Constitutive and Induced Expression of Growth Factors in Normal and Chronic Phase Chronic Myelogenous Leukemia Ph1 Bone Marrow Stroma

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ABSTRACT

Study of growth factor mRNA levels in the stromal cells derived from the adherent layer of long-term bone marrow culture demonstrated constitutive expression of transforming growth factor β (TGF-β) and macrophage colony-stimulating factor. These cells did not express granulocyte colony-stimulating factor, granulocyte-monocyte colony-stimulating factor, interleukin (IL) 1α, IL-1β, IL-3, and IL-6. However, granulocyte colony-stimulating factor expression could be induced by recombinant human IL-1β; while IL-6 could be induced by both IL-1β and tumor necrosis factor-α. No differences could be detected between adherent layers established from normal and benign phase Ph1 chronic myelogenous leukemia bone marrow. The induced expression of TGF-β, a potent hematopoietic cell growth inhibitor, suggests that stromal cells play an inherent role in regulating the proliferation of adjacent bone marrow hematopoietic progenitor cells. However, a defect in stromal TGF-β production cannot account for the profoundly expanded myeloid compartment in chronic phase chronic myelogenous leukemia. In contrast to the constitutive expression of TGF-β, and macrophage colony-stimulating factor, hematopoietic growth factors are only expressed following a proper stimulation.

INTRODUCTION

Long-term bone marrow culture, developed by Dexter et al. in 1974, provided experimental hematologists with a vehicle to examine the interplay between hematopoietic progenitor cells and bone marrow stroma (1). The adherent layer in such a system is necessary to maintain in vitro long-term hematopoiesis (2, 3), and appears therefore to possess part of the regulatory "machinery" which maintains hematopoiesis. It may also harbor some of the hematopoietic stem cells. Thus, study of the adherent layer allows further analysis of the cellular and molecular mechanisms regulating blood cell production.

Chronic myelogenous leukemia is a clonal neoplasm in which malignant transformation occurs in a pluripotent hematopoietic progenitor cell (4). As a result, there is a massive expansion of myeloid stem cells and their progeny. Comparative studies of the in vitro growth characteristics of normal and CML progenitor cells have suggested that discordant maturation is the primary biological defect in CML (5). When the long-term culture system has been established from Ph1-positive CML bone marrow, it became evident that the malignant cell population declines in the adherent layer, whereas the cytogenetically normal progenitor cells remain in the system (6).

Recent investigations suggest that the interaction between bone marrow stroma and hematopoietic cells may be mediated by the release of growth factors from the stromal compartment (7). Therefore, in the current studies, we have characterized the long-term bone marrow culture system obtained from normal and chronic phase CML patients, and utilized it to examine the effects of cytokines on the production of growth factors by bone marrow stromal cells.

MATERIALS AND METHODS

Bone Marrow Culture. Bone marrow samples from normal donors and CML Ph1 patients were collected by posterior iliac crest aspirations following skin infiltration with 0.5% Xylocaine for local anesthesia. All CML patients were either untreated or at least 4 weeks off any form of therapy. As controls, samples were taken from untreated metastatic breast cancer patients without marrow involvement when marrow was collected for autologous transplantation. Sample collections were performed at M. D. Anderson Cancer Center in accordance with institutional guidelines; all donors gave informed consent.

Long-term bone marrow cultures were established as described (8). Light density bone marrow cells were isolated on 1.077 g/ml Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ; Winthrop Laboratories, New York, NY) density gradient centrifugation. Bone marrow cells were suspended at a final plating concentration of 5 x 10⁶/ml in 150/75/25-cm² tissue culture flasks (Corning Glass Works, Corning, NY), each containing 40/20/10 ml, respectively, of long-term culture growth medium. This consisted of α medium (Hasleton, Denver, CO) with 15% fetal calf serum (Whittaker, Walkersville, MD), or 12.5% fetal calf serum and 12.5% horse serum (Whittaker), with or without 1.7 x 10⁻⁷ M methylprednisolone (Upjohn, Irving, TX). Cultures were maintained at 37°C in humidified 5% CO₂ in air environment with complete replacement of medium weekly with the removal, at each medium change, of all the nonadherent cells. When cultures reached confluency (between 4 and 6 weeks), they were refed, and 24 h later, they were washed twice with Ca²⁺-, Mg²⁺-free phosphate-buffered saline (Gibco, Santa Clara, CA) to remove loosely adherent cells and were treated with 0.25% trypsin (Gibco) (9).

