Interleukin-6 and Granulocyte-Macrophage Colony-Stimulating Factor Are Candidate Growth Factors for Chronic Myelomonocytic Leukemia Cells

By M.P. Everson, C.B. Brown, and M.B. Lilly

Previous studies suggest that malignant cells from some patients with myeloid leukemias produce colony-stimulating factors (CSFs) that can function as autocrine growth factors in vitro. We have examined the roles of interleukin-6 (IL-6) and granulocyte-macrophage CSF (GM-CSF) in the proliferation of myeloid leukemia cells. IL-6 activity was assessed in conditioned medium (CM) from myeloid leukemia cell cultures or cell lysates using IL-6-dependent KDB3 and 7TD1 murine cell lines. Media conditioned by cells from patients with chronic myelomonocytic leukemia (CMMoL), but not by normal monocytes, chronic myelogenous leukemia (CML), or acute myelogenous leukemia (AML) cells, contained substantial levels (50 to 1,000 U/10^6 cells) of IL-6. The IL-6 content of CM correlated directly with donor peripheral blood WBC count. CM from two of five CMMoL samples also contained >350 pg/mL GM-CSF. Moreover, CMMoL cells spontaneously formed colonies in semisolid medium. CMMoL colony formation could be partially inhibited by antibodies to IL-6 or GM-CSF, whereas combination of these antibodies gave additive, and nearly complete (>93%), inhibition of spontaneous colony formation. Cell lysates from uncultured CMMoL cells from one patient contained abundant GM-CSF protein but no detectable IL-6. These data suggest that IL-6 and GM-CSF act in vitro as autocrine growth factors for CMMoL cells, and that CMMoL cells in vivo may represent a GM-CSF–dependent autocrine growth system.

Proliferation of normal myeloid cells is regulated by a variety of cytokines, including the colony-stimulating factors (CSFs) tumor necrosis factor (TNF), transforming growth factor-β, interleukin-4 (IL-4), and IL-6. Moreover, considerable effort has been focused on defining possible roles for these mediators in the proliferation of leukemic myeloid cells.

The pleiotropic cytokine IL-6, previously termed interferon-β, B-cell stimulatory factor 2, hybridoma/plasmacytoma growth factor, or 26-Kd protein, is a soluble mediator produced by activated monocytes or T cells and fibroblasts. IL-6 acts on T cells to induce growth, IL-2 secretion, and generation of cytotoxic T lymphocytes; on B cells to induce terminal differentiation to immunoglobulin secretion; on B-cell hybridomas and plasmacytomas to induce growth; and on hematopoietic cells as a co-factor for colony proliferation. Granulocyte-macrophage CSF (GM-CSF) is a soluble mediator produced by T cells, macrophages, and fibroblasts that functions, at least in part, to induce growth and maturation of hematopoietic cells to granulocytes and macrophages. Although these two factors possess growth potential for certain myeloid leukemia cells, the roles of IL-6 and GM-CSF in chronic myelomonocytic leukemia (CMMoL) have not been addressed. Therefore, we have investigated the roles of IL-6 and GM-CSF in CMMoL for the following reasons: (1) IL-6 and GM-CSF have been reported to function as growth or growth-enhancing factors for certain leukemic cells (eg, AML blast cells); (2) IL-6 is a product of activated monocytes; and (3) malignant cells from some patients with myeloid leukemias produce CSFs that function as autocrine growth factors in vitro. The data presented herein indicate that IL-6 and GM-CSF are produced by CMMoL cells in vitro, and that IL-6 and GM-CSF function as in vitro growth factors for CMMoL cells. Furthermore, GM-CSF is produced by CMMoL cells in vivo. Therefore, these data suggest regulatory roles for IL-6 and GM-CSF in the pathogenesis of CMMoL.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PBMC) were isolated from patients with the myeloid leukemias, AML, CML, and CMMoL. Blood samples were collected after obtaining informed patient consent as required by the local Institutional Review Board for human use. All three AML patients were M1-2 by the French-American-British classification. All eight CML patients were Philadelphia chromosome positive. All seven CMMoL patients had leukocytosis with >20% monocytes without intermediate myeloid cells, and of three examined patients, none had a Philadelphia chromosome. Some CML (two of eight) and CMMoL (six of seven) patients were receiving hydroxyurea, but all patients had stable or increasing WBC counts. Mononuclear leukemia cells were isolated from PBMC by density gradient centrifugation followed by depletion of T cells by rosetting with AET-treated sheep RBCs. Adherent, normal peripheral blood monocytes were similarly isolated, then further depleted of T cells by repeated washings to remove non-adherent cells. The final cell populations contained <3% T cells as assessed by immunofluorescence against Leu-4 cell surface antigens and >90% monocyte/monoblast-like cells as assessed by morphology.

