Development of Biological Diversity and Susceptibility to Chemotherapy in Murine Cancer Metastases

James E. Talmadge,1 Karen Benedict,2 John Madsen,3 and Isaiah J. Fidler3

Preclinical Screen Laboratory, Program Resources, Inc. [J. E. T., K. B.], and Cancer Metastasis and Treatment Laboratory, Basic Research Program-LBI [J. M., I. J. F.], National Cancer Institute-Frederick Cancer Research Facility, Frederick, Maryland 21701

ABSTRACT

We studied the development of biological heterogeneity in a spontaneous melanoma metastasis of clonal origin as demonstrated by karyotypic analysis. The metastatic potential and sensitivity to different chemotherapeutic agents varied both among and within clones of this metastasis isolated either in vitro or in vivo. This finding indicates that, even within a metastasis of clonal origin, cellular heterogeneity for chemotherapeutic or metastatic potential can develop rapidly and provides a mechanism for the emergence of resistance to therapy. Since most cancer deaths result from metastases that do not respond to treatment, the implications of these findings for the treatment of cancer are clear. Treatment modalities must be designed that circumvent the biological heterogeneity that can develop rapidly within each metastasis and among metastases.

INTRODUCTION

Primary malignant neoplasms are not uniform in composition but consist of subpopulations of cells that differ in their biological characteristics, including invasiveness and metastatic potential (5-7, 14-20). Whether these neoplasms are unicellular or multicellular in origin, the development of variant cells during progressive growth results in heterogeneity. Studies from several laboratories have shown that metastases can result from the selective growth of metastatic subpopulations that preexist within the primary neoplasm (7, 14, 23-25). Many spontaneous (26) and experimental (20) metastases can have a clonal origin, and multiple metastases can result from the proliferation of different progenitor cells (26). The findings that metastatic cells have higher mutation rates than do nonmetastatic cells (3) and that heterogeneity develops more rapidly in tumors containing few subpopulations of cells (17) suggest that tumor evolution and progression (15) will result in the rapid development of biological diversity in solitary metastases, even when a metastasis is of clonal origin (based on karyotypic markers).

The phenotypic diversity that has been observed within primary tumors (1, 10, 21, 27, 28) has serious implications for the treatment of neoplastic disease. Furthermore, different metastatic foci, as a tumor population, vary in their sensitivity to cytostatic agents (2, 8, 21, 27-29) and provide major impediment to the successful chemotherapy of systemic disease. The suggestion that the cell population within individual lesions could progress (15), developing variant sensitivities to common therapeutic modalities, has serious implications for the development of therapeutic regimens. In the present study, we report on our investigations of the development of phenotypic diversity within spontaneous metastases of clonal origin.

MATERIALS AND METHODS

Animals. Adult C3H/HeN mammary tumor virus-negative mice, 6 to 8 weeks old, were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility. Tumor. The original K-1735 melanoma (11) was a gift from Dr. Margaret L. Kripke, National Cancer Institute-Frederick Cancer Research Facility. The K-1735-M2 line has been derived from a spontaneous pulmonary metastasis produced by the K-1735 parent tumor growing at a s.c. site (25). The K-1735-M2 cells were exposed to 650 rads of X-radiation to induce random chromosome breakage and rearrangements. These irradiated cells were implanted into the footpads of syngeneic mice. When the resulting s.c. tumors reached an average diameter of 1 cm, the tumor-bearing leg, including the popliteal lymph node, was resected at midfemur. Six weeks after resection, the mice were necropsied, and numerous well-isolated solitary, spontaneous pulmonary metastases were aseptically removed, grown in culture as individual lines, and karyotyped (26). As reported previously, within certain metastases, the same chromosomal abnormalities were detected in all of the cells examined, suggesting that these metastases were of clonal origin. One such cell line, X-met-21, was used in the present study.

