p210BCR-ABL inhibits SDF-1 Chemotactic Response via Alteration of CXCR4 Signaling and Down-regulation of CXCR4 Expression

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Abstract

It has been shown that p210BCR-ABL significantly impairs CXCR4 signaling. We report here that the migratory response to SDF-1 was profoundly altered in blast crisis, whereas chronic-phase CD34+ cells migrated normally to this chemokine. This migratory defect was associated with a low CXCR4 membrane expression. In vitro STI-571 treatment of CD34+ cells from patients in blast crisis markedly increased the CXCR4 transcript and CXCR4 membrane expression. Because p210BCR-ABL frequently increases with disease progression, we determined the effects of high and low p210BCR-ABL expression on CXCR4 protein in the granulocyte macrophage colony-stimulating factor–dependent human cell line MO7e. p210BCR-ABL expression distinctly alters CXCR4 protein through two different mechanisms depending on its expression level. At low expression, a signaling defect was detected with no modification of CXCR4 expression. However, higher p210BCR-ABL expression induced a marked down-regulation of CXCR4 that is related to its decreased transcription. The effect of p210BCR-ABL required its tyrosine kinase activity. Collectively, these data indicate that p210BCR-ABL could affect CXCR4 by more than one mechanism and suggest that down-regulation of CXCR4 may have important implications in chronic myelogenous leukemia pathogenesis. (Cancer Res 2005; 65(7): 2676-83)

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease arising at the level of a pluripotent stem cell characterized by the presence of a Philadelphia chromosome generated by the translocation of the c-ABL gene located on chromosome 9 in the specific breakpoint region (bcr) region of the BCR gene on chromosome 22 (1). This translocation results in synthesis of the chimeric fusion protein p210BCR-ABL with a constitutive tyrosine kinase activity. The fact that human CML is due to the presence of p210BCR-ABL protein has now well been established in both experimental and clinical conditions. Indeed, introduction of the p210BCR-ABL cDNA into murine stem cells by retrovirus induces a disease that shares many common features with CML (2, 3). In human cells, as well as in the mouse model, hematopoietic cells expressing the p210BCR-ABL oncoprotein display reduced growth factor requirement, differentiation arrest, resistance to apoptosis, altered adhesion, and homing properties (4–7). The oncogenic properties of p210BCR-ABL are caused by its high constitutive tyrosine kinase activity recruiting and activating multiple biochemical pathways, partly via protein-protein interactions and tyrosine phosphorylation of target substrates (4, 8). Consequently, p210BCR-ABL regulates the expression of several key proteins involved in cell cycle (9) and DNA reparation, both at a transcriptional and post-transcriptional level. For example, p27Kip and DNA PKCs are post-transcriptionally regulated in CML through their accelerated degradation by the proteasome (10, 11). In contrast, the granulocyte colony-stimulating factor (G-CSF) receptor is down-regulated at the transcriptional level by a suppression of CAAT/enhancer binding protein α (C/EBPα) expression, whereas overexpression of SOCS2 in p210BCR-ABL–expressing cells is related to increased transcript levels mediated through STAT5 activation (12, 13).

CXCR4 belongs to a family of seven transmembrane-spanning proteins, the vast majority being receptors coupled to, or signaling via, heterotrimeric guanine nucleotide-binding proteins (G-proteins; ref. 14). The CXCR4 natural ligand is stromal-derived factor-1α (SDF-1α), a CXC chemokine constitutively produced by bone marrow stromal cells. SDF-1α was initially characterized as a powerful chemoattractant for T cells and monocytes (15), but it was subsequently shown that SDF-1α also displays similar properties on early hematopoietic cells (16–18). The major in vivo processes regulated by the SDF-1α/CXCR4 couple have been highlighted by the phenotype of mice in which one or the other genes have been deleted (19–21). Mice lacking SDF-1α or CXCR4 die perinatally and display profound defects in the nervous system, vascular development, cardiogenesis, and hematopoiesis (21, 22). Notably, in addition to an abnormal early B-cell lymphopoiesis, SDF-1α−/− or CXCR4−/− mice show a profound defect in marrow myelopoiesis, whereas myelopoiesis in the fetal liver is only slightly impaired, indicating that SDF-1α and CXCR4 are involved in migration and colonization of bone marrow by hematopoietic progenitors during fetal development (21, 23). Moreover, an inhibition of CXCR4 function with blocking antibodies results in a considerable delay in bone marrow engraftment by human hematopoietic stem cells in the NOD-SCID model (24). On the other hand, CXCR4 expression is also crucial for the retention of maturing hematopoietic cells in the marrow microenvironment, as revealed by the early release of CXCR4−/− hematopoietic precursors and mature cells into the circulation (23). Similarly, normal circulating stem cells and progenitor cells are less responsive to SDF-1α than their marrow counterparts, this difference being correlated with a lower expression level of CXCR4 suggesting that loss of CXCR4 signaling may be involved in progenitor cell mobilization.

