Growth Factor Stimulation Reduces Residual Quiescent Chronic Myelogenous Leukemia Progenitors Remaining after Imatinib Treatment

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Abstract

The BCR/ABL tyrosine kinase inhibitor imatinib mesylate is highly effective in the treatment of chronic myelogenous leukemia (CML) but fails to eliminate all leukemia cells. Residual leukemia stem and progenitor cells persist in imatinib-responsive patients and may be a potential source of relapse. Previous studies indicate that imatinib preferentially targets dividing cells, and nondividing progenitor cells are resistant to imatinib-mediated apoptosis. We investigated whether growth factor stimulation of progenitor proliferation could reduce the number of residual nondividing cells remaining after imatinib treatment. CML and normal CD34$^+$ cells were labeled with 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to track cell division and cultured in low or high concentrations of growth factor to determine effects of growth factor stimulation on nondividing cells. High growth factor concentrations significantly enhanced CML proliferation with or without imatinib treatment and significantly reduced the number of viable, nondividing CFSE bright cells remaining after imatinib exposure. Stimulation with high growth factor before imatinib treatment further reduced the number of residual nondividing CML CD34$^+$ cells. Importantly, clinically achievable concentrations of granulocyte macrophage colony-stimulating factor alone or in combination with granulocyte colony-stimulating factor also significantly reduced nondividing CML CD34$^+$ cells. These results support the potential efficacy of growth factor stimulation in reducing the residual leukemia progenitor population in imatinib-treated patients. [Cancer Res 2007;67(3):1113–20]

Introduction

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell malignancy characterized by the t(9;22) chromosomal translocation that generates the BCR/ABL oncogene (1, 2). Imatinib mesylate (imatinib, Gleevec), a small-molecule inhibitor of the BCR/ABL tyrosine kinase, has proven to be highly effective for treatment of CML. Imatinib results in cytogenetic responses in the majority of chronic phase CML patients and a substantial proportion of patients in accelerated phase and blast crisis and has emerged as the front-line treatment for CML (3–7). Clinical studies indicate that the response to imatinib is sustained in the majority of chronic phase CML patients >5 years, although the long-term durability of response is still unclear.

Because CML originates in a hematopoietic stem cell, it is important to understand the effects of imatinib on malignant stem and progenitor cells. We have detected persistent, residual BCR/ABL$^+$ progenitors in patients achieving CCR on imatinib (8). In addition, we and others have shown that imatinib effectively inhibits proliferation of CML primitive progenitors but only modestly increases progenitor cell apoptosis (9, 10). Mathematical modeling of the effect of imatinib on the different hematopoietic cell compartments in CML also suggests that malignant hematopoietic stem cells are resistant to elimination by this drug (11). We have shown that nondividing CML CD34$^+$ cells are insensitive to imatinib-induced apoptosis and that apoptosis is restricted to dividing cells (12). Inhibition of progenitor proliferation by imatinib coupled with resistance of nondividing progenitors to imatinib-induced apoptosis may contribute to retention of a population of nondividing malignant progenitors in imatinib treated patients.

We hypothesized that interventions to enhance cycling of nonproliferating, primitive CML progenitors could enhance elimination of this imatinib-insensitive population. Growth factor stimulation offers a potential approach to activate progenitor cells into cycle. However, growth factor stimulation may also enhance survival signaling and protect progenitors from apoptosis, necessitating careful evaluation of the relative effects of growth factor stimulation on proliferation and apoptosis in CML and normal progenitors. Here, we investigated whether growth factor stimulation could activate CML progenitors into cell cycle and reduce the number of viable undivided CML progenitors that persist after imatinib treatment.

Materials and Methods

Subjects. Heparinized bone marrow samples were obtained from 11 CML patients and peripheral blood stem cell (PBSC) samples were collected from five normal donors after informed consent was obtained using guidelines approved by the Institutional Review Board of the City of Hope National Medical Center. CML patients ranged in age from 19 to 41 years and included six males and four females (one donor was unidentified). CML patients were in either chronic phase (n = 10) or accelerated phase (n = 1). The time since diagnosis ranged from 0 to 25 months. One patient had previously received IFN but had been off therapy for 4 months. Patients had not received prior imatinib therapy at the time the sample was collected, with the exception of one patient who was newly diagnosed and had received imatinib for 1 week.

Selection of CD34$^+$ progenitors. Bone marrow mononuclear cells were isolated by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation (specific gravity 1.077) for 30 min at 400 × g. CD34$^+$ cells were selected using immunomagnetic column separation (Miltenyi Biotech, Inc., Auburn, CA).

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***In vitro* cell culture.** CML and normal CD34$^+$ cells were cultured in tissue culture plates in serum-free medium (StemPro, Life Technologies, Gaithersburg, MD) supplemented with growth factors in the presence or absence of imatinib (1 μmol/L) at 37°C in a humidified atmosphere with 5% CO_2. Growth factor conditions were as specified in Results. Following culture, cells were harvested and assayed for proliferation, apoptosis, and colony-forming cells (CFC).

Progenitor assays. CFC assays were done by plating CD34$^+$ cells in semisolid methylcellulose progenitor culture for 14 to 18 days followed by enumeration of granulocyte-macrophage colony-forming unit and blast-forming unit colonies as described previously (9). Long-term culture initiating cell (LTCIC) assays were done by plating cells on M2-10B4 feeders in 96-well plates as described previously (9). Following 6-week culture period, wells were overlaid with CFC growth-supporting medium. After 14 days, wells were scored as positive or negative for the presence of CFCs. The frequency of LTCIC was calculated with L-Calc software (StemCell Technologies, Vancouver, British Columbia, Canada) based on the reciprocal of the concentration of test cells that yielded 37% negative wells.