All tumor cell lines were maintained in tissue culture in complete minimal essential medium (Eagle’s complete minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution; Flow Laboratories, Rockville, MD). Cell cultures were maintained on plastic and were incubated in 5% CO2 at 37°C. Cultures were routinely monitored for and found free of Mycoplasma and the following murine viruses: reovirus type 3; pneumonia virus; K virus; Thielers encephalitis virus; Sendai virus; minute virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

Experimental Metastasis Assay. Tumor cells in exponential growth phase were harvested by a brief exposure to 0.25% trypsin-0.02% EDTA. The flask was tapped sharply to dislodge the cells, complete minimal essential medium was added, and the cell suspension was pipetted again to prevent aggregation. The cells were washed and resuspended in Ca2+ and Mg2+-free Hanks’ balanced salt solution to a cell concentration of 1.25 x 106 cells/ml. Cell viability was determined by trypan blue exclusion, and only suspensions exhibiting more than 90% viability were used. All suspensions were composed of single cells, as determined during hemocytometer counts, and mice were given injections of 0.2-ml aliquots of these suspensions in a lateral tail vein. Three weeks after tumor cell injection, the mice were killed, and the lungs were removed, washed, and fixed in Bouin’s solution to differentiate the neoplastic lesions from the organ parenchyma. The lung nodules were counted with the aid of a dissecting microscope.

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2 Recipient of support from the National Cancer Institute, Department of Health and Human Services, under Contract NO1-CO-23910 with Program Resources, Inc.
3 Recipient of support from the National Cancer Institute, Department of Health and Human Services, under contract NO1-CO-23909 with Liton Bionetics, Inc. Present address: Department of Cell Biology, M. D. Anderson Hospital and Tumor Institute, The University of Texas System Cancer Center, HMB-173, Houston, TX 77030.

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In Vitro Cloning. The K-1735 X-met 21 line was cloned in vitro by a limiting dilution technique described previously (7). Briefly, viable single tumor cells were suspended in supplemented medium at 5 cells/ml. Each 38-sq cm well of a 96-well plate was seeded with 0.2 ml of suspension. After a 24-hr incubation period, the wells were examined, and those wells containing a single attached cell were marked clearly. Fourteen days later, those cultures showing cell growth were harvested by a brief trypsinization, and the individual cloned populations were expanded on plastic.

Clonal Assay of Sensitivity to Chemotherapeutic Agents. Three hundred single viable cells from cultures of the indicated tumor cell lines were suspended in supplemented culture medium and plated into each 38.5-sq cm well of a 6-well plate. Chemotherapeutic drugs at the following concentrations were added to the individual plates 4 hr later, after the cells had attached VCR (1, 2, 5, 7, and 10 ng/ml), ADR (1, 5, 10, 50, and 100 ng/ml), and BLEO (100, 500, 1000, 2500, and 5000 ng/ml). These drugs and dosages were based on previous studies from this laboratory with the K-1735 melanoma tumor (28). Twelve hr thereafter, the cultures were washed twice to remove the drug and refed with fresh medium. After a 10-day incubation period, the dishes were washed, and tumor colonies were fixed with absolute methanol and stained with méthylène blue. The number of colonies with more than 100 cells was determined with the aid of an automated colony counter. This assay was repeated 3 times for each cell line.

The relative sensitivity of the cell lines was estimated as follows: the number of colonies formed following exposure to one of the 4 drugs was divided by the average of the number of colonies formed by untreated cells and was expressed as the proportion surviving. The dose-dependent relationship of these proportions was tested with a linear regression, using the proportion surviving as the dependent variable and the logarithm of the dose as the independent variable. In the great majority of cases (73 of 76), a highly significant dose-response relationship was observed. Based on the regression equation, the logarithms of the IC<sub>50</sub> and IC<sub>90</sub> estimates of the individual cell lines were then compared to the corresponding estimates of their parent control using Cochran's approximation to the Behrens-Fisher solution (22). The chemotherapeutic drugs used in these studies were generously provided by Dr. John Douros of the National Cancer Institute.

Experimental Design. Immediately after the X-met-21 line was established in culture, we cloned it in vitro to produce 10 clones (C-1 to C-10) using a limiting dilution technique (see Chart 1 for a scheme of the experimental design). To examine how cells within a metastasis can diversify with regard to their metastatic potential and response to anticancer drugs, we implanted X-met-21 cells, immediately after establishment of the cell line, into footpads of syngeneic mice. Sixty days later, a tumor growing s.c. was reestablished in culture (SC-60 line). At the same time that SC-60 was established as a cell line, 7 individual spontaneous lung metastases (from 3 different mice) were harvested and established in culture as individual lines (SM-A1 to SM-A3, SM-C1, and SM-D1 to SM-D3). We examined all of the isolated cell lines (C1-C10 and SM-A1 to SM-A3, SM-C1, and SM-D1 to SM-D3) with regard to their ability to produce experimental lung metastases following injection into the venous circulation, sensitivity to several chemotherapeutic agents, and the expression of the unique marker chromosome. These studies were undertaken simultaneously for each group of variants; thus, the metastatic potential, sensitivity to chemotherapeutic agent, and karyotype were examined for all of the clones or spontaneous metastases at one time point.

