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Microenvironment Revisited: Time for Reappraisal of Some Prevailing Concepts of Cancer Metastasis

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Abstract The development and biological characteristics of tumor metastasis are influenced by multiple cell and host-associated factors. To study these factors experimentally, the necessity of choosing adequate in vivo model systems for human tumor metastasis is emphasized. Recent research has provided results that highlight the role of the microenvironment in determining important characteristics of the metastatic cells, including their degree of differentiation and sensitivity to drugs. Furthermore, evidence is presented as background for discussing the general validity of the notion of clonal selection of metastatic cells, and whether the metastatic phenotype is acquired through the last of a series of mutational events occurring during tumor progression. © 1994 Wiley-Liss, Inc.

Key words: cancer, metastasis, microenvironment, clonal selection, tissue specificity, tumor models, chemosensitivity, nude mice, nude rats

The complexity of the metastatic process makes metastasis research one of the most challenging areas in tumor biology. Much of the recent progress in understanding mechanisms involved in metastasis formation may be ascribed to the development of novel methods in molecular biology and to the availability of good in vivo models for human tumor metastasis. To elucidate metastasis mechanisms, attempts have commonly been made to relate alterations in a single feature of the neoplastic cell to the appearance of overt metastases in an animal model, i.e., the end result of a long series of sequential steps involved in the metastatic process. Each of these steps can be rate limiting [Fidler, 1990], all depending on a finely tuned interplay between the tumor cells and multiple host factors. On this background, and because of the heterogeneity in the metastatic patterns observed clinically, the need for comprehensive in vivo studies in relevant model systems becomes obvious.

Previous studies involving rodent tumor lines have helped to establish several basic concepts of the metastatic process. Nevertheless, it should be noted that most of these tumors are not spontaneous, but were originally induced by carcinogens, and that the cell lines may have undergone significant changes during years of in vitro and in vitro passaging. When examining specific cell-associated factors involved in metastasis mechanisms, the clinical validity of results obtained with such cell lines might be questioned. This pertains particularly to studies on tumor heterogeneity and clonality, as it is likely that genetic instability and phenotypic plasticity of these cells may differ greatly from that of the average solid human cancer. Therefore, the use of well-characterized human tumor lines or freshly biopsied tissue from patients should be preferred.

Immunodeficient nude and SCID mice, as well as nude rats, provide important tools for tumor biology research. One major drawback has been the very low frequency of spontaneous metastasis observed when human tumors are grown subcutaneously in such rodents [Liotta, 1986; Fodstad, 1991a]. However, during the last decade an increasing number of investigators have reported on models for spontaneous and experimental human tumor metastasis. The health status of the animals [Fidler, 1986; Fodstad, 1991a], the nature of their immunodefiency [Fodstad, 1991b], the preparation of tumor material, and the site or route of cell administration [Fidler, 1990; Kjønniksen et al., 1990; Hoffman 1992] seem to have a strong impact on both the development of metastatic tumor lesions and on the pattern of metastasis.

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Orthotopic implantation, i.e., inoculation of the tumor material in the same tissue as that of origin of the human tumor, may be advantageous in establishing progressive local growth and in facilitating metastasis formation in clinically relevant organs. Thus, evidence has been provided on the advantage of inoculating e.g., colon cancer cells to colon mucosa, renal cell carcinomas to the subrenal capsule, melanomas into the skin, and bladder carcinomas to the bladder wall [for review, see Fidler, 1990]. To determine the extent to which these observations reflect a growth- and metastasis-promoting activity truly specific for each tumor type would require a higher number of control experiments than those reported. Nevertheless, the findings emphasize the importance of the local microenvironment for tumor cell growth and biological behavior. The importance of such interaction has been underestimated, and the nature and magnitude of its ramifications are yet to be established.

