Alteration in Bone Marrow Adherent Layer Growth Factor Expression: A Novel Mechanism of Chronic Myelogenous Leukemia Progression

By Meir Wetzler, Razelle Kurzrock, David G. Lowe, Hagop Kantarjian, Jordan U. Gutterman, and Moshe Talpaz

Philadelphia chromosome\(^1\) positive (Ph\(^+\)) chronic myelogenous leukemia (CML) is characterized by metaphors of the chronic phase to blastic crisis. However, cellular events associated with this transition are poorly understood. To examine the possible participation of hematopoietic growth factors in this process, we studied growth factor expression in adherent layers of bone marrows derived from CML Ph\(^+\) patients in various stages of the disease. Interleukin-1 \(\beta\) (IL-1\(\beta\)) and IL-6 mRNA were expressed in five of six patients, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in one of six patients with myeloid/undifferentiated blast crisis. In addition, leukemia inhibitory factor (LIF) expression was increased in four of six patients with myeloid/undifferentiated blast crisis phase of the disease. IL-1\(\beta\) was also detected in bone marrow adherent layer conditioned medium from two of these patients. These results were in sharp contrast to the lack of detectable levels of uninduced IL-1\(\beta\), IL-6, and GM-CSF mRNA, in samples derived from 4 patients in lymphoid blastic crisis, 3 in accelerated, and 11 in chronic phases of the disease, or from normal controls. The possibility of a paracrine loop formation, whereby the adherent layers representing the bone marrow stroma are induced to express hematopoietic growth factors, was supported by our finding IL-1\(\beta\) mRNA expression in the leukemic blast cells in three of four studied patients in blast crisis and IL-1\(\beta\) protein production in seven of eight patients studied. Finally, coculturing CML blast crisis cells onto pre-established adherent layers induced the expression of both IL-1\(\beta\) and IL-6 genes. From this preliminary study, it appears that abnormal expression of growth factors is a common event with CML Ph\(^+\) progression. We hypothesize that IL-1\(\beta\) generated by the transformed malignant clone stimulates the marrow stroma to produce various growth factors, and that this process may play a role in disease progression.

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C HRONIC MYELOGENOUS leukemia (CML) is a paradigm of tumor progression. The disease starts in a chronic phase characterized by marked hyperplasia of myeloid cells that maintain their maturation capacity, and is easily controlled with therapy.\(^{1,2}\) This is followed by an accelerated phase in which the myeloid cells gradually lose their capacity for terminal differentiation. Basophilia, thrombocytosis, and cytogenetic clonal evolutions also appear. Finally, the disease terminates in a blastic phase, which is characterized by the rapid accumulation of cells that retain a "blast" morphology and is highly resistant to therapy.\(^{3-5}\) Blastic crisis can be classified as myeloid, lymphoid, or undifferentiated, based on morphologic, cytochemical, and immunologic features of the cells.\(^{6,7}\)

Genetic instability of the malignant clone is a characteristic of CML.\(^8\) Although the chronic phase is characterized in most cases by the Philadelphia chromosome (Ph) and the resultant BCR-ABL hybrid gene,\(^8\) it is generally believed that acquisition of additional genetic molecular abnormalities within the malignant clone herald phenotypic transformation. However, the information on specific acquired molecular abnormalities is rather limited. Sporadic reports have shown alteration in H-ras,\(^{10,11}\) myc,\(^12\) and P53\(^{13,14}\) during the progression of CML.

Long-term bone marrow culture, developed by Dexter and Lajtha in 1974, provides a means to examine the interplay between hematopoietic cells and bone marrow stroma.\(^{15}\) The adherent layer in such a system modulates in vitro long-term hematopoiesis\(^{16,17}\) and therefore appears to possess part of the regulatory "machinery" that maintains blood cell production. To date, there has been no data accrued which suggest bone marrow stromal involvement in CML progression. In this report we provide evidence for such changes occurring in the advanced stages of the disease.

MATERIALS AND METHODS

Bone marrow and peripheral blood samples. Bone marrow samples from normal donors and CML Philadelphia chromosome\(^1\) positive (Ph\(^+\)) patients were obtained by posterior iliac crest aspirations after skin infiltration with 0.5% lidocaine for local anesthesia. Control 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 (Houston, TX) in accordance with institutional guidelines, and following obtaining an informed consent from all participants. Light-density bone marrow cells and peripheral blood cells were isolated on 1.077 g/mL Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, NJ; Wintrop Laboratories, New York, NY) centrifugation. To avoid activation by purification procedure, samples were lysed with 1.22% ammonium oxalate and cell pellets collected. For granulocyte and lymphocyte purification, cells were further separated on Percoll (Pharmacia) discontinuous gradient.

