Biological Diversity in Metastatic Neoplasms: Origins and Implications

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Summary. Whether neoplasms are unicellular or multicellular in their origin, the process of tumor evolution and progression can rapidly generate biological diversity. Metastases result from the survival and proliferation of specialized subpopulations of cells within the parent tumor. Metastases may have a clonal origin and different metastases may develop from different progenitor cells. However, as with the primary tumor, the origin of metastases is unimportant since the process of tumor evolution and progression can generate biological diversity within and among different metastatic foci.

The most fearsome and devastating aspect of cancer is the propensity of cells from malignant neoplasms to disseminate from their primary site to distant organs and there to develop into metastases. Despite remarkable advances in surgical treatment of primary neoplasms and aggressive adjuvant therapies, most cancer patients die of metastatic disease. There are several reasons for the present lack of success in treating metastases. Metastasis probably already has occurred at the time of diagnosis and initial treatment in most patients with solid tumors, excluding those suffering from various forms of skin cancer. Moreover, the metastases may be located in organs that are difficult to treat with effective concentrations of therapeutic agents without causing undesirable toxic effects. However, the most formidable obstacle to the successful treatment of disseminated cancer may well be the fact that the cells of a tumor are biologically heterogeneous. This phenotypic diversity, which allows selected variants to develop from the primary tumor, means not only that primary tumors and metastases can differ in their responses to treatment but also that individual metastases differ from one another. This diversity can be generated rapidly even when the tumors originate from a single transformed cell. A further complication arises because metastatic lesions are fairly large by the time they are diagnosed. A tumor mass at the lower limit of radiographic detection, say 1 cubic centimeter, may contain as many as 10^9 cells; eradication of 99.9 percent of these cells, a remarkable therapeutic achievement, still leaves 10^6 cells to proliferate, thus providing a large base for the further generation of biological heterogeneity. One of the important goals of today's cancer research is to better understand the mechanisms responsible for the spread of neoplastic cells and the generation of phenotypic diversity in primary and secondary neoplasms.

The Pathogenesis of Metastasis

The process of metastasis is a dynamic event that can be described as a sequence of interrelated steps. If the disseminating tumor cell fails to complete one of these steps, it is eliminated (Fig. 1). Thus, malignant cells that eventually develop into metastases have survived a series of potentially lethal interactions, whose outcome is dependent on both the responses of the host and on the intrinsic properties of the tumor cells [for a review, see (*1*)]. Metastasis begins with the local invasion of the host stroma by cells from the primary tumor. Among the mechanisms believed to play a role in this process are the generation of mechanical pressure, the release of lytic enzymes from either tumor cells or host inflammatory cells, and an increased motility of individual tumor cells (*2*). The tumor cells penetrate the vascular or lymphatic channels and then either grow at the site of penetration or detach and circulate as an individual cell embolus or as small embolic aggregates. In the circulatory system, tumor cells must evade host immune and nonimmune defenses such as blood turbulence, lymphocytes, monocytes, and natural killer cells (*3*). The tumor cells then arrest in the capillary beds of distant organs either by adhering to endothelial cells or by attaching to exposed basement membrane at the sites of endothelial cell retraction (*4*). They extravasate into the organ parenchyma probably by means of many of the same mechanisms that determine initial invasion (*2*). Growth in the organ parenchyma requires the development of a vascular network and continued evasion of the host immune system (*5*). Finally, the established metastases may themselves give rise to other metastases (*6*), and in a short period of time a small primary tumor can produce a large number of metastases.

Few cells survive this arduous and dangerous process to establish secondary foci and therefore metastasis can be regarded as an inefficient process (*7*). For example, the presence of tumor cells in the circulation does not predict the eventual formation of metastases (*8*). Using radiolabeled murine B16 melanoma cells injected directly into the venous circulation of mice, we have shown that less than 0.1 percent of the original inoculum survives to proliferate into secondary growths (*9*).