Evaluation of apoptosis in dividing versus nondividing cells. CD34$^+$ cells were labeled with 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) before culture. At the end of the culture period, cells were labeled with Annexin V-phycoerythrin or Annexin V-Cy5, and divided versus undivided cells were analyzed by flow cytometry as described previously (12). A proliferation index was calculated based on analysis of the CFSE fluorescence profile of the sample compared with that of fixed undivided control cells using ModFit software (Verity, Topsham, ME). The percentage of Annexin V–positive cells was calculated for the total population as well as for nonproliferating cell subsets. Nonproliferating cells were defined based on fixed undivided control cells using ModFit software. The number of viable, undivided cells that remained at the end of the culture period was expressed as a percentage of the number of cells used to initiate the culture.

Statistics. Results of data obtained from multiple experiments were reported as the mean ± one SE. Significance levels were determined by ANOVA or Student's *t* test analysis as indicated in the figure legends.

Results

We investigated the effect of exposure to high concentrations of growth factor on the response of CML CD34$^+$ cells to imatinib treatment. CML and normal CD34$^+$ cells were cultured in high (100×) or low (1×) growth factor concentrations with or without imatinib (1 μmol/L) for 48 h and assayed for changes in cell proliferation and apoptosis. Low growth factor conditions [1.0 ng/mL stem cell factor (SCF), 1.0 ng/mL Flt-3 ligand (FL3), 0.2 ng/mL interleukin (IL)-3, 0.2 ng/mL IL-6, and 0.2 ng/mL granulocyte colony-stimulating factor (G-CSF)] are capable of maintaining LTCIC for up to 10 days (13, 14). High growth factor conditions, a 100-fold increase in the same growth factors (100 ng/mL SCF, 100 ng/mL FL3, 20 ng/mL IL-3, 20 ng/mL IL-6, and 20 ng/mL G-CSF), can support near maximal expansion of LTCIC numbers (14). In the absence of imatinib, CML progenitors were significantly more proliferative than normal progenitors in low growth factor conditions. High growth factor conditions significantly increased proliferation of both normal and CML CD34$^+$ cells (Fig. 1*A, left*). Imatinib-mediated proliferation suppression was significantly greater for CML progenitors compared with normal progenitors in low growth factor conditions (54 ± 3 versus 26 ± 6; $P = 0.002$). CML progenitor proliferation was effectively suppressed by imatinib in high growth factor conditions (Fig. 1*A, right*) but the amount of suppression was decreased compared with low growth factor conditions (54 ± 3% versus 45 ± 2%; $P = 0.022$). Interestingly, culture with high growth factor increased imatinib-mediated suppression of normal CD34$^+$ cell proliferation (45 ± 3 versus 26 ± 6; $P = 0.053$).

Culture with high growth factor significantly reduced baseline apoptosis of both CML and normal progenitors compared with low growth factor (Fig. 1*B*). CML progenitors were more sensitive to imatinib than normal progenitors in low growth factor (increase in apoptosis of 23 ± 2% for CML versus 5 ± 3% for normal CD34$^+$ cells; $P = 0.0006$). Imatinib-mediated apoptosis of CML