Adherent Layer Cell Characterization. To define the cellular composition of the adherent layer, and to define their analog to those described by others (2, 3, 7, 8), indirect immunofluorescence and cytochemistry were used. Tryptsinized adherent layer cells were placed on slides by cytocrontifugation, and were morphologically evaluated by using May-Grünwald-Giemsa stain. Monocytes were identified by butyrate esterase cytochemistry (10). The immunological phenotype of the cells was determined by using indirect immunofluorescence. For better visualization of cell outlines, the adherent layers of confluent cultures were detached with trypsin and recultured at 36°C in humidified 5% CO₂ in air after adjusting to 5 x 10⁶ cells in 0.5 ml of α medium, 15% fetal calf serum, and 1.7 x 10⁻⁷ M methylprednisolone in tissue culture chamber slides (Miles Scientific, Naperville, IL). Slides were fixed after coverage reached 50-70% confluence. The following monoclonal antibodies were used: a polyclonal rabbit antiserm to human fibronectin (Capell Laboratories, West Chester, PA), produced by both endothelial cells and fibroblasts (11); antisemur to human factor VIII-related antigen (DAKO Corp., Santa Barbara, CA) to iden-
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tify endothelial cells (12), and anti-Leu-M3 (Becton-Dickinson Monoclonal Center, Inc., Mountain View, CA) for monocytes/macrophages identification (13). For indirect immunofluorescence, the trypsinized cells were washed with phosphate-buffered saline (Gibco) and 10 μl of the first-stage antibody were then added to the cells for 30 min at 4°C. Cells were then washed in cold phosphate-buffered saline and incubated for 30 min with the second-stage fluorescein isothiocyanate-conjugated antibody. The cells were then counted in an immunofluorescence microscope. Irrelevant similar isotype antibodies were used as negative controls.

Analysis of bcr Rearrangement Studies. The molecular hallmark of CML Ph1 is the aberrant genomic configuration, bcr rearrangement (14). Therefore to evaluate the presence of leukemic hematopoietic cells in the long-term bone marrow cultures, DNA was prepared, as previously described (15), from CML Ph1 hematopoietic cells in the chronic phase of the disease, and from the cultured adherent layers of the same patients. Ten μg of DNA were digested with BamHI and BglII restriction endonucleases in conditions recommended by the supplier of the endonucleases (International Biotechnologies, Inc., New Haven, CT), electrophoresed in 0.8% agarose gel, blotted, and hybridized according to the method of Southern (16). The universal probe (Ph1- bcr-3' probe) was obtained from Oncogene Science, Inc. (Manhattan, NY) and labeled by oligoprimere extension to a specific activity of 1-3 x 109cpm/μg of DNA (17). After hybridization, the filters were washed at 60°C for 1 h in 0.1 x standard saline citrate solution (1 x standard saline citrate is 0.15 mol/liter sodium chloride, 0.015 mol/liter sodium citrate) containing 0.1% sodium dodecyl sulfate, dried, and autoradiographed.

