CONCISE REPORT

Secretion of Interleukin-1 by Acute Myeloblastic Leukemia Cells In Vitro Induces Endothelial Cells to Secrete Colony Stimulating Factors

By James D. Griffin, Alessandro Rambaldi, Edo Vellenga, Diane C. Young, Diana Ostapovicz, and Stephen A. Cannistra

The interaction of acute myeloblastic leukemia (AML) cells with stromal cells was investigated by adding AML-conditioned media to cultures of human endothelial cells. This conditioned media contained factors that induced expression of both the granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF) genes and release of colony stimulating activity from endothelial cells. The conditioned media contained interleukin-1 (IL-1) bioactivity and the endothelial cell stimulatory activity was partially neutralized by anti-IL-1 antiserum. Constitutive expression of the IL-1-beta gene was detected in ten of 17 AML cases analyzed. These results suggest that the unregulated secretion of IL-1 by AML cells can induce stromal cells in vitro to overproduce CSFs. This could contribute to the unrestricted growth of AML cells.

A\nCUTE MYELOBLASTIC LEUKEMIA (AML) is a highly lethal hematopoietic malignancy characterized by excessive proliferation and aberrant differentiation of myeloblasts. A fraction of leukemic cells from most AML patients will also proliferate in vitro in semi-solid medium to form colonies of blasts. In most cases, this requires the addition of colony-stimulating factors (CSFs), and recent studies with recombinant human CSFs have demonstrated that AML cells will proliferate in response to granulocyte CSF (G-CSF), granulocyte macrophage CSF (GM-CSF), and interleukin-3 (IL-3). Thus, unlike most human myeloid leukemia cell lines such as HL-60, primary human leukemic cells remain factor dependent at least in vitro and presumably in vivo as well. The cells that supply hematopoietic growth factors in the marrow microenvironment are incompletely understood, but are likely to include endothelial cells, fibroblasts, macrophages, and T lymphocytes. While it is possible that the marrow microenvironment would normally supply adequate amounts of growth factors for a rapidly expanding leukemic clone, it is also possible that leukemic cells have developed mechanisms to augment their sources of growth factors. In this study, we demonstrate that AML cells constitutively secrete factors in vitro that induce stromal cells (endothelial cells) to secrete CSFs and that one of these factors is IL-1.

MATERIALS AND METHODS

Leukemic cells. Pretreatment bone marrow mononuclear cells cryopreserved in DMSO were thawed in the presence of human serum, depleted of T cells by rosetting with sheep erythrocytes, and depleted of monocytes by plastic adherence at 37°C for one hour. All specimens contained >95% blasts. Conditioned media was prepared by incubating leukemic cells at a concentration of 1 x 10^6 cells/mL in RPMI 1640 (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS) for 48 hours.

CSF assay. CSF activity was measured using a standard double layer agar assay for granulocyte-macrophage colony-forming units (CFU-GM) in which 5 x 10^4 light density, nonadherent, T depleted human marrow cells were cultured in a 0.3% agar overlay and sources of CSFs or AML-conditioned media (AML-CM) (final concentration 10%) were in a 0.5% agar underlayer. GCT medium (Gibco) was used as a positive control. Normal marrow cells were obtained from volunteers who gave informed consent approved by the institutional review board. CFU-GM in duplicate or triplicate cultures were counted on day 7.

Endothelial cells. Human umbilical vein endothelial cells (a gift of Dr Douglas Fallar, DFCI) were grown in medium 199 (Gibco) containing 20% FBS, heparin 2 U/mL, and endothelial cell growth factor 25 µg/mL (Sigma, St Louis). AML-CM or control media was added to endothelial cells in a final concentration of 20% (vol/vol) and incubated for an additional nine hours before RNA extraction or 24 hours for CSF assay. In some experiments, purified anti-IL-1 antibody (Endogen Inc, Boston) was added to conditioned media (10 neutralizing U/mL) for one hour at 25°C before incubation with endothelial cells.

IL-1 assay. IL-1 activity of CM was assessed by measuring incorporation of tritiated thymidine by the IL-1 dependent AKR/J T cell clone D10.G4.1 (a gift of Dr Abul Abass, Harvard Medical School, Boston) in triplicate assays. AML-CM was added to 2 x 10^6 D10.G4.1 cells in 150 µL containing 2 µg/mL concanavalin A (Con A) (Sigma). Recombinant IL-1-beta (Genzyme Corporation, Boston) was used to generate a standard curve. Duplicate wells without Con A served as a negative control. After 48 hours (37°C, 5% CO2), the cells were pulsed with [3H]-TDR (1.9 Ci/mmol, Schwartz-Mann, Spring Valley, NY) and harvested as previously described. Data were expressed as IL-1 activity in units per milliliter from the standard curve.