Consideration of this inefficiency has led us to question whether metastasis is a random or a selective process. That is, given the quantity of neoplastic cells that can be shed into the blood (*10*), does the development of metastases represent the fortuitous survival of a few tumor cells or the selection from the parent tumor of a subpopulation of metastatic cells endowed with properties that enhance their survival. Our studies, and data reported by others, support the latter possibility. Metastasis appears to be a highly selective process that is regulated by a number of imperfectly understood mechanisms (*11*). Surprisingly, this view may be more optimistic than one that postulates that cancer metastasis is a random event. A random event cannot be characterized or manipulated; however, a selective event is governed by rules that can be studied with the goal of developing improved therapeutic interventions. Putting our subjective feelings aside, the weight of evidence supports the view that metastasis is a selective process.

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The Metastatic Heterogeneity of Malignant Neoplasms

At the time of diagnosis, most animal and human neoplasms are composed of subpopulations of cells with distinctly different phenotypes. Cells isolated from one tumor have been shown to differ with respect to their growth rate, karyotypes, cell surface receptors for lectins, hormone receptors, immunogenicity, response to cytotoxic drugs, and capacity for invasion and metastasis (*12, 13*).

Two approaches have been used to isolate populations of cells with differing metastatic capacities from parent tumors. In the first, tumor cells are selected in vivo. These cells are implanted subcutaneously or intramuscularly or injected intravenously into mice, and metastasis is allowed to occur. The metastatic lesions are collected, and the cells that are recovered are used to repeat the process. The cycle is repeated several times. The behavior of the cycled cells is compared with the cells of the parent tumor to determine whether the selection process enriched the lines for cells with enhanced metastatic capacity. This procedure was originally used to obtain the B16-F10 line from the unselected B16 melanoma (*14*). The increase in metastatic capacity does not result from the adaptation of tumor cells to preferential growth in a particular organ (*15*). This procedure has been used successfully to produce tumor cell lines with increased metastatic capacity from many (*14, 16, 17*), but not all (*18*), of the experimental tumors tested.

In the second type of approach, cells are selected in vitro for the enhanced expression of a particular phenotype believed to be important in one step of the metastatic process. Again, the behavior of the cells in the appropriate host is assessed to determine whether the metastatic capacity is greater or less than that of the parent cells. This method has been used to examine whether tumor cell properties such as resistance to T lymphocytes (*19*), antibody-complement–dependent cytotoxicity (*20*), adhesive interactions (*16, 21*), lectin resistance (*22, 23*), invasive capacity (*24*), and resistance to natural killer cells (*3*) are instrumental in metastasis.

One obvious criticism of these studies is that the surviving variant line may have arisen as a result of adaptive rather than selective processes. To determine whether differences between metastatic capacities of tumor cell populations are preexistent or arise as a result of adaptation, we applied the classical fluctuation analysis of Luria and Delbruck (*25*) to our studies of the B16 melanoma (*26*). We reasoned that if a tumor was populated by cells with uniform metastatic capacity, isolated clones would produce equal numbers of metastases, whereas if a tumor was populated by cells with diverse metastatic capacity, different clones would produce varying numbers of metastases. We therefore performed the following experiment. A cell culture of the B16 melanoma was established from a subcutaneous mass grown in a syngeneic C57BL/6N mouse and was divided into two portions. One portion was maintained as a mass culture; the other portion was cloned to produce several cell lines, each one established from an individual cell. After incubation for the same period of time, equal numbers of tumor cells in suspension from each of the cloned lines and from the parent tumor were injected into syngeneic mice. The groups of animals injected with the uncloned, mixed line all produced a similar number of lung metastases, whereas the cloned sublines differed markedly from the parent tumor and among themselves in the numbers of metastases produced. Control subcloning experiments showed that this variability was not introduced by the process of cloning per se. Clearly, populations of cells with differing metastatic capacity existed within the original tumor (*26*).