Bone marrow cultures. The bone marrow cultures were established to support the adherent layers only.\(^{18}\) Bone marrow cells were suspended at final plating concentration of 5 \( \times 10^7 \) cells/mL in 150/75 cm\(^2\) tissue culture flasks (Corning Glass Works, Corning, NY), each containing 40/20 mL, respectively, of adherent layer culture growth medium. This consisted of a medium (Hazleton, Denver, CO) with 15% fetal calf serum (Whittaker, Walkersville, MD). Cultures were maintained at 37°C in humidified 5% CO\(_2\)-in-

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air environment with complete replacement of medium weekly with the removal, at each medium change, of all nonadherent cells. When cultures reached confluency (between 4 and 6 weeks), they were washed twice with Ca2+/Mg2+-free phosphate-buffered saline (GIBCO, Santa Clara, CA) to remove loosely adherent cells and treated with 0.25% trypsin (GIBCO).

Co-culture experiments. These experiments were designed to study the effect of the CML cells on stromal elements. Accordingly, 5 x 10^5 peripheral blood cells from patient F, with mixed phenotype blasts (Table 1), and from a patient in chronic phase were seeded onto adherent stromal elements. Accordingly, 5 x 10^5 peripheral blood cells from patient F, with mixed phenotype blasts (Table 1), and from a patient in chronic phase were seeded onto adherent stromal layers for the expression of bcr exon 2-ABL exon 2 and bcr exon 2-ABL exon 2 junctions, respectively, by polymerase chain reaction, as previously described.

Expression of cytokines. To analyze interleukin-1α (IL-1α), IL-1β, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), monocyte-CSF (M-CSF), leukemia inhibitory factor (ILF), transforming growth factor β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and IL-3 mRNA expression, adherent and leukemic cells were lysed by 4 mol/L guanidinium isothiocyanate (Bethesda Research Laboratories, Bethesda, MD) solution, and total RNA was prepared. To prepare a positive control for IL-1α, IL-1β, IL-6, GM-CSF, G-CSF, M-CSF, ILF, TGF-β1, and TNF-α expression, RNA was extracted from fresh peripheral blood mononuclear cells isolated on 1.077 g/mL Ficoll-Hypaque density gradient centrifugation and cultured with 1% phytohemagglutinin (GIBCO) for 24 hours at 37°C under humidified 5% CO2-in-air conditions. The 5637 bladder carcinoma cell line (American Type Culture Collection [ATCC], Rockville, MD) expresses IL-3 and served as control for this cytokine expression. Total RNA derived from adherent layer cells (40 µg per lane) and poly (A)+ selected mRNA (4 µg per lane) from leukemic cells were size separated in 1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized to P-labeled cDNA probes. Preparation of cDNA probes and hybridization conditions used for the Northern analysis have been previously described. The relative density of the signals was determined by laser densitometry using the Joyce Loebel Densimeter, Epphoretic (Vickers Instruments Inc, Malden, MA). The results are presented as percentage of maximum, after subtracting the reading of a chronic phase sample, from the chronic crisis sample performed on the same blot.

IL-1β enzyme-linked immunosorbent assay (ELISA). To demonstrate IL-1β protein in the adherent layers, the confluent adherent layers were refed with the standard culture medium 24 hours before harvesting, and 24 hours later the conditioned medium was pooled and 10-fold concentrated with an Amicon apparatus (Amicon Corp, Danvers, MA). To demonstrate IL-1β protein in fresh leukemic cells, peripheral blood cells, bone marrow cells, granulocytes, and lymphocytes were frozen and thawed three times in low-concentration phosphate-buffered saline, and supernatants were pooled. All samples were studied in duplicates by an ELISA kit (Cistron, Pine Brook, NJ) which uses a monoclonal antibody specific for IL-1β. The assay was read in a Molecular Devices' microplate reader (Palo Alto, CA).

DNA probes. cDNA probes for human (h) GM-CSF, hG-CSF, hIL-1α and hIL-1β, hIL-3 (COS Lab, Denton, TX), hILF, hTGF-β1, hIL-6 (Genentech Inc, South San Francisco, CA), hM-CSF (Genetics Institute, Cambridge, MA), hTNF-α, and β-actin (ATCC) have been previously described.

