CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells

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Abstract
Chronic myelogenous leukemia (CML) is driven by constitutively activated Bcr-Abl tyrosine kinase, which causes the defective adhesion of CML cells to bone marrow stroma. The overexpression of p210Bcr-Abl was reported to down-regulate CXCR4 expression, and this is associated with the cell migration defects in CML. We proposed that tyrosine kinase inhibitors, imatinib or INNO-406, may restore CXCR4 expression and cause the migration of CML cells to bone marrow microenvironment niches, which in turn results in acquisition of stroma-mediated chemoresistance of CML progenitor cells. In KBM5 and K562 cells, imatinib, INNO-406, or IFN-α increased CXCR4 expression and migration. This increase in CXCR4 levels on CML progenitor cells was likewise found in samples from CML patients treated with imatinib or IFN-α. Imatinib induced G0-G1 cell cycle block in CML cells, which was further enhanced in a mesenchymal stem cell (MSC) coculture system. MSC coculture protected KBM-5 cells from imatinib-induced cell death. These antiapoptotic effects were abrogated by the CXCR4 antagonist AMD3465 or by inhibitor of integrin-linked kinase QLT0267. Altogether, these findings suggest that the up-regulation of CXCR4 by imatinib promotes migration of CML cells to bone marrow stroma, causing the G0-G1 cell cycle arrest and hence ensuring the survival of quiescent CML progenitor cells. [Mol Cancer Ther 2008;7(1):48–58]

Introduction
Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease characterized by the translocation of the c-Abl gene located on chromosome 9 to the specific breakpoint region (Bcr) on chromosome 22 (1). This translocation results in the synthesis of the chimeric fusion protein p210Bcr-Abl, which has constitutive tyrosine kinase activity. Hematopoietic cells expressing p210Bcr-Abl display reduced growth factor requirement, resistance to apoptosis, and altered adhesion and homing properties; this endows p210Bcr-Abl with oncogenic properties when constitutively expressed (2). High constitutive tyrosine kinase activity in turn causes multiple biochemical pathways to be activated via protein-protein interactions and the tyrosine phosphorylation of target substrates (3). Consistent with this, imatinib mesylate (imatinib, STI571), specific tyrosine kinase inhibitor that inhibits Bcr-Abl, Tel-Abl, V-Abl, and c-Kit kinase activity, as well as the growth and viability of cells transformed by any of the Abl genes (4), achieves durable responses in most patients with newly diagnosed CML in the chronic phase. However, a small percentage of patients, as well as most patients in the advanced phase of their disease, are resistant to imatinib therapy (5). Mechanisms for this resistance include p210Bcr-Abl protein overexpression and point mutations of the Bcr-Abl kinase in the ATP-binding pocket, which prevents the binding of imatinib (6). To overcome imatinib resistance, higher doses of imatinib and the combination of imatinib with other agents have been used with some success (7, 8). However, optimal therapy in patients with CML depends on a greater understanding of the mechanisms of imatinib resistance. Recent reports suggest that quiescent primitive CML CD34+ cells are insensitive to imatinib, and these cells persist even when complete responses are achieved after imatinib therapy (9). In addition, the quiescent primitive CML CD34+ cells are, in general, more resistant to a wide variety of proapoptotic stimuli (10), implying that these cells have unique mechanisms of protection from imatinib and other insults inducing cell death.
The extensive interactions necessary for proper growth and maturation of normal hematopoietic precursors may help to shed some light on this problem. These properties depend on the cells being in close contact with bone marrow stromal cells, with chemokines playing an important role in regulating the retention and movement of these progenitor cells within the bone marrow microenvironment. Stromal derived factor-1 (SDF-1), a member of the CXC subfamily of cytokines, is produced by stromal cells and, acting through the G protein–coupled CXCR4, the sole receptor for SDF-1 (11). SDF-1 functions as both a chemoattractant and a modulator of cellular growth/survival (12). The G protein–coupled CXCR4 receptor, which is expressed on the membrane of normal hematopoietic and acute myelogenous leukemia CD34+ cells, including CD34+38− cells (13), has been reported to mediate the chemotaxis of CD34+ stem cells and to play a critical role in the homing of these cells to the bone marrow microenvironment (14). Integrins represent another essential component of the bone marrow microenvironment that regulate cell survival and promote activation of multiple prosurvival signaling pathways (15). We recently reported the prominent role of integrin-linked kinase (ILK) that directly interacts with β integrins and promotes survival of leukemic cells through phosphorylation of Akt in a phosphoinositide 3-kinase–dependent manner (16). Microenvironment-mediated chemoresistance, including through CXCR4/SDF-1 axis, is now well recognized in different hematologic malignancies, including multiple myeloma, acute myelogenous leukemia, acute lymphoblastic leukemia, and chronic lymphocytic leukemia (17–19). However, its role in CML is less defined, primarily due to the notion of defective leukemia-stroma interactions inherent to CML cells. It has been shown that p210BCR-ABL can inhibit SDF-1α–induced migration and signaling and, hence, interfere with their migration to bone marrow stromal cells (20). Furthermore, spontaneous or SDF-1–mediated adhesion of CML cells to stromal cells and fibronectin is reportedly low (21). These findings suggest a principal role for CXCR4/integrin-dependent homing and retention defects characterized by abnormal release of immature myeloid cells in CML from the bone marrow into the circulation. Downregulation of CXCR4 expression was reported to be reversible by a small-molecule Bcr-Abl inhibitor STI (imatinib) in CD34+ cells from a limited number of samples from CML patients cultured with this agent in vitro (20). In this study, we tested the hypothesis that imatinib via inhibition of Bcr-Abl will restore CXCR4/SDF-1α interaction and subsequently cause CML cells to migrate and attach to supportive milieu of bone marrow microenvironment. We investigated the effects of imatinib on CXCR4 expression, cell migration, and apoptosis in both imatinib-sensitive and imatinib-resistant CML cell lines and primary samples from CML patients. We further used pharmacologic inhibition of CXCR4 and additionally of ILK to pinpoint the role of cell migration and stroma-leukemia interactions on CML survival after imatinib treatment. Our results show that imatinib restores CXCR4 expression under mesenchymal stem cell (MSC) coculture conditions, which result in the enhanced migration of CML cells to bone marrow stromal cells. This is associated with inhibition of proliferation of CML cells concomitant with enhanced survival. Because surviving quiescent CML progenitor cells are postulated to represent the potential source of CML relapse (22), this provides the rationale for the combined use of pharmacologic inhibitors of CXCR4 signaling with imatinib in CML therapy.

