ABSTRACT

In vitro and in vivo invasive capacities of rat ascites hepatoma cells (AH 130) that had been cultured on the feeder layers of rat macrophages were examined. The in vitro invasive capacity of the tumor cells was measured by their ability to form tumor cell colonies underneath cultured mesothelial cell monolayers; in vivo invasive capacity was examined by the implantation of the tumor cells into the rat peritoneal cavity. When the tumor cells were precultured on a macrophage feeder layer, the in vitro invasive capacity of the tumor cells increased almost 10 times as much as that of uncultured control cells. The cocultured tumor cells, when implanted in rat peritoneal cavity, infiltrated extensively in the peritoneum and formed many tumor nodules and enlarged metastatic lymph nodes. Implantation of the uncultured tumor cells did not develop any macroscopically detectable nodules. The effect of macrophages was reversed by subculturing the cocultured tumor cells without macrophages. Treatment of the tumor cells with the medium conditioned by macrophage culture did not result in the increase in invasive capacity. Almost 50% of the macrophage-mediated enhancement of the in vitro invasive capacity was inhibited by the simultaneous addition of superoxide dismutase and catalase at the time of tumor cell-macrophage coculture.

INTRODUCTION

Malignant tumor cells are characterized by their ability to invade surrounding normal tissues and metastasize to distant host sites. Tumor cell metastasis seems to develop through the interaction of tumor with host cells. Although host macrophages have been reported to be tumoricidal (1), a possible involvement of macrophages in enhancing metastatic potentials of certain tumor cells has also been reported. For example, Gorelik et al. (2) observed an augmentation of artificial lung metastasis of B16 melanoma and Lewis lung carcinoma cells by the i.v. injection of elicited host macrophages. Kerbel et al. (3) have presented evidence for hybridization of tumor cells with bone marrow-derived cells in generating highly metastatic tumor cell variants. Larizza et al. (4) have shown that in vitro hybridization of tumor cells with macrophages enhanced metastatic potential.

These investigators have tested the effect of macrophages on the formation of secondary tumors at distant host sites. Metastases establish themselves by a sequential series of steps including invasion by tumor cells of surrounding normal tissues, entrance into microcirculation, implantation in distant host sites, extravasation, and growth. Despite its obvious importance, relatively little is known about the mechanism of tumor cell invasion.

We recently established a culture system for studying tumor cell invasion (5). In this system, rat ascites hepatoma cells (AH 130 cells) were seeded on primary cultured layers of mesothelial cells isolated from rat mesentery. The individual tumor cells penetrated beneath the mesothelial cell layer, grew, and formed flattened tumor cell islands underneath the monolayer (penetrated tumor cell colonies). This in vitro penetration by tumor cells mimics in vivo invasion by the tumor cells morphologically. Moreover, the in vitro invasive capacity of the tumor cells estimated by counting the number of penetrated colonies was found to correspond with the in vivo invasive capability of the tumor cells; our culture system could provide a useful method to investigate tumor cell invasion. By using this system, we recently found that the preculture of AH 130 cells with macrophages greatly potentiated both the in vitro and in vivo invasive capacities of the tumor cells.

MATERIALS AND METHODS

Tumor Cells and Cell Culture. AH 130 cells were maintained by serial i.p. transplantation into male Donryu rats. The cells were obtained usually 7 days after the transplantation. They consisted of about 93% tumor cells, 3% monocytes, and 4% lymphocytes. These cells were cultured for 1 week before use in EM supplemented with 10% calf serum and replated every other day. The tumor cells grew in suspension and did not adhere to plastic culture dishes, whereas monocytes did. By this procedure, host cells were eliminated and virtually not contaminated in the cell suspension after 1 week of culture. The cultured tumor cells were confirmed to be transplantable in rat peritoneal cavity.

Mesothelial cells were isolated and cultured as reported previously (5). Briefly, mesothelial cells were isolated from normal rat mesentery by trypsin digestion and the cells (1 x 10⁶) were inoculated into a 35-mm plastic culture dish and cultured in EM supplemented with 10% calf serum in an atmosphere of 95% air and 5% CO₂ at 37°C. M-cells grew forming a monolayer resembling a "paving stone sheet." When M-cells grew to a confluent state, the monolayer was used for the assay of in vitro tumor cell invasion.