The B16 melanoma has been repeatedly passaged in animals or culture for many times the life-span of its natural host. The observed metastatic heterogeneity of this tumor could be an artifact resulting from its longevity (*27*). However, exactly comparable data have now been obtained with another murine melanoma of much more recent origin. Kripke (*28*) has described the induction and isolation of a new melanoma syngeneic to the C3H/HeN mouse. The primary K-1735 melanoma was established in culture after a single transfer in an immunodeficient mouse. Cells from the fifth passage in vitro were used to produce clones. The clones and the parent tumor line were then analyzed for metastatic capacity in syngeneic mice. The clones differed dramatically from each other and from the parent line in their production of metastases in the lungs, lymph nodes, and other organs. Statistical analysis indicated that 20 of 22 K-1735 clones were significantly different from the parent tumor with regard to metastatic capacity (*29*). In the B16 melanoma system, 15 of 17 clones were significantly different from the parent tumor (*26*), and in a third tumor system (a recently induced fibrosarcoma), 15 of 21 clones were different from the parent tumor (*30*). These figures clearly indicate the degree of heterogeneity of tumors and show that tumors of recent origin are no less heterogeneous with regard to metastatic capacity than the venerable B16 melanoma. Longevity of neoplasms, therefore, is not a prerequisite for the generation of metastatic heterogeneity.

If cancer metastasis leads to the positive selection of cells better able to me-

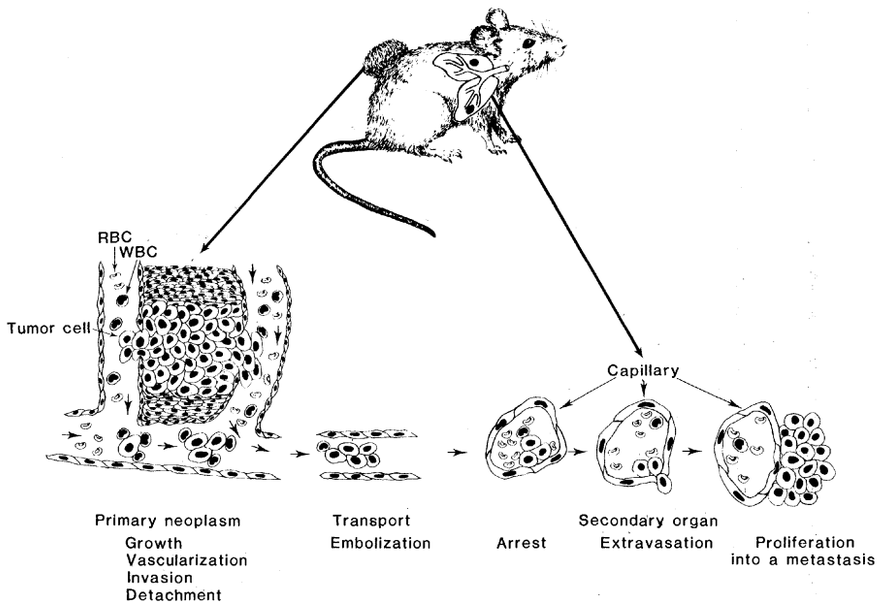

Fig. 1. The pathogenesis of a cancer metastasis. A metastatic cell must complete a complex series of steps in order to spread from the primary tumor to a distant organ and give rise to a clinical metastasis. (*RBC* and *WBC*, red and white blood cells, respectively.)

tastasize, then those cells populating secondary foci should be more metastatic than the cells of the primary tumor. Some support for this possibility comes from the initial selection experiments in vivo already discussed. Serial passaging of B16 melanoma cells produced the significantly more metastatic B16-F10 cell line (*14*). Comparable results have been obtained recently with the several newly developed mouse tumors (*31*). Syngeneic mice were given intramuscular injections into one of their hind footpads of cells derived from the parent tumor. The resulting "primary tumors" were allowed to grow large enough to produce spontaneous pulmonary metastases, and cell lines were established from individual lung nodules. The metastatic capacity of cells from the spontaneous metastases was found to be always higher than that of cells from the parent tumor.

