Abnormal Function of the Bone Marrow Microenvironment in Chronic Myelogenous Leukemia: Role of Malignant Stromal Macrophages

By Ravi Bhatia, Philip B. McGlave, Gordon W. Dewald, Bruce R. Blazar, and Catherine M. Verfaillie

The bone marrow microenvironment supports and regulates the proliferation and differentiation of hematopoietic cells. Dysregulated hematopoiesis in chronic myelogenous leukemia (CML) is caused, at least in part, by abnormalities in CML hematopoietic progenitors leading to altered interactions with the marrow microenvironment. The role of the microenvironment itself in CML has not been well characterized. We examined the capacity of CML stroma to support the growth of long-term culture-initiating cells (LTIC) obtained from normal and CML marrow. The growth of normal LTIC on CML stroma was significantly reduced compared with normal stroma. This did not appear to be related to abnormal production of soluble factors by CML stroma because normal LTIC grew equally well in Transwells above normal stroma as in Transwells above normal stroma. In addition, CML and normal stromal supernatants contained similar quantities of both growth-stimulatory (granulocyte colony-stimulating factor (CSF), interleukin-6, stem cell factor, granulocytemacrophage CSF, and interleukin-1β) and growth-inhibitory cytokines (transforming growth factor-β, macrophage inflammatory protein-1α, and tumor necrosis factor-α). The relative proportion of different cell types in CML and normal stroma was similar. However, polymerase chain reaction and fluorescence in situ hybridization studies showed the presence of bcr-abl-positive cells in CML stroma, which were CD14+ stromal macrophages. To assess the effect of these malignant macrophages on stromal function, CML and normal stromal cells were separated by fluorescence-activated cell sorting into stromal mesenchymal cell (CD14+) and macrophage (CD14+) populations. CML and normal CD14+ cells supported the growth of normal LTIC equally well. However, the addition of CML macrophages to normal or CML CD14+ mesenchymal cells resulted in impaired progenitor support. This finding indicates that the abnormal function of CML bone marrow stroma is related to the presence of malignant macrophages. In contrast to normal LTIC, the growth of CML LTIC on allogeneic CML stromal layers was not impaired and was significantly better than that of normal LTIC cocultured with the same CML stromal layers. These studies demonstrate that, in addition to abnormalities in CML progenitors themselves, abnormalities in the CML marrow microenvironment related to the presence of malignant stromal macrophages may contribute to the selective expansion of leukemic progenitors and suppression of normal hematopoiesis in CML.

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THE BONE MARROW (BM) microenvironment plays an important role in supporting and regulating the proliferation and differentiation of hematopoietic cells. The microenvironment is a complex system consisting of a variety of cell types, including stromal cells of nonhematopoietic, mesenchymal origin as well as hematopoietically derived stromal macrophages producing extracellular matrix components and hematopoietic growth factors. Hematopoietic progenitor cells in the marrow are in close contact with stromal elements and are subject to regulatory influences arising from the marrow microenvironment.1

Chronic myelogenous leukemia (CML) is a malignancy arising from the hematopoietic stem cell. It is characterized at the cytogenetic level by the Philadelphia chromosome (Ph) resulting from a translocation of chromosomes 9 and 22 [(t:9:22)] and at the molecular level by the bcr/abl gene rearrangement.2 Clinically, CML is characterized by an abnormal expansion of malignant bcr/abl-positive myeloid cells. The abnormal hematopoiesis in CML is related, at least in part, to abnormalities in the hematopoietic cells themselves leading to abnormal interactions between CML progenitors and stroma. Normal primitive progenitors adhere well to stroma and are usually quiescent when in contact with stroma.3 In contrast, CML progenitors show reduced adhesion to normal stromal layers4,5 and are continuously proliferating even when in contact with stroma.6 This may result from the abnormal function of important cytoadhesion receptors on CML progenitors.5,6 It is not clear if and how the CML microenvironment contributes to dysregulated hematopoiesis in CML.

Tissue macrophages are derived from monocytes that originate from narrow hematopoietic progenitors. Because monocytes from patients with CML are derived from the malignant bcr/abl-positive clone, it is likely that a proportion of CML stromal macrophages may be of malignant origin. Indeed, pulmonary alveolar macrophages have been shown to be derived from the malignant clone in patients with CML.9 We hypothesized that CML marrow stromal macrophages may be derived from the bcr/abl-positive clone, which may result in abnormalities in the function of CML BM stroma. Consequently, abnormal stromal function in CML may in part underlie the hematopoietic abnormalities observed in CML.

In this report, we characterized the function of the hematopoietic microenvironment in CML. We show that CML marrow stroma has a reduced ability to support growth of normal progenitors cultured in contact with stroma. Cells of malig-
DEFECTIVE MARROW STROMAL FUNCTION IN CML

MATERIALS AND METHODS

BM Samples

Twenty-nine patients with CML and 25 normal healthy volunteers were studied. Patient characteristics are summarized in Table 1. Twenty-eight patients were in the chronic phase of CML. One patient had received interferon-α in the past but had not received any other treatment or was being treated with hydroxyurea only. Five patients had accelerated phase of CML. Twenty-three patients had either never received chemotherapy or whose BM stroma was studied only for cellular composition was in an accelerated phase of CML. Twenty-nine patients with CML and 25 normal healthy volunteers were studied. Patient characteristics are summarized in Table 1.

