Characterization of High- and Low-Metastatic Clones Derived from a Methylcholanthrene-induced Murine Fibrosarcoma

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ABSTRACT

A methylcholanthrene-induced fibrosarcoma (3AM) and several of its clones were evaluated for pulmonary metastasis, growth rate, and chromosome composition. Heterogeneity was observed in the three parameters, and no correlation was found between growth rate and metastatic potential. Furthermore, three clones (10, 34, and 27) were identified with distinctive, high or low, metastatic potential and marker chromosomes. The marker chromosomes characteristic for each clone were identified in the early passages of the parental 3AM line, indicating the preexistence of the different cell types in the original neoplasm. The three clones were then characterized as to tendency to adhere to vascular endothelium, immunogenicity, and antigenic specificity. Clone 10, with two large metacentric markers (T2,4 and T10,13) and the highest metastatic potential (221 foci/lung), expressed the highest endothelial attachment and immunogenicity. Clone 27 was characterized with an extremely low rate of metastasis (nine foci/lung) and a T2,7 large acrocentric marker, while clone 34 was characterized with a moderate rate of metastasis (107.5 foci/lung) and a T4,18 acrocentric marker. Antigenically, clone 10 cross-reacted with clones 34 and 27 and 3AM, while clones 27 and 34 cross-reacted with clone 10 and 3AM but not with each other, suggesting that, within the original tumor, there were common tumor antigens shared by some cells but no universal antigen shared by all cells.

INTRODUCTION

Cloning studies have demonstrated the heterogeneity of primary tumor cell populations with regard to metastatic potential for several murine neoplasms, including the C57BL/6 B16 melanoma (4, 7), a UV radiation-induced C3H fibrosarcoma (5, 10), an autochthonous BALB/cF3H mammary tumor (2), and a sarcoma virus-transformed fibrosarcoma (13). Furthermore, clones of different metastatic potentials were found to have different modal chromosome numbers (2, 5). All these studies indicate that metastasis results from the nonrandom selection of certain preexisting subpopulations of specific hereditary intrinsic properties. However, no specific morphological markers, such as chromosomal rearrangement, have been identified for the individual clones with different metastatic potentials. Therefore, it has not been possible to (a) identify and confirm the existence of each individual clone in the parental tumor cell population, (b) trace the fate of a clone during the metastatic process, or (c) study the possible cell-cell interaction.

In the present study, we report that the tumor cell population of a methylcholanthrene-induced fibrosarcoma syngeneic to C3H mice was heterogeneous in both chromosome composition and metastatic potential. Furthermore, metastases did not result from random survival of cells released from the primary tumor but from the selective growth of specialized cell subpopulations with specific marker chromosomes. Finally, isolated high-metastatic and low-metastatic clones identified by cytogenetic markers differed with regard to in vivo growth rate, endothelial cell attachment, and immunological properties.

MATERIALS AND METHODS

Animals. Eight- to 16-week-old male C3H/HeJ mice obtained from The Jackson Laboratory, Bar Harbor, Maine, were used in all experiments.

Tumors. A fibrosarcoma, 3AM, was originally induced in a male C3H/HeJ mouse by the injection of 0.5 mg of 3-methylcholanthrene dissolved in 0.1 ml of trioctanoic oil. The tumor was maintained in vivo by biweekly s.c. implantation. After 2 passages in the animals, a s.c. tumor was removed aseptically to start an in vitro cell line. Briefly, the whole tumor was excised, minced, and treated with mechanical forces by consistent, constant pipeting. The resulting cell suspension was plated on Falcon tissue culture flasks (Falcon Plastics, Oxnard, Calif.). The cultured tumor cells were maintained in RPMI Medium 1640 containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and were subcultured weekly. After 2 in vitro passages, tumor cells were doubly cloned in microtiter plates (Falcon No. 3040). Briefly, a single-cell suspension obtained by trypsinization of a 3AM monolayer culture was diluted to a final concentration averaging 3 cell/ml. An aliquot of 0.3 ml was then placed in each well of the microtiter plate, and clones recovered from those wells of confirmed single-cell origin were re-cloned in the same manner. All the clones and the parental cell line, in early passages, were banked in liquid nitrogen. To avoid any possible artifact caused by long-term cultivation, tumor cells maintained in tissue culture for over 6 weeks were replaced with new frozen stocks.