The issue of whether metastasis is a selective or random process is somewhat controversial because some investigators who used the in vivo selection technique reported results that were inconsistent with our data (*18*). The discrepancies in the data may have been caused by differences in the relative heterogeneity or homogeneity of the various tumor systems used and by differences in experimental conditions. We attempted to minimize such variables by using three variant lines isolated from the B16 melanoma (*31*): (i) the B16-F1 variant, an unselected tumor line that is heterogeneous and poorly metastatic; (ii) the B16-F10 variant, a tumor line selected in vivo for its high lung-colonizing potential; and (iii) the B16-BL6 variant, a tumor line selected in vitro for its invasiveness and its high metastatic capacity. All tumor lines were implanted subcutaneously in normal mice and allowed to metastasize. The metastases were recovered and established as individual cell lines. The metastatic abilities of these new cell lines were compared with those of the respective parent variant tumors. Metastases produced by the unselected (that is, heterogeneous) and poorly metastatic parent B16-F1 tumor were composed of cells with increased metastatic capacity. In contrast, metastases produced by the previously selected (more homogeneous) B16-BL6 tumor lines were not composed of cells that were more metastatic than the parent variant tumor lines. Thus, even under controlled conditions the process of metastasis could have the appearance of being either selective or random depending on the nature of the starting population (*31*). The heterogeneous nature of unselected cell lines allows selection of variants with higher metastatic capacity (*32, 33*), whereas the homogeneous nature of the selected cell lines restricts further selection.

Tumor Evolution and Progression

These findings raise an interesting question: If cells populating metastases generally are more metastatic than most cells populating the parent tumor, why does development of a new cell line, such as the B16-F10 melanoma line, require ten selection cycles rather than one? We can explain this apparent paradox only by considering specific aspects of tumor evolution and progression. First, it is necessary to understand the origin of the cellular heterogeneity in malignant neoplasms. There are several possibilities. Tumors could have a multicellular origin, for example, clinical cancer often appears to be multifocal (*34*). In this case, the diverse metastatic populations would reflect their diverse parentage (*35*). However, most (*27, 36, 37*), but not all (*35*), human cancers probably result from the proliferation of a single transformed cell. The generation of biological diversity in these tumors of unicellular origin is probably attributable to the process of tumor evolution.

Clinical observations of neoplasms have suggested that tumors undergo a series of changes during the course of the disease. Thus, a tumor that was initially diagnosed as benign (noninvasive, nonmetastatic) can be transformed over a period of many months or even years into a malignant tumor. This phenomenon, termed neoplastic progression, has been defined by Foulds as acquisition of permanent, irreversible qualitative changes of one or more characteristics in a neoplasm (*38*). The loss or acquisition of various characteristics can occur independently and over protracted periods of time. Moreover, since tumor progression in vivo occurs in the host, the phenomenon is influenced by homeostatic factors in the host that act as selective pressures (*39*). Nowell (*27*) has suggested that acquired genetic variability within developing clones of tumor cells together with these selection pressures allow new sublines to emerge with a growth advantage that is manifested by increased malignancy. We explored the issue of whether tumors of unicellular origin are able to generate metastatic heterogeneity by performing the following experiment. Six clones of BALB/c mouse embryo fibroblasts transformed by a cloned murine sarcoma virus were propagated as individual cell lines. Intravenous injection of viable cells from each clone into syngeneic mice demonstrated that there were marked differences among the clones with regard to the ability to form lung metastases. The metastatic capacity of the different clones was unrelated to the degree of virus expression as assessed by level of serum antibodies to mouse sarcoma virus proteins. Because each tumor line was derived from the progeny of a single, transformed cell, the data indicate that multicellular origin of a neoplasm can indeed be responsible for its metastatic heterogeneity. However, when one colony with a high metastatic capacity and another colony with a low metastatic capacity, both originating from single cells, were subcloned 42 days after transformation and evaluated for metastatic capacity, both exhibited striking heterogeneity with regard to this characteristic. These data demonstrate that whether tumors have a unicellular or multicellular origin, they can rapidly become heterogeneous with respect to the metastatic phenotype (*33*).