Tumor necrosis factor assay. Tumor necrosis factor (TNF) activity was assessed by measuring cytotoxicity for WEHI-164 fibrosarcoma cells (a gift of Alberto Mantovani, Mario Negri Institute, Milan, Italy). CM was added to 2 x 10^6 WEHI-164 cells in 100 µL medium containing 4 µg/mL actinomycin D (Sigma). Recombinant TNF (Asahi Chemical Co, Shizuoka, Japan) was used to generate a standard curve. For some experiments purified anti-TNF monoclonal antibody (Asahi) was added (10 µg/mL) for one hour at 25°C before the addition of WEHI-164 cells. After 18 hours (37°C, 5% CO2), 10 µL of MIT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/mL in PBS, Sigma) was added, followed in four hours by addition of acid-isopropanol (100
SECRETION OF IL-1 BY AML CELLS

µL of 0.04N HCl in isopropanol). Absorbance was measured on a Titertek Multiscan Elisa plate reader (Flow Laboratories, McLean, VA) with a test wavelength of 570 nm. In this assay, 0.5 × 10^{-9} g TNF/mL was lethal to 50% of cells.

Northern blots. Four to 50 × 10^{6} cells were lysed in 4 mol/L guanidium isothiocyanate and RNA was extracted, electrophoresed in agarose gels, and blotted as previously described. Membranes were hybridized with 32P-labeled probes as previously described. cDNA probes were labeled with 32P-dCTP by the method of Feinberg and Vogelstein. IL-1-beta, GM-CSF, and G-CSF mRNA were detected with cDNA probes provided by Drs Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA. RNA from AML cells was derived directly from cryopreserved cells for some experiments, and from thawed, adherent-cell depleted, cultured AML cells in others.

RESULTS

AML-CM contains factors that induce CSF release by endothelial cells. Medium conditioned by purified blasts from four patients with AML was added to 1-mL cultures of human umbilical vein endothelial cells (EC) (10^{5} cells) at a final concentration of 20%. The EC were then cultured for 24 hours and the medium removed for CSF assay. Control medium or medium from unstimulated EC did not contain detectable CSF activity in a standard CFU-GM assay (Table 1). The addition of AML-CM to EC cultures resulted in the production of readily detectable CSF activity for three of the AML cases (cases no. 1, 3, and 4). AML-CM from case no. 1 contained baseline CSF, in agreement with our previous observations, but the amount of CSF present was enhanced after addition of this AML-CM to endothelial cells.

Culture of endothelial cells with AML-CM results in the induction of the GM-CSF and G-CSF genes. Northern blot analysis was used to investigate expression of the GM-CSF and G-CSF genes in EC. Cultures containing 4 × 10^{6} EC were incubated for nine hours with control medium or AML-CM, RNA was extracted and analyzed for the presence of GM-CSF and G-CSF transcripts (Fig 1). Unstimulated endothelial cells did not express either CSF gene. However, addition of CM from AML patient no. 4 resulted in the induction of expression of both CSF genes.

The EC-stimulating activity present in AML-CM can be partially neutralized by anti-IL-1 antibodies. IL-1 and TNF have been previously shown to induce GM-CSF gene expression in EC. We therefore measured levels of IL-1 and TNF in AML-CM using appropriate bioassays. CM from AML patients no. 1 to 4 contained 0.5, 0.075, >5, and >5 U/mL of IL-1 activity, respectively. TNF activity was <0.5 × 10^{-9} ng/mL in all four media. In order to test the possibility that one of the factors in AML-CM that induced EC release of CSF was IL-1, we incubated 200 µL aliquots of AML-CM with ten neutralizing units of anti-IL-1 antibody for one hour at room temperature, and then determined the residual ability of this medium to induce release of CSFs by EC. In the same experiments shown in Table 1, the addition of anti-IL-1 to CM from case no. 1 decreased subsequent CFU-GM formation from 99 ± 5 to 47 ± 9 CFU-GM/5 × 10^{6} normal marrow cells. In case no. 4, anti-IL-1 reduced CFU-GM from 81 ± 7 to 17 ± 2. No effect of the antibody was observed in case no. 3 (62 ± 2 without antibody to 71 ± 2 with antibody), which also demonstrates the lack of toxicity of the anti-IL-1 antiserum. These results suggest that some, but perhaps not all, of the EC-stimulatory activity was due to IL-1.

Constitutive expression of the IL-1-beta gene in AML. Expression of the IL-1-beta gene in nonadherent AML blasts cultured for several hours in vitro was determined by Northern blot analysis in 17 cases of AML (Fig 2). RNA from the four AML cases described in Table 1 is shown in lanes 16, 14, 13, and 17, respectively. Expression of the IL-1-beta gene was observed in at least ten of 17 cases.

