Induction of Cytokine Messenger RNA and Secretion in Alveolar Macrophages and Blood Monocytes from Patients with Lung Cancer Receiving Granulocyte-Macrophage Colony-stimulating Factor Therapy

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ABSTRACT

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes the proliferation and differentiation of hematopoietic progenitor cells. Although preliminary data are available from clinical trials, the effect of GM-CSF on gene expression of immunocompetent cells in treated patients has not been studied. We previously demonstrated that in vitro treatment with GM-CSF also enhances maturation-related anti-tumor activities in mononuclear phagocytes. The purpose of the present study was to examine the effects of in vitro recombinant GM-CSF therapy on alveolar macrophages and blood monocytes, to determine if these cells demonstrated differential expression of cytokine genes, cytokine production, and tumoricidal activity. Alveolar macrophages and blood monocytes were isolated from 13 patients receiving a range of GM-CSF doses (60-250 μg/m²/day) by continuous infusion over a 2-week period. Both monocytes and macrophages were isolated prior to therapy and at day 10 of the infusion. Monocytes, in addition, were isolated on day 3 of infusion. Results indicated that GM-CSF therapy enhanced expression of tumor necrosis factor, interleukin 1, and interleukin 6 mRNA in both monocytes and alveolar macrophages. Differential responses, however, were observed in cytokine secretion; monocytes demonstrated enhanced secretion of all three cytokines by day 3 of treatment, but alveolar macrophages showed only enhanced interleukin 6 secretion at day 10. Monocyte tumoricidal activity after in vitro lipopolysaccharide stimulation was also significantly elevated by day 3 of treatment, but at day 10 activity was not statistically different from pretreatment values in either monocytes or alveolar macrophages. These data indicate that GM-CSF exerts striking time-dependent modulatory effects on gene expression and functional activities of monocytes and alveolar macrophages in vivo, although the responses of the two cell types differ with respect to cytokine secretion.

INTRODUCTION

GM-CSF, in addition to promoting proliferation and differentiation of hematopoietic progenitor cells, has been shown to influence function of both monocytes and macrophages (1, 2). We previously reported that GM-CSF exerts a differential effect on alveolar macrophages and monocytes (3). We demonstrated that in vitro exposure to GM-CSF induced tumoricidal activity in human alveolar macrophages, whereas GM-CSF had little effect on the direct antitumor activity of freshly obtained blood monocytes. Maturation of monocytes in vitro, however, resulted in acquisition of tumoricidal activity. Activities associated with tumoricidal activity (e.g., production of superoxide anion, secretion of tumor necrosis factor, and secretion of interleukin 6), however, are up-regulated in freshly obtained monocytes after in vitro exposure to GM-CSF (3, 4). In contrast, we found no enhancement of TNF secretion in human alveolar macrophages by GM-CSF, suggesting differences between monocyte and macrophage regulation of activation.

Clinical trials of biological response modifier therapy have only recently begun to focus on the effect of these agents on monocyte activity. MTP-PE is an agent which has been shown in animal systems to be effective in rendering macrophages tumoricidal in vivo and inducing regression of established tumor metastases (5-7). In a clinical trial of MTP-PE, blood monocytes were found to have enhanced tumoricidal activity and IL-1 secretion (8). Monocytes have also been examined in phase I clinical trials of GM-CSF, where both functional changes (9, 10) and dose-dependent increases in circulating monocytes have been demonstrated (9-17).

Evaluation of the effects of systemic–biological response modifier therapy on alveolar macrophages has not been carried out. Lung cancer is the most common fatal cancer in the United States (18), and other cancers such as renal and colon frequently metastasize to the lung (19, 20). In patients with pulmonary malignancy, the antitumor activities of effector cells present at the site of tumor may be critical. The purpose of the present investigation was to examine the effects of in vivo GM-CSF therapy on alveolar macrophages and blood monocytes, to determine if these cells demonstrated differential cytokine gene expression, cytokine production, and tumoricidal activity.