Evolution and progression occurs not only in the primary tumor but also within the metastases themselves. Tumor progression and evolution can generate both more metastatic and less metastatic variants, and therefore the experimental isolation of an individual metastasis does not guarantee that the cells derived from it invariably will be more metastatic than cells isolated from the parent tumor. Although increased metastasis appears to have been the general finding in the experiment we performed, it is theoretically possible that some metastases could, as a result of generation of diversity, be less metastatic than their parent lines; perhaps this is why selection of the B16-F10 tumor required ten cycles.

Metastases within one host can exhibit heterogeneity with regard to many characteristics beside metastatic capacity, such as hormone receptors (*40*), marker enzymes (*37, 41*), antigenicity or immunogenicity (*13, 42*), and response to various chemotherapeutic agents (*43*). This biological diversity may be a consequence of tumor progression, or it may result from the nature of tumor cell dissemination. There are several unanswered questions: Do tumor emboli that survive the many steps of metastasis to form metastases consist of single cells or cell aggregates? How does the composition of these emboli affect the malignant process? Can embolic clumps survive in the circulatory system and give rise to homogeneous metastases of monoclonal origin?

Pathologists have long recognized that primary tumors are made up of zones of

morphologically distinct cells. Recent studies have demonstrated that these zonal differences are not restricted to morphology alone, but include other biological characteristics as well (*44*). These findings present the possibility that embolic aggregates arise from a single zone of the tumor and thus exhibit a degree of uniformity for specific characteristics. Then, irrespective of whether only the central cell or all the cells of the clump survive, the resulting metastatic growth is analogous to a primary tumor of unicellular origin with regard to the subsequent development of heterogeneity. The same situation could arise if only one cell survived in a mixed embolus derived from an area of zonal junctions. In these situations the origin of the metastasis would be essentially unicellular and diversity would result from tumor evolution and progression. Alternatively many, or all, of the cells forming a mixed embolus may survive to proliferate, in which case diversity would result from the multicellular origin of the neoplasm (Fig. 2).

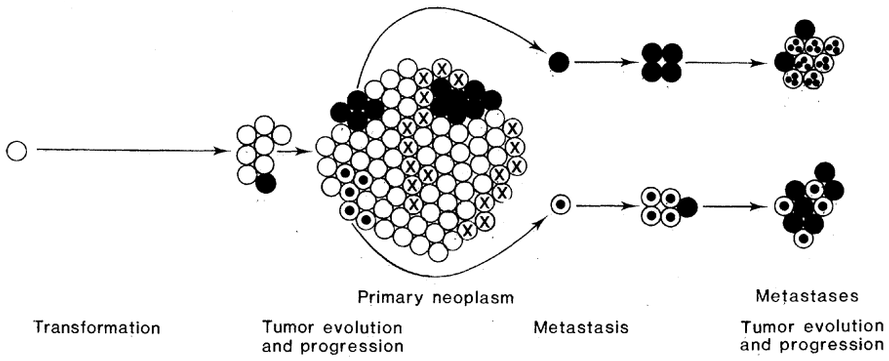

Fig. 2. The origin of tumor heterogeneity. Many human or animal tumors are unicellular in their origin. Tumor evolution or progression may rapidly generate cellular diversity. Metastasis is a process that selects metastatic cells from within a heterogeneous parent tumor. Some metastases can be clonal in origin, and metastases of one parent tumor can develop from different progenitor cells. Regardless of their origin, the process of tumor evolution is responsible for the generation of tumor cell heterogeneity within and among metastases.