Identification of Pulmonary Metastasis. Tumor cell suspensions (0.2 ml containing 5 x 10³ cells) with greater than 90% viability, as judged by trypan blue staining, were injected s.c. into the backs of C3H/HeJ mice with a 25-gauge needle. Five weeks after the injection, the animals were sacrificed and evaluated for the number of pulmonary metastases according to the method of Wexler (20). Briefly, the lungs were insufflated with 15% (v/v) solution of India ink by introduction through the trachea with an 18-gauge needle until the lungs were filled. The lungs were then dissected from the bronchi and esophagus and immersed in tap water for 1 hr to remove excess ink. They were then fixed in Fakete's solution (100 ml of 70% ethanol:10 ml of formalde-
Evaluation of Tumor Growth Rate in Vivo. With an equal inoculum of 5 x 10^6 cells/animal for each clone, all tumors were palpable 1 to 2 weeks after the s.c. injection. The size of each palpable nodule was then measured weekly in 3 dimensions, and the volume was calculated. To determine the growth rate of a clone, the average increment in tumor volume was obtained from 8 to 10 mice for each clone. Using least-square analysis, a linear relationship (r = 0.9) (Table 1) between the tumor size and number of days after the injection was observed for each clone. The slope of the change in tumor volume versus time was used to measure the growth rate of the clone and the parent tumor.

Cytogenetic Analysis. Tumor cells from the first in vitro passage of the parental cell line 3AM and the second in vitro passages of some clones derived from it were harvested for conventional chromosome analysis without Colcemid treatment. Monolayers of 50 to 70% confluency were first trypsinized to obtain single-cell suspension and were then treated hypotonically with 0.064 M KCl or 0.7% sodium citrate for 5 min before fixing in methanol:acetic acid (3:1, v/v). Air-dried slides were prepared on the fixed tumor cells. The parental cell line and the clones having appropriate marker chromosome(s), as identified by conventional karyotypic study, were then submitted to trypsin G-band analysis by the method described previously (19). Briefly, the air-dried slides were heated on a 70°C hot plate for 2 to 3 min, treated with 0.725% trypsin (in 0.9% NaCl solution) for 5 to 10 sec, rinsed with 0.9% NaCl solution, and stained with Giemsa for 3 to 4 min. For each clone, 25 to 30 metaphase cells were analyzed, while 200 metaphase cells were analyzed for the parental cell line 3AM.

Assay for the Tumor Cell Adherence to Endothelium. Human vascular endothelial cells (EC) were harvested from umbilical cord veins and grown to confluence in banks of small plastic wells (15). Immediately prior to their use in the experiments, EC layers were washed twice with culture medium (RPML Medium 1640:4:2-hydroxyethyl)-1-piperazineethanesulfonic acid without serum). Tumor cell monolayers were removed from the culture flasks and dispersed mechanically. Tumor cells suspended in culture medium were incubated at room temperature for 20 min with 0.1% (10 μl/10⁶ tumor cells), washed 5 times with large volumes of 0.9% NaCl solution, and suspended in culture medium at a concentration of 4 x 10⁶ cells/ml. The tumor cell suspension (250 μl; 1 x 10⁶ cells/ml) was layered on EC layers (5 x 10⁴ EC) in quadruplicate wells and incubated at 37°C. After 3 hr, EC layers were washed 6 times with 0.3 ml of 0.3% medium, and the remaining cells were solubilized with 1% sodium dodecyl sulfate. The percentage of tumor cells adhering to EC layers was determined from the radioactivity in the successive washes and that in the sodium dodecyl sulfate-solubilized material.

