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Tumor resistance

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It is suggested that evolution has equipped humans and other species with powerful and, largely non-immunological resistance mechanisms that can nip pre-neoplastic cells, as well as cells disseminating from established tumors in the bud. These mechanisms must operate while maintaining tissue structure, polarity and a large variety of cell-to-cell interactions. Altogether, they are essential for microenvironmental tissue integrity. It has further been postulated that the genes underpinning microenvironmental control are not merely alleles of known cancer susceptibility genes, but constitute sui generis systems.

Introduction

In 1909, Paul Ehrlich wrote that "aberrante Keime" (which we could translate and modernize to "mutant cells") must be arising continuously during the complex process of embryonic and post-embryonic development. Were it not for defense mechanisms of the organism, cancer would arise at an enormous frequency ("in einer ungeheurer Frekvenz").[1]

This was a visionary statement. Two out of three persons never get cancer. Even heavy smokers who continuously bombard their lungs with carcinogens develop lung cancer in only about 25 percent. When inbred mouse strains were developed by serial brother-sister mating and selection for a particular type of cancer, the derived homozygous strains had a high incidence of the specific neoplasm they were selected for, but not of others. Genetic analysis has shown that the cancer proneness of the cancer-susceptible mouse strains is polygenically controlled. Some of the genes involved in this control were later on shown to influence hallmark characteristics of cancer, whereas others act by hitherto unknown mechanisms. Moreover, inbreeding and selection for a certain type of cancer has often fixed non-genetic factors that increase the probability of tumor development, like viruses. Mammary tumor virus (MMTV) and murine leukemia virus (MuLV) are the best known examples of this tendency.

Mouse inbreeding programs for the study of cancer genetics, pioneered by C. C. Little and later on by L. C. Strong, also included the selection of poorly susceptible mouse strains, such as the C57Bl variant. Although selected for a low incidence of breast cancer, these mice display a low frequency of all neoplasms. This points to the existence of a systemic resistance against potentially neoplastic cells.

Systemic resistance against cancer is also indicated by Peto's paradox, namely, the absence of any relationship between cancer propensity and body size. Oncogenes, tumor suppressor genes and many other signaling pathways are highly conserved among mammalians. Given a similar probability of somatic mutations, blue whales, which have 10^{17} cells, would be expected to have a higher number of cancers than mice, which have only 10^{9} cells. This is, however, not so. Whales are species at a low cancer risk. Among rodents, cancer propensity differs, but is not related to body size.[2] The naked mole rat, the most long-lived rodent (up to 28 y) is totally resistant to cancer. In vitro, its fibroblasts show early contact inhibition accompanied by the upregulation of the cell cycle-inhibitory protein p16, which is not seen with either mouse or human fibroblasts that require full confluence for contact inhibition and upregulate p27, but not p16.[3]

Systemic cancer control is also indicated by the extensively studied phenomenon of two-phase carcinogenesis.[4] Suboptimal doses of carcinogens that induce no tumors and even some non-carcinogenic compounds, like urethane, "initiate" pre-neoplastic cells all over the mouse or rat skin. These cells do not develop into tumors, however, unless the skin is exposed to "tumor promoters," like phorbol esters, which are not carcinogenic by themselves. The promoters act by favoring inflammation and tissue proliferation. Thus, the breakdown of local control may be crucial for oncogenesis.

A possibly analogous picture emerges from the study of micrometastases. Disseminated tumor cells are found throughout the body of cancer patients. Most of them, however, never develop into progressively growing metastases.[5] Some tumors, like melanoma, can remain dormant over several decades, but can be awakened by disturbances of the tissue equilibrium, e.g., by irradiation.[6]

How is Microenviromental Control Mediated?

This field of investigation is still in its early stages of development, but it is already apparent that microenviromental control can be mediated in several different ways. The classical experiment of Beatrice Mintz,[7] the normalization of highly malignant teratoma cells in an early embryonic environment, together with subsequent evidence on the induction of differentiation by cytokines and other signal mediators in leukemia, for instance, is closely linked to developmental regulation. Many different approaches have shown that tumor cells exhibit a relative, rather than absolute, degree of autonomy. Forces safeguarding the maintenance of

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normal tissue architecture, boundaries and polarity play important roles. Mina Bissell has launched the notion, both at a conceptual and an experimental level, that the re-establishment of a normal or normal-like microenvironment can curb the neoplastic behavior even of highly malignant cells.[8] This can be achieved by growing tumor cells on appropriate scaffolds or by exposing them to components of the extracellular matrix, such as Matrigel, that can override the effect of highly potent oncogenes like *MYC*.[9] Taken together, such experiments have demonstrated the important notion that phenotype can override genotype.