We performed a series of experiments to determine (i) whether individual metastases are clonal in their origin and (ii) whether the metastases from one tumor derive from different progenitor cells by utilizing the fact that x-irradiation of tumor cells induces chromosomal damage (*45*). We reasoned that all tumor cells populating a single spontaneous metastasis arising from x-irradiated cells would exhibit the same chromosomal arrangement if the metastasis were derived from one cell and that metastases from one tumor would exhibit different chromosomal arrangements if the metastases were derived from different progenitor cells.

In these experiments, cells from a metastatic line of the K-1735 melanoma were exposed to 650 roentgens of x-radiation to induce chromosomal breaks and rearrangements and then were injected into the footpads of syngeneic C3H mice. We performed chromosome analysis on at least 100 spreads of each individual line. In 10 of 21 lines, all the chromosomes were telocentric and, therefore, these metastases were noninformative. In the other 11 lines, single or multiple marker chromosomes (submetacentric, metacentric, minute) were observed. In eight of these lines, unique patterns of chromosomes were found in most spreads, suggesting that each metastasis originated from a single cell. In the remaining three lines, the pattern of markers varied, suggesting a bi- or multimodal origin. However, G-band analysis indicated that these variations probably represented evolution within the individual metastasis. This experiment does not resolve the question of whether metastases arose as a consequence of individual cells surviving in the blood stream or whether homogeneous clumps survived in the circulation, but it does indicate that many secondary foci originate from single cells or the progeny of single cells. Because the metastases each exhibited a unique pattern of marker chromosomes, the data also indicate that different metastases could originate from a different progenitor cell (*45*). These findings help to explain the biological diversity of multiple metastases proliferating in the same host (*13, 37, 40–43*).

We have recently found that highly malignant tumor variants are more likely to undergo rapid evolution and progression than their less malignant counterparts. This finding is compatible with the hypothesis of tumor progression formulated by Nowell (*27*) that states that increasing progression toward malignancy is accompanied by increasing genetic instability of the evolving cells. To test this hypothesis, we examined the metastatic stability of several tumor lines with different capacities for spontaneous and experimental metastasis. Concomitantly, we determined the rates at which paired metastatic and nonmetastatic cloned lines isolated from four different neoplasms became resistant, by mutation, to ouabain or 6-thioguanine (*46*). Poorly metastatic and highly metastatic clones were isolated from the UV-2237 fibrosarcoma (*30*) and were cultivated in vitro for 72 or 60 days, respectively. Simultaneously, both clones were also grown subcutaneously in syngeneic mice. Then, cell cultures were established from these solid tumors, and 1 week later subclones were isolated. The ability of these subclones to form experimental metastases was compared to that of subclones derived from clones grown in culture and to that of subclones isolated and frozen when parent clones were initially established.

The patterns of behavior of all the subclones derived from the poorly metastatic clone were remarkably similar to that of the parent clone, regardless of whether the subclones were derived at the time of isolation or after 72 days of continuous growth in vitro or in vivo. In contrast, the metastatic behavior of the subclones derived from the highly metastatic clone differed considerably from that of the parent clone. Significant diversity had been generated by 60 days after cultivation both in vitro and in vivo, suggesting that the metastatic phenotype of the highly metastatic clone is unstable (*46*). This rapid generation of diversity may have been caused in part by increased genetic instability. We found that in the highly metastatic clone the rate of spontaneous mutation to ouabain- and 6-thioguanine–resistance was 3- and 4.6-fold higher, respectively, than in the poorly metastatic clone. Similar differences in the rates of spontaneous mutation of highly metastatic and poorly metastatic cells were found when these studies were extended to three other mouse tumor systems; in the UV-2237 fibrosarcoma, the K-1735 melanoma, and the spontaneous SF-19 fibrosarcoma, the more highly metastatic cells showed an increase in the rate of spontaneous mutation to ouabain resistance of 6.5-, 7.0-, and 5.8-fold, respectively (*46*).