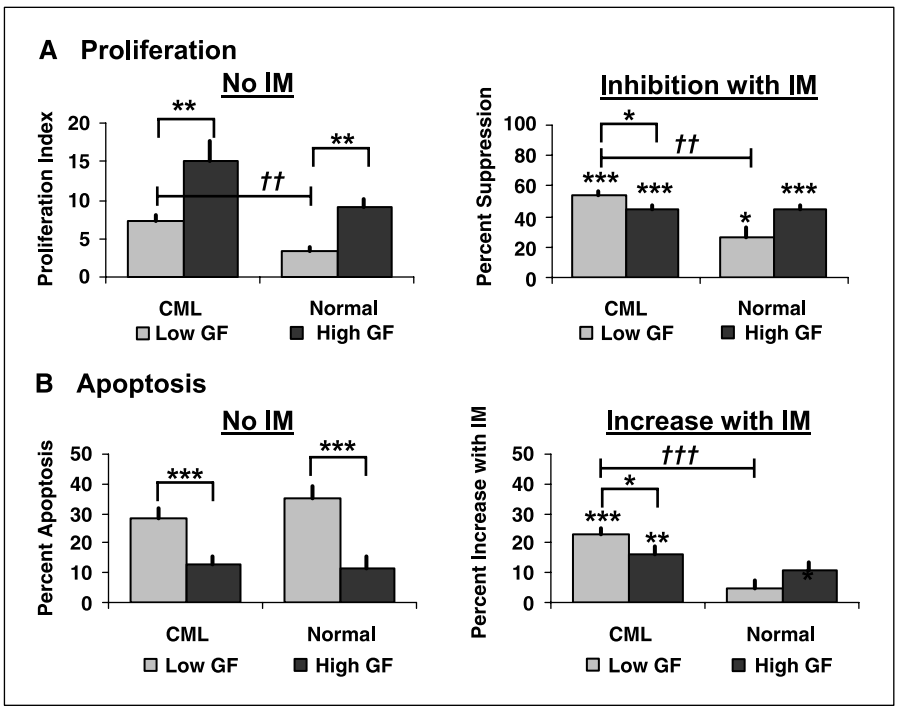

Figure 1. Effects of high growth factor conditions on CML and normal CD34$^+$ cell proliferation and apoptosis. CML and normal CD34$^+$ cells were cultured for 48 h in low or high (100×) growth factor concentrations with or without imatinib (*IM*; 1 μmol/L) and assayed for changes in cell proliferation and apoptosis (*A*). *Left,* proliferation index for CML ($n = 6$) and normal ($n = 4$) CD34$^+$ cells cultured in low (*gray columns*) or high (*black columns*) growth factor; *right,* inhibition of proliferation with imatinib compared with controls for cells cultured in low growth factor (*gray columns*) or high growth factor (*black columns*). *B, left,* percentage of cells positive for Annexin V in the absence of imatinib for CML ($n = 6$) and normal ($n = 4$) CD34$^+$ cells cultured in low growth factor (*gray columns*) or high growth factor (*black columns*); *right,* increase in apoptosis for cells exposed to imatinib. *Columns,* mean; *bars,* SE. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, significant differences between high and low growth factor, or with and without imatinib, as determined by paired *t* tests. †††, $P < 0.001$; ††, $P < 0.01$, significant differences between CML and normal cells, as determined by unpaired *t* tests.

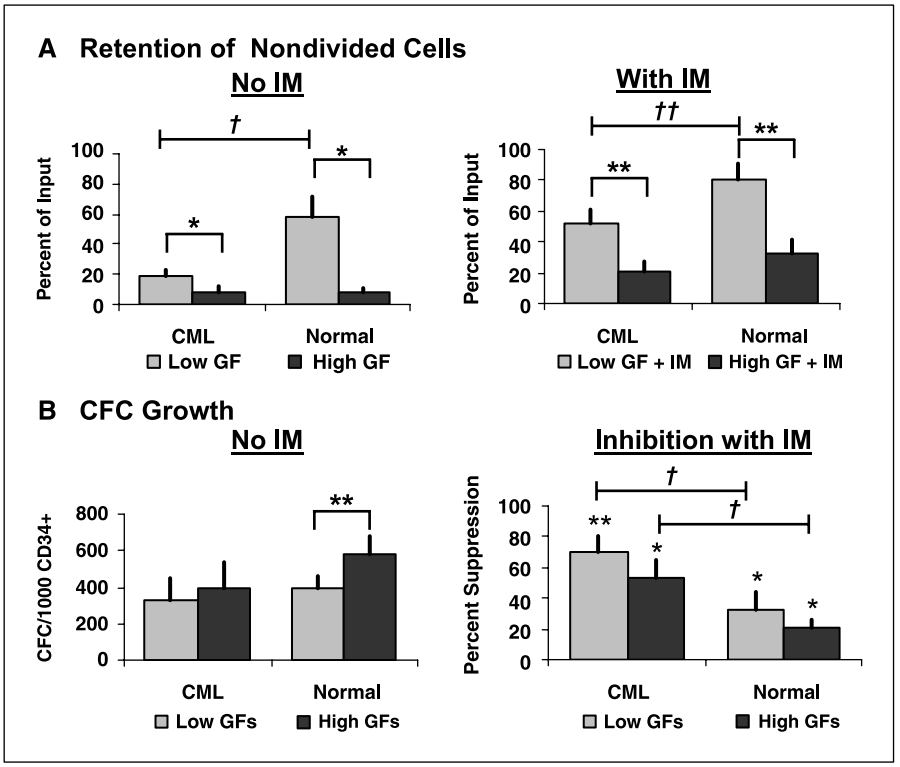

Figure 2. Effect of high growth factor conditions on retention of undivided CML and normal CD34$^+$ cells and on CFC growth. CML and normal CD34$^+$ cells were cultured as described for Fig. 1. *A, left,* percentage of input cells that remain viable and nondivided after culture in low growth factor (*gray columns*) or high growth factor (*black columns*) in the absence of imatinib for CML ($n = 6$) and normal ($n = 4$) CD34$^+$ cells; *right,* percentage of input cells that remain viable and nondivided after 48 h culture in low (*gray columns*) or high (*black columns*) growth factor in the presence of 1 μmol/L imatinib. *B, left,* the CFC frequency for CML ($n = 4$) and normal ($n = 5$) CD34$^+$ cells cultured in low (*gray columns*) or high (*black columns*) growth factor; *right,* inhibition of CFC growth with imatinib compared with controls for cells cultured in low growth factor (*gray columns*) or high growth factor (*black columns*). *Columns,* mean; *bars,* SE. **, $P < 0.01$; *, $P < 0.05$, significant differences between high and low growth factor, or with and without imatinib, as determined by paired *t* tests. ††, $P < 0.01$; †, $P < 0.05$, significant differences between CML and normal cells, as determined by unpaired *t* tests.

CD34$^+$ cells was significantly lower in high compared with low growth factor (16 ± 3% versus 23 ± 2%; $P = 0.015$). Combined CFSE and Annexin labeling to separately evaluate the effect of imatinib on apoptosis of undivided and divided cells showed reduced imatinib-induced apoptosis of dividing cells but no change in apoptosis of undivided CML CD34$^+$ cells in high compared with low growth factor (data not shown). In contrast to CML CD34$^+$ cells, normal CD34$^+$ cells cultured in high growth factor condition showed a significant increase in apoptosis following imatinib treatment compared with low growth factor conditions (13 ± 3% versus 29 ± 5% with imatinib; $P = 0.016$).

