Antitumor Activity of Pleural Cavity Macrophages and Its Regulation by Pleural Cavity Lymphocytes in Patients with Lung Cancer

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ABSTRACT

Antitumor activities of pleural cavity macrophages (PCM) and pleural cavity lymphocytes (PCL) in lung cancer patients were examined. The effect of coculture supernatants of PCL and autologous tumor cells on the cytotoxic activity of macrophages was also examined. Cytotoxic activity of PCM was not affected by an advance of metastasis to regional lymph nodes or increase of tumor size and difference of histological type. However, the cytotoxic activity of PCM was markedly augmented when pleural invasion was limited to within the visceral pleura to the neighboring lobe or chest wall. The mechanisms that activate the antitumor activity of PCM have not been clarified yet; however, soluble lymphokines.

INTRODUCTION

It is well accepted that activated macrophages play an important role in host resistance to tumor growth (1, 2). Lymphokines have been shown to regulate a number of functions of macrophages and to activate their cytotoxic capacity against tumor cells (3–5). However, most of our knowledge relating to the macrophage system comes from animal studies.

Our previous study in lung cancer patients (6) showed that the cytostatic activity of PCM showed a particular pattern of changes especially in relation to the development of pleural invasion by the tumor. The cytostatic activity of PCM was augmented when pleural invasion was limited to within the visceral pleura and suppressed when the pleural invasion extended beyond the visceral pleura to the neighboring lobe or chest wall. The mechanisms that activate the antitumor activity of PCM have not been clarified yet; however, soluble lymphocyte mediators may play an important role because of the coexistence of large numbers of lymphocytes in the pleural cavity.

In this study, we elucidated the role of PCL in macrophage activation resulting in augmented cytostatic activity.

MATERIALS AND METHODS

Patients. Seventy-six patients (48 males and 28 females) with resectable primary lung cancer were included in this study. They had not received any anticancer therapy when they were examined. According to histological classification, there were 42 adenocarcinomas, 17 squamous cell carcinomas, 14 large cell carcinomas, 3 small cell carcinomas, and 1 adenosquamous cell carcinoma.

Stage. The tumor-nodes-metastasis classification system (Union Internationale Contre Le Cancer, 1978) was used for staging of the disease. The 76 patients were classified as Stage I, 3 Stage II, 29 Stage III, and 6 Stage IV.

Degree of Pleural Invasion. The degree of pleural invasion of the tumor was classified as: Grade 0, without any visceral pleural invasion; Grade 1, pleural invasion limited to within the visceral pleura; and Grade 2, invasion extending beyond the visceral pleura to the neighboring lobe or chest wall.

Preparation of PCM and PCL. The pleural cavity was irrigated with 1000 ml of saline immediately after thoracotomy, and the saline was collected and centrifuged at 1000 rpm for 15 min to obtain cell pellets. Contaminated RBC were lysed by Tris-buffered NH4Cl. The cells obtained were washed 3 times with HBSS and suspended in RPMI 1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated pooled human AB serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at a concentration of 1–2 x 10⁶ cells/ml. This cell suspension was placed in a plastic culture dish (No. 150350; Nunc) and incubated for 1 h in a humidified atmosphere containing 5% CO₂. After 1 h incubation, nonadherent cells were collected by gentle washing with the culture medium.

Measurement of Cytostatic Activity of PCM. The cell lines designated as QG-56 and QG-90 (8), derived from a squamous cell carcinoma and normal lung cells, were inoculated into microtest plates at a concentration of 1 x 10⁴ cells/ml in RPMI 1640 containing 10% FBS and 2% sodium bicarbonate. After 24 h incubation, the cells were added to the RPMI 1640 containing 0.01% hyaluronidase (type I-S), 0.25% collagenase (type I-A), and 0.1% DNase (type I) to release adherent cells from the plastic dishes. The fragments were put into a sterile flask with about 10–15 ml of 0.25% trypsin in Dulbecco’s phosphate-buffered saline with EDTA without calcium and magnesium and stirred with a magnetic stirrer for 15 min. The resulting single cells were suspended in the culture medium at a concentration of 1–2 x 10⁶/ml. The nonadherent cell suspension was then divided into 2 parts and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 48 h incubation, the adherent cells in each half were collected and counted by trypan blue exclusion test.

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The abbreviations used are: PCM, pleural cavity macrophages; PCL, pleural cavity lymphocytes; MAF, macrophage-activating factor; AT, autologous tumor cells; ANL, autologous normal lung cells; HBSS, Hanks’ balanced salt solution; [³H]dThd, tritiated thymidine; PBMC, peripheral blood mononuclear cells.
cells was more than 30% of maximal release the data were excluded. Calculated as

\[
\text{Cytostatic activity (\%) = Control (dpm) - test (dpm)} \times 100
\]

The control is a mean dpm of \(^{3} \text{H}\text{dThd}\) incorporated by target cells in the absence of macrophages. Effector cells did not incorporate a significant level of \(^{3} \text{H}\text{dThd}\) throughout the present study.

