Enhancement of Metastatic Capacity of Fibroblast-Tumor Cell Interaction in Mice

Hajime Tanaka, Yoichi Mori, Hiroko Ishii, and Hitoshi Akedo

Departments of Radiology [H. T.], Cell Biology [Y. M., H. I.], and Tumor Biochemistry [H. A.], Research Institute, The Center for Adult Diseases, Osaka, Osaka 537, Japan

ABSTRACT

A low metastatic clone, G6, was isolated from the B16 melanoma cell line by cloning procedure. When the cells were cultured in vitro with fibroblasts from newborn mice, the lung-colonizing potential of G6 cells was substantially increased. The effect of coculture depended on the number of the fibroblasts. The elevated colonizing potential of G6 cells was reversed to the original low potential by subculturing them for 20 days without the fibroblasts. The culture medium conditioned by G6-fibroblast coculture demonstrated an activity to enhance the lung-colonizing potential of G6 cells, whereas the medium from the culture of fibroblasts alone showed only a little activity. The growth rate and plating efficiency of G6 cells cultured with the fibroblasts or in the conditioned medium did not differ from those of uncultured G6 cells. The potentiating activity in the conditioned medium was nondialyzable and stable to heating at 80°C for 10 min, but was lost after heating for 10 min at 120°C, or by the treatment with trypsin. These results indicate that the enhancement of lung-colonizing potential of G6 cells could be mediated by a soluble factor(s) released from cocultured fibroblasts.

INTRODUCTION

Metastasis is a multistep phenomenon including release of malignant cells from the primary tumor, intravasation, circulation of the released tumor cells, and their lodging at the target organs where they proliferate and form secondary tumor foci (1-4). Interruption of the sequence at any of these steps can prevent the formation of metastases. Several factors such as tumor cell-matrix interactions (5, 6), normal cell-tumor cell interactions (7-12), and organ-tumor cell interactions (13-16) may be involved in these steps.

Tumors are often extensively infiltrated by normal host cells (17-21) such as macrophages, lymphocytes, and fibroblasts. When these normal cells are in proximity to neoplastic cells, they are able to have profound effects on the growth and other properties of tumor cells (18-20). Rapid generation of phenotypic heterogeneity of tumor cells has been observed in vivo passage (22-27). For example, the growth of a hepatocarcinoma cell line in vivo resulted in more rapid generation of phenotypic heterogeneity than the same line maintained in vitro (27).

There have been other reports on enhancement of metastatic capacity of tumor cells after a single passage through s.c. transplantation (28, 29) and of tumor cells recovered from their lungs after i.p. injection of 1 ml of thioglycollate medium (Nippon Roche, Tokyo, Japan). Macrophages were collected by peritoneal lavage with 10 ml of PBS, centrifuged, and resuspended in RPMI-FCS. The cell suspension containing an appropriate number of macrophages was inoculated i.v. via the tail vein with an appropriate number of cells in 0.2 ml of PBS. Mice were sacrificed 21 days after the inoculation; the lungs were removed and rinsed in water, and the number of lung tumor colonies (experimental metastases) was counted under a dissecting microscope.

Preparation of Fibroblast Monolayer. Fibroblasts were obtained by treating the minced skin of C57BL/6 newborn mice with 0.25% trypsin for 1 h at 37°C. The cells were cultured as a monolayer in RPMI-FCS at 37°C in a humidified atmosphere of 5% CO₂ in air.

Preparation of Macrophage Monolayer. Peritoneal macrophages were collected 5 days after i.p. injection of 1 ml of thioglycollate medium (Nippon Roche, Tokyo, Japan). Macrophages were collected by peritoneal lavage with 10 ml of PBS, centrifuged, and resuspended in RPMI-FCS. The cell suspension containing an appropriate number of macrophages was inoculated in each flask. The macrophages were allowed to adhere to the bottom of the culture flask at 37°C for 2 h, and the resulting monolayer macrophage was washed with jets of medium from a Pasteur pipet. Macrophages were identified by morphological criteria.