Contact Control

As discussed above, tumor development is influenced by the microenvironment in multiple ways, and part of this influence is dependent on the direct contact between tumor and normal cells. Michael Stoker and his group discovered in the 1960s that normal fibroblasts can inhibit the growth of tumor cells upon direct contact.[10] The mechanism of this phenomenon, referred to as neighbor suppression, has not been clarified, but adherens junctions are suspected to be involved.[11,12] This interpretation is supported by the fact that one important component of adherens junctions, E-cadherin, is downregulated in most carcinomas, usually upon gene methylation, while their catenin and connexin components may be mutated.[13] Re-establishment of E-cadherin expression by transfection can reverse the tumor phenotype. Preservation of the normal integrin structure appears to be important as well.[14] Notch receptors and their ligands may also be involved in contact control, since their deletion in the basal layer of mouse epidermis can lead to hyperplasia and skin tumors.[15]

Such and other physical interactions between normal and tumor cells may be at least partly responsible for the frequent observation that the majority of disseminated tumor cells never grow into metastases. In one experimental model, a significant fraction of injected mouse mammary tumor cells of either high or low metastatic potential persisted as solitary non-dividing cells in the liver.[16] They were fully tumorigenic, when re-inoculated into new hosts. Dormancy of solitary tumor cells has also been demonstrated with melanoma, squamous cell carcinoma and prostatic carcinoma cells in the same model. "Awakening" of such dormant tumor cells could be accelerated by altering the tissue equilibrium, e.g., by exposure to phorbol esters.

The Role of Inflammation and Regeneration

A particularly striking microenvironmental control force has been shown to curb the transforming effect of a powerful oncogene. In the pivotal experiments of Peyton Rous in 1911, the "filterable" agent that was to be called Rous sarcoma virus (RSV), induced tumors at the site of inoculation in newly hatched chicks after a relatively short latency. This transforming activity could later be attributed to the *v-src* oncogene. When chicken embryos were infected with v-src at an early embryonic stage, they developed normally and, after hatching, such birds developed no tumors. On culturing, their fibroblasts grew immediately with a transformed phenotype. Importantly, wounding the *v-src*-carrying birds led to tumor development at the site of tissue regeneration.[17] It was concluded that the puncture wound must have been essential for the induction of tumors in Rous' original experiments. Apparently, the healing process can break down the microenvironmental control that normally inhibits the growth of the transformed cells.

A recent experiment, conducted in a different system, showed that the stroma can be of paramount importance for tumor development. Maffini, et al. used a chemically induced rat mammary tumor system and the direct carcinogen *N*-nitrosomethylurea (NMU).[18] The mammary fat pads of female recipients were cleared of glandular tissue, which was explanted in vitro and then re-implanted into the cleared fat pad, with or without previous exposure to NMU. Exposure of the cleared fat pad to NMU before the implantation of the gland led to tumor development by untreated cells of the mammary epithelium, whereas the implantation of NMU-treated glands to untreated fat pads did not. The results suggest that the carcinogen exposure has affected a microenvironmental control system that would normally prevent the development or progression of pre(cancerous) cells.

The histopathologically observed lack of progression of microscopically detectable cancerous foci in the prostate or the mammary gland of people who never develop clinically apparent tumors may be due to similar control mechanisms. The persistence of epithelial cells with defined genetic lesions in their oncogene and/or oncosuppressor gene equipment that failed to grow while in contact with the normal epithelium is another case in point.[19] Suppression of initiated pre-neoplastic or neoplastic cells by normal cells can be overcome by a variety of non-carcinogenic agents, classed as tumor promoters.[20] There is much clinical and epidemiologic evidence to suggest that inflammatory conditions are associated with an increased cancer risk.[21]

It may be relevant in that context to note that cancer associated fibroblasts (CAFs) are characterized by a pro-inflammatory gene signature.[22] Such CAFs were shown to enhance tumor growth in an NFκB-dependent manner. Conceivably, NFκB may be a main molecular link between inflammation and carcinogenesis.[21]

