, resulting in reversal of their resistance to DNA-damaging drugs, such as adriamycin (ADR) and mitomycin, through changes in the P-gp function (Schuldes *et al.*, 2001). Tetrandrine, an inhibitor of P-gp-mediated drug efflux, was able to decrease cell membrane fluidity in a concentration-dependent manner and reverse the resistance to Dox (21-fold) *in vitro* in human breast adenocarcinoma cell lines and *in vivo* in human xenografts (Fu *et al.*, 2002). Furthermore, membrane-mediated mechanisms of tamoxifen action, through a putative modulation of membrane fluidity, are likely to play an important role in its anticancer action and its ability to reverse MDR (Engelke *et al.*, 2002; Wiseman, 1994), while bryostatin is known to increase the fluidity in the drug-resistant B-cell chronic lymphocytic leukemia (B-CLL) cell line and sensitize the cells to a variety of chemotherapeutic drugs (Liu *et al.*, 1999b). However, because of the often variations in the effect of lipid modifications observed between different cell types, it is not yet possible to make any generalizations or to predict how a given system will respond to a particular type of lipid modification, or to propose a uniform effect on all processes in a single cell line.

In the context of apoptosis induction by modifications in membrane fluidity, we treated PC-3 cells, a human androgen-independent prostate tumor cell line resistant to TNF-related apoptosis inducing ligand (TRAIL), with lipofectamine for transfection. We observed that following treatment there was significant spontaneous induction of apoptosis compared to cells treated with medium alone. Lipofectamine also enhanced the apoptotic potential of

TRAIL in a dose-independent manner (Baritaki *et al.*, 2005). The above findings suggest that lipofectamine may sensitize the tumor cells to immune-mediated apoptosis.

The induction of apoptosis by lipid compounds such as lipofectamine is not unique. Recent and past studies have shown that apoptosis can be induced by various lipid compounds such as ceramide (Obeid *et al.*, 1993), sphingosine (Ohta *et al.*, 1994), ether lipid (Diomede *et al.*, 1993), retinoic acid (Martin *et al.*, 1990), farnesol (Haug *et al.*, 1994), geranylgeraniol (Ohizumi *et al.*, 1995), geranylgeranyl acid (Nakamura *et al.*, 1995), palmitate, and strearate (Paumen *et al.*, 1997). The proposed mechanism of apoptotic induction by lipid compounds seems to be related with the cell membrane dynamics. It is clear that the first event of apoptosis is the partition of added chemical compounds to the plasma membrane of cells due to their amphipathic properties. These compounds significantly perturb the lipid bilayer and alter its fluidity. Similarly, the well-controlled transfer of lipid inducers from the emulsion to the lipid layer of cells can bring about the increase of membrane dynamics that might lead to the induction of apoptosis (Fujimoto *et al.*, 1999). Indeed, synthetic phospholipid analogues, such as ALP (alkylysophospholipids) and APC (alkylphosphocholines), have been shown to interfere with cellular membranes modulating a number of membrane functions, including membrane permeability and fluidity, lipid composition, metabolism of phospholipids, and signal transduction. In leukemic and brain tumor cells lines, the above compounds were able to induce apoptotic cell death independently, or they could exert a synergistic effect with cytotoxic drugs or radiation (Jendrossek and Handrick, 2003).

Further treatment of PC-3 cells with lipofectamine for 6 h and wash, followed by treatment with CDDP or vincristine and TRAIL for 18 h, resulted in significant apoptosis compared to treatment with the drugs and/or TRAIL alone. Apoptosis was measured by activation of the effector caspase-3 (Baritaki *et al.*, 2007). These studies suggested that lipofectamine may sensitize the tumor cells to drug- and immune-mediated apoptosis. The findings corroborate the hypothesis stated above and suggest that tumor cell resistance can be modified by alterations in the membrane lipid composition. Possible mechanisms of how membrane fluidity alterations triggered by apoptotic inducers influence the apoptotic pathways are discussed below.

C. Mechanisms of Apoptosis Induction Related to Plasma Membrane Fluidity Alterations

The mechanisms by which alterations of membrane fluidity are responsible for changes in resistance and cell sensitization to apoptotic cell death are not clear. The apoptotic inducers may mediate their action by different ways

related to the induction of early to late apoptotic events, including phospholipid translocation (mainly translocation of phosphotidylserine), increased fluidity and decreased anisotropy [the increased membrane fluidity associated with apoptosis may represent either a mechanism to cycle phosphatidylserine to the outer leaflet, mediating phagocytic recognition of apoptotic cells, or a consequence of this event (Jourd'heuil *et al.*, 1996)], decreased lipid package, and DNA fragmentation (Jessel *et al.*, 2002). The first three events reveal loss of lipid asymmetry and are characterized as early apoptotic events, while the fourth is a late apoptotic event. Although the different apoptotic inducers can initiate different signal steps to apoptosis that is dependent on inducer characteristics and thus resulting in different reactions among the cells. It seems that the kinetics of early to late apoptosis appear to be a fixed program (Jessel *et al.*, 2002).

It has been proposed that alterations in plasma membrane fluidity, induced by different apoptotic stimuli such as anticancer drugs, could be implicated either in the differential expression of membrane proteins (Muller and Krueger, 1986) and/or in the recruitment of receptor proteins, such as death receptors, to lipid rafts (Bezombes *et al.*, 2003; Dimanche-Boitrel *et al.*, 2005). Induction of death receptor expression, including tumor necrosis factor (TNF)-related receptors Fas (CD95) and DR5, has been shown to occur in various tumor cell lines after treatment with chemotherapeutic agents or other sensitizers (Huerta-Yepez *et al.*, 2004; Kim *et al.*, 2004; Meng and El-Deiry, 2001; Micheau *et al.*, 1997; Nagane *et al.*, 2000; Shankar *et al.*, 2004; Sheikh *et al.*, 1998; Singh *et al.*, 2003; Shankar and Srivastava, 2004; Wu *et al.*, 1997). Similarly, we observed a significant increase in DR5 protein levels after treatment of PC-3 cells with CDDP, etoposide, vincristine, and doxorubicin (Baritaki *et al.*, 2007). Most of the above chemotherapeutic agents have been well demonstrated as potent modulators of membrane fluidity. We can postulate that this increase in DR5 levels of expression could be attributed by either the "syndromic" character of the surface death receptor, that is changes in membrane fluidity cause proportional changes in receptor expression, or to the repression of a negative regulator of DR5 expression induced by CDDP. We have investigated the last hypothesis and we concluded that genotoxic agents like CDDP may possibly inactivate, directly or indirectly, the transcription factor Ying-Yang 1 (YY1) which has previously been shown to act as transcriptional repressor of DR5 expression (Baritaki *et al.*, 2007; Huerta-Yepez *et al.*, 2007) (Fig. 2). The above phenomena may take place additionally or separately to induce apoptosis in target cells.

Recently, many hypothetical models have also been proposed in the context of receptor protein recruitment, including death receptors, to lipid rafts. It has been shown that CDDP could induce redistribution and clustering of CD95 (Fas), together with the death-inducing signaling complex

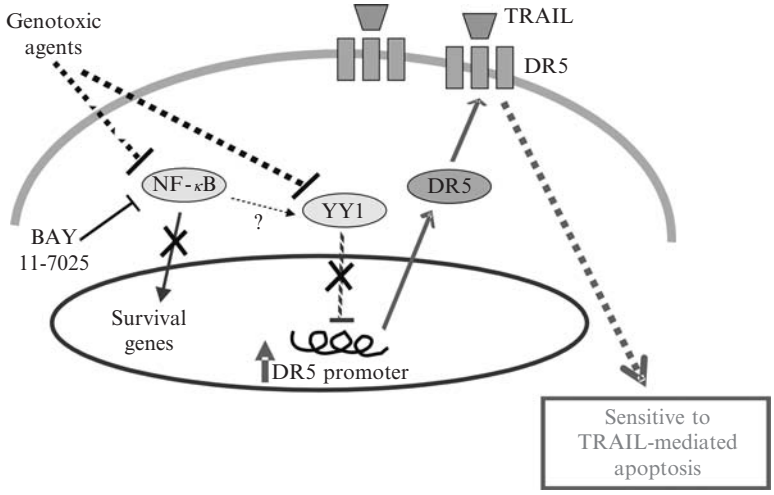


Fig. 2 Schematic delineation of the mechanism proposed to be involved in TRAIL-mediated apoptosis in TRAIL-resistant PC-3 prostate cell line after chemotherapy. Genotoxic drugs, like CDDP or ADR, seem to influence directly or indirectly, via NF-κB downregulation, the protein levels and the DNA-binding activity of the transcription factor YY1. Since direct experimental evidences have demonstrated YY1 as negative regulator of DR5 transcription, repression of YY1 may result in DR5 transcriptional upregulation and receptor overexpression on cell surface. Thereafter, administration of TRAIL may sensitize the TRAIL-resistant PC-3 cells to TRAIL-mediated apoptosis.

(DISC)-forming molecules FADD and procaspase-8, into membrane lipid rafts of human colon cell lines. This could lead to CDDP-induced apoptosis in a ligand-independent manner or to a synergistic cytotoxic effect of CDDP with an agonist anti-CD95 Ab (Lacour *et al.*, 2004). The significant points of the proposed mechanism implicated to CD95 receptor clustering are briefly analyzed: CDDP, like many cytotoxic drugs, increases the membrane fluidity and induces the formation of a CD95 including DISC in a ligand-independent manner. The formation of initial DISCs leads to the activation of the enzyme acid SMase (aSMase). aSMase translocates from an intracellular compartment onto the extracellular leaflet of membrane rafts where it catalyzes the generation of extracellularly oriented ceramides. These surface ceramides are able to induce reorganization and coalescence of the elementary rafts, resulting in colocalization of clustered CD95 receptors. The aggregated receptors may thereby promote amplified CD95 signaling by formation of larger complexes, further recruitment of FADD and procaspace-8, and stabilization of the DISC (Dimanche-Boitrel *et al.*, 2005). However, the mechanisms by which ceramide may exert allosteric effects or controls the physical properties of membranes and submembrane aggregates like rafts are still under investigation.

A recent work conducted in yeast demonstrated that edelfosine, a prototype member of the APC class of cancer chemotherapy, induced the redistribution of ergosterol from the plasma membrane into the cell resulting in the selective loss of Pma1p, an essential proton pump ATPase from plasma membrane lipid rafts and certainly in alteration of the biophysical properties of the remaining lipid raft microdomains (Zaremberg *et al.*, 2005). These data pointed for the first time to a novel mode of action for anticancer drug via the modification of plasma membrane lipid composition, resulting in selective dissociation of an essential protein with lipid raft scaffolds. This modification of plasma membrane lipid composition induced by edelfosine may be related to increase plasma membrane fluidity (Storme *et al.*, 1985) and to translocation of Fas into membrane rafts (Gajate and Mollinedo, 2001).

However, other reports demonstrate that in some cell types exclusion of Fas from lipid rafts by the use of cholesterol-depleting compounds, which results in raft disruption, leads to the spontaneous ligand-independent clustering of Fas in the nonraft compartment of the plasma membrane, formation of Fas–FADD complexes (DISC), activation of caspase-8, and finally apoptosis (Gniadecki, 2004).

Hence, it cannot be excluded that similar mechanisms, as those indicated above, are also activated for DR5 receptor aggregation or overexpression in the case of PC-3 cell sensitization to TRAIL-mediated apoptosis induced after drug or lipofectamine treatment. In the same setting, the anticancer agent resveratrol has been shown to be able to induce redistribution of Fas and other death receptors of the TRAIL death pathway in lipid rafts in drug-resistant cells. Although this redistribution was not sufficient to trigger cell death, it contributed, however, to their sensitization to death receptor agonists such as anti-Fas antibodies or TRAIL (Delmas *et al.*, 2004).

The immuno-mediated induction of cell apoptosis by modulators such as the ligands of death receptors has also been reported to be associated with membrane events. TRAIL has been shown to influence the biophysical membrane properties in expressing cells. In the leukemic Jurkat cell line transfected with the Rev Tet-On TRAIL gene expression system, the expression of TRAIL was reported to cause significant changes in the biophysical properties of the membrane, including reduction of surface charge density, membrane fluidity, and proportion of a helix in membrane protein secondary structure. However, the $K1$ elastic coefficient was increased (Chen *et al.*, 2004). The above changes may promote the apoptotic cell program by TRAIL alone or in combination with chemotherapeutic drugs, or radiation.

Moreover, the role of membrane oxidative injury in apoptosis induction has been demonstrated as a direct or complementary mechanism of apoptosis triggering related to membrane biophysical alterations. Apoptosis induced by oxidative stress may be related to the lipid peroxidation (Ren *et al.*, 1998). As mentioned above, many allylic sphingolipids are able to induce cell apoptosis by generation of ROS after oxidation of the allylic alcohol group

(Radin, 2004). Allylic alcohols are relatively easily oxidized by quinines, thus it becomes obvious that ceramide, the precursor of all sphingolipids, is able to interfere with the oxidative activity of mitochondria via the ubiquinone cycle (Gudz *et al.*, 1997). The direct interaction of ceramide with mitochondria has been documented by early studies on isolated mitochondria from rat hepatocytes (Garcia-Ruiz *et al.*, 1997), where it induced disruption in electron flow at complex III of the respiratory chain, resulting in enhanced ROS generation, release of cytochrome *c*, and caspase activation (Garcia-Ruiz *et al.*, 1997; Gudz *et al.*, 1997). Findings have also proposed that the role of SMase-induced ceramide generation on apoptosis after TNF or FasL signaling seems to be mediated by its conversion to ganglioside, GD3 (Morales *et al.*, 2004). GD3 like ceramide was also shown to interact with mitochondria-inducing ROS generation and release of cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor (AIF); however, the underlying mechanisms responsible for the GD3 effects on the oxidative activity of mitochondria as well as for the GD3 trafficking to mitochondria still remain unclear (Garcia-Ruiz *et al.*, 2000). Hence, the above finding reveals a novel function of this lipid as death effector.

Another lipid, which plays an important role in mitochondria-dependent death pathway, is the cardiolipin. Cardiolipin is a phospholipid uniquely present in the inner membrane of mitochondria that has been involved in apoptosis regulation (Iverson and Orrenius, 2004). Cytochrome *c* must be detached from cardiolipin to be released into the cytosol during apoptosis. Among mechanisms involved in loss of cardiolipin, we can mention oxidative degradation of cardiolipin on p53- (Polyak *et al.*, 1997), Fas-, and radiation-induced apoptosis (Matsko *et al.*, 2001) or altered phosphatidylcholine homeostasis on TRAIL-induced apoptosis (Sandra *et al.*, 2005).

Moreover, recent observations on the crucial role of membrane fluidity in ethanol intoxication have given new input in the field of the use of membrane fluidity modulators in tumor therapeutic approaches related to apoptosis induction. Sergent and colleagues showed in primary rat hepatocytes that ethanol-induced oxidative stress resulting in ROS production, lipid peroxidation, and cell death is directly dependent on the fluidization status of the plasma membrane. Specifically, only increased membrane fluidization was able to enhance the oxidative stress, which was attributed to elevated levels of low molecular weight iron induced by the increased membrane fluidity (Sergent *et al.*, 2005).

D. Role of Intracellular Membranes in Apoptosis Induction by Apoptotic Inducers

In addition to the above observations, it has been shown that some agents can cause direct mitochondrial membrane changes that could lead to complete mitochondrial membrane disruption. For example, Dol-P (Dolichyl

monophosphate) induces apoptosis in the human leukemia U937 cells by reducing the mitochondrial membrane potential and by inducing the translocation of AIF to nuclei, processes necessary for apoptosis triggering (Yasugi *et al.*, 2000). The toxicity of hydrophobic bile salts (such as deoxycholate and glycochenodeoxycholate), especially to hepatocytes, has also been reported to be mediated by cytochrome *c* release, through a mechanism associated with marked direct effects on mitochondrial membrane lipid polarity and fluidity, protein order, and redox status (Sola *et al.*, 2002).

The ability of a number of anticancer agents to induce oxidative stress, via interference with the oxidative activity of mitochondria, may be attributed to the presence of allylic alcohol residues which can be oxidized for ROS production, similarly to some allylic sphingolipids (Radin, 2004). Furthermore, other antitumor drugs may contain allylic ketone moieties able to induce reduction to cellular thiols like glutathione (GSH) (Radin, 2003). Since GSH is an inhibitor of SMases, destruction of GSH by the above drugs may lead to the formation of more ceramide and ROS production. Following a sequence of reactions within a loop, the gradually increasing concentration of ceramide-induced ROS may act on decreasing GSH levels and inducing apoptosis (Radin, 2004).

In contrast, other agents such as ursodeoxycholate (UDC) and tauroursodeoxycholic acid (TUDCA) seem to modulate the apoptotic threshold, in part, by preventing Bax translocation both *in vitro* and *in vivo* (Rodrigues *et al.*, 2002; Sola *et al.*, 2003) and appear to be cell death protective. Spectroscopic analyses of mitochondria exposed to Bax, a potent proapoptotic member of the Bcl-2 protein family that localizes to the mitochondrial membrane during apoptosis, revealed increased polarity and fluidity of the membrane lipid core as well as altered protein order, indicative of Bax binding, together with loss of spin-label paramagnetism, characteristic of oxidative damage. TUDCA markedly abrogated the Bax-induced membrane perturbation, resulting therefore in inhibition of cytochrome *c* release from mitochondria through a mechanism that does not require the permeability transition (Liu *et al.*, 1999a; Rodrigues *et al.*, 2002). Another protective mechanism of ursodeoxycholic acid (UDCA) against induction of apoptosis may be related to its membrane-stabilizing properties since UDCA has been shown to reestablish the normality in membrane fluidity in primary rat hepatocytes following ethanol treatment (Sergent *et al.*, 2005).

