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Granulocyte-Macrophage Colony-stimulating Factor Augments Lymphokine-activated Killer Activity from Pleural Cavity Mononuclear Cells of Lung Cancer Patients without Malignant Effusion

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The role of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) in augmentation of lymphokine-activated killer (LAK) cell induction by interleukin-2 (IL-2) from pleural cavity mononuclear cells (PCMNCs) was examined in sixteen patients with resectable primary lung cancer not associated with malignant effusion. None of the patients had received any anticancer therapy prior to this study. Incubation of PCMNCs of patients without malignant effusion with GM-CSF for 4 days in the presence of IL-2 resulted in a significant increase in LAK activity against natural killer-resistant Daudi cells. This result was obtained by using the 4 h ^{51}Cr-release assay. PCMNCs and blood mononuclear cells (BMNCs) were harvested simultaneously from pleural cavity lavage fluid and peripheral blood in lung cancer patients. The LAK activity developed from PCMNCs and BMNCs following incubation with IL-2 for 4 days, but the LAK activity from PCMNCs was significantly lower than that from BMNCs ($P < 0.05$). Incubation of PCMNCs with GM-CSF augmented the LAK activity from PCMNCs to a level as high as that from BMNCs. These results suggest that the combined use of GM-CSF with IL-2 may result in augmentation of LAK activity developed from PCMNCs of lung cancer patients without malignant effusion.

Key words: GM-CSF — LAK — IL-2 — Mononuclear cell — Lung cancer

GM-CSF[4] is a glycoprotein produced by T-lymphocytes, macrophages, fibroblasts, and endothelial cells.[1] Much attention has been paid to GM-CSF[2] since it affects various monocyte-macrophage functions, such as stimulation of proliferation and maturation of macrophage progenitors,[3] Ia antigen expression,[4] induction of prostaglandin,[5] and production of monokines.[6] *In vitro,* GM-CSF induces proliferation of committed bone marrow precursors of granulocytes and macrophages. Moreover, GM-CSF enhances bactericidal and tumoricidal activities of their mature progeny.[1] Phase I trials with GM-CSF in patients with advanced malignancies have shown similar tumoricidal activities without an antineoplastic response.[7]

Unprimed human lymphocytes, cultured with IL-2 for 4–6 days without prior antigenic stimulation, develop nonspecific antitumor activity that can destroy various fresh autologous and allogeneic solid tumor cells, and are designated as LAK cells.[8,9] LAK cells alone or LAK cells with IL-2 are used for cancer treatment. There are encouraging reports that systemic administration of LAK cells and IL-2 resulted in partial regression of cancer.[10,11]

Recently, we reported that mononuclear cells in the pleural cavity of lung cancer patients without malignant pleural effusion could develop LAK activity after incubation for 4 days with IL-2.[12] PCMNCs may play an important role in protecting the pleural cavity from the invasion and spreading of lung cancer cells. The LAK activity from PCMNCs was, however, significantly lower than that from BMNCs of lung cancer patients without malignant effusion.[12] On the other hand, the LAK activity from PCMNCs of lung cancer patients with malignant effusion was as high as that from BMNCs.[13]

An agent that can augment development of LAK activity from PCMNCs of lung cancer patients might be therapeutically useful to protect the pleural cavity. Induction of LAK activity by IL-2 is augmented by various humoral and cellular factors.[14–18] We previously reported that GM-CSF augmented LAK activity from peripheral blood lymphocyte of healthy donors.[19] In this study, we examined whether GM-CSF could augment the development of LAK activity from PCMNCs of lung cancer patients without malignant pleural effusion for the purpose of determining its potential for cancer treatment.

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[4] Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; LAK, lymphokine-activated killer; IL-2, interleukin-2; PCMNCs, pleural cavity mononuclear cells; BMNCs, blood mononuclear cells; FBS, fetal bovine serum; PBS, phosphate-buffered saline solution; IFN, interferon; TNF, tumor necrosis factor.