Own Experiments

As an experimental approach toward the study of the inhibition of tumor progression by the normal stroma, we have chosen to study the phenomenon of neighbor suppression originally described by Stoker, et al.[10] Our results obtained so far have been published in two papers.[23,24]

In the first study, the effect of 107 samples of low passage number primary normal fibroblasts from pediatric and adult donors was tested on the growth of six human tumor cell lines. The majority of the tested fibroblasts inhibited the proliferation of tumor cells. The antiproliferative effect of fibroblasts differed, depending on the site of origin. Skin fibroblasts were more inhibitory than prostate fibroblasts, which were obtained from donors affected prostatic cancer. Normal hernia fibroblasts were less inhibitory than skin fibroblasts. Inhibition required direct cell contact and such an effect could also overcome the mouse-human species barrier. The second study showed that effective

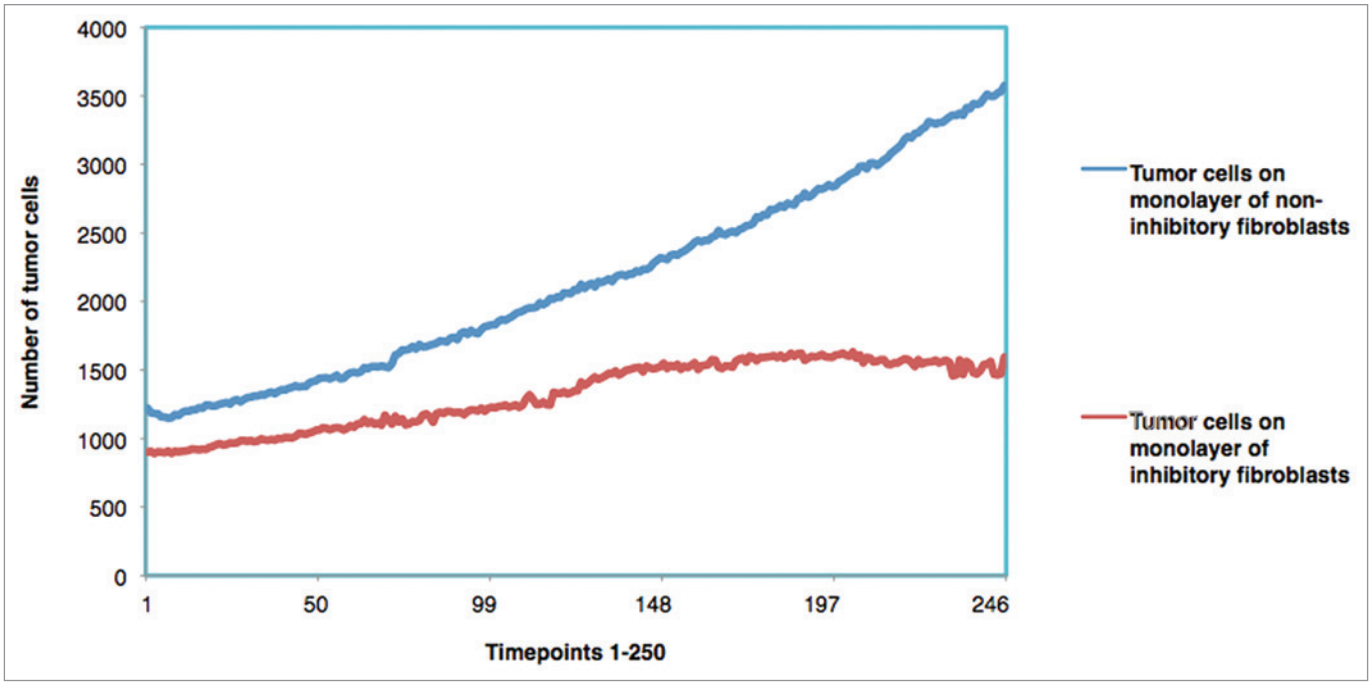

Figure 1. Proliferation of tumor cells during 62.5 h of culture on inhibitory or non-inhibitory fibroblast monolayers. After Flaberg, et al.,[23] courtesy of Dr. Emilie Flaberg.

inhibition of tumor growth by normal fibroblasts requires the formation of a morphologically intact fibroblast monolayer before the seeding of the tumor cells. Interference with the formation of the monolayer impaired inhibition.