Materials and Methods

Cells and Cell Culture Conditions

KBM-5 cells derived from a Ph+ CML patient in blast crisis and its imatinib-resistant subvariant KBM-5/STI571 cells were kindly provided by Dr. M. Beran (Department of Leukemia, M. D. Anderson Cancer Center; ref. 23). KBM-5/STI571 cells was established through the acquisition of a single-point mutation leading to a threonine-to-isoleucine substitution at position 315 of the ABL gene (6). KBM-5 and KBM-5/STI cells were cultured in Iscove’s modified Dulbecco’s, and K562 cells in RPMI 1640, both containing 10% heat-inactivated fetal bovine serum, 1% l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO2. Samples procured from CML patients treated at M. D. Anderson Cancer Center in 1999 to 2000, and normal bone marrow were obtained after informed consent in accordance with institutional guidelines set forth by M. D. Anderson Cancer Center and the Declaration of Helsinki. For analysis of bone marrow biopsy sections, the files of the Department of Hematopathology at M. D. Anderson Cancer Center were searched for CML cases treated with imatinib under Institutional Review Board–approved protocol. MSCs obtained from a normal bone marrow donor were cultured at a density of 5,000 to 6,000 cells/cm² in MEM-a supplemented with 20% fetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin (24). Passage 3 or passage 4 MSCs were used for the coculture experiments.