The number of viable, undivided CD34$^+$ cells remaining at the end of culture was analyzed (Fig. 2*A*). The number of cells remaining undivided, expressed as a percentage of cells used to initiate the culture, was significantly reduced in high growth factor conditions in the absence of imatinib (9 ± 3% in high growth factor versus 19 ± 4% in low growth factor; $P = 0.030$). This likely reflects increased cell cycling in response to high growth factor. The proportion of nondividing cells increased in the presence of imatinib (13 ± 6% increase in high growth factor; 32 ± 6% increase in low growth factor). Importantly, the fraction of cells remaining undivided and viable following exposure to imatinib was significantly lower in high growth factor conditions (21 ± 6%) versus low growth factor (52 ± 9%; $P = 0.002$). Therefore, increased proliferation of CML progenitors in response to high growth factor is associated with reduction in residual undivided CML progenitors remaining after culture. We and others have shown previously that CD34$^+$ cells from patients with active disease are predominantly BCR/ABL positive, both before culture as well as after culture in growth factor containing medium, with and without imatinib (9, 15). We have also shown that undivided cells remaining after growth factor culture with or without imatinib treatment are predominantly BCR/ABL positive by fluorescence *in situ* hybridization and express the BCR/ABL gene as evaluated by quantitative PCR (12). Interestingly, high growth factor conditions were also associated with reduction in nondividing normal CD34$^+$ cells (32 ± 9% in high growth factor versus 81 ± 11% in low growth factor; $P = 0.007$).

Imatinib treatment significantly reduced the CFC growth from CML CD34$^+$ cells in both high growth factor (54 ± 11% decrease with imatinib; $P = 0.017$) and low growth factor (71 ± 10% decrease with imatinib; $P = 0.005$) conditions (Fig. 2*B*). Imatinib also had a small effect on normal CFC growth in both low growth factor (33 ± 11% decrease with imatinib; $P = 0.041$) or high growth factor (21 ± 5% decrease with imatinib; $P = 0.013$) conditions. CFC suppression by imatinib was significantly greater for CML compared with normal samples in both low growth factor ($P = 0.040$) and high growth factor ($P = 0.022$) conditions.

In the next set of experiments, we investigated whether prestimulation with high growth factor to activate proliferation could enhance sensitivity to proliferation inhibition by imatinib. CML CD34$^+$ cells were cultured with high growth factor for 48 h followed by imatinib treatment for 48 h in either low or high growth factor conditions. High growth factor prestimulation significantly reduced imatinib-mediated inhibition of proliferation in both low growth factor (30 ± 4% less inhibition; $P < 0.0001$) and high growth factor (18 ± 4%; $P = 0.002$) compared with cells exposed to imatinib in similar conditions without any prestimulation (Fig. 3*A*). On the other hand, prestimulation also reduced apoptosis of cells exposed to imatinib in both low growth factor (19 ± 2% reduction; $P < 0.0001$) and high growth factor (7 ± 3% reduction; $P = 0.06$; Fig. 3*B*). Importantly, the proportion of viable undivided cells was significantly reduced after high growth factor prestimulation (Fig. 3*C*), both for cells exposed to imatinib in low growth factor (47 ± 11%; $P = 0.0015$) and high growth factor (17 ± 7%; $P = 0.034$). Imatinib treatment significantly inhibited CFC and