Culture conditions. For production of conditioned medium (CM), PBMC were cultured in RPMI 1640 containing 10% iron-supplemented calf serum (Hyclone Laboratories, Inc, Logan, UT) at 10^6 cells/mL for 24 hours. Resultant CM were harvested, filtered, and stored at 4°C until assayed. For assessment of in vitro colony formation, PBMC were cultured in semisolid medium by plating 2 × 10^3 cells/mL in Iscove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY) containing 20% calf serum and 0.3% agar. Cultures were scored for colony formation eight to ten days after plating.
days after culture initiation with an inverted-stage microscope. Using standard methods, cultures were then fixed with glutaraldehyde, dried onto glass slides, stained in situ for \textit{a}-naphthyl-acetate-esterase, and counterstained with hematoxylin for differential assessment of cell types.

**Cytokine assays.** IL-6 activity was assessed using the IL-6–dependent murine myeloma KD83 cell line\textsuperscript{11} or the IL-6–dependent murine hybridoma 7TD1 cell line\textsuperscript{12} (kindly provided by R. Nordan, NIH, Bethesda, MD). These cell lines proliferate in response to recombinant (r) murine (m) and human (h) IL-6 (kindly provided by F. Lee, DNAX Research Institute, Palo Alto, CA).\textsuperscript{13} KD83 cells are not stimulated by IL-1, IL-2, IL-3, IL-4, IFN-\textit{a}, IFN-\textit{g}, G-CSF, or GM-CSF. Since KD83 cells also respond to rmIL-5 (but not rhIL-5), the IL-6 specificity of CMMoL CM was confirmed in KD83 assays using anti–hIL-6 antibodies (see Results). KD83 proliferative assays were performed as previously described\textsuperscript{14} by culturing $5 \times 10^4$ KD83 cells in 7% CO$_2$-containing humidified atmosphere at 37°C in 96-well, flat-bottomed microculture plates (200 \textmu l total volume) with various dilutions of samples or rIL-6 controls. Cultures were pulsed with 1 \textmu Ci $[^{3}H]$Tdr/well (2 Ci/mmol sp. act., Amersham, Arlington Heights, IL) two hours before termination of a 48 to 50 hour culture period. Cells were harvested and assessed for $[^{3}H]$Tdr uptake as an indicator of cellular proliferation as previously described.\textsuperscript{15} For calculation of IL-6 activity contained in CM, CM-induced KD83 cellular proliferation was compared with rhIL-6 dose-response curves, where 10 U/mL IL-6 activity was ascribed to the concentration of rhIL-6 that induced 50% maximal proliferation of KD83 cells. IL-6 assays using 7TD1 cells were performed as previously described\textsuperscript{12} by culturing $2 \times 10^4$ 7TD1 cells as indicated above for KD83 cells. The number of cells was evaluated after four days in culture using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in MTT colorimetric assays as previously described.\textsuperscript{16}