MATERIALS AND METHODS

Patients Studies were made on 16 patients (10 males and 6 females), aged between 46 and 77 (median 66), with resectable primary lung cancer not associated with malignant pleural effusion. None of the patients had received any anticancer therapy prior to the study. Computed tomography and magnetic resonance imaging were used to identify whether pleural effusion was present or not. The clinical characteristics of the patients are summarized in Table I. Histologically, the cancers included 8 squamous cell carcinomas, 6 adenocarcinomas and 2 large cell carcinomas. The degree of pleural invasion by lung cancer was classified as 7 of grade 0, 4 of grade 1, 2 of grade 2, and 3 of grade 3. The TNM classification system (International Union Against Cancer, 1987) was used for classification of the disease. Five patients were classified as stage I, 6 as stage IIIA, one as stage IIIB, and 4 as stage IV. Three of four patients classified as stage IV had pulmonary metastasis to the same lobe and one of these patients had a superior vena cava syndrome and adrenal gland metastasis, but they had no malignant pleural effusion. All patients were confirmed to have no malignant pleural effusion at the time of thoracotomy.

Reagents Recombinant IL-2 was prepared by Takeda Pharmaceuticals (Osaka), and had a specific activity of 3.5×10^4 U/mg, as assayed on IL-2-dependent murine NKC3 cells.[20] Recombinant GM-CSF (specific activity, 1.7×10^7 U/mg of protein) was supplied by Genetics (Brookline, MA). All reagents were free of endotoxins as determined by *Limulus* amebocyte lysate assay (sensitivity limit, 0.1 ng/ml). FBS was purchased from M.A. Bioproducts (Walkersville, MD).

Cell culture Human Burkitt's lymphoma cells (Daudi) (American Type Culture Collection, Rockville, MD) were maintained as stationary suspension cultures in RPMI 1640 medium supplemented with 10% heat-incubated FBS and gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Table I. Clinical Features of Patients

Features		No.
No. of patients		16
Sex	Male	10
	Female	6
Age (yr)	Median	66
	Range	46–77
Histology	Squamous cell	8
	Adenocarcinoma	6
	Large cell	2
P-factor	0	7
	1	4
	2	2
	3	3
Stage	I	5
	IIIA	6
	IIIB	1
	IV	4

Isolation and culture of PCMNCs and peripheral BMNCs Pleural lavage was performed as follows.[12] At thoracotomy, it was confirmed that no malignant pleural effusion was present in the pleural cavity. The pleural cavity was then lavaged with 1,000 ml of 0.9% NaCl solution (saline solution) at 37°C. The lavage fluid was collected aseptically in heparinized (10 U/ml) centrifuge bottles and centrifuged at 1,200 rpm (400*g*) for 10 min. The cell pellets were resuspended in 15 ml of PBS. At the same time, 20 ml of peripheral blood was taken from each patient in a heparinized syringe to obtain samples of BMNCs. MNCs were separated from the lavage fluid and the peripheral blood by means of discontinuous gradient centrifugation in lymphocyte separation medium.[12] Then, as a further check, the absence of malignant cells among washed PCMNCs was confirmed morphologically, using May-Grünwald Giemsa staining. PCMNCs and BMNCs were tested for ability to develop LAK activity. The viability of these MNCs was more than 95%, as judged using the trypan blue exclusion test. The lavage and isolation methods used here yielded about $22.3\pm26.9(SD)\times10^6$ cells per patient. These cells consisted of $56.7\pm13.1\%$ lymphocytes and $43.1\pm14.1\%$ macrophages. The total number of BMNCs recovered by this method was $23.4\pm12.5\times10^6$ cells, which consisted of $68.5\pm14.2\%$ lymphocytes and $31.4\pm14.1\%$ monocytes.