Direct orthotopic injection of suspended tumor cells involves the risk of introducing malignant cells directly into blood or lymph vessels. This might affect the potential for metastasis formation, as has been observed upon subcutaneous injection of tumor cells [Fodstad, 1991a]. An alternative approach is to transplant small pieces of tumor tissue obtained from subcutaneous xenografts or from biopsies of patient tumors [Hoffman, 1992]. The feasibility of this procedure has been demonstrated for several cancer types, and it may allow for rapid establishment of a vascularized, three-dimensionally intact tumor with a high propensity for spontaneous metastasis formation. [Fu et al., 1991, 1992; Hoffman, 1992]. One limitation with the approach might be the requirement for refined, time-consuming microsurgical techniques.

Experimental metastasis formation can be obtained by injecting tumor cells by different routes into the venous or arterial circulation. Although the early steps of the metastatic process in this case are not involved, specific patterns of clinically relevant metastasis formation may be achieved. Thus, after intravenous injections tumor manifestations can develop specifically in the lung [Kerbel et al., 1984; Fodstad et al., 1988a] or lymph nodes [Fodstad et al., 1988b], in bone after intracardial inoculation [Kjønniksen et al., 1990], and in the brain or its meninges after administration of the cells to the internal carotid artery [Schackert and Fidler, 1988; Fodstad, 1993]. The resulting models permit studies of factors determining tissue-preferenced metastatic growth, and the nature of the interactions between the neoplastic cells and the microenvironment. The relevance of such models for the human situation is illustrated by two examples. In one case, cells from a lung metastasis of a human melanoma show a clear preference for lung tumor formation when injected by several different routes in athymic animals [Fodstad et al., 1988a; Fodstad, 1993]. In contrast, cells from a patient with lymphatic spread of a melanoma predominantly give lymph node metastases in nude mice [Fodstad et al., 1988b]. Furthermore, we had the opportunity to compare technetium bone scans of three patients with skeletal metastases with those of nude rats with bone metastases that appeared after intercardial injection of tumor cells originating from the same patients [Kjønniksen et al., 1992b]. A striking similarity between matched pairs of scans was seen, with tumors of sclerotic (Fig. 1), lytic, or mixed patterns of radioactivity uptake. Clearly, the tumor cells had retained characteristics important for their interaction with and growth in bone/bone marrow, and the local microenvironment provided for the tumor cells must have been closely similar in man and in nude rat.

As mentioned above, support for the importance of individual tumor cell features for metastasis formation has commonly been sought by relating differences in a particular characteristic, between cell sublines, cell clones, or different cell lines, to differences in metastatic capacity tested in an animal model. A prerequisite for this approach to be valid would be that the tumor cells were identical in all other aspects than the one examined. Moreover, host factors that might influence metastasis formation should be closely similar to those in man. Since it is not likely that these requirements can be fully met, several tumor cell lines should be tested, preferably in different model systems. One example illustrating possible pitfalls is our finding that the same human cell line shows distinct metastasis patterns in nude mice and rats [Kjønniksen et al., 1991]. Furthermore, host differences have been seen for several cell lines also in threshold levels in the number of cells that give metastases, in growth rate of overt metastases, and in latency times before appearance of tumor lesions. Clearly, interpretation of data obtained in experiments with one cell line might differ with the choice of injection route and the strain or species of the recipient animals.