CM was assayed for GM-CSF content using the anti–GM-CSF monoclonal antibodies 126.2.1.3 and 126.2.2.1 (developed by Dr Charles Hart, Zymogenetics, Inc, Seattle) in a "sandwich" radioimmunoassay as previously described.\textsuperscript{17}

**Antibodies.** An anti–hIL-6 antiserum was purchased from Boehringer-Mannheim (Indianapolis), and its neutralizing titer was determined by adding anti–IL-6 to IL-6–dependent KD83 proliferative assays. Although KD83 cells proliferate in the presence of rhIL-6 or rmIL-6, anti–IL-6 neutralized $>20,000$ U rhIL-6/mL, but did not neutralize rmIL-6–induced KD83 proliferation. A neutralizing monoclonal antibody to rhG-CSF (clone 5.24) was prepared in our laboratory. This preparation was derived from ammonium sulfate-precipitated hybridoma culture supernatant and had a neutralizing titer of $>400$ ng/mL in human bone marrow cultures stimulated with rG-CSF (AMGEN, Thousand Oaks, CA). A neutralizing monoclonal antibody to rhGM-CSF (neutralizing titer of $>5,000$ U/mg) was also purchased (Genzyme, Boston) for use in colony formation inhibition studies. At concentrations used in these experiments, none of these antibodies was toxic to human CFU-GM stimulated with rGM-CSF (anti–IL-6 or anti–G-CSF) or rG-CSF (anti–GM-CSF) (data not shown). For inhibition of colony formation, antibodies were incorporated into the semisolid medium at the time of plating.

**RESULTS**

IL-6 activity was assessed in CM from AML, CML, and CMMoL T-cell–depleted mononuclear cell (TDMC) cultures using the KD83 cell line (Fig 1). Substantial levels of IL-6 (>2 x medium control values) were observed in 0/3 AML, 0/8 CML, and 7/7 CMMoL samples. To confirm that the observed proliferative signals were in fact mediated by IL-6, we tested CMMoL CM for KD83 proliferation-inducing activity in the presence of neutralizing anti–IL-6 antibodies. Anti–IL-6 (ie, anti–hIL-6) effectively neutralized the proliferative effects of rhIL-6 and CMMoL CM, but did not ablate rmIL-6–induced KD83 cellular proliferation (Table 1). The rmIL-6 control used in this assay excluded the presence of nonspecific cytotoxic effects of the anti–IL-6 preparation for KD83 cells.

Substantial variance was observed among patient samples in levels of IL-6 produced by CMMoL TDMC (Fig 1). This observation raised the possibility that IL-6 production might be related to peripheral blood WBC count. Indeed, when IL-6 levels in CM were plotted against WBC counts from donor CMMoL patients, a significant correlation was observed (Fig 2). CMMoL cells have been reported to proliferate spontaneously in semisolid cultures.\textsuperscript{18} Therefore, we examined CM for an additional myeloid growth factor, GM-CSF. Using a radioimmunoassay, elevated levels of GM-CSF were detected in two of five tested CM samples (Table 2). CM from normal, unstimulated monocytes lacked detectable levels of GM-CSF.

We examined the possible roles of IL-6 and GM-CSF in the spontaneous in vitro proliferation of CMMoL cells using a semisolid culture system (Table 3). Mononuclear cells from three CMMoL patients were cultured with or without antibodies to putative myeloid growth factors. All samples lacking antibodies spontaneously formed colonies.
Table 1. Effects of Anti-IL-6 on IL-6- and CMMoL CM-Induced K83 Proliferation

