Effect of Recombinant Granulocyte/Macrophage Colony-stimulating Factor on Human Monocyte Activity in Vitro and following Intravenous Administration

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ABSTRACT

The purpose of these studies was to examine the antitumor properties of blood monocytes from patients undergoing a phase I trial with recombinant granulocyte/macrophage colony-stimulating factor (rGM-CSF). Peripheral blood monocytes from 7 patients receiving various doses of rGM-CSF by continuous infusion were isolated prior to therapy and at various times during the 2-wk infusion. Monocytes/cubic centimeter of blood increased in a dose-dependent fashion in patients receiving rGM-CSF. However, activation of monocyte-mediated tumorilic properties was seen in only 1 of 7 patients. rGM-CSF administration also did not stimulate interleukin-1 or tumor necrosis factor production by blood monocytes. The failure to detect monocyte-mediated tumoricidal activation was not secondary to an inherent "defect" in the patients' monocytes because in vitro incubation with lipopolysaccharide alone or human recombinant γ-interferon plus muramyl dipeptide resulted in monocyte-mediated cytotoxicity and in the secretion of interleukin-1 and tumor necrosis factor. rGM-CSF in vitro also did not stimulate the tumoricidal function of normal monocytes or the secretion of interleukin-1 or tumor necrosis factor.

We concluded that systemic administration of rGM-CSF did not result in routine activation of monocyte-mediated cytotoxicity but did result in a dose-dependent rise in the number of circulating monocytes. Because these monocytes could be activated in vitro, we propose that, in an adjuvant therapy setting, rGM-CSF be combined with other activating agents to increase the pool of potential killer cells.

INTRODUCTION

GM-CSF3 is defined by its ability to stimulate bone marrow precursor cells to proliferate and differentiate into granulocytes and macrophages. In addition, GM-CSF stimulates a variety of functional activities in mature macrophages and granulocytes, including oxidative metabolism, inhibition of migration, antibody-dependent cellular cytotoxicity, and increased phagocytosis of microbes (1-3). The complementary DNA for human T-cell-derived GM-CSF has now been cloned and recombinant GM-CSF purified to homogeneity (4, 5). rGM-CSF is undergoing intense clinical studies to determine its efficacy in the treatment of cancer. In addition, the effect of in vivo administration of rGM-CSF on other biological activities are being extensively studied (6). Recently, rGM-CSF was reported to activate the tumoricidal function of human peripheral blood monocytes in vitro (4). This prompted us to investigate whether demonstrable activation of antitumor properties of blood monocytes would occur following rGM-CSF administration to patients. We also attempted to correlate the antitumor properties with the secretion of IL-1 and TNF.

Patients. Seven patients who were entered in a phase I trial with rGM-CSF were studied for blood monocyte-macrophage function. Patients were ambulatory and had not received any antitumor therapy for a minimum of 3 wk prior to entrance into the study. Diagnoses included myelodysplasia (3 patients), myelofibrosis, breast carcinoma, melanoma, and acute myelogenous leukemia with myelodysplasia. Informed consent was obtained from each patient according to our institutional policy. rGM-CSF was administered at the specified level by continuous infusion for 2 wk, discontinued for 2 wk, then administered again for 2 wk. This constituted 1 treatment cycle. The doses were as follows: level 1, 15 μg/m²/day; level 2, 30 μg/m²/day; level 3, 60 μg/m²/day; level 4, 100 μg/m²/day; level 5, 250 μg/m²/day; and level 6, 500 μg/m²/day.

Sampling. Peripheral blood monocytes were isolated from 20 ml of heparinized blood and assayed for tumoricidal activity. A WBC count was performed in the laboratory prior to the separation procedure. Initial determinations of monocyte activity were conducted before administration of rGM-CSF and also during and after rGM-CSF therapy as indicated. For control studies, blood specimens were obtained from 13 normal donors.

Reagents and Drugs. RPMI 1640, HBSS, FBS, human AB serum, and Eagle's complete minimal essential medium were purchased from Grand Island Biological Co., Grand Island, NY. RPMI 1640, HBSS, RIFN-γ, human recombinant TNF-α, MDP, and lipopolysaccharide were supplied by Genentech Inc., South San Francisco, CA. MDP was the gift of Ciba Geigy, Ltd., Basel, Switzerland. Reagents for the Limulus amoebocyte lysate assay were purchased from W. AT. Bioproducts, Walkersville, MD. pg/ml was determined by the Limulus amoebocyte lysate assay (sensitivity limit of 0.025 ng/ml). Salmonella typhosa LPS was purchased from Difco Laboratories, Detroit, MI.