Treatment of Cells

To study the effect of bone marrow stroma on CML cells, KBM-5, K562, and KBM-5/STI cells at a density of 0.5 × 10⁶ or cells from CML patients at a density of 1 × 10⁶ were cultured with and without a layer of MSCs at a density of 0.2 × 10⁵ cells/cm² under serum-starved conditions (0.5% fetal bovine serum). Cocultured CML cells were separated from MSC monolayer by careful pipetting with ice-cold PBS (repeated twice). After collecting the CML cells, MSC monolayers were observed by microscopy (100×) to confirm that the monolayer was not damaged and that fewer than 10 leukemic cells per vision field remained attached. To study the effects of the soluble factors produced by MSCs, CML cells were plated in 12-well plates containing preestablished (24 h) confluent MSCs in conditioned low-serum medium, either in direct contact or separated by a 0.4-μm porous transwell insert that allowed the passage of soluble growth factors (24). In some experiments, cocultures were done in the presence of...
imatinib (kindly provided by Novartis), INNO-406 (formerly NS-187, kindly provided by Innovive Pharmaceuticals), a novel dual Bcr-Abl/Lyn tyrosine kinase inhibitor, which is 25-fold to 55-fold more potent than imatinib in suppressing Bcr-Abl+ leukemias and with activity against most of imatinib-resistant mutated Bcr-Abl except T315I (25), IFN-α (10,000 units/mL; Schering Co.), stem cell factor (SCF; kindly provided by Amgen or purchased from R&D Systems), or SDF-1α (purchased from R&D Systems). AMD3465, a second generation small-molecule reversible inhibitor of SDF-1α/CXCR4 with an IC_{50} SDF-1 binding of 42 ± 2 nmol/L, was provided by Dr. G. Bridger (AnorMED, Inc.). The ILK inhibitor QLT0267, a submicromolar inhibitor of the phosphotransferase activity of ILK toward a molecule library against the target ILK, was obtained from QLT, Inc. (26, 27).

**CXCR4 Expression in Imatinib-Treated CML**

Plasma levels of SDF-1α in CML patients and normal subjects and SCF levels in conditioned media from KBM-5 cells were detected using ELISA, which were done according to the manufacturer’s instructions (R&D Systems).

**Chemotactic Assay**

The cell migration assay was done using 5-μm pore transwell filters (Costar) as previously described (29). All experiments were done in triplicates. Data are presented as the chemotaxis index, calculated as the ratio of the number of cells migrating to MSCs to the number of cells migrating to medium.

**Western Blot Analysis**

Western blot analysis was done as described previously (24), immunoblotted with phosphorylated MAPK^{Thr202/Tyr204} [phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated Akt^{Ser473}, or Akt antibodies (Cell Signaling Technology), p42MAPK (ERK1/2; Santa Cruz) plus β-actin antibody (Abcam). For the coculture experiments, KBM-5 cells were cultured alone or with MSCs under the indicated conditions. Signals were detected by a luminescence image analyzer (LAS-100 plus; Fujifilm) and quantified by Image Gauge (Fujifilm).

**Statistical Analysis**

Results are shown as the mean ± SD or SE of at least three experiments each. Paired data were analyzed using the paired Student’s t test or Wilcoxon matched pairs test, with P values of <0.05 considered significant.

**Results**

**Imatinib Inhibits Cell Growth by Inducing Apoptosis and G_0 Cell Cycle Arrest**

We first examined the effects of imatinib on cell growth inhibition and apoptosis by exposing KBM-5, K562, and KBM-5/STI cells to incremental concentrations of imatinib. As shown in Fig. 1A, imatinib inhibited cell growth of KBM-5 and K562 but not of KBM-5/STI cells. This finding was concordant with the induction of apoptosis in KBM-5 and K562 cells but not in KBM-5/STI cells, as detected by flow cytometry at 72 h of culture (Supplementary Fig. S1A, B).7

We then did cell cycle analysis to acquire a greater understanding of the mechanisms of cell growth arrest in these cells, in light of the dual function of imatinib as a cell proliferation and apoptosis regulator. To assess the effects of bone marrow stroma, we also conducted experiments in the coculture system of human bone marrow–derived MSCs with KBM-5 and KBM-5/STI cells. Imatinib significantly increased the G_{0-/G_1} fraction of parental KBM-5 cells and decreased proportion of S-phase cells compared with the control cells (Supplementary Table S1).7 Interestingly, MSC coculture promoted increase in the percentage of cells in G_{0-/G_1} phase, and this effect was further enhanced by imatinib. However, no significant G_{0-/G_1} arrest was observed in KBM-5/STI cells under any of these conditions. It was noted, however, that the proliferation of untreated KBM-5/STI cells is slower compared with parental cells.

7 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Figure 1. A, imatinib inhibits growth of KBM-5 and K562 but not KBM-5/STI cells. KBM-5, K562, and KBM-5/STI cells were cultured at an initial cell density of $2.5 \times 10^5$/mL with indicated concentrations of imatinib. Viable cells were counted using the trypan blue exclusion method after 72 h of culture. Columns, mean from three independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$.

B, effect of imatinib on CXCR4 expression in KBM-5, K562, and KBM-5/STI cells cocultured with MSCs. Percentage of CXCR4-expressing cells after 72 h of treatment with 0.5 μmol/L imatinib was determined. Cells were cultured under serum-starved condition (0.5% fetal bovine serum) with or without MSC monolayer and then analyzed by flow cytometry as described in Materials and Methods. Columns, mean from three independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$.

