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

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ABSTRACT

High- (CI-33H) and low- (CI-35L) metastatic clones were established from a methylcholanthrene-induced rat fibrosarcoma (FMQ-100). The modal chromosome numbers of the two clones were different. These clones grew in \textit{in vitro} culture, showing similar growth rate and saturation density. However, in \textit{in vivo} experiments, CI-33H exhibited a higher tumor growth rate, tumorigenicity, spontaneous metastatic potential, and experimental metastatic potential than did CI-35L. Alveolar macrophages obtained from normal syngeneic rats stimulated growth of these clones \textit{in vitro}, as assessed by [H]thymidine uptake. Moreover, this effect was greater on CI-33H than CI-35L. The growth-promoting effect of macrophages was also observed under the \textit{in vitro} condition of lack of direct contact between macrophages and tumor cells. These results suggested the possibility that alveolar macrophage-derived growth-promoting factors play some role in the development of pulmonary metastasis in this tumor system, and the difference of susceptibility to the growth-promoting factors might be one of the causes of the different metastatic potentials of CI-33H and CI-35L.

INTRODUCTION

A malignant tumor may consist of various cellular subpopulations which exhibit heterogeneous metastatic potential. This concept originally proposed by Koch and Klein (1, 2) has resulted in the isolation of a number of variants from established tumor cell lines which differ in their metastatic potential (3–13). These variants may be useful to study mechanisms of cancer metastasis. Hematogenous metastasis involves complex biological processes including invasion of the blood vessel by a primary tumor, release of tumor emboli into the blood stream, arrest of the emboli in distant organs, and their final development into secondary tumors. Furthermore, these steps may be influenced by both host factors and tumor cell properties (14–16).

We established high- and low-metastatic clones from a methylcholanthrene-induced rat fibrosarcoma and examined their characteristics \textit{in vivo} and \textit{in vitro}. In particular, the role of alveolar macrophages on pulmonary metastasis was studied.

MATERIALS AND METHODS

Animals and Tumor. Specific-pathogen-free female F344 rats aged 5–8 wk (Charles River Japan, Inc., Tokyo, Japan) were used for the study. A fibrosarcoma, designated FMQ-100, was originally induced in the hind leg of a female F344 rat by the i.m. injection of 1 mg of 3-methylcholanthrene suspended in 0.2 ml of peanut oil mixed with 0.5 mg of cholesterol. The tumor was maintained \textit{in vivo} by monthly i.m. implantation.

Primary Culture and Cloning. The tumor (40th passage) was excised and minced by scissors after removal of necrotic tissue. After treatment with 0.25% trypsin, cell pellets were washed 3 times with HBSS and resuspended at a concentration of \(2 \times 10^5\) cells/ml in TC Medium 199

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: HBSS, Hank's balanced salt solution; [H]HdThd, tritiated thymidine.)
and stained with cold solution containing propidium iodide (0.05 mg/ml) and sodium citrate (1 mg/ml). After 5 min at 4°C, nuclei were dislodged by repeated pipetting. Normal rat spleen cells were used for the standard. All DNA histograms of these samples were obtained with the use of a Cytofluorograf (System 30L; Ortho Diagnostic Systems, Inc., Raritan, NJ).

Preparation of Macrophages. F344 rats were anesthetized with an i.p. injection of pentobarbital sodium. After exsanguination by severing both femoral arteries, the lungs were lavaged with 50 ml of phosphate-buffered saline instilled through the trachea. The lavaged cells were washed 3 times with HBSS and resuspended in the culture medium. This cell suspension was placed in a plastic culture dish. After 1-h incubation, more than 90% of adherent cells were detached by a jet stream of 10 ml of HBSS from a 26-gauge injection needle. These cells were resuspended in the culture medium to be adjusted to various concentrations. More than 98% of these adherent cells were identified as macrophages by Giemsa stain and nonspecific esterase staining.