Preparation of BM Stromal Layers

BM stromal layers were formed after 4 to 5 weeks of culture and were irradiated at 1.250 cGy using a Cesium irradiator to eliminate hematopoietic cells. Adherent cells, collected after digestion with 0.1% collagenase (Boehringer Mannheim, Indianapolis, IN), were subcultured at a concentration of 350,000 stromal cells/well in 24-well plates or 37,500 cells/well in 96-well plates (Costar, Cambridge, MA).4

Selection of Purified Progenitor Populations

Purified normal and CML progenitor populations were obtained from BMNC using methods previously described.4,11,12 Lineage negative cells were obtained from BMNC by sequential counterflow centrifugation elutriation,11,12 sheep erythrocyte rosetting, and immunomagnetic bead depletion.12 Alternatively, CD34+ cells were selected from BMNC using avdin-biotin immunoabsorption columns (CellPro Inc, Bothell, WA). The resultant populations were labeled with anti-CD34-phycocerythrin (PE) and anti-HLA-DR-fluorescein isothiocyanate (FITC) antibodies (Becton Dickinson, San Jose, CA) and sorted on a FACStarPlus laser flow cytometry system (Becton Dickinson) equipped with a CONSORT 32 computer system. Cells were selected for low vertical and horizontal light scatter properties and for expression of CD34 and HLA-DR antigens based on isotype control stains.

Progenitor Culture

Stromal Cultures

Stroma contact cultures. Normal CD34+HLA-DR- (DR-) or CML CD34+HLA-DR- (DR-) cells were plated in limiting dilutions on normal or stromal stroma subcultured in 96-well plates (300, 100, 33, or 11 cells/well; 22 replicates per concentration). The absolute number of long-term culture-initiating cells (LTC-IC) was calculated as the reciprocal of the concentration of test cells that gave 37% negative wells, using Poisson statistics.15,16 The selection of primitive normal and CML progenitors was also evaluated by plating 5,000 normal DR- or CML DR+ cells in direct contact with normal or stromal stroma layers (or sorted CD14+ and CD14- stromal cells [see below]) subcultured in 24-well plates.4,11 The number of colony-forming cells (CFC) generated after 5 weeks in stroma contact cultures then serves as a measure of the growth of LTC-IC. Results from bulk stroma contact cultures correlated well with the results of limiting dilution analyses.

Stroma noncontact cultures. A collagen-coated transwell insert with a 0.4-μm microporous filter membrane (Costar, Cambridge, MA) was placed above CML or normal irradiated stroma. Five thousand normal DR- or CML DR+ cells were plated in the Transwell insert and cultured for 5 weeks, after which CFC generation was evaluated.17

Maintenance of cultures. Cultures were maintained for 5 weeks in a humidified atmosphere at 37°C and 5% CO2. Weekly media changes were performed by removing half the cell-free supernatant medium from stroma contact cultures and replacing it with fresh LTBMC medium. For stroma noncontact cultures, half the cell-free supernatant from the bottom well only was removed and replaced with fresh medium.

Evaluation of cultures. Cells harvested from stroma contact cultures after brief trypsinization or from transwell inserts of stroma noncontact cultures were replated in methylcellulose progenitor culture media supplemented with 3 IU recombi-

<table>
<thead>
<tr>
<th>Table 1. Characteristics of CML Patients</th>
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<tr>
<td>Characteristic No.</td>
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<tr>
<td>Disease stage</td>
</tr>
<tr>
<td>Chronic phase</td>
</tr>
<tr>
<td>&lt;1 yr</td>
</tr>
<tr>
<td>&gt;1 yr</td>
</tr>
<tr>
<td>Accelerated phase</td>
</tr>
<tr>
<td>Blast crisis</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>Busulfan</td>
</tr>
<tr>
<td>Interferon-α</td>
</tr>
<tr>
<td>BM fibrosis</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Minimal</td>
</tr>
<tr>
<td>Mild-moderate</td>
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*Stopped 4 months before study.
†Stopped more than 1 month before study.
nant erythropoietin (Epoetin; Amgen, Thousand Oaks, CA) and 4 ng/mL recombinant interleukin-3 (IL-3; a kind gift from Dr G. Wong, Genetics Institute, Boston, MA) as previously described. After 14 to 18 days, the cultures were assessed for the presence of colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-Mix colonies.

Measurement of Cytokine Production by Stromal Layers

Multiple aliquots of cell-free supernatants were collected from irradiated CML and normal BM stromal layers and from fluorescence-activated cell sorting (FACS)-sorted normal and CML CD14+ and CD14− stromal populations (see below) and were frozen at −70°C. Standard enzyme-linked immunosorbent assays (ELISAs) for granulocyte colony-stimulating factor (G-CSF), IL-6, stem cell factor (SCF), macrophage inflammatory protein-1α (MIP-1α), IL-3, platelet-derived growth factor AB (PDGF-AB; R&D, Minneapolis, MN), granulocyte-macrophage colony-stimulating factor (GM-CSF; Endogen, Boston, MA), transforming growth factor-β1 (TGF-β1; Genzyme, Cambridge, MA), and tumor necrosis factor-α (TNF-α; Boehringer Mannheim) were performed per the manufacturer’s recommendations.

