Irradiation-induced Marker Chromosomes in a Metastasizing Murine Tumor

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ABSTRACT

We have used irradiation to induce marker chromosome formation in a metastasizing murine tumor with a stable karyotype. The induced recombinant chromosomes then served to determine whether metastases were of clonal or multicellular origin. Cumulative data were obtained from four series of experiments on spontaneous metastases originating from tumors grown from irradiated cells; 20 of these metastases expressed unique chromosomal alterations consistent with a clonal origin. The majority of metastasis-derived cell populations remain stable with respect to their marker chromosomes in culture as well as in successive animal transplantation. In several instances, however, chromosomal instability was sufficient to obscure the cellular origins of individual metastases. A few metastases showed mixed chromosomal patterns initially that were consistent with multicellular origin, but repeat examinations have revealed a chromosomal instability which persisted during propagation in culture.

The frequency of chromosomal recombinants in metastases from the combined series was sufficient to suggest biological and statistical significance. The morphology of recombinants was not associated with radiation dose but appeared as an apparently random response of the tumor population in different experiments. Analysis of chromosomal markers by banding techniques was performed to determine if specific chromosomes or chromosomal sites were associated with tumor cells from metastatic foci (a host-selected subpopulation with a metastatic phenotype). Our results did not reveal preferential involvement of whole chromosomes or intrachromosomal sites in recombinant formation.

INTRODUCTION

Development of chromosomal banding techniques has permitted the relatively precise identification of normal and abnormal chromosomes within tumors and has led to correlations of specific abnormalities with several human and mouse leukemias and solid tumors. In humans, the translocations seen in chronic myelocytic leukemia and Burkitt's lymphoma are perhaps the best known: t(9;22) in chronic myelocytic leukemia (1), and t(8;14) in Burkitt's lymphoma (2). Specific chromosome changes have also been identified in several human solid tumors (3–5). Studies of melanocytic lesions showed clones with multiple chromosomal abnormalities with nonrandom involvement of chromosomes 1, 6, and/or 7 (6). In mice, trisomy 15 occurs in spontaneous lymphomas (7) and in thymic lymphomas induced by chemical and viral carcinogens and X-irradiation (8–10). Murine plasmacytomas show extensive chromosome changes (11), but three cell lines with a translocation involving chromosome 15 had a consistent breakpoint (12). In contrast, spontaneous mouse mammary tumors are associated with trisomy or recombination involving chromosome 13 (13). The increasing reports of malignancies with consistent, stable karyotypic changes has led some investigators to suggest that all malignancies have chromosomal abnormalities which will become more evident as cytogenetic techniques improve (14).

Irradiation, in vivo or in vitro, results in chromosome damage such as breakage, rearrangement, and disruption of mitotic separation. The degree of chromosome damage has been shown to be proportional to the dose of radiation received. Recently, we used irradiation to induce marker chromosome formation in a metastasizing murine tumor, K-1753-M2, with a stable karyotype (15). Stable rearrangements (90% of cells within a metastasis having the same marker chromosome) were interpreted as consistent with clonal origin; unstable chromosome complements (defined as low marker frequency or open breaks) could indicate multiclonality or continuing chromosome instability. Predicated on cumulative data from four experiments, several metastases were also considered as clonal in origin on the basis of minute chromosomes or conversion in ploidy (16). Approximately 20% of metastases originating from irradiated tumor cells showed altered chromosome patterns that were consistent with a clonal origin. The type of recombinant chromosome varied in two groups derived from tumor cells given the same dose of irradiation (650–700 R). In the first series, metacentric and submetacentric chromosomes were seen in the metastases, while in the second series, telocentric marker chromosomes were common. In another series, induced at a higher radiation dose, minute chromosomes marked the metastases which were not characterized by recombinant chromosomes.

We have now analyzed the recombinant chromosomes by banding techniques to determine if specific chromosomes or preferential involvement of chromosome sites were associated with a selective advantage for the metastatic process. In addition, we studied a few metastases after prolonged culture because multiclonal origin could not be ruled out on initial examination. In several metastases, the mixed pattern appeared secondary to inherently high rates of chromosome breakage and recombination. We report the identification of chromosomes involved in recombinant formation and the evolution of cultures with "unstable" recombinants and also present data on the metastatic potential of cultures with "unstable" karyotypes.

