Interleukin-1α Also Induces Granulocyte-Macrophage Colony-Stimulating Factor in Immature Normal Bone Marrow Cells

By Fredrik J. Bot, Pauline Schipper, Lianne Broeders, Ruud Delwel, Kenneth Kaushansky, and Bob Löwenberg

The cytokine interleukin-1 (IL-1) plays a role in the regulation of normal as well as leukemic hematopoiesis. In acute myeloid leukemia (AML), IL-1 induces autocrine granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF) production, and these factors may then synergistically induce proliferation in AML blast cells. In this report, we show that IL-1 stimulates DNA synthesis of highly enriched normal bone marrow blast cells (CD34 positive, adherent cell depleted, CD3/CD14/CD15 negative). The stimulatory effect of IL-1 can be blocked with neutralizing anti-TNFα and anti-GM-CSF antibodies and, most efficiently, by the combination of anti-TNFα and anti-GM-CSF, but not with anti-G-CSF antibody, suggesting that IL-1-induced proliferation was initiated through TNF and GM-CSF release. Concentrations of TNF and GM-CSF increased in the culture medium of normal bone marrow blast cells after IL-1 induction. Of the IL-1-induced cells, 12% were positive for GM-CSF mRNA by in situ hybridization, as opposed to 6% of non-induced cells. Thus, in addition to its effect on leukemic blast cells, IL-1 also acts on normal marrow blast cells. We propose a scheme where IL-1 stimulation of normal bone marrow blast cells leads to the induction of TNFα and GM-CSF, which in association stimulate DNA synthesis efficiently according to a paracrine or autocrine mechanism within the marrow blast cell compartment.

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Boehringer Institute, Vienna, Austria). Anti-TNF antibody at a concentration of 1:10,000 neutralized up to 10,000 U/mL of TNF in vitro proliferation assays of B-cell chronic lymphocytic leukemia (B-CLL) cells (data not shown). This concentration of anti-TNF was chosen to ensure a complete neutralization of all TNF present in the medium. Recombinant human erythropoietin was purchased from Amgen Biologics (Thousand Oaks, CA) and used at 1 U/mL.

Preparation of conditioned media. In three experiments, conditioned media were collected after culture of enriched normal marrow blast cells with and without IL-1 in serum-free medium. In parallel, a medium enriched normal bone marrow blast cells was measured by [3H]-TdR uptake. The cells were cultured for 48 hours in round-bottom 96-well microtiter plates (mean, 8,200 cells per well) in 0.1 mL of a serum-free medium composed of bovine serum albumin (BSA), transferrin, Na-selenite, linoleic acid, cholesterol, insulin, mercaptoethanol, and Iscove's modified Dulbecco's medium (IMDM), supplemented with growth factors and antibodies, exactly as reported.

Preparation of conditioned media. In three experiments, conditioned media were collected after culture of enriched normal marrow cells with and without IL-1 in serum-free medium. In parallel, a control medium with IL-1 but without cells was also prepared. The media were harvested after 64 hours of culture, centrifuged to remove cells, and stored at -20°C. The cells from these supernatants were derived were also examined cytologically after May-Griinwald-Giemsa staining.

TNF and GM-CSF assays. Immune-reactive TNF was measured with the TNFα immunoradiometric assay (IRMAs; IRE-Medgenix, Fleurus, Belgium), which makes use of several MoAbs directed against distinct epitopes of TNFα. The assay showed no crossreactivity with TNFβ, IL-1, IL-2, and interferons α, β, and γ. The detection limit was 2 pg/mL TNF, and the interassay coefficient of variation at a level of 131 pg/mL was 7.2% (n = 10).

GM-CSF was measured by enzyme-linked immunosorbent assay (ELISA; Zymogenetics Inc, Seattle, WA), with a sensitivity threshold of 8 ng/L. For positive release of GM-CSF and TNF in the culture medium, values were required to be ≥10 ng/L.

