Macrophage Colony-stimulating Factor Activity in Malignant Pleural Effusions*  
Its Augmentation by Intrapleural Interleukin-2 Infusions  
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The activity of endogenous colony-stimulating factor (CSF) in malignant pleural effusions of lung cancer patients before and during daily intrapleural infusions of recombinant interleukin-2 (IL-2) was measured quantitatively by colony-forming bioassay and radioimmunoassay (RIA). Before therapy, malignant pleural effusions had various levels of CSF activities, and this CSF activity was neutralized almost completely by anti-M-CSF antibody. RIA also showed that the effusions contained various amounts of M-CSF. Daily intrapleural infusion of recombinant IL-2 caused significant increase in the CSF activities and M-CSF levels in pleural effusions. These results indicate that in vitro treatment with IL-2 induces production of endogenous M-CSF.  

(Materials and Methods)  

LAK cells and/or IL-2 are now widely used in immunotherapy of malignant diseases. The mechanism by which IL-2 mediates tumor regression in vivo is unknown. Induction of human LAK activity by IL-2 was recently found to be up- or down-regulated by monocyte-macrophages, depending upon their states of maturation and activation. It is important to determine whether IL-2 can stimulate the host to induce production of secondary cytokines in vivo. Certain cytokines such as TNF, and IFN-α are produced in vivo by IL-2-stimulated peripheral blood mononuclear cells (MNC). Moreover, in vivo IL-2 infusion was recently found to induce the productions of IFN-α and TNF-α and mRNA coding for TNF, IL-1 and IL-6. Nothing is known, however, about the alteration of endogenous CSF-1 (M-CSF) in cancer patients during IL-2 treatment. In the present study, we showed by both bioassay and EIA that daily intrapleural infusion of recombinant IL-2 into patients with malignant pleural effusions caused increase in CSF activity and in the M-CSF level in pleural effusions.

Materials and Methods

Subjects  
We studied 17 patients with primary lung cancer with malignant pleural effusions (12 adenocarcinomas, three squamous cell carcinomas, one large cell carcinoma and one small cell carcinoma) and 22 lung cancer patients without malignant effusion (five adenocarcinomas, nine squamous cell carcinomas and eight small cell carcinomas) before conventional anticancer therapy. They were all in-patients in Tokushima University Hospital, and ranged in age from 42 to 78 years old. The patients with malignant pleural effusions had received no previous treatment for their pleural effusion. With written informed consent from the patients and/or

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their families, four patients received local daily IL-2 therapy. The study was approved by the Ethical Committee of the Tokushima University Hospital, Tokushima.

**Reagents**

Fetal bovine serum (FBS) was purchased from M.A. Bioproducts, Walkersville, MD. Recombinant human interleukin-2 (IL-2) was kindly provided from Takeda Pharmaceutical Co (Osaka, Japan), and had a specific activity of 3.5×10⁶ U/mg as assayed on IL-2 dependent murine NKC3 cells. Recombinant human macrophage colony-stimulating factor (M-CSF) (specific activity, 0.8×10⁶ U/mg protein) and rabbit antisera against recombinant human M-CSF (neutralizing activity, 250 U/ml) were supplied from the Genetics Inst, Cambridge, MA. All reagents were free of endotoxins as determined by the Limulus amebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

**Bioassay of CSF**

Bone marrow was obtained by posterior iliac crest puncture from hematologically normal volunteers who had given their informed consent. The marrow cells were collected in Dulbecco’s modified Eagle medium (DMEM) with heparin, and layered over Ficoll-Hypaque. After centrifugation, the MNC were harvested, washed twice with DMEM and resuspended in DMEM. Colony-forming assay in methylcellulose was performed as described previously. Cells were inoculated into flasks (Falcon 3001; 35×10 mm²) at 1×10⁶ marrow cells/ml of DMEM with 5×10⁻⁴ M 2-mercaptoethanol, 25 percent FBS and 1.1 percent methylcellulose (wt/vol) in the presence of 100 U/ml of M-CSF or test samples containing 10 percent of effusions. Colonies of more than 50 cells were scored after 14 days of culture at 37°C under 5 percent CO₂ in air.