Heparinized blood was obtained from F344 rats via the abdominal aorta, and the blood from three or four rats was pooled. Mononuclear cells were separated by centrifugation at 400 × g at room temperature for 30 min on Ficoll-Hypaque (LSM; Litton Bioinetics, Kensington, MD). Mononuclear cells were washed 3 times with HBSS and resuspended in the culture medium. Adherent cells were collected as described for alveolar macrophages.

Peritoneal exudate cells of a normal F344 rat were obtained by washing the peritoneal cavity with approximately 50 ml of HBSS. Adherent cells were collected by the method described above.

Growth Inhibition Assay. Tumor cells used as targets were F344, CI-33H, CI-35L, RS-III, and QG-56. RS-III is a methylcholanthrene-induced fibrosarcoma established in a F344 rat, and it does not metastasize to the lung after i.m. inoculation. QG-56 is a human lung cancer cell line (18).

The cytostatic activity of macrophages was estimated by the inhibition of incorporation of [3H]dThd into target tumor cells according to the method previously reported (19). Briefly, 1 × 10⁶ target cells were cocultivated with effector macrophages at various ratios relative to the number of target cells in each well of a microtest plate for 48 h. Thereafter, 0.2 μCi of [3H]dThd were added to each well. After an additional 24-h incubation, the target cells were harvested, and incorporation of [3H]dThd into the target cells was assessed by liquid scintillation counter. The percentage of inhibition or stimulation of [3H]dThd uptake was calculated as (1 - A/K) × 100, where A is the total dpm of target cells alone. All assays were done in triplicate. Isotope uptake by macrophages alone was negligible (<150 dpm) and was not taken into account in the calculation.

To determine the effect of soluble factors secreted by macrophages on tumor growth, a growth inhibition assay was carried out using a specially designed double chamber microtest plate. The chamber, a cross-section of which is shown in Fig. 1, consists of an upper and lower chamber. The lower chamber was filled with 0.2 ml of culture medium containing 1 × 10⁵ target cells. The upper chamber was filled with 0.2 ml of effector cell suspension (5 × 10⁴/ml) or 0.2 ml of the culture medium alone as a control. Following 48-h incubation in a CO₂ incubator, 0.3 μCi of [3H]dThd were added to each well of the upper chamber. Preliminary study showed that [3H]dThd can easily pass through the filter to the lower chamber. After an additional 24-h incubation, the upper chamber was removed. Each well of the lower chamber was washed 3 times with phosphate-buffered saline. The target cells in each well of the lower chamber were harvested by trypsinization. Incorporation of [3H]dThd into the cells was assessed by a liquid scintillation counter. The percentage of inhibition or stimulation of [3H]dThd uptake was calculated as mentioned above.

RESULTS

In Vitro Characteristics of Cultured Tumor Cells. The modal chromosome number of the parent cell population was 69 (38-77), and those of CI-33H and CI-35L were 71 (58-76) and 62 (45-69), respectively. The chromosome number of CI-35L was significantly different from the number of the parent (P < 0.01) and CI-33H (P < 0.001) (Mann-Whitney U test). The difference of distribution of chromosome number between CI-33H and CI-35L may suggest a single cell origin of CI-33H and CI-35L lines. The DNA content of cultured clone cells was analyzed by a flow cyrometer, but a difference in the G1-peak position in the DNA histograms was not detected. When peak positions were standardized by normal spleen G1 peak at Channel 174, the G1-peak positions of parent cell, CI-33H, and CI-35L were 434, 456, and 446, respectively. Studies of in vitro growth of the CI-33H and CI-35L revealed no difference in the growth rate or saturation density. The tumor doubling time of CI-33H was 55.0 h, and that of CI-35L was 56.2 h. The saturation density of CI-33H was 7.6 × 10⁶/cm², and that of CI-35L was 7.3 × 10⁶/cm².