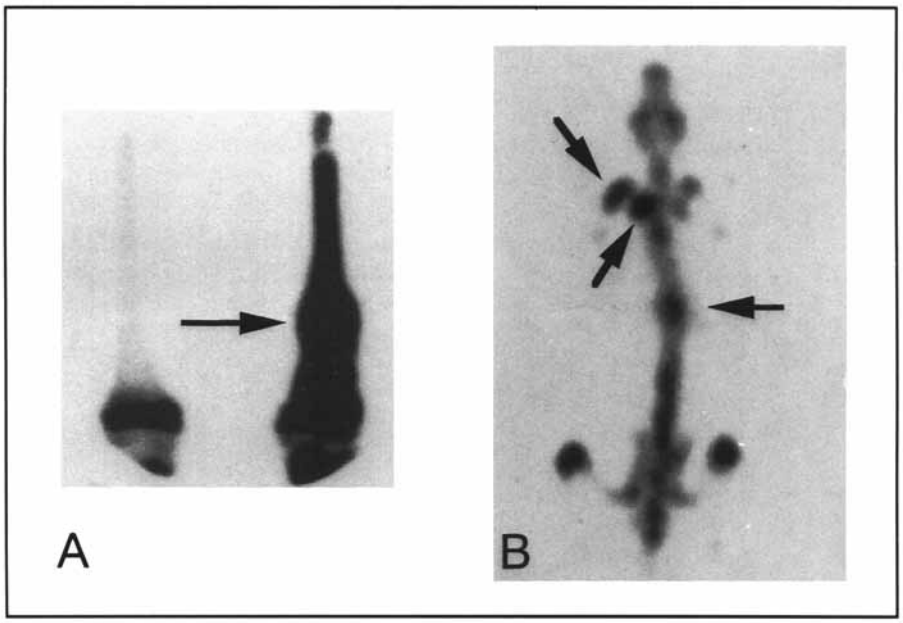

Fig. 1. Technetium scintigrams of (**A**) a patient with a highly sclerotic osteosarcoma (arrow) in his left femur, and (**B**) of a nude rat with similarly sclerotic skeletal metastases (arrows) that developed after intracardial injection of cells from a cell line established from the femoral tumor illustrated in panel (A).

For many years, one of the prevailing concepts of the emergence of cells with the metastatic capacity has been based on Nowell's theory of tumor progression [Nowell, 1976]. According to this, the metastatic cell is the end result of a series of sequential genetic changes, implying that there are fundamental differences between metastatic and nonmetastatic cancer cells with respect to gene regulation and signal transduction [Kerbel, 1990]. This concept implies that the metastatic phenotype is obtained as the ultimate mutation occurring during tumor progression, giving rise to a population of cells with a selective advantage, which is also a permanent feature of its progenies. Experimental evidence has been provided indicating that metastatic cells represent a pre-existing subpopulation of cells within the primary tumor [Fidler, 1990]. Metastatic lesions, therefore, should contain only cells with these characteristics, whereas nonmetastatic cells can acquire the metastatic trait only through the same series of mutational events. Some studies on the clonal orgin of metastasis have supported the concept [Fidler, 1990; Kerbel, 1990]. However, several investigators have reported data that do not fit into this picture, and alternative theories have been brought forward. Thus, the view that cancer metastases are the result of a selection process favoring either escape from or growth dominance in the primary tumor [Kerbel, 1990] has been challenged by results obtained by Weiss et al. [1980], Alexander [1984], Ling et al. [1985], and Vaage [1988]. Ling et al. [1985] introduced the concept of "dynamic heterogeneity," which argues that although the metastatic phenotype is a genetically controlled trait, it is inherently dynamic or unstable.

Evidence for the selective nature of the metastatic phenotype was first obtained by Fidler [1973] in his well-known experiments with murine B16 melanoma cells. However, it was later found that similarly selected metastatic "clones" after some weeks of culture reverted in their metastatic capacity to that of the parent "wild-type" cells [Kerbel, 1990]. This phenotypic drift [Nicolson, 1987] has been explained by assuming that the ability to metastasize might be the result of heritable epigenetic changes [Ling et al., 1985; Kerbel, 1990]. The discrepancy between the concepts of "clonal selection" and "dynamic heterogeneity" is not easily resolved. We would, however, like to draw attention to results in favor of the latter theory. Vaage [1988] reported results on murine mammary tumors transplanted orthotopically to the mammary fatpad. In disagreement with the clonal selection concept, cells from the spontaneous metastases formed were not more aggressively metastatic than those of the parent tumor. Three of

seven tumors gained increased metastatic potential, but, importantly, this occurred in parallel during serial passage of both the parent tumor and the metastases. These results might reflect an instability in the tumor cells, making them prone to acquiring a higher metastatic potential after a certain number of cell divisions.

