Vascular Endothelial Cells and Granulopoiesis: Interleukin-1 Stimulates Release of G-CSF and GM-CSF

By Krisztina M. Zsebo, Victoria N. Yuschenkoff, Susan Schiffer, David Chang, Elaine McCall, Charles A. Dinarello, Melissa A. Brown, Bruce Altrock, and Grover C. Bagby, Jr

Cultured mononuclear phagocytes produce soluble factors that stimulate endothelial cells to release GM-colony-stimulating activity (GM-CSA). One such factor was recently identified as interleukin 1 (IL 1). Studies were designed to determine which types of granulopoietic factors are released by IL 1-stimulated endothelial cells. Supernatants from endothelial cells cultured for 3 days in medium containing IL 1 α and β were tested in both murine and human CFU-GM colony growth assays. The effect of conditioned media on differentiation of WEHI-3B myelomonocytic leukemic cells was also examined. Control media containing IL 1 alone or unstimulated endothelial cell-conditioned media contained no detectable CSA in any bioassay. Medium conditioned by IL 1-stimulated endothelial cells stimulated the clonal growth of both human and murine CFU-GM and induced macrophage differentiation of WEHI-3B cells. Treatment of these conditioned media with a highly specific neutralizing monoclonal G-CSF antibody completely inhibited their activity in the murine CFU-GM assay, but only partially inhibited CM colony growth by human marrow. Treatment of the active conditioned media with a neutralizing rabbit anti-human GM-CSF antibody partially reduced the activity of the media in the human GM-colony growth assay. G-CSF radioimmunoassay of endothelial cell culture supernatants and Northern blot analysis of endothelial cell cytoplasmic RNA for GM-CSF gene transcripts confirmed that IL 1 induced expression of both G-CSF and GM-CSF genes. Because treatment of media with both antibodies abrogated all activity in the human GM colony growth assay, we conclude that IL 1-stimulated endothelial cells release both G and GM-CSF and that these are the only granulopoietic factors detectable in clonogenic assays released by these cells in vitro.

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MATERIALS AND METHODS

Experimental Design

Bioassays were carried out in two steps: (a) Recombinant human IL 1 α or β was added to confluent monolayers of multiply passaged human endothelial cells in vitro and (b) the bioactivity of the endothelial cells-conditioned media was compared with media conditioned in the absence of IL 1 or containing IL 1 incubated for 3 days in the absence of endothelial cells.

Endothelial Cell Cultures

Human umbilical vein endothelial cells were prepared as previously described, using limited collagenase treatment (type I, 0.1% wt/vol in phosphate-buffered saline (PBS); Worthington Diagnostic Systems, Freehold, NJ). The cells were suspended in RPMI 1640 medium supplemented with 25 mmol/L Hepes buffer, 2 mmol/L l-glutamine, 20% fetal calf serum (FCS) antibiotics, and 250 µg/ml endothelial cell growth factor extracted from bovine hypothalami (Pel-Freeze, Rogers, AR.). The endothelial cells were cultured in 16-mm tissue culture wells coated with fibronectin (2 µg/cm² surface area; Collaborative Research, Bethesda, MD) for 3 days at 37°C in 5% CO₂ in air. The subcultured cells exhibited the characteristic “cobblestone” appearance of endothelial cells, and 90% to 98% of these cells contained factor VIII-related antigen as determined by indirect immunofluorescence using rabbit anti-factor VIII and fluorescein-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). The cells did not react with monoclonal antibodies OKM1, MAC 120, or OKT3. In the experiments described below, endothelial cells from the fourth through the fifteenth passage were used. Cell density ranged from 1 to 5 x 10⁴ cells/cm². The endothelial cell culture medium was replaced with medium containing IL 1 or control media, and the cultures were returned to the incubator. Other control wells contained the same amount of IL 1 but no cells. At the end of the 3-day incubation period, the media were aspirated and centrifuged (200 G for 5 minutes), and the supernatants were used in the CSF bioassays described below.

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Bioassays

Human and murine CFU-GM. CSA was measured using bone marrow cells from healthy human donors after obtaining informed consent and from femoral marrow of female BALB-c mice. Theuffy coat fraction of the bone marrow suspension was diluted 1:1 with PBS and separated by density-gradient fractionation on Ficoll-Hypaque. Separated cells were plated in Falcon dishes for 1/2 hours at 37°C. The nonadherent fraction was plated in 0.3% agar with supplemented McCoy's 5a medium and 10% heat-inactivated FCS, as described. Twenty microfilters of endothelial cell supernatants were used in the bioassays. Bioassays were done in triplicate, and results are presented ± SD.

WEHI-3B assay. Differentiation induction of the murine myelomonocytic leukemic cell line WEHI-3B $^1$ was assayed in semisolid agar medium as described.

Radioimmunoassay (RIA). G-CSF in endothelial supernatants was quantitated in a monospecific sandwich immunoassay using human recombinant G-CSF as a standard (D. Chang, H. Hackman, and B. Altrock, manuscript in preparation).

