Proliferation of Myeloid Progenitor Cells in Human Long-Term Bone Marrow Cultures Is Stimulated by Interleukin-1 Beta

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To study the effect of interleukin-1 (IL-1) beta on the proliferation of hematopoietic progenitor cells (HPC) in long-term bone marrow cultures (LTBMC), stromal cell layers were established from normal human bone marrow. Autologous cryopreserved mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells were reinoculated on the stromal layers in fresh culture medium, with or without the addition of human IL-1 beta (30 U/mL). Once a week, half of the culture supernatant was replaced with fresh culture medium with or without IL-1, and all nonadherent cells were returned to the flasks. At weekly intervals during a period of 5 weeks, one culture was sacrificed to determine the total number of cells and hematopoietic progenitor cells, present in the adherent and the nonadherent cell fractions. In IL-1-stimulated cultures, the number of cells recovered during a period of 5 weeks exceeded the number of cells in unstimulated control cultures by 1.5 times. This difference was attributed to a twofold increase in the number of adherent cells. The number of HPC recovered from IL-1-stimulated cultures was not different from that recovered from controls. The levels of colony-stimulating activity (CSA) in supernatants from IL-1-stimulated cultures were significantly higher than those in supernatants from control cultures. These results indicate that IL-1 enhances the recovery of cells in LTBMC by stimulating the proliferation of HPC with the concurrent release of CSA from stromal cells, without diminishing the number of HPC.

EMATOPOIESIS in vivo occurs in association with a variety of cells, collectively called the hematopoietic microenvironment or the bone marrow stroma. Several stromal cell types can be recognized including endothelial cells, marrow fibroblasts, adipocytes, and macrophages. In human long-term bone marrow cultures (LTBMC), hematopoietic progenitor cell (HPC) proliferation and differentiation occur under conditions that closely mimic hematopoiesis in vivo. In these cultures, sustained proliferation of HPC during several weeks is associated with the formation of a heterogeneous adherent cell population that is believed to stimulate the hematopoietic microenvironment. Thus, the LTBMC system provides a suitable model for studying interactions between stromal cells and HPC.

Colony-stimulating factors (CSF) comprise a family of glycoproteins that stimulate colony formation in bone marrow cultures, and are believed to be of physiologic importance in hematopoiesis in vivo. Four human CSF have been purified to homogeneity and their cDNA clones have been identified. Granulocyte-CSF (G-CSF) and macrophage-C SF (M-CSF) are lineage-specific growth factors, whereas the formation of myeloid, erythroid, and multilineage colonies is stimulated by granulocyte-macrophage CSF (GM-CSF) and multi-CSF (interleukin-3, IL-3).

CSFs can be produced by the various cell types present in the hematopoietic microenvironment. Recently, it has been demonstrated that these cells are able to elaborate CSF in response to inflammatory mediators. For instance, interleukin-1 (IL-1) induces the release of CSF by mononuclear phagocytes, fibroblasts, bone marrow fibroblasts, and endothelial cells. We have recently demonstrated that human bone marrow stromal cells in long-term culture release G-CSF and M-CSF following stimulation by IL-1.

In the present report, we describe studies of the influence of IL-1 on proliferation and differentiation of HPC in LTBMC. It was found that IL-1 increases the recovery of neutrophils and mononuclear phagocytes from these cultures with the concurrent release of colony-stimulating activity (CSA) from stromal cell layers, without affecting the total number of progenitor cells.

MATERIALS AND METHODS

Normal human bone marrow was obtained, after informed consent, by aspiration from the posterior iliac crest of hernia pulposi patients, who underwent laminectomy. The cells were collected in Hanks' balanced salt solution with 100 U/mL preservative-free heparin, diluted in RPMI 1640 with 5% fetal bovine serum (FBS: Gibco, Grand Island, NY), separated over Ficoll-Isopaque (1.077 g/mL, 1,000 g; 20 minutes; 20°C), and the interphase cells were harvested and washed three times in RPMI 1640 plus 5% FBS.