Flow cytometry A direct immunofluorescence assay was used to detect surface markers of the pleural cavity and blood lymphocytes. Monoclonal antibodies conjugated with fluorescein isothiocyanate (OKT monoclonal antibodies, Ortho Diagnostics, or Leu monoclonal antibodies, Becton-Dickinson) were added to the cell suspension, and the mixtures were incubated for 30 min at 4°C. These cells were then washed, resuspended in 0.1 ml of PBS, and analyzed using a FACScan. The monoclonal antibodies used were those directed against lymphocyte antigen CD3 (OKT3), CD4 (OKT4a), CD8 (OKT8), CD11b (OKM1), CD16 (Leu11a), CD25 (OKT26), CD57 (Leu7), and human class II HLA-DR (OKDR). Before FACScan analysis, dead cells and non-lymphoid cells were excluded from analysis by means of light scatter gating.

LAK activity assay The LAK activity was assayed by measuring ^{51}Cr release using a method described previously.[12] Briefly, to develop LAK activity, precursor cells (PCMNCs or BMNCs) with or without IL-2 (400 U/ml) and GM-CSF (100 U/ml) were incubated for 4 days in RPMI 1640 medium supplemented with 5% FBS and gentamicin at 37°C under 5% CO_2 in humidified air.

Unless otherwise noted, 400 U/ml of IL-2 and 100 U/ml of GM-CSF were used for development of LAK activity from PCMNCs or BMNCs; we confirmed that these concentrations of IL-2 and GM-CSF are optimal for development and augmentation of maximal LAK activity from BMNCs. There was no significant difference between the numbers of cells after 4 days of culture with and without the presence of IL-2 and GM-CSF. The cytotoxicities of these cultured PCMNCs and BMNCs against ^{51}Cr-labeled Daudi cells (1×10^4) were measured at 20:1, 10:1, and 5:1 effector/target ratios in triplicate cultures. Coculture of the effector cells and target cells was terminated after 4 h, and the radioactivity of the supernatants (0.1 ml per well), separated by brief centrifugation at 65*g*, was determined using a gamma counter. The percentage cytotoxicity was calculated as follows:

$$\%\text{Cytotoxicity} = 100\times\frac{\text{experimental cpm}-\text{spontaneous cpm}}{\text{total cpm}-\text{spontaneous cpm}}$$

The spontaneous release was about 10% (range, 7 to 13%) of total release. The total release was determined from the radioactivity of the supernatant of Daudi cells (1×10^4) destroyed with 2 *N* HCl. One lytic unit (LU) was defined as the number of effector cells required for 20% lysis of target cells.

Statistical analysis The statistical significance of differences between values obtained for test groups was determined by using the paired *t* test.

RESULTS

Phenotypic characterization of PCMNCs In the present study, we examined the phenotypes of MNCs in the pleural cavity and peripheral blood of twelve lung cancer patients without malignant effusion (Table II). The proportions of the $CD4^+$ T-cell subset and the NK cell subset of $CD16^+$ cells were significantly higher in the peripheral blood ($P<0.05$, $P<0.01$). On the other hand, there were more HLA-DR^+ cells in the pleural cavity than in the peripheral blood ($P<0.01$).

***In vitro* effect of stimulation of GM-CSF on LAK development by IL-2 from PCMNCs** Significant LAK activity was developed from PCMNCs incubated for four days with IL-2 (the presence of IL-2 vs. the absence of IL-2, $P<0.01$). PCMNCs of 10 of these 14 patients developed significant LAK activity. But the PCMNCs of four patients did not develop LAK activity. Stimulation with GM-CSF (100 U/ml) alone failed to develop LAK activity from PCMNCs, though GM-CSF could significantly augment LAK activity developed from PCMNCs by IL-2 ($P<0.05$). The LAK activities from PCMNCs of six of these 14 patients were augmented remarkably by stimulation with GM-CSF. In the other eight patients, stimulation with GM-CSF caused no significant change (Fig. 1).