Macrophages. Glycogen-elicited peritoneal exudate cells were obtained from normal rats given i.v. injections of 10 ml of 0.1% glycogen in PBS 4 days before harvesting. BCG-activated peritoneal exudate cells were obtained 3 weeks after i.p. injection of 6 x 10⁶ viable BCG (Japan BCG Manufacturing Co.) per rat as reported (6). Peritoneal exudate cells were harvested by washing the peritoneal cavity with EM supplemented with 10% calf serum and 10 units/ml sodium heparin (Novo, Ind. A/S, Denmark). The cells were washed with PBS, plated on culture dishes, and incubated at 37°C for 140 min to obtain adherent cells. To isolate splenic monocytes, rat spleen was cut into pieces and the cells released from the tissue pieces were centrifuged at 400 x g for 30 min in Ficoll-metrizoate solution (Muto Pure Chem., Ltd., Japan). The cells fractionated at the interface were collected and washed once with PBS. Monocytes from rat peripheral blood were fractionated in Ficoll-metrizoate solution as for isolating splenic monocytes. The monocyte fractions were seeded in plastic culture dishes containing EM with 10% calf serum and cultured for 7 days to obtain adherent cells. During the culture, the medium was refreshed every other day. Under histological examinations and nonspecific esterase staining more than 95% of the adherent cells were macrophages. These cells were used for macrophage feeder layer.

Coculture of AH 130 Cells with Macrophages. AH 130 cells (2.4 x 10⁶/dish) were seeded on the macrophage feeder layer (7 x 10⁶–1.7 x 10⁷ macrophages/dish) and cultured for various periods of time. After the coculture, the medium was gently pipetted and the tumor cells in suspension were harvested, washed with the culture medium, and tested...
for their invasive capacities. Staining the cocultured tumor cell population for macrophage esterase showed that the contamination of macrophages was less than 1.8%. To test the effect of indomethacin, \(10^{-4}\) M indomethacin was added to the culture medium during the coculture.

**Medium Conditioned by Macrophage Feeder Layers.** Conditioned medium was obtained from 3 days of culture of splenic macrophages. The medium was filtered through a Millipore membrane (0.45 \(\mu\)m in diameter).

Assay and Histological Examinations. The *in vitro* invasive capacity of the tumor cells was assayed by counting tumor cell colonies formed underneath the M-cell monolayer by penetrated tumor cells as reported previously (7). Briefly, \(2.4 \times 10^5\) AH 130 cells were seeded on an M-cell monolayer and cultured for 48 h in EM supplemented with 10% calf serum. The number of penetrated tumor cell colonies in 60 different visual fields (1.13 mm\(^2\) each) was counted under a phase contrast microscope with the guide of a grid drawn on the lid of culture dish and expressed as penetrated colonies/cm\(^2\). SOD and CAT were purchased from Boehringer Mannheim, West Germany, and cytochrome c (type IV) was from Sigma Chemical Co. For histological examinations, tissues were fixed in 10% formalin and embedded in paraffin, and thin sections were stained with hematoxylin and eosin.

**RESULTS**

**AH 130 Cells Cocultured with Macrophages.** When AH 130 cells were cultured on macrophage feeder layers, the tumor cells tended to aggregate together on Day 1 and formed tumor cell clumps (10–20 cells/clump) on Day 3 (Fig. 1). The cells in the clumps did not bind tightly together; they could easily be disaggregated by gentle pipeting. Generation time of the cocultured tumor cells did not differ significantly from the uncultured tumor cells, unless the number of macrophages exceeded that of the tumor cells.

**In Vitro Invasion by AH 130 Cells Precultured with Macrophages.** The formation of penetrated tumor cell colonies (*in vitro* invasion) was measured with AH 130 cells precultured with macrophages obtained from rat spleen and peripheral blood. As shown in Table 1, preculture of the tumor cells on macrophages resulted in an appreciable increase in the formation of penetrated tumor cell colonies, irrespectively of the source of macrophages. Both glycogen-elicited and BCG-activated peritoneal macrophages showed a similar effect. The stimulation by macrophages depended on the time period of preculture; the effect increased with time up to about 48 h (Fig. 2). Fig. 3 shows that the degree of potentiation depended on the number of macrophages seeded per dish. More than \(3 \times 10^4\) macrophages/dish had a stimulatory effect.