Isolation of Human Monocytes. MNL were isolated from peripheral blood on lymphocyte separation medium (Litton Bionetics, Kensington, MD), washed twice with HBSS, and then suspended in RPMI 1640 containing 5% human AB serum. The percentage of monocytes in the MNL layer was assessed by morphology and peroxidase stain, and the cell suspension was adjusted to contain 2 x 10⁶ monocytes/ml. Into each well of a 24-well flat-bottomed Microplate II plate (Falcon Plastics, Oxnard, CA), 2 x 10⁶ monocytes were added and allowed to adhere for 1 h at 37°C. Nonadherent cells were removed by 3 washes with HBSS. The purity of the adherent monocyte monolayers was >97% as assessed by the following tests: India ink ingestion, morphology, and peroxidase staining. Puriﬁed monocytes were then incubated at 37°C for 18–24 h with 0.2 ml control medium, medium containing rIFN and MDP, or medium containing LPS. After this incubation period, the adherent monocyte cultures were washed with HBSS, and [³¹P]labeled target cells were added as described below.

Target Cell Cultures. The cell line cell A375, derived from a human melanoma (7), was used as human tumor target cells exactly as described previously (8). Monolayer cultures were maintained in plastic in medium supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, twice concentrated vitamin solution, and L-glutamine (M. A. Bioproducts) in a humidified atmosphere of 5% CO₂ in air. All cultures were free of Mycoplasma and pathogenic murine viruses.

Assay of Monocyte-Macrophage-mediated Cytotoxicity against Tumor Target Cells. Macrophage-mediated tumor cytotoxicity was assessed by a modification of a radioactive-release assay as previously described (8–

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2To whom requests for reprints should be addressed.

3The abbreviations used are: GM-CSF, granulocyte/macrophage colony-stimulating factor; rGM-CSF, recombinant granulocyte/macrophage colony-stimulating factor; MNL, mononuclear leukocytes; IL-1, interleukin-1; TNF, tumor necrosis factor-α; FBS, fetal bovine serum; WBC, white blood cell.
10). Target cells in exponential growth phase were incubated for 24 h in supplemented medium containing [125I]iododeoxyuridine (0.3 μmol; New England Nuclear, Boston, MA). The A375 cells were washed twice to remove unbound radiolabel and harvested by a 1-min trypsinization with 0.25% trypsin (Difco) and 0.02% EDTA. The labeled cells were washed with medium and resuspended in supplemented medium, and 1 x 10^6 cells were plated into the culture wells with the macrophage cultures to obtain an initial target:effector cell ratio of 1:20. Radiolabeled target cells were also plated alone as an additional control group. After 24 h, the cultures were washed to remove the nonadherent target cells, refed with fresh medium, and then cultured for an additional 48 h. The cultures were then washed twice with HBSS, and the adherent viable cells were lysed with 0.1 ml of 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter. Percentage of monocyte-mediated cytolysis of tumor cells was calculated by two different formulas. In the first, we calculated the cytolysis mediated by monocytes collected from cancer patients without further in vitro treatment.

\[
\text{% of cytotoxicity} = \frac{A - B}{A} \times 100
\]

where \(A\) is counts per minute in target cells cultured alone, and \(B\) is counts per minute in target cells cultured with monocytes. Values of <0% cytotoxicity were expressed simply as 0% cytotoxicity. We determined previously that natural killer cell activity does not contribute to the cytolysis assay (10), and that this assay measures lysis and not target cell detachments (8).

In the second formula, for assays measuring the effects of in vitro incubation of monocytes with LPS or a combination of rIFN-7 and MDP, we calculated the percentage of generated cytotoxicity by the formula

\[
\text{% of generated cytotoxicity} = \frac{A - B}{A} \times 100
\]

where \(A\) is counts per minute of target cells cultured with untreated monocytes, and \(B\) is counts per minute of target cell cultures with in vitro-treated monocytes.

**Statistical Analysis.** Experimental results were analyzed for their statistical significance using the Student's t test.

**Supernatants for IL-1 and TNF assay.** Patient monocytes were incubated separately with RPMI 5% AB serum or the indicated immunostimulant for 24 h. The supernatants were harvested, centrifuged at 225 g for 5 min to remove residual cells and cellular debris, and stored at -20°C.

**IL-1 Assay.** The murine helper T-cell clone D10.G4.1 kindly provided by Dr. C. Janeway was used to assay IL-1 activity. The cell line was carried in IL-2-supplemented Click's medium (11) passed on provided by Dr. C. Janeway was used to assay IL-1 activity. The cell line was carried in IL-2-supplemented Click's medium (11) passed on

\[
\text{EFFECT OF rGM-CSF ON MONOCYTES IN VITRO AND IN VIVO}
\]

where \(\Delta\) is counts per minute of target cells cultured with untreated monocytes, and \(B\) is counts per minute of target cell cultures with in vitro-treated monocytes.