Table II. Distribution of Lymphocyte Subpopulations of PCMNCs and BMNCs in Lung Cancer Patients without Malignant Effusion (n=12[a])

	Percentage of cells expressing surface markers (mean±SD)	
	Pleural cavity	Peripheral blood
CD3	59.9±19.5	64.5±9.2
CD4	30.6±13.6[b]	49.6±7.6[b]
CD8	27.7±16.9	27.7±11.2
CD11b	29.4±11.6	30.7±11.2
CD16	4.6±3.8[c]	18.6±7.5[c]
CD25	3.2±3.1	3.1±1.0
CD57	17.4±8.1	23.9±12.7
HLA-DR	56.2±19.2[d]	23.7±7.7[d]

a) Numbers in parentheses indicate number of patients tested. Statistical significance of the differences between the values observed in pleural cavity versus peripheral blood: *b*) $P<0.05$. *c, d*) $P<0.01$.

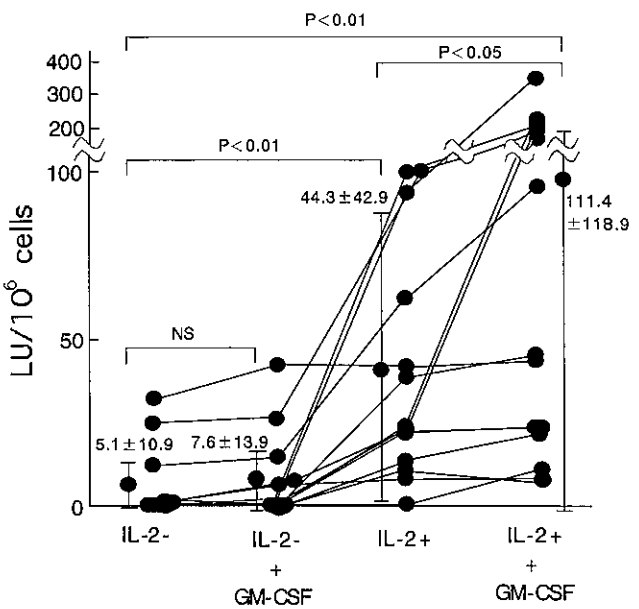

Fig. 1. Effect of stimulation with GM-CSF on LAK development by IL-2 from PCMNCs. Freshly separated PCMNCs (10^5) of 14 patients with lung cancer without malignant effusion were incubated for 4 days in medium with or without IL-2 (400 U/ml) and/or GM-CSF (100 U/ml) in 96-well plastic plates before LAK assay. Lytic unit (LU) was determined as described in "Materials and Methods." The line represents the LU of PCMNCs of each lung patient against allogeneic tumor cell lines of Daudi cells. The results represent the mean±SD (NS; no significance).

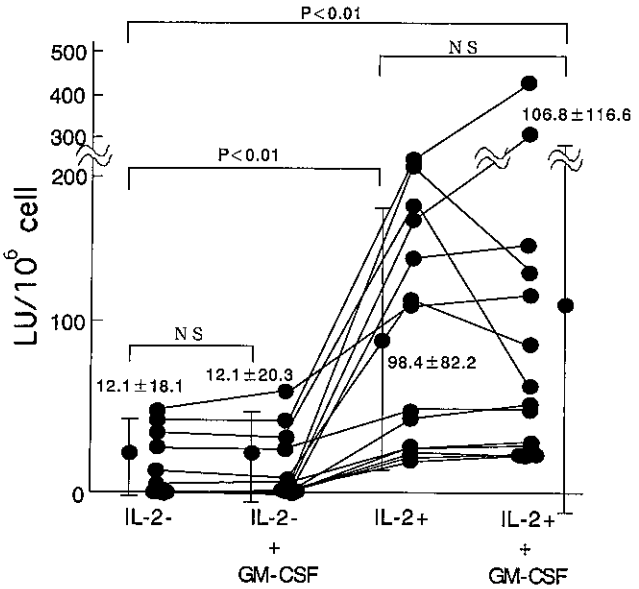

Fig. 2. Effect of stimulation with GM-CSF on LAK development by IL-2 from BMNCs. Freshly separated BMNCs of 14 patients with lung cancer without malignant effusion were incubated for 4 days before LAK assay. The results represent the mean±SD (NS; no significance).