Evaluation of Cellular Composition of CML and Normal Stroma

CML and normal irradiated stromal cells were harvested by collagenase treatment. Cytospin preparations were stained with Wright-Giemsa to examine the general morphology and with cytochemical stains for nonspecific esterase, alkaline phosphatase, and Oil-Red-O. Stromal macrophages, fibroblasts, and adipocytes were identified on the basis of cellular morphology and cytochemical staining characteristics. Stromal macrophages were identified as cells with round, heterochromatic nuclei and vacuolated cytoplasm that stained brightly positive with nonspecific esterase. Stromal mesenchymal cells were identified as cells with larger, irregular euchromatic nuclei and blue cytoplasm that showed positive membrane staining with alkaline phosphatase. Adipocytes were identified as cells containing fat which stained positively with Oil-Red-O.

Evaluation for bcr/abl Gene Rearrangement in CML Stromata

Polymerase Chain Reaction (PCR) Analysis

Irradiated CML stromal layers containing 300,000 cells each were harvested by treatment with Trypsin and frozen at −70°C in phosphate-buffered saline (PBS). Total cellular RNA was extracted from thawed samples using the method of Chomczynski and Saachi. After reverse transcription, amplification of bcr/abl mRNA was performed by PCR using nested primers. Amplified samples were size separated by gel-electrophoresis, ethidium bromide stained, and photographed. Beta-actin controls were run for each sample. Amplified cDNA fragments were also electrophoretically transferred to a Nytran nylon membrane (Schleicher and Schuell, Keene, NH). Antisense oligonucleotide probes complementary to the bcr exon 2/abl exon 2 (B2/A2) and bcr exon 3/abl exon 3 (B3/A3) were end-labeled using TDT (Bethesda Research Laboratories, Gaithersburg, MD) and 32P-adenosine triphosphate (DuPont/Nexgene Nuclear, Wilmington, DE) and hybridized to the membrane. After initial hybridization with the B2/A2 probe, membranes were stripped and reprobed with B3/A2. CML-derived K562 cells were used as positive controls and Raji cells and normal stromal cells were used as negative controls.

Fluorescent In Situ Hybridization (FISH) Analysis

Irradiated CML stromal layers were harvested by collagenase treatment. In some experiments, collagenase digested stromal layers were stained with anti-CD14 monoclonal antibody (Becton Dickinson) and FACS sorted to obtain CD14+ (macrophage-depleted stromal cell) populations (see below). FISH analysis for the rearranged bcr/abl fusion gene was performed using probes for the abl oncogene and the Mbc region (Oncor, Gaithersburg, MD) to detect t(9;22)(q34;q11.2) in interphase cells. The abl probe consisted of two overlapping biotin-labeled cosmids that hybridize to 9q34. The Mbc probe consisted of three overlapping digoxigenin-labeled cosmids that hybridize to the Mbc at 22q11.2. The hybridization, washing, and analysis procedures have been described elsewhere. With this method, the abl probe had a green signal, the bcr probe had a red signal, and the bcr/abl fusion resulted in a fused red-green signal, which sometimes appeared yellow. Only interphase nuclei in which it was possible to account for both copies of the bcr and abl probes were scored. For each specimen, up to 200 consecutive qualifying interphase nuclei were scored and the final result was expressed as the percentage of cells with an apparent bcr/abl fusion signal. We have previously established the normal range for this probe mixture and used a cutoff of ≥10% interphase cells with apparent bcr/abl fusion to classify any specimen as abnormal.

Separation of Stromal Macrophage and Mesenchymal Cell Populations

Stromal cell suspensions were prepared by collagenase treatment of nonirradiated stromal adherent layers from CML and normal LTBMCS. Cells were suspended in IMDM supplemented with 5% fetal bovine serum and EMDA (2.5 mmol/L), incubated with mouse Ig for 30 minutes to block nonspecific antibody binding, and then incubated for 30 minutes with either anti-CD14PE (Becton Dickinson) or control mouse IgG2bPE. Antibody-labeled cells were filtered through a Nitex mesh (100-μm pure size; Teto Inc, Monterey Park, CA) to remove cell aggregates and then FACS sorted into CD14+ and CD14− populations. Normal and CML CD14+ and CD14− cells were either plated separately in wells of 24-well plates at a concentration of 50,000 cells per well or 50,000 CD14+ and 50,000 CD14− cells were mixed together to reconstitute normal or CML stroma. Alternatively, 50,000 normal CD14+ cells were mixed with 50,000 CML CD14+ cells and 50,000 CML CD14− cells to make hybrid stromal layers. The cells were grown in LTBMCS medium for 3 to 4 weeks until CD14+ cells reached maximal confluence and were then irradiated at 1,250 cGy.