Working with human tumor xenografts, we followed an approach similar to that used by Fidler with B16 cells, in attempts to select sublines of a malignant melanoma with increased experimental metastatic capacity. As schematically shown in Figure 2, single cell suspensions prepared from an FEMX human melanoma xenograft in a nude mouse were injected intravenously *into* recipient animals. When a subcutaneous metastasis developed, cell suspensions were prepared and injected directly intravenously into new mice, avoiding in vitro culture before reinoculation. Surprisingly, the latency time before the appearance of measurable metastases increased with each passage, and after the 5th passage no new tumors developed [Fodstad et al., 1988c]. At each generation of FEMX metastasis, some of the tumor tissue was both transplanted subcutaneously in other groups of mice and used for establishing in vitro sublines. Both the in vivo and in vitro growth rates decreased with the passage number, in keeping with the increased delay in metastasis formation after intravenous injection. Moreover, the cells acquired the characteristics of increased differentiation. Importantly, whereas the in vitro cultivated cell sublines retained the distinct metastatic capacity typical for each passage, cells from the 5th generation of selected metastatic tumors grown subcutaneously for a period of less than 3 months reverted to that of the original tumor.

Since identical results were obtained when the entire experiment was repeated, we are confident that in the case of the FEMX tumor the selection pressure favored the development of metastases originating from more differentiated cells that concurrently had decreased proliferation rates. Based on cytogenetic studies, and also on experiments with individual and mixed cloned sublines, we interpret the data to demonstrate an example of how alterations in the metastatic phenotype can occur without mutations, and the reversibility of these changes supports the importance of epigenetic mechanisms. Among several possibilities in this regard, differences in gene methylation patterns [Kerbel, 1990; Kern, 1993], and in extrachromosomal gene amplification in double-minute minichromosomes [Kerbel, 1990], may be mentioned. Also differences in transcriptional regulation of some essential genes in response to exogenous signals might conceivably be involved. In a recent editorial, Kern [1993] concluded that the

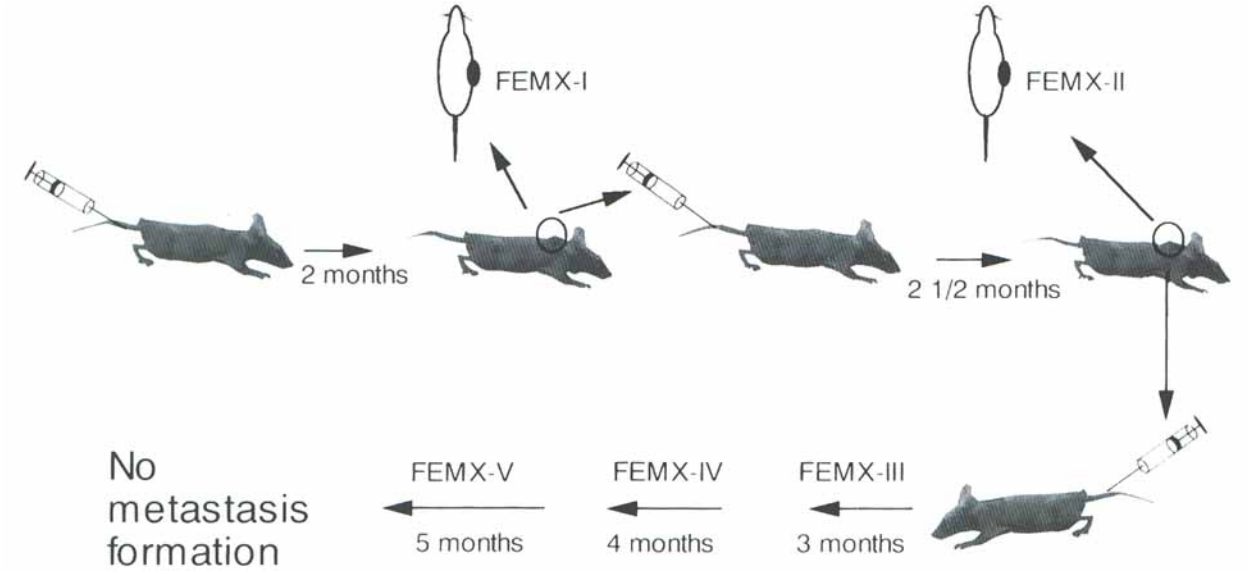

Fig. 2. In vivo selection of metastatic human melanoma cell variants. Intravenous injection of FEMX cells resulted in subcutaneous metastases after 2 months. Cells from such tumors (circles) were for each new generation of metastasis used for direct injection in new recipient animals, as well as for subcutaneous transplantation (drawings of mice with FEMX-I and -II tumors) and for establishment of in vitro sublines (not shown). The latency time for metastases to develop increased with each generation, from 2 to 5 months, until no metastases appeared in mice injected with cells from the 5th passage.