Expression of Cytokines. To analyze cytokine (IL-1α, IL-1β, IL-6, GM-CSF, G-CSF, M-CSF, TGF-β1, and IL-3) mRNA expression, adherent cells were lysed either with 4 M guanidinium isothiocyanate (Bethesda Research Laboratories, Bethesda, MD) solution (18) or with Nonidet P-40 (Sigma, St. Louis, MO), efficient extraction of protein from the postnuclear cytoplasmic lysate was ensured by denaturation with sodium dodecyl sulfate and urea (19), and total RNA was prepared. To analyze GM-CSF mRNA expression, cultures were harvested 6 and 24 h after refeeding (20). To prepare a positive control for cytokine expression, RNA was extracted from fresh peripheral blood lymphocytes isolated on 1.077 g/mm2 Ficoll-Hypaque density gradient centrifugation and cultured with 15% phytahemagglutinin (Gibco) for 24 h at 37°C under humidified 5% CO2 in air conditions. The positive control for IL-3 expression was the 5637 bladder carcinoma cell line (ATCC, Rockville, MD). Preparation of complementary DNA probes and hybridization conditions used for the Northern analysis have been previously described (21).

To examine the modulation of growth factor expression in stromal cells, various cytokines were added to the cultures, 24 h before harvesting. These experiments were done with medium consisting of α medium with 15% fetal calf serum and without methylprednisolone. Recombinant α-interferon (Hoffmann-La Roche, Nutley, NJ) at a concentration range of 100 to 200 units/ml was added to duplicate samples of 4 normal and 5 CML bone marrows. This concentration was chosen because it has been previously demonstrated that 100 units/ml of interferon is sufficient to produce growth inhibitory effects (22). Recombinant human IL-1α (Hoffmann-La Roche) at a concentration of 50 units/ml, or hIL-1β (Cistron, Pine Brook, NJ) at a concentration of 50 units/ml were added to duplicate samples of 3 normal and 2 CML bone marrow cultures; these concentrations are optimal for stimulation of human endothelial cells to release GM-CSF and G-CSF (23-27). hTNF-α (Genentech, Inc.) at a concentration of 200 units/ml was added to duplicate samples of 4 normal and 2 CML bone marrows. It has been previously demonstrated that hTNF-α, at this concentration, stimulates GM-CSF mRNA and protein production in normal human lung fibroblasts and vascular endothelial cells (28, 29).

DNA Probes. Complementary DNA probes for hGM-CSF (30), hG-CSF (31), hIL-1α and hIL-1β (32), hIL-3 (33) (all from COS Laboratory, Denton, TX), h2'-5'-oligo(A) synthetase (34) (Dr. Chebath, Weizmann Institute, Israel), hTGF-β1 (35), hIL-6 (36) (both from Genentech, Inc., South San Francisco, CA), hMSCF (37) (Genetics Institute, Cambridge, MA) and β-actin (ATCC, Rockville, MD) have been described.

RESULTS

Long-term Bone Marrow Culture Conditions. Formation of an adherent layer was associated with rapid cell number decrease during the first week, followed by steady increase through weeks 2-5, when confluency was reached.

We examined long-term bone marrow cultures under conditions optimal for myelopoiesis, which require horse serum (39), or for B-lymphopoiesis, requiring fetal calf serum (40). Duplicate samples of adherent layers from one normal donor and one CML patient bone marrow with and without the addition of horse serum did not reveal any difference regarding morphological, cytochemical, or antigenic characterization as well as constitutive cytokine mRNA expression. We also examined adherent layers from 3 CML and 1 normal bone marrow cultures with the addition of methylprednisolone, which is known to enhance myelopoiesis (41, 42) and induce adipose cells (43), and found increased percentage (up to 20%) of adipocytes, examined by light microscopy, in comparison to the adherent layers cultured without methylprednisolone. In order to investigate the stromal layer alone, we elected to continue our experiments with α medium and 15% fetal calf serum without methylprednisolone.

Morphological and Cytochemical Assessment of Adherent Cell Layer. Three types of cells could be distinguished by Giemsa staining and routine light microscopy at the time of trypsinization: (a) an elongated cell, which accounted for 50-70% of the cells at the time of trypsinization; (b) a large, mononuclear cell with gray vacuolated cytoplasm, which accounted for 20-30% of the cells; and (c) adipocytes, visible on light microscopy and electron microscopy, which constituted 5-15% of the cell population; 5-20% of the cells stained positive for butyrate esterase, indicative of monocytic lineage; the elongated cells were negative for this stain.

