Granulocyte/Macrophage Colony-Stimulating Factor Stimulates Monocyte and Tissue Macrophage Proliferation and Enhances Their Responsiveness to Macrophage Colony-Stimulating Factor

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a specific humoral growth factor that stimulates both neutrophilic granulocyte and macrophage production by bone marrow hematopoietic progenitor cells. GM-CSF also stimulates the proliferation and clonal growth of both tissue macrophages and blood monocytes. Although at low concentrations GM-CSF was unable to support the long-term growth of tissue macrophages, it greatly enhanced their responsiveness to macrophage CSF (M-CSF, or CSF-1). This effect was also observed by treating macrophages with GM-CSF for a short time. GM-CSF did not compete with M-CSF for binding to M-CSF receptors nor was it inactivated by treatment with anti-M-CSF antiserum. Treatment of tissue macrophages with GM-CSF led to a rapid but transient downregulation of M-CSF receptors; prolonged incubation at 37°C, however, resulted in a restoration and upregulation of M-CSF receptors. Identical effects were observed with both native or recombinant GM-CSF. This study suggests that GM-CSF regulates tissue macrophage production by two modes of action: (a) direct stimulation of macrophage proliferation, and (b) enhancement of their responsiveness to M-CSF.

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The production of mature granulocytes and macrophages is regulated by a group of hematopoietic growth factors referred to as colony-stimulating factor (CSF). CSFs are heterogeneous; at least four CSF subclasses have been described in terms of the type of mature cells produced in semisolid cultures of bone marrow cells. One of them, multi-CSF (also known as interleukin 3, IL 3), appears to act on very primitive progenitors responsible for most of the hematopoietic cell lineages, including macrophage, granulocyte, megakaryocyte, mast cell, and erythrocyte lineages. Others, such as M-CSF (also known as CSF-1) and G-CSF, appear to stimulate predominantly the proliferation of progenitors committed to either macrophage or granulocyte lineages, respectively, and GM-CSF stimulates the proliferation and differentiation of precursor cells for both lineages. It is now clear that there is a hierarchy of hematopoietic factors, which act during different stages of differentiation. Thus, as a hematopoietic cell matures, it may lose its predominant responsiveness to one factor but in turn become predominantly responsive to another. Despite their similarity in biologic activities and functional overlap on hematopoietic precursors, there is no sequence homology or direct competition for receptor binding among these molecules.

Recent studies showed that M-CSF appears to act primarily on precursors committed to the monocytic lineage. This conclusion is drawn from the following observations: (a) M-CSF stimulates predominantly macrophage production from bone marrow precursor cells; (b) bone marrow monocytic precursors possess only minimal M-CSF receptor activity that increases as they become more mature, and (c) a subpopulation of M-CSF receptor-bearing tissue macrophages derived from various sources, including blood monocytes, can be stimulated by M-CSF to undergo clonal growth in vitro. Although the role of M-CSF in the induction of tissue macrophage proliferation in vitro has been well established, the effect of GM-CSF in the process is less clear.

Previous studies have shown that, at higher concentrations, GM-CSF was also capable of stimulating megakaryocyte, eosinophil, and erythroid colony formation by bone marrow precursors. In this study, the effect of GM-CSF on the proliferation of blood monocytes and tissue macrophages was investigated. Our results presented in this study further extend the biologic effect of GM-CSF in vitro to include the support of long-term growth of tissue macrophages, including bone marrow-derived macrophages and, at lower concentrations, the enhancement of their responsiveness to the action of M-CSF.

Materials and Methods

Mice. Male or female C3H/HeJ mice 8 to 12 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME). Cells obtained from this strain of mice are refractory to the stimulation of endotoxin in vitro. All mice were fed standard lab chow and water ad libitum.

CSF and reagents. Both native and recombinant murine GM-CSF were obtained from Genzyme Biochemicals, Boston. Highly purified native GM-CSF (specific activity >6.6 x 10^4 U/mg protein) was prepared from serum-free supernatants of murine T-lymphoma LBRM-33-5A4 cells by ion-exchange chromatography and high-pressure liquid chromatography (HPLC). Recombinant GM-CSF (>15 x 10^5 U/mg) was prepared from yeast containing a plasmid expression vector that contains the GM-CSF gene cloned from LBRM-33-5A4 cells and was purified to homogeneity by HPLC.