Depletion of mononuclear phagocytes from bone marrow cell suspensions was performed by carbonyl-iron incorporation, as previously described. T lymphocytes were removed from the Ficoll interphase cells by rosetting with 2-aminoethylthiouronium bromide (AET)-pretreated sheep erythrocytes and sedimentation of the rosetted cells through Ficoll-Isopaque. The nonrosetting mononuclear phagocyte-depleted interphase cells contained <2% T lymphocytes; this was determined with indirect immunofluorescence microscopy using monoclonal antibody Leu-4 (anti-CD3; Becton Dickinson, Oxnard, CA) and fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin antisera (Nordic Immunological Laboratories, Tilburg, The Netherlands). These cells also...

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IL-1 induces proliferation of progenitor cells

contained <2% mononuclear phagocytes, as determined by alpha-naphthylbutyrate esterase staining. For cryopreservation, mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells were resuspended in a medium consisting of 70% RPMI 1640, 20% FBS, and 10% dimethylsulphoxide (DMSO) at 0°C. These cells were then frozen, using a computer-controlled freezer (Cryogen; Middenbeemster, The Netherlands) at a rate of 1°C per minute from 0°C to −50°C, and at a rate of 4°C per minute from −50°C to −90°C. The cells were stored in liquid nitrogen. Before reinoculation onto stromal cell layers, these cells were thawed for one minute in a 37°C water bath, diluted in HEPES-buffered RPMI 1640 plus 20% FBS at 0°C, washed twice in the same medium at 0°C, and resuspended in culture medium.

Interleukin-1

Electrophoretically pure human IL-1 beta was obtained from crude human lymphokine preparations by a four-step purification schedule as previously reported. The preparation used in all experiments contained 10,000 U/mL, as tested in a standard assay for lymphocyte-activating factor (LAF) or for antiviral activity. It did not contain other cytokine activities tested, including IL-1 alpha, IL-2, IL-6, interferons and CSA. It was found to be free of bacterial endotoxins, as tested by a limulus amoebocyte lysate assay able to detect levels of endotoxin as low as 60 pg/mL.

Radioimmunoassay for IL-1 Alpha and Beta

Radioimmunoassays (RIAs) for IL-1 beta and alpha were performed according to Lisi et al. and Lonneeman et al., respectively, using recombinant human IL-1 alpha (Hoffmann la Roche, Nutley, NJ) or IL-1 beta (Biogen, Geneva, Switzerland) as standards (10⁵ LAF or antiviral units per milligram protein). The anti–IL-1 alpha antiserum used in the RIA for IL-1 alpha was a kind gift from Dr C.A. Dinarello (Tufts University School of Medicine, Boston). The antiserum was prepared in rabbits, using a preparation of human recombinant IL-1 alpha. The anti–IL-1 beta antiserum used in the RIA for IL-1 beta was prepared by Dr J. Van Damme in goats, using a preparation of electrophoretically pure human natural IL-1 beta.

The anti–IL-1 alpha antiserum did not crossreact with IL-1 beta and vice versa. The limit of sensitivity of the assay for IL-1 alpha was 1 U/mL and for IL-1 beta, 2 U/mL.

Establishment of Stromal Layers

Quantities of 1.5 to 2.0 x 10⁶ Ficoll-interphase bone marrow cells were suspended in 4 mL of culture medium consisting of HEPES-buffered alpha-modified Eagle’s minimal essential medium (alpha-MEM; Flow Laboratories, Irvine, UK) with 25% fresh (frozen) AB human heparin plasma and 10⁻⁴ mol/L hydrocortisone. The cells were cultured in 50 x 20 mm polystyrene tissue culture flasks (Nunc Plastics, Roskilde, Denmark) at 33°C in fully humidified air containing 5% CO₂. Once a week, half of the culture medium and nonadherent cells were harvested and centrifuged (1,000 g, ten minutes). The supernatants were harvested and stored at −80°C until used. The pellet, containing the nonadherent cells, was resuspended in fresh culture medium with or without IL-1 (30 U/mL), and returned to the culture flask. At weekly intervals during a period of 5 weeks, one culture was sacrificed and the total number of cells and hematopoietic progenitor cells, present in the adherent and the nonadherent cell fractions, were determined. For this purpose, the nonadherent cells were harvested first. The adherent cells were subsequently detached from the bottom of the flasks by the use of a rubber policeman. Single cell suspensions were obtained by vigorous and repeated shaking.

As controls, cultures were included that were manipulated identically, but from which either the stromal layer or the added cryopreserved bone marrow cells were omitted.