In terms of oxidative stress induction, other apoptotic mediators such as bilirubin and ascorbic acid–ferrous system have been found to have as a primary target the plasma membranes interacting directly with them and influencing lipid polarity by enhancing the membrane permeability, fluidity, protein order by inducing protein mobility, and redox status characterized by significant oxidative injury to membrane lipids (Ren *et al.*, 1998; Rodrigues *et al.*, 2002). This oxyradical-induced damage may be partially

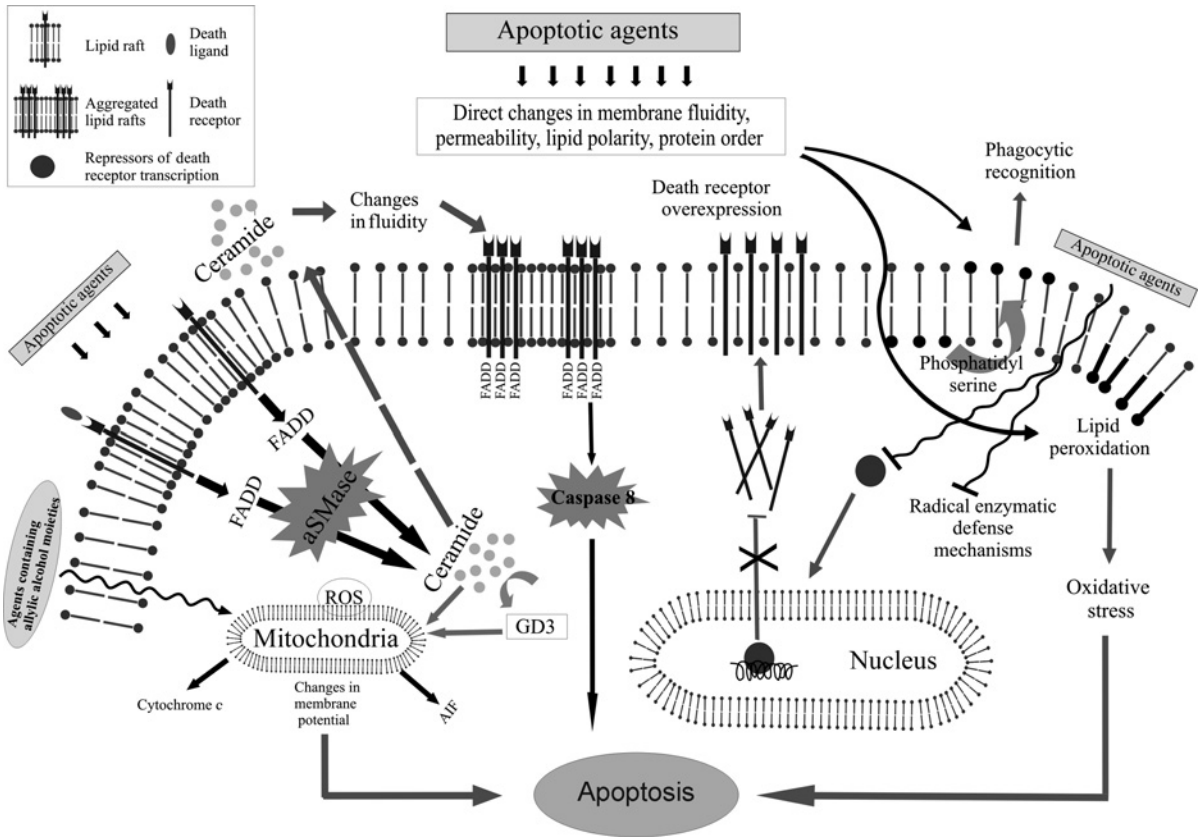
related to decreased oxyradical enzymatic defense mechanisms derived by the drug action (Galeotti *et al.*, 1986). Furthermore, the critical role of calcium changes has been proven in a variety of tissues under various apoptotic-inducing conditions. A correlation between membrane oxidative damage and calcium signaling has been observed in the induction of apoptosis in murine splenocytes (Agrawal *et al.*, 2003; Bhosle *et al.*, 2005; McConkey and Orrenius, 1997).

In conclusion, despite the well-established implication of both plasma membrane and mitochondria in drug-induced cytotoxicity, however, it still remains under investigation which of these two targets is more vital for restoring the cell's former drug sensitivity to cell death. The proposed mechanisms of apoptosis induction related to membrane biophysical alterations are summarized schematically in Fig. 3.

VIII. THERAPEUTIC INTERVENTIONS AND NOVEL APPROACHES IN CANCER THERAPY

Alterations in physical aspects of cell membranes are currently used in therapeutic and cancer management practises. These approaches tend to sensitize tumor cells to chemotherapeutic drugs through induction of apoptosis, or to reverse their existing resistance to the available antitumor agents and radiation. Alternatively, changes in physical parameters of plasma membranes may enhance the efficiency of immunotherapeutic approaches by modulation of T-cell-dependent cytotoxic-signaling cascades or by enhancing the tumor response to monoclonal antibodies against cancer epitopes. Moreover, synergistic effects of compounds able to alter membrane fluidity could also be exerted with cytotoxic drugs or radiation leading finally to apoptotic cell death.

In terms of tumor resistance to drugs due to nonefficient drug delivery, conventional and stealth liposomes have been considered as one of the most broadly studied modern drug delivery systems. For an effective drug delivery, temperature and target sensitive liposomes have already been designed (Voinea and Simionescu, 2002). The above liposomes are able to crumble and to release, in the surrounding area of the cell, their content, part of which permeates the plasma membrane. An exchange of liposome lipids with membrane lipoproteins, especially HDL, can also take place, leading therefore to liposome destabilization and release of encapsulated molecules in the plasma (Yuan *et al.*, 1994). In addition, pH-sensitive liposomes have been constructed so as to fuse with endosomes and release their content in cytosol avoiding lysosomal degradation. Liposome-encapsulated doxorubicin and


Lipid raft
Death ligand
Aggregated lipid rafts
Death receptor
Repressors of death receptor transcription
Apoptotic agents
Direct changes in membrane fluidity, permeability, lipid polarity, protein order
Phagocytic recognition
Death receptor overexpression
Changes in fluidity
Ceramide
Apoptotic agents
Apoptotic agents
FADD
FADD
FADD
FADD
FADD
FADD
Phosphatidyl serine
Lipid peroxidation
FADD
FADD
aSMase
Caspase 8
Ceramide
Agents containing allylic alcohol moieties
Radical enzymatic defense mechanisms
Oxidative stress
ROS
Mitochondria
GD3
Nucleus
Cytochrome c
Changes in membrane potential
AIF
Apoptosis

daunorubicin formulations are already under clinical trials with good results in Kaposi's sarcoma (Lasic, 1998). These liposomes circulate in the vasculature of patients for several days, and therefore have increased chances of extravagating at sites of increased permeability. Immunoliposomes (Ahmad *et al.*, 1993; Huwyler *et al.*, 1997) and termosensitive liposomes (Maruyama *et al.*, 1993; van Bree *et al.*, 1996) have also been reported as alternative well-designed approaches in the field of the liposomal anticancer therapy. However, compared to stealth liposomes, the above liposomal constructs do not seem to exert better efficiency in anticancer drug delivery to solid tumors (Goren *et al.*, 1996; Vingerhoeds *et al.*, 1996).

A novel application of Pluronic block copolymers in drastic chemosensitization of MDR tumors to various anticancer agents, particularly anthracycline antibiotics, has been discovered (Kabanov *et al.*, 2002). Pluronic block copolymers are known to induce changes in the microviscosity of cell membranes (Melik-Nubarov *et al.*, 1999). It has been demonstrated that exposure of cells to Pluronic block copolymers, such as P85, results in fluidization of the cellular membranes of both normal and malignant cells expressing high levels of P-gp (Regev *et al.*, 1999). Since membrane fluidization is known to contribute to inhibition of P-gp efflux function (Regev *et al.*, 1999), Kabanov and colleagues proposed the induction of membrane fluidization as a mechanistic model of Pluronic block copolymers action on P-gp-induced efflux inhibition (Kabanov *et al.*, 2002). However, further studies and experiments led the above authors to conclude that membrane fluidization by itself may not be adequate for inhibition of the P-gp-mediated drug efflux in MDR cells

Fig. 3 Diagrammatic summary of the various interventions to reverse chemoresistance, based on alterations in membrane fluidity of tumor cells. All the mechanisms are associated with induction of early to late apoptotic processes. The apoptotic stimuli including conventional and novel chemotherapeutic agents may cause direct changes in membrane fluidity, permeability, lipid polarity, and protein order. These alterations may trigger a series of cellular events such as translocation of phosphatidylserine to the outer membrane leaflet, lipid peroxidation, repression of radical enzymatic defense mechanisms, as well as differential expression of death-related molecules, including death receptors, and possibly suppression of intracellular pathways involved in death receptor downregulation. Moreover, apoptotic stimuli, such as death ligands, chemotherapeutic drugs, or ionizing radiation, could activate the SMases, accounting for the ability of inducing stimuli to generate ceramide and gangliosides (e.g., GD3), which may be associated with lipid raft reorganization and death receptor aggregation amplifying therefore the apoptotic signals, or alternatively may interact with mitochondria for ROS generation and release or activation of apoptosis-related molecules. The induction of oxidative stress could also be enhanced by agents carrying allylic ethanol moieties which could be easily oxidized. Known agents such as bile salts and Dol-P may directly interfere with intracellular membranes, causing reduction in mitochondrial membrane potential resulting again in enhanced release of cytochrome *c* and AIF. All the above series of events singularly, or in interreaction with each other, or moreover in combination with other unspecified mechanisms finally result in significant potentiation of apoptosis and reversal of drug- or immunoresistance in tumor cells.

and inhibition of P-gp ATPase activity, presumably, through nonspecific membrane changes in lipid and protein conformation and mobility, has a major complementary contribution to the inhibition of P-gp efflux function. On the same setting, P85 exclusion from MDR cells suggested that energy depletion by itself, in the absence of direct interaction of the block copolymer with the P-gp-enriched membranes, might be insufficient to inhibit the efflux system. Thus, it is more likely that these Pluronic block copolymers have a dual effect on MDR tumor cells: through ATP depletion and membrane fluidization, which both have a combinational contribution to the potent inhibition of P-gp. Moreover, there is mounting evidence suggesting that the effects of Pluronic block copolymers might trunk beyond inhibition of the P-gp efflux pump. It has been shown that P85 was able to inhibit drug efflux in tumor cell lines expressing other transporters like MRP1 efflux pump, and possibly some other organic anion transporters including MRP2 (Miller *et al.*, 1999). The effects of Pluronic block copolymers on apoptosis triggering, however, are not adequately studied at present.

Since ATP depletion seems to play a crucial role in the function of ATP-dependent efflux pumps, we can speculate, as future prospective, that if we can knock out just one of the ATP-binding sites on these transporters, we could knock out their effectiveness. Alternatively, the possibility of reversing the polarity of these efflux pumps so that drugs could be delivered more effectively into cells making the problem of chemoresistance almost nonexistent. However, as more is learned about the genome, more ATP-dependent transporters than previously thought are being discovered, making the problem of chemoresistance more complex.

Decades ago, we have reported that alterations in membrane fluidity enhanced NK-mediated cytotoxicity (Roozemond and Bonavida, 1985; Roozemond *et al.*, 1987). The biophysical parameters of the membrane also seem to play an important role in immunogenicity. The identification of tumor-associated antigens has provided the basis for new concepts in immunotherapy. Tumor-associated antigens are present in the membrane of tumor cells, but most of them are usually concealed from the immune system (cryptic antigens). Therefore, by altering membrane fluidity and affecting microviscosity, these antigens can be exposed to the outer domain thus resulting in an increase in immunogenicity and increase in humoral and cellular responses *in vivo*. Indeed, increase in immunogenicity was correlated with the decrease in membrane fluidity as demonstrated by Shinitzky and colleagues for both lymphoma and mammary adenocarcinoma cells (Shinitzky and Henkart, 1979; Shinitzky *et al.*, 1979). The efficiency of exogenous administration of monoclonal antibodies against cryptic epitopes could also been enhanced after exposure of the tumor targets.

However, increasing membrane fluidization to sensitize to chemo-, radiation- or immune-cell death could have some other effects. In fact, it has been

reported that hyperfluidization of cell membrane induced by membrane fluidizers, benzyl alcohol, or heptanol initiates a stress response as the heat shock protein response (Balogh *et al.*, 2005), which may modulate positively or negatively apoptotic cell death (Garrido *et al.*, 2001).

Overall, we suggest that the fluidity variable could be used as an independent additional factor for the efficacy of combinational therapies, including chemotherapy, immunotherapy and radiation. However, for further successful development of novel approaches in the field of anticancer therapy based on alterations in physical aspects of tumor cell membranes, promising trends must be identified and exploited, albeit with a clear understanding of the limitations of these approaches.

IX. CONCLUDING REMARKS

In conclusion, the integrity of plasma membrane is essential for cell homeostasis and survival. Alteration of membrane structure or the membrane's physical properties can seriously manipulate the cell's viability or even provoke apoptotic pathways leading to cell death. Membrane fluidity is one of the most obvious physical properties of a membrane, but till recently the absence of quantitative measurements resulted in underestimation of its biological importance. Recent data nevertheless indicated that alteration in membrane fluidity is probably an important event during apoptotic pathways but also that environmentally induced changes in membrane fluidity can influence the cell's susceptibility to apoptosis. The fact that cells can be killed or become resistant to death through alteration of their membrane's physicochemical properties is undoubtedly a tempting idea in the field of disease management and especially cancer therapy. In this chapter, we have presented a large amount of available data indicating that several anticancer agents alter membrane fluidity and that, in some cases, this is the main way of provoking their cytotoxic effects while, in other cases, this is an essential coaction that enhances the induction of apoptotic pathways. Furthermore, the observed differences in membrane fluidity between resistant and sensitive tumor cells indicate that membrane fluidity is an important modulator of MDR, but also, and most importantly, alteration of membrane fluidity could result in increased cell chemo- or immunosensitivity. The important issue to be addressed is how to apply gained knowledge in cancer management practises. We believe that findings made both *in vitro* and *in vivo* that have already demonstrated remarkable results using changes in membrane fluidity and hybrid liposomes against tumor cells and resulting in the reversal of resistance suggest their potential clinical application. Further, we are confident

that we will soon witness the development of new therapeutic strategies based on membrane-induced apoptosis.

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Mutant Transcription Factors and Tyrosine Kinases as Therapeutic Targets for Leukemias: From Acute Promyelocytic Leukemia to Chronic Myeloid Leukemia and Beyond

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Mutations in transcription factors (TFs) and protein tyrosine kinases (PTKs), which result in inhibition of differentiation/apoptosis or enhanced proliferative/survival advantage of hematopoietic stem/progenitor cells, are two classes of the most frequently detected genetic abnormalities in leukemias. The critical roles for mutant TFs and/or PTKs to play in leukemogenesis, and the absence of mutant TFs/PTKs in normal hematopoietic cells, suggest that the two types of aberrant molecules may serve as ideal therapeutic targets. The great success of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) in treating acute promyelocytic leukemia through modulation of the causative PML-RARα oncoprotein represents the first two paradigms of mutant TFs-targeting therapeutic strategies for

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leukemia. More recently, tyrosine kinase inhibitor STI-571/Imatinib mesylate/Gleevec in the treatment of Breakpoint Cluster Region-Abelson (BCR-ABL) positive leukemia elicits paradigm of mutant PTKs as ideal antileukemia targets. Thus to further improve clinical outcome of leukemia patients, elucidation of pathogenesis of leukemia, screening for oncoprotein-targeting small molecules, as well as rationally designed combination of drugs with potential synergy are of importance.

I. INTRODUCTION

Leukemia is a group of phenotypically and genetically heterogeneous diseases. In the last two decades, significant progress had been achieved in understanding the fundamental genetic abnormalities in leukemia cells. In general, two categories of genetic abnormalities are involved in the leukemogenesis: anomalies in hematopoietic transcription factors (TFs) resulting in impairment of hematopoietic differentiation and subsequent apoptosis, and alterations in protein tyrosine kinases (PTKs) leading to uncontrolled proliferation and survival advantages of hematopoietic cells. So far, a large body of evidence has been emerging, particularly in mouse model, suggesting that the involvement of both classes of abnormalities is required for a full-blown acute myeloid leukemia (AML) to occur (Gilliland, 2002; Gilliland *et al.*, 2004; Kelly and Gilliland, 2002; McKenzie, 2005; Fig. 1).

Molecular and genetic approaches to understanding leukemia biology not only shed entirely new lights into the leukemogenesis, but also provide novel therapeutic strategy, the targeted therapy, to restore the molecular defects in leukemia cells (Bernasconi *et al.*, 2004). This therapeutic strategy targets the

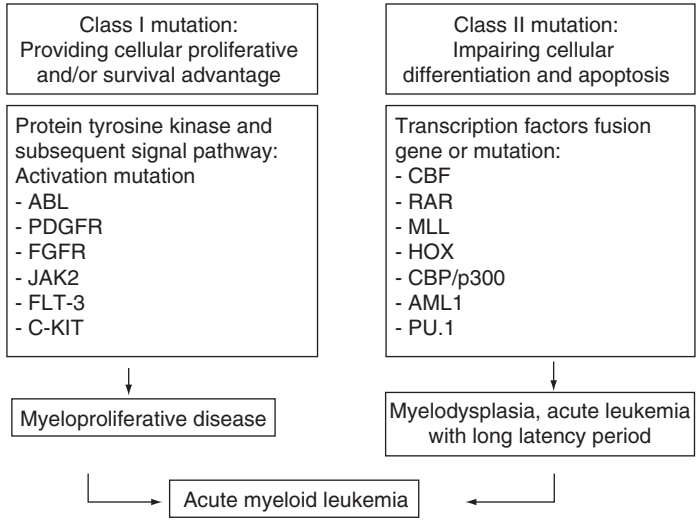

Fig. 1 Model of genetic cooperation in myeloid leukemogenesis.

biological properties of leukemia but not healthy cells, thus may fulfill the maximal efficacy with minimal adverse effects, as already well illustrated in the models of acute promyelocytic leukemia (APL) and chronic myeloid leukemia (CML) over past two decades (Deininger *et al.*, 2005; Huang *et al.*, 1988; Schindler *et al.*, 2000; Tallman, 2004).

This chapter will review the development and clinical benefit of differentiation/apoptosis agents against aberrant TFs, including all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) and small molecule kinase inhibitors such as Imatinib, which set applaudible examples for the molecular target therapy of leukemia.

II. PML-RARα AS A THERAPEUTIC TARGET FOR DIFFERENTIATION THERAPY

A. Retinoids: Differentiation Therapy in Promyelocytic Leukemia

APL was identified over 1950s to 1970s as a distinct subtype of AML characterized by three distinguished features: (1) the presence of an accumulation of abnormal promyelocytes unable to differentiate into mature granulocytes, (2) the occurrence of fibrinogenolysis and disseminated intravascular coagulation (DIC) that is often worsened by chemotherapy, and (3) the presence of the specific chromosomal translocation t(15;17)(q22;q21). APL accounts for 10–15% of all cases of AML and remains as high-risk leukemia before the advent of differentiation therapy. Anthracycline-based chemotherapy was associated with a complete remission (CR) rate of 60–76% and a 5-year event-free survival rate of 23–35% (Chen and Wang, 2003; Zhou *et al.*, 2005).

ATRA was first introduced to clinical treatment of APL in early 1980s in Shanghai Institute of Hematology (SIH) based on *in vitro* study, which demonstrated that ATRA could induce terminal differentiation of leukemia cell line with promyelocytic features (HL-60) and fresh leukemic cells from APL patients. In the pilot study from 1985 to 1988, 24 APL patients received ATRA at 45–100 mg/m^2 daily. Twenty-three patients (95.8%) obtained CR without developing bone marrow hypoplasia. Morphological maturation of bone marrow promyelocytes was found in all patients during the ATRA treatment (Huang *et al.*, 1988). These results were confirmed by large-scale randomized studies worldwide and ATRA was then adopted as standard treatment of newly diagnosed APL with daily dose of 45 mg/m^2 until remission (Wang, 2003).