In situ hybridization with cDNA GM-CSF. In situ hybridization was done as described, with minor modifications. Enriched bone marrow blast cells (approximately 2 × 10^6 per group) were cultured with or without IL-1 in serum-free medium, harvested after 18 hours, washed twice with α-minimal essential medium (α-MEM; Gibco, Grand Island, NY), and resuspended in 0.050 mL. Then, 0.010 mL of this cell suspension was applied to fibronectin precoated (4 μg/mL in PBS; 1 hour) glass slides and permitted to stick for 10 minutes. Subsequently, the slides were washed in PBS followed by fixation with formaldehyde (3.7% diluted in PBS) for 2 minutes, dehydration in 70% ethanol for 5 minutes and air-dried. The 0.8 kb cDNA GM-CSF probe (obtained from Genetics Institute) and a control 1.8 kb cDNA EGFR-receptor probe were nick-translated using 32P-dATP, resulting in a specific activity of 2 to 6 × 10^6 cpm/μg, with a fragment size of ≈300 bp. Prehybridization (1 hour) and hybridization (18 hours) were done at 37°C applying 1 ng of radiolabeled probe per slide. After autoradiography (using NTB-2 Kodak emulsion) for 6 to 28 days, the slides were stained with May-Grünwald-Giemsa and examined at 1,000× magnification.

RESULTS

IL-1 stimulation of DNA synthesis of purified normal bone marrow blasts. The results of stimulation of highly enriched blast cells from 16 different normal bone marrow donors with IL-1 in the presence or absence of neutralizing antibodies against TNFα, GM-CSF, and G-CSF are shown in Table 1. IL-1 stimulates DNA synthesis significantly above background [3H]-TdR incorporation in all cases (mean ± SD stimulation index [SI]: 3.5 ± 2.1). The addition

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Factor</th>
<th>IL-1 Without Antibody</th>
<th>SI</th>
<th>αTNF</th>
<th>αGM-CSF</th>
<th>αGM-CSF</th>
<th>αG-CSF</th>
<th>αG-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,269 ± 699</td>
<td>5,792 ± 527</td>
<td>2.6</td>
<td>5,708</td>
<td>6,630</td>
<td>4,877*</td>
<td>---</td>
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<tr>
<td>2</td>
<td>3,721 ± 1,589</td>
<td>8,124 ± 2,047</td>
<td>2.2</td>
<td>6,790</td>
<td>8,601</td>
<td>3,788*</td>
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<td>---</td>
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<tr>
<td>3</td>
<td>3,503 ± 673</td>
<td>7,333 ± 1,584</td>
<td>2.1</td>
<td>6,241</td>
<td>4,857</td>
<td>4,502*</td>
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<td>---</td>
</tr>
<tr>
<td>4</td>
<td>512 ± 289</td>
<td>2,488 ± 197</td>
<td>4.9</td>
<td>1,971</td>
<td>2,260</td>
<td>1,668*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>3,305 ± 175</td>
<td>5,781 ± 884</td>
<td>1.7</td>
<td>5,077</td>
<td>5,411</td>
<td>4,126*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>687 ± 115</td>
<td>1,990 ± 102</td>
<td>2.9</td>
<td>1,087*</td>
<td>1,666</td>
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<tr>
<td>7</td>
<td>3,404 ± 157</td>
<td>4,926 ± 606</td>
<td>1.4</td>
<td>2,985*</td>
<td>3,812</td>
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<td>---</td>
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<tr>
<td>8</td>
<td>528 ± 141</td>
<td>4,684 ± 445</td>
<td>8.9</td>
<td>3,675*</td>
<td>3,336*</td>
<td>4,771</td>
<td>---</td>
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<tr>
<td>9</td>
<td>3,304 ± 192</td>
<td>8,443 ± 532</td>
<td>2.6</td>
<td>5,925</td>
<td>3,710*</td>
<td>4,126*</td>
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<td>---</td>
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<tr>
<td>10</td>
<td>378 ± 138</td>
<td>1,891 ± 564</td>
<td>5.0</td>
<td>815*</td>
<td>1,019</td>
<td>504*</td>
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<td>---</td>
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<tr>
<td>11</td>
<td>722 ± 173</td>
<td>2,873 ± 396</td>
<td>4.0</td>
<td>1,916*</td>
<td>2,004</td>
<td>1,840*</td>
<td>2,816</td>
<td>1,646*</td>
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<tr>
<td>12</td>
<td>618 ± 298</td>
<td>4,423 ± 884</td>
<td>7.2</td>
<td>3,164</td>
<td>2,842*</td>
<td>1,914*</td>
<td>4,542</td>
<td>3,286*</td>
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<tr>
<td>13</td>
<td>3,262 ± 708</td>
<td>4,970 ± 584</td>
<td>1.5</td>
<td>3,292*</td>
<td>3,260*</td>
<td>2,247*</td>
<td>4,871</td>
<td>3,664*</td>
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<tr>
<td>14</td>
<td>387 ± 109</td>
<td>776 ± 81</td>
<td>2.0</td>
<td>596*</td>
<td>477*</td>
<td>349*</td>
<td>712</td>
<td>562*</td>
</tr>
<tr>
<td>15</td>
<td>731 ± 399</td>
<td>3,010 ± 593</td>
<td>4.1</td>
<td>1,586*</td>
<td>1,827*</td>
<td>856*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>16</td>
<td>1,252 ± 245</td>
<td>2,877 ± 256</td>
<td>2.3</td>
<td>2,092*</td>
<td>1,721*</td>
<td>1,652*</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Trisilicate cultures of accessory cell-depleted/CD34+ normal bone marrow cells were set up with or without IL-1 (100 U/mL) and in the presence of anti-TNFα, anti-GM-CSF, and anti-G-CSF antibodies (α) in several combinations. After 48 hours of culture, DNA synthesis was assessed. Values are expressed as mean disintegrations per minute (dpm) ± SD. The stimulation index (SI) is defined as the quotient of dpm of IL-1-stimulated cultures and dpm of nonstimulated cultures.