Mouse L cell M-CSF was purified by a five-step procedure developed by Stanley and Heard with some modification. Iodination of purified M-CSF was carried out by the chloramine T method described in detail elsewhere. We used radioactively labeled M-CSF within 6 weeks of iodination since its biologic and binding activities decreased with time (half-life, t½, ~8 weeks).

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CSFs were determined from the linear portion of the dose–response curve, assigning 50 U/mL to the concentration, causing the formation of 50% of maximal colony numbers when 5 × 10^4 bone marrow cells were cultured in 1 mL soft agar.

**Cells.** Peritoneal exudate cells were harvested by peritoneal lavage with 5 mL α-minimal essential medium (α-MEM) (GIBCO, Grand Island, NY), three days after one intraperitoneal (IP) injection of 1.5 mL thiglycollate medium (Difco Laboratories, Detroit). The yields of exudate cells varied from 10 to 25 × 10^6 cells/lavage of which >75% were mononuclear phagocytes. Murine blood monocytes were isolated by the Ficoll-Paque technique described in detail previously. Bone marrow cells were obtained from femoral shafts by flushing with 3-ml α-MEM containing 10% fetal calf serum (FSC, Sterile Systems, Logan, UT) (α-10). The cell suspensions were passed up and down five times through an 18-g needle in α-10 to disperse cell clumps. 

125I-M-CSF binding assay. The details of the binding assay for M-CSF receptors is described elsewhere. Nonspecific binding, determined as the amount of radioactivity bound in the presence of a 200-fold excess of unlabelled M-CSF, was subtracted from all results to give specific binding. Nonspecific binding was always <3% of the total binding.

**Preparation of gelatin-coated flasks.** Gelatin-coated flasks were prepared by adding 3 mL sterile gelatin solution (Travenol Laboratories, Cambridge, MA) (0.1% in distilled water) to each Falcon flask (25 cm², Falcon Labware, Oxnard, CA). After 24 hours at 4°C, the excess gelatin solution was removed and the flasks were allowed to dry at room temperature inside a Laminar airflow hood for 48 hours with caps loosened.

**Mouse bone marrow-derived macrophages.** To obtain bone marrow-derived nonadherent (NA) cells, 10^7 bone marrow cells in 10 mL growth medium (α-MEM containing 15% FCS and 3,000 U/mL M-CSF) were cultured in T-25 gelatin-coated Falcon tissue culture flasks. After 24 hours at 37°C, or as indicated, nonadherent cells were harvested, centrifuged, and resuspended in 3 mL cold α-10 containing 20 mmol/L MOPS, pH 7.2, instead of sodium bicarbonate. The cell mixture was further centrifuged through 7 mL cold FCS at 400 g for 25 minutes to remove dead cells and debris, after which the cell pellet was resuspended in 1 mL cold α-10. To obtain the adherent cell population, culture flasks were washed extensively with cold phosphate-buffered saline (PBS) before removal of NA cells and replenished with 4 mL 10 mmol/L lidocaine solution in α-10 (Antra Pharmaceutical Products, Westborough, MA). After 5 minutes at 37°C, adherent cells were then dislodged from the flask by vigorous shaking. Cells were washed once in cold α-10 and stored at 4°C for later use.

**Assay for colony-forming cells.** The number of colony-forming cells was determined according to the method described previously with some modifications. Cells were incubated at 37°C in a fully humidified incubator with 7.5% CO2 in air for either 7 to 10 days (bone marrow and nonadherent cells) or 14 to 21 days (tissue macrophages and monocytes). Colonies were fixed, stained, and counted with a dissecting microscope (40x). Cell aggregates having ≥50 cells were considered colonies.

**Cell counting.** The number of cells were determined by the hemocytometer counting technique described previously. In brief, cultures were depleted of medium by aspiration, washed once with warm PBS, and then replenished in 1 mL warm cetridme solution (30 g cetridme, 0.37 g disodium EDTA, and 8.5 g NaCl in 1,000 mL water, pH 5.0) at 37°C for 3 minutes. This was followed by a thorough rinsing with warm cetridme solution (final volume 10 mL/culture) to completely remove and lyse the adherent cells. This treatment readily lysed the adherent cells and liberated intact nuclei, which were then counted by a Coulter Counter (Model ZM).