In the subsequent laboratory study, several groups including the SIH identified in 1990s the underlying mechanism of the dramatic differentiation induction of ATRA in APL (de The *et al.*, 1990; Kakizuka *et al.*, 1991; Melnick and Licht, 1999). The characteristic chromosomal translocation involving the retinoic acid receptor alpha (*RARα*) locus on chromosome 17q21 and *PML* (for promyelocytic leukemia) gene on chromosome 15q22 generates a fusion gene, the *PML-RARα*. The RARα, its paralogs RARβ and RARγ, as well as retinoid X receptors (RXRα, RXRβ, and RXRγ) belong to the steroid/thyroid/retinoid nuclear receptor superfamily of ligand-inducible TFs. RARα forms a heterodimer with RXR and binds to retinoic acid response element (RARE) to control the expression of target genes in the presence of physiological concentrations (10^{-9}–10^{-8} M) of retinoids such as ATRA with ability of binding to RARs and RXRs. The PML-RARα oncoprotein binds to target genes through typical RARE or newly identified response elements (REs) unique for chimeric receptors. In both situations, PML-RARα interacts with RE either on its own as homodimer or with RXR as complex, which exerts a dominant negative effect over wild-type RAR by sequestering RXR and other RARα cofactors, recruitment of high amount of the corepressor and histone deacetylase complexes, leading to repression of transcription (de The *et al.*, 1990; Kakizuka *et al.*, 1991; Melnick and Licht, 1999).

It is now well established that pharmacological concentration of ATRA (10^{-7}–10^{-6} M) binds to PML-RARα homodimer or to complex with RXR in a more efficient way, triggering change in the configuration with resultant dissociation of the corepressor complexes and engagement of coactivation complexes, as well as subsequent degradation of the oncoprotein. Promyelocytes are then able to undergo terminal hematopoietic differentiation program and ultimately enter into the programmed cell death (Avvisati and Tallman, 2003; Nervi *et al.*, 1998; Zhu *et al.*, 1999).

Although the therapeutic effect of ATRA in APL is dramatic, this compound can induce hyperleukocytosis to different extension in most patients and a major life-threatening adverse "retinoic acid syndrome (RAS)" has been reported in as high as 20–25% of patients in Caucasian population and about 5% in Asian one (Chen and Wang, 2003; Tallman, 2004). Besides, patients receiving long-term ATRA treatment develop resistance and relapse eventually without chemotherapy. To overcome these problems, clinical trials were performed in SIH to optimize the therapeutic protocol. A clinical trial was conducted to evaluate the therapeutic effect and side effects of low-dose ATRA (40–60% decrease of ATRA dose). A total of 27 patients with *de novo* APL were treated with oral ATRA at the dose of 15–20 mg/m^2/day and 24 of 26 evaluable cases (92%) achieved clinical CR. Pharmaceutical data showed that maximal plasma concentration (Cpmax) with oral 15 mg/m^2 ATRA was high enough (10^{-6} M) to induce APL cell differentiation while no

patient experienced RAS and exaggerated DIC (Chen *et al.*, 1996). Hence, low-dose ATRA may provide advantages through decreased hyperleukocytosis and other side effects compared to the standard dose ATRA. In a large-scale national study, the results demonstrated that combination of ATRA and chemotherapy as postremission treatment improved the long-term survival compared to ATRA monotherapy (Sun *et al.*, 1993). With a more recent retrospective analysis and prognostic study based on long-term follow-up of 120 *de novo* APL patients, it has been identified that low-dose ATRA is as effective as the standard dose in terms of survival and the combination of chemotherapy and ATRA is superior to chemotherapy or ATRA alone as postremission therapy in terms of disease-free survival (DFS: 55.8 ′±10.4% vs 26.7 ′± 9.4%) (Hu *et al.*, 1999).

Subsequently, these results were confirmed by randomized trials. In European APL91 trial, 101 patients had been randomized between ATRA followed by 3 courses of daunorubicin-AraC chemotherapy (ATRA group) and the same chemotherapy alone (chemotherapy group). Long-term follow-up resulted a Kaplan–Meier estimate of 4 year event-free survival (EFS) and relapse rate of 63% and 31%, respectively in the ATRA group as compared to 17% and 78% in the chemotherapy group ($p = 0.0001$). The Kaplan–Meier estimated overall survival (OS) at 4 years was 76% in the ATRA group and 49% in the chemotherapy group ($p = 0.026$) (Fenaux *et al.*, 2000). In the North American Intergroup study, APL patients were treated with ATRA plus daunorubicin-based chemotherapy as induction followed by high-dose cytarabine plus daunorubicin for consolidation, and then daily maintenance of ATRA versus observation. The best outcome was observed in patients who received ATRA in both induction and maintenance therapy with a 5-year DFS of 74% (Tallman *et al.*, 2002). These results confirmed the superiority of the combination of ATRA and chemotherapy over chemotherapy alone in newly diagnosed APL, and that ATRA should be incorporated in the frontline treatment of APL. These data together with ours shaped out the basic principles for the treatment of *de novo* APL: (1) differentiation therapy with ATRA is responsible for the high remission rate, (2) addition of anthracycline-based chemotherapy to the ATRA can improve long-term survival, and (3) maintenance with ATRA or low-dose chemotherapy or both improves outcome (Tables I and II).

B. Arsenic: Induction of Partial Differentiation and Apoptosis in Promyelocytic Leukemia Cells

Treatment of APL by arsenic compounds represents another successful development of differentiation/apoptosis induction therapy in APL (Zhu *et al.*, 2002). Arsenic had been long used in traditional Chinese medicine (TCM)

Table I Evolution of Differentiation/Apoptosis Therapy in APL in Shanghai Institute of Hematology

Year	Publication	Number of patients (*n*)	Diagnosis	Treatment	Comment
1988	Blood (Huang *et al.*, 1988)	24		ATRA ± chemotherapy	23 achieved CR
1996	Leukemia (Chen *et al.*, 1996)	27		ATRA (15–20 mg/m^2/day)	Same effect as 45 mg/m^2/day
1997	Blood (Chen *et al.*, 1997)	15	Relapsed APL	ATO (0.16 mg/kg/day) ± ATRA	14/15 CR
1997	Blood (Niu *et al.*, 1999)	47	Relapsed APL	ATO (0.16 mg/kg/day)	40/47 CR; 1- and 2-year DFS 63.6% and 41.6%
		11	Newly diagnosed	ATO (0.16 mg/kg/day)	8/11 CR
2001	Leukemia (Shen *et al.*, 2001)	20	Relapsed APL	ATO (0.08 mg/kg/day)	16 CR; 2-year OS/RFS 61.5 ± 15.8% and 49.1 ± 15.1%
2004	PNAS (Shen *et al.*, 2004)	61	Newly diagnosed	ATRA ± chemotherapy ATO ± chemotherapy ATO ± ATRA ± chemotherapy	CR rates >90% in all 3 groups; early remission and more significant molecular response and superior survival in ATRA + ATO group

Table II Complete Remission Rate and Survival in APL Patients Treated with ATRA

Trial	Number of patients (*n*)	Induction	CR (%)	ED (%)	OS/DFS/ RFS (%)
APL91 (Fenaux *et al.*, 1993)	54	ATRA (+chemo)	91	9	79 ± 7 (12 months)
	47	Chemo	81	8	50 ± 9 (12 months)
SKCRC (Warrell *et al.*, 1994)	79	ATRA	84.8	12.7	—
JALSG (Kanamaru *et al.*, 1995)	109	ATRA ± chemo	89	8	81 (21 months)
GIMEMA (Mandelli *et al.*, 1997)	240	ATRA + chemo	95	5	79 ± 3.2 (2 years)
Intergroup (Tallman *et al.*, 1997)	172	ATRA	72	11	69 (3 years)
	174	Chemo	69	14	18
MRC (Burnett	119	ATRA (5d) chemo	70	23	52 (4 years)
et al., 1999)	120	ATRA + chemo	87	12	71
PETHEMA (Sanz *et al.*, 1999)	123	ATRA + chemo	89	11	92 (2 years)
APL93 (Fenaux	413	ATRA ± chemo	92	7	
et al., 1999)	109	ATRA chemo	95	—	77 (2 years)
	99	ATRA + chemo	94	—	84
German AML Cooperative Group (Lengfelder *et al.*, 2000)	51	ATRA + HD AraC	92	8	88 ± 9 (2 years)
JALSG (Asou *et al.*, 2001)	369	ATRA ± chemo	90	10	52 (6 years)

—, Data not available.

for various diseases including cancers. In the early 1970s, a crude solution of ATO was reported to be effective in treating APL in approximately two-thirds of patients while 5-year and 10-year survival rates were at 50% and 18.8%, respectively (Sun *et al.*, 1992; Zhang *et al.*, 1995). To confirm the above data, studies were carried out in SIH to test the ATO monotherapy in relapsed APL patients resistant to ATRA treatment. ATO was given intravenously at a dose of 0.16 mg/kg/day and CR was obtained in 14 of 15 patients after 28–54 (median 38) days (Chen *et al.*, 1997). In subsequent serial of newly diagnosed patients, remission was obtained in 8 of 11 (72.7%) patients (Niu *et al.*, 1999). These data were further confirmed by large-scale

clinical trials (Lazo *et al.*, 2003; Soignet *et al.*, 1998, 2001). Additionally, in follow-up study it was found that ATO could significantly increase the remission rate and improve the survival of relapsed patients previously treated with ATRA and chemotherapy (Niu *et al.*, 1999; Shen *et al.*, 2001). Now ATO is considered as the first choice for the treatment of patients with relapsed or refractory APL based on multiple clinical studies.

On the basis of the clinical efficacy of arsenic in APL treatment, SIH further explored the potential mechanism of specific antileukemia activity of arsenic in treating APL. It was demonstrated that ATO exerts a dose-dependent dual effect on APL cells. At low concentrations (0.1–0.5 μM), ATO drove APL cells to commit a partial granulocytic differentiation while at higher concentrations (0.5–1.0 μM), ATO induced apoptosis of APL cell with typical morphological changes, DNA fragmentation, and increased expression of annexin V. Increase in reactive oxygen species, collapse of mitochondrial transmembrane potential, and activation of caspases triggered by ATO could contribute to its apoptotic effect (Cai *et al.*, 2000, 2003; Dai *et al.*, 1999; Davison *et al.*, 2002; Shen *et al.*, 1997).

More recently, a large body of evidence demonstrated that the rapid modulation and degradation of PML-RARα proteins which was induced by ATO at 0.1–2 μmol/liter could contribute to the differentiation/apoptosis effects (Lallemand-Breitenbach *et al.*, 2005). It has been shown that besides the recruitment of transcriptional corepressor and subsequent modification of histone and DNA to repress transcription by PML-RARα oncoprotein, the PML domain also plays an important role in the leukemogenesis (Jing *et al.*, 2001; Lallemand-Breitenbach *et al.*, 2001; Lin *et al.*, 1998; Zhu *et al.*, 2005). The PML is modified by sumolation at amino acid K160, which recruits additional transcriptional repressor such as death-associated protein 6 (DAXX) and contributes also to the impairment of myeloid differentiation. ATO targets the PML moieties by inducing changes in the posttranslational status of amino acid K160 and releasing transcription repression. Furthermore, ATO can also promote the small ubiquitin-like modifiers (SUMO) conjugation at amino acid K160 which results in 11S proteasome activator complex recruitment and PML-RARα degradation (Jing *et al.*, 2001; Lallemand-Breitenbach *et al.*, 2001, 2005; Lin *et al.*, 1998; Sternsdorf *et al.*, 1999; Zhu *et al.*, 2005).

C. Combining ATRA and Arsenic: A Cure for APL?

Since both ATRA and ATO exert their differentiation/apoptosis induction effects on APL cells through modulation and/or catabolism of PML-RARα oncoprotein, can an additive or synergetic effect be attained when two drugs are combined in treating APL? In mouse models, an increased potential in eradicating the APL disease by sequential and/or combinatory

ATO and ATRA treatment was reported, suggesting the combined protocol may bring benefit to APL patients (Jing *et al.*, 2001; Lallemand-Breitenbach *et al.*, 1999). We also applied an approach integrating cDNA microarray, two-dimensional gel electrophoresis, and methods of computational biology to study the effects of ATRA and ATO combination on APL cell line (Zheng *et al.*, 2005). Numerous features were revealed, which indicated the coordinated regulation of molecular networks from various aspects of granulocytic differentiation and apoptosis at the transcriptome and proteome levels such as modulation of an array of TFs and cofactors, activation of calcium signaling, stimulation of the interferon (IFN) pathway, activation of the proteasome system, degradation of the PML-RARα oncoprotein, restoration of the nuclear body, cell-cycle arrest, and gain of apoptotic potential. These data suggested that ATRA exerts its effects on APL cells mainly through nuclear receptor-mediated transcriptional regulation, whereas ATO exercises its impact through targeting multiple pathways/cascades such as proteasome and stress response. Although complete understanding of the complexity and dynamics of *in vivo* synergy between ATRA and ATO is beyond the scope of the study, these data clearly provided a framework to reach that goal (Zheng *et al.*, 2005).

Meanwhile, a randomized clinical trial was conducted in SIH to test the benefit of ATRA and ATO combination in APL patients. A total of 61 newly diagnosed APL patients were randomized into 3 treatment groups treated by ATRA, ATO, and the combination of the 2 drugs. Although CR rates in three groups were all above 90%, the time to achieve CR was significantly shorter in the combination group compared to ATRA or ATO monotherapy. Of note, the level of *PML-RAR*α transcripts at remission decreased more significantly with combined therapy as compared with other groups ($p < 0.01$). Importantly, all 20 cases in the combination group remained in CR, whereas 7 of 37 cases treated with monotherapy relapsed after a median follow-up of 18 months ($p < 0.05$) (Shen *et al.*, 2004). Thus, the primary data clearly demonstrated the synergism of ATRA and ATO and its clinical benefit. On the basis of these data, we developed an ATRA–ATO–chemotherapy triad protocol for all newly diagnosed APL: induction therapy with ATRA and ATO followed by three cycles of chemotherapy as consolidation and sequential maintenance with sequential ATRA, ATO, and low-dose chemotherapy. With more recent follow-up of 61 patients treated so far, 58 (95.1%) patients achieved CR and 56 patients remained relapse free with a median follow-up at 43 months. The estimated 4-year relapse-free survival (RFS) and OS were both over 90%. (Liu, Y. F., Hu, J., Chen, S. J., and Chen, Z., unpublished data) These data highly suggested a potential benefit of frontline combination of differentiation/apoptosis induction therapy and conventional chemotherapy, which might translate into a better chance of curing the disease.

III. TYROSINE KINASES AS TARGET FOR APOPTOSIS INDUCTION THERAPY

In leukemogenesis, abnormal activation of signal transduction pathways, which confers proliferative and/or survival advantage to hematopoietic progenitors has been well documented (Gilliland *et al.*, 2004; Kelly and Gilliland, 2002). Examples include activation of PTKs by chromosomal translocations involving *ABL*, platelet-derived growth factor receptor (*PDGFR*), and fibroblast growth factor (*FGF*) genes and point mutations of *C-KIT*, *FLT-3*, and *JAK2* genes (Goldman and Melo, 2003; Guasch *et al.*, 2003; Kralovics *et al.*, 2005; Levitzki, 2004; Longley *et al.*, 2001; Reilly, 2003; Wong and Witte, 2004). A common thesis of genetic abnormality involving tyrosine kinases is the acquirement of constitutive tyrosine kinase activity, which activates multiple signal transduction cascades leading to increased proliferation and/or survival advantage of leukemic cells. These genetic abnormalities at the nodal points of hematopoietic regulation provide potential drug targets for therapies to be developed to inhibit the tyrosine kinase activity and ultimately induce cell proliferation arrest and apoptosis (Chalandon and Schwaller, 2005; Kelly and Gilliland, 2002). The development of efficient small molecule tyrosine kinase inhibitors is another milestone in the targeted anticancer therapy (Chalandon and Schwaller, 2005). More and more compounds targeting tyrosine kinases and their downstream-signaling pathways are currently entering clinical trials for hematological diseases (Table III).

A. Selective Inhibition of BCR-ABL as a Model in Targeting Aberrant Tyrosine Kinase

CML is characterized by the presence of *BCR-ABL* fusion gene, which is the result of reciprocal translocation between chromosomes 9 and 22 t(9;22)(q34;q11) (Melo *et al.*, 2003). It has been established that BCR-ABL is causal to the pathogenesis of CML and that constitutive tyrosine kinase activity of BCR-ABL is central to the capacity to transform hematopoietic cells *in vitro* and *in vivo*. The activation of multiple signal transduction pathways by BCR-ABL leads to increased proliferation, reduced growth factor dependence and cell apoptosis, and perturbed interaction with extracellular matrix and stroma (Deininger *et al.*, 2000; Lugo *et al.*, 1990; Melo *et al.*, 2003; Ren, 2005; Sawyers, 1999).

Table III Small Molecule Inhibitors for Tyrosine Kinases in Leukemias

Target	Disease	Compound	Producer
ABL	CML/ALL	Imatinib mesylate	Novartis
		AMN-107	
ABL/SRC	CML/ALL	BMS-354825	Bristol–Myers–Squibb
PDGFαR/PDGFβR	HES/CMML	Imatinib mesylate	Novartis
KIT	AML/mast cell leukemia	Imatinib mesylate	Novartis
		BMS-354825	Bristol–Myers–Squibb
		MLN-518	Millennium
FLT-3	AML/ALL	PKC-412	Novartis
		MLN-518	Millennium
		Cep-701	Cephalon
		SU-11248 (SU-5614)	Sugen
FGFR	EMS	PKC-412	Novartis

CML, chronic myeloid leukemia; HES, hypereosinophilic syndrome; CMML, chronic myelomonocytic leukemia.

1. THERAPEUTIC EFFECT OF GLEEVEC AND OTHER ABL KINASE INHIBITORS

The essential role of BCR-ABL tyrosine kinase activity for leukemogenesis provided the rationale for targeted therapy by specific inhibitor. Enormous efforts have been focused on the identification of compounds with inhibitory activity. Imatinib (Gleevec or Glivec formerly known as CGP57148B, STI-571) emerged as one promising compound for clinical development with significant selectivity for growth inhibition of BCR-ABL-expressing cells (Beran *et al.*, 1998; Druker *et al.*, 1996). *In vitro* study demonstrated that Imatinib inhibits ABL kinase activity with 50% inhibitory concentration (IC_{50}) values ranging between 0.1 and 0.5 μM in cells expressing constitutively active forms of ABL such as v-ABL, p210BCR-ABL, p185 BCR-ABL, and translocated ets leukemia (TEL)-ABL (Beran *et al.*, 1998; Druker *et al.*, 1996). Consistent with its *in vitro* profile, Imatinib also inhibited signaling of ligand-activated PDGFR, constitutively activated TEL-PDGFR fusion protein, KIT receptor activated by binding to its ligand, stem cell factor (SCF), or by activating mutation (Buchdunger *et al.*, 2000; Carroll *et al.*, 1997). Subsequently, potential antiproliferative activity and apoptosis induction activity of Imatinib was demonstrated in leukemia cells: exposure to Imatinib led to apoptotic cell death in fresh leukemic cells from CML patients while little effect on normal hematopoiesis at concentrations up to 1 μM was reported. In the following study using

transfection–transplantation model of CML, treatment with Imatinib lead to prolonged survival (Beran *et al.*, 1998; Druker *et al.*, 1996; Thiesing *et al.*, 2000).