C, CXCR4 expression of KBM-5 cells is induced independently of direct contact with MSCs. KBM-5 cells in the absence or presence of 0.5 μmol/L imatinib and/or 5 μmol/L AMD3465 were separated from MSCs by a porous transwell insert or cultured on top of the MSC monolayers. After 72 h of coculture, KBM-5 cells were harvested by extensive washing and CXCR4 expression was assessed by flow cytometry. CXCR4 expression was up-regulated in coculture, both under noncontact and cell-to-cell contact conditions, and further increased by imatinib or INNO-406. AMD3465 did not affect CXCR4 expression. Columns, mean for triplicate experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$.

D, SCF-induced CXCR4 expression in KBM-5 cells and primary CML progenitor cells. Bone marrow mononuclear cells from CML patients were treated with or without 100 nmol/L recombinant SCF, stained with CD34-PerCP and CXCR4-PE antibodies and analyzed by dual-color flow cytometry. Data represent percentage of CXCR4(+) cells after electronic gating on CD34+ progenitor CML cells.
Taken together, these results indicate that KBM-5 cells are sensitive to the growth-inhibitory effects of imatinib and respond by initiating apoptosis and accumulating in G0-G1. The presence of bone marrow stroma might enhance the antiproliferative effects of imatinib with a further increase in the proportion of cells in the quiescent phase.

**Imatinib Induces CXCR4 Expression and Triggers Migration of KBM-5 Cells**

We next examined changes in CXCR4 expression in KBM-5 and KBM-5/STI cells in response to imatinib in cells cultured with or without MSCs. As shown in Fig. 1B, CXCR4 expression was moderately but significantly increased by MSC coculture in both KBM-5 and KBM-5/STI cells. Whereas imatinib did not affect CXCR4 expression in KBM5 cells cultured without stromal support, it significantly enhanced the CXCR4 levels induced by MSC cocultures (Fig. 1B; Supplementary Fig. S2B). In contrast, no change in CXCR4 expression was induced by imatinib in KBM-5/STI cells cultured either with or without MSCs (Fig. 1B; Supplementary Fig. S2B). CXCR4 expression was similarly induced by dual Bcr-Abl/Lyn tyrosine kinase inhibitor INNO-406 in KBM-5 cells but not in KBM-5/STI cells (Supplementary Fig. S2). Likewise, imatinib or INNO-406 enhanced CXCR4 expression in imatinib-sensitive K562 cells growing on MSC layer, similar to KBM-5 cells.

Next, to determine whether the ability of MSCs to induce CXCR4 expression depends on direct cell-to-cell interactions or is mediated by soluble factors secreted by MSCs, we cultured KBM-5 and KBM-5/STI cells that were separated from MSCs by transwell inserts. CXCR4 expression was up-regulated by MSCs under both noncontact and cell-contact conditions (Fig. 1C), suggesting that soluble factors secreted by MSCs induce CXCR4 expression. Imatinib and INNO-406 further induce CXCR4 expression in K562 cells when cocultured with MSC under both conditions, with or without direct leukemia-MSC contact. This increase in CXCR4 expression was unperturbed by the small-molecule inhibitor of SDF-1/CXCR4 AMD3465.

In an attempt to identify factors secreted by MSC that are capable of inducing CXCR4 expression, we treated KBM-5 cells and CD34+ bone marrow cells from primary CML samples with SCF. SCF is secreted by bone marrow stroma and is known to increase CXCR4 expression (30). In KBM-5 cells, SCF alone or combined with imatinib induced minimal up-regulation of CXCR4. In contrast, SCF with imatinib induced significant increase in CXCR4 expression under conditions of MSC coculture (Supplementary Fig. S3), with ~80% of cells expressing CXCR4. We next examined whether imatinib affects SCF production by MSC, but observed no significant changes in SCF levels in MSC conditioned medium (data not shown).

In CD34+ cells from primary CML patients, SCF up-regulated CXCR4 levels (Fig. 1D), indicating that primary CML cells are more sensitive to the effects of SCF on CXCR4 induction than KBM-5 cell line. These results indicated that SCF produced by MSCs may trigger moderate induction of CXCR4 expression, which is further stimulated by imatinib.