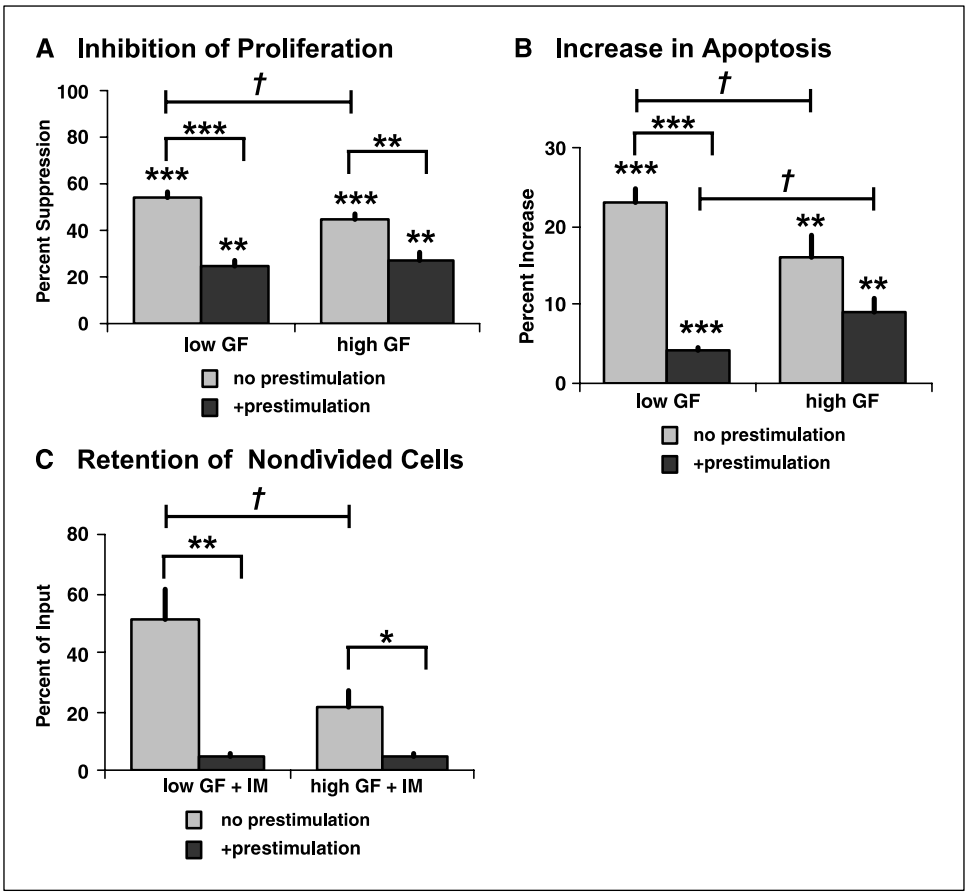

Figure 3. Effect of prestimulation with high growth factor on imatinib responsiveness of CML CD34$^+$ cells. CML CD34$^+$ cells were prestimulated with high growth factor for 48 h and then cultured for an additional 48 h in either low or high growth factor conditions with or without 1 μmol/L imatinib (n = 5; *black columns*). These results are compared with those obtained in the previous experiments for CML CD34$^+$ cells cultured under the same conditions with no prestimulation (n = 6; *gray columns*). *A,* effect of prestimulation on imatinib-mediated inhibition of CML CD34$^+$ cell proliferation. *B,* effect of prestimulation on imatinib-mediated increase in CML CD34$^+$ cell apoptosis. *C,* effect of prestimulation on the proportion of cells that remain viable and nondividing after exposure to imatinib. *Columns,* mean; *bars,* SE. Statistical significance for the effect of prestimulation was determined using unpaired, two-tailed t tests. Statistical significance for imatinib effects or high versus low growth factor effects was determined using paired, two-tailed t tests. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, significant differences with prestimulation or with imatinib. †, $P < 0.05$, significant differences with addition of imatinib or between low and high growth factor.

LTCIC growth from cells prestimulated with high growth factor (20 ± 6% in low growth factor, P = 0.03; 30 ± 7% in high growth factor, P = 0.01; Fig. 4*A* and *B*). Prestimulation with high growth factor decreased imatinib-mediated inhibition of CFC from cells cultured in low growth factor (34 ± 9%; P = 0.006) but not in high growth factor (15 ± 11%; P = 0.19). A similar comparison is not available for LTCIC.

Finally, we tested whether the clinically available growth factor, G-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF), affected CML progenitor response to imatinib. CML CD34$^+$ cells were cultured for 96 h with or without 1 μmol/L imatinib in low growth factor conditions similar to those found in bone marrow stromal conditioned medium (250 pg/mL G-CSF, 10 pg/mL GM-CSF, 200 pg/mL SCF, 1.0 ng/mL IL-6, 200 pg/mL macrophage inflammatory protein 1α, and 50 pg/mL leukemia inhibitory factor; refs. 9, 16). We used this basal growth factor combination for these experiments to simulate physiologic conditions and added G-CSF or GM-CSF to these basal conditions to model what may occur in patients treated with these agents. Cultures were supplemented with G-CSF (50 ng/mL), GM-CSF (10 ng/mL), and G-CSF and GM-CSF in combination or with low growth factor alone (controls). The G-CSF and GM-CSF concentrations chosen were representative of those that could be achieved in patients receiving therapeutic doses of these agents (17, 18). CML CD34$^+$ cell proliferation was significantly increased with GM-CSF alone (P = 0.010) or GM-CSF plus G-CSF (P = 0.021; Fig. 5*A, left*). Imatinib significantly suppressed progenitor proliferation for all growth factor conditions tested (Fig. 5*A, right*). The addition of G-CSF, GM-CSF, or both led to modest but significant decreases in apoptosis of CML CD34$^+$ cells in the absence of imatinib (Fig. 5*B*). There was no significant difference in imatinib-induced apoptosis between the different growth factor conditions. A significant reduction in the number of viable nondividing cells seen following imatinib treatment was observed when cells were cultured with G-CSF (P = 0.047), GM-CSF (P = 0.036), or G-CSF plus GM-CSF (P = 0.027) compared with control conditions (Fig. 6*A*). Addition of G-CSF or GM-CSF did not significantly affect imatinib-mediated inhibition of CML CFC growth (data not shown). However, significantly increased inhibition of CML LTCIC by imatinib was observed following addition of GM-CSF (P = 0.024) or G-CSF plus GM-CSF (P = 0.0008) compared with control conditions (Fig. 6*B*). These results indicate that reduction in nondividing cells is associated with inhibition of primitive progenitor growth.