MATERIALS AND METHODS

Animals. Adult C3H/HeN mammary tumor virus-negative mice, 6–8 weeks old, were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility.

Tumors. The original K-1735 melanoma (17) was a gift from Dr. Margaret L. Kripke, NCI-Frederick Cancer Research Facility. The K-1735-M2 line was derived from a spontaneous pulmonary metastasis produced from the K-1735 parent tumor (18). Irradiated K-1735-M2 cells were implanted into the footpads of syngeneic mice. When the resulting footpad tumors reached an average diameter of 1 cm, the tumor-bearing leg, including the popliteal lymph node, was resected at midfemur. Five weeks after resection, the mice were necropsied and multiple well-isolated solitary spontaneous metastases were aseptically
removed, grown in culture as individual lines, and karyotyped (15).

All cultures were maintained in tissue culture in complete minimum essential medium (Eagle's complete minimum essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and 2-fold vitamin solution; all from Flow Laboratories, Rockville, MD). Cell cultures were maintained on plastic and incubated in 5% CO₂ at 37°C. They were subcultured at weekly intervals. Cultures were routinely monitored for and found to be free of Mycoplasma and murine viruses as reported previously (15, 16). In series II, III, and IV chromosome harvests were obtained between 7 and 14 days after culture initiation. Repeat examinations for persistent instability were harvested after an additional 8 weeks of growth and subculture.

Radiation. The parental tumor cells in culture were exposed to X-irradiation in a biological irradiator (Ridge Instrument Co., Inc., Atlanta, GA) at 500 rads/min (300 KV at 10 mA).

Chromosome Studies. Confluent cultures of lung metastases were split 1:3 and harvested 48 h later. Cultures were exposed to Colcemid (0.5 μg/ml) for 1 h. Cells were trypsined, centrifuged at 800 rpm for 10 min, swollen in 0.075 M potassium chloride, and fixed twice in methanol:acetic acid (3:1). Cells were dropped on cold, wet slides and analyzed by GTG banding. Briefly, slides were exposed to 0.1% trypsin, rinsed in 50% and 100% ethanol, stained in 4% Giemsa stain in phosphate buffer (pH 6.8), and rinsed in two changes of buffer. When dry, slides were dipped in xylene and coverslipped with Canada Balsam. For analysis of C-bands, slides were exposed to 0.2 N hydrochloric acid for 30 min and to 0.07% barium hydroxide for 7 min at 37°C, rinsed with distilled water, incubated in 2x standard saline-citrate at 65°C for 2 h, and stained in 4% Giemsa stain in phosphate buffer for 5 min. When dry, slides were dipped in xylene and coverslipped in Canada Balsam. Karyotypes were prepared according to the recommendations of the Committee on Standardized Genetic Nomenclature for Mice (19).

RESULTS

Metastases with Stable Recombinants. The K-1735-M2 line has a stable karyotype with a modal number of 44 (87% of cells at the mode) and all chromosomes are telocentric. No recombinant chromosomes have been identified. However, trisomies are consistently present for chromosomes 1, 8, and 15 and frequently for other chromosomes. In four separate experiments (Table 1), metastases derived from irradiated K-1735-M2 cells were analyzed for stable and unstable chromosome aberrations. Of 92 metastases analyzed (Table 1), 20 were characterized by chromosomal alterations, some of which were not recombinant chromosomes, i.e., minutes, telomere deletions, etc. While 13 metastases showed either recombinant chromosomes in a small proportion of cells or unstable chromosome aberrations, such as open breaks, dicentrics, etc. Seventeen of the 20 metastases contained stable recombinant chromosomes (the recombinant occurring in 90% or more of the cells analyzed). These stable recombinant chromosomes were identified by GTG banding (Fig. 1, Table 2). In two series (I and II), using the same radiation dose, 13 distinct rearrangements were found in 14 karyotypically stable metastases from 10 animals although 2 could not be identified. Thirteen of the 19 autosomes were involved in recombinants. Only chromosomes 2, 3, 4, 5, 6, and 13 were involved in recombinant formation more than once. Chromosome 5 was a component in 4 recombinants while chromosome 6 was involved in at least 3 and possibly a fourth.