Staining of Cells

To assess the granulocytic, eosinophilic, or monocyte-macrophage lineage, cells harvested from long-term cultures were stained with naphthol-AS-D-chloroacetate, Luxol fast blue, and alpha-naphthylbutyrate esterase, respectively.

CFU-GEMM Cultures

The harvested adherent or nonadherent cells were cultured for CFU-GM (defined as aggregates of more than 20 granulocytic, eosinophilic, or mononuclear cells), BFU-E (defined as bursts of colonies consisting of hemoglobinized cells), and CFU-GEMM (defined as colonies containing at least both erythroid and myeloid cells), at a concentration of 10⁶ cells/mL in 1 mL medium consisting of 30% fresh (frozen) ABO-compatible human heparin plasma, 7.5% phytohemagglutinin leukocyte-conditioned medium (PHA-CM), 5% 10⁻¹ mol/L 2-mercaptoethanol, 5% deionized bovine serum albumin, 5% transferrin, 7.5% Iscove's modified Dulbecco's medium (IMDM), with 2 U/mL human recombinant erythropoietin (a kind gift from Organon Teknika B.V., Turnhout, Belgium), and 40% of a methylcellulose solution (2.8%) in IMDM, in 35-mm plastic dishes (37°C, fully humidified atmosphere, 5% CO₂). The number of CFU-GM, BFU-E, and CFU-GEMM colonies were counted on day 18.

Assay for CSA

The medium used for assays of CSA consisted of 30% alpha-MEM, 30% of a methylcellulose solution (3%) in alpha-MEM, 20% FBS (Rehatuin, Kankakee, IL), and 20% of the additives, as indicated in the Results section (ie, leukocyte-conditioned medium [LCM] or LT.BMC-conditioned medium or IL-1). The cultures were incubated at 37°C in fully humidified air with 5% CO₂. Sets of six replicate 0.1 mL aliquots, each containing 0.5 x 10⁶ mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells, were plated into the wells of flat bottom microtiter plates (Greiner K.G., Nüttlingen, West Germany). After ten days, the number of CFU-GM colonies were scored. Cultures, to which 20% crude LCM was added as a source for CSA, were included as positive controls, in order to define the 100% colony growth level. The number of colonies obtained in the presence of LCM were within the normal limits of our laboratory. To study the effect of stromal cell-conditioned media on colony formation, LCM was replaced by IL-1 or media conditioned by stromal cells in the presence or absence of IL-1. As a negative control, no LCM, stromal cell-conditioned medium, or IL-1 was added to the culture medium. Differences between paired observations were evaluated using the signed rank test.
RESULTS

Effect of IL-1 on Reinoculated Long-Term Bone Marrow Cultures

Number of cells. Reinoculated LTBMCs were established by adding T lymphocyte-depleted and mononuclear phagocyte-depleted bone marrow cells to stromal cell layers. These cultures were grown in the presence or absence of 30 U/mL of IL-1. The cumulative number of cells recovered over a 5-week period after the establishment of the cultures, exceeded the initial number of reinoculated cells by a factor of 8.13 ± 2.47 (mean ± SE, n = 3) in IL-1-stimulated cultures, and by a factor of 5.29 ± 1.34 in unstimulated ones. Thus, significantly more (P = .0002; Fig 1A) cells were recovered from IL-1-stimulated cultures; the increment was due to the adherent cell population (P = .0002; Figs 1B and C). Cytochemical staining of the cells recovered from the adherent cell fraction in reinoculated cultures indicated that the relative number of myeloid cells, eosinophilic cells, and mononuclear phagocytes were similar for IL-1-stimulated and unstimulated control cultures (data not shown).

Cultures established from equal numbers of mononuclear phagocyte- and T lymphocyte-depleted cryopreserved bone marrow cells in flasks without pre-established stromal cell layers yielded numbers of cells that were less clearly affected by stimulation with IL-1 (difference not significant; Table 1). Furthermore, significantly fewer cells (P = .003) were recovered from these cultures than from those established on stromal cell layers (Table 1).