Using time-lapse imaging combined with extended field live-cell microscopy we could follow a large number of tumor cells growing on confluent and morphologically intact fibroblast monolayers over time. **Figure 1** illustrates the differences in proliferation of tumor cells growing on inhibitory or non-inhibitory fibroblasts during 62.5 h (250 time-points). Differences were also found in the motility of tumor cells. The motility of tumor cells on an inhibitory monolayer started to decrease after 25 h with minimal movement in the last time intervals, whereas the monolayer from non-inhibitory fibroblasts had no effect on tumor cell motility (**Fig. 2**).

Telomerase-immortalized human fibroblasts were good inhibitors. Based on morphological criteria, subclones with variable inhibitory capacity could be selected from the telomerase-immortalized cell line. By comparing highly inhibitory and poorly inhibitory fibroblasts from the in vitro immortalized line and of inhibitory and poorly inhibitory from ex vivo explants, we identified a set of genes that co-segregated with the inhibitory phenotype. This was taken to suggest that our model system may reveal molecular mechanisms involved in contact-mediated microenvironmental surveillance.

Discussion

Studying the fibroblast-mediated inhibition of tumor cell growth in vitro, termed neighbor suppression, three different levels of variation may be discussed: epigenetic, tumor related and genetic.

Epigenetic variation. Epigenetic variation came through clearly, both in our ex vivo and in vitro systems. The former was illustrated by the different strength of inhibitory effects exerted by hernia vs. skin fibroblasts obtained from the same donor. In the in vitro system, it was exemplified by the difference between the morphologically distinguishable whirly and crossy subclones, derived from the same telomerase-immortalized cell line.

Tumor-related variation. Tumor-related variation was manifested by the high inhibitory effects of skin-derived as compared with the poor inhibitory and sometimes stimulatory effects of the prostate-cancer associated fibroblasts derived from the same patient. This ties in with the extensive literature on CAFs.

Genetic variation. The third and perhaps most interesting question concerns the possibility of genetic variation. In contrast to other antitumor surveillance mechanisms, such as the immune response against virus-associated tumors, DNA repair, apoptotic propensity and epigenetic imprinting, for which genetic variation has previously been shown to influence tumor propensity, there is no information on the question whether the microenvironmental control, and stroma-tumor interactions in particular, can vary genetically. Together with epidemiologists, we are presently exploring this question by twin studies. Looking for possible prognostic correlations, we are also involved in a coded clinical study. In correlation with prostatic carcinoma surgeons, we are comparing fibroblasts from prostatic cancer patients with quiescent vs. progressive tumors for their ability to inhibit the growth of prostatic carcinoma cells in vitro.

Are there specific tumor resistance genes? At a more general level, the question may be raised as to whether tumor resistance has genetics of its own or whether it merely reflects a variant on the theme of tumor susceptibility. Has evolution favored specific