**RESULTS**

Effect of rGM-CSF Infusion on WBC Counts and Percentage of Monocytes. Similar to other colony-stimulating factors, rGM-CSF has been shown to stimulate the in vitro proliferation of granulocytes and macrophages. We therefore sought to determine the effect of rGM-CSF administration on the WBC count and yield of monocytes obtained from peripheral blood of patients receiving rGM-CSF by continuous infusion. For this study, blood samples were obtained prior to therapy and on days 1, 8, and 15 (day 15 corresponds to the end of the 2-wk treatment cycle). Patients were off therapy for 2 wk and then studied again during cycle 2. A total of 13 normals served as controls. As shown in Table 1, the percentage of monocytes from the MNL layer obtained from normal peripheral blood ranged from 7 to 35% (mean, 24%). The number of monocytes yielded per cubic centimeter of whole blood in all individuals was <4.0 x 10^9/cc. Three individuals (Nos. 11, 12, and 13) were studied on 2 occasions, 1 wk apart. As shown in Table 1, both the percentage of monocytes composing the MNL layer and the yield of monocytes/cubic centimeter did not change significantly after 1 wk. We previously showed that normal monocyte function also did not fluctuate when studied sequentially over time (15, 16). Table 2 shows the number of MNL obtained following separation on lymphocytic separation medium, percentage of monocytes composing this MNL layer, and the yield of monocytes obtained from the patient samples. Prior to therapy, all patient values were not significantly different from the normal values shown in Table 1. The percentage of monocytes from the MNL layers ranged from 15 to 35% (mean, 25%), and the yield of monocytes was also <4 x 10^9/cc whole blood. However, at 60 μg/m^2/day (level 3), the monocyte yield increased 1–8 days after the beginning of therapy. This increase was more pronounced at levels 5 and 6 (250 μg/m^2/day and 500 μg/m^2/day). Blood samples obtained after 2 wk off therapy showed values similar to pretreatment levels. Thus, the data suggest that the increases observed were directly related to drug administration. Indeed, when rGM-CSF therapy was reinstated, the yield of monocytes once again increased. The data also suggest that the increases observed were dose related. For example, Patient 7 showed an increase of 1.1 x 10^10 to

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**Table 1 MNL and monocyte counts from normal controls**

<table>
<thead>
<tr>
<th>MNL layer</th>
<th>Normal MNL/cc</th>
<th>% of monocytes</th>
<th>Monocytes/cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.4 x 10^5</td>
<td>26</td>
<td>1.7 x 10^9</td>
</tr>
<tr>
<td>2</td>
<td>10.5 x 10^5</td>
<td>22</td>
<td>2.3 x 10^9</td>
</tr>
<tr>
<td>3</td>
<td>11.5 x 10^5</td>
<td>20</td>
<td>2.3 x 10^9</td>
</tr>
<tr>
<td>4</td>
<td>3.0 x 10^5</td>
<td>30</td>
<td>0.9 x 10^9</td>
</tr>
<tr>
<td>5</td>
<td>10 x 10^5</td>
<td>15</td>
<td>1.5 x 10^9</td>
</tr>
<tr>
<td>6</td>
<td>10.9 x 10^5</td>
<td>22</td>
<td>2.4 x 10^9</td>
</tr>
<tr>
<td>7</td>
<td>11.9 x 10^5</td>
<td>32</td>
<td>3.8 x 10^9</td>
</tr>
<tr>
<td>8</td>
<td>11.4 x 10^5</td>
<td>7</td>
<td>0.8 x 10^9</td>
</tr>
<tr>
<td>9</td>
<td>8.2 x 10^5</td>
<td>17</td>
<td>1.4 x 10^9</td>
</tr>
<tr>
<td>10</td>
<td>6.9 x 10^5</td>
<td>21</td>
<td>1.5 x 10^9</td>
</tr>
<tr>
<td>11</td>
<td>Sample 1</td>
<td>3.8 x 10^5</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Sample 2^*</td>
<td>6.3 x 10^5</td>
<td>1.9 x 10^9</td>
</tr>
<tr>
<td>12</td>
<td>Sample 1</td>
<td>10.7 x 10^5</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Sample 2^*</td>
<td>13.3 x 10^5</td>
<td>2.4 x 10^9</td>
</tr>
<tr>
<td>13</td>
<td>Sample 1</td>
<td>4.3 x 10^5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Sample 2^*</td>
<td>3.0 x 10^5</td>
<td>1.0 x 10^9</td>
</tr>
</tbody>
</table>

- MNL were isolated from 10–20 cc whole blood on lymphocyte separation medium. Percentage of monocytes was determined by morphology and peroxidase staining.
- Obtained 1 wk after sample 1.