Number of progenitor cells. The total number of hematopoietic progenitor cells (HPC), ie, CFU-GM, BFU-E, CFU-GEMM, that was recovered from the adherent and nonadherent cell fractions were similar for both IL-1-stimulated and unstimulated cultures (Table 1). The number of CFU-GM declined steadily with time, both in control and in IL-1-stimulated cultures. At week 1 after reinoculation, the number of adherent plus nonadherent CFU-GM that were recovered was 99% ± 92% (mean ± SD, n = 3) of the number of CFU-GM present in the reinoculated bone marrow for IL-1-stimulated cultures, and 78% ± 94% for unstimulated control cultures. At week 2 after reinoculation, the number of CFU-GM had declined to 20% ± 10% and 21% ± 12%, and at week 3 to 15% ± 5% and 13% ± 1%, respectively. The same was true for the number of BFU-E and CFU-GEMM (data not shown). These results indicate that the increased proliferation in IL-1-stimulated cultures did not occur at the expense of the number of progenitor cells. Results of control cultures showed that <10% of the total number of cells recovered were accounted for by adherent cells that originated from the stromal layer, before reinoculation (Table 1). In addition, stromal layers to which no bone marrow cells were added yielded no hematopoietic progenitor cells (Table 1), indicating that all hematopoietic progenitor cells originated from the reinoculated marrow cells. Cultures established from cryopreserved depleted bone marrow cells in flasks without pre-established stromal cell layers, yielded a number of HPC that were not significantly different, regardless of the addition of IL-1 (Table 1).

Levels of CSA. As expected, the levels of CSA in culture supernatants were significantly (P = .002) higher in supernatants from reinoculated cultures stimulated with IL-1, than in supernatants from unstimulated control cultures (Fig 2). Both in the presence of IL-1 and in its absence, supernatants from cultures recharged with mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells yielded CSA levels that were similar to those from the unrecharged cultures (data not shown). The CSA levels in supernatants from cultures of depleted bone marrow cells in the absence of pre-established stromal cell layers, were significantly higher in the presence of IL-1 than in unstimulated controls (P = .01), and similar to those in supernatants from unstimulated cultures, with a pre-established stromal cell layer (Fig 2).
Table 1. Effect of IL-1 on Cumulative* Recovery of Cells and Hematopoietic Progenitor Cells From Long-Term Bone Marrow Cultures, Established From Mononuclear Phagocyte- and T Lymphocyte-Depleted Bone Marrow Cells Seeded in Flasks With or Without Pre-established Stromal Layers

<table>
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<tr>
<th>Culture Conditions</th>
<th>Pre-established Stromal Layer Present</th>
<th>Bone Marrow Cells Added</th>
<th>IL-1 Present (30 U/mL)</th>
<th>Total Number of Cells</th>
<th>Cell Recovery†</th>
<th>Cell Recovery†</th>
<th>Cell Recovery†</th>
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<td>42 ± 10</td>
<td>20 ± 5</td>
<td>24 ± 6</td>
<td>39 ± 34</td>
</tr>
</tbody>
</table>

*Cumulative number of cells or hematopoietic progenitor cells recovered at weeks 1, 3, and 5 after (re)inoculation of depleted bone marrow cells.
†Percent of inoculum; (mean ± SE; n = 3).

Endogenous Production of IL-1 by Stromal Cells

To study the endogenous production of IL-1 in the culture system, supernatant fluids from unstimulated cultures were assayed for IL-1 alpha and beta. One week after the addition of cryopreserved bone marrow cells, IL-1 beta could be detected in the culture supernatants, regardless of the presence of a pre-established stromal layer (Table 2). In one supernatant containing the highest concentration of IL-1 beta, IL-1 alpha could also be detected. In contrast, no IL-1 alpha or beta was measured in supernatants from unstimulated control stromal layers that were not reinoculated (Table 2). During the second to fourth week after the addition of the cryopreserved bone marrow cells, no IL-1 was detected in the culture supernatants (Table 2).

DISCUSSION

In the present report, we studied the effect of IL-1-induced CSA production by bone marrow stromal cells on the proliferation of HPC in long-term bone marrow cultures. Active cell proliferation occurred in cultures recharged with mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells, both in IL-1-stimulated and in unstimulated cultures. The cumulative number of cells recovered during a 5-week period after reinoculation, exceeded the initial number of reinoculated cells by a factor of 8 or 5, depending on whether IL-1 had been present or not. The differences in the number of cells between IL-1-stimulated and control cultures was due to the adherent cell fraction, indicating that IL-1 stimulates proliferation of HPC in the stromal cell layer. Indeed, visual inspection of the cultures showed an increased cellularity in the IL-1-stimulated stromal layers. Control cultures without pre-established stromal cell layers showed that the reinoculated cryopreserved cells that had been depleted of accessory cells able to produce CSF had a limited proliferative capacity in the absence of stromal cells.