Northern blot analysis. In two separate experiments using endothelial cells from two different umbilical cords, adherent cells were lifted from the culture dish using limited trypsin exposure and RNA was isolated from the cytoplasmic fraction after NP-40 lysis according to previously published methods.

RNA was dissolved in loading buffer (50% formamide, 2.2 mol/L formaldehyde, 1 x MOPS) heated to 70°C for 5 minutes and electrophoresed (120 V, 3 hours). The ethidium bromide-stained gel was photographed and destained in 10x SSC (1 x SSC consists of 8.8 g/L NaCl, and 4.4 g/L sodium citrate, pH7). After overnight capillary transfer of RNA onto nitrocellulose, the nitrocellulose membrane was dried and baked in a vacuum oven for 2 hours, prehybridized (40% deionized formamide, 5 x SSC, 10 mmol/L Tris pH8, 1 x Denhardt's and 50 µg salmon sperm DNA) at 42°C then hybridized in the same solution (containing 10% dextran sulfate) with $^{32}$P by the random hexamer primer method using an oligolabeling reaction protocol according to the manufacturer's (Phar- macia, Piscataway, NJ) instructions.

RESULTS

As reported recently, endothelial cells stimulated with IL-1 released CSA active on human marrow cells (Fig 1). Approximately 7 to 10,000 U CSA was released into the medium. IL-1 alone did not directly stimulate colony growth. In human cultures, the colonial cell morphology included neutrophils, eosinophils, and mononuclear phagocytes.

Neutralizing antibodies against G and GM-CSF were used to determine which CSFs were released by IL-1-stimulated endothelial cells. Results presented in Table 1 illustrate the neutralizing effect of anti-GM and anti-G-CSF antibodies against the recombinant proteins in human CFU-GM assays. These antibodies alone had no inhibitory effect on colony formation (Table 1).

Endothelial cell supernatants were tested in the CFU-GM assay with combinations of anti-G and anti-GM-CSF anti-

<p>| Table 1. Inhibition of Human Bone Marrow Colony Formation by Anti-CSF Antibodies |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Antibody</th>
<th>rGM-CSF</th>
<th>rG-CSF</th>
<th>rGM-CSF</th>
<th>rG-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti--</td>
<td>Number of Colonies</td>
<td>Inhibition (%)</td>
<td>Number of Colonies</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>GM-CSF (µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23 ± 3</td>
<td>39 ± 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>18 ± 2</td>
<td>—</td>
<td>81</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>41 ± 3</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>G-CSF monoclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB 75A (µg IgG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>21 ± 3</td>
<td>28 ± 3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>24 ± 3</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>23 ± 4</td>
<td>0</td>
<td>100</td>
<td></td>
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</tbody>
</table>

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bodies. As shown in Table 2, both anti-G-CSF and anti-GM-CSF antibodies decreased total colony formation; however, neither antibody alone abolished all colony growth. The inclusion of both antibodies completely abolished all colony-forming activity from endothelial supernatants stimulated with IL-1. To document further that G-CSF is released by endothelial cells exposed to IL-1, the supernatants were tested in a murine CFU-GM assay (an assay that detects only G-CSF). As shown in Table 3, supernatants of endothelial cells exposed to IL-1 released a CSF that stimulated murine cells to form colonies and was neutralized by anti-G-CSF antibody. Because h-CSF-1 stimulates murine cells to form macrophage colonies, and because anti-G-CSF antibody completely inhibited all colony formation by endothelial cells stimulated by IL-1, we conclude the CSF-1 is not released by endothelial cells stimulated by IL-1 at the levels detected by the bioassay, which is ~10 U/mL.

The differentiation-inducing capacity of endothelial supernatants was examined by exposing the murine myelomonocytic leukemia cell line WEHI-3B to these media. WEHI-3B cells undergo differentiation to granulocytes and macrophages when exposed to G-CSF but not GM-CSF. As shown in Fig 2, IL-1-stimulated endothelial supernatants induced WEHI-3B differentiation. Preincubation of the medium to the anti-G-CSF antibody reduced the number of differentiated cells by 80%. The residual differentiation activity present in unstimulated endothelial cell supernatants is not sensitive to neutralization by anti-G-CSF antibody.

The phenomenon under study was specifically the granulopoietic effects of IL-1 on endothelial cells. The results in Tables 2 and 4 also confirm our previously reported results indicating that the addition of recombinant and native IL-1 to medium conditioned by uninduced endothelial cells does not induce more bone marrow colony growth than does endothelial cell conditioned medium alone. There remained at least two explanations for our observations: first that the endothelial cells were induced by IL-1 to express the G-CSF and GM-CSF genes or, alternatively, that the endothelial cells release another as yet undefined factor that could cause the release of GM-CSF and G-CSF by auxiliary cells in the GM-CSA bioassays. Consequently, we performed radioimmunoassay for G-CSF using supernatants from IL-1-induced and IL-1-uninduced endothelial cells. In addition, we performed Northern blot analysis of cytoplasmic RNA derived from IL-1-induced and IL-1-uninduced endothelial cells using as a probe 32P-labeled GM-CSF cDNA. The results (Table 4 and Fig 3) indicate that IL-1 induces endothelial cells to express G-CSF and GM-CSF genes. The tissue culture medium, unstimulated endothelial cells supernatants, and IL-1 all lacked measurable G-CSF activity in the RIA assay, the sensitivity of which is 100 pg/mL. IL-1-induced endothelial supernatants contained ~4 ng/mL G-CSF.