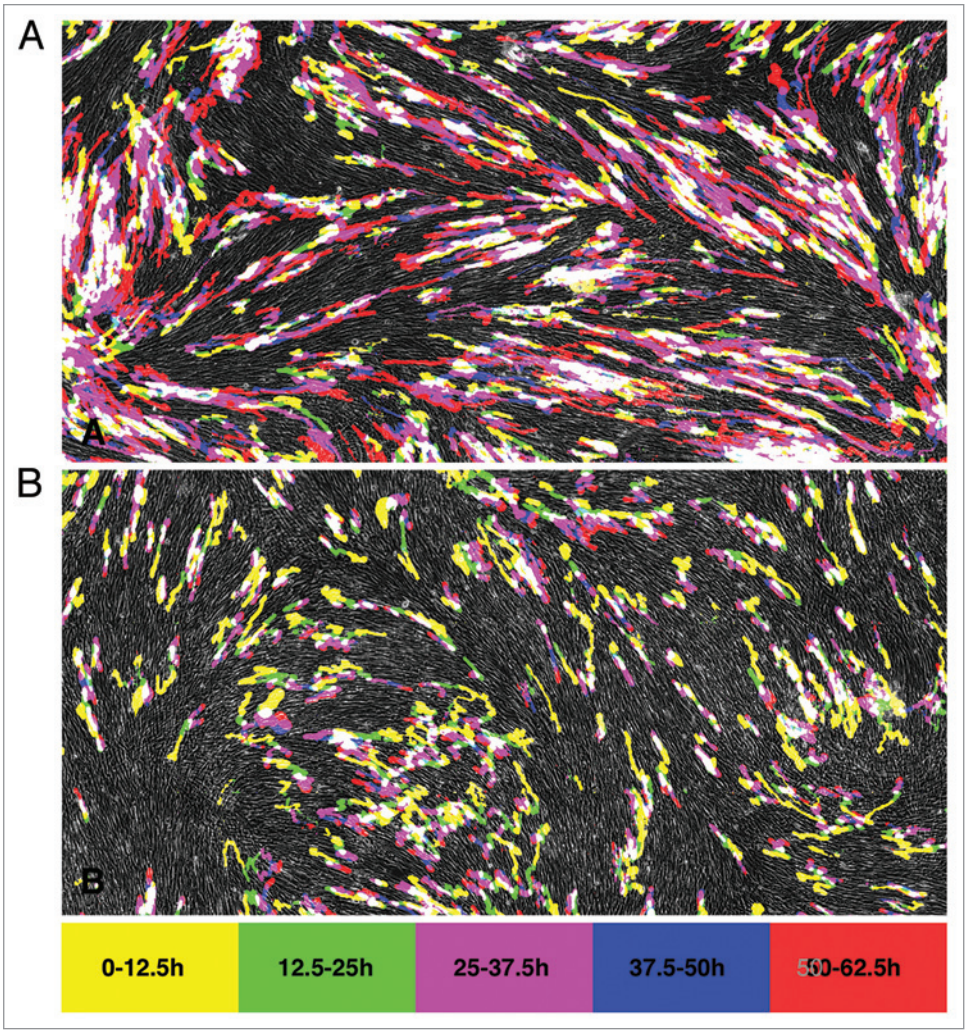

Figure 2. Color-coded trajectories of tumor cells during 62.5 h of culture on inhibitory or non-inhibitory fibroblast monolayers. Each color represents projections of 12.5 h intervals. **(A)** Tumor cells growing on a monolayer of non-inhibitory fibroblasts. **(B)** Tumor cells growing on a monolayer of inhibitory fibroblasts. After Flaberg, et al.,[23] courtesy of Dr. Emilie Flaberg.

resistance genes that prevent the outgrowth of potentially tumorigenic cells?

Specifically-designed epidemiological studies are needed to approach this question. Twin studies may be the most straightforward ones. Do identical and fraternal twins of index subjects, who have reached an old age without cancer, differ in concordance with regard to tumor incidence? Another approach is to compare tumor incidence in first-degree relatives of people who reached an old age without cancer, with appropriate controls.

It is noteworthy, in this context, that most tumors that develop in adults show a basically similar age incidence curve. Steep rise in tumor incidence with advancing age is followed by a decline in the very old. The decline starts around 80–85 years of age.[25] Very few if any tumors appear in centenarians. They may represent a population selected, inter alia, for cancer resistance. As the experimental studies on cancer resistance develop by gene profiling of putative effector cells, the expression of potentially relevant genes may be compared at different ages, with particular focus on the centenarians.

Are cancer susceptibility and resistance genes functionally different? Known tumor susceptibility genes belong to different categories. Some of them influence the hallmark characteristics of cancer, such as the activity of proliferation-driving oncogenes, the functional integrity of tumor suppressor genes, the role of pro- or anti-apoptotic genes and, in the case of virus-induced tumors, the ability of the virus to replicate as well as antiviral or immune responses. Sui generis tumor resistance genes may act by different mechanisms. They may influence the microenvironmental recognition of incipient tumors or that of disseminated cancer cells. They may nip tumor development in the bud, by recognizing cellular changes associated with the neoplastic transformation.

The postulated contrast between specific cancer susceptibility and general cancer resistance is also in line with the fact that mouse strains that exhibit a high propensity to develop cancer, as established by serial brother-sister mating and selection, only do so for the selected cancer, not for tumors in general. In contrast, strains selected for tumor resistance, such as C57Bl mice, have a low incidence of all tumors.

The existence of evolutionarily fixed tumor resistance mechanisms that would provide a survival advantage has often been doubted. It has been argued that most cancers occur beyond the reproductive age and resistance against them would therefore not provide any selective advantage to the species. It is easy to see the fallacy of this argument. The "nipping in the bud" type of resistance would protect the species during its entire lifetime, not merely at an old age, and could have given a strong survival advantage also to the young. Species-specific variation of the resistance may also explain Peto's paradox, the lack of any correlation between body size and cancer incidence in different mammalian species.[26]

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