Statistical Analysis

Results of data obtained from multiple experiments were reported as the mean ± 1 SEM. Significance levels were determined by two-sided Student’s t-test analysis.

RESULTS

Preparation of Stromal Layers

Stromal layers were prepared by plating normal or CML BM in LTBMCS for 4 to 5 weeks until a confluent adherent stromal layer was formed. The formation of confluent stromal layers from CML BM required a higher number of cells (1.6 × 10^7/cm^2) than was required for the formation of normal stromal layers (1 × 10^7/cm^2). Confluent layers were irradiated at 1,250 cGy to eliminate hematopoietic cells. Irradiated stromal layers were harvested with collagenase and subcultured in 24-well and 96-well plates using a constant number of stromal cells per well (350,000 and 37,500 cells per well, respectively) to ensure comparability between normal and CML stroma.

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Growth of Normal Primitive Progenitors on CML Stroma

To assess the growth of normal LTC-IC on CML stroma, normal DR<sup>-</sup> cells were plated in limiting dilutions on CML stroma and normal stroma subcultured in 96-well plates and grown in LTBMC for 5 weeks to assess the absolute number of LTC-IC that could be induced to grow. These studies showed that the number of LTC-IC induced to differentiate/proliferate by CML stroma was significantly lower than that induced by normal stroma (P < .03; Fig 1). In addition, 5,000 normal DR<sup>-</sup> cells were plated on CML and normal stromal layers subcultured in 24-well plates and grown in LTBMC for 5 weeks. A significantly reduced number of CFC was produced after 5 weeks of culture on CML stroma compared with normal stroma (P < .001; Fig 1). The average number of CFC generated from each LTC-IC (calculated by dividing the number of CFC generated from 5,000 DR<sup>-</sup> cells after 5 weeks of LTBMC by the number of LTC-IC detected by LDA within 5,000 DR<sup>-</sup> cells) for normal stroma (5.0 ± 0.9) was not significantly different than that for CML stroma (5.7 ± 1.0). The presence of CML stromal cells did not affect the growth of normal CFC in the methylcellulose progenitor assay (134.0 ± 20.7 and 141.6 ± 17.1 BFU-E, 83.9 ± 4.5 and 81.3 ± 7.0 CFU-GM, and 18.9 ± 1.2 and 19.4 ± 0.8 CFU-Mix per 5,000 DR<sup>-</sup> cells in the presence of CML and normal stroma, respectively). Although there was variability in the LTC-IC frequency in progenitor populations obtained from different normal donors, normal progenitors from a single donor showed similar growth on different normal stromal layers. Experiments evaluating the hematopoietic supportive capacity of CML stroma were therefore always performed concomitantly with normal stroma controls. The progenitor growth on each individual CML stroma studied was expressed as a percentage of that on the corresponding normal stroma control for that experiment. These studies suggest therefore that growth of normal primitive progenitors is impaired when cocultured with stroma from certain CML patients. There was considerable variability in the LTC-IC supportive capacity of individual CML stromal layers (Fig 1). In this limited sample size, we did not find a correlation
Transwell inserts with a 0.4-μm microporous membrane, rise to similar numbers of CFC when cultured in Transwells which prevents cell contact with the underlying CML or of marrow fibrosis with the functional capacity of the stromal layers.

Interestingly, normal DR- cells gave stroma noncontact cultures than in stroma contact cultures were generated when normal DR- cells were cultured in fresh LTBMC medium, with the exceptions of TGF-α, IL-1, and PDGF were undetectable in both (Table 2). The sensitivities of the cytokine assays were as follows: IL-6, 0.35 pg/mL; G-CSF, 10.9 pg/mL; GM-CSF, 5 pg/mL; SCF, 3 pg/mL; TNF-α, < 10 pg/mL; IL-1β, 0.3 pg/mL; PDGF, 8.4 pg/mL; TGF-β, 50 pg/mL; MIP-1α, 2 pg/mL/L. Cytokines were undetectable by ELISA in fresh LTBMC medium, with the exceptions of TGF-β (2.1 ng/mL) and GM-CSF (6 pg/mL/L). Values are the mean ± SEM with the range in parentheses.

Table 2. Cytokine Production by CML Stroma as Compared With Normal Stroma

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CML Stroma</th>
<th>Normal Stroma</th>
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<tr>
<td>IL-6 (ng/mL)</td>
<td>1.4 ± 0.3 (0.6-2.7)</td>
<td>0.8 ± 0.3 (0.1-2.2)</td>
</tr>
<tr>
<td>G-CSF (pg/mL)</td>
<td>300 ± 197 (0.1-194)</td>
<td>231 ± 176 (0-2.315)</td>
</tr>
<tr>
<td>GM-CSF (pg/mL)</td>
<td>0 (n = 11)</td>
<td>0 (n = 13)</td>
</tr>
<tr>
<td>SCF (pg/mL)</td>
<td>201 ± 39 (33-534)</td>
<td>300 ± 59 (74-704)</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>10 ± 3 (0.04-7)</td>
<td>22 ± 8 (0-73)</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0 (n = 7)</td>
<td>0 (n = 7)</td>
</tr>
<tr>
<td>PDGF (pg/mL)</td>
<td>0 (n = 7)</td>
<td>0 (n = 7)</td>
</tr>
<tr>
<td>TGF-β (ng/mL)</td>
<td>2.1 ± 0.3 (1.4-4.3)</td>
<td>2.2 ± 0.2 (1.5-2.9)</td>
</tr>
<tr>
<td>MIP-1α (pg/mL)</td>
<td>16 ± 5 (0-75)</td>
<td>34 ± 9 (0-111)</td>
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</table>