Immunization. Tumor cells (5 x 10⁶) in a volume of 0.2 ml were inoculated s.c. into a hind limb of normal mice, and the tumor-bearing limb was amputated 6 days later. The mice were used 2 weeks after amputation in this standardized test for immunization.

In Vivo Assay of Tumor Rejection. Normal or tumor-immune mice were challenged with various doses of tumor cells in 0.1 ml RPMI Medium 1640 by s.c. injection. Tumor development was monitored weekly for 6 weeks, and tumor incidence was expressed as the percentage of the mice developing tumors.

RESULTS

Evaluation of Growth Rate and Metastatic Potential. The growth rate and metastatic potential of the parental tumor, 3AM, and 21 clones are summarized in Table 1. The growth rate of the parental cell line was 382 cu mm/day, while that of tumors ranged from 106 cu mm/day (clone 3) to 656 cu mm/day (clone 30), suggesting that the tumor cell population is heterogeneous with respect to the growth rate. Furthermore, a good linear correlation (r = 0.917 to 0.991) existed between the tumor size and the duration of growth in vivo. This indicates that the growth rate of each clone was constant for the 5-week period. At the end of the 5 weeks, the animals were sacrificed and evaluated for the numbers of pulmonary metastases. The median number of pulmonary metastatic foci for the parental cell line 3AM was 42, while that for the clones ranged from zero (clone 7) to 221 (clone 10), showing that these clones varied enormously with respect to metastatic potential. The metastatic potential of clones 10 and 27 was evaluated on the frozen sample of different in vitro passages and on the fresh sample of different in vivo passages and was found quite stable in both in vivo and in vitro systems. Table 2 shows the results of the study during 30 in vivo passages. In order to find out if there was a correlation between the metastatic potential and the in vivo growth rate, statistical analysis was performed on these 2 parameters. A correlation coefficient of 0.101 was obtained, indicating that there was no significant correlation between the metastatic potential of a clone and its growth rate. This conclusion coincides with what has been reported by Dexter et al. (2) and Fidler and Cifone (5).

Cytogenetic Evidence for the Nonrandom Selection of Metastasis. In order to obtain morphological markers on clones of high- and low-metastatic potential, conventional chromosomal analysis was performed on the clones which produced either <10 or >100 foci of pulmonary metastasis. After primary screening, apparent marker chromosomes were found in clones 27, 34, and 10. The chromosomal compositions of the
tumor. The homogeneity of the chromosome composition within clones existed in the very early in vivo passage of the parental was also studied by the trypsin G-banding analysis. Through this analysis was markedly heterogeneous with respect to degree of somal number specific for each clone, it is evident that the 3 clones were detailed by the trypsin G-banding analysis.

<table>
<thead>
<tr>
<th>No. of in vivo passages</th>
<th>Median no. of pulmonary metastases</th>
<th>Metastatic foci/individual mouse*</th>
<th>Median no. of pulmonary metastases</th>
<th>Metastatic foci/individual mouse*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0, 0, 0, 0, 1, 1, 5, 6, 11, 51</td>
<td>109</td>
<td>4, 5, 51, 59, 109, 130, 332, 335, 350</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>0, 0, 0, 0, 0, 0, 1, 3, 4, 5, 5, 8, 22, 40</td>
<td>90</td>
<td>1, 33, 35, 66, 89, 90, 100, 130, 150, 260, 332</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0, 0, 0, 0, 0, 0, 1, 2, 5, 9, 18, 36</td>
<td>109</td>
<td>2, 3, 4, 39, 54, 89, 109, 200, 235, 260, 350</td>
</tr>
</tbody>
</table>

*Evaluated 25 days after s.c. injection of 5 x 10^6 tumor cells.