Multiple clinical trials have also demonstrated the clinical benefit of Imatinib in CML patients at different stages (Branford *et al.*, 2003; Kantarjian *et al.*, 2002; Talpaz *et al.*, 2002). Over 90% patients received complete hematological response (CHR) with complete cytogenetic response (CCR) over 40% in patients after treatment failure of IFN-α or as high as 80% in newly diagnosed patients. Monitoring of residual disease by reverse transcription-polymerase chain reaction (RT-PCR) in complete cytogenetic responders showed that patients with CCR can achieve a profound reduction of *BCR-ABL* mRNA and may have low risk of disease progression (Branford *et al.*, 2003). Although complete molecular remission defined by the undetectable *BCR-ABL* mRNA was achieved in a proportion of patients, residual disease usually remains detectable with RT-PCR, indicating that disease eradication may remain as a significant challenge. Despite durable responses in most chronic phase patients, relapses have been observed and are much more prevalent in patients with advanced disease. The most common mechanism of acquired Imatinib resistance is the *BCR-ABL* kinase domain mutations with decreased Imatinib sensitivity (Gorre *et al.*, 2001). For patients in chronic phase with prolonged follow-up treated with Imatinib, development of *BCR-ABL* mutations leading to resistance and disease progression also emerged as potential clinical problem. To overcome the resistance, several approaches have been studied *in vitro* and *in vivo*. These include dose escalation of Imatinib, alternative BCR-ABL inhibitors, and combination of Imatinib with chemotherapeutic drugs, interferon, or other therapeutic agents (La Rosee *et al.*, 2002b; Martinelli *et al.*, 2005).

Alternate BCR-ABL kinase inhibitors that have activity against Imatinib-resistant mutants would be useful for those patients relapsed while on Imatinib therapy. Two such BCR-ABL inhibitors were developed and being evaluated in clinical trials: AMN-107, a selective ABL inhibitor with improved potency and BMS-354825, a highly potent dual Src/Abl inhibitor (Shah *et al.*, 2004; Weisberg *et al.*, 2005). In *in vitro* study, AMN-107 and BMS-354825 are more potent than Imatinib against cells expressing wild-type BCR-ABL and most of reported mutants BCR-ABL with the exception of T315I. These two compounds also inhibit proliferation of BCR-ABL-positive bone marrow progenitor cells from patients with Imatinib-sensitive and Imatinib-resistant CML. In *in vivo* study, BMS-354825 also prolongs survival of mice with BCR-ABL-driven disease (Shah *et al.*, 2004; Weisberg *et al.*, 2005). Phase I/II primary clinical trials have demonstrated the safety and potential efficacy of BMS-354825 and AMN-107 in CML patients resistant to Imatinib (Giles *et al.*, 2004; Talpaz *et al.*, 2004).

2. ARSENIC COMPOUNDS TARGET BCR-ABL AND EXERT SYNERGISTIC EFFECT WITH IMATINIB

Arsenic-containing solution has been used as therapeutic agent since ancient times for CML and the early experience suggested that arsenic might have some inhibitory effect on proliferation of BCR-ABL-expressing cells (Forkner and Scott, 1931). Several groups including ours have shown that arsenic could induce apoptosis in BCR-ABL-positive leukemia cells (La Rosee *et al.*, 2002a, 2004; Nimmanapalli *et al.*, 2003; Perkins *et al.*, 2000; Porosnicu *et al.*, 2001; Puccetti *et al.*, 2000; Yin *et al.*, 2004). More interestingly, the enforced expression of BCR-ABL oncoprotein in U937 cells dramatically increased the sensitivity to ATO. Thus, these data suggested that the inhibitory effect of ATO is not simply due to a nonspecific toxicity to the BCR-ABL leukemia cells (La Rosee *et al.*, 2004). Instead, ATO-induced apoptosis is genetically determined by the presence of specific BCR-ABL fusion gene products, though the mechanism by which BCR-ABL mediates ATO-induced apoptosis remains unclear. Perkins *et al.* (2000) reported that following treatment with ATO of clinically achievable concentrations (0.5–2.0 μmol/liter) for 7 days, growth inhibition (0.8–1.5 μmol/liter) and apoptosis (2.0 μmol/liter) were observed in BCR-ABL-positive acute leukemia HL-60/BCR-ABL and K562 cells (Perkins *et al.*, 2000). There was a declined BCR-ABL protein level due to the inhibition of translation mediated by inhibition of ribosomal p70S6 kinase activity and/or acetylation of histones H3 and H4.

Study at SIH showed that an oral arsenic compound As_4S_4 and Imatinib exerted synergistic effects in inhibition of growth and clonogenic ability on K562 cells and fresh CML cells (Yin *et al.*, 2004). The effective concentrations on fresh CML cells were pharmacokinetically available *in vivo* but had much less inhibitory effect on normal CD34+ hematopoietic cells. Using a number of parameters such as morphology, annexin V/propidium iodide (PI), mitochondrial transmembrane potential, caspase-3 activity, and Fas/FasL, the synergistic effects were revealed on induction of cell apoptosis, largely through the mitochondrial pathway. These two drugs also exhibited a synergistic effect in targeting BCR-ABL protein. While As_4S_4 reduced its protein level and Imatinib inhibited its tyrosine kinase activity, the combination of these two drugs led to a much lower protein/enzymatic activity levels of BCR-ABL (Yin *et al.*, 2004). These *in vitro* data strongly suggest that arsenic and Imatinib can target the leukemia cell-specific BCR-ABL at complimentary but different level: arsenic at the protein level and Imatinib at the kinase activity thus making the Imatinib + arsenic a reasonable combination for potential clinical application in the treatment of CML.

Recently, a pilot study was conducted in CML patients in accelerated phase ($n = 5$) and blast crisis ($n = 5$). All patients received Imatinib at

400–600 mg daily combined with As_4S_4 at 150 mg/kg daily and the results were compared to those of 26 patients from Glivec International Patient Assistance Program (GIPAP) with Imatinib 600 mg daily. Similar toxicities were met in both study and GIPAP groups, which were generally moderate. With regard to the efficacy of treatment, the CHR rates were similar between the two groups; however, the CCR and molecular response were slightly higher in study group (40% and 30%) than GIPAP group (30.8% and 10%). There was a trend toward better OS and PFS in favor of combination therapy for patients without cytogenetic clonal evolution. Although it is difficult to make any conclusion based on this nonrandomized study with limited patients number, our primary data suggested that achievement of CHR, CCR, or even molecular remission was documented with combination therapy, which is also a feasible regimen for CML in terms of toxicity profile and potentially enhanced the therapeutic effects (Li, J. M., Wang, A. H., Shen, Z. X., Chen, S. J., Chen, Z., and Hu, J., unpublished data).

B. PDGFR as a Therapeutic Target

PDGFR belongs to the type III receptor PTK family including KIT, CSFR1, and FLT-3 that are characterized by a transmembrane domain, a juxtamembrane domain, and a split kinase domain (Chalandon and Schwaller, 2005; Levitzki, 2004). Involvement of PDGFR in the pathogenesis of leukemia is well-characterized by balanced translocation from patients with chronic myelomonocytic leukemia (CMML) or atypical CML (Philadelphia chromosome-negative), which leads to fusion of various partner genes to the tyrosine kinase domain of *PDGFβR* such as *TEL/PDGFβR* in t(5;12)(q33;p13), *HIP1/PDGFβR* in t(5;7)(q33;q11), *RAB5/PDGFβR* in t(5;17)(q33;p13), and *H4/PDGFβR* in t(5;10)(q33;q21) (Golub *et al.*, 1994; Kulkarni *et al.*, 2000; Magnusson *et al.*, 2001; Ross *et al.*, 1998). The identification of the *FIP1L1-PDGFαR* fusion gene provides a molecular explanation for the pathogenesis of approximately half of the patients with hypereosinophilic syndrome (HES) and subsequently reclassified as chronic eosinophilic leukemia (CEL) based on the availability of molecular diagnosis (Cools *et al.*, 2003). This fusion gene is not the result of a translocation, but the consequence of a cryptic interstitial deletion, which fuses the kinase domain of the *PDGFαR* gene located on 4q12 to *FIP1L1a* gene (*FIP1-like1* gene). More importantly, these fusion genes transformed the hematopoietic cell line to growth factor independence and caused a rapid development of fatal myeloproliferative disease (MPD) in mouse retroviral bone marrow transplant model. Detailed mutational analysis demonstrated that constitutive tyrosine activation of PDGFR tyrosine kinase domain due to an oligomerization domain in the 5′ fusion partner is a critical element in the transforming

property. These results emphasize the critical importance of the activated tyrosine kinase in the disease phenotype and the ideal target for the molecular-targeting therapy. *In vivo* study has shown that Imatinib is highly effective against cells transformed with *PDGFR* fusion oncogene and a dose–response study for Imatinib reveals that PDGFR is approximately tenfold more sensitive than BCR-ABL with regard to IC_{50}. Various clinical reports also confirmed the significant clinical efficacy of Imatinib at lower daily dose (~300 mg daily) in the treatment of atypical CML, CMML, and HES patients, which harbor the *PDGFR* fusion oncogenes (Magnusson *et al.*, 2002; Pardanani and Tefferi, 2004).

C. C-KIT as a Therapeutic Target

A number of observations documented the role of C-KIT in the development of a range of malignant disease such as gastrointestinal stromal tumors (GIST) and leukemia (Blanke *et al.*, 2001; Dematteo *et al.*, 2002, Demetri, 2001, 2002). The C-KIT protein is a transmembrane receptor with tyrosine-kinase activity mediated by its physiological ligand SCF. The *C-KIT* gene, located on the long arm of chromosome 4, encodes for a member of the type III receptor tyrosine kinase family. KIT shares extensive structural homologies with receptor of macrophage colony-stimulating factor (M-SCF) or PDGFR (Blume-Jensen and Hunter, 2001). The mutated and/or activated forms of C-KIT tyrosine kinase receptor (CD117) have been demonstrated as an early event in malignant transformation of GIST (Blanke *et al.*, 2001; Dematteo *et al.*, 2002). At protein level, there are two categories of mutations: (1) mutations occurred in the regulatory regions that affect the extracellular domains of the molecule, or juxtamembrane dimerization domains and (2) mutations occurred in the kinase domain, generally in parts of the protein encoded by exon 13 or 17. Since 2001, Imatinib has been shown to induce 60–70% objective responses in GIST patients with 1-year survival rate of patients with advanced GIST close to 90%, thus making GIST as a model of molecular target-based therapy in solid tumor (Blanke *et al.*, 2001; Dematteo *et al.*, 2002; Demetri, 2001, 2002).

A number of observations suggested a role of *C-KIT* gene in the oncogenesis in various types of hematological malignancies. High expression of *C-KIT* was documented in AML (60–80%) and point mutations have been identified in 33.3–45% of AML (Beghini *et al.*, 2000, 2004; Care *et al.*, 2003; Cole *et al.*, 1996). In a study, we identified 11 types of *C-KIT* gene mutations (*mC-KIT*) in 26 of 54 (48.1%) cases of *de novo* AML with t(8;21) associated with *AML-ETO* fusion gene (*AE*) (Wang *et al.*, 2005). Most leukemic cells at diagnosis harbored both genetic alterations, whereas during CR only *AE* but not *mC-KIT* could be detected by allele-specific PCR.

Therefore, *AE* could be considered as primary genetic abnormality and *mC-KIT* as a subsequent event in the leukemogenesis. More importantly, induction expression of *AE* in U937 cells significantly upregulated the expression of *C-KIT* at both mRNA and protein levels. This may lead to an alternative way of C-KIT activation and may explain not only the significantly higher expression in 81.3% of patients with t(8;21), but also serve as a selection basis for the development of *C-KIT* mutation (Wang *et al.*, 2005). Combined with the other *in vitro* and *in vivo* data showing that *AE* is necessary but not sufficient to induce leukemia phenotype in transgenic mice model, our findings strongly suggest that t(8;21) AML may follow a stepwise and multiple hits model in leukemogenesis, that is *AE* represents the first, primary genetic event to initiate the defect in hematopoietic differentiation, whereas activation of the C-KIT pathway may be a secondary but also crucial hit for the acquirement of proliferation or survival advantage for a full-blown leukemia (Wang *et al.*, 2005).

More importantly, our *in vitro* study demonstrated that Imatinib significantly suppressed the C-KIT activity, induced a prominent proliferation inhibition and apoptosis of hematopoietic cells bearing *C-KIT* mutation or overexpression. Furthermore, Imatinib exerted a synergic effect with cytarabine, a chemotherapy frequently used for the AML in induction of leukemia cell apoptosis, suggesting thus combination or sequential use of Imatinib with chemotherapy might be a potential therapeutic strategy for *de novo* t(8;21) leukemia or other leukemia carrying mutant *C-KIT* (Wang *et al.*, 2005). Hence, prove-of-principle clinical trials should be warranted.

D. FLT-3 as a Therapeutic Target

FLT-3 is the most commonly mutated gene in AML with activation mutation detected in approximately 30–35% of AML (Gilliland and Griffin, 2002; Reilly, 2003). Most *FLT-3* mutations are characterized by an internal tandem duplications (ITDs) in the juxtamembrane domain ranging in size from several to >50 amino acids. These mutations are always in frame and the diversity of mutations among patients suggested that they may be loss-of-function mutations in an autoinhibitory domain. In addition, mutations also occur in the activation loop of FLT-3 in about 5–10% of AML, which result in folding out of the activation loop, providing access of the catalytic site to ATP and substrate. Thus in all these cases, the consequence of the mutation is constitutive activation of FLT-3 tyrosine kinase activity. The FLT-3 ITD confers IL-3 independent growth to the murine hematopoietic cell line Ba/F3. Furthermore, FLT-3 ITD also induces a MPD in primary hematopoietic progenitors in a murine transfection/transplantation model

(Kelly *et al.*, 2002a; Kiyoi *et al.*, 1997; Rombouts *et al.*, 2000; Thiede *et al.*, 2002; Yamamoto *et al.*, 2001).

Selective inhibitors of FLT-3 have been identified and these include PKC412 (Novartis), MLN518 (Millennium), SU11248, SU5614, and SU5416 (SuGen), and CEP-701 (Cephalon) (Clark *et al.*, 2004; O'Farrell *et al.*, 2003, 2004; Schmidt-Arras *et al.*, 2004; Smith *et al.*, 2004; Weisberg *et al.*, 2002). All these agents can inhibit the proliferation and induce apoptosis in Ba/F3 cell lines harboring FLT-3 ITD, and several inhibitors can also induce apoptosis of human AML cell lines with FLT-3 ITD or overexpression of wild-type FLT-3. In murine bone marrow transplant models, FLT-3 inhibitors demonstrate statistically significantly prolonged survival indicating the *in vivo* efficacy. More importantly, several studies demonstrated that the simultaneous or sequential use of chemotherapy with FLT-3 inhibitor might have additive to synergistic effect in the antileukemia potential (Levis *et al.*, 2004; Yee *et al.*, 2004). On the basis of these data, Phase I/II trials of FLT-3 inhibitors in the treatment of AML were undergoing and most trials involved patients with relapsed AML carrying mutant *FLT-3*. Although it is still too early to make a conclusion, reports suggest that FLT-3 inhibitor have antileukemia activity in clinical setting (Fiedler *et al.*, 2003, 2005; Stone *et al.*, 2005). Additional testing will be necessary to determine whether the FLT-3 inhibitor can be used in combination or sequentially with available therapies for *de novo* and refractory/relapsed AML.

In a recent study, we analyzed FLT-3 mutations in 104 APL patients and found 20.2% (21/104) patients carrying FLT-3 ITD and 3.85% (4/104) with D835 point mutation. The FLT-3 ITD was also associated with clinical and biological features such as leukocytosis at diagnosis ($p < 0.01$) and short/variant form of *PML-RARα* fusion gene (Zhu, Y. M., Hu, J., Chen, Z., and Chen, S. J., unpublished data). Previous study of FLT-3 ITD in murine model demonstrated that FLT-3 ITD induced AML-like disease in cooperation with PML-RARα oncoprotein, thus provided experimental evidence for a potential cooperation of FLT-3 ITD conferring the proliferation/survival advantage with PML-RARα, blocking hematopoietic differentiation in leukemogenesis (Kelly *et al.*, 2002b). On the basis of these data, we suggest that FLT-3 inhibition combined with PML-RARα-targeting strategy (e.g., ATRA) might be ideal in induction of both differentiation and apoptosis in leukemia cells. The therapeutic efficacy of this new combination-targeting strategy should be explored further in laboratory and potentially clinical settings.

E. FGFR as a Therapeutic Target

FGFs are a large family of pleiotropic heparin-binding growth factors, which exert the functions through four related receptor tyrosine kinase domains and play a role in the regulation of cellular proliferation, migration,

and differentiation during embryogenesis (Guasch *et al.*, 2003; Ornitz and Itoh, 2001). Several studies have proposed that the FGF/FGFR system may play a role in the early hematopoiesis, while the evidence of FGF/FGFR involvement in leukemogenesis came from cases of Philadelphia chromosome-negative myeloproliferative syndrome named as 8p13 myeloproliferative syndrome (EMS) characterized by eosinophilia, generalized lymphadenopathy, and an unusually high incidence of T-cell lymphoblastic leukemia/lymphoma. Molecular characterization of the reciprocal translocation t(8;13)(p11;q12) present in some EMS cases revealed a fusion of *ZNF198* gene to the intracellular part of the *FGFR1* (Macdonald *et al.*, 2002; Roumiantsev *et al.*, 2004). Subsequently, there were reports of translocations leading to CML-like myeloproliferative disorder without the development of T-cell leukemia/lymphoma. In mouse retroviral bone marrow transplant model, *ZNF-FGFR1* induces significant proliferation of myeloid cells and development of T-cell lymphomas, which was closely mimicking the human EMS while another fusion gene, the *BCR-FGFR1*, induced an aggressive MPD similar to *BCR-ABL*, suggesting that the fusion partner gene may have an important effect on the disease phenotype (Roumiantsev *et al.*, 2004). Interestingly, there is also strong experimental evidence that FGFR can be targeted by small molecule inhibitors (Chalandon and Schwaller, 2005).

F. JAK2 as a Therapeutic Target

Janus kinase 2 (JAK2) is a cytoplasmic protein tyrosine kinase that catalyzes the transfer of the gamma-phosphate group of adenosine triphosphate to the hydroxyl groups of specific tyrosine residues in signal transduction molecules. JAK2 mediates signaling downstream of cytokine receptors after ligand-induced autophosphorylation of both receptor and enzyme. The main downstream effectors of JAK2 are a family of TFs known as signal transducers and activators of transcription (STAT) proteins. Recently, several different studies have independently described a close association between an activating JAK2 mutation (JAK2V617F) and the classic BCR-ABL-negative MPD (i.e., polycythemia vera, essential thrombocythemia, myelofibrosis with myeloid metaplasia) as well as the less frequent occurrence of the same mutation in both atypical MPD and the myelodysplastic syndrome (Baxter *et al.*, 2005; James *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005; Zhao *et al.*, 2005). These particular findings emphasize the importance of genetic abnormality in downstream signal pathway of PTK in the leukemogenesis and its potential in the target therapy.