### RESULTS

GM-CSF stimulates the clonal growth of both bone marrow-derived adherent and nonadherent cells. When bone marrow-derived cells from cultures containing M-CSF for 1 and 4 days were further cultured with native GM-CSF, both AD and NA populations responded by undergoing clonal growth in semisolid medium (Table 1). More than 95% of the AD cells were identified morphologically as monocytes and macrophages, and NA cells were composed primarily of monocyctic precursors, monocytes, and granulocytes (granulocyte number decreased rapidly in prolonged cultures as a result of cell death). A longer incubation period (14 to 21 days) was needed for colony formation in agar cultures containing AD cells as compared with 7 to 10 days for the NA cells and normal bone marrow cells. The number of colony-forming cells from both populations responding to M-CSF appeared to be higher than that responding to GM-CSF. Because AD cells derived from bone marrow cultures represent a relatively mature macrophage population, we asked whether macrophages derived from peripheral tissue sources also respond to GM-CSF.

**GM-CSF stimulates proliferation and colony formation by tissue-derived macrophages.** Freshly obtained mouse peritoneal exudate macrophages (PEMs), monocytes, and day 3 bone marrow-derived adherent cells were cultured in the continuous presence of GM-CSF and periodically the number of cells were determined. As shown in Fig 1, GM-CSF stimulated the growth of both PEMs and monocytes. Compared to that of bone marrow-derived macrophages (BMDMs), the mode of growth by PEMs and monocytes was slower and had a much longer lag period than did BMDMs, in a manner very similar to that induced by M-CSF. Morphologically, cells derived from GM-CSF containing cultures were smaller and more rounded up with very little cytoplasm, whereas cells derived from M-CSF cultures were larger and more stretched (data not shown).

GM-CSF also caused colony formation by PEMs and monocytes in agar cultures with a dose–response curve similar to that of bone marrow GM-CFC but requiring

| Table 1. GM-CSF-Induced Clonal Growth of Bone Marrow-Derived AD and NA Cells in Semisolid Agar Medium |
|---|---|---|---|---|---|
| **Additions** | **U/mL** | **No. of Colonies per 10^5 NA Cells** | **No. of Colonies per 2 × 10^5 AD Cells** | **(Day 1)** | **(Day 4)** |
| None | — | 0 | 0 | 0 | 0 |
| GM-CSF | 300 | 76 ± 8 | 99 ± 5 | 23 ± 2 | 28 ± 9 |
| GM-CSF | 3,000 | 79 ± 4 | 104 ± 8 | 30 ± 6 | 29 ± 12 |
| M-CSF | 300 | 88 ± 6 | 176 ± 23 | 44 ± 6 | 36 ± 6 |
| M-CSF | 600 | 86 ± 3 | 203 ± 13 | 60 ± 6 | 50 ± 5 |

C3H/HeJ bone marrow cells were cultured with 1,500 U/mL M-CSF for either 24 or 96 hours at 37°C, after which both adherent (AD) cells and nonadherent (NA) cells were removed from culture, washed, and further cultured in semisolid agar medium containing native GM-CSF or highly purified stage IV M-CSF. After 7 days (NA cells) and 14 days (AD cells), respectively, colonies were fixed and scored. Cell aggregates having ≥50 cells were considered colonies. Data are means from duplicate cultures ± SD.
longer incubation periods (14 to 21 days) than bone marrow culture (7 days) (Fig 2). In liquid cultures, GM-CSF-induced macrophage colonies were very diffuse as compared with those induced by M-CSF, rendering the quantitation of colony formation unreliable.

**GM-CSF enhances the responsiveness of tissue macrophages to M-CSF.** When cultured concomitantly with M-CSF, GM-CSF enhanced the responsiveness of both PEMs and monocytes to M-CSF. This effect was most obvious when a very low concentration of GM-CSF (<30 U/mL) was used together with an optimal concentration of M-CSF (Fig 3). The enhancing effect was similar regardless of whether native or recombinant GM-CSF was used and was always greater in PEM cultures than in monocyte or BMDM cultures. At higher GM-CSF concentrations (>300 U/mL), however, the synergistic effect was abolished and in some cases growth inhibition was noted (data not shown). The continuous presence of GM-CSF was not required for the induction of the synergistic effect. As shown in Fig 4, exposure of PEM to various concentrations of GM-CSF for 2 hours was sufficient to trigger an increased proliferative capacity in response to M-CSF. In this case, the synergistic effect was observed even with higher GM-CSF concentrations (300 U/mL).

