Abstract

Purpose: Primitive quiescent chronic myeloid leukemia (CML) cells are biologically resistant to imatinib mesylate, an inhibitor of the p210BCR-ABL tyrosine kinase. The present study was designed to investigate whether either continuous or intermittent exposure of these cells to granulocyte-colony stimulating factor (G-CSF) in vitro can overcome this limitation to the effectiveness of imatinib mesylate therapy.

Experimental Design: CD34⁺ leukemic cells were isolated from six newly diagnosed chronic phase CML patients and cultured for 12 days in serum-free medium with or without G-CSF and/or imatinib mesylate present either continuously or intermittently (three cycles of G-CSF for 0, 1, or 4 days ± imatinib mesylate for 0, 3, or 4 days). Every 4 days, the number of residual undivided viable cells and the total number of viable cells present were measured.

Results: Intermittent but not continuous exposure to G-CSF significantly accelerated the disappearance in vitro of initially quiescent CD34⁺ CML cells. This resulted in 3- and 5-fold fewer of these cells remaining after 8 and 12 days, respectively, relative to continuous imatinib mesylate alone (P < 0.04). Cultures containing imatinib mesylate and intermittently added G-CSF also showed the greatest reduction in the total number of cells present after 12 days (5-fold more than imatinib mesylate alone).

Conclusion: Intermittent exposure to G-CSF can enhance the effect of imatinib mesylate on CML cells by specifically targeting the primitive quiescent leukemic elements. A protocol for treating chronic-phase CML patients with imatinib mesylate that incorporates intermittent G-CSF exposure may offer a novel strategy for obtaining improved responses in vivo.
Taken together, these observations prompted us to look for a treatment that might enhance the rate of entry into cycle of primitive quiescent CML cells and thereby improve responsiveness to imatinib mesylate. Previous in vitro studies of leukemic cell lines from patients with acute myeloid leukemia have shown that the susceptibility of these cells to killing by chemotherapeutic agents, especially cell cycle–specific agents such as cytarabine (14–16), is enhanced by prior exposure to growth factors, such as granulocyte-colony stimulating factor (G-CSF), and that the acute myeloid leukemic cells are more sensitive in this regard than their normal counterparts (17). In addition, G-CSF has been safely and successfully used for peripheral blood stem cell mobilization in healthy donors and in CML patients treated with imatinib mesylate with no significant increase in BCR-ABL transcript levels by quantitative reverse transcription-PCR (18, 19). G-CSF is currently being used in patients with CML to overcome imatinib mesylate–induced neutropenia as a myelosuppression during imatinib mesylate therapy has been found to be associated with a poorer cytogenetic response (20, 21). In this setting, it has been postulated that the improved cytogenetic responses observed result from an increased exposure to imatinib mesylate (22–24). However, another effect of pharmacologic doses of G-CSF given to CML patients might be to stimulate the entry of their quiescent CML stem cells into cycle and, hence, increase the sensitivity of these cells to imatinib mesylate (25). The present in vitro study was designed to directly test this possibility experimentally.

Materials and Methods

Patient cells and isolation of specific subsets. The CML cells used in this study were all from peripheral blood samples, usually leukapheresis samples, collected as part of the routine management of previously untreated, newly diagnosed patients with chronic phase CML (n = 6; Table 1). Samples of normal adult human bone marrow cells were either from harvests taken for allogeneic transplants or were from cadaveric donors (Northwest Tissue Center, Seattle, WA). All samples were obtained with written informed consent. An enriched population of CD34+ cells (49-98% CD34+) was obtained either by fluorescence-activated cell sorting (FACS) for G-CSF immunomagnetic removal on a column of more mature cells or by positive selection of CD34+ cells (Isolex, Nexell International, Brussels, Belgium; ref. 27). These CD34+–enriched cell populations were then cryopreserved. Populations of isolated by fluorescence-activated cell sorting (FACS) for G-CSF receptor mRNA analyses from thawed CML peripheral blood and normal bone marrow samples directly after staining with Hoechst 33342 (Sigma Chemicals, St. Louis, MO), Procion Y (Sigma Chemicals), anti–CD34-phycocyanin (Becton Dickinson, Oxford, United Kingdom), and propidium iodide (Sigma Chemicals), as described (9). Cell culture experiments were initiated with thawed CD34+–enriched CML cells. In the second series of experiments, these were first stained with carboxy fluorescein succinimidyl ester (CFSE, Molecular Probes, Invitrogen Ltd., Paisley, United Kingdom) and cultured overnight in serum-free medium as described below.