Antigenic Characterization of Adherent Layer Cells. Endothelial cells and fibroblasts comprised 60-80% of the cells as demonstrated by the presence of a fine network of fibronectin coating the cells. The presence of factor VIII-related antigen indicated that 10-20% of the cells in the adherent layer were of endothelial origin. Monocytes were shown to comprise 3-5% of the adherent layer cells as demonstrated by staining with anti-Leu-M3. In summary, by morphological, specific cytochemical, and immunological methods the adherent layer was found to contain 60-70% fibroblastic cells, 10-20% endothelial cells, 3-5% monocyte/macrophages, and 5% fat-laden adherent cells.

Cytokine mRNA Expression in Adherent Layer Cells. Cytokine mRNA expression was studied in 8 normal and 11 CML chronic phase bone marrow cultures. TGF-β1 and M-CSF mRNA were constitutively expressed in all samples. This expression was not affected by culture conditions, with or without horse serum, or with or without methylprednisolone (Fig. 1). IL-3, GM-CSF, G-CSF, IL-1α, IL-1β, and IL-6 gene expression were not detected in adherent layers grown to confluency, and 24 h after medium replacement. GM-CSF gene expression was not detected even 6 h after medium replacement. GM-CSF, G-CSF, IL-1α, IL-1β were expressed in phytahemagglutinin-stimulated lymphocytes which served as a positive control and hIL-3 was expressed in RNA extracted from the bladder carcinoma 5637 cell line. There was no difference between normal and chronic phase CML regarding cytokine mRNA expression (Fig. 1).

Effect of Exogenous Cytokines on Hemopoietic Growth Factor Gene Expression by Adherent Cell Layer. Factors were added for 24 h after the adherent layer reached confluency. The results...
Expression of Growth Factors in Bone Marrow Stromal Cells

**Fig. 1.** Representative Northern blot analysis of RNA samples from long-term bone marrow stromal cells. The blots were hybridized with TGF-β (A) and with M-CSF (B). Lane 1, normal bone marrow stroma grown with 15% fetal calf serum; Lane 2, CML Ph1 bone marrow stroma grown with 15% fetal calf serum; Lane 3, normal bone marrow stroma grown with 12.5% fetal calf serum and 12.5% horse serum; Lane 4, normal bone marrow stroma grown with 15% fetal calf serum and 1.7 x 10⁴ units methylprednisolone. kb, kilobase.

Table 1: Effect of Cytokines on Bone Marrow Stromal Growth Factor Expression

<table>
<thead>
<tr>
<th>Cytokine added</th>
<th>Interferon</th>
<th>TNF-α</th>
<th>IL-1β</th>
<th>IL-1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>CE</td>
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<td>CE</td>
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*+, induced expression; -, not expressed; CE, constitutively expressed.

Fig. 2. Northern blot analysis of RNA samples from IL-1β-induced (Lane 2) and uninduced (Lane 1) long-term bone marrow stromal cells. Probes used were hG-CSF (top panel), hIL-6 (second panel), hIL-1β (third panel), and β-actin (bottom panel). kb, kilobase.

Fig. 3. Rearrangements in Adherent Layers and Hematopoietic Cells. bcr Rearrangements were not found in the hematopoietic or stromal cell DNA of two CML patients (data not shown). DNA extracted from two CML Ph¹-positive hematopoietic cells demonstrated bcr rearrangement with two restriction endonucleases, whereas no bcr rearrangement could be found in DNA extracted from the adherent layers of the same patients (Fig. 3). Since about 1–2% of bcr rearrangement-positive cells can be detected by this methodology (44), these results suggest that either Ph¹-positive hematopoietic progenitors constitute only a very small proportion of the population or that they are not present in the adherent layer.