MATERIALS AND METHODS

Patients. Thirteen patients with unresectable lung cancer (mean age, 60 ± 10 years) were entered in a phase I trial of GM-CSF. All patients provided written informed consent approved by the Cleveland Clinic Foundation Institutional Review Board. Six patients were active smokers, four had quit 1 year or more prior to diagnosis, and three had never smoked. The mean cigarette consumption was 38 ± 17 (mean ± SD) pack years, with a range of 3-60 pack years. Twelve patients were diagnosed with non-small cell cancer and one with small cell cancer. Patients were ambulatory and had not received any antitumor therapy for a minimum of 4 weeks prior to entrance into the study. GM-CSF was administered by continuous infusion for a total of 14 days. The dose levels were as follows: level 1, 60 μg/m²/day (four patients); level 2, 125 μg/m²/day (four patients); and level 3, 250 μg/m²/day (five patients). Monocytes were isolated from blood on one to three different occasions before study, on day 3 and day 10 of GM-CSF treatment, and 2 weeks after treatment. Total WBC count and absolute monocyte numbers were determined on four occasions prior to therapy and on days 3 and 10 of therapy. Alveolar macrophages were isolated from

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2 To whom requests for reprints should be addressed, at Department of Pulmonary Disease, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195-5038.

3 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; MTP-PE, muramyl tripeptide phosphatidylethanolamine; ELISA, enzyme-linked immunosorbent assay; IL-1, interleukin 1; IL-6, interleukin 6; IL-10, interleukin 10; TNF-α, tumor necrosis factor α; TNF, tumor necrosis factor; SSC, standard saline citrate; LPS, lipopolysaccharide.
patients before study and on day 10 of treatment. Due to limitations in cell yields, not all assays were performed on each individual's cells.

Preparation of Alveolar Macrophages. Alveolar macrophages were obtained by fiberoptic bronchoscopy, as previously described (21). The tip of the bronchoscope was wedged into a noninvolved segment of the lung. Aliquots of 75 ml of sterile saline were alternately instilled by gravity and withdrawn by gentle aspiration. A total volume of 300 ml was usually employed. All lavage fluid was passed through a blood filter (McGaw, Sabana Grande, Puerto Rico) and then centrifuged at 400 x g for 10 min. The cell pellet was then washed with Hank's balanced salt solution (M. A. Bioproducts, Walkersville, MD). Cell number was determined on a hemocytometer and differential cell counts were performed with either a modified Wright's stain (Diff-Quik; American Scientific, McGaw Park, IL) or nonspecific esterase stain (22). The cells were resuspended in RPMI 1640 medium (M. A. Bioproducts) supplemented with 5% human AB serum (GIBCO, Grand Island, NY), L-glutamine, and antibiotics (complete medium). The cell concentration was adjusted to 5 x 10^5/ml, and 0.1 ml was added to each well of 96-well microtiter plates (Falcon Laboratories, McLean, VA). Cells were allowed to adhere for 60 min at 37°C in a moist 5% CO2 incubator, and nonadherent cells were then removed by washing of the wells with warmed RPMI. By nonspecific esterase cytochemistry, over 99% of adherent mononuclear cells were characterized as macrophages. Endotoxin contents of culture reagents did not exceed 0.3 ng/ml, as determined by the Limulus amebocyte assay (Associates of Cape Cod, Inc., Woods Hole, MA).

Preparation of Peripheral Blood Monocytes. Mononuclear leukocytes were isolated from EDTA-treated whole blood by centrifugation over Nycodenz monocytes (Accurate Chemical & Scientific, Westbury, NY) and were resuspended in complete medium. The cell concentration was adjusted to 1 x 10^6/ml and 0.1 ml/well was added to 96-well microtiter plates. Cells were adhered as described above for macrophages. By nonspecific esterase cytochemistry, over 99% of adherent mononuclear cells were characterized as monocytes.

Reagents and Drugs. Recombinant GM-CSF was supplied by the National Cancer Institute and produced by Immunex Corporation. Purified GM-CSF had a specific activity of approximately 5 x 10^7 units/mg and a purity level of >95%. Salmonella typhimurium LPS from Sigma Chemical Co. (St. Louis, MO) was used in indicated in vitro experiments.

Tumoricidal Assay. The tumoricidal assay was based on that described by Kleinerman et al. (23). Adherent monocytes or macrophages were cultured in microtiter plates, as described above. The cells were incubated in complete medium with or without LPS. After 20 h, the cells were washed with warm medium. Target cells labeled for 24 h with 5 μCi/ml tritiated thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, MA) were added at a target to effector ratio of 1:10 [previously determined to be the optimal ratio (24)]. Target cells were co-cultivated with macrophages or monocytes for 24 to 96 h, after which time the tritium contents of respective gene expression in comparison to that of actin.

