Growth Factor-mediated Terminal Differentiation of Chronic Myeloid Leukemia

Atul Bedi, Constance A. Griffin, James P. Barber, Milada S. Vala, Anita L. Hawkins, Saul J. Sharkis, Barbara A. Zehnbauer, and Richard J. Jones

Johns Hopkins Oncology Center, Baltimore, Maryland 21287-8967

Abstract

Expression of the BCR-ABL chimeric gene in chronic myeloid leukemia results in the inhibition of apoptosis, a genetically programmed process of autonomous cell death. BCR-ABL and other genetic factors that suppress apoptosis confer cross-resistance to cytokotoxic agents with diverse mechanisms of action. Eradication of the chronic myeloid leukemia clone requires strategies that circumvent this inherent resistance to cytokotoxic therapy. We have determined that BCR-ABL expression augments the sensitivity of hematopoietic cells to growth factor-mediated signals of differentiation; hematopoietic growth factors induce the selective terminal differentiation of chronic myeloid leukemia progenitors at concentrations that allow optimal growth of normal progenitors. Hematopoietic growth factors may be an effective strategy for the elimination of cytokotoxic therapy-resistant leukemic cells by inducing their terminal differentiation while allowing concomitant expansion of coexistent normal hematopoietic progenitors.

Introduction

The Philadelphia chromosome (Ph), t(9;22), is the cytogenetic hallmark of CML (1). This balanced translocation results in the creation of the chimeric BCR-ABL gene that expresses an 8.5-kilobase hybrid mRNA transcript and a Mr 210,000 fusion protein (P210BCR-ABL) (2). Expression of the BCR-ABL chimeric gene in CML results in the inhibition of apoptosis, a genetically programmed process of autonomous cell death (3, 4). BCR-ABL (4, 5), as well as other genetic factors that suppress apoptosis (6, 7), confers cross-resistance to cytokotoxic agents with diverse mechanisms of action. Eradication of the CML clone requires strategies that overcome this inherent resistance to cytokotoxic therapy. Because cellular differentiation is uninterrupted in chronic phase CML, it might be possible to induce terminal differentiation of CML progenitors leading to their elimination. We studied the effect of BCR-ABL expression on CML progenitor differentiation in response to hematopoietic growth factors.

Materials and Methods

Cell Lines. The murine IL-3-dependent myeloid cell line, FDC-P1 (8), and the cell line FDC-P1(P210) induced to constitutively express P210 by retroviral infection with a full-length BCR-ABL complementary DNA sequence (provided by S. Cory) (9) were maintained in complete serum-free media (Serum-Free Medium; Sigma Chemical Co., St. Louis, MO) in 5% CO2 at 37°C. WEHI-conditioned medium (10%) was added to the parental cell line as a source of IL-3. For the growth and differentiation experiments, log phase cells (5 x 105) were cultured in serum-free medium supplemented with recombinant murine IL-3 (R & D Systems, Minneapolis, MN).

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2 To whom requests for reprints should be addressed, at Room 2-127, The Johns Hopkins Oncology Center, 600 North Wolfe Street, Baltimore, MD 21287-8967.

The abbreviations used are: CML, chronic myeloid leukemia; IL, interleukin; rhu, recombinant human; CFU-GM, granulocyte-macrophage colony-forming units; GM-CSF, granulocyte-macrophage colony-stimulating factor; FISH, fluorescent in situ hybridization.

Inhibition of BCR-ABL Expression by Antisense Oligonucleotides. FDC-P1(P210) cells were exposed to BCR-ABL junction-specific (b,a) 18-base antisense (5'-GAAGGGGTTTGTGACTCT-3') or 2-nucleotide-mismatched nonsense (5'-GAATGCTTGTTGAACTCT-3') oligodeoxynucleotides using the sequences and conditions described previously (3). Oligonucleotides were added at a concentration of 15 µM 12 h prior to culture in IL-3, again at the initiation of culture, and then at 24-h intervals thereafter for 4 days. We have shown previously that these conditions effectively inhibit BCR-ABL expression (3).