Discussion

Our current studies provide evidence for the constitutive expression of TGF-β1 and M-CSF, but not of hematopoietic growth factors, G-CSF, GM-CSF, IL-1α, IL-1β, IL-3, and IL-6, in adherent layers from normal and chronic phase CML Ph¹ bone marrows. However, expression of IL-1α and IL-1β, G-CSF, and IL-6 could be induced by exposure of the stromal...
cells to specific cytokines (Table 1). TGF-β1, is known to be a potent inhibitor of hematopoietic progenitor proliferative response to hematopoietic growth factors (45). Further, exogenous addition of TGF-β1, in long-term bone marrow culture inhibits primitive hematopoietic progenitor cells from progressing in cell cycle following the addition of horse serum, myeloid growth factors, or other cell activators (46). It has also been reported that the high proliferative potential colony-forming cells, CFU-GEMM, BFU-E, and CFU-GM are inhibited by TGF-β1, whereas the more differentiated colony-forming cells, CFU-G, CFU-M, and CFU-E, are not (46). Therefore, our findings of uninduced TGF-β expression may be consistent with the concept, suggested by Eaves et al. (47), that the stromal cells have an important role in keeping the pluripotent stem cells in quiescent state and preventing exhaustion of the stem cell pool (48).

Our findings of the constitutive expression of M-CSF in bone marrow stromal cells is supported by previously published data (49) showing expression of this factor in monocytes, endothelial cells, and fibroblasts. Although the precise role of this molecule in the cytokine network is not clear, it has been demonstrated that M-CSF is essential for the survival of macrophages (50), and its continuous production by the adherent layer could provide sufficient M-CSF to fulfill this role (50).

hIL-1 has been reported to stimulate the transcription and release of GM-CSF and G-CSF in human dermal fibroblasts (51), as well as in stimulated human stromal fibroblast cell lines (52). Similarly, hTNF-α has been shown to elicit G-CSF and GM-CSF gene expression and activity in human lung fibroblasts (43) and in human endothelial cells (53). However, in our culture system we were unable to detect induction of GM-CSF or G-CSF after exposure to IL-1α or TNF-α. The discrepancy in results between our current experiments and prior publications is probably attributable to the study of fibroblasts cultured from fresh bone marrow samples rather than those derived from skin or lung tissues or from established cell lines. The restricted and only partially overlapping induction of myeloid growth factors by IL-1α, IL-1β, and TNF-α is suggestive of a system with “specific” roles for various cytokines.

Our findings concur with the model proposed by Eaves et al. (54), in which the stromal layer regulates hemopoiesis by a constant release of a growth inhibitory cytokine, TGF-β1, the effect of which can be overridden by the transient secretion of myeloid growth-stimulating peptides, which, however, is dependent on proper stimuli. Factors such as IL-1β may provide these putative stimuli and are likely to be generated during an infection or other inflammatory condition.

Different culture conditions with or without horse serum and methylprednisolone did not affect constitutive gene expression in the adherent layers. Two possible mechanisms may explain the myeloid growth-promoting effect of these agents. The first is a direct effect on myeloid progenitor cells, and the other is that while not affecting constitutive expression of myeloid growth factors, they may modulate release of these proteins from the stroma after specific stimuli.

We failed to find differences between the adherent layers of CML Ph1 in the chronic phase and normal controls as far as cellular composition and both constitutive and induced growth factor gene expression. It is therefore unlikely that the growth advantage of chronic phase CML Ph1 cells over normal hematopoietic cells stems from unregulated growth factor “support” by the bone marrow stroma. Although presumably the defect in CML is within the progenitor cells of the malignant clone, our studies do not rule out mechanisms such as in vivo tumor cell stimulation of stroma-generated cytokines, either directly or via release of other cytokines or an autocrine release of growth factors by the malignant clone. Alternatively, another growth factor, yet unknown, or altered adherence of Ph1 cells to bone marrow stroma may be operative in the deranged Ph1 cell growth (55).

Finally, α-interferon, a cytokine active in the clinical management of CML Ph1 (56), failed to induce changes in the adherent layers. Therefore, this agent probably mediates its salutary effect via direct growth inhibition of the malignant Ph1 cells, rather than through indirect effect on the stromal cells.

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