**Radioimmunoassay of M-CSF**

For quantitative measurement of M-CSF (sensitivity limit, 0.1 ng/ml), duplicate samples (100 μl) were mixed with ¹²⁵I-M-CSF (1,000 cpm/100 μl) and then 20,000 dilutions of anti-M-CSF antisera (200 μl). The anti-M-CSF antibody binding reaction attained equilibrium within 20 h at room temperature or within 48 h at 4°C. After incubation, bound antibody was separated from free ¹²⁵I-M-CSF by addition of 100 μl of normal rabbit serum diluted 400 times with PBS. 100 μl of anti-rabbit IgG serum diluted 40 times with PBS, and 1.0 ml of 6 percent polyethylene glycol (MW 8,000) in PBS. The tubes were shaken and centrifuged at 1,000 g for 15 min at 4°C. The supernatants were removed by aspiration, and the precipitates were counted for 1 min in a automated gamma spectrometer.

**Administration of IL-2**

Recently we showed that pleural effusion MNC generated LAK activity when cultured in vitro with recombinant IL-2 at concentrations of 0.1 to 1.0 U/ml. When the cell-free effusions obtained from the patients 24 h after local injections of IL-2 (1,000 U/person) were assayed on the NKC3 cell line, IL-2 levels in the effusions ranged from 0.3 to 1.1 U/ml. We also found that daily intrapleural injections of IL-2 (1,000 U/person) were effective for inducing LAK activity in the effusion of lymphocytes in association with rapid disappearance of cancer cells from the effusion. For these reasons, IL-2 at a daily dose of 1,000 U/person was injected for 14 days into the pleural cavity through a Silicone-coated, 500 mm long, 7 French catheter (Hanako Medical Co, Tokyo, Japan). Administration of antipyretics against fever and temporary drainage to avoid increase of pleural effusions were performed as conservative therapy. More detailed clinical data have been reported elsewhere.

**Statistical Analysis**

The statistical significance of differences between test groups was analyzed by Student's t test.

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**Table 1—CSF Activities in Malignant Pleural Effusions of Lung Cancer Patients**

<table>
<thead>
<tr>
<th>Case*</th>
<th>% Control value (No colonies)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
</tr>
<tr>
<td>rGM-CSF (100 U/ml)</td>
<td>100 (74)</td>
</tr>
<tr>
<td>Medium alone</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
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<tr>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
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<tr>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>14</td>
<td>37</td>
</tr>
</tbody>
</table>

*RM-CSF = recombinant granulocyte-macrophage colony-stimulating factor; ND = not determined.

**RESULTS**

**M-CSF Activity in Malignant Pleural Effusions**

First, we examined whether malignant pleural effusions possessed CSF activity, by a colony-forming bioassay using normal human bone marrow cells. Examination of cell morphology indicated that a high concentration of effusion was toxic to bone marrow cells to form colony (data not shown). However, with effusions diluted 1:10, many clusters of more than 50 cells were seen, and scored as colonies. Table 1 shows that levels of CSF activity in 10 percent malignant pleural effusions were variable. Morphologic analysis showed that the colonies induced by three different pleural effusions consisted of 85.6 percent macrophage-type, 9.8 percent granulocyte-macrophage-type, 2.8 percent eosinophil-type and 1.7 percent granulocyte-type colonies. In a parallel experiment, we examined whether anti-M-CSF antibody neutralized the CSF activities of pleural effusions. Treatment of recombinant M-CSF (1,000 U/ml) with anti-M-CSF antibody resulted in its almost complete removal. Under the same experimental conditions, treatment

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**Table 2—Presence of M-CSF Activity in Malignant Pleural Effusions**

<table>
<thead>
<tr>
<th>Effusion of donor*</th>
<th>Medium</th>
<th>Anti-M-CSF antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>rM-CSF</td>
<td>41</td>
<td>4 (90)</td>
</tr>
<tr>
<td>1</td>
<td>38</td>
<td>9 (76)</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>15 (60)</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>9 (70)</td>
</tr>
</tbody>
</table>

*rM-CSF = recombinant macrophage colony-stimulating factor.
Previously, we found that lung cancer was associated with blood monocytosis,27 suggesting an increase in the serum M-CSF level. To test this possibility, we measured the levels of M-CSF in the sera of 22 lung cancer patients with various cell types without malignant pleuritic effusions. Their average levels of M-CSF in patients with squamous cell carcinoma, small cell carcinoma and adenocarcinoma were 3.9 ± 1.6 ng/ml, 4.7 ± 1.7 ng/ml and 4.6 ± 1.5 ng/ml (mean ± SD), respectively, which were approximately three times that in the sera of 50 normal healthy donors (1.4 ± 0.5 ng/ml; mean ± SD). Next, we measured levels of M-CSF in malignant pleural effusions of 17 lung cancer patients before therapy and the results are also shown in Figure 1. The level of M-CSF varied, but the mean M-CSF level in the malignant pleural effusions was significantly higher than that in the sera of healthy subjects (3.0 ± 1.2 ng/ml vs 1.4 ± 0.5 ng/ml).