Real-time reverse transcription-PCR analysis of G-CSF receptor transcripts. Real-time reverse transcription-PCR analyses were done as described (28, 29) on total RNA extracted using the Absolutely RNA MicroPrep kit (Stratagene, La Jolla, CA) or TRIzol (Invitrogen, Burlington, ON, Canada). Briefly, the reverse transcription reaction was carried out on a 20 μL aliquot using Superscript II reverse transcriptase (Invitrogen) and random hexamer oligonucleotides (Amerham Pharmacia, Piscataway, NJ) and real-time PCR was then done using 1 to 2 μL of the cDNA product plus 25 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). 1 μL of 20 pmol/L of specific primers, and water to obtain a final volume of 50 μL. Forward and reverse primers to produce ~100 bp amplicons for optimal amplification in the real-time PCR of human G-CSF receptor cDNA were 5′-CATCACAGGCTCTGGAATGCT-3′ and 5′-CTGAAGCTCTGCTC-3′, and for glyceraldehyde-3-phosphate dehydrogenase were 5′-CCATACACATCTTCAGGAG-3′ and 5′-CTTCTTCATGGTTGAAGC-3′. Real-time PCR and data analysis were done on an iCycler iQ system, using iCycler iQ Real-Time Detection Software (Bio-Rad, Hercules, CA). Optimal reaction conditions for coamplification of human G-CSF receptor and glyceraldehyde-3-phosphate dehydrogenase CDNAs were as follows: 40 cycles of three-step PCR (94°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds with a single fluorescence measurement) after initial denaturation (94°C for 5 minutes). Human G-CSF receptor transcript levels were quantified relative to glyceraldehyde-3-phosphate dehydrogenase as described (30).

Culture experiments. Cells were cultured at 37°C in an atmosphere of 5% CO2 in air in a serum-free medium consisting of Iscove’s modified Dulbecco’s medium (Sigma-Aldrich Ltd., Irvine, United Kingdom) supplemented with a serum substitute (Bitt, StemCell Technologies), 1 mmol/L glutamine, 1 mmol/L streptomycin, and penicillin, 40 μg/mL low density lipoproteins (Sigma-Aldrich), and 10−5 mol/L 2-mercaptoethanol (Life Technologies/Invitrogen) and other additives as indicated. The concentration and time of exposure to G-CSF that would give a maximal proliferative response was assessed by measuring the total number of viable (trypan blue excluding) cells present after varying periods of time (up to 6 days) in cultures initiated with 5 × 105 CD34+–enriched cells in 1 mL of serum-free medium and different concentrations of G-CSF (0-200 ng/mL; Chugai Pharma, London, United Kingdom). These CD34+–viable, quiescent (G0), and proliferating (G1-S/G2-M) CD34+ cells were then cryopreserved. Populations of enriched cell populations were then cryopreserved.

Table 1. Patient characteristics

<table>
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<tr>
<th>UPN</th>
<th>Sex</th>
<th>Disease stage</th>
<th>Prior treatment</th>
<th>WBC at leucapheresis (× 10^9/L)</th>
<th>Preselection %CD34</th>
<th>Post selection %CD34</th>
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</table>

Abbreviations: Ph, Philadelphia chromosome; D-FISH, dual-color fluorescence in situ hybridization; CP, chronic phase; UPN, unique patient number.
Kingdom) but no imatinib mesylate. Cultures containing a cocktail of five growth factors [interleukin (IL)-3 (Novartis Pharmaceuticals), IL-6 (Cangene, Mississauga, ON, Canada), Flt3 ligand (Immunex Corporation, Seattle, WA), stem cell factor (StemCell Technologies), and G-CSF; ref. 9] were used as a positive control.