Preparation of Lymphocytes. Mesenterial lymph nodes were minced with fine scissors in PBS. The resulting cell suspensions were then filtered through 150-mesh stainless steel wire screens. The cell suspensions were centrifuged, and the pellets were resuspended in RPMI-FCS. Cell viability determined by the trypsin blue exclusion test was about 95%.

Coculture of G6 Cells with Normal Cells. Before the coculture, normal tumor cells. We found that coculture with fibroblasts increased the lung-colonizing potential of a low metastatic subclone of B16 melanoma and that this increase was probably mediated by a putative factor(s) released from the host fibroblasts.

MATERIALS AND METHODS

Animals. Specific-pathogen-free female C57BL/6 mice, 6 to 8 wk old, were obtained from Shizuoka Laboratory Animal Center, Shizuoka, Japan. Mice were age and weight matched for each experiment.

Tumors and Culture Conditions. The B16 melanoma cell line was supplied by Dr. K. Ootsu, Biological Research Laboratories, Takeda Chemical Industries, Ltd., Osaka, Japan. Cells and clones were grown in 25-cm² plastic tissue culture flasks (Corning Glass Works, Corning, NY) in RPMI-FCS at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cloning Procedures. Clones were isolated from B16 melanoma cells by a slight modification of the method of Fedler and Kripke (32). Briefly, clones were isolated by plating 0.01 ml of tumor cell suspensions in RPMI-FCS (100 cells/ml) into 96-well sterile plastic microwell dishes (No. 3072; Becton Dickinson, Oxnard, CA). Wells containing a single cell were identified under a phase-contrast microscope, and colonies formed in these wells were transferred subsequently to vessels of increasing size and then passaged routinely in RPMI-FCS. The clones obtained differed in their ability to produce experimental lung metastasis. A poorly metastatic clone, G6, was used for the present studies.

Experimental Pulmonary Metastasis. Tumor cells were harvested from subconfluent monolayer cultures in exponential growth phase by overlaying the cells with 0.25% trypsin and 0.02% EDTA for 2 min. The detached cells were then washed and resuspended in PBS. Only those cell suspensions composed of single cells with viability of 95% or greater were used. Groups of 8 to 10 unanesthetized mice were inoculated i.v. via the tail vein with an appropriate number of cells in 0.2 ml of PBS. Mice were sacrificed 21 days after the inoculation; the lungs were removed and rinsed in water, and the number of lung tumor colonies (experimental metastases) was counted under a dissecting microscope.

Preparation of Fibroblast Monolayer. Fibroblasts were obtained by treating the minced skin of C57BL/6 newborn mice with 0.25% trypsin for 1 h at 37°C. The cells were cultured as a monolayer in RPMI-FCS at 37°C in a humidified atmosphere of 5% CO₂ in air.

Preparation of Macrophage Monolayer. Peritoneal macrophages were collected 5 days after i.p. injection of 1 ml of thioglycollate medium (Nippon Roche, Tokyo, Japan). Macrophages were collected by peritoneal lavage with 10 ml of PBS, centrifuged, and resuspended in RPMI-FCS. The cell suspension containing an appropriate number of macrophages was inoculated in each flask. The macrophages were allowed to adhere to the bottom of the culture flask at 37°C for 2 h, and the resulting monolayer macrophage was washed with jets of medium from a Pasteur pipet. Macrophages were identified by morphological criteria.

Preparation of Lymphocytes. Mesenterial lymph nodes were minced with fine scissors in PBS. The resulting cell suspensions were then filtered through 150-mesh stainless steel wire screens. The cell suspensions were centrifuged, and the pellets were resuspended in RPMI-FCS. Cell viability determined by the trypsin blue exclusion test was about 95%.