G. Other Potential Benefits of Targeting Tyrosine Kinase Downstream Pathway

Intensive research provided evidence that oncogenic PTK is critical for the leukemogenesis and the transforming activity is mediated by activation of downstream signaling pathways (Chalandon and Schwaller, 2005). In general, multiple phosphorylated tyrosine residues of PTKs serve as a docking site for multiple adapter or signaling mediators to become themselves activated through phosphorylation by the constitutive active PTK. The complex signaling network results in the translocation of phosphoproteins to the nucleus as transcriptional regulators and thus activating the target genes. Several important signaling pathways in cancer cells have been demonstrated, which include JAK/STAT, RAS/MAPK, PI3K/AKT, and/or NF-κB and interference with the downstream signaling pathway, especially combined with small molecule PTK inhibitors, may improve the antileukemia therapy or overcome the resistance (Chalandon and Schwaller, 2005; Irish *et al.*, 2004; Schindler, 2002). For example, RAS/ERK signaling pathway is considered as one of the major effector cascades mediating oncogenic activities of deregulated PTK. Interference with farnesylation is able to downregulate the RAS/ERK pathways. Farnesyltransferase inhibitors such as R-115777 had been shown to inhibit proliferation of BCR-ABL-expressing cells (Daley, 2003; Miyoshi *et al.*, 2005). Several studies also proposed that constitutive activation of the NF-κB transcriptional regulator might represent other effectors of the oncogenic effects of a mutated PTK. Although it is not well understood how deregulated PTK activity leading to constitutive activation of NF-κB, there is evidence of activation of NF-κB as downstream event in the transformation of BCR-ABL fusion protein (Kirchner *et al.*, 2003; Reuther *et al.*, 1998). For example, Imatinib can effectively inhibit growth of the Ba/F3 murine hematopoietic cell line transformed with the *PDGFβR*- or *ABL*-associated fusion oncogenes while this effect can be salvaged by the addition of IL-3. Inhibition of NF-κB translocation combined with Imatinib can successfully inhibit cell growth of transformed Ba/F3 with or without IL-3. (Hu, J., Magnus, M., Robyn, J., and Dunbar C. E., unpublished data). The proteasome inhibitor PS-341 (bortezomib or Velcade), a compound known to interfere with deregulated NF-κB activity, is able to suppress cell growth and induce apoptotic cell death in BCR-ABL-positive cell lines that were either sensitive or resistant to Imatinib (Gatto *et al.*, 2003). All these data thus provided primary evidence of synergetic target therapy against the leukemogenetic molecule and its downstream pathway might have therapeutic potential, though detailed molecular mechanisms and efficacy remain to be fully elucidated.

IV. PERSPECTIVES

A large body of evidence suggested that therapy based on targeting the leukemogenetic molecule is effective with significant clinical benefit as witnessed by the tremendous improvement of clinical outcome in the treatment of APL by ATRA and/or ATO and CML by Imatinib. Of note, primary studies of combined target therapy (ATRA + ATO for APL and As_4S_4 + Imatinib for CML) also set applaudible examples of combined differentiation/apoptosis target therapy which might translate into a better chance of curing the disease.

Acute myeloid leukemia is characterized by class I and class II mutations (Fig. 1). The current list of known leukemogenic class I mutations contains more than 10 different PTKs that undergo constitutive activation either by chromosome translocation leading to the fusion with different partner genes or by activating point mutation in the kinase domain or ITD in the juxtamembrane domain. Expression of class I mutations confers proliferation and/or survival advantage to hematopoietic cells. When analyzed for their transforming potential, overexpression of class I mutations is generally sufficient to transform hematopoietic cells to growth-factor independence *in vitro* and to induce a lethal leukemia-like myeloproliferative disorder in mice. As to the class II mutations the targets are mostly TFs which are normally critical for hematopoietic differentiation. The dominant negative formation or gain-of-function by chromosome translocation or point mutation of these TFs leads to the impairment of hematopoietic differentiation but not directly provides proliferation and/or survival advantage. Although mutations of TFs were detected mostly in acute leukemia, they are necessary but not sufficient to induce acute leukemia in mice model while induce a myelodysplasia syndrome. These observations suggest a multigenetic hit model for the leukemogenesis of AML, which is supported by several lines of experimental evidence that two classes of mutations cooperate to cause acute leukemia in mice model which include BCR-ABL with NUP98-HOXA9 or AML1-EVI1 and FLT-3 ITD with PML-RARα (Dash *et al.*, 2002; Kelly *et al.*, 2002b). A stepwise leukemogenesis model has been proposed for AML-M2 with t(8;21) in clinical setting where AML1-ETO was considered as a primary event, whereas activation of C-KIT pathway could represent a second hit (Wang *et al.*, 2005).

Since acute leukemia is characterized by class I and class II mutations, development of prove-of-principle studies by combining treatment agents targeting synergistically class I and II mutations will be of interest. However, at the moment, treatment targeting class II mutations available is still limited compared to the small molecule PTK inhibitors which target mostly class I mutations. Thus, further efforts to identify the mutational status and

exploration of new targeting agents will be important and may have significant impact on the development of new therapeutic regimens in leukemia.

Witnessing the tremendous advances in understanding the pathogenesis and development of target therapeutics for APL and CML in the last two or three decades, conventional chemotherapy for many types of leukemia often fail because of treatment-induced mortality or resistance to therapy and the genetic abnormalities underlying the leukemias remain to be explored. However, the launch of Leukemia Genome Anatomy Project (LGAP) at SIH and other centers may accelerate the dissection of leukemogenic mechanism and identification of therapeutic targets, and this may also facilitate the screening of promising specific molecular-targeting agents, leading eventually to improve clinical outcome or even conquer leukemias.

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The Effect of Cell–Matrix Interactions and Aging on the Malignant Process

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The malignant process, transformation of normal cells, proliferation, and metastasis formation, was considered as if originating from one single cell. Although the intrinsic mechanisms of transformation from the normal to the malignant state were both confirmed, an increasing body of evidence points to the surrounding matrix and cell–matrix interactions as major players in this process. Some of the most important arguments in favor of this contention are cited and commented in this chapter. Another important question concerns the relationship between the aging process and malignant transformation. A few decades ago, the frequency of clinically manifest tumors of several organs and tissues appeared to increase with age. As, however, average life expectancy increased rapidly over the last decades, clinical frequency of malignant tumors did not follow this tendency. It was argued that late in life the malignant process appears to decline. This justly inspired several teams to study the relationship between cellular senescence and malignant transformation. This is now an actively growing field which deserves special attention. Some of the pertinent experimental and theoretical arguments in favor of an antioncogene-mediated switch between these two processes are also reviewed with the caveat that this important and new subject of basic and clinical research on the malignant process is just at its beginning. It will certainly take an increasing importance during the coming years and decades with the hope to contribute to answer one of the most burning questions concerning the aging process: will life expectancy continue to increase linearly as predicted by some gerontologists, or will life expectancy level off or even decline as

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predicted by other epidemiologists. The relationship between cellular senescence and malignant transformation will play in this respect an important role.

I. INTRODUCTION

Looking through the cancer literature over the last decades one can clearly notice an increasing tendency to consider this class of *diseases* as the result of the interaction of abnormal cell(s) with their environment. The classical concept in cancer research was and still is to localize the causation of the disease within the cell, possibly in one single cell (Weinberg, 1998), without taking care to replace the cancer cell in its environment. There should be no blame for this tendency followed by the multistage mechanism of tumor progression (Klein, 1998) because a number of important experimental facts and derived concepts originated from this theory were adopted in many laboratories. The other line of research, which originated sporadically during the last decades of the twentieth century, follows a more holistic approach, replacing the malignant cell in its environment in order to better understand the progression of the disease as a result of interaction of the malignant cell and its environment, essentially the surrounding matrix, its cells and its macromolecules (the stroma). Looking on histological sections of malignant tissues, the pathologist concentrates its attention on two major changes: the general pattern of the tissue as far as cells and extracellular matrix (ECM) are concerned and the presence and distribution of "abnormal" cells recognized by their karyotype. The deviation of tumor tissue pattern from normal tissues is essentially the result of the relationship of malignant cells to other surrounding cells, and, especially, to the surrounding ECM. Although its abundance varies among tissues, ECM is always present and appears to play an important role in the malignant process also. Interactions between malignant cells and surrounding, apparently normal, cells were recognized over the last decades as a potentially important factor (Rubin, 2003, for review). The possible or even probable implication of ECM components in the malignant process is of more recent origin, although some authors did mention "connective tissues" as of importance in this respect (van den Hooff, 1988). The purpose of this chapter is to present some of the experimental facts and arguments in favor of this new tendency in cancer research with no ambition to be exhaustive. As a matter of fact, this tendency increased rapidly over the last years and the limits between modern matrix biology [an abbreviation for biology and pathology of ECM (Balazs, 1970)] and cancer biology are not clearly delimited as will appear also from this chapter. Therefore, we shall start with a short recall of some relevant aspects of matrix biology before considering its implication in the malignant process. Aging intruded so to say spontaneously in this picture because of the continuous modification of the ECM with age. Cells also

change with age as shown by their serial culture (*in vitro* aging) with, however, the caveat of no easy extrapolation from *in vitro* experiments to the *in vivo* situation. Such more holistic considerations of the malignant process will presumably prevail over the coming years, both for theoretical and practical reasons. The theoretical arguments will emerge as we present some recent data on cell–matrix interactions and their relation to the malignant process. The practical arguments in favor of such a more integrated approach were drastically summarized by Kibestis and Travis (2006) in the introduction of a series of articles on cancer by the statement "An examination of the annual statistical data compiled by the American Cancer Society quickly reveals that the rate of mortality from cancer has changed very little over the past 50 years." Even if, as forseeable not all physicians involved in the treatment of cancer patients would subscribe to this statement, the fact that cancer might progressively replace cardiovascular diseases as the leading cause of death in the elderly population, at least below 70–80 years (because later in life, a cancer incidence decreases, see Section IX), can be interpreted as the result of a faster progress in the prevention and treatment of cardiovascular diseases than malignant diseases. Most therapeutical strategies directed to malignant diseases tend to target exclusively the malignant cells. This is the case of most, more or less, specific chemotherapies. Radiation therapies adopted also this principle. It cannot be excluded that a better knowledge of the role of cell–matrix interactions in the malignant process might increase the efficacy of treatment strategies. We shall come back later to this possibility. Although in this respect also evolution of socioeconomic factors might reserve some surprises for physicians and biologists as shown by the pandemic increase of obesity in recent decades (Olshansky *et al.*, 2005). This new fact might well play an increasing role in "prepositioning" cardiovascular diseases to much earlier generations as shown by the occurrence of diabetes type II (the metabolic syndrome) in young adults, and not only anymore in aging individuals (Smyth and Heron, 2005). However, according to recent statistics, cancer incidence also increases with obesity (Adami and Trichopoulos, 2003). These observations open another important chapter on the role of cell–cell and cell–matrix interactions and interfering endocrine factors in the malignant process. But before considering the role of cell–matrix interactions in the malignant process, we shall summarize some relevant aspects of matrix biology.

II. THE EXTRACELLULAR MATRIX

The classical pathology books separately considered "connective tissues" and "parenchymatous tissues." The connective tissues are rich in ECM which is however present in variable proportions in all tissues, in the

so-called parenchymatous tissues also. The proportion of ECM components and especially of collagens increases with age to the detriment of "parenchymatous" cells such as hepatocytes. This change of proportions becomes prevalent in liver cirrhosis often precursor of malignant transformation of the liver. Up to the 1950s ECM was considered as of relatively simple composition. Besides collagen, the major component (about 30% of all body proteins), "acid mucopolysaccharides" and elastin were known to be present. Today, there are at least 28 different collagen types recognized only in vertebrates (Ricard-Blum and Ruggiero, 2005) with many more types in invertebrates. Collagen(s) appeared during evolution with the first organized pluricellular organisms, the sponges. Proteoglycans (PGs) and glycosaminoglycans (GAGs) replaced "acid mucopolysaccharides." Elastin (and other ECM) components were shown to be present in most tissues with predominance in large vessels, lung, and skin. Elastin presents microheterogeneity as a result of alternative splicing of the primary transcript of the gene coding for tropoelastin, its subunit (Robert and Hornebeck, 1989, for review). Besides these three families of ECM components, (collagens, elastin, proteoglycans and glycosaminoglycans, hyaluronan), a fourth family of macromolecules was identified during the last decades of the twentieth century, designated initially as "structural glycoproteins" (Labat-Robert *et al*., 1986, for review). These glycoproteins, first identified in corneal stroma, were shown to be synthesized locally by connective tissue cells and not in the liver as are most circulating glycoproteins as for instance α-foetoprotein shown to play some role in the malignant process (Abelev, 1971). Their isolation and characterization led first to the recognition of the important role of fibronectin and laminin in cell–matrix interactions and progressively to the identification of an increasing number of such glycoproteins endowed with "structural" roles in tissue patterning and cell–matrix interactions. These discoveries increased considerably the number of ECM components and, consequently, the potential role of ECM in normal and pathological processes. As ECM components were shown to appear very early during embryonal development (laminin and collagen type IV are present from the four-cell gastrula stage in chick embryos), their important role in morphogenetic processes was rapidly recognized. Knowledge about their pathological role progressed also rapidly first in diseases where "connective tissues" are primarily involved as in osteoarticular diseases, followed by the "collagen diseases" of Klemperer comprising an autoimmune factor as well as in dermatology, and, mainly in cardiovascular diseases, essentially atheroarteriosclerosis (Robert *et al*., 1995, for review). Over the last decades the importance of ECM components in the malignant process started also to attract increasing attention. With the rapid increase of the number of ECM components, the complexity of this interactive

pattern between ECM and cells, the scope of cell–matrix interactions increased also considerably. Another complicating fact emerged with the identification of cell membrane receptors mediating the interactions of cells with ECM macromolecules. This problem will be discussed in the next section because of its potential role in the malignant process. But let us first consider the role of aging.

III. AGE-DEPENDENT CHANGES OF TISSUES

Tissues are made of cells and ECM in varying proportions. The nature of differentiated cells as well as the macromolecular composition of the ECM vary from one tissue to the next and change progressively with age. For cell aging the *in vitro* model, standardized by the work of L. Hayflick (Macieira-Coelho, 1988, for review), progressively prevailed as compared to more difficult *in vivo* procedures. The extrapolation of *in vitro* results to the *in vivo* situation is however more difficult than supposed originally. This came to the forefront of discussions since the demonstration of telomere shortening during serial culture of fibroblasts and its interpretation as the "molecular clock" limiting the "life" of proliferating cells by telomere shortening (Allsop *et al.*, 1992). It became however rapidly clear that this view is an oversimplification, the "real" situation is more complex (Macieira-Coelho, 2000), without even mentioning the *in vivo* complication where, at least, some postmitotic cells remain biologically viable for the whole human life cycle (a large part of neurons) and their eventual decay appears to be of a different nature, attributed at least partially to free radical damage (Ceballos-Picot, 1997; Emerit and Chance, 1992, for review).

The situation is less complicated for ECM components; they could be and were studied *ex vivo* according to chronological aging (Robert, 1994, for review). Among the first seminal observations, one has to cite the contribution of Fritz Verzar who demonstrated from the 1950s the age-dependent exponentially increasing cross-linking of collagen fibers (Verzar, 1964). Attributed essentially to the Maillard reaction, this process, nonenzymatic glycosylation of proteins and nucleotide bases, became progressively recognized as an important process of tissue aging (Baynes *et al.*, 2005; Robert, 2006, for review). We could show that advanced glycation end products (AGEs) can produce cell death by a receptor-mediated process (Péterszegi *et al.*, 2006). It could be shown that AGEs might well be involved in the angiogenic process through phosphatidylethanolamine glycation (Nakagawa *et al.*, 2005). Some AGEs exhibit genotoxic activity (Schupp *et al.*, 2005) as well as proinflammatory potential (Uribarri *et al.*, 2005), indications that the Maillard reaction might well play a role in the malignant

process also. A further confirmation of the potential role of AGE-products in carcinogenesis is the demonstration of the presence of such compounds in human tumor tissues by a team in the Netherlands (Van Heijst *et al.*, 2005). Using specific antibodies, they detected at least two of such Maillard products, *N*-ε-carboxymethyllysine (CML) and argpyrimidine. These authors attribute the accumulation of AGEs in tumor tissues to the possible prevalence of the glycolytic pathway over the mitochondrial oxidative pathway of glucose utilization: an old theory (the Warburg effect) which was recently implicated again in the tumorigenic process and shown to be under the control of p53 (Matoba *et al.*, 2006). The prevalence of glycolysis together with increased glucose uptake is claimed to accelerate the Maillard reaction in tumor tissues. No need to say that the presence of two of the well-known AGEs in human tumor tissues represents a strong incentive to look for other related compounds, among them heterocyclic, nonnatural molecules. One important source of such AGEs is the consumption of food, some of them, as bread crust or french fries rich in Maillard products. Their penetration in the blood circulation and accumulation in tissues was convincingly demonstrated (Uribarri *et al.*, 2005). One important aspect of these findings is their postgenetic (epigenetic, posttranslational) character (Robert and Labat-Robert, 2000). We shall come back later to these considerations.

Nonenzymatic glycosylation represents however only one aspect of the age-changes of connective tissues of potential importance for the malignant process, as well as for aging. Another discovered similarity between the aging process and cancer is the frequent mutational inactivation of the WRN gene in many cancer cases (12 different types of tumors screened in more than 600 individuals) as in the Werner syndrome, characterized by premature aging and higher cancer prevalence. This gene, coding for a helicase, is essential among others for DNA repair, silenced in tumors by methylation of its active site (Agrelo *et al.*, 2006).

Among the important mechanisms of the age-dependent modifications of the structure and composition of ECM with age are the progressive modulation of their relative rates of biosynthesis and the slowdown of their turnover. The biosynthesis of some ECM components is progressively upregulated and others downregulated. Fibronectin and collagen type III belong to the first category (Labat-Robert *et al.*, 1992) and some proteoglycans and glycosaminoglycans to the second (Robert and Moczar, 1982). Progressive cross-linking of collagen-rich tendons, skin, and other tissues as described by Verzar is a major factor for the slowdown or even the arrest of the removal of "old" collagen, essentially of type I and its replacement by freshly synthesized molecules. In some tissues, as the cornea, collagen turnover is relatively rapid (Kern *et al.*, 1991) and measurable in skin or aorta explant cultures also. These modifications affect the age-dependent

modulation of cell phenotype observed for mesenchymal cells most actively involved in matrix biosynthesis such as fibroblasts and vascular smooth muscle cells. The slowdown of the turnover of macromolecules has several important consequences, among them their postsynthetic modifications not observed for "young" tissues and involving several different mechanisms besides the one described by Verzar are mentioned above. This fact was revealed by the seminal observation of the accumulation of inactive enzymes in tissues by the Gershons at the Haifa Technion (Gershon and Gershon, 1970). Confirmed and extended by a number of laboratories, this process was first attributed to increasing "error rate" in their biosynthesis with age [the celebrated "error Catastrophe Theory" of aging by Leslie Orgel (1963)]. No such "errors" could be detected, at least at the level of amino acid incorporation in proteins, the less attractive explanation progressively prevailed with the demonstration of postsynthetic modifications of macromolecules as for instance polyadenylation (Adelman and Roth, 1983), carboxylation, and other oxidative modifications by reactive oxygen species (ROS), besides their nonenzymatic glycosylation. Such modifications (as cross-linking by Maillard products) further decrease or even abolish the degradation of modified macromolecules arresting progressively their renewal. Some of these nonnatural molecules as for instance the AGEs were shown to exhibit harmful effects, acting directly on cells or mediated by receptors (such as RAGE for instance, Péterszegi *et al*., 2006) with mutagenic properties. These modifications of cell and tissue structure do certainly play an important role in the age-dependent modifications of cell–matrix interactions and might well play a role in the malignant process which remains to be explored.