Northern Blot Analysis. RNA was analyzed according to a modification of the methods of Maniatis et al. (26). Total RNA (10–20 μg) in 60% formamide, 1 x 3-(N-morpholino)propanesulfonic acid buffer, 11% formaldehyde, was heat denatured (65°C, 15 min), quenched on ice, and size fractionated by agarose gel electrophoresis. Following electrophoresis, ethidium bromide staining identified RNA molecular weight standards or 28S and 18S bands of total RNA run in control lanes. The RNA was transferred overnight to a GeneScreen (Dupont, Boston, MA) hybridization membrane that was pre-treated with transfer buffer. Following overnight transfer, the membrane was briefly air dried and then baked at 80°C for 2 h in a vacuum oven. The membrane was then hybridized with [α-32P]dCTP (specific activity, 300 Ci/mmol; Amersham, Arlington, IL) per probe, using a nick translation kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. Enhanced expression of cytokine genes was determined as a qualitative increase in respective gene expression in comparison to that of actin.

Slot Blot Analysis. When sufficient RNA was not obtained for Northern blots, slot blot analyses were carried out. To compare results, both Northern and slot blots were performed with RNA from six patients and results were comparable (alveolar macrophage RNA from three patients and monocyte RNA from three patients were analyzed by both). Total RNA (5–10 μg) was heat denatured (65°C, 15 min) in 6x...
Cytokine ELISA assays, measurements below the detectable level of the vacuum oven. Hybridization was carried out as described above. Each sample was washed twice with 500 μl of 10x SSC. The membrane was briefly air dried and then baked at 80°C for 2 h in a vacuum oven. Hybridization was carried out as described above.

Statistical Analysis. The results were analyzed for their statistical significance by Wilcoxon signed rank test. For analysis of data from cytokine ELISA assays, measurements below the detectable level of the assay were considered equivalent to zero.

RESULTS

GM-CSF Effects on Monocyte/Alveolar Macrophage Populations. In all patients, circulating monocytes were increased at all dose levels, compared to baseline (level 1, 1.8 ± 0.4-fold increase, mean ± SD; level 2, 2.7 ± 0.6; level 3, 3.5 ± 1.2). Bronchoalveolar lavage yields of alveolar macrophages were not increased in 10 of 11 patients, and differential lavage leukocyte counts remained unchanged in 10 of 11 patients (96.0 ± 1.7% alveolar macrophages, mean ± SD, before study versus 97.4 ± 2.8 on day 10). In one patient the absolute number of alveolar macrophages recovered from bronchoalveolar lavage increased 2-fold, while in another neutrophil counts increased (95% alveolar macrophages and 2% polymorphonuclear leukocytes before study versus 70% alveolar macrophages and 21% polymorphonuclear leukocytes on day 10).

Monocyte/Macrophage Products. Both TNF and IL-1 were elevated in supernatants obtained from monocytes cultured in medium alone, during therapy at all three dose levels, in 12 of 13 patients, compared to either prestudy or poststudy supernatants (all P values were ≤0.03) (Fig. 1). The single exception was a patient at dose level 3, who did not show elevated IL-1. Increases in cytokine secretions ranged from 6- to 39-fold for TNF and 2- to 29-fold for IL-1. In addition, secretion of both cytokines was further enhanced by culture of monocytes with LPS (data not shown). Spontaneous IL-6 secretion was enhanced 2–56-fold from monocytes of 9 of 12 patients tested; the three patients who did not show enhanced IL-6 secretion had preexisting high levels before treatment (Table 1). IL-6 secretion was not measured after LPS stimulation.

Results with supernatants obtained from alveolar macrophages varied somewhat but generally showed little spontaneous increase in TNF or IL-1 secretion (Fig. 1). In 5 of 8 patients, however, supernatants obtained from LPS-stimulated macrophages demonstrated increased TNF and/or IL-1 levels (data not shown). Spontaneous IL-6 secretion by alveolar macrophages was enhanced 4–30-fold in 4 of 7 patients; the three patients who did not show enhanced secretion had high pre-study IL-6 levels (Table 1).