RESULTS

Bone marrow culture conditions. Variations in conditions used to establish long-term bone marrow cultures result in differences in the pattern of hematopoiesis observed. We have previously examined bone marrow adherent layer cultures under conditions optimal for myelopoiesis, which require horse serum and methylprednisolone, or

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**Table 1. Growth Factor Expression in CML Ph' Bone Marrow Culture**

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<th>Patient</th>
<th>Blastic Crisis Characterization</th>
<th>Growth Factor Expression</th>
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for B lymphopoiesis, which require fetal calf serum. We found no differences regarding morphologic, cytochemical, or antigenic characteristics. We have since studied bone marrow culture with fetal calf serum only, because under these conditions hematopoiesis decreases rapidly, enabling us to study the adherent layer itself. Formation of the adherent layer was consistent with rapid cell number decrease during the first week, followed by steady increase through week 5, when confluency was reached. This method was applied to study bone marrow adherent layers from 10 blastic crisis, 3 accelerated-phase, and 11 chronic-phase CML Ph' patients.

Coculture experiments. Low-density mononuclear cells obtained from patient F (Table 1) in blast crisis and from a patient in the chronic phase were seeded onto pre-established adherent layers derived from a normal donor. In neither case did the cells localize in the adherent layers or proliferate in culture, as observed by inverted microscopy. In addition, no cells were found in the nonadherent component of the culture after the first medium removal. Cultures established from the peripheral blood cells without the support of adherent layers died out after the first medium removal.

Morphologic diagnosis of the leukemic cells. Patients A through C (Table 1) were classified to have undifferentiated blast crisis, and patients D and E were classified as having myeloid blast crisis. Patient F was classified in mixed phenotype blast crisis, and patients G through J were classified in lymphoid blast crisis.

Analysis of bcr rearrangement studies. bcr rearrangement was not found in DNA derived from the adherent layers of patients A and D (data not shown) or in stromal cell DNA derived from 10 chronic-phase CML, whereas DNA extracted from the leukemic cells of the corresponding patients showed bcr rearrangement with two restriction endonucleases (data not shown). Unfortunately, no DNA was available on the other blast crisis patients. Likewise, no bcr rearrangement was found in the adherent layers derived from the coculture experiments (Fig 1). Because about 1% to 5% of bcr rearrangement-positive cells can be detected by this methodology, we used the polymerase chain reaction technique to show that the mRNA coding for bcr exon 2-ABL exon 3 was detected in the adherent layer derived from the coculture experiment (data not shown). These results suggest that Ph'-positive hematopoietic progenitors constitute only a very small proportion (<1% to 5%) of the population present in the adherent layer.

Cytokine mRNA expression in blast crisis adherent layer cells. IL-1β mRNA was expressed in five of six adherent layers established from CML myeloid/undifferentiated blast crisis samples (Fig 2), patients A through D, and F. In contrast, IL-1β was not discerned in adherent layers established from four individuals with lymphoid blastic crisis, nor from the 3 accelerated-phase, 11 chronic-phase, and 11 normal patient samples. Similarly, IL-6 mRNA was expressed in adherent layers of five patients in myeloid/undifferentiated blast crisis (A through D, and F), but not in the adherent layers of normal individuals nor in those of chronic- or accelerated-phase patients (Fig 3). Interestingly, the patient with undifferentiated blastic crisis who expressed the highest levels of IL-1β (patient A) also expressed GM-CSF. Constitutive LIF expression was found in all adherent layers from normal volunteers and chronic phase CML patients, in agreement with our previous results. However, four of five blast crisis patients who

![Fig 1. Southern blot analysis. The samples were hybridized with the universal bcr probe (Ph'/bcr, Oncogene Science, Inc). Restriction enzymes were BamHI (lanes 1 and 2) and BglII (lanes 3 and 4). Lanes 1 and 3, adherent layer from the coculture experiments; lanes 2 and 4, hematopoietic cells derived from the patient whose cells served for the coculture experiments. Kb, kilobase.](image)