IV. CELL–MATRIX INTERACTIONS

An important step was accomplished toward the understanding of tissue structure and function with the demonstration of cell membrane receptors mediating cell–matrix interactions. Among these receptors, integrins concentrated most attention over the last decades followed by several others as for instance the elastin-laminin receptor(s) (ELR) (Robert, 1999, 2005, for review). We shall shortly discuss some aspects of the role of integrins because of several more or less recent reviews discussing exclusively these receptors (Defilippi *et al*., 1997; Eble and Kühn, 1997) and shall then discuss the role of ELRs because of their age-dependent modifications studied more intensely than those concerning integrins. Although about 5000 references appear on the computer screen only for the role of integrins in cancer, very little was done on the age-dependent modifications of integrin

expression and function. The demonstrated roles of integrins go well beyond the mediation of cell–matrix interactions as related to the malignant process. Therefore, we shall limit this chapter to some aspects of integrin function only. Integrins were first considered as essentially involved in cell–matrix adhesions. This can be considered as a static role although of potential importance for the adhesion of cells to ECM. Progressively, it could be demonstrated that integrins act not only as static adhesion molecules but as receptors capable of signal transduction from matrix components to the cell interior as well as from the cell to the ECM (outside-in or inside-out signaling) (Defilippi *et al*., 1997; Eble and Kühn, 1997, for reviews). This important function, first attributed mainly to fibronectin, became more complex by the recognition of integrins as more or less obligate mediators of cell–matrix interactions. These observations definitively rendered obsolete the ancien dual conception of tissues as made of "living" cells and "inert" matrix. A further argument to this concept came from the observations that anchoring of normal cells to the surrounding matrix is a prerequisite for their survival and for entering the cell cycle to proliferate. This important function, first attributed mainly to fibronectin, became more complex by the recognition of integrins as mediators also of cell–fibronectin interactions. When this process, cell–matrix adhesion, is inhibited, normal cells cannot survive and proliferate. They die mostly by apoptosis or sometimes necrosis. Fibronectin was first shown to play a crucial role in this respect. One simple experiment to demonstrate this fact was carried out in our laboratory by Péterszegi *et al*. (2002) by adding increasing concentrations of ascorbic acid to fibroblast cultures. At low concentrations, up to about 0.5-mM ascorbic acid stimulated cell proliferation. Above this critical concentration, proliferation, as measured by ^{3}H-thymidine incorporation, suddenly decreased and cells became detached and died as shown by the uptake of the vital dye, Trypan blue, excluded by living cells. The cell membrane was damaged as shown by electron microscopy. It could be shown that ascorbic acid above a limiting concentration inhibited fibronectin synthesis in a dose-dependent way, as shown by immunostaining and quantitative determination of fibronectin. Among the early characteristics of malignant "transformed" cells, anchorage-independent proliferation was one of the general characteristics. Seeded in soft gel transformed cells survive. An extreme demonstration of this capacity of malignant cells was the selection for survival and proliferation of malignant cells (Ehrlich ascites tumor cells) in ascitis fluid in mouse peritoneum by the Klein and Klein (1955). Among nontransformed cells only leukocytes are able to survive in similar conditions, without anchoring in the ECM. Among early studies concerning the role of fibronectin in mediating cell–matrix interaction was the observation of its progressive loss from the surface of malignant cells also in solid human tumors (Labat-Robert *et al*., 1981b). Immunostaining

for fibronectin could distinguish between benign mammary tumors and incipient malignancy. Benign adenomas kept the continuous fibronectin staining all around the epithelial cells in mammary glands. Immunostaining for fibronectin showed discontinuity followed by disappearance even at very early stages in malignant mammary tumors. Several different mechanisms can underlie this progressive "disappearance" of cell membrane-anchored fibronectin. One of them is the inhibition of fibronectin biosynthesis, as shown above by using increasing concentrations of ascorbic acid. Another possibility is the decrease or absence of integrins mediating the adhesion of fibronectin to the cell membrane, essentially integrins $\alpha5\beta1$ and $\alpha4\beta1$. A third explanation of the loss of cell membrane-localized fibronectin is its proteolytic degradation. Fibronectin was shown to be particularly sensitive to proteolytic attack, possibly because of its subunit structure made of compact peptides separated by short sequences easily accessible to proteases. We shall come back to these observations in the next section. The crucial role of fibronectin and corresponding integrins in the mediation of cell–matrix interaction as well as the loss of fibronectin from tumor cell membranes could be extended to a number of solid human tumors (Labat-Robert *et al.*, 1981b). These observations, completed by the demonstration of the two-way communication between cells and the surrounding ECM by integrins, represent a coherent demonstration of adhesion and communication between cells and the surrounding matrix, an important process of information transmission which appears seriously impaired during the malignant process. Some other functions of integrins should still be mentioned as related to the topic of this chapter: the deposition in culture of laminin-1, -5, and -10 and the attachment of tumor cells appear to be mediated by $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins. In a model of invasion using penetration through Matrigel, expression of $\alpha6\beta4$ integrin increased migration. The role of this integrin shifts apparently during tumor progression from its contact-stabilization role in hemidesmosomes to promotion of migration (Mercurio and Rabinovitz, 2001). Qualitative and quantitative differences in laminin and integrin expression were reported in some specific tumors. Integrin $\alpha7\beta1$ is absent in normal melanocytes, but strongly expressed in malignant melanoma cells. The expression of $\alpha3\beta1$ integrin was claimed to be associated with tumor progression. Laminin-1 is specifically involved in the polarity of epithelial cells. This function cannot be fulfilled by other laminin isoforms such as laminin-5 or -10/11. This function is disturbed in transformed cells. Overexpression of laminin $\alpha1$ chain in colon cancer is accompanied by an increase in tumor growth. This situation is quite different in glioblastoma cells which synthesize laminin-8 that promotes the migration of these cells, using the $\alpha3$ and $\alpha6$ integrins (Fujiwara *et al.*, 2001). Human glial tumors were shown to overexpress $\alpha4$ laminin chains (Ljubimova *et al.*, 2001). The tumor suppressor DAPK inhibits cell motility

by interfering with the integrin-mediated polarity pathway (Kuo *et al.*, 2006). This death-associated protein kinase inhibits random cell migration by interfering with the directional-motility-regulating mechanism based on cell polarization. This is achieved by the talin-head domain association with integrin, inhibiting thereby the integrin-cdc42 polarity pathway. This mechanism, inhibition of malignant cell migration, appears to operate independently from the apoptosis-inducing pathway as shown with a highly invasive adenocarcinoma cell line. This is an original mechanism of tumor suppression. As a matter of fact integrin complexes were shown to be actively involved in actin polymerization, a necessary step for cell shape change toward the migratory phenotype (Butler *et al.*, 2006). Another observation was made by Weaver *et al.* (1997) showing that integrin β1-blocking antibodies could mediate the reversion of the malignant phenotype of human breast cells in a three-dimensional culture. On the contrary, nonmalignant cells treated with antibodies to α6 or β4 integrins continue to proliferate forming irregular, disorganized colonies reminding those of malignant cells. These observations confirmed the important role of $\alpha 6\beta 4$ integrins in regulating cell polarity, cell patterning, and cell–cell interactions. The authors concluded that ECM and its receptors determine epithelial cell phenotype and, at least in their model the tissue phenotype also, dominating thus above the cell genotype. Several important observations were published on the role of the platelet-integrin, αIIbβ3 in tumor growth (Döme *et al.*, 2005; Raso *et al.*, 2005; Trikha *et al.*, 2002a,b). Human melanoma biopsies showed an increase of expression of this integrin with increasing tumor thickness, indicative of metastatic potential. Transfection of this integrin in αvβ3-expressing human melanoma cells conferred to these cells an increased ability to adhere, spread, and migrate on fibrinogen but not on vitronectin. Implanted in SCID-mice these cells developed a fourfold larger tumor with a reduced rate of apoptosis. Parallel expression of these two integrins, αIIbβ3 and αvβ3 in human melanoma cells upregulated the FGF expression accompanied by an increased angiogenesis. Blocking these (αIIbβ3 and αvβ3) integrins with specific antibodies resulted in decreased angiogenesis and tumor growth. Such antibodies (ligand mimetic) to αIIbβ3 inhibited tyrosine signaling, proliferation, and lung colonization of melanoma cells, as shown by the team of J. Timar in Budapest. Some more examples of integrin signaling will be mentioned in Section X.

V. ROLE OF PROTEOLYTIC ENZYMES AND ROS

Among the (at least) three possibilities underlying interruption of cell–matrix interactions, especially as related to aging, involving fibronectin, and integrin expression and/or matrix degradation, this last mechanism (matrix

degradation) was favored by several authors. The role of proteolytic enzymes was proposed since years as an explanation of tumor progression, metastasis formation, and modified cell–cell interactions (for a recent review, see Wallach and Hornebeck, 2005). Instead of reviewing this huge literature, we shall limit this review to a few special cases. One concerns our early observations showing the age- and passage-dependent upregulation of proteolytic activity in fibroblasts and smooth muscle cells (Robert, 2002; Robert *et al*., 1995, for review). This process, accentuated by the activation of the ELR, is discussed in the next section and might well play an important role in tumor progression. In a number of such specific cases, normal cells were shown to limit the growth of malignant cells, capable even of killing transformed cells by normal cells (Rubin, 2003, for a review). Although the role of ECM components was not systematically investigated in such cell–cell interactions, their role cannot be excluded. The normal, nontransformed cells used in these experiments were the fibroblasts known to start the biosynthesis of ECM components as soon as seeded in the culture dish, among them several collagen types, fibronectin, and proteoglycans as well as hyaluronan. Even at low concentrations, such macromolecules could play an important role in interactions between normal and malignant cells. The expression of proteolytic enzymes, essentially of the Matrix Metallo Proteinase (MMP) family was shown to be influenced by ECM components. Several proteolytic degradation products of ECM macromolecules were shown to play a crucial role in modifications of cell–matrix interactions. We should however mention another possibility insufficiently explored for the role of cell–cell interactions. The example we shall shortly cite was described by the team of Bauer in Freiburg, Germany, and demonstrates the role of ROS and especially of peroxynitrite ($ONOO^-$) (Heigold *et al*., 2002). Nitric oxide ($NO^\bullet$) was shown to mediate apoptosis in oncogene-transformed fibroblasts, normal cells being unaffected. This selective sensitivity of transformed cells to $NO^\bullet$ appears to be mediated by extracellular superoxide anion generation ($O_2^{-\bullet}$) by transformed cells only as shown by the inhibition of apoptosis by blocking NADPH oxidase or by scavenging the $O_2^{-\bullet}$ and also by rapid decomposition of $ONOO^-$. Extracellular $O_2^{-\bullet}$ produced by transformed cells combines with $NO^\bullet$ liberated by nontransformed cells to yield $ONOO^-$, this highly toxic anion inducing apoptosis by activating the mitochondrial permeability transition pores. The regulation of intracellular glutathione level plays in this respect a crucial role. In the above-mentioned necrotic death of normal fibroblasts in presence of higher concentrations of ascorbic acid, the role of ROS production could also be substantiated by the protection of cells when SOD and/or catalase were added to the culture medium (Péterszegi *et al*., 2002). But, by far, the most toxic ROS is $ONOO^-$ capable of killing cells in the millisecond range, mostly by apoptosis.

Although these experiments point to the role of ROS as the active mediators of cell death, among other situation when nontransformed cells are in contact with transformed cells, this does not exclude the role (simultaneous or alternative) of proteolytic enzymes. Some of these enzymes and especially the serine elastase of leukocytes were shown to induce apoptosis, as will be shown later when we will discuss the role of the ELR. The relationship between the mediation of cell anchorage to the ECM and ROS production and scavenging deserves further attention in the realms of tumor biology and especially as related to aging. As mentioned above, in our experiments on ascorbate-induced cell detachment and cell death, increased ROS production (and/or insufficient scavenging) could be demonstrated as the origin of cell death. However, pericellular regulation of the activation of MMP-type proteolytic enzymes acting on ECM components was also shown to play important roles in tumor progression (Wallach and Hornebeck, 2005). Limitation of the mobility of normal cells by ECM components such as basement membranes and escape from such inhibition by transformed cells across ECM barriers during metastasis formation is mostly protease-mediated. The most active proteases capable of attacking most matrix components are MMP-2 and MMP-9, expressed among other cells by fibroblasts (Isnard *et al.*, 2002). Its activation from an inactive pro-form to the proteolytically active form is a cell membrane-localized process involving interaction of its pro-form with TIMP-2 and with its activating enzyme, MT1-MMP as well as with $\alpha V\beta 3$ integrin (Hornebeck *et al.*, 2002). MMP-2 activation was repeatedly implicated in the metastatic potential of cancer cells. It is also involved in angiogenesis, another important factor in tumor progression. ECM components were shown to be directly involved in the regulation of MMP-2 expression. A noncollagenous domain of the $\alpha 3$ chain of collagen type IV binds $\alpha V\beta 3$ integrin and thus inhibits MT1-MMP-dependent activation of pro-MMP-2 (Hornebeck *et al.*, 2005). On the contrary, some ECM components can stimulate MT1-MMP increasing thereby pro-MMP activation. This is the case for some elastin-derived peptides as will be recalled later.

The other mechanisms regulating the invasive behavior of cancer cells by cell–matrix interactions were reviewed by Macieira-Coelho (2002). Modulation of cell attachment through the modification of the structure, conformation, and charge of ECM components can modulate migration and metastasis formation of transformed cells. The survival of mice injected with wild-type L-1210 leukemia cells grown in suspension is strongly limited. Injection of 10^5 cells per mouse limits to 30 days survival. However, when the same number of cells adapted to grow in monolayer is injected the mice survive. The conformation of cell membrane-localized macromolecules can apparently be modulated by the charge distribution of the substrate on to which the cells are attached. This on its turn appears to modulate gene

expression and cell migration. Such close cell–matrix interactions are crucially modified by local activation of cell membrane-localized proteolytic enzymes. The more specific role of some MMPs and especially of stromelysin (MMP-3) was abundantly discussed in recent literature and will not be reviewed here (Hornebeck and Maquart, 2003).

VI. THE ELASTIN-LAMININ RECEPTOR

The search for a receptor recognizing elastin started in our laboratory from the observation concerning the involvement of this fibrous matrix component in vascular and pulmonary functions, their age-dependent decline and accompanying pathologies, but also during the malignant process, essentially of the human mammary gland (Lapis and Timar, 2002; Timar *et al*., 1995). At the beginning of the 1980s, the role of cell–matrix interactions and their mediation by fibronectin and integrins started to be intensively studied and concerned most ECM components, except elastin, because it was believed to be "inert." We could demonstrate the adhesion of fibroblasts and vascular smooth cells to elastic fibers by video-filming cell cultures (fibroblasts and vascular smooth muscle cells) overlaid by micronized and ^{3}H-labeled elastin fibrils (Hornebeck *et al*., 1986). This process appeared to be inducible (accelerated and not inhibited) by elastin-derived peptides, acting on this receptor. The activation of this receptor was shown to induce a Ca-signal and to stimulate the biosynthesis and membrane localization of an adhesive protein, designated elastonectin. A number of the cellular processes, triggered by elastin peptides, were shown to be mediated by this receptor as the regulation of ion fluxes (Jacob *et al*., 1987), release of free radicals and proteolytic enzymes (Fülöp *et al*., 1986), chemotactic movements to an elastin peptide gradient (Mecham and Hinek, 1996), as well as cell proliferation (Ghuysen-Itard *et al*., 1992). These observations were later extended to malignant cells also (Lapis and Timar, 2002; Timar *et al*., 1995). The nature and function of a receptor "recognizing" elastin sequences were intensively studied over the following decade in our laboratory (Robert, 1999, 2005, for review). As far as the present topic is concerned, we shall concentrate here only on two aspects of this receptor, its implication in the malignant process and its age-related modifications. The ELR appears to be composed of three subunits, one of them, an approximately 65- to 67-kDa subunit which "recognizes" elastin sequences. As this protein (designated also as the elastin-binding protein or EBP) is only loosely bound to the cell membrane, its association with the two other subunits is supposed to mediate the transmission of "messages" triggered by the reaction of this subunit with elastin-derived peptides. This same subunit (EBP) carries also a

second binding site specific for oligosaccharides or polysaccharides of the galactose conformation. The occupation of this site lowers the affinity of the elastin peptides recognizing site, therefore galactose-type oligo- or polysaccharides can be considered as antagonists of this receptor. This was shown to be the case for melibiose also (Péterszegi *et al*., 1999). A similar (or identical?) 67-kDa protein was shown to react also with laminin, justifying the designation of this receptor as ELR (Mecham and Hinek, 1996, for review). Its transmission pathway, comprising a G_i-protein, the mobilization of the inositol phosphate pathway, activation of PKC was first elucidated in leukocytes (Varga *et al*., 1989) and later in vascular endothelial cells (Faury *et al*., 1998). These two pathways are similar but not identical, suggesting a phenotypic variability of ELR coupling. Among the functions mediated by ELR, several concern the malignant process also (Lapis and Timar, 2002; Timar *et al*., 1991, 1995). Such functions recognized in several malignant cell types are migration, proliferation, release of lytic enzymes, and free radicals. Among the proteases released by triggering the ELR with elastin peptides are pro-MMP-1 and -3 and MMP-2, potential mediators of tumor cell detachment from the surrounding ECM and matrix remodeling. Extensive elastin degradation and peptide production were shown at the invasive front of melanoma (Hornebeck *et al*., 2005; Ntayi *et al*., 2004). Elastin peptides were also shown to upregulate IL-1β via NF-κB activation, also mediated by ELR (Debret *et al*., 2006). Increased $NO^{\bullet}$ production, demonstrated in endothelial cells in presence of elastin peptides (Faury *et al*., 1995), can be involved in the generation of the highly toxic $ONOO^{-}$ mentioned in the previous section, inducing apoptosis (Heigold *et al*., 2002). The mediation of cell death by sustained activation of ELR was further substantiated in our laboratory (Péterszegi and Robert, 1998) by showing that the addition of increasing concentrations of elastin peptides to human lymphocyte cultures induced the expression of ELR in several lymphocyte subtypes such as memory and helper cells (Péterszegi *et al*., 1997). Further increasing the concentrations of elastin peptides added to these cultures first increased cell proliferation, but also the secretion of a potent serine protease indistinguishable from PMN elastase. Increasing further elastin peptide concentration led to cell death, first by necrosis and at higher elastin peptide concentration by apoptosis. As the circulating elastin peptides can reach comparable concentrations in human blood serum (Bizbiz *et al*., 1997), the above observation might well be of pathophysiological importance.