Coculture of G6 Cells with Normal Cells. Before the coculture, normal...
FIBROBLAST-MEDIATED ENHANCEMENT OF METASTASIS

Table 1 Lung-colonizing potentials of G6 cells cocultured with host cells

<table>
<thead>
<tr>
<th>Host cells</th>
<th>No. of host cells</th>
<th>Median no. of lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3 (0-11)*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>10^4</td>
<td>13 (5-39)</td>
</tr>
<tr>
<td>5 x 10^4</td>
<td>133.5 (62-229)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10^5</td>
<td>347 (327-417)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Macrophages</td>
<td>10^4</td>
<td>9 (1-21)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10^4</td>
<td>13 (6-41)</td>
</tr>
<tr>
<td></td>
<td>10^5</td>
<td>5 (0-14)</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>5 (3-10)</td>
</tr>
</tbody>
</table>

* Probability of no difference in numbers of lung colonies from the uncocultured G6 cells.
* Numbers in parentheses, range.
* NS, not significant.

Table 2 Stability of lung-colonizing potential of G6 cells cocultured with fibroblasts

<table>
<thead>
<tr>
<th>Days of subculture</th>
<th>Median no. of lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>260 (214-343)*</td>
</tr>
<tr>
<td>3</td>
<td>317 (287-417)</td>
</tr>
<tr>
<td>5</td>
<td>294 (217-386)</td>
</tr>
<tr>
<td>8</td>
<td>233 (196-271)</td>
</tr>
<tr>
<td>14</td>
<td>38 (13-72)</td>
</tr>
<tr>
<td>20</td>
<td>8 (6-21)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, range.

Table 3 Effect of the conditioned medium on the induction of lung-colonizing potential of G6 cells

<table>
<thead>
<tr>
<th>Culture medium conditioned by</th>
<th>Median no. of lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6 cells (control)</td>
<td>4 (2-16)*</td>
</tr>
<tr>
<td>X-irradiated fibroblasts</td>
<td>23 (0-32)</td>
</tr>
<tr>
<td>X-irradiated fibroblasts with</td>
<td>155 (129-208)</td>
</tr>
<tr>
<td>G6 cells</td>
<td>127 (25-198)</td>
</tr>
</tbody>
</table>

* Probability of no difference in numbers of lung colonies from the control cells.
* Numbers in parentheses, range.

RESULTS

The lung-colonizing potential of a low metastatic clone (G6) did not appreciably change at least for 6 mo, when the clone was maintained by culturing in vitro.

Coculture of G6 Cells with Normal Component Cells. When G6 cells (2 x 10^4/dish) were cocultured with X-irradiated newborn fibroblasts (10^5-10^6/dish) for 8 days, the tumor cells showed increased lung-colonizing potentials, depending on the number of fibroblasts cocultured (Table 1). Coculture of G6 cells with macrophages also gave an increase in the colonizing potential, though to a much lesser extent. Lymphocytes had no appreciable effect. G6 cells cultured in the presence of 20% heat-inactivated serum from both normal and G6-bearing C57BL/6 mice did not show any increment of the colonizing potential (data not shown). To eliminate the possibility that fibroblasts contaminated in the G6 cell population, if any, might have contributed to the increase of lung colonization, G6 cells and X-irradiated fibroblasts (without coculture) were i.v. cojected into mice. No appreciable difference in lung colonization was found between the injection of G6 cells alone and G6 cell-fibroblast mixture. The colonizing potential of G6 cells began to increase in 5 days of coculture with fibroblasts, reaching a maximum level at the eighth day (median, 288), and the level was maintained thereafter (median, 293 at the 15th day). Coculture of G6 cells with the fibroblasts did not alter the platelet activity on a plastic dish and in vitro growth rate in RPMI-FCS of the tumor cells (data not shown). The 8-day-cocultured G6 cells (1 x 10^9), when s.c. inoculated into 20 individual mice, did not develop any detectable metastases in the lungs at the time of autopsy. The control G6 cells formed no spontaneous metastases either.

Stability of the Induced Metastatic Capacity. To examine the stability of the induced metastatic capacity, G6 cells that had cocultured with fibroblasts for 8 days were transferred into fresh RPMI-FCS, cultured without fibroblasts, and tested for their metastatic capacity at intervals. As shown in Table 2, the induced metastatic capacity of G6 cells fell gradually to the level for the original G6 cells in 20 days of subculture.