Synergism was also observed in the clonal growth of both monocytes and PEMs. In the continuous presence of an
whether GM-CSF may affect the expression of M-CSF. A synergistic effect was more apparent in PEM cultures than in monocyte cultures. At 37°C, GM-CSF induced a rapid but transient downmodulation of M-CSF receptors on PEMs, reaching a nadir within 2 hours (Fig 5). Prolonged incubation, however, resulted in a restoration and upregulation of M-CSF receptors on PEMs. The degree of M-CSF receptor downmodulation was dose and temperature dependent; treatment with GM-CSF at 4°C failed to induce receptor downmodulation on PEMs (Table 3). On the other hand, treatment with M-CSF inhibits the binding of 125I-M-CSF to PEMs at both 37°C and 4°C.

**DISCUSSION**

The experiments described in this report were undertaken to investigate the role of GM-CSF in the regulation of tissue macrophage production. Early studies have shown that several classes of tissue macrophages including inflammatory macrophages and blood monocytes can be induced by M-CSF to undergo extensive proliferation in vitro. The present study shows GM-CSF alone is also capable of doing so, thus suggesting that GM-CSF has a wider range of action than was previously believed. In addition to tissue macrophages, GM-CSF also supports the long-term growth of bone marrow-derived macrophage progenitors induced by M-CSF. Unlike bone marrow GM-CSFs, which are bipotential, both PEMs and monocytes are unipotential and give rise to macrophages exclusively under the influence of GM-CSF. In this regard, GM-CSF, which shares no structural similarity to that of M-CSF, acts like M-CSF in supporting the growth of tissue macrophages. Morphologically, however, cells derived from GM-CSF cultures were smaller and more rounded up, whereas those derived from M-CSF cultures were usually elongated and more stretched out. The significance of the morphological variation is not understood at present; however, M-CSF may exert more “differentiation-inducing activity” on the target cells than GM-CSF does.

In addition to supporting tissue macrophage proliferation, GM-CSF also enhanced the proliferative activity of tissue macrophages when cultured concomitantly with an optimal concentration of M-CSF. Such synergistic effect is most evident when low concentrations of GM-CSF (<30 U/mL) were used with a degree of enhancement that varied from twofold to more than threefold over control cultures containing M-CSF only. Results obtained from recombinant GM-

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**Table 2. Effect of Recombinant GM-CSF on the Clonal Growth of PEMs and Blood Monocytes in Response to M-CSF**

<table>
<thead>
<tr>
<th>GM-CSF (U/mL)</th>
<th>Colonies PEMs</th>
<th>Increase (%)</th>
<th>Colonies Monocytes</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>28 ± 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.8</td>
<td>34 ± 5</td>
<td>21</td>
<td>62 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>7.5</td>
<td>37 ± 5</td>
<td>32</td>
<td>76 ± 8</td>
<td>95</td>
</tr>
<tr>
<td>15</td>
<td>42 ± 2</td>
<td>50</td>
<td>74 ± 4</td>
<td>95</td>
</tr>
<tr>
<td>30</td>
<td>41 ± 4</td>
<td>46</td>
<td>74 ± 14</td>
<td>95</td>
</tr>
</tbody>
</table>

Blood mononuclear cells (3 x 10⁷/well) and PEM (10⁵/well) were cultured with 1,500 U/mL M-CSF (~10 ng) and various doses of purified recombinant GM-CSF as indicated for 12 days at 37°C. Data are means from duplicate cultures ± SD.

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**Table 3. Down Modulation of M-CSF Receptor on PEMs by Murine Recombinant GM-CSF at 4°C and 37°C**

<table>
<thead>
<tr>
<th>Treatment (U/mL)</th>
<th>37°C</th>
<th>4°C</th>
<th>Incubation Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF 600</td>
<td>5,225 ± 85</td>
<td>19.3</td>
<td>25,916 ± 511</td>
</tr>
<tr>
<td>150</td>
<td>9,686 ± 4200</td>
<td>35.9</td>
<td>ND</td>
</tr>
<tr>
<td>40</td>
<td>21,244 ± 782</td>
<td>78.7</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>26,980 ± 2135</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>M-CSF 1,000</td>
<td>1,568 ± 199</td>
<td>5.8</td>
<td>1,493 ± 449</td>
</tr>
</tbody>
</table>

C3H/HeJ PEM (8 x 10⁵/dish) were treated with various concentrations of murine recombinant GM-CSF or L-cell M-CSF for 1 hour at either 37°C or 4°C. After exposure, cells were chilled, washed, and labeled with 125I-M-CSF (85,000 cpm/dish) at 4°C. Data are means from duplicate determinations ± SD. ND, not determined.
GM-CSF STIMULATES TISSUE MACROPHAGE PROLIFERATION

CSF were essentially the same as that from native GM-CSF.