In the first series over 50% of the metastases expressed marker chromosomes. Three metastases (x-met-9, -10, -11), individual lung nodules from a single host, probably originated from a common stem cell. All three metastases show high frequency of the same submetacentric (80–100%) and metacentric (60–100%) markers; the submetacentric consists of a chromosome 3 and a chromosome 15 apparently joined by centric fusion. The metacentric marker appears to be an isochromosome of chromosome 12. Two additional metastases from different host animals, x-met-13 and x-met-14, also contain the identical marker chromosomes (Fig. 1). All metastases in series I were initiated from the same pool of irradiated K-1735-M2 cells. Either parallel evolution or selection for certain recombinants could explain these observations and would, in contrast to other possible explanations, support biological significance for the preferential involvement of certain chromosomes. However, because of the time of propagation of these metastases in culture prior to karyotypic analysis the possibility of cross-contamination could not be eliminated. Further, since recombinant chromosomes also are known to arise in culture de novo, we could not rule out artifactual origin of these recombinant events secondary to propagation prior to study. In later studies metastasis-derived cells were harvested for chromosome analysis within 14 days of initiation of culture which reduced the possibilities of cross-contamination or origin of new mutations in culture.

Three other metastases from series I are clearly of independent origins. x-met-16 contains a submetacentric chromosome consisting of a portion of a chromosome 13 forming the short arm and a chromosome 4 comprising the long arm. Its telocentric marker is as large as the submetacentric chromosome in those cells, and is composed of chromosome 2 (centromere to E4) and the distal portion of chromosome 5 (E2 to terminus). The most complex, x-met-21, contains three marker chromosomes: a submetacentric, a telocentric, and a medium-sized metacentric chromosome. The submetacentric is a centric fusion product of chromosome 11 and chromosome 17. The telocentric is formed from a portion of chromosome 1 (centromere to D) and the distal half of chromosome 16 (B5 to terminus). We were unable to determine the origins of the metacentric chromosome from x-met-21 and the small metacentric that characterized x-met-12.

In series II, cell cultures derived from 38 metastases were analyzed; 6 metastases each contain a marker consisting of a long unmatched telocentric chromosome. The markers in B1 and B2, two separate metastases from the same animal, are identical. However, the other telocentric chromosomes differed from each other and from B1 and B2 by banding pattern. The telocentric marker of B1 and B2 is composed of portions of a No. 6 and a No. 2 chromosome. Two other marker-containing metastases were obtained from a single host animal. Their recombinant chromosomes appear similar when not stained differentially but are clearly derived from different chromosomes when G-banded (Fig. 1). The marker in P2 is formed from a No. 13 and a No. 5 chromosome. The paraacentric portion of the marker found in P1 is part of a No. 9 chromosome but the origin of the remainder was not definitively identified. C-banding, which often helps to localize the recom-

<table>
<thead>
<tr>
<th>Series</th>
<th>Radiation dose</th>
<th>No. of metastases</th>
<th>Total</th>
<th>No. of metastases showing chromosomal alteration</th>
<th>Recombinant/stable*</th>
<th>Unstable</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>650-700</td>
<td>21</td>
<td>11</td>
<td>8/8</td>
<td>3</td>
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</tr>
<tr>
<td>II</td>
<td>650-700</td>
<td>21</td>
<td>13</td>
<td>6/7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>700</td>
<td>9</td>
<td>3</td>
<td>0/1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>900</td>
<td>24</td>
<td>6</td>
<td>3/4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>92</td>
<td>33</td>
<td>17/20</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* Stable metastases marked by chromosome recombinants/total stable clonally distinctive metastases.
Series I

Fig. 1. Marker chromosomes from individual metastases. a, submetacentric and telocentric from x-met-5; b, submetacentric and telocentric character of x-met-9, -10, -11, -13, and -14; c, submetacentric, telocentric, and metacentric from x-met-21; d, telocentric from C1; e, telocentric of P1; f, telocentric characteristic of B1 and B2; g, telocentric of 14; h, telocentric from P2.

Series II

Table 2. Chromosome composition and occurrence of stable recombinants

<table>
<thead>
<tr>
<th>Marker identification</th>
<th>No. of events</th>
<th>No. of animals</th>
<th>No. of metastases</th>
</tr>
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<tr>
<td>t(3;15), iso(12)</td>
<td>2 or 6*</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>t(4;13), t(2:5)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>t(11;17), t(1;16)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>t(2;6)</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>t(5;13)</td>
<td>1</td>
<td>1*</td>
<td>1</td>
</tr>
<tr>
<td>t(9;?)</td>
<td>1</td>
<td>1*</td>
<td>1</td>
</tr>
<tr>
<td>t(4;5;6)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>t(3;3;6 or X)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Minimum 11

* These recombinants were identified in five metastases from three animals. Whether this represents parallel evolution or possible cross-contamination is discussed in the text.