current understanding of the clonal evolution in neoplasms is clearly inadequate. He commented on the results obtained by Shibata et al. [1993], who showed intratumor heterogeneity in c-K-ras mutations in some colorectal adenomas, suggesting multiple and genetically distinct neoplastic clones. Although most of the adenocarcinomas examined had a homogenous composition of c-K-ras mutation, one carcinoma in situ had no detectable mutation in spite of its presence in the associated adenoma. It was concluded that the strong oncogenic mutation in the small adenomas did not allow dominance over the other cells. Our findings with FEMX-cells, which are contradictory to the concept that metastatic competence is a trait acquired during the final step of malignant progression of tumors, is in keeping with Kern's view. There seems to be an emerging feeling that Nowell's theory might be too mechanistic to be fully applicable to solid human cancers.

The tissue-specific metastasis patterns seen clinically and in experimental models highlight the importance of the local microenvironment for the development of metastases [Fidler, 1986; Liotta, 1986; Cavanaugh and Nicolson, 1991a; Fodstad et al., 1988b; Hoffmann, 1992]. In addition to what has already been mentioned, the work of several groups has helped to elucidate this issue. The response to growth factors that are differentially expressed in different organs might determine whether tumor cells homing to these tissues will proliferate and give rise to overt metastasis. Specific in vitro growth enhancement has been observed with conditioned media obtained from different normal tissues when added to tumor cells that in vivo have shown preferential growth in the same tissues [Cavanaugh and Nicolson, 1991a]. The net effect of growth stimulatory and inhibitory factors present in these media may explain such in vitro/in vivo correlations. Similar relationships were seen for human melanoma cells incubated with conditioned medium from the lungs of nude mice or nude rats [Kjønniksen et al., 1991]. Cavanaugh and Nicolson [1991b] isolated a lung-derived factor that stimulated growth of lung-metastasizing tumor cells and identified it as transferrin. Interaction between the tumor and normal cells may, however, also result in alterations in several important tumor cell characteristics other than proliferation. One example is that the melanin production of murine melanoma cells seemed to be influenced by organ microenvironment [Price et al., 1988; Fidler, 1990]. Moreover, in the interaction with the microenvironment, tumor cells may also induce the normal cells to secrete proteases facilitating the development of the metastatic tumor [Basset et al., 1990].

Of particular interest is the recent data from several groups showing how growth conditions may significantly affect the chemosensitivity of tumor cells. It is well known from the clinic that metastases residing in different organs may differ in their response to therapy. We found in nude rat experiments that human tumor metastases that developed after inoculation of a human melanoma cell line showed differential sensitivity, depending on the site of growth [Kjønniksen et al., 1992a; Fodstad, 1993]. Thus, bone marrow tumors were much less sensitive to alkylating agents than lung and subcutaneous lesions. This finding might be ascribed to tissue-dependent differences in expression of the DNA-repair enzyme O^6-methyl-guanine-DNA-methyl transferase, presumably induced in the tumor cells through their interaction with the normal cells in the bone marrow. Teicher et al. [1990] have demonstrated that the drug resistant properties of murine EMT-6 tumor cells that were present in vivo could not be detected in monolayer tissue culture experiments. As a follow-up of this, it was recently shown that the same tumor sublines re-expressed their resistance to alkylating agents when grown as three-dimensional tumor spheroids [Kobayashi et al., 1993]. Evidence supporting the effect of the microenvironment on chemosensitivity has also been obtained with other murine [Wilmanns et al., 1992] and human tumors [Furukawa et al., 1993]. Altogether, these data demonstrate that the interaction between tumor cells and their microenvironment have profound effects of great biological and practical importance. The need for elucidation of the underlying mechanisms may have considerable implications on the direction of tumor metastasis research. Moreover, such work can lead to new approaches that may improve the therapeutic armamentarium available for clinical management of cancer patients.

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