**In Vivo Invasion by AH 130 Cells Precultured with Macrophages.** When AH 130 cells that had been cultured for 5 days on a macrophage feeder layer were implanted in rat peritoneal cavity, the tumor cells invaded extensively in the peritoneum and formed many tumor nodules (Fig. 4a). Enlarged metastatic lymph nodes were found in the mediastinum and the retroperitoneal space near the renal pelvis. Occasionally, the tumor cells infiltrated in the liver and pancreas. In contrast, the implantation of tumor cells that had been cultured for 5 days without macrophages did not develop any macroscopically detectable tumor nodules in the peritoneum, or infiltrated in the liver and pancreas (Fig. 4b).

**Table 1 Potentiation of *in vitro* invasive capacity of AH 130 cells by macrophages and the macrophage-conditioned medium**

<table>
<thead>
<tr>
<th>Macrophages or conditioned medium</th>
<th>No. of penetrated colonies/cm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(110.7 \pm 15.3^a)</td>
</tr>
<tr>
<td>Peritoneal macrophages</td>
<td>(1004.0 \pm 164.6)</td>
</tr>
<tr>
<td>Glycogen-elicited</td>
<td>(1112.8 \pm 64.2)</td>
</tr>
<tr>
<td>BCG-activated</td>
<td>(795.3 \pm 56.0)</td>
</tr>
<tr>
<td>Splenic macrophages</td>
<td>(625.8 \pm 35.6)</td>
</tr>
<tr>
<td>Macrophages from peripheral blood</td>
<td>(147.4 \pm 3.1)</td>
</tr>
<tr>
<td>Conditioned medium</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD of at least 3 determinations.

![Fig. 1. AH 130 cells cocultured with and without macrophages.](image)

- **a.** AH 130 cells from the 3 days of coculture with splenic macrophages. Note a number of large tumor cell clumps; **b.** AH 130 cells cultured for 3 days without macrophages. Phase contrast, \(\times 130\).
POTENTIATION BY MACROPHAGES OF TUMOR CELL INVASION

Fig. 4. In vivo invasion of AH 130 cells cocultured with macrophages. AH 130 cells that had been cultured for 7 days on the feeder layers of splenic macrophages were implanted in rat peritoneal cavity. Rats were sacrificed 10 days later. a, tumor nodules (arrows) formed in the peritoneum of rats that received AH 130 cells that had been cocultured with macrophages; b, a macroscopic view of the visceral organs of a control rat that received AH 130 cells that had been cultured without macrophages.

Reversibility of the Effect of Macrophages. To examine the stability of the macrophage-induced invasive capacity, AH 130 cells that had been precultured on a macrophage feeder layer were transferred into a fresh culture medium, cultured for another 15 days, and tested at intervals for their ability to form penetrated colonies. As shown in Fig. 5, the in vitro invasive capacity of the tumor cells gradually decreased to a half-maximal level in 2 days and thereafter approached the original potential.

Effect of the Culture Medium Conditioned by Macrophage Feeder Layers. The medium conditioned by macrophages was tested for its ability to potentiate the in vitro invasive capacity of AH 130 cells. The tumor cells were cultured for 4 days in the conditioned medium, washed, and seeded on M-cell monolayer. Only a slight increase in the penetrated colony formation over the untreated control was observed (Table 1).

Effect of Indomethacin and Prostaglandin E2. Young and Newby (8) reported that the in vitro migration of a Lewis lung carcinoma variant was stimulated by the culture supernatant of macrophages, in which PGE2 had been excreted. Therefore, we tested the effect of indomethacin and PGE2 on the in vitro invasion by AH 130 cells. Indomethacin (10^-6 M) added in the medium of AH 130 cell-macrophage coculture had no inhibitory effect on the macrophage-induced potentiation of penetrated colony formation (Table 2). When AH 130 cells were seeded on M-cell monolayer in the presence of 25-2500 pg/ml PGE2, no stimulatory effect of the prostaglandin on the penetrated colony formation was observed. Instead, indomethacin was slightly stimulatory and the prostaglandin was rather inhibitory.