Two independent metastases from the same animal host.

Markers in all series were minutes, nonrecombinant chromosomes, or of unidentifiable origin.

Metastases with Unstable Markers. Of 92 metastases derived from irradiated K-1735-M2 tumor cells, 13 were defined as unstable either because chromosome recombinants were detected in only a small percentage of cells or because a significant number of cells had unstable chromosome aberrations, i.e., breaks or unstable recombinants such as multicentrics or rings. Eight unstable and seven stable metastases were sampled on repeated occasions in culture to determine the extent of evolution to, or retention of, a dominant pattern as opposed to retention of a mixed or unstable pattern. Eight of the unstable metastases were analyzed 8 weeks after their first harvests. Two of the metastases which exhibited breakage on first examination showed increase of breakage. Unstable metastases with low frequencies of markers showed retention of a mixed population (with and without recombinants) although the markers often differed (Table 3). Seven initially stable metastases with unique recombinants in greater than 90% of their cells continued to exhibit the same markers at high frequency after 8 weeks in culture. However, in one of the stable metastases, a subpopulation of tetraploid cells arose during the culture period.

The relevance of instability to metastatic ability was also examined briefly. Cells from three metastasis-derived cultures of series III were inoculated into animal hosts as described previously (18). We then analyzed the median number of metastases generated by each. Cells from met-1, met-5, and met-7 had medians of 132, greater than 300, and 145 metastases,
respectively (nonsignificant differences by the Mann-Whitney U test). However, cultures of met-1 and met-5 did not show chromosomal recombinants or evidence of instability, while met-7, with no obvious difference in metastasizing capability, was highly unstable karyotypically. It should be remembered that all these cultures were derived from cells capable of metastasis, as were those of the parent line.

**DISCUSSION**

Nowell (20) proposed that mutant cells are produced in an expanding tumor population as a result of genetic instability, which permits stepwise selection of variant sublines and underlies tumor progression. Cifone and Fidler (21) showed that increasing instability of clones isolated from murine neoplasms was associated with increasing metastatic potential. In contrast, DiRenzo et al. (22) identified a common marker chromosome in highly metastasizing subclones selected in vitro from the nonmetastasizing B77-3T3 line. The frequency of this marker chromosome was concomitant with the expression of a highly metastatic phenotype. These two lines of evidence are divergent with respect to the expectations of chromosomal aberrations to be found in metastatic tumors. The first suggests, due to the randomness of mutation, that metastases might arise with different karyotypic lesions; while the second example indicated that a specific lesion, common to all malignant cells, could be expected. To address this dichotomy we utilized irradiation, which randomly induces chromosomal abnormalities, to determine if a specific karyotypic aberration was associated with metastatic expression.

In the series reported here, of 92 metastases analyzed, 20 had stable and characteristic chromosome alterations indicative of clonal origin. Two experiments at the same radiation dose (650–700 R) induced recombinants that differed both in the frequencies of chromosomal recombinants in the metastases and in the types of markers found. Earlier results raised the possibility that induction of specific chromosomal rearrangements could provide tumor cells with a selective advantage in the metastatic process. Although DiRenzo et al. (22) associated a marker chromosome with a high frequency of metastasis, that same marker was found in a small percentage of cells in the parental nonmetastasizing fibrosarcoma cell line. In the present study, analysis of metastasis-derived cultures revealed 13 distinctive recombinant chromosomes. In series I characterized by metacentric and submetacentric chromosomes, five metastases from three animals had common markers. Several explanations are possible. The recombinant chromosomes could confer a selective advantage for metastasis. The occurrence of the same recombinants in three metastases from a single animal could have resulted from clonal expansion of the primary tumor; i.e., many cells containing the recombinant were present and were the source for several metastases. Alternatively, the occurrence of the same markers could reflect selective effects of irradiation. Kano and Little (23) found nonrandom radiation-induced rearrangements in a normal human foreskin-derived cell line. The possibility of cross-contamination in vitro cannot be excluded but was not supported by results from the second series. Two metastases from a single animal were identical, while two from a second animal were nonidentical. Other metastases derived from the same parental population had different markers involving different chromosomes indicating that those particular markers probably did not confer a selective advantage. In fact, in 14 metastases with recombinant chromosomes, 13 distinctive recombinants were identified.