3 clones were detailed by the trypsin G-banding analysis. Clone 27 (modal chromosome number 42), a low-metastatic clone, had a large acrocentric marker chromosome resulting from the translocation between chromosomes 2 and 7 (Fig. 1A). Clone 34 (modal chromosome number 40), a clone of intermediate metastatic potential, had a large metacentric marker chromosome resulting from a translocation between chromosomes 4 and 16 (Fig. 1B). Clone 10 (modal chromosome number 37), the clone with the highest metastatic potential, contained 2 large metacentric chromosomes, denoted as M1 and M2. M1 is the result of centromeric fusion between chromosomes 2 and 4; M2 is the result of centromeric fusion between chromosomes 10 and 15 (Fig. 1C). The chromosome composition of the 3 clones has been checked periodically between chromosomes 10 and 15 (Fig. 1C). The chromosome composition of the clones has been checked periodically during a 15-month period after different freeze-thaw cycles and been found genetically stable. In addition to these clones, the chromosome composition of the parental tumor 3AM at the first in vitro passage (derived from the second in vivo passage) was also studied by the trypsin G-banding analysis. Through identification of the marker chromosomes and the chromosomal number specific for each clone, it is evident that the 3 clones existed in the very early in vivo passage of the parental tumor. The homogeneity of the chromosome composition within each clone, furthermore, confirmed the single-cell origin of the cloned cell population.

**Endothelial Cell Attachment.** In order to find out if the metastatic potential of a clone correlates with its adherence to endothelial cells, an in vitro assay for evaluation of such adherence was performed on the parental line and the different clones. The results again indicated that the tumor cell population was markedly heterogeneous with respect to degree of attachment to endothelium. As shown in Chart 1, clone 10 demonstrated a high degree of attachment after a single wash, and relatively few cells were dislodged by further washing. In contrast, clones 25 and 34 showed much lower initial attachment, and subsequent washings continued to remove a significant number of tumor cells. Clones 27 and 29 and the parental cell line, 3AM, were intermediate in terms of initial attachment and in terms of numbers of cells dislodged by multiple washings. There was no apparent correlation between degree of endothelial cell attachment and potential for metastasis (Chart 1). However, clone 10, the clone with the highest metastatic potential, did have a significantly high degree of adherence to endothelial cells, especially after multiple washes.

**Tumor Immunogenicity.** The extensive heterogeneity of cell subpopulations in a primary tumor raised questions concerning antigenic specificity and immunogenicity of the variant cells in a tumor. To study this, C3H mice were immunized against the parental tumor, 3AM, or the cloned tumors. Immune mice given injections i.d. with various doses of tumor cells were compared with normal control mice for their capacity to reject tumors. Tumor incidence was expressed as a percentage of mice developing tumors at each dose. The number of tumor cells required to cause 50% tumor incidence was calculated from these dose-incidence curves.

The parental tumor and clone 10 were highly immunogenic. Mice immunized against these tumors were 7 to 8 times more resistant to their respective tumors than were normal control mice (Chart 2). On the other hand, clone 34 and clone 27 tumors were comparatively less immunogenic, since the immune mice were only 2 to 3 times more resistant to these tumors than were control mice. The data obtained demonstrated the heterogeneity of the tumor cell population with regard to immunogenicity.

**Tumor Antigenic Specificity.** To study antigenic specificity, mice immunized against the parental tumor, clone 10, clone 34, and clone 27 were tested for their ability to reject each of these tumors. As shown in Chart 3, A and B, mice immunized against parental tumor or clone 10 were equally capable of rejecting all 4 tumors. On the other hand, mice immunized against clones 34 or 27 showed a significantly higher capacity to reject the parental tumor than to reject clone 10 or their own tumors (Chart 3, C and D). Furthermore, clones 34-immune mice or clone 27-immune mice were not able to reject the tumors of each other.