**DISCUSSION**

Mononuclear phagocytes play an important "accessory" role in the formation of granulocyte and macrophage colonies in vitro. Initially, mononuclear phagocytes themselves

**Table 2. Endothelial Cell Supernatants in Human CFU-GM**

<table>
<thead>
<tr>
<th>Factor Added</th>
<th>no Ab</th>
<th>anti-G-CSF</th>
<th>anti-GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF (3 ng)</td>
<td>67 ± 2</td>
<td>62 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>G-CSF (3 ng)</td>
<td>57 ± 5</td>
<td>0</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>ECM*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ECM + rIL-1† (100 ng/mL)</td>
<td>59 ± 6</td>
<td>20 ± 4</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>ECM + rIL-1† (3 ng/mL)</td>
<td>67 ± 4</td>
<td>32 ± 9</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>rIL-1† (100 ng/mL)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rIL-1† (100 ng/mL)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Endothelial cell-conditioned media (ECM).
†Conditioned media from endothelial cells exposed to IL-1.

**Table 3. Endothelial Supernatants in Murine CFU-GM**

<table>
<thead>
<tr>
<th>Factor Added</th>
<th>no Ab</th>
<th>anti-G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>rG-CSF</td>
<td>16 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>ECM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ECM + rIL-1*</td>
<td>17 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>ECM + rIL-1*</td>
<td>19 ± 5</td>
<td>0</td>
</tr>
</tbody>
</table>

*Conditioned media from endothelial cells exposed to IL-1.

**Table 4. Detection of G-CSF by RIA in Endothelial Supernatants Treated With rIL-1**

<table>
<thead>
<tr>
<th>Medium</th>
<th>G-CSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rIL-1 (50 U/mL)*</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>ECM†</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>ECM + rIL-1 (50 U/mL)†</td>
<td>3.9 ± 0.9</td>
</tr>
</tbody>
</table>

*IL-1 tested with endothelial cell-conditioned medium.
†Endothelial cell-conditioned medium.
‡Conditioned medium from endothelial cells exposed to IL-1.
were believed to provide CSFs that induce proliferation of granulocyte/macrophage colony forming units (CFU-GM) \textsuperscript{35,36} Although stimulated monocytes may produce CSF, recent studies\textsuperscript{4,7,11,12,18,20} demonstrated that monocytes unstimulated by mitogens or endotoxin require the aid of accessory cells to exert their colony-stimulating effects. Specifically, unstimulated mononuclear phagocytes produce and release IL 1 into culture medium. The IL 1 released by these cells stimulates endothelial cells and fibroblasts to release not only granulopoietic factors but other multilineage hematopoietic growth factors as well.\textsuperscript{15,19} Recent reports have documented that another monokine, tumor necrosis factor \(\alpha\), also stimulates the release of granulopoietic factors by endothelial cells.\textsuperscript{27,38} Because macrophages, fibroblasts, endothelial cells, and colony-forming cells are components of hematopoietic organs, hematopoiesis in general, and granulopoiesis in particular, may be under the regulatory control of monokines released in the locale of the hematopoietic microenvironment.

Although both G-CSF and GM-CSF stimulate colony growth of human marrow, there are substantial differences between the two. No nucleotide or amino acid sequence homologies other than a small conserved 3' untranslated region are shared.\textsuperscript{39} The two proteins bind to unique receptors in the murine model.\textsuperscript{40} Bioactivity differs as well. Murine cells are sensitive to G-CSF in both binding assays\textsuperscript{41} and bioassays. Human GM-CSF stimulates colony growth of human CFU-GM, but not clonal growth of murine CFU-GM or differentiation of WEHI-3B cells.\textsuperscript{41} In conjunction with highly specific antibodies and Northern blot analysis, this study used techniques that exploited these biologic differences to determine that both G-CSF and GM-CSF are expressed by IL 1-induced endothelial cells. The complete neutralization observed using the combination of monospecific antirecombinant GM-CSF rules out the possibility of a third as yet uncharacterized CSF in these culture supernatants at least at currently detectable levels. Accordingly, we suggest that either these two molecules are the only GM-CSFs expressed by these cells or that other forms (eg, M-CSFs) are expressed at levels too low to detect in the bioassays we used.

IL 1 stimulates other responses in endothelial cells, including synthesis of inhibitors of plasminogen activation,\textsuperscript{42} release of procoagulant activity\textsuperscript{43} and expression of molecules that increase leukocyte adherence to endothelium.\textsuperscript{44} In view of the known enhancing effects of CSFs on neutrophil function,\textsuperscript{45,46} further studies on the mechanisms by which IL 1 affects expression of GM-CSF and G-CSF genes by endothelial cells may shed light on issues related not only to the regulation of hematopoiesis, but also to the linkage of leukocytes with vascular injury and inflammation.

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