Cell-free supernatants from CML and normal stromal layers were collected 2 days after media change and assayed by ELISA for the above cytokines. The sensitivities of the cytokine assays were as follows: IL-6, 0.35 pg/mL; G-CSF, 10.9 pg/mL; GM-CSF, 5 pg/mL; SCF, 3 pg/mL; TNF-α, < 10 pg/mL; IL-1β, 0.3 pg/mL; PDGF, 8.4 pg/mL; TGF-β, 50 pg/mL; MIP-1α, 2 pg/mL/L. Cytokines were undetectable by ELISA in fresh LTBMC medium, with the exceptions of TGF-β (2.1 ng/mL) and GM-CSF (6 pg/mL/L). Values are the mean ± SEM with the range in parentheses.

between the stage or duration of CML or presence or absence of marrow fibrosis with the functional capacity of the stromal layers.

Normal primitive progenitors were also grown in Transwell inserts with a 0.4-μm microporous membrane, which prevents cell contact with the underlying CML or normal stroma but allows free diffusion of soluble factors. As we have previously described,17 significantly more CFC were generated when normal DR- cells were cultured in stroma noncontact cultures than in stroma contact cultures using normal stroma. Interestingly, normal DR- cells gave rise to similar numbers of CFC when cultured in Transwells above CML as compared with normal stroma (Fig 1), suggesting that reduced growth of normal DR- cells cultured in contact with CML stromal layers may not be related to abnormal production of soluble growth factors.

Growth Factor Production by CML Stroma

Growth factor production by CML and normal stroma was assessed by standard ELISAs of stromal supernatants. G-CSF, IL-6, and TGF-β levels in CML and normal stromal supernatants were not significantly different, whereas GM-CSF, IL-1, and PDGF were undetectable in both (Table 2). Although reduced levels of TNF, SCF, and MIP-1α were observed in CML stromal supernatants, these differences were not found to be statistically significant. Where available, we compared cytokine levels and the capacity of the stromal feeders to support normal primitive progenitor growth. In this limited number of samples we did not find a correlation between cytokine production and the ability of individual stromal layers to support normal primitive progenitor growth (n = 12). These results further suggest that reduced growth of normal progenitors on CML stroma may be related to mechanisms other than reduced production of growth-stimulatory cytokines or increased production of growth-inhibitory cytokines by CML stromal layers.

Cellular Composition of CML Stroma

We next investigated whether the defect in hematopoietic supportive capacity of CML stroma was the result of abnormalities in its cellular composition. CML or normal stromal layers were harvested with collagenase to obtain single-cell suspensions. The relative number of stromal macrophages and nonhematopoietic stromal mesenchymal cells, including adipocytes, was assessed by differential cell count after staining cytospin preparations with Wright-Giemsa stain and various cytochemical stains.18 No significant differences in the cellular composition of CML and normal stromal layers were seen (Table 3).

bcr/abl Rearrangement Is Present in CML Stromal Cells

Irradiated CML stromal layers were harvested and analyzed for the presence of the bcr/abl gene rearrangement, characteristic of the malignant clonal population in CML. Lack of colony growth in methylcellulose progenitor cultures confirmed the absence of hematopoietic progenitors (data not shown). Reverse transcription-PCR (RT-PCR) of irradiated stromal layers indicated the presence of malignant cells (n = 4; Fig 2). These results were confirmed by FISH analysis. These results showed the presence of a large proportion of bcr/abl-positive cells in irradiated CML stroma (56.3% ± 6.5%, n = 7; Table 4). The proportion of CD14+ macrophages in CML stroma (57.3% ± 3.1%, as detected by FACS) was similar to the proportion of cells with bcr/abl gene rearranged cells (56.3% ± 6.5%, as detected by FISH). To further demonstrate that the bcr/abl rearranged cells were macrophages, we depleted CML stroma of macrophages by FACS sorting. CD14+ cells were reevaluated for the bcr/abl rearrangement by FISH. The percentage of bcr/abl-rearranged cells in the CD14+ population was 6% and 13%, as compared with 46% and 53%, respectively, in the undepleted population in two separate experiments. These studies indicate that CD14+ stromal macrophages are malignant.