Blocking is considered positive when dpm ± SD are significantly less (P < .05 by Student's t test) in cultures with antibody as compared with cultures without antibody. Underlined values are not significantly different from the background incorporation (ie, complete blocking).
of antibodies against TNFα to the cultures abrogated IL-1-dependent proliferation ("blocking") in 9 of 16 cases partially and in 2 of 16 cases completely. Similarly, among those cases, the addition of anti-GM-CSF antibody suppressed IL-1 induced proliferation in 4 of 16 instances partially and in 3 of 16 cases completely. In 5 of 16 cases DNA synthesis was not reduced when either anti-GM-CSF or anti-TNFα was added to IL-1 stimulated cultures.

The combination of anti-TNFα and anti-GM-CSF suppressed DNA synthesis significantly in 13 of 14 cases, and complete suppression was evident in 7 of those. In the five cases where the single antibodies had not reduced DNA synthesis, the combination of anti-TNF and anti-GM-CSF was clearly active; and in two of the latter cases, blocking was complete. In 11 of the 14 cases, DNA synthesis was suppressed more efficiently in the presence of both anti-TNFα and anti-GM-CSF antibodies than with either antibody alone, indicating that the effects of the antibodies were additive.

Anti-G-CSF antibody did not diminish ^3H-TdR uptake in four cases tested. Anti-TNFβ had no inhibitory effect on IL-1-induced DNA synthesis in two experiments (no. 12 and no. 16 of Table 1) with significant suppression by anti-TNFα (data not shown).

Finally, in comparison with IL-1, the ability of lipopolysaccharide (LPS) to stimulate DNA synthesis of normal bone marrow blast cells was also assessed. LPS (Escherichia coli serotype 0127:B8; Sigma, St Louis, MO) added to cultures in concentrations of 0.01 μg/mL up to 1 μg/mL did not induce any stimulation of DNA synthesis, indicating that the IL-1 effect could not be the result of LPS addition.

**TNFα and GM-CSF production by enriched bone marrow blast cells.** Conditioned media were prepared from accessory cell-depleted, CD34+ blast cells from three donors, and the concentrations of immunoreactive TNFα and GM-CSF in those supernatants were determined. No GM-CSF and low levels of TNFα were demonstrated in medium conditioned by those cells without IL-1. Both TNFα and GM-CSF concentrations increased significantly in the supernatants of marrow blasts that had been cultured with IL-1 (Table 2).

When 10% IL-1 marrow blast-derived conditioned medium was added to a standard culture system, no myeloid colonies were induced; however, an increase in the number of erythropoietin (Epo)-induced burst-forming units-erythroid (BFU-E) from 100% to 193% noted (data not shown). This suggested the presence of burst-promoting activity, ie, a well-known function of GM-CSF,15-17 in those supernatants.