Growth Characteristics in Vivo. The data shown in Table 1 demonstrate that CI-33H has significantly higher metastatic potential than CI-35L or the parent cell line. The metastatic potential of CI-35L was somewhat lower than that of the parent cell, but this difference was not significant statistically. The difference of metastatic potential between CI-33H and CI-35L was stable and reproducible as shown in Experiments 1 and 2. During these experiments, no distant metastasis to organs other than the lung was observed. Pulmonary metastasis induced i.v. injection of tumor cells showed the significantly greater metastatic potential of CI-33H than CI-35L.

The tumorigenicity of CI-33H was somewhat higher than that of CI-35L as shown by the difference in tumor appearance after i.m. inoculation of varying numbers of tumor cells (Table 2). The difference of the tumorigenicity between these two clones was reproducible as shown in Experiments 1 and 2.

![Fig. 1. Cross-section of the double chamber system for assessing the effect of soluble factors secreted by macrophages on tumor growth. a, upper chamber; b, lower chamber; c, 3.0 μm Millipore filter.](image-url)
showed that alveolar macrophages also stimulated [3H]dTThd uptake of CI-33H and CI-35L in the absence of direct contact with tumor cells. CI-33H showed more accelerated growth than did CI-35L. Peripheral blood monocytes did not show significant effect on these two clones under that condition (Table 4).

**DISCUSSION**

Clonal analyses of tumors with heterogeneous potential for metastasis have been carried out extensively by many investigators (3–5, 7–9, 11–13). Correlations between metastatic potential and properties of tumor cells such as the growth rate (20), chromosome number (5, 11, 20), anchorage-independent growth (21), adhesive property (22, 23), immunogenicity (24), and susceptibility to lymphocyte-mediated cytolysis (20, 25) have been studied.

We established high- and low-metastatic clones derived from a methylcholanthrene-induced rat fibrosarcoma. The high-metastatic clone (CI-33H) and the low-metastatic clone (CI-35L), adapted to growth in culture, showed similar growth rates and saturation density under in vitro conditions. As opposed to these in vitro features, differences of growth rate and metastatic potential between these clones became apparent in in vivo experiments. It has been reported that the eventual outcome of the metastatic process depends on both host factors and tumor cell properties (14–16), and the balance of these relative contributions may vary among tumor systems. The results presented here indicate that the major factor affecting metastasis in this tumor system is a sensitivity of tumor cells to host factors rather than the growth potential of tumor cells themselves. The difference of experimental metastatic potential following i.v. inoculations between CI-33H and CI-35L was found to be the same as that in spontaneous metastasis. This suggests that a major factor may be involved in the late stage of the metastatic process, that is, after the release of tumor emboli into the blood stream. Additionally, spontaneous metastases were recognized only in the lung, suggesting that the two cell lines may have organ affinity to the lung.

The importance of macrophages as effector cells in tumor cell destruction has been shown by many investigators (26, 27). In particular, the role of alveolar macrophages as effector cells against pulmonary metastasis has also been reported (28–30). We established high- and low-metastatic clones derived from a methylcholanthrene-induced rat fibrosarcoma. The high-metastatic clone (CI-33H) and the low-metastatic clone (CI-35L), adapted to growth in culture, showed similar growth rates and susceptibility to lymphocyte-mediated cytolysis (20, 25) have been studied.

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The importance of macrophages as effector cells in tumor cell destruction has been shown by many investigators (26, 27). In particular, the role of alveolar macrophages as effector cells against pulmonary metastasis has also been reported (28–30). In contrast to these findings, the present study showed that alveolar macrophages of normal syngeneic rats exerted growth-promoting effects on CI-33H and CI-35L in vitro, and this effect was more marked in CI-33H than on CI-35L.

**Table 2 Tumorigenicity of CI-33H and CI-35L after inoculation of varying numbers of tumor cells**

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
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<tbody>
<tr>
<td>Dose</td>
<td>CI-33H</td>
</tr>
<tr>
<td>3 x 10⁴</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>1 x 10⁴</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>3 x 10⁴</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>3 x 10⁵</td>
<td>4/5 (80)</td>
</tr>
</tbody>
</table>

* Evaluated at 30 days after i.m. inoculation of tumor cells.
* Number of animals with tumor/number of animals tested.
* Numbers in parentheses, percentage of animals with tumor.