Effect of the Culture Medium Conditioned by G6-Fibroblast Coculture. To investigate whether the induction of metastasis capacity by fibroblasts was due to the direct contact of G6 cells with fibroblasts or was mediated by a factor(s) released from fibroblasts, the media conditioned by fibroblast culture and G6-fibroblast coculture were tested for their abilities to induce the metastatic capacity of G6 cells. As shown in Table 3, the medium conditioned by fibroblasts alone had only a little inducible ability. However, the treatment of G6 cells with the medium conditioned by G6-fibroblast coculture gave a significantly greater increase in the metastatic capacity of G6 cells, suggesting the release of a factor(s) capable of inducing the metastatic potential from either G6 or fibroblasts when both were cocultured. A possibility that the cocultured G6 cells could release the factor(s) is less likely, because the conditioned media conditioned by fibroblasts did not alter the plating efficiency of G6 cells (data not shown). The 8-day-cocultured G6 cells (1 x 10^9), when s.c. inoculated into 20 individual mice, did not show any increment of the colonizing potential (data not shown). The 8-day-cocultured G6 cells (1 x 10^9), when s.c. inoculated into 20 individual mice, did not show any increment of the colonizing potential (data not shown).
FIBROBLAST-MEDIATED ENHANCEMENT OF METASTASIS

Table 4 Heat stability and trypsin sensitivity of the potentiating activity in the conditioned medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median no. of lung metastases</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>180 (82-225)</td>
<td></td>
</tr>
<tr>
<td>Heat treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80°C, 10 min</td>
<td>197 (118-304)</td>
<td>NS*</td>
</tr>
<tr>
<td>100°C, 10 min</td>
<td>118 (31-128)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>120°C, 11 min</td>
<td>11 (2-16)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trypsin treated</td>
<td>13.5 (6-39)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Probability of no difference in numbers of lung colonies from the control cells.
* Numbers in parentheses, range.
* NS, not significant.

Recently, acquisition of the invasive and metastatic phenotype by tumor cells under their possible interactions with normal cells has been reported (7–12). In particular, Picard et al. (11) have shown increased lung colonization of a poorly metastatic subclone from RMS9-4/0 cojected with fibroblasts. Unfortunately, however, they did not conclude whether the fibroblasts acted directly on the tumor cells or whether they elevated lung colonization through a mechanism by which they modified host environmental conditions. In the present study we have demonstrated tumor cell-fibroblast interaction in modulating lung-colonizing potential of the tumor cells.

The molecular mechanism by which fibroblasts enhanced the lung-colonizing potential of G6 cells is not clear at present. A possible depletion of some medium components by fibroblasts seems less likely, because (a) substitution of the conditioned medium by PBS (nutritionally much poorer than the conditioned medium) had no potentiating activity, (b) extensive dialysis against RPMI-medium of the conditioned medium from G6-fibroblast coculture did not eliminate its potentiating ability, and (c) the effect of the conditioned medium was lost after the treatment of the medium with trypsin. These results suggest the release in the cocultured medium of a nondialyzable substance of a protein nature capable of inducing lung-colonizing ability. Koopman and Cotton (34) have reported a polypeptide-like substance in the medium conditioned by STO mouse fibroblast cells that inhibited the differentiation of NG2 and F9 embryonal carcinoma cells.

The putative substance in the G6-fibroblast coculture capable of potentiating the lung-colonizing ability of G6 cells was most likely released from fibroblasts, because (a) the conditioned medium from the culture of G6 cells alone had no potentiating activity, whereas that from the culture of fibroblasts alone had a similar activity, though a little; and (b) the conditioned medium from G6 cells sub cultured from G6-fibroblast coculture had no activity. The putative substance appears to be released from the fibroblasts efficiently, when they are in contact with the tumor cells. A possible relation of this substance to matrix components remains to be determined. Biswas (35) demonstrated that rabbit fibroblasts could be stimulated by a soluble factor released from mouse tumor cells of epithelial origin to produce collagenase specific for type I collagen, which could potentiate metastatic behavior of the tumor cells.