Irradiation damage is expected to be random with respect to chromosomal site. If randomly formed marker configurations did not confer preferential growth advantage, one would expect the chromosomes involved in marker formation to be evenly distributed (with the possible exception of chromosomes present in triplicate (chromosomes 1, 8, and 15) in the parent cell line). In fact, 13 of the 19 autosomes were involved in marker formation and there was not selective involvement of the trisomic chromosomes. However, one or two chromosomes (chromosome 5 and possibly 6) are overrepresented. Chromosome 5 was the most frequently involved chromosome, occurring in four markers. While no known oncogene sites have been mapped to chromosome 5 of the mouse, c-K-ras-2 has been mapped to chromosome 6 (24, 25). In 1961, Ohno et al. (26) suggested that fusion of nucleoli could bring NORs of different chromosomes into proximity and might predispose chromosomes with NORs to centric fusion resulting in Robertsonian translocations. Miller et al. (27) showed, in three murine cell lines from two inbred strains, that the NOR-bearing chromosomes were overrepresented in Robertsonian translocations. In the present study, five markers resulted from centric fusion involving seven chromosomes, none of which have been shown to have NORs in C3H mice by silver staining (28).

The persistence of unstable markers in cultured metastases was somewhat unexpected since one would assume that these markers by their very nature would be lost during subsequent cell divisions. This is in striking contrast to the rapid loss of unstable markers and chromosomal breaks after irradiation in primary tumors of K-1753-M2 (16) and in human fibroblasts (23). Documentation of persistent unstable aberrations as well as stable rearrangements is unusual (29, 30). Some of our metastases were designated unstable because a relatively small proportion of cells had recombinant chromosomes. If recombinant chromosomes conferred growth advantage, the marker-bearing cells should have increased in the population. Conversely, if the marker did not confer a growth advantage, then the proportion of such cells should have declined. Neither effect was observed and the proportion of cells with recombinants remained at a fairly constant level during the culture period. Similarly, Kano and Little (23) showed that radiation-induced rearrangements in human fibroblast cultures persisted for 10 subcultures at approximately the same proportions.

The inability to demonstrate an association between specific chromosomal rearrangements, stable or unstable, with metastatic propensities suggests that Nowell's hypothesis is oper-

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**Table 3 Persistence of unstable chromosome aberrations in individual metastases in culture over time**

<table>
<thead>
<tr>
<th>Series</th>
<th>Metastasis designation</th>
<th>Type of aberration</th>
<th>First examination</th>
<th>Second examination</th>
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<tbody>
<tr>
<td>I</td>
<td>x-met-5</td>
<td>Breaks markers M; SM T; M (15); SM (57); T (10); SM (40)</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x-met-6</td>
<td></td>
<td>Breaks markers SM T; M (20); SM (50)</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>x-met-17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>14</td>
<td>Markers SM T; M (50); SM (30); SM (40)</td>
<td>T (50)</td>
<td>T (50); M (30); SM (10)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3B</td>
<td>Markers SM T; M (20); SM (50)</td>
<td>M (10); SM (20); SM (10)</td>
<td>M (20); M (10); T (50)</td>
</tr>
</tbody>
</table>

* The second examination was 8 weeks after the first. 
* Recombinant chromosome markers were identified as: M, metacentric; SM, submetacentric; T, telocentric.
ative for the K-1735-M2 cell line. Because the parent cell line was metastatic it is possible that the metastatic "genome" was fully expressed allowing the random induction and expression of aberrant chromosomes. It is possible that either the two effects (metastasis and karyotypic expression) are unrelated or insufficient time had elapsed for the preferential expression and/or selection of random but clonal markers. We conclude, therefore, that at least within a cell line with a stable karyotype and metastatic phenotype, the random induction of karyotypic aberrations, as an expression of induced mutation, does not support a common genomic lesion that is associated with the metastatic phenotype. This observation is consistent with the complexity of the metastatic process which would, in theory, require the expression of multiple genotypes rather than a single gene or promoter region for the complete expression of a metastatic phenotype. While metastasis may require the uniform expression of a specific gene, the complete expression of metastasis is still likely to require multiple gene families.

REFERENCES

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