**Imatinib Stimulates Cell Migration to Bone Marrow Stroma**

Based on the finding that tyrosine kinase inhibitors increase CXCR4 levels, we next analyzed the migratory ability of imatinib-treated KBM-5 and KBM-5/STI cells toward MSC monolayer. In untreated KBM-5 cells, ~5% of cells migrated in response to MSCs. In contrast, imatinib-treated KBM-5 cells efficiently migrated to MSCs as illustrated by the in vitro migration assays (Fig. 2A). Similar results were obtained in K562 cells. Conversely, AMD3465, a specific CXCR4 antagonist that inhibits the cell surface binding of SDF-1α to its cognate receptor CXCR4 (31), abrogated imatinib-induced cell migration. In contrast, KBM-5/STI cells treated with imatinib only minimally migrated to MSCs, and AMD3465 had no effect on this migration (Fig. 2A). INNO-406 had the same effects in KBM-5 and K562 (Supplementary Fig. S4) but not in KBM-5/STI cells (data not shown).

These findings collectively suggest that the CXCR4 down-regulation mediated by p210BCR-ABL is restored by imatinib or INNO-406, which in turn enhances the homing of CML cells to the bone marrow microenvironment.

**Effects of MSC Coculture on Imatinib-Induced Growth Inhibition of CML Cells**

Because we observed that imatinib enhanced KBM-5 cell migration to MSCs, along with the significant induction of G0-G1 phase arrest, we hypothesized that molecular effects of imatinib within bone marrow microenvironment may paradoxically promote survival of CML cells and hence provide nonpharmacologic resistance to Bcr-Abl inhibition strategies. We therefore investigated the effects of imatinib on KBM-5 cells grown on MSC monolayers. As shown in Fig. 2B, whereas imatinib induced significant inhibition of cell growth and moderate apoptosis of KBM5 cells, these growth inhibitory effects were diminished by MSC cocultures. The cotreatment with imatinib and CXCR4 inhibitor AMD3465, however, significantly enhanced imatinib-induced growth inhibition and apoptosis only in CML cells cocultured with MSC (Fig. 2B).

Because integrins play a critical role in regulation of cell survival through direct cell-to-cell interactions, we next examined the effects of small-molecule inhibitor of ILK QLT0267 (26, 27) on imatinib-treated CML cells under MSC coculture conditions. QLT0267 blocked KBM5 cell proliferation with moderate induction of cell death. In turn, combination of QLT0267 with imatinib induced further growth inhibition and apoptosis induction (Fig. 2B). These combined effects were again seen only in MSC cocultures, suggesting specific inhibition of leukemia-stroma interactions by these agents.

Altogether, these findings indicate that imatinib-induced CXCR4 expression, at least, partially contributes to survival of KBM-5 cells growing in contact with MSCs, via enhanced migration and integrin-dependent adhesion of CML cells to bone marrow–derived stromal cells.
Figure 2. A, effects of imatinib on migration of KBM-5, K562, and KBM-5/STI cells to MSCs. Columns, mean percentage of migrated cells after 24 h of treatment with indicated inhibitors cultured with or without MSCs; bars, SD. MSC-induced migration of KBM-5 and K562 cells was increased by imatinib and partially abrogated by AMD3465. In contrast, imatinib had no significant effect in KBM-5/STI cells. *, P < 0.05; **, P < 0.01. B, the effects of CXCR4 and ILK inhibition on imatinib-induced apoptosis of KBM-5 cells in MSC coculture. Viable KBM-5 cells were counted using the trypan blue exclusion method after 72 h of culture (i) and Annexin V determined after 72 h of coculture in low serum (0.5% fetal bovine serum) medium with the indicated agents (0.5 μmol/L imatinib, 5 μmol/L AMD3465, or 10 μmol/L QLT0267; ii). Columns, mean from more than two independent experiments; bars, SD. *, P < 0.05; **, P < 0.01. C, effects of imatinib on phosphorylation of Akt and ERK in KBM-5 cells. KBM-5 cells were cultured alone or with MSCs at the indicated conditions (with or without 0.5 μmol/L imatinib and 5 μmol/L AMD3465) for 24 h. Clarified cell lysates were probed with antibodies to phosphorylated Akt (Ser473). Akt, phosphorylated ERK (Thr202/Tyr204), ERK1/2, and β-actin in Western blots. Results shown are representative of two experiments.
Inhibition of Phosphorylated ERK Signaling by Imatinib Persists Despite CXCR4 Up-Regulation