The most surprising observation we could make concerning this receptor is its "uncoupling" with age. Using several different tests, such as free radical release, $NO^{\bullet}$ production (measured by vasodilation), and regulation of cellular cholesterol synthesis, it occured that in cells (monocytes, vascular endothelial cells) obtained from "old" donors (>65 years) ELR behaved

differently from those obtained from "young" donors (<45 years). The G_i-protein, mediating cell acivation triggered by elastin peptides, could no more be inhibited by pertussis toxin as measured by $O^{-}_{2}\cdot$ release which was shown to increase in "old" cells. The age-dependent uncoupling of ELR from the $NO^{\bullet}$-generating pathway produced a decrease of $NO^{\bullet}$ release by endothelial cells in presence of elastin peptides, decreasing progressively the vasorelaxing effect of elastin peptides with age (Faury *et al*., 1997). In "young" cells (monocytes), ^{14}C-acetate incorporation in cholesterol could be inhibited in a dose-dependent fashion by elastin peptides. This effect was no more present in "old" cells (Robert, 1999, for review). All these processes, taken together, showed an age-dependent loss of regulatory functions of ELR by uncoupling it from its intracellular transmission pathway. Curiously, most physiologically "positive" functions decreased with age such as vasodilation, inhibition of $O^{-}_{2}\cdot$ release and of cholesterol synthesis. Only the harmful effects remained or even increased as ROS release and protease production. This age-dependent change in ELR function may well have a role in the modifications of ECM, favoring the malignant process. The underlying mechanism affecting the modifications of most if not all these cellular functions is the progressive uncoupling of ELR activation by elastin peptides from its transmisssion pathway which starts with the increase of intracellular free calcium in presence of elastin peptides. In "young" cells, addition of elastin peptides triggers a typical Ca-transient, a sudden increase followed by its rapid normalization. In mononuclear cells from old and even more old-pathological persons, increase was much attenuated and, most important, the return to normal values strongly delayed or even absent (Ghuysen-Itard *et al*., 1993). Such "old" mononuclear cells appear morphologically normal (important for the cell count) but functionally in a premortal state. The encounter of such cells with malignant cells or bacteria, instead of triggering an aggressive defense reaction, will produce cell death. This process, demonstrated also for another agonist of mononuclear cells, formylmethionylleucylphenylalanine (FMLP) a bacterial peptide, might play a role in the age-dependent decline of cellular surveillance processes, affecting also defense against transformed cells (Fülöp and Larbi, 2002; Ghuysen-Itard *et al*., 1993).

VII. MODIFICATIONS OF ECM-PATTERNING BY THE NEOPLASTIC PROCESS

The progressive modifications of tumor cell–matrix interactions formed the most obvious arguments for pathologists in the diagnostic process, distinguishing a normal tissue from a malignant tissue (Van den Hoof, 1988).

This change of pattern of cell–matrix relations is necessarily followed by the recognition of cellular anomalies such as cell shape and modified nuclear and chromatin patterns. Since the early years of microscopic diagnostic of this tissue-pattern change, it was designated as the "stromal reaction" (Hornebeck and Birembaut, 2004, for historical data). In this context, "stroma" means "abnormal" connective tissue with recognizable collagen-staining fibrous bundles and fibroblast-like cells but also capillaries of crucial importance for tumor progression. The prevailing concept was that the "active" role for the malignant process should be ascribed only to the transformed cells, the modifications of the surrounding "stroma" being a secondary "passive" process. This one-sided consideration of the malignant process survives in the still prevailing dogma that one transformed cell starts and ends the development and progression of tumors. This view was, however, seriously challenged over the last decades by a number of observations, only some of them will be mentioned. Among the early observations concerning the abnormal phenotype of "stromal" fibroblasts was the recognition of their "myofibroblast" nature (Hinz and Gabbiani, 2003; Sappino *et al.*, 1990). But even fibroblasts far removed from the tumor site were shown to exhibit abnormal characteristics as continued proliferation in successive subcultures, irrespective of the "Hayflick limit" as shown by the team of Macieira-Coelho (2005, for review). This crucial observation which might be attributed to the derepression of telomerase—observed in most malignant cells—in the skin fibroblasts of cancer patients, of apparently normal phenotype. Another observation, also by Macieira-Coelho (1988, for review), concerns the progressive aneuploidy of serially cultured normal fibroblasts. Other age-related changes of chromatin structure, described by Macieira-Coelho and Puvion-Dutilleul (1989), concern the progressive decrease of density of the 30-nm chromatin fibers with cell population life span. During the last phase of *in vitro* aging, this spacing increased abruptly. The age (passage)-dependent decline of density and increased spacing of the 30-nm chromatin fibers was accompanied by the decline of entrance in S phase of the fibroblasts. These age-dependent modifications of chromatin were amplified and accelerated in cells from Werner syndrome patients, a pathology mimicking several aspects of accelerated aging.

Such modifications of chromatin structure which might well affect the regulation of gene expression were confirmed. *In vitro* "aging" reveals thus some aspects of chromosomal changes observed also in malignant cells. These changes of chromatin structure might also be related to the age-dependent increase of apparently stochastic variation of gene expression (Bahar *et al.*, 2006).

These and similar observations strengthened a new concept attributing to cells of the "stromal reaction" a more active role in the malignant process than believed originally with a possible age-dependent amplification.

A convincing demonstration of the crucial importance of stromal fibroblasts in the malignant transformation was published by Maffini *et al.* (2004). These authors showed that the carcinogenic effect of *N*-nitrosomethyl urea is directed to stromal fibroblasts and not to the epithelial cells. Isolated cells of both types exposed to the carcinogen and replaced in the mammary tissue devoid of one or the other cell type did present the signs of the malignant process only when the fibroblasts were treated with the carcinogen, and not the mammary epithelial cells (Sonnenschein and Soto, 1999, for comments). This important aspect of modified behavior of fibroblasts during the malignant process was extensively reviewed by Brouty-Boyé (2005). Several other authors insisted on the role of a three-dimensional matrix on cell behavior (Grinnell, 2003), also on the role of some serum factors (amyloidP) on differentiation (Pilling *et al.*, 2003) or on the high rate of diversificational reversal of transformed fibroblasts (Rubin *et al.*, 1992). The modulation of fibroblast phenotype in the stromal reaction appears to play an important role in the malignant process (Schurch *et al.*, 1981). One often neglected aspect of the tumor-induced modulation of fibroblast phenotype is the mechanoregulatory factor attributed to the prevailing myofibroblasts (Tomasek *et al.*, 2002). Mechanical forces were shown to modulate gene expression and might well play a role in tumor progression also. The stromal reaction was claimed also to be involved in the conversion of normal epithelial cells to the malignant phenotype (Ronnov-Jessen *et al.*, 1996). As cell–matrix interactions are of crucial importance in expressing and maintaining normal cell phenotype (Cunha *et al.*, 1985), it could be anticipated that pathological (as well as age-dependent) modifications of ECM are crucial factors in cell phenotype conversion during the malignant process. A number of matrix components were shown to be involved in signaling during this process. A recent, relevant example was published by Teodoro *et al.* (2006) showing that the p53-mediated inhibition of angiogenesis is the result of the activation of the prolyl-4-hydroxylase of collagen type II [α(II)PH]. This results in the extracellular release of antiangiogenic fragments of collagen types IV and VIII. Conditioned media from cells expressing ectopically either p53 or α(II)PH selectively inhibited the growth of primary human endothelial cells. The intracellular expression or addition to the media of α(II)PH significantly inhibited tumor growth in mice. These experiments further confirm the importance of antiangiogenic collagen fragments to limit tumor growth. They also document a new aspect of p53 action through coupling with antiangiogenic collagen fragments synthesis. Some other examples will be mentioned later, concerning especially fibronectin, elastin, proteoglycans, and their receptors.

We should however mention a more recent development of these cell–cell interactions between transformed cells and stromal fibroblasts.

These observations concern the apparently "active" action of tumor cells on stromal cells, exerting a sort of "selective pressure" by inducing mutations. Such mutations can suppress the expression by stromal cells of tumor supressor genes as p53. Using a mouse model for prostate cancer, Hill *et al.* (2005) showed that after the introduction of a fragment of the SV40 large T-antigen it was expressed selectively in the epithelial cells, inactivating the tumor suppressor retinoblastoma (pRB) and related p107 and p130 proteins. When crossed with p53 null mice, the loss of this tumor supressor mainly influenced tumor stroma more than the epithelium. The p53-deficient animals developed tumors with an extensive stromal reaction, earlier than the wild-type, p53-carrying heterozygous mice. It appears that the loss of p53 expression is correlated with the rapidity and extent of the stromal reaction. This proliferative microenvironment was apparently selected for as a result of these cell–cell interactions and might well play a crucial role in tumor progression, apparently as a result of the tumor-induced loss of tumor suppressor gene expression by stromal cells.

VIII. ROLE OF ECM MACROMOLECULES

As far as the ECM macromolecules of normal tissues as well as the tumor stroma are concerned, recent data point to their active role in signaling processes during normal cell–matrix and tumor–stroma interactions. Although it is assumed that fibrous collagen types I, III, and V are the main components of tumor-surrounding matrix, a number of other matrix macromolecules are also present. Among such ECM components, fibronectin and proteoglycans (as well as hyaluronan, glycosaminoglycans) were the most intensively studied. A few examples will suffice to show their importance. Among the early reports are those of Mundy *et al.* (1981) showing that collagen types I and III and derived peptides are chemotactic to tumor cells (Walker, 256 rat carcinosarcoma cells derived from a breast tissue as well as HeLa cells). Tripeptides from these collagens were shown to produce unidirectional cell migration. As most tissues as well as tumor stroma are rich in collagens and in collagenolytic enzymes, this observation might well be of general importance in malignant cell migration and metastasis formation.

Contemporary observations concern the progressive disappearance of fibronectin around the transformed cells, mentioned in a previous section of this chapter, its strong expression in tumor-surrounding stroma was noticed using immunofluorescence for the study of a number of human solid tumors (Labat-Robert *et al.*, 1981b). As proteolytic enzymes are also increasingly expressed, an intensive fragmentation of fibronectin can take

place with consequences to be discussed later. Another interesting aspect of the involvement of fibronectin in the malignant process is the expression of a truncated isoform of fibronectin, characterized by Schor and Schor (2001) and by Schor *et al.* (2003) as a migration-stimulating factor (MSF). This 70-kDa isoform of fibronectin is apparently obtained by a read-through mechanism involving a normally nonexpressed intron sequence. The mitogenic activity of this oncofetal form of fibronectin, produced by stromal fibroblasts, is claimed to play an important role in tumor progression. Fibronectin is also required for $\alpha v\beta 6$ integrin-mediated activation of latent TGF-β complexes containing LTBP-1, involved in the activation of the biosynthesis of several ECM components (Fontana *et al.*, 2005). Besides fibronectin, the role of several of the large number of proteoglycan family members was also intensively studied as far as their role in the malignant process is concerned (Timar *et al.*, 2002). Vlodavsky *et al.* (1980) were among the first to draw attention to the importance of ECM and especially of proteoglycans in the malignant process. More recently, they studied the specific role of heparan sulfate proteoglycan and degrading enzyme heparanase (Vlodavsky and Friedman, 2001) and especially in epithelial–mesenchymal interactions. Hyaluronan overproduced in several tumors as well as some other glycosaminoglycans were shown in our laboratory to upregulate MMP-type protease expression (Bernard *et al.*, 1994; Isnard *et al.*, 2001). More recently tenascin, another ECM component of the structural glycoprotein family, was studied in human breast cancers (Tökés *et al.*, 1999a,b). Its expression coincides with tumor vascularization as shown by its colocalization with or around CD-31-positive cells, a marker of stromal angiogenesis. Tenascin is involved in a number of ECM functions in normal and tumor tissues (Shrestha and Mori, 1997).

As another example, we shall mention the role of elastin in breast tumors. The scirrhous type of breast cancers were shown to be rich in elastic fibers (Kadar *et al.*, 2002, for review). A great deal of attention was devoted to this elastogenic reaction, considered as a potential predictive marker of the evolution of the disease. Since the generalization of early screening for breast tumor however, nearly no such advanced tumors were anymore reported. As elastogenesis is restricted to a few tissues such as major blood vessels, lung, and skin (although most other tissues also contain small amounts of elastic fibers), the important overproduction of elastin in breast tissue could be considered as a special case of the stromal reaction. Its pathological significance was debated with no final consensus. Some pathologists considered breast elastosis as a sign of more differentiated tumor cells, with a more favorable prognosis. But this opinion was not universally accepted (Kadar *et al.*, 2002). We tried to understand the mechanism of this tissue elastogenic reaction using tumor extracts of different origins and rabbit aorta explant cultures as the target organ. Incorporation of

^{3}H-proline and ^{14}C-lysine in ECM macromolecules synthesized by the smooth muscle cells of the aorta explants was determined in macromolecular fractions in order to identify the action of factors present in tumor extracts on the biosynthesis of individual ECM macromolecules. The use of aorta explant cultures was indicated because of the capacity of vascular smooth muscle cells to synthesize elastin in higher amounts than other cell types as for instance fibroblasts together with most other matrix components. All tumor extracts strongly stimulated collagen biosynthesis as shown by the incorporation of ^{3}H-proline in radioactive peptide-bound hydroxyproline. Incorporation in immune precipitable tropoelastin was however only stimulated by extracts from mammary tumors with strong elastogenic reactions. As this experiment could not be reproduced using MCF-7 cells derived from mammary carcinoma, it had to be assumed that overproduction of elastin in scirrhous type of breast tumors might well be attributable to the secretion of some specific stimulatory factors by tumor cells acting on stromal fibroblasts or myofibroblasts inducing an increased synthesis and cross-linking of tropoelastin yielding mature elastic fibers which could be purified using the harsh 0.1-N NaOH extraction procedure at 100°C preserving only cross-linked elastin. These studies remained unpublished and had to be interrupted after the introduction of early screening program for breast tumors. According to Masters *et al.* (see in Kadar *et al.*, 2002), a positive correlation exists between the presence of oestrogen receptors in tumor cells and the elastotic reaction, accompanied by a more favorable outcome of endocrine treatment. The lymph node metastasis of these tumors showed no signs of elastin deposition, confirming the above-cited view that increased elastin production is the result of specific interactions between transformed breast-epithelial cells and stromal fibroblasts. The degree of elastosis more than the receptor status of transformed epithelial cells showed significant positive correlation with endocrine therapy as shown by Mohacsy *et al.* (1985). A discordant finding was reported by Anastassiades *et al.* (1979) between tumor progression and elastosis. These authors claimed a somewhat higher metastatic incidence in tumors with periductal elastosis (66%) than in tumors without elastosis (45%). According to these authors, elastosis could not be considered as of favorable prognostic value. Significant differences in the amino acid composition between breast tumor elastin and aorta elastin (see Table I in Kadar *et al.*, 2002) might be interpreted as a result of the production of splice variants of tropoelastin mRNA in these tumor tissues. Of interest was also the strong correlation between elastin in breast tumors and elastase production determined in breast tumor extracts (Fig. 3 in Kadar *et al.*, 2002).

The other tumor where ECM production was extensively investigated is the EHS sarcoma (Engelbreth-Holm-Swarm) which secretes most constituents of basement membranes as collagen type IV, laminin, and heparan

sulfate proteoglycans. Laminins were originally characterized in this tumor by Timpl, Martin, and colleagues (Ekblom and Timpl, 1996; Patarroyo *et al.*, 2002, for review). Extracts of this tumor are commercialized (Matrigel®) for favoring adhesion of cells in culture.

Another ECM constituent, lysyl oxidase (LOX) was shown recently to be involved in hypoxia-induced metastasis formation (Erler *et al.*, 2006). LOX is of crucial importance in ECM morphogenesis as the enzyme responsible for cross-linking collagen and elastin fibers. Hypoxia is often associated with metastasis formation and poor prognosis. Hypoxic tumor cells overexpress LOX, its expression being regulated by the hypoxia-inducible factor (HIF), expressed often in human breast, head, and neck tumors. Inhibition of LOX in mice decreases metastasis formation of orthotopically grown breast cancers. LOX appears to act through focal adhesion kinase activation influencing cell–matrix interaction. HIF, shown by these authors to be involved in LOX overexpression, is a pleiotropic gene involved in tumor survival and spread (Pouysségur *et al.*, 2006) and might well be a promising target for therapeutical interventions.

IX. EFFECT OF CELL-AGING AND OF MODIFIED CELL–MATRIX INTERACTIONS ON THE MALIGNANT PROCESS

Up to the 1980s, tumor incidence was considered to progress exponentially with age and some authors still adhere to this opinion. This was however based on statistics obtained before the recent increase of life expectancy (Ponten, 1977). Although the incidence of some tumors as for instance prostate cancer continues to increase with age, for most other tumors the situation changed, their maximal occurrence is reached at intermediary ages (age-windows) followed by a decline (Macieira-Coelho, 1986, 2003; Pompei, 2002). One reason for tumor incidence to be still considered as continually increasing with age is the decrease and postponement to later years of cardiovascular death as a result of more efficient therapeutical measures. An increasing number of seniors contract cancers apparently because they escaped earlier cardiovascular death. Aging tissues are less likely to develop a pronounced stromal reaction because of a decrease of cell number in most tissues, a decrease of ECM production, and a more prolonged clinical development of tumors. The autopsies on centenarians carried out by some authors (Haranghy, 1965) showed malignancies unnoticed clinically and apparently not directly responsible for the death of the examined centenarians. The statistics showing a decline of cancer incidence at later ages are based on clinical data. Systematic autopsies, no more

practised in most countries since decades, might change this conviction. Signs of cardiovascular pathologies were however always present and considered as directly responsible for the death of these centenarians (Robert, 2000, for review).