Table 1. AML-CM Induces Endothelial Cells to Release CSFs

<table>
<thead>
<tr>
<th>Source of CSFs</th>
<th>Day 7</th>
<th>CFU-GM/5 × 10^{6} NBM cells</th>
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<tbody>
<tr>
<td>Medium alone</td>
<td>1 ± 0</td>
<td></td>
</tr>
<tr>
<td>AML-1 medium</td>
<td>39 ± 5</td>
<td></td>
</tr>
<tr>
<td>AML-2 medium</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>AML-3 medium</td>
<td>4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>AML-4 medium</td>
<td>5 ± 4</td>
<td></td>
</tr>
<tr>
<td>EC medium</td>
<td>1 ± 0</td>
<td></td>
</tr>
<tr>
<td>EC + AML-1</td>
<td>99 ± 5</td>
<td></td>
</tr>
<tr>
<td>EC + AML-2</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>EC + AML-3</td>
<td>62 ± 2</td>
<td></td>
</tr>
<tr>
<td>EC + AML-4</td>
<td>81 ± 7</td>
<td></td>
</tr>
<tr>
<td>GCT</td>
<td>43 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NBM, normal bone marrow; GCT, conditioned medium from GCT cell line (positive CSF control).

*Control medium (RPMI 1640 with 10% FBS), media conditioned by AML cells or EC alone, or media conditioned by EC cultured for 24 hours with AML-CM were used at a final concentration of 10% in a CFU-GM assay.
There was no apparent correlation with the French-American-British (FAB) classification (four of four M1 cases were positive, as were two of three M2 cases, two of four M4 cases, and one of four M5 cases, one unclassified case was also positive). RNA was also extracted directly from cryopreserved AML cells from five patients in order to determine if the plastic adherence step induced IL-1 beta expression. Four of five cases had IL-1 beta mRNA (data not shown).

**DISCUSSION**

Growth of clonogenic AML cells in semi-solid media generally depends on the addition of CSFs. Recent studies from our laboratory have demonstrated that although expression of the genes for GM-CSF, G-CSF, and M-CSF in AML cells can be frequently detected, the amount of CSF secreted is generally not sufficient to support in vitro leukemic colony growth by itself.\(^5\) Also, many cases have been identified in which no CSF gene activation or secretion of CSF can be detected. We therefore considered the possibility that AML cells might secrete factors that could induce cells of the marrow microenvironment to release CSFs. AML-CM of four patients was tested for CSF activity and for its effects on CSF release by human umbilical vein EC. AML-CM of three of the four cases contained factors that induced expression of both the GM-CSF and G-CSF genes and release of CSF activity from EC. These results indicate that (1) there is a possible humoral interaction in vitro between AML cells and stromal cells that results in an enhanced supply of CSFs to leukemic cells, and (2) that these AML factors promote CSF production by enhancing gene expression rather than by inducing release of preformed CSFs by EC.

Reports from many laboratories have recently indicated that two monokines, IL-1 and TNF, can induce EC and fibroblasts to secrete CSFs.\(^6,7\) We therefore assayed IL-1 and TNF levels in AML-CM. The results indicated that high levels of IL-1 activity could be detected in some, but not all samples, while the levels of TNF were below our detectable range. Further, some of the endothelial cell-inducing activity in AML-CM could be neutralized by a polyclonal antisera to IL-1. These data suggest that one of the endothelial cell activating factors is likely to be IL-1. This was confirmed by Northern blot analysis, which demonstrated the presence of IL-1-beta mRNA in ten of 17 AML cases depleted of adherent cells and four of five cases in which there was no in vitro culture. IL-1 is known to be released by activated normal monocytes,\(^13\) and production of IL-1 has also been observed by the monocytic “leukemic” cell lines U937\(^13\) and THP-1.\(^19\) Furukawa et al\(^17\) recently showed that AML patients of the M4 or M5 subtype may secrete a factor that induces murine thymocyte proliferation and is presumably IL-1; Lachman et al\(^18\) previously described the characterization of a “lymphocyte-activating factor” from acute myelocytic and myelomonocytic leukemia cells.

The results presented here suggest that IL-1-beta is one of the factors released by AML cells in vitro that can promote CSF release by EC. Expression of the IL-1 gene in AML cells is aberrant in the sense that it is “constitutive” and thus unregulated. Unregulated secretion of IL-1 by AML cells in vivo (possibly with other factors such as TNF) could result in unregulated production of CSFs by marrow stromal cells. We suggest that constitutive release of IL-1 by AML cells could contribute to the unrestricted growth of these cells, particularly in cases that do not produce their own CSFs.

**REFERENCES**

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