***In vitro* effect of stimulation of GM-CSF on LAK development by IL-2 from BMNCs** Significant LAK activity was developed from BMNCs incubated for four days with IL-2 (the presence of IL-2 vs. the absence of IL-2, $P<0.01$). BMNCs of seven of these patients developed significant LAK activity. But the BMNCs of seven patients failed to develop LAK activity. Stimulation with GM-CSF alone did not develop significant LAK activity from BMNCs. Stimulation with GM-CSF caused no significant augmentation of LAK activity from BMNCs developed by IL-2 (Fig. 2). The LAK activities from BMNCs of two of these 14 patients were augmented about 70% by stimulation with GM-CSF. In 10 patients, the LAK activity showed no change after stimulation with GM-CSF. In two patients, the LAK activity was strongly suppressed by GM-CSF. Their LAK activities were suppressed by about 40 and 60%. The LAK activities from PCMNCs of these two patients, whose LAK activity from BMNCs was suppressed by stimulation with GM-CSF (60% decrease, 40% decrease), were augmented by stimulation with GM-CSF (60% increase, 60% increase). Although the LAK activity from PCMNCs was significantly lower than that from BMNCs (44.3±42.9 LU vs. 98.4±82.2 LU, $P>0.05$), stimulation with GM-CSF augmented the LAK activity from PCMNCs to a level as high as that from BMNCs (111.4 ±118.9 LU vs. 106.8±116.6 LU, no significance).

DISCUSSION

In the present study, we examined the ability of the LAK activity developed from PCMNCs of lung cancer patients without malignant pleural effusion to be augmented by stimulation with GM-CSF.

PCMNCs of lung cancer patients without malignant effusion could develop LAK activity on incubation with IL-2 for four days.[12] The LAK activity from PCMNCs, however, was significantly lower than that from BMNCs.[12] PCMNCs of lung cancer patients with malignant effusion could develop LAK activity as potently as BMNCs could with IL-2.[13] These facts indicated that PCMNCs might play an important role in protecting the pleural cavity of lung cancer patients from invasion and spreading of lung cancer cells. It is important, therefore, to augment LAK activity from PCMNCs for the purpose of protecting the pleural cavity. Several cytokines, such as IFN γ,[14–16, 18] TNF-α[17] and IL-1β[16] could augment LAK activity. GM-CSF could also augment LAK activity from peripheral blood lymphocytes of healthy donors.[19] In addition, treatment[21, 22] with GM-CSF could reduce chemotherapy-induced neutropenia and thrombocytopenia in patients with advanced malignancies and allow intensification of anticancer drug therapy clinically. In this study, LAK activity from PCMNCs was also significantly lower than that from BMNCs ($P<0.05$) and PCMNCs of four patients could not develop LAK activity with IL-2. Histologically, these four patients included 3 squamous cell carcinomas and one adenocarcinoma. Their pathologic n-factor was n-2. BMNCs of these four patients also could not develop LAK activity with IL-2. Considering these results, mediastinal lymph node metastasis of lung cancer cells may impair development of LAK activity from PCMNCs and BMNCs. BMNCs of seven patients failed to develop LAK activity by IL-2. Four of these patients had mediastinal lymph node metastasis of lung cancer cells as described above. Histologically, the other three patients included one squamous cell carcinoma and 2 adenocarcinomas. Their pathologic n-factor was classified as two of n-0 and 1 of n-3, and these three patients had pleural invasion by lung cancer cells. The reason why pleural invasion by lung cancer cells impaired the development of LAK activity from BMNCs but not PCMNCs, is not clear. The patients whose PCMNCs could not develop LAK activity had no pleural invasion by lung cancer cells.