Expression of Cytokine Genes. Monocyte expression of TNF-α, IL-1β, and IL-6 mRNA was enhanced at dose levels 1 and 2, compared to pretreatment values (see Figs. 2 and 3 and Table 2). Minimal change was found at dose level 3. Alveolar macrophages showed enhanced expression of TNF, IL-1, and IL-6 genes at dose level 1 (Fig. 2), but gene expression was not changed from pretreatment values at dose levels 2 and 3 (Table 2). Monocyte TNF, IL-1, and IL-6 gene expression generally correlated with secretion of the respective cytokines at dose level 1. At dose levels 2 and 3, however, enhanced secretion was not always accompanied by enhanced gene expression. Alveolar macrophage TNF and IL-1 gene expression from two patients at dose level 1 was enhanced without concomitant enhancement of TNF and IL-1 secretion. No enhancement of macrophage TNF and IL-1 secretion or gene expression was detectable at dose levels 2 and 3. IL-6 secretion by alveolar macrophages did not correlate with gene expression at any dose level.

Cytotoxicity. Evaluation of spontaneous tumoricidal activity of monocytes and alveolar macrophages from GM-CSF-treated patients indicated enhanced activity in only two cases. Monocytes from one patient at dose level 2 demonstrated enhanced spontaneous cytotoxicity at day 10 (32% cytotoxicity) of treatment and one patient at dose level 3 demonstrated enhanced cytotoxicity at day 3 (46%) and day 10 (58%) of treatment. In 12 of 13 patients, in vitro stimulation with LPS significantly (P = 0.0005) elevated the antitumor activity of monocytes har-

Table 1 Monocyte and alveolar macrophage IL-6 secretion

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<th>Day 10</th>
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* ND, not done.
INDUCTION OF CYTOKINE mRNA AND SECRETION

Table 2 Expression of cytokine mRNA in monocytes from patients receiving GM-CSF

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<th>Dose Level</th>
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<td>5/3</td>
<td>1/5</td>
<td>1/5</td>
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<td></td>
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<td>2/3</td>
</tr>
<tr>
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<tr>
<td>3</td>
<td>10</td>
<td>0/2</td>
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</tr>
</tbody>
</table>

* Enhanced mRNA expression on day 3 or day 10 of therapy, as compared to pretreatment. Monocyte RNA from four patients was analyzed by Northern blots and that from seven patients by slot blots. Alveolar macrophage RNA from three patients was analyzed by Northern blots and that from three patients by slot blots.

Fig. 2. Northern blot analysis of monocyte and alveolar macrophage RNA. RNA was harvested from monocytes of patient 2 (dose level 1) before study and on days 3 and 10 of GM-CSF treatment and from alveolar macrophages before study and on day 10.

Fig. 3. Slot blot analyses of monocyte RNA isolated from patient 5 (dose level 2) at indicated times, with corresponding cytokine secretion (pg/ml).

Fig. 4. Monocyte (A) and alveolar macrophages (B) tumoricidal activity results from patients at dose levels 1-3 of GM-CSF treatment. Monocytes were harvested before study (mean of one to three results), on days 3 to 10 of treatment, and 2 weeks after treatment. Alveolar macrophages were harvested before study and on day 10 of treatment. Both monocytes and alveolar macrophages were incubated overnight in 0.5 μg/ml LPS, radioactively labeled tumor targets (SK-MEL-28) were added, and tumoricidal activity was measured. * Patient 4 was hypercalcemic (calcium > 12.0 mg/ml) on day 10 of therapy.

DISCUSSION

The present study demonstrates that GM-CSF therapy produces dramatic time-dependent changes in the biological activity of both monocytes and alveolar macrophages. Enhanced expression of mRNA coding for TNF, IL-1, and IL-6 was observed in monocytes and macrophages. With respect to cytokine secretion, differential responses were observed, with monocytes demonstrating enhanced secretion of all three cyto-

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kines and alveolar macrophages showing only enhanced IL-6 secretion. These results suggest that, although both monocytes and alveolar macrophages respond to systemic administration of GM-CSF, the nature of the response may differ.