![Fig 2. Expression of IL-1β mRNA in adherent layers established from CML patients. (a) The relative density of the IL-1β mRNA signals from adherent cells cultured from patients A through D, and F (Table 1) as determined by laser densitometry. Each lane was correlated to a control lane on the same blot. (b) RNA blot analysis of total RNA from adherent layers probed for hIL-1β. The dark area in the 1.0-kb region of lane 2 represents background noise and not an extra band, as verified by rehybridization of this blot (data not shown).](image)
had increased IL-1β expression also showed significantly increased LIF expression as compared with the chronic-phase patients (Fig 4). TNF-α, IL-3, IL-1α, or G-CSF were not detected in any of our samples, whereas TGF-β, and M-CSF mRNA were constitutively and equally expressed in chronic, accelerated, and blastic crisis CML, as well as in the adherent layer established from normal bone marrow (data not shown). No significant differences in β-actin signal intensity were observed, indicating that: (1) equal amounts of RNA had been loaded onto each lane, and (2) no RNA degradation has occurred (data not shown).

Bone marrow adherent cells from the coculture experiments with cells derived from patient F (Table 1) expressed IL-1β (data not shown) and IL-6 mRNA (Fig 5, lane 1), whereas control cultures with the same adherent layers maintained for the same length of time did not express these genes (Fig 5, lane 2). Likewise, the adherent layers cocultured with hematopoietic cells from a patient in the chronic phase did not express IL-1β or IL-6. In addition, both perturbed and control adherent layers expressed TGFβ, M-CSF, and LIF, whereas TNF-α, IL-3, IL-1α, and G-CSF were not detected. No significant differences in β-actin signal intensity were observed (Fig 5B).

IL-1β protein secretion from the bone marrow cultures. Patients A and B, who expressed the highest IL-1β mRNA levels, had 50 pg/mL and 17 pg/mL of IL-1β protein in 10X concentrated conditioned medium. IL-1β protein was not discerned in the media from any other patient, including the three other patients who also expressed IL-1β mRNA, probably because the protein levels were below the limits of sensitivity of this assay.

Cytokine expression and production in the hematopoietic blast cells. IL-1β mRNA was expressed in peripheral blood leukemic cells of three (A, B, and C) of four (A through D) patients in myeloid/undifferentiated blast crisis, while none expressed G-CSF, GM-CSF, IL-1α, LIF, or IL-6 (data not shown). No hematopoietic cells were available on any of the other patients for mRNA evaluation. However, cell pellets were available from patients A through C and E through H for protein determination. Interestingly, patients A and B and E through H (Table 1) produced high levels of IL-1β ranging from 500 to 1,000 pg/2.4 x 10⁷ cells. Patient C produced 14 pg/2.4 x 10⁷ cells of IL-1β. All patients in blastic crisis had 60% to 90% blasts in their peripheral blood at the time of sample collection; no correlation was found between blast percentage and IL-1β levels. Twenty-five normal controls, including nor-

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Fig 3. Expression of IL-6 mRNA in adherent layers established from CML patients. (a) The relative density of the IL-6 mRNA signals from adherent layer cells cultured from patients A through D, and F (Table 1), as determined by laser densitometry (for details see legend for Fig 2). (b) RNA blot analysis of total RNA from adherent layers bone marrow cultures probed for hIL-6.

Fig 4. Expression of LIF mRNA in adherent layers established from CML patients. (a) The relative density of the LIF mRNA signals from adherent layer cells cultured from patients A through D (Table 1), as determined by laser densitometry (for details see legend for Fig 2). (b) RNA blot analysis of total RNA from adherent layers bone marrow cultures probed for hLIF.

Fig 5. Northern blot analysis of RNA samples derived from adherent layer exposed to a coculture experiment with hematopoietic cells derived from patient F (lane 1) and the same adherent layer that was kept for the same length of time, without any perturbation, as control (lane 2). Probes used were hIL-6 (A) and β-actin (B). Kb, kilobase.
DISCUSSION

Interactions between hematopoietic stem cells and the bone marrow microenvironmeent play a major role in the proliferation and differentiation of blood cells. The present system was devised to evaluate the possible role of the microenvironment in myeloproliferator disorder progression. The fact that stromal cells produce growth factors, either uninduced or after stimulation, is known. Clonal cell lines derived from murine bone marrow stroma were found to produce pre-B cell growth factor(s) and human stromal cells were found to produce TGF-β and M-CSF. Normal human bone marrow fibroblast-conditioned medium suppresses proliferation of undifferentiated leukemia cells and stimulates growth of differentiated (M1 of French-American-British classification) leukemia cells. Furthermore, we have previously shown that adherent layers from CML patients in the chronic phase are indistinguishable from normal adherent layers.