These results are in accord with the hypothesis that tumor progression can occur as a result of acquired genetic alterations. However, the evolution of metastatic heterogeneity did not progress in a unidirectional manner (some subclones were less metastatic than the

parent clone), and therefore the results are not incompatible with Nowell's hypothesis (*27*). Perhaps the lack of selection pressure that is characteristic of conditions in vitro and the relatively benign milieu of the subcutis combined with the increased rate of mutation led to the emergence of both less metastatic and more metastatic variants. Indeed, recent experiments on mutagenesis in other murine neoplasms support this explanation. Mutagenesis of malignant cell lines has produced clones incapable of progressive growth in normal hosts (*47*) and variants with metastatic capacities more than (*48*), less than, or equal to the parent tumor (*23, 49*). We conclude from our studies, that increased metastatic capacity frequently is associated with an increased rate of spontaneous mutation, a state of affairs that is reflected in the relative lack of stability of the metastatic phenotype and the generation of variants of both higher and lower metastatic capacity.

Relative Phenotypic Stability of Polyclonal Populations

Considering that malignant cells have higher mutation rates than benign cells, the fact that cells populating metastases so frequently exhibit greater metastatic capacity than cells of primary tumors is remarkable. The question arises, how are degrees of difference maintained between various tumor populations? For example, the B16 melanoma sublines B16-F1 and B16-F10 have maintained their relative metastatic capacities for over 8 years (*50*). What has prevented these differences from being obliterated by the creation of new variants in the manner we have proposed to explain the generation of diversity in tumors of unicellular origin? One reason may be that individual tumor cells or specific subpopulations within the tumor mass are not autonomous units but interact with, and are regulated by, other neoplastic cells. Different subpopulations of tumor cells have been shown to affect the growth patterns and chemosensitivity of other groups of cells (*51*). Similar regulatory control may exist for the metastatic phenotype of different cells residing within a tumor (*52*).

We explored this idea by comparing the stability of the metastatic phenotype of cloned, uncloned, and polyclonal tumor populations. Two poorly metastatic clones and two highly metastatic clones were isolated from the B16-F10 melanoma line. These clones were cultured in vitro or in vivo for 10, 20, or 40 passages (5, 10, or 20 weeks) and then subclones derived at each of these intervals were reassessed for their metastatic ability. After only ten passages in vitro, many subclones that differed significantly from the parent clone were isolated. Continued cultivation introduced more variability such that by 20 and 40 passages, most clones tested differed significantly from their respective parent clone. In marked contrast, the metastatic phenotype of the uncloned B16 melanoma lines B16-F1 and B16-F10 remained remarkably stable over 30 passages in vivo or 60 passages in vitro. Similar stability was attained when different clones were mixed together and cocultivated as polyclonal populations; however, removal of all clones but one then led to the rapid generation of biological diversity in the remaining clone (*52*).

Collectively, these data suggest that different subpopulations of tumor cells act to stabilize their relative proportions and thus impose an equilibrium on the combined population. Removal of the stabilizing effect, by isolating clones or by applying a strong selection pressure such as chemotherapy, leads to rapid diversification in the resurgent populations. Once these populations become relatively heterogeneous they again stabilize, thus achieving equilibrium (*52, 53*). Although we do not as yet understand the nature of these stabilizing influences and their mode of action, their very existence argues against random tumor development. On the contrary, the society of tumor cells imposes regulatory constraints upon its individual members. The maintenance of cell heterogeneity may have the advantages of multiformity without the disadvantages of overspecialization. Certainly, this phenomenon, irrespective of its underlying mechanisms, further complicates attempts to understand the metastatic process.