Table 3. Cellular Composition of CML Stromata

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Normal Stroma (n = 7)</th>
<th>CML Stroma (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>62.9 ± 1.8</td>
<td>61.7 ± 5.9</td>
</tr>
<tr>
<td>Mesenchymal cells</td>
<td>27.7 ± 1.5</td>
<td>31.4 ± 4.3</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>9.4 ± 2.1</td>
<td>6.9 ± 2.0</td>
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Cell morphology was assessed on Wright-stained cytospin preparations made from CML and normal stromal layers. Cytochemical stains were performed to confirm the identity of macrophages (nonspecific esterase), mesenchymal cells (alkaline phosphatase), and adipocytes (Oil-Red-O). Values are percentages.
DEFECTIVE MARROW STROMAL FUNCTION IN CML

CML Macrophages Are Responsible for the Impaired Stromal Function in CML

To evaluate whether the malignant macrophage population was responsible for the defective function of CML stroma, CML and normal stromal layers were separated into CD14+ (stromal macrophage) and CD14- (stromal mesenchymal cell) populations by FACS sorting. The separated populations were cultured over a period of 3 to 4 weeks, allowing CD14- cells to grow to maximum confluence, irradiated at 1,250 cGy, and subsequently evaluated for their ability to support the growth of normal LTC-IC. As was seen for unmanipulated normal and CML stromal layers, CML stromal layers reconstituted by mixing CD14- and CD14+ populations together supported normal LTC-IC growth significantly less well than reconstituted normal stromal layers (216.5 ± 41.8 and 243.8 ± 19.5 CFC per 2,500 normal DR- cells on unmanipulated and reconstituted normal stromal layers and 154.5 ± 29.8 and 157.0 ± 18.0 CFC per 2,500 normal DR- cells on unmanipulated and reconstituted CML stromal layers). In contrast, FACS-purified CML and normal CD14+ mesenchymal cells supported the growth of LTC-IC equally well (Fig 3). The ability of CD14+ cells from CML or normal stroma to support normal LTC-IC growth was low. However, no differences were seen between CML and normal stroma-derived CD14+ cells (136.8 ± 26.9 CFC per 2,500 DR- cells on normal CD14+ cells and 157.3 ± 23.3 per 2,500 DR- cells on CML CD14+ cells). Reconstituted stroma containing a mixture of CML CD14+ cells and normal CD14- cells had significantly impaired hematopoietic supportive ability, whereas the mixture of normal CD14+ cells and CML CD14+ cells had normal hematopoietic supportive function. These results suggest that CML macrophages in the context of CD14- stromal mesenchymal cells are responsible for the impaired function of CML stroma.

No significant differences were found in the amounts of IL-1β, PDGF, TGF-β, and MIP-1α produced by macrophages from CML or normal stroma (Table 5). IL-6 and TNF-α production by CML macrophages was higher than that for normal stromal macrophages, but the differences were not statistically significant. Production of the above-mentioned cytokines by CML or normal CD14+ stromal cells or by mixtures of CML and normal CD14+ and CD14- cells was not significantly different. Therefore, the observed adverse effect of CML macrophages on CML or normal CD14+ stromal cell function cannot be readily explained by significantly altered cytokine production.

Growth of CML Primitive Progenitors on CML Stroma

Finally, we studied the ability of CML stroma to support the growth of CML LTC-IC. We have previously shown
Role of CML stromal macrophages in the reduced growth of normal DR- cells on CML stroma. CML and normal stromal layers were placed in suspension after collagenase treatment, stained with anti-CD14 monoclonal antibody, and FACS sorted into CD14+ (macrophage) and CD14- populations. The sorted normal and CML CD14+ and CD14- cells were either cultured separately or were mixed as shown. Sorted CML CD14+ macrophages were mixed with normal CD14- cells and normal CD14+ macrophages were mixed with CML CD14- cells. A total of 2,500 normal DR- cells were cultured in LTBMC media on sorted CML and normal stromal cells for 5 weeks. Cells were harvested at week 5 and CFCs were assayed in methylcellulose progenitor culture. For all cultures, the number of CFCs generated from DR- cells after culture was expressed as a percentage of the number of CFCs generated on normal controls (mixture of normal CD14+ and normal CD14- stromal cells) for each experiment. Results represent the mean ± SEM of pooled experiments (n = 4). Significance levels compared with colony output on normal controls; *P < .05. The average colony output per 2,500 DR- cells on normal stroma controls was 243.8 ± 19.5 at week 5.

Normal DR- cells cultured in stroma-free wells in LTBMC media yielded no CFC at week 5.

that, in contrast to normal progenitors, malignant CML LTC-IC and CFC are present within the DR2 BM fraction. Therefore, we compared the growth of CML DR2 cells on allogeneic CML stromal layers to their growth on normal stromal layers. We showed that, in contrast to normal LTC-IC, the growth of CML LTC-IC in limiting dilutions in contact with CML stroma was not reduced compared with normal stroma (Fig 4). Similar results were obtained when CML DR2 cells were cultured in contact with CML and normal stromal layers subcultured in 24-well plates for 5 weeks and the number of CFC generated was evaluated (Fig 4). The growth of CML LTC-IC in contact with CML stroma was significantly better than the growth of normal progenitors on the same CML stromal layers (P = .006). As for normal progenitors, CML LTC-IC cultured in Transwells above CML and normal stromal layers gave rise to similar numbers of CFC after 5 weeks. These results suggest that CML LTC-IC are not subject to similar inhibitory influences as are normal progenitors whose growth is impaired when cultured in contact with CML stroma.