The significance of our current results lies in the differences in stromal growth factor expression between the various forms of CML blast crisis and the various stages of the disease. Adherent layers derived from myeloid/undifferentiated blast crisis phase expressed IL-1β, IL-6, and GM-CSF, and showed increased expression of LIF, but adherent layers derived from lymphoid blast crisis, accelerated-phase, and chronic-phase bone marrow samples did not.

Of particular importance is the expression of IL-1β. This cytokine has pleiotropic activities, among which are induction of cytokines and adhesion molecules in fibroblasts and endothelial cells and increased expression of class I major histocompatibility complex antigens. It is also a potent inducer of hematopoietic growth factor expression in bone marrow adherent layers and augments its own expression. A possible role for IL-1β in the pathogenesis of myeloid neoplasia was first suggested with the detection of its secretion by some acute myelogenous leukemia cells. Here we show that bone marrow adherent cells from patients in myeloid/undifferentiated blast crisis express IL-1β, whereas adherent layers from patients in the chronic phase of the disease or from normal controls do not express IL-1β. Furthermore, the finding that Ph1-positive leukemic cells constitute less than 1% to 5% of the adherent layers, as determined by Southern blotting and polymerase chain reaction, indicates that IL-1β gene expression was derived from the adherent layers themselves and not from the hematopoietic cells residing in the adherent layer. Therefore, we conclude that either the neoplastic hematopoietic cells produce molecules that activate the adherent layer cells to release various cytokines or that the adherent layer cells are inherently defective in advanced disease. The first possibility is supported by the finding that IL-1β expression could be induced in normal adherent layers cocultured for 1 week with the leukemic cells derived from a patient in blast crisis. The second possibility, which we feel is less likely, nevertheless derives some support from findings in the literature that tumor stromal cells can acquire malignant phenotypic characteristics, ie, the stroma of cervical tumors has been previously observed to have some dysregulated growth patterns and a decreased serum dependency. In addition, human tumor cell lines in culture have been found to release TGFs that confer the transformed phenotype on untransformed fibroblasts.

The other growth factors that are expressed in the blast crisis adherent layers might have been induced by IL-1β as the initial stimulus. For instance, we have recently shown that LIF is constitutively expressed in adherent layers from normal volunteers, and that this expression can be significantly increased by exposure to IL-1β. Interestingly, in our system exposure of adherent layers derived from normal volunteers or chronic-phase CML patients to IL-1β results in induction of G-CSF, but not GM-CSF, mRNA. The presence of GM-CSF, but not G-CSF, message in our IL-1β-producing adherent layers from a blast crisis patient is unexplained, but could further reflect a perturbed growth factor expression pattern of these cells that is related to the disease process. Each induced cytokine may have an independent role in modulating hematopoiesis. For instance, infection of a factor-dependent murine cell line with a retroviral construct containing the GM-CSF cDNA led to the emergence of autonomous, GM-CSF-producing sublines that were uniformly leukemogenic when transplanted to syngeneic recipients. IL-6 provides a proliferative stimulus to several types of hematopoietic cells and may act as a growth factor in multiple myeloma. Finally, LIF inhibits growth and induces macrophage differentiation of the M1 leukemic cell line, but stimulates growth of IL-3-dependent DA-1 leukemic cells.

Our results showing expression of IL-1β, IL-6, and GM-CSF and increased expression of LIF mRNA in adherent layers established from blast crisis CML possibly suggest a new mechanism of CML progression. This model envisages the leukemic cells to be in intimate contact with regulatory stromal cells of nonhematopoietic mesenchymal origin. Factors generated by the leukemic cells may influence the activity of the microenvironment and, in turn, direct interactions between the regulatory mesenchymal cells, and the leukemic cells determine the proliferative and differentiation state of the latter. The fact that samples derived from lymphoid blast crisis patients were devoid of growth factor expression, which was detected in the myeloid blast crisis samples, suggests that disease progression in the various phenotypic subsets of the disease may be governed by a different mechanism.

Finally, it has been recently shown that, in allogeneic marrow transplantation, host bone marrow stromal cells may regenerate and repopulate the bone marrow or exist for protracted periods after transplantation. Our current results, suggesting a possible role for the stroma in blast crisis phase of CML, might also provide an explanation for the poor results of marrow transplantation in this phase of the disease.

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