RESULTS

The X-met-21 line was established from a solitary pulmonary metastasis in a mouse bearing a footpad tumor from K-1735-M2 tumor cells. Prior to the injection, the K-1735-M2 cells were X-irradiated, which randomly induces chromosome breakage and rearrangements. One of the resultant spontaneous metastases, X-met-21, had a chromosome mode of 42, and all of the cells (>1000 spreads) expressed the same new and unique submetacentric chromosome marker arm length ratio, (3:4). This finding clearly indicates that the X-met-21 metastasis was clonal in its origin (26).
The experimental metastatic potential of cells from the original X-met-21 line (parent) and 10 clones isolated in vitro is shown in Table 1. In this experiment, 6 of 10 clones differed significantly from the parent tumor in their capacity to produce lung tumor colonies (Mann-Whitney U test). The experimental metastatic potential of cells from the X-met-21 population did not change following s.c. growth for 60 days (SC-60) as compared to the parent line X-met-21 (p = 0.45) cultured in vitro (Table 2). This experiment suggests that, as a cellular population, X-met-21 expresses a stable experimental metastatic phenotype. However, the tumor cells from 6 of 7 spontaneous metastases differed significantly in their metastatic potential as compared to 60, the variant of X-met-21 which had been carried at a primary s.c. site for 60 days (Table 2). Moreover, the cells from the cloned lines and from spontaneous metastases differed greatly within each group (cloned or spontaneous metastases) in their ability to produce experimental metastases (p = 0.0001; Kruskal-Wallis test, 3 approximation). In contrast to this diversity in metastatic potential, all cells examined stably expressed the unique submetacentric chromosome marker, as well as the modal number of chromosomes.

In the next set of experiments, we used an in vitro colony-forming inhibition assay to determine the relative sensitivity of tumor cells from the parent line, the cloned lines, and the spontaneous metastases to various chemotherapeutic drugs. The results, summarized in Table 3, report the IC$_{50}$ and IC$_{90}$ of the cell lines to these chemotherapeutic agents, as determined by linear regressions. Statistical analysis (based on the IC$_{50}$) of the differences in drug sensitivity revealed that, for AMSA, 9 of 10 clones and 2 of 7 metastases, for ADR, 8 of 10 clones and 3 of 7 metastases, for BLEO, 5 of 10 clones and 7 of 7 metastases, and for VCR, 2 of 10 clones and one of 7 metastases differed significantly from the parent tumor line in their sensitivity to the drugs. This variability in sensitivity to chemotherapeutic agents was reproducible and was not associated with artifacts of the cloning or selection procedures. We base this conclusion on a study in which 5 subclones isolated from a benign clone of the K-1735 melanoma were indistinguishable from the parent clone in their response to cytotoxic drugs (data not shown). In Table 4, we demonstrate 4 experiments with the parent cell line (X-Met-21) in which we examined their sensitivity to AMSA, ADR, BLEO, and VCR. In these experiments, the sensitivity of the parent tumor line to the agent did not differ significantly between the first experiment and subsequent experiments (AMSA, p = 0.241; ADR, p = 0.1954; BLEO, p = 0.2571; VCR, p = 0.171), demonstrating that the assay is highly reproducible.

### DISCUSSION

Despite impressive advances in the surgical resection of primary neoplasms and aggressive adjuvant therapies, most cancer deaths are attributable to metastases. There appear to be several reasons for this lack of success in controlling metastatic foci. (a) By the time cancer is diagnosed, metastases may already be present in several organs of the patient's body, making surgical resection or destruction by radiotherapy or chemotherapy unlikely. (b) In spite of the development of promising anticancer drugs and regimens, their efficiency is hindered by the occurrence of drug-resistant variants within tumors. This tumor cell resistance to conventional therapy is probably the single most important factor responsible for the refractory response of tumor therapy. This phenotypic diversity, which allows selected variants to develop within the primary tumor, means not only that primary tumors and metastases can differ in their responses to treatment but also suggests that individual tumor cells within a metastasis may differ from one another. This diversity can be generated even when the tumors originate from a single transformed cell. This complication appears to arise in part because metastatic lesions are fairly large by the time they are diagnosed. A tumor mass at the lower limit of radiographic detection, for example, 1 cm, may contain as many as 10$^8$ cells. The eradication of 99.9% of these cells, a remarkable therapeutic achievement, still leaves 10$^3$ cells to proliferate, thereby providing a large tumor base for the outgrowth of a resistant population and the further generation of biological heterogeneity.