Differences in CSA between supernatants from reinoculated cultures stimulated with or without IL-1, paralleled those in cell numbers, i.e., CSA levels in the latter cultures were significantly lower. Similarly, cultures made without pre-established stromal cell layers yielded higher levels of CSA in the presence than in the absence of IL-1; the levels in the presence of IL-1 were similar to those in supernatants from unstimulated cultures reinoculated on stromal cell layers. In spite of that, significantly more cells were recovered from the latter cultures. These findings illustrate that the increased proliferation in IL-1-stimulated recharged cultures cannot merely be attributed to an increased release of CSA by bone marrow stromal cells. It was considered that residual HPC, originating from the first inoculum of bone marrow cells, had contributed to the total cell proliferation in the recharged cultures. However, we were unable to culture HPC from the stromal cell layers. Furthermore, we have previously demonstrated that irradiation of the stromal cell layers before reinoculation did not reduce the recovery of

![Fig 2. CSA in supernatants from long-term marrow cultures over a period of 5 weeks after reinoculation of mononuclear phagocyte- and T lymphocyte-depleted autologous cryopreserved bone marrow cells on pre-established stromal cell layers, in the presence (○) or absence (●) of IL-1 (30 U/mL). Triangles indicate CSA in supernatants from cultures without pre-established stromal cell layers in the presence (△) or absence (▴) of IL-1 (30 U/mL). Data are expressed as a percentage (mean ± SE; n = 3) of the number of colonies obtained in the presence of 20% leukocyte-conditioned medium.](image-url)
HPC from the cultures. These findings indicate that all HPC originated from the re inoculated marrow.

There are several additional influences that may also have contributed to the increased cell proliferation in the LTBMC system after stimulation by IL-1. One factor relates to possible cellular interactions between membrane-bound CSF on stromal cells, or CSF retained in the extracellular matrix and HPC. Another hematopoietic activity of IL-1 has been described in mice as hematopoietin-1, and consisted of a synergistic effect with the lineage-specific factor CSF-1 (M-CSF) on early HPC that could not yet respond to CSF-1 alone. In humans, preliminary data suggest that IL-1 may have a similar synergistic effect with GM-CSF or M-CSF on undifferentiated blast cell precursors that have an ability for self-renewal. Therefore, it should be considered that synergy between IL-1 and CSF has contributed to an increased cell recovery without compensatory decrease in the size of the HPC compartment. Production of other cytokines, eg, interleukin-6 (IL-6), are induced by IL-1 and that may modulate in vitro hematopoiesis, represent a third possible influence. IL-6 was found to act synergistically with IL-3 on the formation of murine spleen-cell derived blast cell colonies. Preliminary data suggest a similar synergistic effect of IL-6 on human multipotent progenitor cells. Since IL-6 production by fibroblasts and mononuclear phagocytes may be induced by IL-1, both the possible synergy of IL-1, with CSF and its CSF-inducing properties may be mediated by IL-6. Further studies are needed to test these hypotheses.

One week after reinoculation of unstimulated cultures, low levels of CSA were found in the culture supernatants. To study whether this initial CSA production could be due to endogenous production of IL-1, we measured IL-1 levels in culture supernatants, using specific radioimmunoassays. No IL-1 alpha or beta could be detected in supernatants from unstimulated, uncharged stromal layers. In contrast, 1 week after the addition of the cryopreserved bone marrow cells, IL-1 beta could be detected in the culture supernatants, independent from the presence of a pre-established stromal layer. However, since week 2, no IL-1 production could be demonstrated. Thus, IL-1 beta appears to be produced endogenously shortly after the addition of the cryopreserved bone marrow cells, suggesting a role for IL-1 in controlling hematopoiesis in the LTBMC system. This is in accordance with our previous observations that G-CSF production after initiation of long-term cultures diminished in the presence of an anti-IL-1 beta antiserum.

In conclusion, our studies show that IL-1 beta stimulates proliferation of HPC in long-term marrow cultures with the concurrent release of CSA from stromal cells, without diminishing the number of HPC. This further supports the role of IL-1 as an important regulator of hematopoiesis.

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