Granulocyte-Macrophage Colony-stimulating Factor Induces Cytokine Secretion by Human Polymorphonuclear Leukocytes

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Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known as an inducer of proliferation and functional activation of myeloid cells. This study was carried out to characterize the effects of GM-CSF on polymorphonuclear leukocytes (PMN) more extensively. Using Northern blot analysis, we show that PMN are able to accumulate mRNAs for different cytokines, including tumor necrosis factor-α (TNF-α); G-CSF, and M-CSF, all of which are involved in inflammation and hematopoiesis. Biological assays and immunosassays demonstrate that PMN translate these mRNAs, except TNF-α, into secretory proteins. However, the expression of these cytokines is dependent on stimulation by exogenous signals, preferentially provided by the T cell-derived lymphokine GM-CSF. Stimulation of hematopoiesis and amplification of defense mechanisms after T cell activation thus might involve not only monocytes but also PMN, a cell type previously believed to be biosynthetically inactive.

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a T lymphocyte–derived glycoprotein of 22 kD, that was first identified by its capacity to induce hematopoietic progenitor cells to proliferate and differentiate to granulocytes and macrophages (1). Cloning of the GM-CSF gene, located on chromosome 5q2 (2) by Wong et al. (3) enabled large-scale production of this lymphokine and facilitated further investigation of its functional repertoire in vitro and in vivo settings.

Several recent studies have shown that GM-CSF is more than a hematopoietic growth-inducing molecule, because it also affects a multitude of functions of mature granulocytes, monocytes, and some mesenchymal cells. PMN respond to GM-CSF by decreased migratory activity (4), enhanced phagocytosis, antibody-dependent cellular cytotoxicity (5), and superoxide anion production in response to chemoattractant FMLP (6). In monocytes, GM-CSF also modulates tumoricidal activity (7) and induces mRNA accumulation and protein synthesis of M-CSF (8) and G-CSF (9).

PMN share a number of common properties with monocytes, including their phagocytic activity, similar membrane receptors, and a common progenitor cell. PMN have also been implicated in the generation and secretion of regulatory mediators like plasminogen activator (10), and an IL 1 inhibitor (11), as well as IL 1-α and IL 1-β, as shown recently by our group (12). Given the relatedness of PMN and monocytes, we asked whether PMN similar to monocytes would respond to GM-CSF by synthesis of a broader spectrum of cytokines like M-CSF, G-CSF, and TNF-α. The data reported herein, demonstrate inducible cytokine gene expression by PMN at the RNA and protein level. These findings may provide the basis for understanding some mechanisms of T cell/PMN collaboration.

Methods

Cell purification. PMN were isolated from heparinized peripheral blood from normal consenting healthy volunteer donors by Ficoll-Hypaque gradient centrifugation (Pharmacia Laboratories, Uppsala, Sweden; density 1.077 g/dl). PMN were recovered from cells present in the pellet by further sedimentation through dextran sulfate (0.4% vol/vol in HBSS; Gibco Laboratories, Grand Island, NY) as described (13). Residual red blood cells were eliminated by hypotonic lysis. PMN preparations were shown to be > 99% pure by morphology, cytochemistry, and reactivity with PMN-specific Ab ID3 (kindly provided by Dr. J. Griffin, Dana-Farber Cancer Institute, Boston, MA). Monocytes from consenting healthy donors' buffy coats were separated on Ficoll-Hypaque gradients and purified by repeated adherence steps after removal of T cells by rosetting with 2-aminoethyl-isothiouronium bromide hydrobromide-treated sheep RBC (5% vol/vol solution). Individual cell fractions assessed by morphology (Wright-Giemsa and α-naphthyl-acetate esterase [ANAEE] staining) and immunofluorescence analysis, using MAb to the T11 (T cells), Mo2 (monocytes), B1 (B cells) (14), and ID3 antigens (granulocytes) revealed preparations of cells with > 98% purity.