Cytolytic Activity of PCL. Cytolytic activity of PCL against AT, QG-56, K562, and ANL were assessed by 4 h \(^{51} \text{Cr}\) release assay. K562 (9) was included as a target cell line with an extremely high sensitivity to lysis by the naturally cytotoxic cells. Target cells (3 \(\times 10^{5}\)) in 0.4 ml of the culture medium were labeled with 0.1 mCi of \(^{51} \text{Cr}\text{O}_{4}\) (Japan Atomic Energy Research Institute, Ibaragi, Japan) for 60 min at 37°C. After three washings with the culture medium, target cells were prepared at a concentration of 5 \(\times 10^{5}\) cells/ml. A constant number of 5 \(\times 10^{3}\) \(^{51} \text{Cr}\)-labeled target cells were incubated with various numbers of effector cells (10 \(\times 10^{3}\), 25 \(\times 10^{3}\), 50 \(\times 10^{3}\)) in round-bottomed microtest plates (No. 163320; Nunc). Cultures in triplicate were incubated at 37°C for 4 h. Then 0.1 ml of supernatants was collected from each well and counted in a gamma counter. The percentage of cytotoxicity was calculated as

\[
\% \text{ of cytotoxicity} = \frac{\text{Experimental (dpm)} - \text{spontaneous (dpm)}}{\text{Maximum (dpm)} - \text{spontaneous (dpm)}} \times 100
\]

Target cells without effector cells were mixed with 0.1 ml of the culture medium to obtain spontaneous \(^{51} \text{Cr}\) release and with 0.1 ml of 0.1 N hydrochloric acid to obtain maximal \(^{51} \text{Cr}\) release. When maximal release was less than 1000 cpm or the spontaneous release from target cells was more than 30% of maximal release the data were excluded from the study.

Preparation of Co-culture Supernatant of PCL and Tumor Cells. Stimulating cells used in this co-culture to obtain supernatants were AT, QG-56, and ANL as a control. PCL (1 \(\times 10^{6}\)/ml) and these stimulating cells (2 \(\times 10^{5}\)/ml) were cocultivated for 72 h. Supernatant fluid from duplicate cultures were pooled, filtered through a 0.22-\(\mu\)m filter (Millipore Co., Bedford, MA), and stored at -20°C until use.

Preparation of PPD-induced Human MAF. PBMC were obtained by standard Ficoll-Hypaque density gradient centrifugation of heparinized venous blood from normal healthy volunteers who were PPD skin test positive. PBMC were cultured at a concentration of 2.5 \(\times 10^{5}\)/ml in the complete medium with PPD (25 \(\mu\)g/ml; purified protein derivatives of Mycobacterium tuberculosis H37Ra; Mitsui Pharmaceutical Co., Tokyo, Japan) at 37°C for 48 h. The cell-free supernatants were harvested and filtered through a 0.22-\(\mu\)m filter, which were used as human MAF. Control supernatants were prepared from cultures of PBMC without PPD.

Effect of Co-culture Supernatants or PPD-induced Human MAF on Cytostatic Activity of Macrophages. Approximately 10\(^{5}\) PBC were incubated for 1 h in plastic dishes at 37°C in a 5% CO\(_{2}\) incubator. Nonadherent cells were removed and plastic-adherent cells were washed three times with HBSS. The adherent cells were dislodged by a jet stream of HBSS and resuspended in the complete medium. Peritoneal

exudate cells of a normal guinea pig were obtained by washing the peritoneal cavity with HBSS and resuspended in the complete medium. Guinea pig peritoneal exudate cells or human monocyte suspension (2 \(\times 10^{5}\)/ml) was placed in each well of a microtest plate (0.1 ml/well) and incubated for 2 h. Then each well was washed three times with HBSS to remove nonadherent cells. The purity of macrophages/monocytes was about 90%, as assessed by morphology and nonspecific esterase staining. Cocultured supernatants or human MAF/control supernatants (0.1 ml) diluted 1/8 with fresh medium were added to each well. After 4 h incubation, the supernatants were withdrawn and the wells were washed once with the medium before addition of tumor target cells (QG-56, 1 \(\times 10^{5}\)/well). Cytostatic activity of macrophages was estimated as described above.