In the second series of experiments, 5 × 10^5 CD34+ CFSE+ cells were cultured in 1 mL serum-free medium F20 ng/mL G-CSF 5 μmol/L imatinib mesylate (Novartis Pharmaceuticals) for a total of 12 days; the experiments are made up of three cycles of treatment of 4 days each (shown schematically in Fig. 1A and B). This way, the effects of a total of 10 different treatment protocols (including controls) were compared as follows: (a) no G-CSF or imatinib mesylate, (b) intermittent imatinib mesylate alone (imatinib mesylate added for the final 3 days only of each 4-day cycle), (c) continuous imatinib mesylate alone, (d) intermittent G-CSF alone (G-CSF added for first day of each 4-day cycle and then removed), (e) intermittent G-CSF (first day only of each 4-day cycle) followed by pulsed imatinib mesylate (last 3 days of each 4-day cycle), (f) continuous imatinib mesylate with intermittent G-CSF (first day only of each 4-day cycle), (g) continuous G-CSF with intermittent imatinib mesylate (imatinib mesylate added for the final 3 days only of each 4-day cycle), (h) simultaneous continuous G-CSF and imatinib mesylate, and (i) a colcemid control [100 ng/mL colcemid (Life Technologies/Invitrogen) added for 4 days].

Flow cytometry. At the end of each treatment cycle, viable dividing and quiescent populations were identified based on their CFSE, propidium iodide, and anti–CD34-phycoerythrin fluorescence using a FACSCalibur (Becton Dickinson). The peak of fluorescence (CFSE_max) exhibited by CFSE+ cells treated with colcemid to arrest cell cycle progression was used to identify the level of fluorescence to be ascribed to cultured cells that had remained quiescent (undivided). To measure the overall effect of each test condition on cell survival and on the number of undivided cells remaining after each cycle, the percentage recovery of viable CD34+ cells in the undivided peak was calculated as previously described (10). Briefly, this is the absolute number of viable, undivided CD34+ cells expressed as a percentage of the total input number of viable CD34+ cells. This calculation permitted an assessment of the relative efficacy of the different in vitro treatments tested to eliminate primitive CML cells. In addition, on a separate aliquot of cells, surface G-CSF receptor expression was assessed at 0, 24, 48 and 72 hours using a phycoerythrin-conjugated primary antibody (BD Biosciences PharMingen, Oxford, United Kingdom).

Cells were stained for apoptosis commitment at 24, 48, and 72 hours using a standard intracellular staining protocol for active caspase-3. Briefly, CFSE-stained cells were fixed and permeabilized according to the instructions of the manufacturer using a Fix and Perm Cell Permeabilization kit (Caltag Laboratories) before staining with rabbit anti-human active caspase-3 antibody (BD Biosciences PharMingen). High-resolution cell cycle analysis was done using Ki-67 (BD Biosciences PharMingen) and 7-aminoactinomycin D (BD Biosciences PharMingen) as previously described (31).

A small aliquot of the CFSE_max, CD34+ propidium iodide-negative cells present at the end of the different in vitro treatment protocols were also sorted directly onto a microscope slide using a FACSVantage (Becton Dickinson) for subsequent fluorescence in situ hybridization analyses.
Fluorescence in situ hybridization. In the second series of experiments, a portion of the CD34+-enriched cells used to initiate the cultures and later sorted CFSE
max, CD34+ propidium iodide-negative cells were placed onto poly-L-lysine–coated microscope slides and then swollen in prewarmed hypotonic solution (0.075 mol/L KCl) before fixation in freshly prepared methanol/acetic acid (3:1) and staining with the LS1 BCRABL Dual Colour Fusion Translocation Probe (Vysis Abbott Laboratories, Maidenhead, United Kingdom) according to the instructions of the manufacturer. A minimum of 200 interphase nuclei per group were evaluated using a fluorescence microscope with a triple band pass filter for 4',6-diamidino-2-phenylindole, Spectrum Green, and Spectrum Orange to determine the frequency of BCR-ABL + cells.

Statistical analyses. Differences between different test conditions were evaluated using the Student's t test. A level of P < 0.05 was considered statistically significant.