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Cytotoxicity of Monocytes from Patients Receiving rGM-CSF. Because rGM-CSF has previously been reported to activate the tumoricidal function of normal monocytes in vitro (4), we also evaluated the antitumor response of blood monocytes from these patients during the systemic administration of rGM-CSF. Once again, samples were obtained before therapy, at 1 and 4 h postadministration, and on days 1, 8, and 15. Thus, 5 samples were obtained on each patient after the commencement of rGM-CSF therapy. Four of 7 patients failed to show any activation of tumoricidal function at any time point tested (Fig. 1). Monocytes isolated from one patient (Patient 2) exhibited antitumor cytotoxic properties as early as 1 h (prevalue; 0%; 1 h, 34% cytotoxicity), which persisted throughout the 15-day drug administration (Fig. 1). In the remaining 2 patients, monocyte-activated tumor cytotoxic properties were exhibited at single isolated time points only (Patient 3 on day 1; Patient 7 on day 15). All other time points tested showed cytotoxic values ≤15% (Fig. 1; data for 1 and 4 h not shown). We previously determined that cytotoxic values ≤15% are not significant in this assay (8, 10) and therefore do not represent tumoricidal function.

The antitumor activity demonstrated in 2 of these 3 patients (Patients 2 and 3) correlated with a small but significant secretion of IL-1 and TNF by these monocytes on the appropriate day (Patient 7 was not tested). Supernatants collected from the monocyte samples from Patient 2 on day 1 contained 3 units/ml IL-1, and TNF activity could be detected in this supernatant using the L929 assay (Fig. 2). The monocytes from Patient 3 were secreting 1 unit/ml IL-1 on day 1, but 0 units/ml on days 8 and 15. Similar to IL-1, TNF activity was found on day 1, but not on days 8 and 15. Monocytes showing cytotoxic values <15% (Fig. 2) were tumoricidal by these in vitro activation techniques. In addition, these monocytes with LPS or a combination of IFN-γ and MDP. Monocytes could be activated in vitro to kill tumor cells by incubating with either LPS or the combination of IFN-γ and MDP. Monocytes harvested from all patients before therapy were secreting 1 unit/ml IL-1 on day 1, but 0 units/ml on days 8 and 15. Similar to IL-1, TNF activity was found on day 1, but not on days 8 and 15. Monocytes showing cytotoxic values ≤15% were not secreting either IL-1 or TNF (Fig. 2). Because monocyte tumoricidal activity has recently been shown to correspond to IL-1 and TNF secretion (17–19), these data further substantiate our findings that cytotoxic values of ≤15% are to be considered negative (Fig. 2).

To assure that our failure to detect activation of tumoricidal activity following rGM-CSF administration was not secondary to an inherent “defect” in the patients' monocytes, we incubated these monocytes with LPS or a combination of IFN-γ and MDP in vitro. Monocytes harvested from all patients before therapy could be activated in vitro to kill tumor cells by incubating with either LPS or the combination of IFN-γ and MDP. Monocytes harvested on therapy (days 8 and 15) could also be rendered tumoricidal by these in vitro activation techniques. In addition, incubation with LPS stimulated the release of both IL-1 and TNF (data not shown). Together these data indicate that rGM-CSF therapy was not toxic to the monocytes.

Effect of rGM-CSF on Normal Blood Monocytes. The rGM-CSF that was administered to patients in vivo was also tested for its ability to activate the tumoricidal properties of normal monocytes in vitro. Peripheral blood monocytes from 6 normal volunteers were isolated as previously described (8) and incubated with rGM-CSF (500 to 5 units/ml) for 24 h, then washed prior to the addition of radiolabeled A375 melanoma cells (8). As shown in Table 3, monocytes from all donors were activated by both LPS and IFN-γ plus MDP to kill tumor cells. The effect of 500 units/ml rGM-CSF is also shown in Table 3. Significant cytotoxicity is seen in only 1 of 6 when treated with rGM-CSF (Normal D). We previously showed that LPS and lymphokine-activated monocytes will lyse HT-29 colon carcino-
These findings are similar to those reported using continuous infusion of human rGM-CSF in normal monkeys (6). Like monocytes of normal donors (8) and those in previous reports from patients with a variety of malignancies (16), peripheral blood monocytes from these patients could be activated in vitro to lyse allogeneic tumor cells in a 72-h cytotoxicity assay (Table 3). Thus, no inherent “defect” in the patients’ monocytes could be demonstrated prior to therapy.