Culture conditions. PMN and monocytes purified as described above, were cultured at 0.5–5 × 10⁶ cells/ml in standard culture medium (RPMI 1640 medium supplemented with 2 mM glutamine, 100 ng/ml streptomycin, 100 U/ml penicillin, 1% sodium pyruvate, and 5% low endotoxin FCS) in 24-well flat-bottom plates (Corning Medi- cal, Corning Glass Works, Medfield, MA) in the presence or absence of various concentrations of yeast-expressed recombinant human GM-CSF (kindly provided by Dr. F. R. Seiler, Behringwerke, Marburg, FRG; specific activity is 4 × 10⁶ U/mg of protein; tests for endotoxin using the Limulus amebocyte lysate assay revealed < 10 pg/mg of protein). After various periods of time, cell-free supernatants of PMN

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1. Abbreviations used in this paper: ANAE, α-naphthyl acetate esterase; ANLL, acute nonlymphocytic leukemia; CAE, naphost AS-D-chloroacetate esterase; CFU-GM, granulocyte-macrophage colony-forming units; CSF, colony-stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte-macrophage-CSF; h, human; M-CSF, macrophage-CSF; PMN-CM, PMNL conditioned medium; r, recombinant; TNF-α, tumor necrosis factor-α.

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cultures (PMN-conditioned medium; PMN-CM) were collected and stored at –70°C until assayed. For RNA analysis, PMN and monocytes were incubated at 1–2.5 × 10^6/ml in standard culture medium under identical conditions.

**Murine CFU-GM colony assay.** Murine CFU-GM were assayed in a double-layer agar culture system as described previously (15). Briefly, 0.5-ml underlayers were composed of 0.5% agar (Agar Noble; Difco Laboratories, Inc., Detroit, MI) in Iscove’s modified Dulbecco’s minimum essential medium supplemented with l-glutamine, penicillin/streptomycin, 20% FCS, and 5 × 10^{-3} M 2-mercapto-ethanol. Culture supernatants to be assayed for CSF biological activity were added to the underlayers at 10% vol/vol final concentrations. Control cultures received medium alone, 250 ng/ml recombinant human (rh) G-CSF (kindly provided by Dr. L. Souza, Amgen Corp., Thousand Oaks, CA) or 1,000 U/ml rhM-CSF (specific activity is 2 × 10^8 U/mg of protein; kindly provided by Dr. P. Ralph, Cetus Corp., Emeryville, CA). 0.5-ml overlayers were composed of 0.3% agar in the same medium and contained 5 × 10^5/ml murine (BDF-1 mice) bone marrow cells. CFU-GM were enumerated at day 7 and lines of colonies were detected by cytochemical in situ staining of whole agar cultures (16). Naphthol-AS-D-chloroacetate esterase (CAE) staining detects granulocyte colonies; ANAE staining detects macrophage colonies.

**TNF cytotoxic assay.** Biological activity of TNF was assayed with the WEHI 164/actinomycin D (AcD) system essentially as described (17). In brief, WEHI 164 target cells sensitized with 1 µg/ml AcD (Calbiochem, Frankfurt, FRG) were labeled for 1 h with 100 µCi of ^{51}Cr (NaCrO_4, Amershams Buchler, Braunschweig, FRG) washed twice in fresh HBSS (2.5% FCS) and seeded in standard culture medium at 5 × 10^4/ml. The cytotoxic assay was performed in sterile U-bottomed microtiter plates (Costar Corp., Cambridge, MA) by incubation of 100 µl of target cells in standard culture medium supplemented with GM-CSF induced PMN-CM followed by centrifugation (at 5 g for 2 min) and incubation for 6 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Half of the SN was then harvested and assessed by 50 µl of the labeled WEHI 164 cells. Sensitivity of the assay was 1 ng/ml.

**TNF-RIA.** A TNF-specific RIA was kindly provided by Dr. G. Lambelin, IRE-Medgenix, Fleurus, Belgium. Sensitivity was shown to be 0.1 ng/ml TNF at 10% tracer binding inhibition. In selected experiments, cell lysates of GM-CSF-induced PMN were generated by repeated freezing/thawing cycles and were assayed for TNF activity as described above.