The in vivo growth rate of CI-33H tumors was also significantly higher than that of CI-35L, the latter showing a growth pattern similar to the parent tumor (Fig. 2).

**Growth-promoting Effect of Alveolar Macrophages.** As shown in Table 3, alveolar macrophages of normal F344 rats inhibited [3H]dTThd uptake of RS-III and QG-56, but they apparently stimulated that of parent, CI-33H, and CI-35L. The values of stimulation at each effector-target ratio indicated a dose-response relationship of this effect. Furthermore, this growth-promoting effect was significantly stronger on CI-33H than on CI-35L for each effector-target ratio.

Peripheral blood monocytes and peritoneal exudate macrophages of normal F344 rats showed a cytostatic effect on QG-56 as did alveolar macrophages. Peripheral blood monocytes stimulated the growth of CI-33H slightly, but not that of CI-35L and parent. However, peritoneal exudate macrophages of normal F344 rats showed a growth-promoting effect on CI-33H and CI-35L to the same extent.

The results obtained using the double chamber system showed that alveolar macrophages also stimulated [3H]dTThd uptake of CI-33H and CI-35L in the absence of direct contact with tumor cells. CI-33H showed more accelerated growth than did CI-35L. Peripheral blood monocytes did not show significant effect on these two clones under that condition (Table 4).

**Table 3 Effect of macrophages on in vitro tumor growth**

<table>
<thead>
<tr>
<th>Macrophages obtained from</th>
<th>E/T ratio</th>
<th>% of inhibition (-) or stimulation (+) of [3H]dTThd uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Parent CI-33H CI-35L RS-III QG-56*</td>
</tr>
<tr>
<td>Alveolar macrophages of normal F344 rats</td>
<td>10</td>
<td>+31.5 ± 3.9&lt;sup&gt;a&lt;/sup&gt; +102.7 ± 12.2&lt;sup&gt;b&lt;/sup&gt; +33.2 ± 10.2 -30.0 ± 3.7 -43.9 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+19.9 ± 7.9&lt;sup&gt;c&lt;/sup&gt; +60.8 ± 9.0&lt;sup&gt;d&lt;/sup&gt; +14.7 ± 6.5 -18.4 ± 6.5 -22.6 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+18.4 ± 5.6&lt;sup&gt;e&lt;/sup&gt; +26.2 ± 6.1&lt;sup&gt;f&lt;/sup&gt; +3.0 ± 5.3 +0.7 ± 2.9 -4.6 ± 2.4</td>
</tr>
<tr>
<td>Peripheral blood monocytes of normal F344 rats</td>
<td>10</td>
<td>+4.2 ± 3.0&lt;sup&gt;g&lt;/sup&gt; +25.3 ± 7.4 -0.8 ± 7.0 -46.2 ± 6.2 -26.3 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-1.1 ± 6.7&lt;sup&gt;h&lt;/sup&gt; +17.3 ± 8.1 -3.8 ± 2.8 -46.2 ± 6.2 -5.8 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+9.9 ± 5.2&lt;sup&gt;i&lt;/sup&gt; +11.2 ± 7.2 -4.6 ± 3.8 -5.8 ± 5.4 -5.8 ± 5.4</td>
</tr>
<tr>
<td>Peritoneal exudate macrophages of normal F344 rats</td>
<td>10</td>
<td>+26.1 ± 7.2&lt;sup&gt;j&lt;/sup&gt; +19.7 ± 6.6 -58.0 ± 1.5 -47.4 ± 1.8 -28.9 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+18.0 ± 6.8&lt;sup&gt;k&lt;/sup&gt; +17.5 ± 10.6 -47.4 ± 1.8 -28.9 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+8.4 ± 4.5&lt;sup&gt;l&lt;/sup&gt; -9.5 ± 5.4 -28.9 ± 5.8 -28.9 ± 5.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> E/T, effector/target cell ratio.
<sup>b</sup> Syngeneic fibrosarcoma cell line.
<sup>c</sup> Human lung cancer cell line.
<sup>d</sup> Mean ± SE.
<sup>e</sup> Significantly different from parent (P < 0.001) and CI-35L (P < 0.001) (Student's t test).
<sup>f</sup> Significantly different from parent (P < 0.001) and CI-35L (P < 0.001) (Student's t test).
<sup>g</sup> Significantly different from CI-35L (P < 0.001) (Student's t test).
Growth-promoting effects of macrophages on tumors were previously reported with lymphoid tumors (31-33) and nonlymphoid tumors (34-37). Evans showed that depletion of macrophages by X-ray treatment was associated with a delay in the appearance of a murine nonmetastasizing, strongly immunogenic fibrosarcoma and also with a decrease in the continuous growth of established tumors (34). He suggested that tumor-associated macrophages may stimulate the growth of the tumor.