Conclusions

The heterogeneous nature of tumors has many ramifications for studies of tumor biology, in general, and studies of metastasis, in particular. However, the complexity of the metastatic process makes it difficult to provide generalized explanations. Bearing this in mind we offer the following synopsis: By the time of diagnosis, many malignant neoplasms are heterogeneous and contain subpopulations of cells with different biological characteristics. Tumor heterogeneity with regard to numerous phenotypes, including metastatic capacity, may be the consequence of the multicellular origin of a neoplasm or it may be the result of continuous evolution and progression in tumors of unicellular origin. Either of these modes of diversification may be operative in the primary or the metastatic tumor. Metastatic cells appear to be less stable genetically and phenotypically than nonmetastatic cells. This acquired instability means that in the presence of strong selection pressures, such as those that occur during tumor spread, it is possible that the metastatic clones are more likely to survive and emerge as the progenitors of secondary tumors. The process of metastasis is not random but rather is selective for metastatic subpopulations of cells within a heterogeneous malignant neoplasm. Some metastases may be clonal in their origin, and metastases proliferating in the same host can originate from different progenitor cells (Fig. 2).

The acquisition of phenotypic heterogeneity by populations of tumor cells imposes a degree of stability on the tumor as a whole. The mechanisms that produce this equilibrium within the parent mixed tumor are completely unknown at present but seem to prevent random behavior of heterogeneous cell populations; tumors therefore profit from the benefits of diversity.

The generation of biological diversity in malignant neoplasms within and among their metastases has profound implications for both studies on the pathogenesis of cancer metastasis and the design of any successful approach to the treatment of this disease (*53*).

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14. Tumor cell variants of the B16 melanoma were

selected by injecting syngeneic C57BL/6N mice intravenously with parent tumor cells. After 2 to 3 weeks, grossly visible pigmented lung tumor colonies were removed, and the tumor cells were adapted to tissue culture. The B16 cells that grew in culture after the first selection in vivo were established as a continuous line and were designated B16-F1. Cells from this line were reinjected into new syngeneic animals, and 3 weeks later a new group of lung tumor colonies was removed and cultured to yield B16-F2. With each succeeding cycle in vivo the ability of the selected B16 lines to implant, survive, and form lung tumors increased [I. J. Fidler, *Nature (London)* **242**, 148 (1973); *Cancer Res.* **35**, 218 (1975)]. After ten such selections the B16-F10 tumor line was obtained. This cell line forms significantly more gross lung tumors per input of cells than the B16-F1 tumor after intravenous or intracardiac injection into syngeneic C57BL/6 mice [I. J. Fidler and G. L. Nicolson, *J. Natl. Cancer Inst.* **57**, 1199 (1976)].
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How the Law of the Sea Treaty Will Affect U.S. Marine Science

David A. Ross and John A. Knauss

Negotiations concerning marine science and other issues at the third United Nations Conference on the Law of the Sea (UNCLOS III) began in 1974. On 30 April 1982 a Law of the Sea Treaty (*1*) was approved by a vote of 130 to 4—the United States, Venezuela, Turkey, and Israel voted against it and there were 17 abstentions. Eventually 60 nations must ratify the treaty for it to enter into force. The United States, in spite of its negative vote, can still eventually sign and later ratify the treaty, but the present Reagan Administration seems to be firmly against this option. U.S. marine scientists must understand, however, that once the treaty enters into effect, coastal states which have ratified it can, and probably will, enforce its regulations on all those who wish to do marine scientific research in their waters. The new regime for the ocean resulting from these negotiations will change markedly the way in which marine scientists and marine scientific research operate. If the treaty enters into force, the marine science articles will restrict many activities of U.S. marine scientists, as well as offer certain opportunities, whether or not this country signs or ratifies the treaty.

The history of marine science negotiations during UNCLOS III has already been discussed (*2*). Most countries supported restrictions on marine research. Its staunchest supporters were the United States, the Soviet Union (until 1976), West Germany, the Netherlands, and occasionally Japan (*3*).

The Law of the Sea Treaty

The treaty recognizes several distinct juridical regions of ocean space including internal waters, territorial seas, straits used for international navigation, archipelagic waters, exclusive economic

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