Discussion

Imatinib treatment results in significant inhibition of CML progenitor cell proliferation but only a modest increase in apoptosis (9, 10, 12). Apoptosis is restricted to dividing cells, whereas nondividing cells resist apoptosis (10, 12). Imatinib-induced inhibition of CML progenitor proliferation together with the resistance of nondividing CML progenitors to imatinib-mediated apoptosis likely contribute to incomplete elimination of malignant progenitor cells in patients otherwise responding well to this agent. Undivided CML progenitors remaining after imatinib

treatment represent either dormant, noncycling cells, or cells that are inhibited from entering cell cycle by the antiproliferative effects of imatinib. The antiproliferative effect of imatinib enhances this population of nondividing cells and potentially interferes with elimination of malignant progenitors by apoptosis. We have shown that the undivided population is BCR/ABL positive, is not enriched for BCR/ABL negative cells, and expresses the BCR/ABL gene (12). The nondividing CML CD34$^+$ cell fraction is also resistant to elimination following treatment with several therapeutic agents (12). Here, we explored whether stimulation with high concentrations of growth factor could enhance proliferation of CML progenitors and reduce the number of residual nondividing cells remaining after imatinib treatment. Several important conclusions can be made from the results of the current studies.

First, we show that growth factor stimulation enhances proliferation of not only untreated but also imatinib-treated CML CD34$^+$ cells and that imatinib-mediated proliferation inhibition is reduced in high growth factor conditions. These observations suggest that high growth factor stimulation can at least partially overcome imatinib-mediated inhibition of CML progenitor proliferation. The effects of growth factor exposure on hematopoietic progenitors are multifaceted. In addition to stimulating proliferation, high growth factor may also enhance progenitor viability and promote differentiation. We show that culture in high growth factor led to lower basal levels of apoptosis in CML CD34$^+$ cells cultured without imatinib. In addition, imatinib-induced apoptosis was reduced in high growth factor compared with low growth factor conditions. Although the antiapoptotic effects of growth factor partly mitigates the proapoptotic effects of imatinib on CML progenitors, this protective effect is limited to proliferating cells, a population effectively targeted by imatinib. However, growth factor stimulation also enhances the proportion of cells that are proliferating and are susceptible to inhibition by imatinib, through both inhibition of cycling and induction of apoptosis (albeit reduced). The net result of high growth factor stimulation is enhanced inhibition of proliferation and significant reduction in nondividing CML progenitor cells, which are otherwise poorly targeted by imatinib treatment. Therefore, antiapoptotic effects do not prevent growth factor stimulation from reducing the nondividing CML progenitor subpopulation. The reduction in nondividing cells following growth factor stimulation may reflect both reduced imatinib-mediated inhibition of cell cycle as well as increased recruitment of dormant cells into cell cycle.

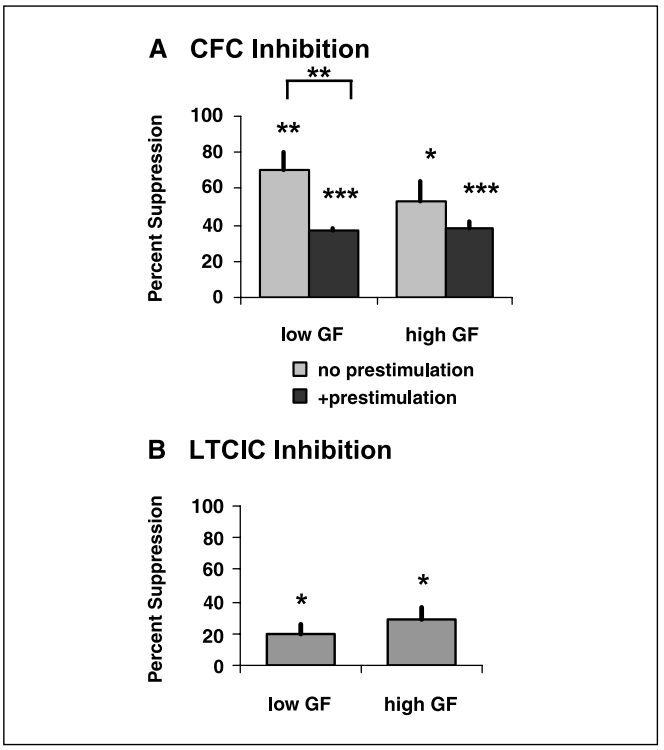

Figure 4. Effect of prestimulation with high growth factor on CFC and LTCIC inhibition by imatinib. CML CD34$^+$ cells were cultured as described for Fig. 3. *A,* effect of prestimulation on imatinib-mediated suppression of CML committed progenitors (*CFC*). *B,* suppression of CML primitive progenitors (*LTCIC*) by imatinib. LTCIC data are only for prestimulated cells as the comparable assay was not done in the original experiments. *Columns,* mean; *bars,* SE. Statistical significance for the effect of prestimulation was determined using unpaired, two-tailed *t* tests. Statistical significance for imatinib effects or high versus low growth factor effects was determined using paired, two-tailed *t* tests. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, significant differences.