MAF Activity. MAF activity was expressed as

\[
\text{MAF activity} = \frac{\text{Cytostatic activity of guinea pig macrophages pretreated with coculture supernatant of PCL and ANL}}{\text{Cytostatic activity of guinea pig macrophages pretreated with coculture supernatant of PCL and ANL}}
\]

Statistical Tests. Statistical difference of population means was determined by means of Student's t test. Linear regression was calculated by least-squares method.

RESULTS

Antitumor Activity of PCL. Correlation among the cytostatic activity of PCL, prognostic factors, and histological types is shown in Table 1. Cytostatic activity of PCL was not affected by an advance of metastasis to regional lymph nodes, increase of tumor size, and difference of histological type. However, in relation to the development of pleural invasion by the tumor, the cytostatic activity of PCL showed a particular pattern of changes against both target cells. Cytostatic activity of PCL against QG-56 was 40.3% \(\pm\) 5.0 (SE) when pleural invasion was Grade 0. It was markedly augmented as high as 76.1% \(\pm\) 5.5 when pleural invasion was Grade 1; however, it decreased to 35.8% \(\pm\) 14.2 when pleural invasion extended beyond the visceral pleura.

Antitumor Activity of PCL. Any cytolytic activity of PCL against AT, QG-56, K562, and ANL was not detected as shown in Table 2.

Table 1 Cytostatic activity of PCL according to prognostic factors and histological types

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>% of cytostatic activity against</th>
</tr>
</thead>
<tbody>
<tr>
<td>QG-56</td>
<td>QG-90</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
</tr>
<tr>
<td>(&lt;3 \text{ cm})</td>
<td>48.5 (\pm) 6.7* (23)*</td>
</tr>
<tr>
<td>(&gt;3 \text{ cm})</td>
<td>48.5 (\pm) 5.3 (52)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>(N_0)</td>
<td>53.6 (\pm) 4.7 (43)</td>
</tr>
<tr>
<td>(N_1)</td>
<td>49.2 (\pm) 12.9 (7)</td>
</tr>
<tr>
<td>(N_2)</td>
<td>39.4 (\pm) 9.0 (25)</td>
</tr>
<tr>
<td>Pleural invasion grade</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>40.3 (\pm) 5.0 (30)*</td>
</tr>
<tr>
<td>1</td>
<td>76.1 (\pm) 5.5 (19)</td>
</tr>
<tr>
<td>2</td>
<td>35.8 (\pm) 14.2 (15)*</td>
</tr>
<tr>
<td>Histological types</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>49.5 (\pm) 4.5 (41)</td>
</tr>
<tr>
<td>Squamous</td>
<td>44.4 (\pm) 6.8 (23)</td>
</tr>
<tr>
<td>Moderate</td>
<td>57.1 (\pm) 6.1 (112)</td>
</tr>
<tr>
<td>Poor</td>
<td>66.8 (\pm) 13.1 (4)</td>
</tr>
<tr>
<td>Squamous</td>
<td>35.5 (\pm) 12.8 (17)</td>
</tr>
<tr>
<td>Large cell</td>
<td>59.7 (\pm) 8.3 (14)</td>
</tr>
</tbody>
</table>

* Mean \(\pm\) SE.
* Numbers in parentheses, number of patients tested.
* Significantly different from Grade 1 (P < 0.001).
* Significantly different from Grade 1 (P < 0.05).
* Significantly different from Grade 1 (P < 0.01).
MAF ACTIVITY OF PLEURAL CAVITY LYMPHOCYTES

Table 2 Cytolytic activity of PCL against various target cells

<table>
<thead>
<tr>
<th>Effector:</th>
<th>% of cytolytic activity against</th>
<th>AT</th>
<th>QG-56</th>
<th>K562</th>
<th>ANL</th>
</tr>
</thead>
<tbody>
<tr>
<td>target cell ratio</td>
<td></td>
<td>20</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-2.6 ± 1.4* (16)</td>
<td>-1.8 ± 0.8 (19)</td>
<td>1.0 ± 2.9 (19)</td>
<td>0.7 ± 5.9 (6)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>-2.0 ± 2.4 (6)</td>
<td>-2.5 ± 2.0 (9)</td>
<td>3.8 ± 4.9 (9)</td>
<td>-2.0 ± 4.8 (6)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>-1.3 ± 2.1 (6)</td>
<td>-1.2 ± 1.0 (8)</td>
<td>2.8 ± 3.2 (7)</td>
<td>7.8 ± 7.6 (3)</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Numbers in parentheses, number of patients tested.