BCR-ABL activates Ras, which leads to the phosphorylation and activation of multiple signaling proteins, including p42/44 mitogen-activated protein kinase (ERK1/2) and phosphoinositide 3-kinase/Akt (32, 33). Imatinib down-regulates ERK and Akt phosphorylation by abrogating BCR-ABL tyrosine kinase activity (33). On the other hand, SDF1-α–CXCR4 signaling was reported to activate Akt and ERK implicated in the regulation of the migration of hematopoietic progenitor cells (34, 35). To examine signaling events in CML cells cultured with or without MSC support, we examined the change in phosphorylation of Akt and ERK induced by imatinib and AMD3465 in the presence and absence of MSCs. As shown in Fig. 2C, AKT and ERK were constitutively phosphorylated and hence activated in KBM-5 CML cells. Imatinib inhibited Akt and ERK activities in KBM-5 cells in the presence or absence of MSCs. MSC coculture did not further increase baseline phosphorylation levels of Akt and ERK, and CXCR4 inhibitor AMD3465 did not significantly alter the phosphorylation of Akt and ERK that was diminished by imatinib treatment.

Imatinib and IFN-α Restore Deficient CXCR4 Expression in Primary CD34+ CML Cells

To determine whether CXCR4 expression is down-regulated in primary CML progenitor cells and whether imatinib can enhance the migration properties of CML cells by restoring CXCR4 expression, CXCR4 levels were measured in bone marrow CD34+ cells from primary CML patients. As shown in Fig. 3A and Supplementary Fig. S5,7 bone marrow progenitor cells from newly diagnosed CML patients and from CML patients in blast crisis expressed markedly lower CXCR4 levels on cell surface compared with CD34+ normal bone marrow progenitor cells. This drastically diminished CXCR4 expression translated into a deficient migration of CD34+ CML cells compared with normal CD34+ cells (Fig. 3B). On the contrary, SDF-1α levels were similar in plasma obtained from CML patients (both in untreated chronic phase and in blast crisis) and from hematologically healthy individuals (Fig. 3C).

To determine if imatinib can induce CXCR4 expression in vivo, we analyzed longitudinal peripheral blood samples from five CML patients in blast crisis treated with imatinib. Remarkably, imatinib induced CXCR4 expression in CD34+ and CD34+38 progenitor cells within a few days after initiation of treatment (Fig. 4A). This coincided with drop in WBC count and absolute blast counts in these patients. CXCR4 levels of CD34+ peripheral blood CML cells, however, decreased frequently, even below the starting levels, after prolonged (range, 3–10 days) exposure to imatinib. We next examined CXCR4 immunostaining on blasts/immature cells in bone marrow biopsy sections from a separate set of patients treated with imatinib (patients 6–12; one patient in chronic phase and seven patients in myeloid blast-phase of CML; Table 1). Before initiation of imatinib therapy, CXCR4 positivity was detected in two of seven samples (patients 8 and 11, both from patients in
blast phase). From five patients whose bone marrow immature cells did not express CXCR4 before therapy, four became CXCR4 positive and one remained CXCR4-negative after imatinib treatment. Examples of CXCR4 staining in bone marrow sections from patients 6 and 9 are presented in Supplementary Fig. S6.7 While patients in blast phase had short-lived hematologic responses to imatinib, Ph+ clone remained predominant in the bone marrow, and all patients ultimately relapsed with recurrence of blasts in the bone marrow.

We also examined the effects the IFN-α, formerly the standard potentially curative treatment in CML, on CXCR4 levels. Comparative analysis of CXCR4 expression on bone marrow CD34+ cells in newly diagnosed CML patients and from CML patients in chronic phase post 3 mo of Hydrea or IFN-α therapy (n = 5). Points, mean; bars, SE. **, P < 0.01. C, CXCR4 expression on bone marrow CD34+ cells in sequential samples from CML patients treated with IFN-α. CXCR4 expression on bone marrow CD34+ cells in sequential samples from CML patients treated with IFN-α for 3 to 8 mo. Percentage of Ph(+) bone marrow metaphases post-IFN treatment: patient 13, 100%; patient 14, 100%; patient 15, 15%; patient 16, 100%; patient 17, 5%.