In vivo Induction of M-CSF Production by IL-2

For determination of whether intrapleural instillation of recombinant IL-2 influences the CSF activity and the M-CSF level in malignant pleural effusions, four lung cancer patients with malignant pleural effusions were given daily local injections of recombinant human IL-2 (1,000 U/day) for 14 days. All these patients showed a clinical response to IL-2 therapy as judged by disappearance of cancer cells and rapid

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### Figure 1

M-CSF levels in sera of lung cancer patients without malignant pleuritis (○) and in pleural effusions of lung cancer patients with malignant pleuritis (●). Points and bars are means ± SD. The shaded area indicates the mean value ± 2 SD in sera of 50 healthy donors.

### Figure 2

M-CSF levels and CSF activities in malignant pleural effusions of patients with malignant pleuritis before and during daily intrapleural infusion of IL-2 (1,000 U/day).
decrease of effusion after the end of IL-2 infusion. Samples of the pleural effusions were obtained before and at various times during local IL-2 therapy for EIA and bioassay. As shown in Figure 2, the CSF activities in pleural effusions reached maximum after daily intrapleural administration of IL-2 for about ten days. Increase in the M-CSF level in pleural effusions of the patients was also seen during IL-2 therapy.

**DISCUSSION**

In the present study we observed that various levels of endogenous M-CSF with colony-forming activity in malignant pleural effusions of lung cancer patients, and found that daily local instillation of IL-2 increased the CSF activities and M-CSF levels in the effusions. Malignant pleurisy, which is frequent in patients with lung cancer, is of particular immunologic interest, because pleural exudate cells are thought to be important in defense of the host against invading cancer cells. Most of the exudate cells are known to be macrophages. M-CSF produced in malignant pleural effusions might be important in determining the migration and functional expression of macrophage progenitors to the pleural cavity into which cancer cells invade, because M-CSF is known to be involved in differentiation and maturation of monocyte-macrophage progenitor cells. M-CSF is also a chemoattractant of blood monocytes. We found that the presence of lung cancer was associated with blood monocytosis. This blood monocytosis might be due to an increased level of serum M-CSF, because the mean endogenous level of M-CSF in the sera of lung cancer patients was three times that of healthy donors (Fig 1).

Moreover, we found that malignant pleural effusions induced colony formation of macrophage-type cells from normal bone marrow cells (Table 1). This CSF activity was almost completely neutralized by anti-M-CSF antibody (Table 2), although CSF activity in the malignant effusions might be due to additive and/or synergistic actions of M-CSF with other CSF. The results of bioassays were confirmed by immunoassays, which indicated relatively high levels of M-CSF in malignant pleural effusions. The role of M-CSF in malignant effusions is not understood, but its activity extends beyond the induction of colony formation from immature precursors. For example, M-CSF was found in murine systems to influence various functions of mature mononuclear phagocytes, including IL-1 and IFN. Moreover, it enhances human monocyte-mediated tumoricidal activity. Thus, M-CSF in malignant effusions may promote important mechanisms for local resistance against cancer.

In previous studies, the ability of IL-2 to induce in vitro production of M-CSF was not examined. In the present study, the four lung cancer patients with malignant pleural effusions showed good clinical responses, as defined by disappearance of malignant cells in the effusion and then rapid loss of effusion after local injections of IL-2. Recently, we found that significant LAK activity was also generated by lymphocytes in pleural effusion by local IL-2 injections. Interestingly, in the present study we found that the M-CSF levels in pleural effusions were augmented by local infusions of IL-2. M-CSF is produced by human natural killer (NK) cells, fibroblasts, macrophages and mesothelial cells, but the cellular source of the increased M-CSF in the malignant effusions after local instillation of IL-2 is unknown. In vivo induction of M-CSF after IL-2 infusion may result from secondary cytokine production by cells capable of responding to IL-2. For example, IL-2 was found to induce secondary releases of cytokines such as IFN-γ and TNF in vitro from lymphocytes. We found that IFN-γ in the malignant effusions was not detectable before therapy, but increased during daily local injections of IL-2, and that TNF-α was not detected before or during IL-2 therapy (data not shown). These cytokines, as well as IL-3 and GM-CSF, are known to stimulate various cells to produce M-CSF, suggesting that IL-2 may induce CSF production through the action to cells capable of producing M-CSF. Investigations into the capacities of these cells to produce M-CSF and of the kinetics of M-CSF in patients with lung cancer will provide information about the regulatory mechanisms for distribution and function of blood monocytes and tissue macrophages which are important in defense against cancer.

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**REFERENCES**