Results

Primitive quiescent CML cells express G-CSF receptors. G-CSF receptor transcript levels in the cycling (G1-S/G2-M) and quiescent (G0) subsets of FACS-purified CD34+ chronic phase CML cells (n = 6) and normal CD34+ bone marrow cells (n = 3) were measured by quantitative real-time reverse transcription-PCR. The results showed G-CSF receptor gene expression to be up-regulated in the cycling fraction of the CML as well as the normal CD34+ cells. The levels in both fractions of the leukemic CD34+ cells were also significantly higher than in their normal counterparts (2- to 4-fold, P < 0.02; Fig. 2A).

G-CSF receptor protein expression is increased in CD34+ CML cells. G-CSF receptor protein expression was measured in CD34+-enriched chronic phase CML and non-CML cells by flow cytometry at 0, 24, 48, and 72 hours culture in the presence or absence of exogenous G-CSF at a dose of 20 ng/mL. At baseline (t = 0 hours), both CML and non-CML cells had equally low G-CSF receptor expression (~1%). Thereafter, G-CSF receptor protein expression began to increase in chronic-phase CD34+ CML cells in both the G-CSF-treated and untreated cells at each time point compared with the non-CML CD34+ cells, which remained between 0% and 2% G-CSF receptor positive for all time points. However, adding exogenous G-CSF at a dose of 20 ng/mL did not affect G-CSF receptor expression in either CML or non-CML cells (Fig. 2B). In addition, there was no significant difference in cell cycle profile between CML and non-CML cells in response to exogenous G-CSF (Fig. 2C).

Characterization of the proliferative response of primitive CML cells to G-CSF in vitro. Immunomagnetically enriched populations of CD34+ CML cells were cultured for 6 days with and without growth factors and viable cell counts done after 1, 2, 3, and 6 days. The results are shown in Fig. 3. Even in the absence of added growth factors, the total number of cells increased in the first 2 days (~3-fold) and then remained constant for the next 4 days, indicating a continuing rate of cell production sufficient to balance but no longer exceed cell death, as expected from the known autocrine IL-3/G-CSF phenotype of these cells (32). Exogenously added G-CSF did not further enhance the expansion seen during the first 2 days but then, over the next 4 days, did stimulate a continued increase in total cells. This effect of G-CSF was saturated at a concentration of 20 ng/mL, although a further increase in cell output could be obtained by the further addition of other growth factors (IL-3, IL-6, FL, and stem cell factor).
Intermittent G-CSF enhances the imatinib mesylate–mediated elimination of CML cells in cultures initiated with CD34+ leukemic cells. In a second series of experiments, we then examined the effect of 5 μmol/L imatinib mesylate ± 20 ng/mL G-CSF given according to different exposure protocols (as described in Materials and Methods; Fig. 1). Cultures to which no imatinib mesylate was added showed the anticipated expansion in total viable cells, although, unexpectedly, this expansion was less in cultures given exogenous G-CSF intermittently than in cultures to which no G-CSF was added (Fig. 4A). In contrast, all cultures to which imatinib mesylate was added showed a net reduction in the total number of cells present after 12 days and this effect was consistently greater when the imatinib mesylate was present continuously than when it was omitted for the first day of each 4-day cycle. As illustrated in Fig. 4B, the pattern of the effect of imatinib mesylate [nil, intermittent (72 hours) or continuous (96 hours)] across the cycles on the total cell number remaining was similar for each set of G-CSF conditions [nil, intermittent (24 hours) or continuous (96 hours)]. Therefore, after 12 days, intermittent exposure of the cells to 20 ng/mL of imatinib mesylate treatment arms compared with the arms lacking imatinib mesylate. Similarly, the cells exposed to exogenous G-CSF continuously were reduced in number by 4- and 9-fold relative to input in the intermittent and continuous imatinib mesylate treatment arms, respectively.