Second, our observation that concurrent imatinib treatment and high growth factor stimulation can enhance the elimination of malignant progenitors from CML patients supports the application of growth factor stimulation to reduce residual malignant progenitors in imatinib-treated CML patients. It is not clear that any available anticancer agents can effectively and selectively target nondividing cancer cells or cancer stem cells. Blagosklonny (19) postulated recently that response to cancer treatment is determined by the proliferating cell compartment. It was argued that cancer treatment needs to target proliferating cells and that resting cancer stem cells require to be targeted only when they start to proliferate. In this context, the current study and our previous reports suggest that it is proliferating cells that are responsive to inhibition by imatinib and other antileukemia treatments. We show here that growth factor stimulation induces a subset of nondividing cells into cycle and thereby allow their targeting by imatinib. Importantly, for the clinical translational application of these observations, growth factor currently approved for clinical use, GM-CSF and G-CSF, also enhanced proliferation and reduced residual nondividing progenitors after imatinib treatment. GM-CSF or the combination of GM-CSF with G-CSF had a greater effect than G-CSF alone. GM-CSF and/or G-CSF treatment did not significantly affect inhibition of CML committed progenitors by imatinib but significantly enhanced inhibition of primitive progenitors analyzed in LTCIC assays. This is consistent with the known quiescent characteristics of primitive progenitors and lends further support to this approach to reducing residual primitive CML progenitors in imatinib-treated patients. In contrast to normal primitive progenitors that require stimulation with multiple growth factor to proliferate, CML primitive progenitors can be induced to proliferate in response to stimulation by a single growth factor and may therefore show enhanced and selective susceptibility to this approach (20–22). Interestingly, administration of GM-CSF has been reported previously to both improve the activity of IFN therapy and have a direct antileukemia effect in CML patients (23, 24). Prestimulation with high growth factor before imatinib treatment resulted in enhanced reduction in inhibition of CML progenitor proliferation by imatinib compared with concurrent exposure to high growth factor and imatinib and further reduction in the percentage of nondividing cells retained in the presence of imatinib. However, the prestimulation approach necessitates short-term discontinuation of imatinib while growth factor are administered, the safety of which would have to be

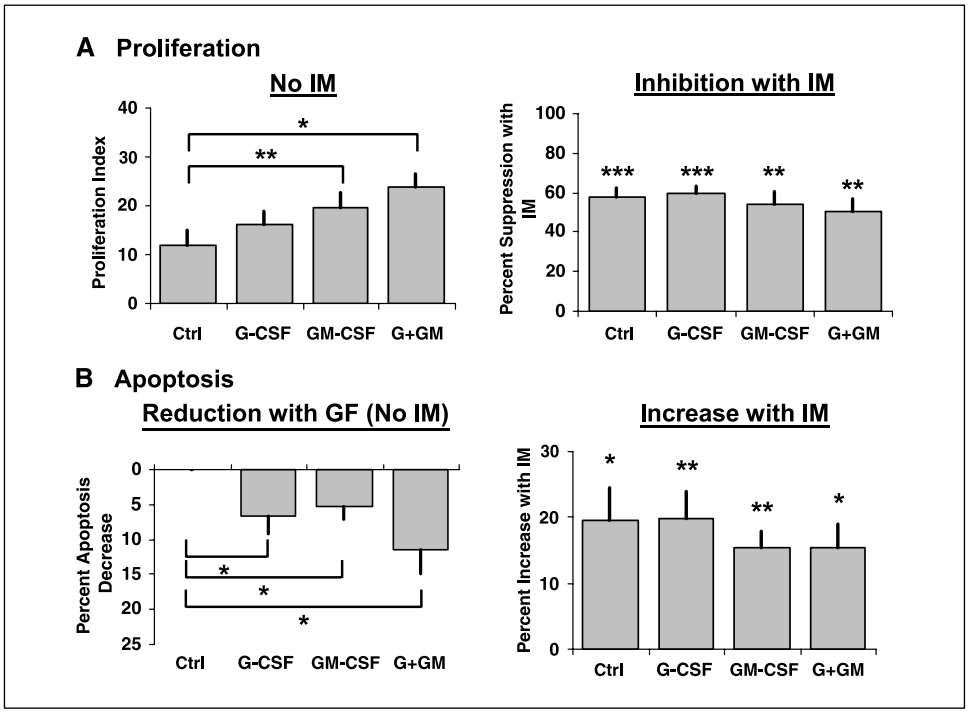

Figure 5. Effects of G-CSF and GM-CSF on CML CD34$^+$ cell proliferation and apoptosis. CML CD34$^+$ cells (n = 5) were cultured for 96 h in low growth factor conditions, as described in the text, with or without supplementation with G-CSF (50 ng/mL), GM-CSF (10 ng/mL), or both G-CSF and GM-CSF (*G+GM*) and with or without treatment with imatinib (1 μmol/L). *A, left,* proliferation index; *right,* amount of proliferation suppression observed for cells exposed to 1 μmol/L imatinib. *B,* treated cells were assessed for apoptosis by flow cytometry assessment following Annexin V labeling. *Left,* increase viability compared with control cells; *right,* increase in imatinib-mediated apoptosis for each growth factor condition. *Columns,* mean; *bars,* SE. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, significant differences, as determined using paired, two-tailed t tests.

considered in the clinical setting. We expect that if growth factor treatment were to be applied clinically, that it is likely to initially involve administration of short courses of growth factor treatment to patients receiving imatinib with careful assessment of effects on residual leukemia cells and toxicity toward normal bone marrow cells. The effects of repeated cycles of growth factor treatment would be investigated. If these studies show promise and show safety, then interruption of imatinib treatment during growth factor treatment may be considered in the second generation of trials.