Effect of SOD and CAT on Macrophage-induced Enhancement of Invasive Capacity. Because the medium conditioned by macrophages was found to be almost unable to affect the tumor cells, we assumed that either the potentiative effect by macrophages is mediated by a direct contact of the tumor cells with macrophages or the effector(s), if any, is labile when excreted by macrophages. Phagocytes are known to produce active oxygen species when exposed to appropriate stimuli (9, 10). Such oxygen species are highly reactive. Therefore, we tested the effect of scavengers, SOD and CAT, on the macrophage-induced potentiation of invasiveness. As shown in Table 3, SOD

Table 2 Effect of indomethacin and prostaglandin E2 on in vitro invasive capacity of AH 130 cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment of AH 130 cells with Macrophages</th>
<th>Addition at invasion assay</th>
<th>No. of penetrated colonies/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Macrophages</td>
<td>None</td>
<td>808.1 ± 27.3*</td>
</tr>
<tr>
<td></td>
<td>Macrophages + indomethacin</td>
<td>None</td>
<td>1063.8 ± 100.8</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>None</td>
<td>114.7 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>PGE₂, 25 pg/ml</td>
<td>66.2 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>PGE₂, 250 pg/ml</td>
<td>56.3 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>PGE₂, 2500 pg/ml</td>
<td>24.0 ± 7.5</td>
</tr>
</tbody>
</table>

* In Experiment 1, AH 130 cells (2.4 x 10⁶/dish) were precultured on macrophage feeder layers with and without 10^-6 M indomethacin and then tested for in vitro invasive capacity. In Experiment 2, AH 130 cells were tested for their invasive capacity in the presence and absence of PGE₂ in the assay system. # Mean ± SD of at least 3 determinations.

Days after coculture

Fig. 5. Reversibility of the effect of macrophages. AH 130 cells (2.4 x 10⁶/dish) were precultured with splenic macrophages (1.7 x 10⁷/dish) for 6 days. Then, the tumor cells were washed with the culture medium, transferred to a fresh medium, and cultured without macrophages. At intervals, the tumor cells were tested for their invasive capacities. Parallel run of the tumor cell preculture without macrophages (control) was done.

% of potentiation

\[
\text{% of potentiation} = \left[\frac{\text{No. of colonies formed by the cocultured AH 130 cells}}{\text{No. of colonies formed by control AH 130 cells}} - 1\right] \times 100
\]
and CAT, which had been added in the culture medium during the coculture of AH 130 cells with macrophages, inhibited the effect of macrophages by about 50%. Heat-denatured enzymes had no inhibitory effect. SOD and CAT also reduced the tendency of the tumor cells to clump, although not completely.

### DISCUSSION

Recent studies have shown that tumor-host cell interaction may result in an expression of higher metastatic capacity of the tumor cells. Among these, a possible fusion of tumor cells and host cells in generating a metastatic variant was first reported by Goldenberg et al. (11). De Baetselier et al. (12) reported organ-specific metastases produced by hybridomas of plasmacytoma cells with spleen B-lymphocytes. Intensive investigations by Kerbel et al. (3) and Larizza et al. (4) have indicated that hybridization of tumor cells with host macrophages and subsequent chromosomal segregations may well be responsible for the progression towards high metastatic capacity at least in certain neoplastic cells.

The present study was aimed at elucidating a possible involvement of macrophages in tumor cell invasion by using an *in vitro* invasion model that we have established. AH 130 cells that had been cultured with macrophages (macrophage/tumor cell ratio, less than 1.0) for at least 24 h expressed high invasive capability both *in vitro* and *in vivo*. Because the growth of the tumor cells on macrophage feeder layers did not differ significantly from the tumor cells that had been cultured without macrophages, selection for highly invasive cells during the coculture is less likely. Furthermore, it is not probable that the apparent increase of the invasive capacity is the result of the increased tumor cell growth. Instead, host macrophages could be involved in the tumor cell penetration.