Isolation of Hematopoietic Cells. Bone marrow cells were obtained by posterior iliac crest aspiration of patients with chronic phase CML and normal volunteers after informed consent approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions. Bone marrow mononuclear cells (density, <1.074) were recovered by Ficoll-Hypaque density centrifugation. CD34+ cells were selected by incubating plastic nonadherent mononuclear marrow cells for 30 min at 4°C with biotin-labeled mouse anti-human CD34 monoclonal antibody (Ceptrate LC34-biotin; Ceptrate, Bothell, WA). Biotin-labeled CD34+ target cells were captured on avidin-coated beads using a continuous flow avidin-immunoadsorption column (Ceptrate).

Hematopoietic Cell Cultures. CFU-GM were cultured from the CD34+ cells before and after their incubation in serum-free medium for 48 h at 37°C in 5% CO2 with graded concentrations (0–1000 units/ml) of rhu IL-3 or rhu GM-CSF (R & D Systems). CFU-GM were assayed as we described previously (10). The long-term growth of progenitors in liquid culture was studied by placing CD34+ cells in 25-cm2 tissue culture flasks containing McCoy’s 5A medium, 10% fetal bovine serum, 10-4 M hydrocortisone sodium hemisuccinate, and the following combination of cytokines: 100 units/ml rhu IL-3; 100 units/ml rhu GM-CSF; 10 units/ml rhu IL-6; and 10 ng/ml rhu c-kit ligand (R & D Systems). The cultures were incubated at 37°C in 5% CO2 for 6 weeks. At 7-day intervals, one-half of the culture volume was removed and replaced with fresh cytokine-supplemented media; the harvested nucleated cells from both the CML patients and normal subjects were counted and assayed for CFU-GM content.

Assessment of Myeloid Differentiation. Cell surface antigen expression was analyzed by flow cytometry (11). FDC-P1 cells (105) cultured for 5 days in serum-free medium ± 100 units of IL-3 were labeled with monoclonal antibodies against macrophage differentiation antigens Mac-1 (12) and F4/80 (13) (American Type Culture Collection, Rockville, MD) followed by incubation with fluorochrome-conjugated goat anti-rat IgG. CD34+ cells (105) were labeled with fluorescein isothiocyanate-conjugated CD34 (HPCA2) and phycoerythrin-conjugated CD34 (Leu-M9) monoclonal antibodies (Becton-Dickinson Immunochemistry Systems, San Jose, CA) before and after culture in serum-free medium supplemented with 100 units/ml of IL-3 or GM-CSF for 72 h in 5% CO2 at 37°C. The cells were sorted on a Coulter Epics 753 dual-laser flow cytometry system (Coulter Electronics, Inc., Hialeah, FL). Cells considered positive for cell surface antigen expression exhibited fluorescence intensity that was greater than the intensity exhibited by 99% of control cells. Cytocentrifuged FDC-P1 cell preparations were also stained with Giemsa and α-naphthyl esterase using a standard kit (Sigma). Phagocytosis was assessed by incubation with 0.4-µm latex beads at 37°C and microscopic analyses of xylene-treated cytocentrifuge preparations as described (14).

FISH. All of the CFU-GM colonies cultured each week from the long-term liquid cultures of CD34+ cells isolated from the CML patients were harvested, pooled, and analyzed by dual-color FISH for detection of the BCR-ABL fusion gene as described previously (15). Metaphase and interphase cell nuclei were hybridized with commercially available fluorescein isothiocyanate-labeled ABL and digoxigenin-labeled BCR probes (Oncor, Gaithersburg, MD).
Hybridization of both probes was observed simultaneously with a fluorescence microscope equipped with a double band-pass filter set. The typical FISH pattern for normal cells showed random placement of two red and two green signals separated by >1 μm (15). CML cells showed one green and one red randomly located signal from hybridization to the normal ABL and BCR genes, respectively, on the uninvolved 9 and 22 chromosomes as well as one red-green (separated by <1 μm) or yellow doublet signal from hybridization to the translocated BCR-ABL fusion gene. The incidence of false positive fusion signals in normal cells was approximately 2% (4 of 200 cells from normal marrow mononuclear cells).

**Determination of the BCR-ABL Gene Rearrangement by Southern Blot.** CFU-GM colonies cultured from the CD34+ cells isolated from the CML patients at the initiation of long-term liquid culture and at 1 week in culture were harvested and analyzed for BCR-ABL gene rearrangement as described previously (10). A Hoefer GS300 scanning densitometer and GS360 data system were used to quantitate autoradiographic signals as we described previously (10).