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Chart 1. Tumor cell adherence to endothelium, expressed as percentage of tumor cells remaining on confluent endothelial monolayers after the number of endothelial washes indicated. Points, mean result for 2 experiments performed 3 months apart and each done in quadruplicate. There was little variability between replicates or between different experiments; for most points plotted, S.D. is less than ±3%. For reference, removal of normal erythrocytes (RBC) (which are nonadherent to endothelium) is also shown.
The aforementioned observation was demonstrated more clearly when the antigenic specificity of these tumors was expressed as a percentage of tumor rejection of immune mice at a single challenge dose of $2.5 \times 10^4$ tumor cells. As shown in Table 3, mice sensitized against 3AM or clone 10 tumors were cross-reactive with each of the 4 tumors, with a high percentage of tumor rejection, ranging from 82 to 100%. In contrast, mice immunized against clone 34 showed 65, 57, and 100% tumor rejection for clone 34, clone 10, and 3AM, respectively, while no cross-reaction was found with clone 27. Similarly, clone 27 did not cross-react with clone 34 but expressed a 56, 71, and 100% tumor rejection for clone 27, clone 10, and parental 3AM, respectively.

**DISCUSSION**

Heterogeneity in metastatic potential of a tumor cell population was first demonstrated by Fidler and Kripke (7) in a malignant murine melanoma, B16, with application of the statistical approach fluctuation test. Since then, similar findings have been confirmed in 4 more murine tumor systems (2, 4, 10, 13). However, direct evidence has not been available to completely rule out the possibility that the heterogeneity expressed by the clones was due to artifacts of cloning instead of intrinsic properties of the original tumors. Table 4 summarizes the findings in the present study. Through identification of the marker chromosomes, it is evident that clones of differing metastatic potentials preexisted in the early passages of the original tumor cell population. Furthermore, correlation studies for each of the 21 clones were performed between the growth rate and the metastatic potential, and no such correlation was found. This is in agreement with the observation of Fidler and Cifone (5) on UV-2237 fibrosarcoma and Hager et al. (8) on spontaneous mammary tumor. Evaluation of the adherence ability of different clones indicated that clone 10, the clone of highest metastatic potential, expressed the highest degree of

### Table 3

Tumor specificity expressed as percentage of tumor rejection of immune mice at a challenge dose of $2.5 \times 10^4$ tumor cells

<table>
<thead>
<tr>
<th>% of tumor rejection</th>
<th>% of tumor incidence from normal control mice</th>
<th>% of tumor incidence from immune mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\times 100$</td>
<td></td>
</tr>
<tr>
<td>Mice immunized against tumor</td>
<td>3AM</td>
<td>Clone 10</td>
</tr>
<tr>
<td>3AM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Clone 10</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>Clone 34</td>
<td>100</td>
<td>57</td>
</tr>
<tr>
<td>Clone 27</td>
<td>100</td>
<td>71</td>
</tr>
</tbody>
</table>

### Table 4

Summary table for the characterization of clones 10, 34, and 27, and the parental line 3AM