Preincubation of AH 130 cells in the macrophage-conditioned medium did not result in an appreciable enhancement of the formation of penetrated tumor cell colonies. This raises a possibility that the invasion-potentiating effect of macrophages may be mediated by a direct contact of macrophages with the tumor cells. Alternatively, the effector(s), if any, may be labile when excreted from macrophages. We observed that the addition of active oxygen scavengers, SOD and CAT, appreciably inhibited the effect of macrophages. Phagocytes like leukocytes and macrophages are known to produce O$_2^-$ when exposed to appropriate stimuli (9, 10). In fact, O$_2^-$ was generated when AH 130 cells were cocultured with macrophages.

We have shown previously that the pretreatment of AH 130 cells with O$_2^-$ that had been generated by hypoxanthine and xanthine oxidase resulted in the potentiation of *in vitro* invasive capacity of the tumor cells (7). The amount of O$_2^-$ generated extracellularly by hypoxanthine and xanthine oxidase was far below cytotoxic level. The treated cells tended to aggregate together, as we found when AH 130 cells were cocultured with macrophages, suggesting that the tumor cells had been affected by a similar effector(s). These observations together with the present results seem to imply that the enhancement by macrophages of the invasion of AH 130 cells could mostly be mediated by certain species of oxygen radicals generated by the host cells. Aggregation of tumor cells cocultured with macrophages is likely to be an indication of cell surface modification leading to the acquisition of high invasive potential. Sacks et al. (13) and Bowman et al. (14) have presented an evidence for the generation of active radicals by polymorphonuclear leukocytes or hyperoxia capable of damaging endothelial cell membranes. AH 130 cell membranes might also be modified as the result of the interaction with activated macrophages.

Young and Newby (8) recently reported that PGE$_2$ that had been excreted from macrophages enhanced experimental migration of metastatic Lewis lung carcinoma variant cells. In our study, however, indomethacin did not inhibit the macrophage-induced enhancement of invasion. Moreover, PGE$_2$ added extracellularly had no stimulatory effect. The reason for this discrepancy is not known at present; the mechanism by which macrophages act may not be the same for all tumor cells.

Clonal heterogeneity has been shown in a wide variety of tumors of different organ sites and histological types. The genetic instability arising from either mutational or epigenetic mechanisms (or combination of both) could result in the production of tumor cell variants, which then provides the basis for malignant progression. Increasing the frequency of variant formation would be expected to increase the chance of invasion and metastasis (15). Genetically stable variant formation by the action of macrophages has been reported recently by Yamashina and Heppner (16). This induction was shown to have been mediated by oxygen radicals generated by the macrophages. In our study, increment of invasive capacity of AH 130 cells caused by the tumor cell-macrophage coculture was reversed by subculturing the affected tumor cells without macrophages, indicating instability of the induced invasiveness of the tumor cells. Similar instability of the induced metastatic phenotype in a population of TA3 mammary carcinoma cells has already been reported by Kerbel et al. (17), who suggested that hypomethylation of DNA was probably involved in the evolution of heterogeneity in a tumor cell population (18).

Tumors are known to contain a variety of normal cells, including infiltrating lymphocytes and macrophages. These host cells have possible roles in host defense mechanisms against malignancy. However, increasing evidences have suggested that macrophages may even stimulate tumor cell proliferation (19, 20) and might rather augment tumor progression and metastasis (2–4). Our present result provided an evidence for unfavorable involvement of macrophages in tumor cell invasion. However, this and already reported evidences are based on the experimental results; much more work must be done before concluding that macrophages are actually involved *in vivo* in the potentiation of tumor progression. The same caution must also be taken in discussing the role of oxygen radicals in tumor progression. Nevertheless, active oxygen species produced by stimulated phagocytes have been shown to cause chromosomal damage and transformation (21–23). Potentiation of host defense mechanisms and therapeutic procedures that result in the generation of oxygen radicals, such as certain anticancer drugs and radiation (24, 25), seem necessary to be reevaluated for the reason that the radical formation...
might paradoxically result in an acceleration of tumor progression.

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Macrophage Potentiation of Invasive Capacity of Rat Ascites Hepatoma Cells

Mutsuko Mukai, Kiyoko Shinkai, Ryuhei Tateishi, et al.


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