Constitutive gene expression of TNF, IL-1, and IL-6 was detected in freshly isolated adherent monocytes and macrophages from patients prior to GM-CSF therapy. Other investigators have also reported constitutive gene expression in both adherent and nonadherent monocytes from normal volunteers; however, enhancement of cytokine gene expression is associated with the process of adherence (4, 27–29). Despite gene expression, we detected little cytokine secretion in patient monocytes before treatment. In patients treated with GM-CSF at dose level 1, both gene expression and secretion of all three cytokines were enhanced, compared to pretreatment. At dose levels 2 and 3, monocyte cytokine secretion was uniformly detected, but gene expression was enhanced in monocytes from only a few patients. The failure to detect enhanced gene expression at higher doses of GM-CSF may result from a more rapid time course of gene transcription and translation. Suppression from high dose GM-CSF is unlikely, since no significant differences in cytokine secretions were observed over the dose range evaluated. Similar events may also occur in alveolar macrophages, since enhanced cytokine gene expression was demonstrated only at dose level 1. The lack of macrophage cytokine secretion may be due to cytokines remaining cell associated, as has been demonstrated by others following stimulation with LPS (30, 31). Alternatively, regulation of translation may differ in monocytes and alveolar macrophages. Nevertheless, the differences in gene expression between monocytes and macrophages obtained before treatment and during therapy indicate modulation of activity by GM-CSF therapy. As observed in our previous studies (3), monocytes and alveolar macrophages demonstrated differential cytokine responses to in vitro treatment with GM-CSF (e.g., GM-CSF enhanced monocyte but not alveolar macrophage TNF secretion). Monocyte secretion of IL-1, however, was not detected after in vitro GM-CSF exposure, and its occurrence in patients receiving GM-CSF treatment suggests that there may be additional regulatory signals in vivo.

IL-6 secretion varied somewhat, but greater responses to GM-CSF therapy were found in both monocytes and alveolar macrophages with low prestudy levels. The exact role of IL-6 in antitumor activity is not clear. IL-6 has been shown to have multiple regulatory functions in both immune and acute phase responses (32) and has been reported to inhibit the growth of some human tumor cell lines (33). Induction of circulating levels of IL-6 by administration of TNF and IL-2 has been reported in both humans and animals (34, 35), and it is possible that the elevated monocyte TNF induced by GM-CSF may participate in secondary induction of IL-6, although the mechanisms have not been identified. In several animal tumor models, serum levels of IL-6 have correlated with tumor burden (35). All patients in the present study had advanced disease and thus the pretreatment elevations of IL-6 observed in monocytes and alveolar macrophages of a few patients may relate to tumor burden and/or location.

As with other GM-CSF clinical trials (9–17), absolute monocyte counts increased in a dose-dependent manner. The absolute number of alveolar macrophages recovered from bronchoalveolar lavage did not increase; however, bronchoalveolar lavage is not a quantitative technique (36).

Only a few of the GM-CSF clinical trials have evaluated monocyte activities, and no study has examined alveolar macrophages. Wing et al. (9) demonstrated enhanced TNF and interferon secretion from monocytes of GM-CSF-treated patients after monocyte stimulation in vitro with LPS. Kleinerman et al. (10) found no enhancement of IL-1 and TNF secretion by unstimulated monocytes of GM-CSF-treated patients, but most of these individuals had various myeloid disorders. The varied responses observed in GM-CSF therapy may reflect differences in the method of GM-CSF administration or dosage, timing of sample collection, sensitivities of cytokine detection assays, and/or differences in patient populations.

Monocyte tumoricidal responses during GM-CSF therapy have also been examined. We did not detect significant enhancement of spontaneous tumoricidal activity of monocytes or macrophages, although LPS-stimulated cytotoxicity of monocytes was significantly enhanced at day 3 but not day 10 of treatment. This decrease in monocyte cytotoxicity during treatment suggests that time of sampling may be important. Kleinerman et al. (10) also did not detect significant enhancement of spontaneous tumoricidal activity in monocytes from patients treated with GM-CSF. Conversely, Wing et al. (9) demonstrated enhanced antibody-dependent cytotoxicity. However, it is likely that the mechanisms of antibody-dependent and -independent cytotoxicity are different (37).

GM-CSF treatment exerts striking time-dependent modulatory effects on both monocytes and alveolar macrophages, as demonstrated by enhanced gene expression for TNF, IL-1, and IL-6. However, cytokine secretion between the two cell types differed, suggesting inherent differences between the two cell types. Furthermore, the increase in the number of circulating monocytes and enhanced cytotoxic activity when coupled with a second signal (LPS) suggest that GM-CSF in combination with other agents such as γ interferon or MTP-PE should be investigated for its effects in patients with cancer. Additional studies are underway to evaluate these possibilities.

REFERENCES

INDUCTION OF CYTOKINE mRNA AND SECRETION


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