**Northern blot analysis.** Total cellular RNA was isolated using the guanidinium/cesium chloride method (18). 10 µg of glyoxylated RNA, obtained from 1–1.5 × 10^6 monocytes and 5–10 × 10^5 granulocytes, were fractionated on a 1.2% agarose gel and blotted onto nylon membranes (Schleicher & Schuell, Dassel, FRG). IL 1 mRNA was detected by endlabeled oligonucleotide probes (19) (N19–48 for IL-1α, and N75–786 for IL-1β [20]) as described (21). Specific cDNA probes were obtained for M-CSF by Dr. R. P. Ralph, Cetus Corp., Emeryville, CA, for G-CSF by Dr. L. Souza, Amgen Corp., and for TNF-α by Dr. G. Adolph, Bender, Vienna, Austria, and for v-fms by Dr. J. S. Sherr, Department of Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, TN. Radiolabeling and hybridization including 5% dextran sulfate (500,000 mol wt; Sigma Chemical Co., St. Louis, MO) were performed as described (22). All filters were reprobed with an α-actin cDNA (kindly provided by Dr. R. J. Schwarz, Department of Cell Biology, Baylor College of Medicine, Houston, TX) that also hybridizes with nonmuscle β actin, expressing 70% sequence homology to α actin (23). Blots were developed on Kodak X-OMat films with intensifying screens.

**Results**

**GM-CSF induces M-CSF and G-CSF secretion by PMN.** G-CSF and M-CSF biological activity present in conditioned medium of GM-CSF-induced PMNL was detected using a murine CFU-GM assay. Murine CFU-GM are known to respond to hG-CSF and hM-CSF, although unresponsive to hGM-CSF, thus providing a suitable means to specifically detect G- and M-CSF activity. Supernatants from PMN cultured in the absence of GM-CSF for 48–72 h did not induce any detectable growth of day 7 murine CFU-GM derived colonies. However, conditioned medium of GM-CSF activated PMNL induced considerable growth of both granulocyte and macrophage lineage colonies (Table I). Similarly, total cellular RNA of GM-CSF-induced PMN was found to strongly hybridize to M-CSF and G-CSF specific cDNA probes in a similar way as activated monocytes (Fig. 1 and 2). Maximum expression of G-CSF and M-CSF mRNA was found after 12–24 h of culture. Unstimulated PMN did not reveal any detectable signal. As seen in Fig. 1 hybridization of mRNA of LPS- and IFN-γ-stimulated monocytes and GM-CSF-stimulated PMN revealed multiple transcripts when probed with a M-CSF-specific cDNA probe. This is well in line with recent observations of RNA obtained from the PMA stimulated pancreatic carcinoma line MIA PaCa exhibiting six different M-CSF messages, due to alternate splicing of exon six and alternatively used 3′-untranslated sequences (24). Similarly lectin-stimulated PBMC were shown to express several M-CSF transcripts (25). Different conditions of stimulation may, however, favor the expression of the most abundant 4.2-kb message, as likewise the assay procedure (stringency of hybridization, time of exposure) influences detection of less abundant messages. To exclude any contamination of monocytes in the PMN preparations, the filters were reprobed with a v-fms-specific cDNA, revealing a specific signal in the monocyte RNA-containing lanes only.

**Table I. Effect of rhGM-CSF on Secretion of G- and M-CSF by PMN**

<table>
<thead>
<tr>
<th>Source of CSF</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>5±3</td>
<td>11±2</td>
<td>4±1</td>
<td>4±2</td>
<td>7±2</td>
<td>3±1</td>
</tr>
<tr>
<td>PMN-CM</td>
<td>5±3</td>
<td>9±1</td>
<td>3±1</td>
<td>4±2</td>
<td>8±2</td>
<td>4±1</td>
</tr>
<tr>
<td>GM-CSF PMN-CM</td>
<td>57±6</td>
<td>59±9</td>
<td>47±3</td>
<td>48±3</td>
<td>72±3</td>
<td>49±3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>4±2</td>
<td>9±1</td>
<td>ND</td>
<td>4±2</td>
<td>7±3</td>
<td>ND</td>
</tr>
<tr>
<td>G-CSF</td>
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<td>14±2</td>
<td>ND</td>
<td>79±3</td>
<td>78±4</td>
<td>ND</td>
</tr>
<tr>
<td>M-CSF</td>
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<td>89±2</td>
<td>ND</td>
<td>3±2</td>
<td>6±2</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Purified PMN were cultured at 2.5 × 10^6/ml for 48–72 h in the presence or absence of 250 ng/ml GM-CSF. Cell-free culture supernatants were used as potential stimuli for murine CFU-GM at final concentrations of 20% vol/vol: PMN-CM, in the presence of GM-CSF (GM-CSF PMN-CM); rh CSFs at concentrations of 250 ng/ml GM-CSF, 250 ng/ml G-CSF, and 1,000 U/ml M-CSF served as controls.