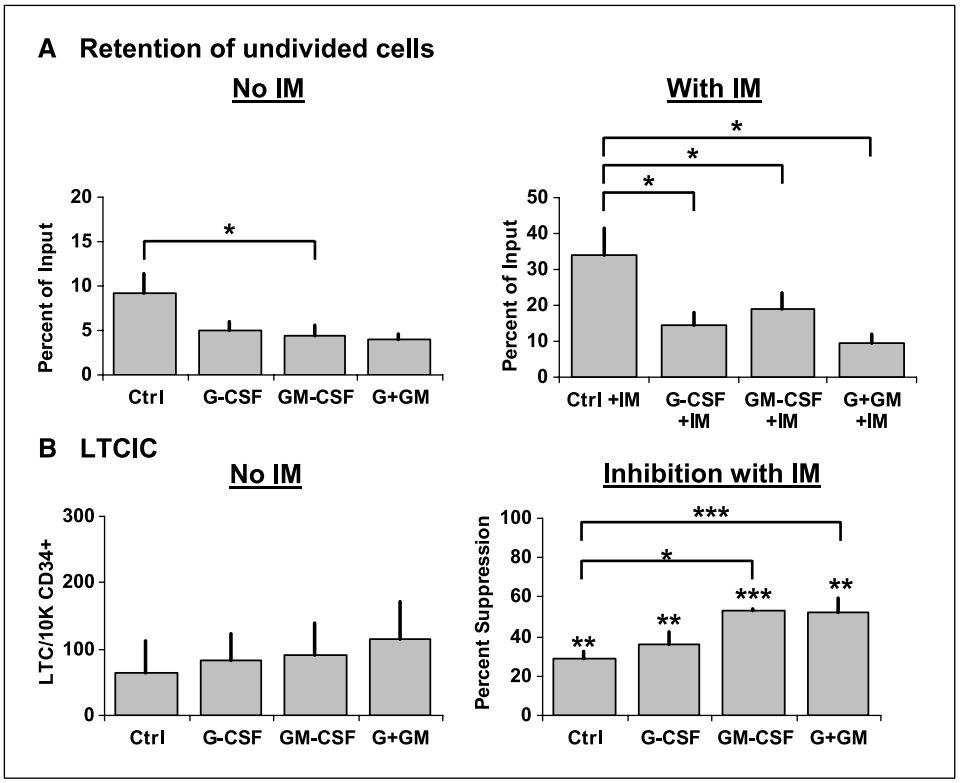

Figure 6. Effect of G-CSF and GM-CSF on retention of undivided CML CD34$^+$ cells and on imatinib-mediated inhibition of CML primitive progenitor growth. CML CD34$^+$ cells (n = 5) were cultured for 96 h in low growth factor conditions (as in Fig. 3) with or without supplementation with G-CSF (50 ng/mL), GM-CSF (10 ng/mL), or both and with or without treatment with imatinib (1 μmol/L). *A, left,* percentage of input cells that remain as viable and undivided in the absence of imatinib; *right,* percentage of input cells that remain undivided in the presence of imatinib. *B, left,* total number of LTCIC per 10,000 CD34$^+$ cells cultured in the absence of imatinib; *right,* percentage suppression of LTCIC following treatment with imatinib. *Columns,* mean; *bars,* SE. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, significant differences, as determined by paired t tests.

Third, we make the interesting observation that growth factor stimulation also alters imatinib effects on normal progenitors. Normal progenitors are less proliferative than CML progenitors under low growth factor conditions but this difference was eliminated in high growth factor conditions, possibly indicating enhanced responsiveness to low growth factor concentrations in CML. We show that growth factor stimulation of proliferation in normal $CD34^+$ cells enhances their sensitivity to inhibition of proliferation by imatinib and reduces the number of nondividing progenitors. This may reflect inhibition of tyrosine kinases other than BCR/ABL, such as c-Kit, because SCF was included in growth factor combination used in these experiments (25). These observations do raise the possibility of toxicity to normal hematopoietic cells with this approach. However, clinical application of this approach would target patients who achieve remission on imatinib treatment to reduce the small population of residual leukemia progenitors. In this setting, the overwhelming majority of hematopoietic cells are BCR/ABL negative and partial inhibition of these normal hematopoietic cells may be acceptable. In addition, we observed that CFC suppression by imatinib was significantly greater for CML compared with normal samples even with high growth factor stimulation. Evidence to date indicates that it is safe to administer growth factor to CML patients receiving imatinib. G-CSF can be safely administered alongside imatinib to stimulate myelopoiesis to resolve imatinib-induced neutropenia (26–30). G-CSF has also been safely used to mobilize autologous PBSC products from CML patients in CCR on imatinib (27–30). In neither of these applications was G-CSF administration associated with increased risk of disease progression or relapse.

While the current studies were in progress, Jorgenson et al. (31) reported that intermittent exposure to G-CSF during imatinib treatment reduced the number of quiescent CML progenitors *in vitro*. These effects of G-CSF are consistent with those made in the present study, which additionally shows the significantly greater efficacy of GM-CSF compared with G-CSF in reducing the nondividing CML progenitors. The current study further explores mechanisms underlying growth factor effects on CML and normal progenitors through a detailed analysis of the effects of high and low growth factor concentrations on imatinib-induced proliferation inhibition and apoptotic induction and the effects of dissociating growth factor and imatinib exposure on residual nondividing progenitors and confirms efficacy of this approach in targeting primitive progenitors using functional assays.

The results of the current study provide a strong rationale for further clinical evaluation of the effectiveness of GM-CSF alone or in combination with G-GSF in reducing residual disease in CML patients treated with imatinib. Future studies will also explore the development of alternative approaches to enhance the activity of imatinib by selectively enhancing cell cycle entry of dormant CML progenitors without potentially enhancing viability, for example by targeting cell cycle inhibitors (32–35). These studies will be guided by results of careful evaluation of differences in cell cycle regulatory mechanisms in CML compared with normal progenitor cells.

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