### Table 1. CXCR4 immunostaining in bone marrows from patients before and after imatinib therapy

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<th>Patient no.</th>
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<td>Status</td>
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<td>12</td>
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NOTE: CP, chronic phase; BC, blast-phase CML; ND, not done.
five newly diagnosed patients started on IFN-α therapy documented gradual increase in CXCR4 expression measured on CD34+ bone marrow CML cells during treatment (Fig. 4C). These findings suggest that IFN-α, which was reported to down-regulate p210BCR-ABL protein (36), restores cell adhesion, inhibits the proliferation of CML progenitors (37), and induces up-regulation of CXCR4. Whereas IFN therapy resulted in normalization of WBC in all five patients (mean ± SE, 29.5 ± 7 for pre-IFN WBC and 4.4 ± 1.1 for post-IFN), analysis of cytogenetic responses in these patients showed persistence of 100% Ph(+) bone marrow metaphases in three of the five patients (patients 13, 14, and 16) and partial cytogenetic responses in the remaining two patients (patients 15 and 17).

Taken together, these results indicate that imatinib or IFN-α treatment is associated with the up-regulation of CXCR4 expression, which may have a protective effect on a population of imatinib-refractory quiescent CML cells persisting in the bone marrow.

Discussion

In this study, we investigated the effects of the Bcr-Abl tyrosine kinase inhibitors imatinib and INNO-406 (25), as well as IFN-α (36), on CXCR4 expression, migration to bone marrow stroma cells, and survival of CML cells. Our in vitro experiments showed that imatinib and INNO-406 induced the expression of CXCR4 only in the imatinib-sensitive KBM-5 and K562 cells but not resistant KBM-5/STI cells. These results suggest that the ability of these tyrosine kinase inhibitors to up-regulate CXCR4 is linked to their inhibitory effects on BCR-ABL activity, hence indirectly suggesting that p210BCR-ABL is critical for the deregulation of CXCR4 expression. Our findings that primary CML progenitor cells, not only in blast crisis phase but also in chronic phase, exhibit low CXCR4 expression indicate that CXCR4 is deficient in all phases of CML. These results are perhaps at variance with those recently reported by Geay et al. (20), in which CXCR4 levels were diminished only in CD34+ cells from patients in blast crisis CML, but not in chronic phase. These discrepancies likely stem from the fact that Geay et al. found no difference between CXCR4 expression measured in normal apheresis products and peripheral blood from patients with chronic phase CML, whereas our studies were based on the comparison between normal CD34+ cells and CML CD34+ cells harvested from the bone marrow. Of note, studies in normal donors reportedly showed different density of CXCR4 on mobilized peripheral blood neutrophils and CD34+ cells with respect to bone marrow–derived CD34+ cells (38, 39).

In the in vitro systems of KBM-5 cells cocultured with bone marrow–derived stromal cells, restoration of CXCR4 expression by tyrosine kinase inhibitors was associated with the increased migration of CML cells to MSCs. Conversely, cell migration was partially, but significantly, inhibited when CXCR4 function was blocked with the small-molecule antagonist AMD3465 in imatinib-sensitive KBM-5 cells. The fact that the cytokine SCF and coculture with MSCs also induced CXCR4 expression in CML cells suggests that cytokines secreted by bone marrow stroma, including SCF, are factors in bone marrow that induce CXCR4. This is also supported by the findings that MSC-conditioned medium induces CXCR4 expression as efficient as MSC grown in direct contact with leukemic cells. Cotreatment of imatinib with SCF enhanced CML cells CXCR4 expression without affecting SCF production by MSC, further indicating that SCF and imatinib regulate CXCR4 induction via different mechanisms.

In KBM-5 cells, low-dose imatinib treatment inhibited cell growth mainly by inducing G0-G1 cell cycle block and by moderately inducing apoptosis. Notably, KBM-5 cells cocultured with MSCs were less sensitive to imatinib-induced apoptosis. The protective effects of bone marrow stroma and imatinib on leukemic cells were likewise seen in p190BCR-ABL-expressing lymphoblasts (40). In our system, the prosurvival effects of MSC on imatinib-induced growth inhibition of KBM-5 cells were blocked by AMD3465, suggesting that the CXCR4 up-regulation induced by imatinib and bone marrow stroma promotes survival of CML cells. Hence, the synergistic chemotaxis effects of bone marrow stroma–secreted cytokines, together with imatinib, may paradoxically favor CML cell survival. Similarly, imatinib was recently shown to be incapable of eradication of quiescent leukemia stem cells (41, 42). From this, it seems that imatinib has dual effects on CML cells in the bone marrow microenvironment; it may induce growth inhibition followed by apoptosis in most cells, but at the same time, it may enable some CML cells to migrate to and become sequestered in the bone marrow environment through up-regulation of CXCR4, hence selecting bone marrow–resident, imatinib-refractory CML cells. This hypothesis was further substantiated in our findings of restoration of CXCR4 expression in CML patients treated with imatinib or IFN-α. Whereas our measurements do not allow differentiation between CML and normal CD34+ cells, the steady increase in CXCR4 levels was seen in bone marrow cells of IFN-α-treated patients despite persistence of CML clone, supporting our hypothesis of homing of CXCR4-expressing CML cells to the bone marrow upon prolonged IFN-α treatment. Imatinib induced transient up-regulation of CXCR4 in peripheral blood CD34+ CML cells, followed by decrease in the number of CXCR4-expressing cells. Although imatinib-induced apoptosis could plausibly explain these results, data from other groups suggest that, unlike in cell lines, imatinib primarily suppresses proliferation rather than induces apoptosis of primary CML progenitor cells (43). Hence, these observations are more consistent with decrease of CML CD34+ cells from circulation secondarily to migration of high CXCR4-expressing cells to the bone marrow. This is supported by the immunohistochemical analysis data of bone marrow biopsies from a limited number of CML patients demonstrating conversion to CXCR4 positivity after imatinib therapy. Although further studies are required to convincingly show a link between...
higher CXCR4 expression in bone marrow–resident CML cells and refractoriness to imatinib, these preliminary results support the hypothesis of bone marrow homing of CML cells after imatinib-induced CXCR4 up-regulation.