**DISCUSSION**

Ordered hematopoiesis is believed to depend on the interaction of primitive and more differentiated hematopoietic progenitors with specific cellular and extracellular elements of the BM hematopoietic microenvironment. However, regulatory mechanisms operative in normal hematopoiesis do not appear to be operative in CML. Malignant CML hematopoietic progenitors clearly have intrinsic defects leading to abnormal interactions with normal stroma. Surprisingly, there is little information available regarding the function of the BM microenvironment in CML. The present study suggests that marrow stromal function is abnormal in CML as a result of the presence of malignant stromal macrophages. Our results suggest that microenvironmental dysfunction may contribute to the selective outgrowth of malignant cells and suppression of normal hematopoiesis in CML.

We show that marrow stroma from a significant proportion of CML patients has reduced ability to support the growth of normal LTC-IC. In contrast, growth of progenitors at

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**Table 5. Cytokine Production by CML and Normal Stromal Macrophages**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CML</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>155 ± 74 (43-294)</td>
<td>37 ± 13 (12-54)</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>25 ± 4 (18-33)</td>
<td>15 ± 2 (12-17)</td>
</tr>
<tr>
<td>PDGF (pg/mL)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TGF-β (ng/mL)</td>
<td>2.9 ± 0.1 (2.5-2.7)</td>
<td>2.6 ± 0.06 (2.5-2.7)</td>
</tr>
<tr>
<td>MIP-1α (ng/mL)</td>
<td>1.9 ± 0.2 (1.5-2.2)</td>
<td>1.6 ± 0.3 (1.1-1.9)</td>
</tr>
</tbody>
</table>

Stromal macrophages were separated from CML and normal stromal cell suspensions by selecting CD14+ cells by FACS, cultured in LTBMC medium for 2 to 3 weeks, and then irradiated at 1,250 cGy. Culture supernatants were collected 2 days after a scheduled weekly media change and assayed for the above cytokines by ELISA (n = 3). Cytokines were undetectable by ELISA in fresh LTBMC medium, with the exception of TGF-β (2.1 ng/mL). Values are the mean ± SEM, with the range in parentheses.
DEFECTIVE MARROW STROMAL FUNCTION IN CML

Fig 4. Growth of CML DR+ cells cultured with CML stroma. (A) CML DR+ cells were plated in limiting dilutions on normal and CML stromal layers grown in 96-well plates. Cells were cultured in LTBMC for 5 weeks, followed by methylcellulose progenitor culture as described in the Materials and Methods. The frequency of LTC-IC was calculated using Poisson statistics. The LTC-IC frequency on CML stroma for each experiment was expressed as the percentage of the LTC-IC frequency on normal stromal controls for that experiment. Results of individual experiments are shown (n = 4). The number of LTC-IC per 1,000 CML DR+ cells plated on normal stromal layers was 1.9 ± 0.2. (B) Five thousand CML DR+ cells were grown in LTBMC for 5 weeks, in contact with CML and normal stromal layers subcultured in 24-well plates followed by methylcellulose progenitor culture as described in the Materials and Methods. The number of CFCs generated from DR+ cells after culture on CML stroma was expressed as a percentage of the number of CFCs generated on normal stromal controls for each experiment. Results of individual experiments are shown (n = 8). The average colony output from 5,000 CML DR+ cells plated in contact with normal stromal layers was 194.8 ± 89.5. (C) Five thousand CML DR+ cells were plated in Transwell inserts placed above CML and normal stromal layers. Cells were harvested from the Transwell after 5 weeks of culture and assessed for CFC content by replating in methylcellulose progenitor culture. Results for each individual experiment were expressed as the ratio of the number of CFC in stroma noncontact cultures on CML stroma relative to that in a control stroma noncontact culture on normal stroma. Results of individual experiments are shown (n = 8). The average colony output per 5,000 CML DR+ cells for normal stromal layers was 267.4 ± 115.8 at week 5.

a distance from CML stroma was normal, indicating that production of soluble hematopoietic growth factors by CML stroma may be normal. Although there were trends towards reduced levels of TNF, SCF, and MIP-1α in CML stromal supernatants, these differences were not found to be statistically significant. This finding is consistent with previous studies indicating that stromal cells from patients with chronic-phase CML produce normal levels of various growth-stimulatory and growth-inhibitory cytokines.24-26 The observed decreased growth of LTC-IC, occurring only under conditions of close contact between stroma and progenitors, may reflect either imbalances in growth factors bound to stroma or abnormal expression of adhesive ligands in CML stroma. Although our preliminary unpublished observations failed to demonstrate significant differences in the expression of extracellular matrix components such as fibronectin, thrombospondin, and collagen type 4 or cell surface adhesion molecules such as ICAM, VCAM, and ELAM between CML and normal stroma, differences in the conformational states7 or alternatively spliced forms of these components28,29 that have not yet been assessed may account for the observed altered stromal function. In addition, other extracellular matrix components such as specific proteoglycans30 may be abnormal or absent from CML stroma. Furthermore, a variety of growth factors are either membrane anchored31 or extracellular matrix bound.32 These immobilized cytokines can bind and activate receptors on adjacent cells and result in the delivery of growth regulatory signals in a spatially localized manner to adherent cells. Altered capacity of extracellular matrix components in CML stroma to bind important hematopoietic growth factors or altered expression of membrane-anchored cytokines may then lead to abnormal growth signaling despite normal levels of cytokines being present in stromal supernatants.