Intermittent G-CSF enhances the rate at which undivided CD34+ CML cells disappear in vitro by cell division and not apoptosis. We next asked whether the differential effects of intermittent and continuous G-CSF on total cell outputs in 12-day cultures of CD34+ CML cells might be explained by matching effects on the quiescent subset present in the cells used to initiate the cultures. To address this question, the rate at which these quiescent cells disappeared was also determined. Their number in the input cells was defined as the number of viable (propidium iodide-negative) undivided (CFSEmax) CD34+ cells still present after an initial 4 days in cultures to which no G-CSF or imatinib mesylate was added. Changes in the size of this population were then calculated for each treatment protocol after each 4-day cycle for each CML sample tested (Fig. 1). The combined results are shown in Fig. 5A. As predicted from previous experiments (10), in the absence of G-CSF, imatinib mesylate treatment had no significant effect on this population, which slowly decreased over time (~10-fold after 12 days). Interestingly, continuous exposure of the cells to 20 ng/mL of imatinib mesylate treatment arms compared with the arms lacking imatinib mesylate. Points and columns, mean; bars, SE. G, G-CSF; w/o, washout; cont, continuous.
G-CSF had little effect on the rate at which the input quiescent CD34+ CML cells decreased. Moreover, these cells also did not decline any faster when 5 μmol/L imatinib mesylate was present (either intermittently or continuously), indicating that they had also not been sensitized to imatinib mesylate. The persistent insensitivity of the CD34+ undivided cells remaining after 12 days of continuous exposure to G-CSF and imatinib mesylate was not attributable to the emergence of a normal population because fluorescence in situ hybridization analysis showed that these were still BCR-ABL+ (>99% positive, e.g., 205 of 206).

Importantly, intermittent G-CSF markedly accelerated the pace at which the pool of undivided CD34+ cells decreased, in contrast to the lack of an effect of continuous G-CSF on this compartment. Thus, by the end of the second 4-day cycle of intermittent G-CSF treatment, the number of undivided CD34+ CML cells was reduced ~12-fold (i.e., ~3-fold more than in the cultures to which no G-CSF was added, although this difference was not significant) and, by the end of the third 4-day cycle, they had decreased 50-fold (~5-fold more than in the absence of G-CSF, P < 0.04). Of interest, the number of undivided CD34+ cells persisting in these cultures was not further reduced when imatinib mesylate was present either intermittently or continuously. Thus, the undivided CD34+ CML cells retained an imatinib mesylate-resistant phenotype as long as they persisted regardless of their growth factor treatment but rapidly became imatinib mesylate sensitive when stimulated to proliferate. Therefore, intermittent G-CSF offers the potential to increase the number of proliferating cells and reduce the pool of undivided CD34+ CML cells.

To determine the fate of cells leaving the undivided (CFSEmax) CD34+ cell population, caspase-3 activity was assessed at 24, 48, and 72 hours to determine if those cells leaving the undivided gate were undergoing apoptosis. The CFSEmax cells remaining at each time point were exclusively caspase-3 negative (Fig. 5B), showing that those cells that had escaped the CFSEmax gate had done so through cell division and not cell death.

Discussion

This study set out to evaluate the potential of using G-CSF stimulation and interruption of imatinib mesylate therapy to overcome the innate imatinib mesylate resistance that is characteristic of the most primitive quiescent CML cells. The first step was to characterize the response of primitive CML cells to elevated levels of G-CSF in vitro either maintained continuously or provided intermittently. It is well established
that in chronic-phase CML patients, an increased proportion of the CD34+ leukemic cells are already proliferating in vivo and we have shown that these cells will also continue to grow in vitro in the absence of added growth factors due to the activation of an autocrine G-CSF/IL-3 mechanism (33). Thus it could be inferred that cycling CD34+ CML cells possess functional G-CSF receptors. However, the question of whether the quiescent subset of CD34+ CML cells also express functional G-CSF receptors was an issue because these cells do not appear to respond rapidly to G-CSF stimulation in vitro (9). Here, we show for the first time that G-CSF receptor transcripts are present in both the quiescent and cycling fractions of CD34+ CML cells and at levels that are significantly higher than in their counterparts in normal bone marrow, with G-CSF transcript levels being higher in both cases in the proliferating populations.