The demonstration that spontaneous metastases result from the clonal expansion of highly specialized cells and that clonal cells (17), as well as cells from metastases, have a high rate of spontaneous mutation (3) as compared with nonmetastatic tumorogenic cells suggests that clonal metastases may rapidly become heterogeneous. We were interested, therefore, in investigating whether tumor evolution and progression could occur within a clonal metastasis. The demonstration of clonal origin of spontaneous metastases (26), which was supported by kary-
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Tumor cell sensitivity to chemotherapeutic drugs

Three hundred single viable cells from cultures of the indicated lines suspended in supplemented tissue culture medium were plated into 38.5-sq cm culture wells (6 wells/test). AMSA, ADR, BLEO, or VCR was added to the dishes 4 hr later. Twelve hr thereafter, the cultures were washed twice and refed with fresh medium. After 10 days of incubation at 37° in a humidified incubator with a 5% CO₂ atmosphere, the cultures were washed, and the tumor colonies were fixed with absolute methanol and then stained with methylene blue. The number of colonies (>100 cells) was determined with the aid of an automated bacterial colony counter. This assay was repeated 3 times.

<table>
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<tr>
<th>Tumor variant</th>
<th>ADR</th>
<th>AMSA</th>
<th>BLEO</th>
<th>VCR</th>
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<tr>
<td>X-Met-21 (parent)</td>
<td>IC₀</td>
<td>33.1</td>
<td>102.6</td>
<td>1.1</td>
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<td>C1-1</td>
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<td>63.2</td>
<td>1.2</td>
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* The significant difference in the log IC₅₀ and IC₆₀ estimates of the individual cell lines as compared to the parent line (X-met-21) by the t test using Cochran’s approximation to the Behrens-Fisher solution (22). The sensitivity of the cells to VCR formed a relatively flat linear regression, and no IC₅₀ could be accurately determined.

Reproducibility of the assay for chemosensitivity

The parent cell line (X-met-21) from a spontaneous metastasis was examined in 4 separate experiments to determine its sensitivity to the cytostatic agents AMSA, ADR, BLEO, and VCR. Three hundred single viable cells from cultures of the indicated lines suspended in supplemented tissue culture medium were plated into 38.5-sq cm culture wells (6 wells/test). ADR, AMSA, BLEO, and VCR at the indicated concentrations were added to the dishes 4 hr later. Twelve hr thereafter, the cultures were washed twice and refed with fresh medium. After 10 days of incubation at 37° in a humidified incubator with a 5% CO₂ atmosphere, the cultures were washed, and the tumor colonies were fixed with absolute methanol and then stained with methylene blue. The number of colonies (>100 cells) was determined with the aid of an automated bacterial colony counter.

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<td>IC₀</td>
<td>33.1</td>
<td>102.6</td>
<td>1.06</td>
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<td>X-Met-21</td>
<td>IC₀</td>
<td>31.3</td>
<td>104.8</td>
<td>1.31</td>
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* No significant difference compared to the first analysis of the parent tumor (X-met-21). The log IC₀ and IC₆₀ estimates of the individual cell lines as compared to the parent line (X-met-21) using Cochran’s approximation to the Behrens-Fisher solution (22). The sensitivity of the cells to VCR formed a relatively flat linear regression, and no IC₅₀ could be accurately determined.