<table>
<thead>
<tr>
<th>Sample</th>
<th>U/mL or Dilution</th>
<th>None</th>
<th>1:5,000 Dilution</th>
<th>1:2,500 Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rhIL-6</td>
<td>10</td>
<td>13,989 ± 1,1396</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5,491 ± 3,749</td>
<td>1,438 ± 25</td>
<td>1,783 ± 98</td>
</tr>
<tr>
<td>rmIL-6</td>
<td>3</td>
<td>26,624 ± 2,259</td>
<td>32,989 ± 1,465</td>
<td>30,444 ± 4,329</td>
</tr>
<tr>
<td>CMMoL CM (Patient 3)</td>
<td>1:2</td>
<td>21,696 ± 380</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>9,071 ± 83</td>
<td>1,990 ± 247</td>
<td>1,600 ± 167</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>2,382 ± 254</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CMMoL CM (Patient 4)</td>
<td>1:2</td>
<td>27,593 ± 744</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>32,115 ± 2,867</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>17,686 ± 1,997</td>
<td>7,153 ± 566</td>
<td>2,399 ± 63</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*[^3H]Tdr uptake measured using IL-6-dependent K83 cells.
†Anti-IL-6 and samples were pre-incubated for one hour at 37°C before initiation of cell culture.

Colonies were predominantly monocytic (65%), with fewer monocytic-granulocytic (35%) and granulocytic (5%) colonies (pooled data from three patients). Incorporation of anti-G-CSF inhibited colony formation ≤35% as assessed by colony count comparisons. In contrast, anti-IL-6 alone reduced colony formation 26% to 71% and anti-GM-CSF inhibited colony formation 82% to 94%. Combination of anti-G-CSF plus anti-IL-6 did not augment inhibition relative to that observed with anti-IL-6 alone. In contrast, there was additive inhibition mediated by the combination of anti-GM-CSF plus anti-IL-6. The morphologic appearance and differentials of surviving colonies in antibody-treated cultures did not differ from that of untreated cultures (data not shown). These data indicate that, unlike anti-G-CSF, anti-GM-CSF and anti-IL-6 (alone or in combination) can significantly inhibit spontaneous in vitro colony formation by CMMoL cells.

Because myeloid leukemia cells can be induced to secrete cytokines by in vitro culture, we examined fresh, uncultured CMMoL cells from one patient for secretion of IL-6 and GM-CSF protein. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation, then immediately prepared for assay. No IL-6 protein was identified by immunoblotting detergent lysates of cells using either polyclonal (Genzyme) or monoclonal (kindly provided by Immunoex Corp, Seattle) antibodies to hIL-6. Similarly, lysates of 4.4 x 10^8 CMMoL cells/0.5 mL phosphate-buffered saline containing 1 mmol/L phenylmethylsulfonyl fluoride contained no detectable IL-6 biologic activity or inhibitors when used at a 1:400 dilution in IL-6 assays employing 7TD1 cells, ie, with an assay detection limit of 0.006 U/mL rhIL-6, the undiluted sample contained <2.5 U/mL IL-6 activity. In contrast, lysates of fresh CMMoL cells (4.4 x 10^6 cells in 10 mL buffer containing 10 mmol/L Tris, 0.15 mol/L NaCl, 1% Triton X-100, 0.1% SDS, and 5 mmol/L ethylenediaminetetraacetic acid, 1.0% Na deoxycholate) contained abundant (>40 ± 10 ng/mL; mean ± SEM for two experiments, each performed in duplicate) GM-CSF protein as measured by radioimmunoassay. These levels are similar to those detected in monkey kidney (COS) cells transfected with a variety of GM-CSF-encoding plasmids, or in normal monocytes stimulated with lipopolysaccharide. In contrast, similarly treated human myeloid leukemia cells (HL60 and KG1) and untransfected COS cells contained no detectable GM-CSF protein.