One possible mechanism by which GM-CSF enhances the proliferative responsiveness to M-CSF may be mediated through the modulation of M-CSF receptor activity. GM-CSF causes a rapid (2 hour) but transient downmodulation of M-CSF receptors on PEMs; prolonged incubation, however, resulted in a recovery and eventually upregulation of M-CSF receptors. Both effects are dose dependent and occur only at 37°C. Similar effects have been reported previously on mouse bone marrow cells. The biologic significance of growth-promoting activities, it has been proposed that GM-CSF and M-CSF may stimulate the production by cultured macrophages of multiple cytokines including interleukin 1,11,12, and T cells.13,14,15 The concept of this M-CSF receptor "trans-modulation" by GM-CSF in the process of a synergistic effect is a matter of speculation at present. Based on the observation that ligands that induce growth factor receptor trans-modulation generally synergize (rather than antagonize) with the growth factor in its growth-promoting activities, it has been proposed that GM-CSF can lead to a mitogenic signal by binding to its own receptor and, at the same time, provokes a "differentiation signal" to the target cells by receptor trans-modulations.15 Thus, the binding of GM-CSF on PEMs will not only stimulate cell proliferation but also activate M-CSF receptors and facilitate their responsiveness to M-CSF. The concept of the activation of M-CSF receptors is mediated through receptor downmodulation was also supported by the finding that short-term exposure (2 hour) of PEMs to GM-CSF was sufficient to cause a synergistic response to M-CSF. This observation also raised the concern regarding the underlying mechanism leading to the activation of M-CSF receptors and enhanced proliferative capacity in PEMs. Clearly, the relationship between receptor downmodulation and transduction of proliferative signal in the process needs further studies. Alternatively, GM-CSF may trigger more macrophage precursors, which are normally unresponsive to M-CSF into active cell cycles. We found that the number of colonies responding to M-CSF increased 50% and 100%, respectively in monocyte and PEM cultures when a low dose of GM-CSF was added concurrently with an optimal concentration of M-CSF, indicating that more cells were capable of colony formation than in the presence of M-CSF alone.

Interpretation of these data is complicated by several facts: (a) the expression of M-CSF receptors on PEMs is measured at one time point, whereas macrophage proliferation and colony formation are measured over a period of 14 days; (b) the relationship between receptor occupancy and the concentrations of CSFs required to produce a biologic effect cannot be directly correlated; and (c) GM-CSF and M-CSF may stimulate the production by cultured macrophages of multiple cytokines including interleukin 1,12,13 in the process. Indeed, we observed the synergistic effect only at low concentrations of GM-CSF (<30 U/mL). At high concentrations, GM-CSF failed to enhance cell proliferation in response to M-CSF. This seemingly difficult discrepancy could have several explanations. We performed studies in which PEMs were exposed to GM-CSF for a short period (2 hours); thereafter, cells were washed to remove GM-CSF and replenished with medium containing M-CSF only. In this case, we found that GM-CSF always exerts a synergistic effect to the response of M-CSF. At high concentration of GM-CSF and M-CSF, cell proliferation may be inhibited by a "feedback" mechanism. Furthermore, CSFs are also known to exert functional and differentiative activities on both macrophages and granulocytes in cultures.16,17,18 High concentrations of GM-CSF and M-CSF may cause an activation and/or differentiation in macrophages, thus leading to a diminished proliferative capacity.

The physiologic role of GM-CSF in the regulation of tissue macrophage production in vivo is not clear at present. GM-CSF is known to be produced primarily by stimulated endothelial cells, fibroblasts, and T lymphocytes in cultures.19 Other researchers have shown that organ GM-CSF levels were increased considerably in mice injected with endotoxin, an indirect but convincing piece of evidence supporting a regulatory role of GM-CSF in granulocyte and macrophage formation in vivo.20 Several lines of evidence show that exudate macrophages are derived from bone marrow by the circulating monocytes21 as well as by the local proliferation of newly arrived cells and/or resident macrophages in response to an inflammatory stimulation.22,23 Because mouse organs appear to be a major source of GM-CSF during an immune reaction, the elevated GM-CSF levels are probably responsible for, at least in part, the expansion of local macrophage populations by acting directly on nearby macrophage progenitors and/or newly recruited cells and, at the same time, enhancing their responsiveness to M-CSF in the process.

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BD Chen, CR Clark and TH Chou