The presence of functional G-CSF receptors on CD34+ CML cells was indicated by a dose-dependent ability of G-CSF (up to 20 ng/mL) to enhance the growth of these cells after 2 days in vitro and confirmed by flow cytometry for surface G-CSF receptor expression. Direct evidence that G-CSF could activate the quiescent subset of BCR-ABL+ CD34+ cells was also obtained by measuring the increased rate at which these cells disappeared over a 12-day period when cultured in serum-free medium to which 20 ng/mL G-CSF was added for the first day every 4 days. Thus, primitive quiescent CML cells seem to be responsive to G-CSF at concentrations similar to those that maximally stimulate primitive, quiescent normal bone marrow cells (34–36).

Interestingly, the effect of G-CSF on primitive quiescent CML cells was seen here only when they were exposed to the growth factor intermittently, compared with cultures in which the same concentration of G-CSF was either present continuously or was not added at all. Similarly, the total output of cells in the same cultures was consistently severalfold lower when the G-CSF was added intermittently, compared with an uninterrupted exposure to either the same concentration of G-CSF or the much lower levels obtained from the autocrine G-CSF produced (i.e., in the absence of exogenously added G-CSF). This suggests that intermittent exposure of primitive quiescent CML cells to G-CSF may elicit a unique mitogenic response initially that is unnecessary. However, whereas agents other than imatinib mesylate (e.g., hydroxyurea, 1-β-D-arabinofuranosylcytosine) could be used clinically to control the peripheral WBC count and avoid leukostasis, to date imatinib mesylate is the only drug that has the proved ability to reduce progression by decreasing mutagenesis presumed to be controlled through reduction of BCR-ABL kinase–driven reactive oxygen species production and subsequent decline in the rate of inappropriate repair of DNA double-strand breaks (37).

Previous studies have shown that imatinib mesylate reversibly blocks proliferation of primitive CML cells but does not induce apoptosis of these cells (6, 10, 38). Our group has also recently showed that whereas BCR-ABL kinase activity can be more effectively inhibited with novel agents within the progenitor compartment, primitive CML cells remain viable. As a subset, they may not have BCR-ABL activity inhibited rather the kinase remains active (39). Therefore, a further aspect of the present study was to determine whether exposure of primitive CML cells to G-CSF would sensitize these cells to the effects of either intermittent or continuous imatinib mesylate. We now show that this intrinsic imatinib mesylate insensitivity of primitive quiescent CML cells is not altered by either intermittent or continuous G-CSF exposure. However, the enhanced reduction of imatinib mesylate–resistant quiescent cells resulting from intermittent exposure to G-CSF is additive with the imatinib mesylate effect and, thus, in combination, a greater overall reduction in CML cells is achieved. Notably, this outcome was obtained with an in vitro concentration of 20 ng/mL of G-CSF and 5 μmol/L imatinib mesylate, both of which are at the upper limit of those achieved with doses currently considered safe for use in CML patients (18, 19, 40). Improved kill of quiescent CD34+ CML cells exposed to imatinib mesylate in combination with high concentrations of a cocktail of growth factors has been recently reported by another group (41).

Taken together, these findings provide a strong rationale for further evaluation of the potential clinical use of growth factors in combination with imatinib mesylate to improve long-term outcomes in chronic-phase CML (25). In fact, a multicenter clinical trial to examine this hypothesis has now been initiated in the United Kingdom and France. Such an approach is additionally supported by several recent reports that administration of G-CSF to patients who have become neutropenic on imatinib mesylate therapy may improve their cytogenetic response without evidence of stimulating the development of accelerated or blast phase disease (22–24). Administration of granulocyte macrophage-CSF has been reported to improve the activity of IFN therapy in CML patients (42) and a recent randomized study examining the effect of priming with G-CSF before chemotherapy showed this significantly improved disease-free survival in acute myeloid leukemia patients with standard risk disease (43). The studies here suggest that G-CSF in combination with imatinib mesylate may offer a novel strategy for improving responses in patients with chronic-phase CML by enhancing the elimination of imatinib mesylate–resistant CML stem cells.

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References

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Intermittent Exposure of Primitive Quiescent Chronic Myeloid Leukemia Cells to Granulocyte-Colony Stimulating Factor In vitro Promotes their Elimination by Imatinib Mesylate

Heather G. Jørgensen, Mhairi Copland, Elaine K. Allan, et al.


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