Table 2. GM-CSF Levels in CM From CMMoL Cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>GM-CSF (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,120 ± 590</td>
</tr>
<tr>
<td>2</td>
<td>&lt;350</td>
</tr>
<tr>
<td>3</td>
<td>&lt;350</td>
</tr>
<tr>
<td>4</td>
<td>&lt;350</td>
</tr>
<tr>
<td>5</td>
<td>840 ± 198</td>
</tr>
</tbody>
</table>

Normal monocytes <350

GM-CSF values represent means ± SD for two-to-three determinations performed using duplicate samples.
CMMoL is a myeloproliferative disorder with distinct clinical, genetic, and biologic characteristics. CMMoL differs from CML in several ways, including cytologic criteria, lack of Philadelphia chromosome, and spontaneous colony formation by CMMoL cells in semisolid medium. CML cells rarely form colonies without an exogenous source of CSFs.

Inasmuch as activated monocytes are able to secrete a wide variety of cytokines and growth factors, the possibility was raised that CMMoL cells secrete their own growth factors that might subsequently lead to their spontaneous in vitro proliferation through an autocrine growth factor-mediated mechanism. Our in vitro data demonstrate that cultured CMMoL cells spontaneously secrete two myeloid growth factors, IL-6 and GM-CSF. In contrast, normal monocytes cultured under identical conditions did not secrete detectable levels of these cytokines. Thus, the CMMoL cells most closely resemble activated monocytes. Moreover, the antibody neutralization studies suggest that both IL-6 and GM-CSF contribute to the spontaneous in vitro proliferation of CMMoL cells. This is consistent with previous reports describing a synergistic interaction between these two growth factors for normal and other leukemic myeloid cells.

In our spontaneous colony formation inhibition studies, GM-CSF appears to be the primary stimulant, even though three of five tested CM samples contained less-than-detectable levels of GM-CSF. This apparent discrepancy may be due to the relative insensitivity of the GM-CSF immunoassay (lower limit of detection = 350 pg/mL) and/or the possible uptake of GM-CSF in vitro by CMMoL cells. Thus, biologically active amounts of GM-CSF could certainly be undetected. CMMoL cells could also exhibit an exaggerated sensitivity to GM-CSF, possibly through abnormalities of second messenger systems such as protein kinase C. In this regard, it is interesting to note that CMMoL has been associated with deletions or inversions of chromosome 16, which contains the gene for protein kinase C-b.

While IL-6 and GM-CSF appear to be involved in the spontaneous in vitro proliferation of CMMoL cells, we cannot exclude the possible participation of other cytokines in the in vivo proliferation of CMMoL cells. Activated macrophages can produce a host of positive and negative cytokines for myeloid cell proliferation, and it is logical to expect that some of these factors, such as IL-1 (recently observed to be produced by AML cells) or TNF, might be secreted by CMMoL cells as well.

Of prominent concern is the relationship, if any, of the spontaneous in vitro proliferation of CMMoL cells to the in vivo biology of the disease. Several reports have described the expression of myeloid cytokines by AML cells and have suggested an autocrine role for some of these factors in AML cellular proliferation. However, there have been data presented recently that suggest that GM-CSF secretion by AML cells is an artifact of in vitro culture. In this regard, although we have observed in vitro production of IL-6 by cultured CMMoL cells, we have not been able to demonstrate IL-6 protein in uncultured CMMoL cells by either immunoblotting or bioassay, and thus cannot exclude that IL-6 secretion may be an artifact of in vitro culture. A more sensitive assay for IL-6 message or protein might clarify this issue. The in vitro proliferative data do suggest, however, that IL-6 from other sources, such as stromal cells, could be contributing to the proliferation of CMMoL cells in vivo. The direct relationship between in vitro IL-6 secretion and patient WBC count support this provocative possibility. Moreover, these observations raise the possibility of other cytokines (such as IL-1 and TNF) acting, perhaps in a cascade or in concert with other cytokines, to promote proliferation of CMMoL cells. In contrast to these IL-6-related findings, our data clearly identify abundant GM-CSF protein in uncultured CMMoL cells and strongly suggest that the observed in vitro autocrine system involving GM-CSF and CMMoL cells may also be functioning in vivo. Taken together, our data support further study of the roles of myeloid cytokines in CMMoL pathogenesis.

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