**Results**

We studied the effect of BCR-ABL expression on the ability to differentiate the growth factor-dependent myeloid cell line, FDC-P1 (8). As reported previously (14, 16), we found that FDC-P1 cells are refractory to differentiation; in the presence of IL-3, these cells maintained the appearance of uniform nonadherent blasts without markers of myeloid differentiation (Table 1). FDC-P1 cells induced to constitutively express P210 after retroviral infection with a full-length BCR-ABL complementary DNA sequence (FDC-P1P210) exhibited cytokine-independent survival and growth (Fig. 1A) as described previously (3, 9). However, although IL-3 produced a dose-dependent stimulation of parental FDC-P1 cell growth, concentrations of IL-3 exceeding 10 units/ml suppressed the growth of FDC-P1P210 cells (Fig. 1A); by day 7 of culture in 100 units/ml of IL-3, there was loss of cell proliferation and eventual death of all FDC-P1P210 cells (Fig. 1B). IL-3 induced the FDC-P1P210 cells to terminally differentiate into cells with a pleiotropic, vacuolated morphology that had acquired markers of macrophage differentiation including Mac-1 (C3bi receptor), F4/80, α-naphthyl acetate esterase, plastic adherence, and phagocytic ability (Table 1).

We (3) and others (4, 17) have demonstrated that BCR-ABL expression is effectively inhibited by antisense oligonucleotides complementary to BCR-ABL mRNA. Exposure of FDC-P1P210 cells to BCR-ABL antisense oligonucleotides partially reversed the IL-3-induced suppression of growth (Fig. 1B) as well as the IL-3-mediated induction of differentiation (Table 1; Fig. 1C). BCR-ABL nonsense oligonucleotides had no effect on the growth or differentiation of FDC-P1P210 cells. Therefore, BCR-ABL expression facilitated the growth factor-mediated differentiation of cell lines that are otherwise refractory to signals for differentiation.

To determine if BCR-ABL expression produces similar effects on growth factor-mediated differentiation of CML progenitors, CD34+ cells were isolated from the bone marrow of six patients with CML and five normal volunteers. The CD34+ cells were cultured for CFU-GM before and after serum-free incubation for 48 h with graded concentrations of growth factors. Normal progenitors survived poorly without growth factors and displayed a dose-dependent increase in progenitor survival with either GM-CSF or IL-3 (Fig. 2A). Conversely, the effects of hematopoietic growth factors on CML progenitors mirrored those seen in the FDC-P1 cells expressing BCR-ABL. As we described previously (3), CML progenitors exhibited prolonged growth factor-independent survival. However, concentrations of IL-3 or GM-CSF exceeding 10 units/ml resulted in a paradoxical reduction in the recovery of CML progenitors (Fig. 2A). Analysis of cell surface antigen expression confirmed that the loss in colony-forming ability was a consequence of the accelerated kinetics of CML progenitor differentiation; there was a substantially more rapid loss of the CD34 antigen with a reciprocal acquisition of the more differentiated CD34-CD33+ phenotype in the CML progenitors when compared to normal (Fig. 2B).

Since CML CFU-GM displayed a hypersensitivity to growth factor-induced differentiation, we examined whether more primitive CML progenitors capable of initiating long-term growth in liquid culture exhibit a similar hyperresponsiveness to cytokine-mediated signals of differentiation. Hematopoietic progenitors capable of producing CFU-GM for 1–2 months in cytokine-supplemental stroma-free liquid culture appear to represent primitive progenitors that are similar to those that initiate stroma-based long-term marrow cultures (18). CD34+ cells isolated from the six patients with chronic phase CML and the five normal donors were placed in stroma-free culture supplemented with a combination of cytokines that allow long-term growth of normal primitive hematopoietic progenitors (18). CFU-GM production by the primitive progenitors in long-term liquid cultures was analyzed weekly for 6 weeks. Normal CD34+ cells showed an initial increase in CFU-GM production followed by continued generation of CFU-GM for at least 6 weeks (Fig. 3A). In contrast, there was a rapid depletion of CFU-GM from the long-term liquid cultures of CML CD34+ cells with few CFU-GM evident beyond 3 weeks (Fig. 3A). CFU-GM harvested from the long-term CML cultures were also analyzed by FISH (15) and recombinant DNA analysis (10) to quantify the fraction of CFU-GM harboring the BCR-ABL gene rearrangement. The BCR-ABL gene rearrangement was present in 99 ± 1% (SEM) of the CFU-GM produced by the CD34+ cells prior to initiation of liquid culture (Fig. 3B). With increasing time in culture, the fraction of BCR-ABL+ CFU-GM progressively declined; after 3 weeks in culture, the majority (66 ± 13%) of CFU-GM did not harbor the BCR-ABL gene rearrangement (Fig. 3B). Cytokines that allowed optimal growth of normal progenitors capable of initiating and sustaining long-term culture induced preferential differentiation of BCR-ABL+ pre-CFU-GM.