The growth rate and plating efficiency of the cocultured G6 cells did not differ from those of un cocultured G6 cells, suggesting that the apparent increase in lung colonization of the cocultured G6 cells could not be the result of selective growth advantage of the tumor cell population. Resistance to natural and specific immune defenses, increase of homotypic or heterotypic aggregation, and release of lytic enzymes from the potentiated G6 cells remain to be determined. Fusion of certain tumor cells with host lymphocytes or macrophages has been reported to enhance metastatic capacity (36–38). Although we have not examined any possible fusion of G6 cells with fibroblasts, fusion seems less likely to participate in the elevation of lung-colonizing potential, because the conditioned medium from G6-fibroblast coculture gave a similar degree of potentiation.

Instability of malignant phenotypes, including metastatic properties of clonal neoplastic cells during in vitro and in vivo growth, has been reported (24, 26, 39, 40). Environmental changes, such as transfer from in vivo growth to in vitro culture, were reported to modify metastatic ability (41). Neri and Nicolson (42) observed a large variation in the stability of metastatic phenotype within the clones of mammary adenocarcinoma cells during growth in vitro. Some of the clones were...
phenotypically stable in prolonged cultivation. In our study, G6 cells were stable in its lung-colonizing potential during the prolonged cultivation. However, the potentiated colonizing ability of G6 cells that had been cocultured with fibroblasts or cultured in the conditioned medium was unstable and reversed by subculturing the tumor cells without fibroblasts or the conditioned medium. Such instability of the acquired capacity resembled the ones reported by Raz and Ben-Ze’ev (43), who studied metastability of B16 melanoma cells under different culture conditions, and by Takenaga (44), who studied the effect of dimethyl sulfoxide treatment on Lewis lung carcinoma cells.

Metastasis is the result of a highly complex chain of events; no single mechanism is likely to be sufficient to guarantee its success. Aukerman et al. (4) suggested a single or multiple different deficiencies in low metastatic clones. G6 cells cocultured with fibroblasts produced a large number of lung colonies when injected i.v., whereas, when inoculated s.c., the potentiated G6 cells failed to develop lung metastases. This may mean that coculture with fibroblasts repaired the deficiency in pulmonary arrest of the tumor cells.

Stackpole (28) demonstrated that several cell lines derived from B16 melanoma that were initially weakly colonizing became more strongly colonizing after a single s.c. passage. In our earlier study, G6 cells were found to increase lung-colonizing ability after a single in vivo passage, and the acquired metastatic potential was lost after subculturing the cells for at least 20 days in vitro.

Whether the induction of lung-colonizing ability of G6 cells by in vitro coculture with fibroblasts is an in vivo counterpart and whether it has any relevance to the mechanism of tumor invasion and metastasis remain matters of speculation. The lung-colonizing ability of G6 cells potentiated by fibroblasts in vitro resembled that of the in vivo pasaged G6 cells (data not shown). However, the density of fibroblasts in the coculture may be much higher than that in the growing G6 tumor in vivo. Proliferating (cultivated) fibroblasts may act on tumor cells differently from the seemingly quiescent fibroblasts at the implantation site (11), although one cannot eliminate the possibility that the fibroblasts in the tumor mass may be active and in a proliferating state. Additional studies are necessary to understand the relationship between the in vitro and possible in vivo interactions of tumor cells with host normal cells.

REFERENCES

15. Nicolson, G. L., and Dulsik, K. M. Organ specificity of metastatic coloniza

Downloaded from cancerres.aacrjournals.org on May 3, 2017. © 1988 American Association for Cancer Research.
Enhancement of Metastatic Capacity of Fibroblast-Tumor Cell Interaction in Mice

Hajime Tanaka, Yoichi Mori, Hiroko Ishii, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/6/1456

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.