The mechanisms involved in the p210\textsuperscript{Bcr-ABL}–induced disruption of CXCR4 expression and the events downstream of CXCR4 signaling are not well understood. Imatinib and IFN\textalpha{} have been known to have overlapping effects on intracellular signaling and cell function in CML, such as interacting with some signaling pathways that are influenced by p210\textsuperscript{Bcr-ABL}, in particular the components of the phosphoinositide 3-kinase signaling (44). We found in this regard that imatinib caused inhibition of Akt and ERK phosphorylation in KBM-5 cells, a finding consistent with those in previous studies (33, 45). Although phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase signaling have been reported to be downstream targets of SDF-1\textalpha{} and the SCF-induced activation of the CXCR4 pathway (34, 35), AMD3465 alone failed to affect phosphorylated Akt and phosphorylated ERK expression in CML cells. These findings suggest that although imatinib suppresses Akt and ERK phosphorylation through BCR-ABL-Ras inactivation, the factors other than AKT and mitogen-activated protein kinase signaling are responsible for sensitizing effects of CXCR4 inhibitor in MSC cocultures.

In addition to chemokine receptors, integrins and other components of extracellular matrix are strongly implicated in the stroma-mediated chemoresistance (46). Both CXCR4 and integrin signaling are implicated in adhesion and chemoresistance of other cell types (47). Recently, the α6β1-integrin–mediated adhesion has been reported to up-regulate CXCR4 in pancreatic cancer cells (48). To interrogate the functional significance of ILK, which is activated proximally in response to integrin ligation (16, 48), we studied the combined effects of imatinib and selective ILK inhibitor QLT0267 (26, 27). Similar to the effects of AMD3465, QLT enhanced imatinib-induced growth inhibition only in MSC cocultures, indicating specificity of these effects to stroma-mediated survival. VLA-4/VCAM-1 interactions represent cooperative and compensatory molecular pathways guiding bone marrow homing of adult bone marrow hematopoietic cells in addition to SDF-1/CXCR4, with secondary contributions by β2-integrins and endothelial selectins (37). Furthermore, SDF1/CXCR4 interactions have been implicated in the impaired binding of VLA-4 and VLA-5 to their endothelial/stromal and extracellular matrix ligands in Ph\textsuperscript{+} progenitor cells (24). Although further studies are clearly warranted to characterize changes in ILK function in CML, these preliminary studies suggest contribution of both chemokines and integrin-linked signaling pathways in the supportive effects of bone marrow microenvironment, likely predisposing CML cells to imatinib resistance. We propose the model whereby CXCR4 expression in CML cells is induced through intracellular signals initiated by imatinib and bone marrow stroma–produced cytokines, such as SCF. In turn, SDF1/CXCR4 interactions in the bone marrow microenvironment trigger integrin engagement and activation of the downstream signaling cascades, such as ILK, which further promote survival of CML cells (Fig. 5).

Taken together, our results provide the rationale for interfering with the protective effects of bone marrow stroma cells by inhibiting CXCR4 and/or integrin signaling, which could be of benefit in eradicating CML cells that represent a potential source of relapse in patients with CML.

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References
CXCR4 Expression in Imatinib-Treated CML

CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells

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