**Discussion**

The effect of growth factors on a progenitor cell population is determined by a balance of their pleiotropic effects of survival, self-renewal, and differentiation. Colony formation in direct clonogenic assays results from some combination of self-renewing cell divisions and cell divisions that result in terminal differentiation; assessing colony numbers by direct clonogenic assays may not

### Table 1 Effect of BCR-ABL expression on IL-3-mediated differentiation of FDC-P1 and FDC-P1P210 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>FDC-P1 + IL-3</th>
<th>FDC-P1P210 - IL-3</th>
<th>FDC-P1P210 (NS) + IL-3</th>
<th>FDC-P1P210 (AS) + IL-3</th>
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</thead>
<tbody>
<tr>
<td>Mac-1</td>
<td>0</td>
<td>2.7 ± 1</td>
<td>74 ± 2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>F4/80</td>
<td>0</td>
<td>18 ± 9</td>
<td>93 ± 1</td>
<td>19 ± 2</td>
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<tr>
<td>Esterase</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Plastic adherence</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
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Cells were cultured for 5 days in serum-free medium ± 100 units/ml of IL-3. The results are the mean ± SEM of three separate experiments for each cell line. NS, nonsense oligonucleotides; AS, antisense oligonucleotides; ND, not done.
Fig. 1. Growth of FDC-P1 and FDC-P1_p210 cells (A) cultured for 36 h in graded concentrations (0–1000 units/ml) of IL-3 and (B) cultured for 7 days in 100 units/ml of IL-3. After 5 days in 100 units/ml of IL-3, parental FDC-P1 cells continued to proliferate with a doubling time of approximately 19 h. (C) Differentiation of FDC-P1 and FDC-P1_p210 after 5 days of culture in 100 units/ml of IL-3. Each data point represents the mean ± SEM (bars) of viable cells (determined by trypan blue dye exclusion) from three experiments for each cell line. NS, nonsense oligonucleotides; AS, antisense oligonucleotides.

distinguish between self-renewal and differentiation because both processes can lead to increased numbers of progeny. In fact, previous studies, including our own (3, 19), show that CML progenitors display a normal proliferative response to IL-3 or GM-CSF in direct clonogenic assays. In contrast, assessing colony formation at serial intervals during liquid culture measures the net effect of growth factors on the progenitor cell population; progenitor self-renewal will increase colony numbers in liquid culture, while progenitor differentiation will decrease colony numbers. In response to growth factors in liquid culture, CML progenitors produced markedly decreased numbers of CFU-GM colonies compared to normal as a result of augmented differentiation. In fact, the selective depletion of BCR-ABL progenitors that has been observed in long-term stromal cultures of CML bone marrow (20) may be the result of terminal differentiation induced by growth factors produced by stroma-adherent layers.

Growth factor levels have been reported to be depressed in CML patients (21), possibly as a result of feedback inhibition from the clonal myeloid cell expansion. We reported previously that BCR-ABL expression promotes the growth factor-independent survival of cells by inhibition of apoptosis (3); low levels of cytokines would favor the progressive accumulation and eventual dominance of the leukemic clone in CML. Low growth factor levels also may allow the leukemic clone to escape terminal differentiation and may be an important
mechanism of normal hematopoietic progenitor suppression in CML. Hence, growth factor levels appear to be a critical determinant of whether normal or CML progenitors predominate in vitro or in vivo. Since the cumulative effect of hematopoietic growth factors favors the differentiation of CML progenitors, growth factor-mediated differentiation may be a novel and effective strategy for the treatment of CML.

Acknowledgments

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References

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