Stimulation of GM-CSF could augment LAK activity from PCMNCs to a level as high as that from BMNCs. Degrees of change below 40% fall within the standard deviations. So, we evaluated increases and decreases of LAK activities from PCMNCs and BMNCs of below 40% as no change. Stimulation of GM-CSF augmented

the LAK activities from PCMNCs of six patients significantly. Their pathologic n-factor was classified as three of n-0 and 3 of n-2, and the degree of pleural invasion was two of grade 0, 2 of grade 1, 1 of grade 2 and 1 of grade 3. The LAK activities from BMNCs of two of these 14 patients were augmented significantly by stimulation with GM-CSF. Their n-factors were n-0 and n-2, and they had no pleural invasion by lung cancer cells. On the other hand, in two patients, the LAK activity from BMNCs was suppressed significantly by GM-CSF. Their n-factors were n-2, and their degrees of pleural invasion were 0 and 2. In patients with mediastinal lymph node metastasis of lung cancer cells, LAK activity from BMNCs was suppressed significantly by stimulation with GM-CSF. Suppression of LAK activity from BMNCs by stimulation with GM-CSF might be caused by mediastinal lymph node metastasis of lung cancer cells. On the other hand, augmentation of LAK activity from PCMNCs and BMNCs by stimulation with GM-CSF showed no relation to the pathologic factors of individual patients. IL-2-dependent immune responses were regulated by nylon-wool-adherent suppressor cells[23] and serum[24] of some advanced cancer patients. We should examine other factors that affect augmentation of LAK activity by GM-CSF. Although several cytokines, such as IFN γ,[14-16, 18] TNF-α[17] and IL-1β,[16] could augment LAK activity, GM-CSF is an excellent agent that is used for not only myeloprotection, but also augmentation of LAK activity from PCMNCs.

The proportion of HLA-DR$^+$ cells was significantly higher in the pleural cavity without malignant effusion than in peripheral blood ($P<0.01$). The proportions of CD4$^+$ and CD16$^+$ cells were significantly lower in pleural cavity without malignant effusion than in peripheral blood ($P<0.05$, $P<0.01$). High potential ability of CD16$^+$ cells to develop LAK activity has been reported.[25, 26] In the present study, the proportion of CD16$^+$ cells in the pleural cavity without malignant effusion was found to be significantly less than in peripheral lymphocytes. The reduction of LAK activity from PCMNCs, therefore, may be caused by the difference of the population of CD16$^+$ cells. But, as far as changes of LAK activity by stimulation of GM-CSF was concerned, there was no relation in individual patients between augmentation or suppression rate of LAK activity and proportions of CD3$^+$, CD4$^+$, CD8$^+$, CD16$^+$, CD25$^+$ and HLA-DR$^+$ cells of PCMNCs and BMNCs (data not shown). Further, the degree of development of LAK activity from PCMNCs and BMNCs by IL-2 also had no relation to the proportions of these cells in this study (data not shown).

In all lung cancer patients, LAK activity from PCMNCs was not suppressed significantly by stimulation with GM-CSF. In about 20% of lung cancer patients, LAK activity from BMNCs was significantly suppressed by stimulation with GM-CSF. Stimulation of GM-CSF could augment LAK activity from PCMNCs more efficiently than that from BMNCs of lung cancer patients. In healthy donors, stimulation with GM-CSF significantly augmented LAK activity from BMNCs,[19] but in some advanced lung cancer patients, GM-CSF may suppress the LAK activity from BMNCs. These changes of immunological behavior induced by stimulation with GM-CSF may be caused by mediastinal lymph node metastasis of lung cancer cells. When we use GM-CSF with anticancer drugs for some advanced lung cancer patients with mediastinal lymph node metastasis of cancer cells, we must monitor the biological function of GM-CSF for each patient individually.

In conclusion, the use of GM-CSF could protect myelocytic cells from anticancer drugs, and could also protect the pleural cavity from invasion of lung cancer cells by augmentation of LAK activity developed from PCMNCs.

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