**In situ demonstration of GM-CSF mRNA in normal marrow blasts.** Enriched normal bone marrow progenitor cells were incubated with and without IL-1 for 18 hours in serum-free medium. In situ hybridization of the cells with a GM-CSF cDNA probe revealed GM-CSF mRNA in 6% of unstimulated and in 12% of IL-1-stimulated cells (Table 3). The percentage of strongly positive cells (greater than 20 grains per cell) was 3% in noninduced cultures, and 7% in IL-1-induced cultures. Figure 1 shows a cytospin slide of bone marrow blast cells after incubation with IL-1 and probed with GM-CSF cDNA.

**DISCUSSION**

We report that IL-1 may induce proliferation of highly enriched immature normal bone marrow cells. The stimulating effect of IL-1 is apparently the result of a two-stage process that includes the induction of TNFα and GM-CSF as a first step and a cellular response of DNA synthesis as a second event. The notion that IL-1 induces cytokines in normal blast cells to elicit proliferation is supported by three sets of experimental findings: (1) the inhibition of IL-1-induced DNA synthesis with specific antibodies against TNFα and GM-CSF; (2) the increased release of immunoreactive

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Blast Cells + IL-1</th>
<th>Blast Cells - IL-1</th>
<th>Cell-Free Medium + IL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF (ng/L)</td>
<td>GM-CSF</td>
<td>TNF (ng/L)</td>
</tr>
<tr>
<td>1</td>
<td>373</td>
<td>ND</td>
<td>186</td>
</tr>
<tr>
<td>2</td>
<td>638</td>
<td>61</td>
<td>400</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>12</td>
<td>18</td>
</tr>
</tbody>
</table>

Accessory cell-depleted/CD34+ normal bone marrow cells were incubated for 64 hours with (+) or without (−) IL-1 (100 U/mL), and the supernatants were collected. Control medium without cells but with IL-1 was incubated similarly. The TNF and GM-CSF concentrations are expressed as ng/L.

Abbreviations: −, negative, (ie, values of 10 ng/L or less); ND, not done.
active TNFα and GM-CSF by bone marrow blasts into the culture supernatant after IL-1 induction; and (3) the increase of GM-CSF mRNA positive cells among normal marrow blasts and the appearance of cells strongly positive for GM-CSF mRNA (greater than 50 grains per cell) in response to IL-1.

The role of IL-1 in the regulation of human hematopoiesis is diverse. As yet, direct effects of IL-1 upon blast cells have not been recognized. However, indirect effects of IL-1 are well-established. For instance, GM-CSF and G-CSF production is induced in fibroblasts and endothelial cells after stimulation with IL-1. The enriched blast cell population that was separated from normal marrow and used in the experiments described here was carefully depleted of accessory cells and consisted almost exclusively of cells with blast morphology. After 64 hours in culture, there was no cytological change in the cell population. Nevertheless, we cannot absolutely exclude that minimal numbers of accessory cells were admixed with the immature cell fraction and responsible for the IL-1 effect. However, we do not consider this a likely explanation, since up to 12% of IL-1–induced cells expressed GM-CSF mRNA, and this fraction does not correlate with the number of morphologically recognizable accessory cells. Our observations suggest that certain normal marrow blast cells may produce GM-CSF and TNFα. In analogy, leukemic blast cells are able to produce GM-CSF in response to IL-1 and, thus, stimulate their own proliferation. It follows that autocrine stimulation of blast cells may be a physiologic event, rather than being leukemia-specific (pathophysiologic).

Observations that TNF may strongly enhance GM-CSF–induced proliferation of human AML-cells suggest an important role for TNF and its inducer IL-1 in the regulation of hematopoiesis. Even at low levels of GM-CSF, significant proliferative activity can be induced in immature marrow cells when TNF is present as a costimulus. Thus, positive interactions between TNF and GM-CSF permit powerful stimulatory effects that appear under control of the common inducer IL-1. In addition, it is conceivable that as an extension of the stepwise mechanism of positive regulation primed by IL-1, TNF may in turn trigger the release of GM-CSF and G-CSF in tertiary blast cells in analogy to its ability to induce GM-CSF and G-CSF in fibroblasts and endothelial cells, thereby further augmenting the hematopoietic response. Furthermore, it has been shown that TNFα induces the production of IL-1 in endothelial cells and fibroblasts. The evidence that IL-1 and TNFα may reciprocally stimulate the production of each other in different target cells implies that both molecules may significantly amplify their positive effects on CSF production, providing a powerful mechanism for expansion of hematopoiesis at times of stress.

ACKNOWLEDGMENT

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REFERENCES