In the present studies, distinct differences in metastatic properties and drug sensitivity were found in many of the in vitro and in vivo isolated clones which were rapidly established from a spontaneous metastasis of clonal origin. In contrast to the heterogeneous sensitivity to chemotherapeutic drugs and metastatic potential which we observed in our studies, the expression of the marker chromosome was extremely stable both in vitro and in vivo. Furthermore, the intralex differences in chemosensitivity and metastatic potential could not be attributed to in vivo fusion of tumor cells with each other or normal host cells, since tetraploid karyotypes were only rarely observed. It appears, therefore, that the progressive growth of metastases results in the development of biological intralexional heterogeneity. The studies of heterogeneity and sensitivity to chemotherapeutic agents revealed both increased resistance and sensitivity to the drugs under investigation. However, neoplastic lesions in patients receiving chemotherapy may not contain variants with increased sensitivity to specific drugs, presumably due to the selective pressure of chemotherapy. Siracky (21) performed a sequential study of in vitro drug sensitivities and karyotypic analysis of ascites cells from patients undergoing chemotherapy for ovarian carcinoma, as well as from cell suspensions of metastatic lesions from a number of the same primary tumors. He concluded that chemotherapy resulted in the emergence of preexistent drug-resistant cell clones in tumors which were initially of heterogeneous character. This conclusion was based on differences observed in cell suspensions from primary and metastatic lesions from the same patient, as well as changes in the karyotype of ovarian cancers sampled successively during cytostatic treatment (21).

Recent studies of human tumors, including an ovarian carcinoma (12), a neuroblastoma (4), and a neurofibrosarcoma (9), have addressed the clonal versus multicellular origin of metastatic foci utilizing karyotypic analysis. In all of these studies, the evidence, while not definitive, supports the observation of a clonal origin for metastases. Furthermore, as was observed in the present studies, the marker chromosomes of these metastases were also stably expressed within all of the lesions.

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In the study of clonal origin of experimental metastasis by Poste et al. (20), the metastatic properties of several tumor cell clones isolated from individual B16 melanoma pulmonary metastases at different stages during the evolution of metastasis were investigated. They found that, during the early stages of metastatic growth following i.v. injection of tumor cells, the majority of metastatic lesions contain cells with indistinguishable metastatic phenotypes (intralesional clonal homogeneity). In contrast, the progressive growth of metastatic lesions was accompanied by the emergence of variant tumor cells with altered metastatic properties within clonal homogeneous lesions (intralesional heterogeneity).

Taken together, the growing body of experimental and clinical evidence suggests that cells within the same cancer exhibit different susceptibilities to the broad range of conventional treatments; therefore, the successful therapy of malignant tumors will require the development of new approaches capable of overcoming this variation and against which resistance is unlikely to develop. Given the extraordinary level of cell diversity apparently present in many tumors, the probability is small that a single anticancer drug or any other treatment used alone will be capable of killing all of the cancer cells in a malignant tumor and its metastases. New treatment strategies must be developed which will slow the potential of tumor cells that survive initial waves of treatment(s) to generate new variants. To achieve this with existing therapeutic modalities, we have reduced the interval between successive administration of different anticancer agents in an attempt to destroy the cancer cell subpopulations that survive each successive treatment before the generation of large numbers of new variants could occur. Furthermore, there is a growing trend in cancer medicine to use a combination of anticancer treatments for the patients with malignant tumors.

The problem of selecting effective therapy for heterogeneous tumors may be further compounded by the existence of interactions between tumor subpopulations, as well as between tumor cells and normal cells, which affect the measured sensitivity of the whole tumor, a concept that has received extensive study by Heppner and Miller (10). Heppner and coworkers have shown that subpopulations of a mouse mammary tumor, which differ in sensitivity to chemotherapeutic agents, can interact in such a way that the apparent sensitivity of one subpopulation is changed in the presence of the other (10, 13).

The heterogeneity of tumors, however it originates, has important implications for the study and treatment of metastases. For example, cells obtained from a primary tumor are not necessarily representative of cancer cells populating metastases or even cells in different regions of the same primary tumor. Thus, experimental efforts must be concentrated on the identification of the features that permit malignant cells to metastasize. In addition, the test systems for new therapeutic agents or modalities must address the problem of tumor cell heterogeneity. More effort must be devoted to testing the efficacy of combination therapies with the objective of circumventing the problem of cellular diversity within tumors. The short-term therapeutic goal must be to choose the combination of antitumor agents and to determine the sequence of administration that will be most effective against a particular tumor. Nonetheless, the only successful treatment of metastatic disease will be one that circumvents the different phenotypes of tumor cells within individual metastases of a patient and probably will require multiple therapeutic agents and multiple therapeutic modalities.

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REFERENCES


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