Interactions between senescent cells and malignant cells were also investigated by some authors (see Campisi, 2001, 2005, for review). The most remarkable observations concern the modifications of expression of tumor suppressor genes as a result of such cell–cell interactions. Classified in two broad categories by Kinzler and Vogelstein (1997), the tumor suppressor genes were designated as "caretakers" or "gatekeepers," their essential task being the protection of the genome in two slightly different manners. Caretakers tend to prevent DNA damage and/or supervise its repair. An apparent "side effect" of this activity is to fight against shift of phenotypes to aging and age-related pathologies (Hasty *et al*., 2003). In this sense, these genes act in favor of increased longevity. Gatekeeper genes act essentially by inducing apoptosis in cells shifting to the malignant phenotype or by inducing cellular senescence. Thereby these genes also act in favor of increased longevity. However, as remarked by Campisi (2005) this type of preventive action is not risk-free. The cumulative apoptotic and senescence responses might well produce also deleterious effects contributing to late-life pathologies. The question Campisi tries to answer is about the mechanisms of the age-promoting effects of gatekeeper genes. Both apoptosis and senescence might deplete tissues of potentially renewable cells (stem cells) decreasing the possibility of tissue repair and regeneration. Gatekeeper tumor supressor genes could be considered as an example of evolutionary antagonistic pleiotropy as proposed by Williams (1957). Such genes are considered as favorable early in life and detrimental in aging organisms. Campisi's review considers the causes and consequences of the senescence response which indirectly contributes to age-related pathologies, especially to cancer. Among the arguments in favor of this hypothesis, some deserve to be mentioned. Cell-aging is accompanied by telomere shortening at least in the proliferative cell pool. Telomeres contribute to the maintenance of genome integrity and might be considered in this sense as cancer protective. The withdrawal of senescent cells from the proliferating pool can therefore be considered as protective against cancer. A number of oncogenic stimuli were shown to induce cellular senescence among them oxidative DNA-lesions, similar to those kept responsible for a number of age changes (Emerit and Chance, 1992, for review). The RAS-mitogenic pathway depends extensively on ROS signaling, involved both in the mitogenic effect as well as in the induction of senescence. The importance of the oxidative stress is further exemplified by the finding that mouse cells can proliferate without restriction in oxygen-poor conditions but do not proliferate at normal oxygen tension in contrast to human cells endowed with a much

more developed defense system against ROS-induced stress (Parrinello *et al*., 2003, 2005). Senescence arrest of murine fibroblasts can be reversed by pRB inactivation (Sage *et al*., 2003). Normal cells can enter senescence by overexpressing some oncogenes as those of the RAS-RAF-MEK signaling pathway. This contrasts with the mutational activation of RAS without overexpression favoring transformation but not senescence (Tuveson *et al*., 2004). Most senescence-inducing stimuli converge essentially to two cancer protective gene-expression pathways related either to p53 or pRB. In her review, Campisi (2005) tries to answer the question about the mechanisms concerning the role of these gatekeeper tumor suppressors in the balance between senescence and the malignant process. Among the mechanisms mentioned is the loss of p53 which abrogates cellular senescence, at least in cells expressing p21 but not in those expressing p16. Senescent cells express mostly one or the other of these two genes (Herbig *et al*., 2004). Some strains of cells reaching the state of replicative senescence are either p16 or p21 positive, the p16-positive cells being devoid of foci with markers of DNA damage. This might be one way of coupling loss of the senescent phenotype with increased oncogenic risk through p53 dysfunction. In cells keeping their senescent phenotype even with impaired p53 function, the expression of p16 may be the explanation. p16 expression is considered by some as a possible marker of cell senescence. In favor of this view is the coexpression of p16 with the senescence-marker SA-β gal described by the team of Campisi (2005, for review). Caloric restriction which retards senescence in rodents and some other species (Roth *et al*., 2004, for review) attenuated the expression of p16 in correlation with its transcriptional activator Ets-1 (Krishnamurthy *et al*., 2004). These authors consider Ink 4a/Art also as a marker of senescence. As p16 activates the pRB pathway, this "gatekeeper" like p53 can also act in favor of the senescent phenotype. This gene is a positive regulator of the other tumor supressor, pRB. Recent experiments reported by Pinkston *et al*. (2006) also conclude to the antagonism between cellular changes which lead to senescence and tumor growth. These authors studied a germ line tumor of *Caenorhabditis elegans* associated with mutations of the gld-1 gene. A wide variety of mutations that extend the life span of *C. elegans* confer resistance to these tumors. Several mutations of the daf-e/insulin receptor confer increased life expectancy to these worms, unaffected by gld-1 mutations. The authors attribute this tumor-resistance conferred by the insulin-receptor mutations to a decrease of cell divisions and an increased DAF-16/p53-dependent incidence of apoptosis within the tumors. Other mutations which increased life span by decreasing food intake, calorie restriction, or decreasing respiration correlated with decrease of ROS production did not affect apoptosis but inhibited tumor cell division. Surprisingly, none of the longevity mutations modified the rate of mitosis in normal germ lines. All the above summarized

data emphasize the role of genes and mutations in the balance between senescence and the malignant process. Epigenetic processes were also shown to play an important role in the above-mentioned regulatory processes, conforted also by the clustering of the concerned genes (Zhang *et al.*, 2003, 2005). One conclusion which can be proposed concerning both senescence and oncogenesis is that there are (at least) two distinct pathways leading to senescence, induced by one or the other of the above-mentioned gatekeeping mechanisms, mediated either by p53 or pRB pathway (Beauséjour *et al.*, 2003). The p53 mediation could favor senescence through telomere dysfunction and DNA damage, and the p16/pRB-pathway acts by oncogene activation, chromatin modifications, and stress-inducing processes (Campisi, 2005, for review). Although these hypotheses need further confirmation, they trace the way for further studies at the cellular, genetic, and epigenetic levels for the understanding of mechanisms underlying the senescent state and its relation to the malignant process. The conclusion which can be reached from these and similar studies is that p16 and to some extent ARF are part of the few genetic markers of senescence induction as shown also by the coexpression of p16 and SA-β gal, which accumulates in several tissues also at sites of age-related pathologies characterized by altered cell phenotype and cell proliferation as seen in the malignant process. Senescent cells can also contribute to tissue remodeling by stimulating local inflammation and the stromal reaction by the secretion of proinflammatory cytokines, proteolytic enzymes as some MMPs (MMP-3, for instance) disrupting the differentiated phenotype of epithelial cells as shown in three-dimensional cultures (Parrinello *et al.*, 2005). Senescent stromal cells, fibroblasts might thus contribute to the development of the hyperplastic epithelial lesions as seen in the precancerous states. By creating a propice microenvironment, senescent stromal cells can promote the progression of preneoplastic cells toward the overt malignant phenotype.

X. SIGNALING BY ECM MACROMOLECULES AND THEIR PROTEOLYTIC FRAGMENTS

As mentioned previously, the discovery of cell–matrix interactions draws attention to the fact that ECM macromolecules can and do send signals to the cell interior modifying gene expression and cell behavior. This "outside-in" signaling (Defilipi *et al.*, 1997; Eble and Kühn, 1997, for review) draws attention to the nature of matrix macromolecules and their modifications for the regulation of cell behavior. Parallel with the early embryonal development of cell phenotypes there is also a developmentally regulated expression of genes coding for the above-mentioned variety of ECM

macromolecules (Macieira-Coelho, 2005, for review). These coregulatory processes determining cell and matrix phenotypes and their age-dependent modifications are profoundly altered during the neoplastic process. We mentioned already the role of fibronectin, laminin, and other ECM components and their age- and pathologies-dependent modifications (Labat-Robert, 2001, 2003, 2004; Labat-Robert and Robert, 1999, for review). Observations reported from several laboratories over the last decades further complicated this issue by showing that proteolytic degradation products, large peptides derived from ECM macromolecules, play unexpected and important roles in cell–matrix interactions in normal and pathological conditions. Some examples of early signaling by matrix components can be given. The earliest of these phenomena during development is the expression of laminin chains as well as collagen type IV chains and laminin-recognizing integrins ($\alpha 6\beta 1$) from the very early embryonic stage. These ECM components are directly involved in the polarized disposition of epithelial cells and their anchorage in the basement membrane. This elementary process of epithelial–mesenchymal interaction during morphogenesis can be observed from the trophoectoderm to the intestinal epithelium throughout the organism. The splice variants of $\alpha 6$-integrin chain appear to have discrete roles in this respect. Laminin-1 was also shown to act as an autocrine enhancer in epithelial morphogenesis. Mesenchymal cells in a process of epithelial conversion express laminin $\beta 1$ and $\gamma 1$ chains whereas the $\alpha 1$ chain appears only during epithelial polarization (Ekblom *et al.*, 1990). There is a concomitant expression of E-cadherin and $\alpha 6$-integrin chains with $\beta 1$ chains. It appears that laminin is an important component of the signaling environment originating from the surrounding matrix inducing epithelial differentiation. When cultured in a collagen gel, epithelial polarity is reversed, this process being apparently mediated by $\beta 1$ integrins (Wang *et al.*, 1990). Anti-$\beta 1$ antibodies interfere with epithelial cell adhesion to collagen or laminin-1. Similar roles were assigned to laminin and integrin chains in the branching process during epithelial development. The necessary presence of mesenchyme for epithelial differentiation was recognized already by Grobstein (1953, 1956). Epithelial basement membrane components orient the branching pattern of proliferating epithelium. The proteoglycan components of basement membranes, perlecan and others, appear also to play an important role in this process (Bernfield *et al.*, 1984). Epithelial–mesenchymal conversion is also observed during normal development and also during the malignant transformation in cancers, involving other signaling molecules also as EGF-II and the AKT-protein kinase family (Julien-Grille *et al.*, 2003, 2004). These signaling pathways play an important role in cancer progression and metastasis formation during epithelial cell mobilization. The tumor-regulating TWIST gene is switched on in metastatic mouse cancer cells. This same gene is involved

in the regulation of mesodermal development. Inactivation of TWIST inhibits metastasis formation with limited influence on the primary tumor. Transfection of TWIST in normal epithelial cells induced their mesodermal transformation (Yang *et al.*, 2004). A number of other examples can be given for the role of laminin isoforms in signaling to normal and transformed cells (Patarroyo *et al.*, 2002, for review). The increase of laminin $\alpha 4$ chain expression in tumor stromal fibroblasts might be of importance in this respect. Another example is the overexpression of laminin $\alpha 1$ chain in colon cancer accompanied by increased tumor growth. Glioblastoma cells express laminin-8 which appears to promote migration of malignant cells with the help of $\alpha 3$ and $\alpha 6$ integrins (Fujiwara *et al.*, 2001). Laminin isoforms were shown also of importance in tumor-related angiogenesis.

The role of fibronectin in malignant tissues was already mentioned. Studies on this matrix component were the earliest to stimulate interest in cell–matrix interactions as related to the malignant process (Hynes, 1990, for review). We also mentioned the potential role of an oncofetal, truncated form of fibronectin described by Schor and Schor (2001), an MSF, exhibiting "cryptic" behavior, not observed with the integral fibronectin molecule. This is however not a proteolytic degradation product of fibronectin as those which will be mentioned below. These authors showed however also that the isolated gelatin-binding domain of fibronectin (Gel-BD) stimulates also cell migration in the 0.1- to 1.0-pg/ml concentration range, but acting only on fibroblasts adhering to a native (nondenatured) collagen type I substrate. Synthetic peptides containing the IGD motif, present in Gel-BD, also stimulated cell-migration. This action could be inhibited by antibodies to $\alpha v \beta 3$ integrin. The motogenic truncated form of fibronectin, MSF, also contains the same sequences as Gel-BD, suggesting that proteolytic fragments of fibronectin containing this IGD sequence could stimulate cell-migration independently of the production of the truncated oncofetal form (MSF) of fibronectin. Contrary to these harmful effects of fibronectin fragments possibly involved in the malignant process, another form of fibronectin-derived fragment was shown by Yi and Ruoslahti (2001) to inhibit tumor growth, angiogenesis, and metastasis formation. This polymeric form of fibronectin, termed sFN (super fibronectin), can be obtained *in vitro* by treating soluble fibronectin with a 76-amino acid (aa) peptide derived from the first type III repeat of the molecule. This III1-C peptide and sFN inhibit tumor growth in mice, essentially by downregulating neovascularization. The III1-C fragment of fibronectin acts apparently by polymerizing fibronectin (producing sFN) but also fibrinogen (sFBG) exhibiting also antiangiogenic and antitumor activity. These peptide polymers act *in vivo*, reducing in mice tumor development after injection of C8161 melanoma, KRIB osteosarcoma, and MDA-MB-435 breast carcinoma human tumor cell lines, essentially by interfering with neovascularization.

It could be further shown by the team of Ruoslahti (Akerman *et al.*, 2005) that angiostatic peptides—endostatin, antithrombin, anastellin, and anginex—interact with fibronectin or vitronectin when they target angiogenic endothelial cell proliferatiion. Anginex is a 33-aa peptide reproducing the β-sheet structure of the other angiogenic peptides. Anginex initiates fibronectin polarization as does anastellin. This angiogenic peptide–fibronectin interaction is inhibited by the RGD (Arg-Gly-Asp) tripeptide, suggesting a mechanism for their angiostatic action passing by interaction with integrins, mediated by the peptide–fibronectin complex.

A more recent chapter in matrix-derived signaling is the recognition of the role of degradation products of ECM macromolecules. These clearly post-transcriptional regulatory processes were progressively uncovered during the last decades of the twentieth century and reached their maturity in recent years, essentially through the demonstration of their widespread occurrence and importance in aging and related diseases. This led to the recognition of a new paradigm in matrix biology and cell–matrix interactions, of importance also in the malignant process. This field was recently reviewed (Labat-Robert, 2002, 2004), a few examples will suffice to show their relevance to the present topic. Among the earliest observations we can mention the work of Keil-Dlouha and coworkers (Emod *et al.*, 1990; Keil-Dlouha and Planchenault, 1986) at the Paris Pasteur Institute showing that a fragment of fibronectin behaves as a proteolytic enzyme, a function totally absent in the parent molecule. But for the topic of this chapter, the observation of Barlati *et al.* (1986) in Brescia, Italy are of more direct relevance. They first observed the potentiation of malignant transformation mediated by RSV in presence of fibronectin fragments present in the cryoprecipitate of plasma of cancer patients (Barlati *et al.*, 1986). The responsible fragment contains the collagen-binding site of fibronectin. Such fragments were produced by the action of tissue plasminogen activator on fibronectin. Fibronectin fragments were also shown to increase opsonin-independent phagocytosis of human monocytes. They also exhibit chemotactic activity to peripheral monocytes and stimulate DNA synthesis by fibroblasts. The inflammation-inducing activity of other fibronectin fragments was published by Homandberg *et al.* (1992) and Homandberg (2001). Other fragments of fibronectin, carrying the heparin-binding site, are potent inhibitors of endothelial cell proliferation. The Hep-2 domain of fibronectin has a cryptic region endowed with antiadhesive activity (Fukai *et al.*, 1996). A cryptic 10-kDa antiadhesive site was shown to be released from fibronectin by cleavage of a specific sequence (YTIYVIAL) by MMP-2 (reviewed by Labat-Robert, 2004). More to the point of this review is the observation of the inhibition of cell cycle progression by a 25-kDa amino terminal fragment of fibronectin. Another 80-kDa fragment stimulates fibronectin biosynthesis, but also TNF-α production (Lopez-Armada *et al.*, 1997).

A cryptic fragment of fibronectin's type III module localizes to lipid rafts on the plasma membrane and stimulates cell proliferation (Hocking and Kowalski, 2002). As fibronectin production was shown to increase with age (Labat-Robert *et al.*, 1981a) as do also proteolytic enzymes (Robert, 2002, for review), together with the above observations a vicious circle is established, involving stimulation of production of fibronectin, followed by its degradation with the production of peptides modulating several processes of cell growth, differentiation, migration but also inflammation and malignant transformation. This vicious circle exhibits a positive feedback effect with age-dependent upregulation and might thus play an important role in age-dependent pathologies as the malignant process. The study by immunoblotting of plasma samples from patients 70- to 90-year old, suffering from age-related pathologies, in a geriatric service revealed fibronectin fragments (Labat-Robert *et al.*, 2000). No such fragments were detected in the plasma of several centenarians in apparently good health.

Several other ECM macromolecules were also shown to yield proteolytic fragments potentially involved in the malignant process. This is the case for the basement membrane collagen type IV studied by the team of Maquart and Monboisse at the Reims Medical School. Its degradation is observed during the metastatic process involving the cleavage of basement membranes by migrating-invading cancer cells. Several fragments of collagen type IV were shown to promote melanoma cell adhesion, migration, and invasion (Pasco *et al.*, 2005). Some of this activity requires the integrity of the triple helical domain of collagen type IV fragments. A specific sequence however of the α3(IV) NC1 domain was shown by the team of Monboisse and Maquart to inhibit melanoma cell proliferation, migration, and invasion, essentially through the inhibition of MMP production and activation. Other collagen type IV fragments derived from the α1(IV), α2(IV), and α3(IV) chains, designated by the terms: arrestin, canstatin, and tumstatin were shown to inhibit angiogenesis (Pasco *et al.*, 2004a,b, 2005). ECM-derived fragments play a role in angiogenesis also (Bellon *et al.*, 2004). It was proposed to designate such biologically active peptides as *matrikins*. A previous proposition from Davis *et al.* (2000) concerned the transconformational exhibition of proteolytically vulnerable sites (matricryptic sites), followed by their cleavage by proteolytic enzymes with the production of *matricryptins*, peptides endowed with specific biological activities. More similar discoveries can be expected in the near future, increasing the number and importance of such phenomena. For our present purpose, the proteolytic production from ECM components of peptides capable of modulating cell function and behavior directly involved in the malignant process is of importance. Besides their involvement in pathologies, matricryptins and matrikins were shown to play a crucial role in age-dependent ECM remodeling and degradation (Labat-Robert, 2002, 2003, 2004; Maquart *et al.*, 2004, 2005).

XI. CONCLUDING REMARKS

Even if we accept the unicellular origin of the malignant process as proposed by several authors ("one renegate cell," Weinberg, 1998), a proposition still open to discussion (see Maffini *et al*., 2004) very rapidly cells surrounded by ECM enter in the multistep process supposed to lead to the overt malignant disease (Klein, 1998). Without this rapid implication of surrounding fibroblasts and cell–matrix interactions discovered over the last decades, antimitotic therapy would have proven much more efficient. The examples cited in this chapter, although not exhaustive, suffice to make the point. Apparently normal cells far from the tumor participate in the malignant process as shown by the example of skin fibroblasts in breast cancer patients (Brouty-Boyé, 2005, for review). The stromal reaction part of the profound reorganization of tissue structure with the details of underlying molecular mechanisms briefly recalled in this chapter speaks also in favor of a much more general cellular-molecular process than deducible from the proposed single-cell origin of the malignant disease. Among the more theoretical conclusions suggested by the above-discussed experimental findings, we should mention the posttranslational nature of most processes described in Section X (Labat-Robert, 2002, 2004; Robert and Labat-Robert, 2000). The generation of proteolytic fragments from matrix (or other) macromolecules, endowed with a variety of biological activities, is probably one of the most fascinating recent aspects of matrix biology. Age-dependent modifications of tissues appear to play an important role in this respect also. Besides the cell–cell interactions involved in cell-aging as well as in the malignant process and driven by the cancer protective genes (Campisi, 2005), other, apparently unrelated processes were shown to be involved. This is the case of the age-dependent upregulation of fibronectin biosynthesis together with the accompanying upregulation of proteolytic enzymes. This leads to the vicious circle with positive feedback, resulting in increased production of potentially harmful degradation products. It might well be that posttranslational processes, such as biologically active peptide production, interfere with gene regulation circuits further complicating the mechanisms underlying the malignant process. The importance of epigenetic process in malignant transformation and cancer progression was recognized over the last decades (Paz *et al*., 2002; Van Speybroeck *et al*., 2002; Verma *et al*., 2003, for review). Horizontal transfer of DNA by the uptake of apoptotic bodies is another mechanism escaping direct gene control (Holmgren *et al*., 1999). This is further complicated by the intrusion of posttranslational processes such as the proteolytic generation of biologically active peptides. Such processes are apparently not "coded" in the genome, occur "by surprise" as emergent properties of biological systems, object

now of philosophical discussions (Robert and Miquel, 2004, for review). Such conceptual advances further extend the deep insight in the logics of vital processes, triggered by François Jacob's reflections on "tinkering" (Jacob, 1977). Not only did "Nature tinker" during evolution, it did not foresee furthermore some of the potentially harmful consequences of biological processes such as those of harmful effects of proteolytic breakdown products of biologically important structural components of the organism. It might well be that the malignant process is one of the consequences of this short-sighted "creation" of complex living systems. One consolation however is that it might well be easier to interfere therapeutically with posttranslational processes than with modifications at the level of the genome.

NOTE ADDED IN PROOF

A further confirmation of the importance of some tumor suppressor genes in the switch between cellular senescence and malignant transformation was published in the September 28, 2006 issue of *Nature* (Vol. 443) by three different laboratories (p. 421, 453, and 448) and commented by Christian Beausejour and Judith Campisi (p. 404). As stated in this comment "the proliferation of cells must balance the longevity assured by tissue renewal against the risk of developing cancer. The tumor suppressor $p16^{INKA4A}$ seems to act as a pivot of this delicate equilibrium." These reports appeared to confirm the proposition by Campisi (2001, 2005) cited in some detail in this chapter. The November 30, 2006 issue of *Nature* (Vol. 444) carried two further articles by J. Bartkova *et al.* (p. 633: Oncogene-induced senescence is part of the tumorigenesis barrier improved by DNA damage) and by R. Di Micco *et al.* (p. 638: Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication) bringing further arguments for the above thesis.

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