We previously reported that rIFN-γ administered i.v. or i.m. was able to generate tumoricidal blood monocytes in vivo (16). The ability of rIFN-γ to activate blood monocytes in vivo appeared to be dose related. Administration of 0.25 mg/m²/day i.v. or 0.25–0.5 mg/m²/day i.m. was very effective in rendering the patients’ monocytes cytotoxic against tumor cells in vitro (16). Doses below 0.25 mg/m²/day were not effective, whereas higher doses (1 mg/m²/day) were found to be toxic because monocytes from these patients would no longer respond to in vitro activators (16).

Administration of rGM-CSF resulted in the sustained activation of tumoricidal monocytes in only 1 of 7 patients (Fig. 1). One patient showed activation on day 1 that was not sustained on days 8 and 15, and one patient showed an isolated activation point on day 15. This does not represent evidence of significant in vivo activation of tumoricidal function because >85% of the samples tested showed no tumoricidal activity. rGM-CSF administration also did not appear to stimulate IL-1 or TNF production by the monocytes (Fig. 3). Unlike monocytes obtained from high-dose rIFN-γ patients, however, these monocytes could be stimulated in vitro to lyse tumor cells and to secrete IL-1 and TNF (see results). It is therefore unlikely that toxicity from rGM-CSF could account for this lack of tumoricidal activity. We thus concluded that the systemic administration of rGM-CSF did not result in routine activation of monocyte-mediated cytotoxicity but did result in a dose-dependent rise in the number of circulating monocytes.

Grabstein et al. reported that in vitro incubation of rGM-CSF with normal monocytes resulted in the activation of tumoricidal function (4). We have no obvious explanation for the discrepancy between our data and those reported by Grabstein.
EFFECT OF rGM-CSF ON MONOCYTES IN VITRO AND IN VIVO

Fig. 2. Effect of rGM-CSF infusion on triggering monocyte-mediated tumoricidal activity, IL-1 and TNF secretion. Peripheral blood monocytes were isolated separately from 6 patients receiving rGM-CSF therapy. The highest percentage of cytotoxicity (from Fig. 1) is shown with the corresponding results from the IL-1 and TNF assays. A, percentage of cytotoxicity against A375 tumor target cells in 72-h assay. B, IL-1 secretion as measured by the D10.G4.1 assay. C, TNF secretion as measured by percentage of growth inhibition of L1929 cells in a 72-h assay.

Table 3 Effect of rGM-CSF in vitro on triggering of monocyte tumoricidal activity

2 × 10^5 Monocytes were incubated with the indicated agent(s) for 24 h and washed prior to the addition of [125I]iododeoxyuridine-labeled A375 target cells.

<table>
<thead>
<tr>
<th>Treatment of monocytes</th>
<th>% of generated cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>25</td>
</tr>
<tr>
<td>LPS</td>
<td>55</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>35</td>
</tr>
<tr>
<td>500 ng/ml</td>
<td>48</td>
</tr>
<tr>
<td>250 ng/ml</td>
<td>37</td>
</tr>
<tr>
<td>100 ng/ml</td>
<td>0</td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>0</td>
</tr>
<tr>
<td>MDP (100 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>IFN-γ (10^3 units/ml)</td>
<td>0</td>
</tr>
<tr>
<td>rGM-CSF (500 units/ml)</td>
<td>22</td>
</tr>
<tr>
<td>rGM-CSF (100 units/ml)</td>
<td>36</td>
</tr>
<tr>
<td>rGM-CSF (50 units/ml)</td>
<td>29</td>
</tr>
</tbody>
</table>

et al. Their paper reported findings with only 2 normal donors. We have tested 8 normal donors, only one of which showed in vitro activation by rGM-CSF (Tables 3–5). This correlates with our findings that only 1 of 7 patients showed evidence of in vivo activation. Whether this represents donor sampling differences or the fact that we use human serum rather than FBS in our activation phase is unclear at this time. However, since the material used for our in vitro work was identical to that administered to the patients, we feel that there is good correlation between our in vitro and in vivo findings. Correlation between in vitro and in vivo data was found in our previously reported study with IFN-γ (16) as well.

The activation of monocyte tumoricidal properties in vivo by agents such as liposome-encapsulated muramyl tripeptide-phosphatidyl-ethanolamine has been proposed for the treat-
EFFECT OF rGM-CSF ON MONOCYTES IN VITRO AND IN VIVO

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