§ Colony numbers are means of triplicate CFU-GM assays (±SD).

Not done.

Cytokine Secretion by Polymorphonuclear Leukocytes
GM-CSF (250 ng/ml) for 0, 6, 12, 24, and 48 h. Normal human PMN were purified and cultured as described in Methods. Total cellular RNA was analyzed by Northern blotting using M-CSF-, α-actin-, and v-fms-specific cDNA probes. The transcript sizes were 4.2–2.8 (M-CSF), 2.0 (actin), and 4.2 kb (c-fms). RNA of unstimulated PMN extracted after various culture intervals up to 32 h did not hybridize with a M-CSF-specific probe. Control monocytes were cultured for 12 h in the presence (i, induced) or absence (ni, not induced) of 10 ng/ml LPS + 25 ng/ml IFN-γ.

Discussion

PMN are relatively short-lived end-stage cells, considered to have little, if any, biosynthetic capacity. Several recent studies, however, clearly demonstrate that PMN can be induced in vitro to synthesize mRNA and subsequently produce various proteins such as c-fos (26), plasminogen activator (10), IL 1-α and IL 1-β (12). PMN are also shown to share a number of common features with monocytes, including their common cell lineage and their migratory and phagocytic potential. PMN and monocytes have similar receptors and surface antigens and may play a similar role in the nonspecific host defense. In this regard the T cell-derived lymphokine GM-CSF has been identified as a major activation factor for both cell types. As shown in this study, GM-CSF not only recruits factor-mediated regulatory functions of monocytes, but also those of PMN by inducing mRNA accumulation and protein secretion of various cytokines.

In our experiments, the purity of analyzed PMNL samples was > 99% with a contamination of < 0.1% monocytes, as demonstrated by microscopy of ANAE- and CAE-stained aliquots. To further exclude the possibility that cytokine release attributed to PMNL is being executed by contaminating monocytes, PMNL derived mRNA was reprobed with a v-fms-specific cDNA also detecting c-fms (27), without demonstrating a specific hybridization signal, whereas monocyte RNA hybridized to the same probe. It has been previously shown that PBMC expression of c-fms, coding for the M-CSF receptor (28), is restricted to monocyte lineage cells (29). As shown by cell quantities needed to obtain a comparable amount of RNA, the specific capacity per cell is ~ 5–10 times less in PMN than in monocytes. This, however, does not argue against the possible physiological role of cytokine production by PMN in vivo, because of their quantitative predominance in the peripheral blood and particularly at most sites of inflammation.

Major sources of M-CSF and G-CSF so far identified are macrophages, endothelial cells, and fibroblasts. GM-CSF-in-
We have previously shown that GM-CSF stimulates PMNL to secrete TNF, which can induce IL-1 and TNF-α, supplements their functional role in inflammatory reactions, and may additionally be relevant for tumoricidal effects (TNF-α) as well as immune responses via interaction with T cells (IL-1) (36, 37).

Recent reports of hematopoietic growth factor secretion by granulocyte lineage acute nonlymphocytic leukemia (38, 39) relate to our results and suggest that myeloid lineage cells bear the capacity to synthesize various cytokines. A state of dysregulation of the progenitor cell and external stimuli of the mature PMN may trigger this potential. According to preliminary results, aside from GM-CSF, TNF-α and LPS were found to induce IL-1 and TNF-α mRNA in PMNL, whereas G-CSF was found to be inactive in this regard. However, none of the stimulatory molecules was as active as GM-CSF in inducing a broad spectrum of cytokines in PMNL, suggesting a central role of this T cell–derived lymphokine in the recruitment of nonspecific effector cells. More importantly, PMN were shown to mediate regulatory functions by demonstrating biosynthetic activity, thus being responsible for some in vivo functions previously ascribed only to monocytes.

**Acknowledgments**

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**References**