<table>
<thead>
<tr>
<th>Type of tumors</th>
<th>Modal chromosome no.</th>
<th>Cytogenetic markers</th>
<th>Tumor growth rate (mm/day)</th>
<th>Endothelial cell attachment ( % of tumor cells attached after 6 washes)</th>
<th>Pulmonary metastases (median no. of metastatic foci/lung)</th>
<th>Immuno-genicity index (see legend of Chart 2)</th>
<th>Antigenically cross-reacted with</th>
</tr>
</thead>
<tbody>
<tr>
<td>3AM (parental)</td>
<td>41</td>
<td>Heterogeneous</td>
<td>382</td>
<td>31.4</td>
<td>42</td>
<td>8.0</td>
<td>Clones 10, 34, and 27</td>
</tr>
<tr>
<td>Clone 10</td>
<td>37</td>
<td>T2, T10, 15</td>
<td>307</td>
<td>54.6 ± 2.5</td>
<td>221</td>
<td>8.0</td>
<td>3AM, clones 34 and 27</td>
</tr>
<tr>
<td>Clone 34</td>
<td>40</td>
<td>T1, 16</td>
<td>485</td>
<td>17.9 ± 1.5</td>
<td>107.5</td>
<td>3.0</td>
<td>3AM, clone 10</td>
</tr>
<tr>
<td>Clone 27</td>
<td>42</td>
<td>T2, 7</td>
<td>311</td>
<td>28.6 ± 5.2</td>
<td>9</td>
<td>3.0</td>
<td>3AM, clone 10</td>
</tr>
</tbody>
</table>
adherence to endothelium. However, there was no linear correlation between metastatic potential and degree of endothelial adherence. Kramer and Nicolson (9) suggested that, in general, highly malignant or highly invasive cells in vivo were capable of attachment, invasion, and migration under endothelial cells in vitro. The results obtained in the present study indicate that adherence of tumor cells to endothelial cells, although necessary for metastasis, is probably not sufficient for production of metastasis. Therefore, cells with high-metastatic potential may be necessarily adherent to endothelium; however, cells with low-metastatic potential may also exhibit endothelial attachment. As metastasis is a multiple-step process, interruption at any stage can prevent production of visible metastasis (5).

In addition to the in vivo growth rate and in vitro endothelial attachment, immunogenicity and antigenic specificity were also evaluated for clones of different metastatic potential and with cytogenetic markers. The influence of the immune status on metastasis has been demonstrated by Fidler et al. (6) and Seshadri et al. (16) for individual solid tumors induced by radiation and chemicals, respectively. In the present study, immunogenicity of the parental cell line and 3 of its clones (10, 34, and 27) was examined. Clone 10 tumors, which produced the most pulmonary metastases, expressed the highest immunogenicity, whereas clone 27 tumors, which formed the fewest pulmonary metastases, expressed a low immunogenicity. This showed that the tumor cell population was heterogeneous in immunogenicity which may play an important role in metastasis. To verify the correlation between degree of immunogenicity and potential for metastasis, detailed studies of more clones are required. To test the tumor specificity of each clone, mice immunized against the parental, clone 10, clone 34, or clone 27 tumors were evaluated for their ability to reject each of the tumors. The results indicated that clone 10 cross-reacted immunologically with clones 27 and 34, while clone 27 and clone 34 tumors did not cross-react with each other. It is of interest to note that clone 10 shared a common chromosome abnormality with clone 27 (chromosome 2) and clone 34 (chromosome 4), respectively, while clones 27 and 34 shared no common chromosome abnormality with each other.

Antigenic heterogeneity has been demonstrated among the subpopulations originated from different locations in a tumor (1, 14). Furthermore, alteration in antigenicity has been found between pulmonary metastasis and the primary tumors (3, 18). All this information and the antigenic specificity study of clones as reported in this paper suggest that among the selection of tumor antigens on the cell surface, there are "common" or overlapping antigens shared by some cells, but there is no universal tumor antigen shared by all cells.

A simple model of this relationship is shown in Chart 4. The 3 clones tested require a minimum of 4 different antigens to explain the experimental results. Obviously, many more may be involved. The unexplained finding was that each of the clones induced immunity against the parent tumor with its mixture of cells from all the clones. This raises the possibility that a mixed population of cells that are antigenically related but not identical may be treated differently by the immune response from an antigenically pure population. Through the study of cell-mediated immunity (12) and growth interactions (11), Miller et al. obtained similar findings between the subpopulations of a viral-associated mouse mammary tumor. The availability of specific marker chromosomes for the identification of individual clones, as reported here, will facilitate more direct comparison of the immunological and other biological behavior of a clone in a pure and reconstructed mixed population.

ACKNOWLEDGMENTS

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REFERENCES

Heterogeneity of a Methylicholanthrene-induced Fibrosarcoma


Fig. 1. Metaphase spreads of clone 27 (A), clone 34 (B), and clone 10 (C) showing the specific marker chromosomes (arrowed) characteristic for each clone.
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