Mantovani (36) found that macrophages isolated from a weakly immunogenic metastasizing murine sarcoma nonspecifically enhanced the proliferative activity of tumor target cells, and he and his coworkers (37) also showed a growth-promoting effect of tumor-associated macrophages on at least one of two allogeneic and two xenogeneic target cell lines in patients with ascitic ovarian tumors. Gabizon and Trainin (35) reported that normal murine peritoneal macrophages were capable of enhancing growth of a syngeneic fibrosarcoma in vivo transfer tests, and those from tumor-bearing mice also showed a strong tumor-enhancing effect. The present study suggested the possibility that alveolar macrophages may play some role in the promotion of lung metastasis, and that the difference of susceptibility to the growth-promoting effect of the macrophages may be one of the causes of different metastatic potential between CI-33H and CI-35L. In vivo administration of carageenan, which inhibits macrophage function, did not eliminate the difference in the metastatic potential of the CI-33H and CI-35L. Furthermore, in vitro treatment of alveolar macrophages with carageenan did not diminish the growth-promoting effect (data not shown). This suggests that the growth-promoting effect of macrophages is independent of phagocytic or cytotoxic function which was affected by carageenan.

Macrophages secrete a variety of biologically active substances. Secretory products are important in many macrophage functions, such as immunoregulation and the killing of tumors (38, 39). Namba and Hanaoka reported that a humoral factor produced by cultured phagocytic cells stimulated growth of IgM-producing myeloma cells (MOPC-104E), and that the factor was a heat-labile glycoprotein (32, 40). Salomon and Hamburger hypothesized that a macrophage-secreted factor supporting clonal multiplication of antigen-activated B-lymphocytes also incidentally supports the proliferation of neoplastic cells. They also suggested the role of macrophages as a promoter of tumor growth (41). In our study, the growth-promoting effect of macrophages was also observed in vitro under conditions preventing direct contact between effector and target cells, but the culture supernatant of alveolar macrophages did not exhibit any effect on the tumor growth (data not shown). This suggests the relative instability of the growth-promoting factor secreted by macrophages.

Table 4 Effect of soluble factors secreted by macrophages on in vitro tumor growth

<table>
<thead>
<tr>
<th>Macrophages obtained from</th>
<th>CI-33H</th>
<th>CI-35L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar macrophages of normal F344 rats</td>
<td>+80.8 ± 26.0*</td>
<td>+23.2 ± 10.2</td>
</tr>
<tr>
<td>Peripheral blood monocytes of normal F344 rats</td>
<td>−10.7 ± 5.9</td>
<td>−10.8 ± 12.4</td>
</tr>
</tbody>
</table>

a Assessed by using the double chamber system (effector/target cell ratio is 10).
b Mean ± SE.

REFERENCES

29. Sone, S., and Fidler, I. J. In situ activation of tumoricidal properties in rat alveolar macrophages and rejection of experimental lung metastases by


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