In contrast to normal hematopoietic progenitors that adhere well to normal stroma, CML progenitors show reduced adhesion to stroma and proliferate continuously even when in contact with stroma, suggesting that they are not subject to negative regulation by stroma.7 We show here that the growth of CML progenitors in contact with CML stroma is not reduced compared with their growth on normal stroma and is significantly better than that of normal progenitors on CML stroma. In contrast, CML and normal progenitors grow equally well when cultured separated from either CML or
normal stroma. The abnormalities in the CML marrow microenvironment described here may therefore contribute to the abnormal expansion of the malignant Ph' positive clone and suppression of coexisting normal progenitors in CML. Because contact with CML stroma inhibits normal but not malignant progenitor growth, the Ph'-negative progenitors may proliferate less than their malignant counterparts, which may result in a selective growth advantage for the malignant progenitors.

PCR and FISH analysis of CML stroma irradiated to eradicate endogenous hematopoiesis showed the presence of bcr/abl-positive cells. Based on previously published studies, CML stromal fibroblasts are likely to be nonmalignant. However, there is some evidence that macrophages may be derived from the Ph'-positive clone. Separated stromal macrophages and mesenchymal cells by FACS and confirmed that stromal macrophages but not mesenchymal cells are malignant. CML stromal mesenchymal cells, separated from malignant CML macrophages, supported normal hematopoietic progenitors by FACs and confirmed that stromal macrophages resulted in diminished support of normal hematopoietic progenitors by both CML and normal stromal mesenchymal cells. The addition of CML macrophages resulted in diminished support of normal hematopoietic progenitors by both CML and normal stromal mesenchymal cells. This finding shows that the defective hematopoietic supportive function of CML stroma depends on the interaction of malignant stromal macrophages with nonhematopoietic stromal mesenchymal cells. The mechanism(s) by which CML stromal macrophages causes altered stromal function cannot be readily explained on the basis of altered production of stimulatory or inhibitory hematopoietic growth factors or of mesenchymally active cytokines. Altered cell-cell interactions between the malignant macrophages and other stromal cells or cytokines other than those studied here may be responsible for the adverse effect of CML macrophages on mesenchymal cells. Such interactions may then affect cytokine production and/or binding, cell adhesion molecule expression, or extracellular matrix production by neighboring stromal mesenchymal cells, thereby leading to the above described defects. Abnormal function of various adhesion receptors has been described in CML including abnormalities in β1 integrin receptors and the LFA-3 receptor. Such defects may be present in CML macrophages. Because adhesion receptors, including the β1 integrins, play an important role in extracellular matrix deposition and remodeling, such defects may underlie the macrophage-mediated stromal abnormalities observed here.

A higher number of CML BM cells was required to obtain confluent stromal layers. This finding is consistent with the observation that the CFU-F frequency is reduced in CML BM, likely as a result of dilution by the expanded number of myeloid cells. However, because we compensated for this by replating an equal number of CML and normal stromal cells in each experiment, the observed differences in stromal function are not caused by a decreased total number of stromal cells in CML stromal feeders. In contrast to some earlier reports, we did not find a consistent increase or decrease in the number of reticular cells, adipocytes, or macrophages in CML stromal layers. Our results indicate that the observed functional abnormalities of CML stromal layers were not related to alterations in the relative proportions of the stromal macrophages and mesenchymal cells, including adipocytes.

The abnormalities in the CML marrow microenvironment described here should be taken into account when gene insertion into stem cells and/or expansion of stem cells are attempted. Indeed, the use of autologous CML marrow stroma to support CX vivo expansion or retroviral gene transduction of selected benign stem cells in CML may necessitate the removal of CD14+ macrophages to improve the hematopoietic supportive capacity of autologous feeders. In addition, our observation that stromal macrophages are bcr/abl positive needs to be taken into account when analyzing BM samples early post-BMT for minimal residual disease. PCR positivity may reflect persistent malignant stromal macrophages that survive for several months after BM transplantation. This finding may explain why some patients who remain bcr/abl positive by PCR early after BM transplantation do not develop recurrent disease.

In conclusion, we show that the BM microenvironment in CML is abnormal and may contribute to clinical abnormalities observed in this disease. Malignant stromal macrophages appear to be responsible for the observed defects in stromal function. Further studies examining the nature of the defect and the mechanisms by which macrophages affect stromal function may provide significant new insights into the pathophysiology of CML and lead to new therapeutic approaches for this disease.

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DEFECTIVE MARROW Stromal Function in CML


Abnormal function of the bone marrow microenvironment in chronic myelogenous leukemia: role of malignant stromal macrophages

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