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

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Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML) is characterized by metamorphosis 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-1β (IL-1β) 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-1β 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-1β, 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-1β mRNA expression in the leukemic blast cells in three of four studied patients in blast crisis and IL-1β 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-1β 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-1β 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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CHRONIC 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 cyto genetic 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.3,4 Blastic crisis can be classified as myeloid, lymphoid, or undifferentiated, based on morphologic, cytochemical, and immunologic features of the cells.5,6

Genetic instability of the malignant clone is a characteristic of CML.7 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 P5313,14 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 hematopoiesis16,17 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-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 × 10^6/mL in 150/75 cm² 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% CO₂-in
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). When cultures reached confluency (between 4 and 6 weeks), they were seeded onto adherent As control, the pre-established layers were maintained without the addition of the peripheral blood cells without the support of adherent cells. The cultures were treated once a week by removal of all of the growth medium, including the nonadherent cells, and the addition of fresh medium. Cultured adherent layer cells were harvested after 3 weeks of coculturing. Subsequently, the adherent cells were analyzed for mRNA expression and DNA was extracted for bcr rearrangement studies.

Morphologic cytochemical diagnosis of blast cells. To define the cellular characteristics of the blastic crisis cells, biochemical and enzymatic stains were used, as previously described. Myeloperoxidase-positive cells were classified as myeloid blast crisis. Also, a peroxidase-negative but chloroacetate or nonspecific esterase-positive blast cells were classified as myeloid. Peroxidase-negative terminal deoxy nucleotyl transferase (TdT) enzyme-positive blast cells were categorized as lymphoid blasts, whereas undifferentiated blast cells could not be categorized on that basis of cytochemical analysis.

Analysis of bcr rearrangement studies. The molecular hallmark of CML Ph™ is the aberrant genomic configuration: bcr rearrangement. Therefore, to evaluate the presence of leukemic hematopoietic cells in the adherent layers bone marrow cultures, DNA was prepared, as previously described, from CML Ph™ hematopoietic cells in blastic crisis and from the cultured adherent layers of the same patients. Ten micrograms of DNA was digested with BamHI and PstI 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. The universal probe (Ph′/ber-3′ probe) was obtained from Oncogene Science, Inc (Manhasset, NY) and labeled by oligoprimers exten-
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 blast 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 blast 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
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 TGF-β, 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-
We feel is less likely, nevertheless derives some support from a patient in blastic crisis. The second possibility, which disease. The first possibility is supported by the finding that layers cocultured for 1 week with the leukemic cells derived IL-lp expression could be induced in normal adherent cells. Adherent layer cells are inherently defective in advanced hematopoietic cells residing in the adherent layer. derived from the adherent layers themselves and not from chain reaction, indicates that IL-lp gene expression was 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 blastic 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 blastic 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 blastic 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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