Table 3 Cytostatic activities of human monocytes and guinea pig macrophages

<table>
<thead>
<tr>
<th>Treatment of macrophage</th>
<th>Monocyte/macrophage source</th>
<th>% of cytostatic activity against QG-56*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MAF</td>
<td>Human</td>
<td>40.0 ± 2.6*</td>
</tr>
<tr>
<td>Control</td>
<td>Human</td>
<td>5.4 ± 11.5</td>
</tr>
<tr>
<td>Human MAF*</td>
<td>Guinea pig</td>
<td>41.3 ± 1.3*</td>
</tr>
<tr>
<td>Control*</td>
<td>Guinea pig</td>
<td>15.5 ± 3.4</td>
</tr>
</tbody>
</table>

* Supernatants containing human MAF were prepared from cultures of PPD-stimulated PBMC, and control from cultures of nonstimulated PBMC.
* Mean ± SD of triplicates.
* P < 0.05.

Fig. 1. Effect of coculture supernatants of PCL with AT, QG-56, and ANL on cytostatic activity of guinea pig macrophages. The supernatant of PCL and AT rendered macrophages more cytostatic as compared with the supernatant of PCL and QG-56 or PCL and ANL.

**Effect of Coculture Supernatants of PCL and AT on Cytostatic Activity of Macrophages.** To test a number of samples, guinea pig macrophages were used as effector cells. As shown in Table 3, our preliminary experiments revealed that human MAF prepared from PPD-stimulated PBMC is capable of activating not only human monocytes but also guinea pig macrophages. Cytostatic activities of guinea pig peritoneal exudate macrophages pretreated with 1/8 diluted supernatants obtained from coculture of PCL with QG-56 or autologous normal lung cells were below 20%. However, cytostatic activity of guinea pig macrophages pretreated with supernatants obtained from coculture of PCL with autologous tumor cells exceeded 20% in 9 of 17 patients as shown in Fig. 1.

Fig. 2 showed the correlation between cytostatic activity of PCM and degree of MAF activity of the supernatant in individual patients. A significant positive correlation (P < 0.01) was found between MAF activity induced by PCL and cytostatic activity of PCM against QG-56. A similar correlation was also observed when the cytostatic activity of PCM against QG-90 was used as the horizontal parameter (r = 0.692, P < 0.01).

**DISCUSSION**

The antitumor activity of monocyte-macrophage lineage in cancer patients had been reported by several investigators (6, 10–12); however, most of the reports were concerned to PBM. In our previous study, differences in the antitumor activity of PBM among normal healthy persons, patients with lung cancer, benign respiratory diseases, and malignancies other than lung cancer could not be detected (8). Cytostatic activity of PBM in lung cancer patients declined significantly in association with the development of pleural invasion, whereas it was scarcely affected by the presence of metastasis to regional lymph nodes or an increase in tumor size (6). Gerrard et al. (11) also reported that PBM from various cancer patients exhibited the normal level of cytotoxicity which was exhibited by PBM from normal persons. These results may indicate that the antitumor activity of PBM is stable and rarely affected by tumor-bearing states. Pleural cavity in lung cancer patients may be isolated from sites of systemic immunological responses and suitable site for detection of local immune response to the tumor. The marked augmentation of antitumor activity of PCM with Grade 1 pleural invasion may be a result of specific immunological response to the tumor in the pleural cavity.

On the other hand, PCL did not show any antitumor activity against various target cells in vitro (Table 2). What is a role of PCL in immunological response? Previous studies in rodents suggested that in vivo activation of macrophages requires the interaction of specifically sensitized T-lymphocytes with appropriate antigens, and macrophages became cytotoxic by mediators released from sensitized lymphocytes in vitro (3, 4, 13–15). Nakajima et al. (15) showed that macrophages preincubated with supernatant from coculture of tumor-unimmunized normal spleen and lymph node cells plus tumor cells failed to exhibit any significant antitumor effect on unrelated tumor cells. On the other hand, the addition of supernatant from cultures containing immune lymphocytes to macrophages resulted in appreciable augmentation of cytostatic and cytolytic effects on the tumor cells, indicating the generation of MAF in culture supernatant. Recent studies in humans also showed that human macrophages were activated by lymphokines (16–20). It seemed likely that a macrophage-activating factor analogous to that described in rodents was responsible for the augmentation of cytotoxicity by human macrophages.

In this study, coculture supernatant of PCL and allogeneic tumor cell line QG-56 did not augment the cytostatic activity of macrophages. However, coculture supernatant of PCL and...
AT augmented the cytostatic activity of guinea pig macrophages. This may suggest that autologous tumor cells do not share antigenic determinants with the allogeneic lung cancer cell line. Furthermore, significant correlation was observed between the degree of MAF activity induced by PCL and individual cytostatic activity of PCM. Although actions of MAF were reported to have species specificity (21), PPD-induced MAF could activate human and also guinea pig macrophages in a dose-dependent fashion using the cytostatic assay which we utilized as shown in Table 3. These results suggested that antitumor activity of PCM was controlled by specifically sensitized PCL through lymphokines.

REFERENCES

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