p210BCR-ABL was shown to alter CXCR4 signaling, as it can inhibit SDF-1α induced migration and signaling upon its
overexpression in several hematopoietic cell lines (25). A recent study has also reported a cross-talk between p210BCR-ABL and CXCR4 pathway resulting in disrupted chemokine signaling and chemotaxis (26). However, it is not clear whether cells from CML patients exhibited altered responses to SDF-1α. It was reported that leukemic CD34+ cells in CML patients display either diminished (25) or normal chemotactic response to SDF-1α (27, 28). Interestingly, CD34+ cells collected from the blood of some patients treated with hydroxyurea lost their ability to migrate in response to SDF-1α (27). One explanation for the discordant observations between untreated and hydroxyurea-treated chronic-phase CML progenitors or p210BCR-ABL cell lines was that the two later harbored a higher level of p210BCR-ABL protein. Indeed, the level of BCR-ABL expression was shown a crucial determinant in modulating growth factor independence, survival, or resistance to apoptosis (29, 30).

In this study, we analyzed the consequences of elevated expression of p210BCR-ABL on CXCR4 expression and function in the pluripotent human hematopoietic cell line M07e. Our results show that in cells expressing relatively low level of p210BCR-ABL, CXCR4/SDF-1 is only slightly altered with no alteration of CXCR4 expression. In contrast, cells expressing high level of p210BCR-ABL exhibited an important signaling defect, associated with diminution of CXCR4 expression at the transcriptional level. We also assessed CXCR4 expression and function in CD34+ cells isolated from patients at different stage of the disease. We observed that CXCR4 membrane expression and SDF-1 induced migration are down-regulated in CD34+ cells from patients in blast crisis compared with CD34+ cells from either patients at the chronic phase or control individuals. This down-regulation of CXCR4 expression was reversible by STI-571 in both M07e cells and CD34+ cells from CML patients.

Materials and Methods

Plasmid constructs. Migr1-p210BCR-ABL retroviral vector was kindly provided by Dr. Warren Pear (Boston, MA). To obtain the MSCV-Neo-p210BCR-ABL retrovirus, the BCR-ABL cDNA was subcloned in the MSCV-Neo vector, a gift from Dr. Robert Hawley (Toronto Hospital, Toronto, Ontario, Canada). Migr-K1172R-p210BCR-ABL was kindly provided by Dr. R.A. Van Etten (Harvard University, Boston, MA).

Retroviral infectious particles production in 293 EBNA cells. The retrovirus-producing cell line 293 EBNA was maintained in DMEM (Life Technologies, Paisley, United Kingdom) with 10% fetal bovine serum (FBS) and 250 μg/mL of G418 (Life Technologies). The vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped retroviruses were produced by transient transfection of 293 EBNA with three different constructs as previously described (31). Viral titers were determined by limiting dilution assay with NIH-3T3 cells based on GFP fluorescence or G-418 resistance (250 μg/mL) and ranged from 1 to 5 × 10⁹/mL.

Generation of BCR-ABL-expressing cells. M07e cells were infected with the viral supernatant at different multiplicity of infection (MOI) of retrovirus particles per cell in the presence of 4 μg/mL of hexadimethrine bromide (Sigma-Aldrich, St Quentin Fallavier, France). GFP expressing cells were sorted using a FACSVantage (Becton Dickinson, Erembodegem, Belgium). M07e-Neo-p210BCR-ABL and M07e-Neo were obtained after G418 selection (250 μg/mL) during 10 days and maintained in culture in MEM α containing granulocyte macrophage colony-stimulating factor (GM-CSF). Cells and culture conditions. Parental and BCR-ABL-expressing human hematopoietic M07e mass cell populations were maintained in MEM α (Life Technologies) supplemented with 10% FBS, 1 mg/mL l-glutamine, 100 units/mL penicillin G, 100 μg/mL streptomycin (all from Life Technologies) in the presence of 10 ng/mL recombinant human GM-CSF (a gift from Novartis, Basel, Switzerland).

Apheresis products of mobilized peripheral blood patients (MPB), peripheral blood from patients with chronic-phase CML at diagnosis and from patients in accelerated and blastic crisis were obtained after informed consent in accordance with the institutional guidelines of the Committee on Human Investigation. CD34+ cells were separated using a magnetic cell sorting system (miniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with manufacturer’s recommendations. The purity of recovered cells was determined by flow cytometry after staining with the PE-HPCA2 anti-CD34 monoclonal antibody (mAb) and was over 95%. To allow time for resensitization of potentially desensitized CXCR4 receptors, CD34+ cells (3 × 10⁵ cells/mL) were cultured for 6 hours in serum-free medium. Some experiments were done in the presence or absence of STI-571. Upon this short-term culture, the cells were harvested for evaluation of CXCR4 membrane expression.

Antibodies and flow cytometry analysis. FITC and PE-HPCA2 (anti-CD34), FITC-CD33, APC- and PE-12G5 (anti-CXCR4), FITC-, PE- and APC-conjugated IgG1 and IgG2a control mAbs were from BD Biosciences (Le Pont de Claix, France). Cells were suspended in PBS, kept at 4°C, and analyzed on a FACSsort (Becton Dickinson) with the Cell Quest software package.

Chemotactic assay. The migration assay in response to SDF-1α was as previously described (32). All assays were done in triplicate. Data are presented as the percentage of migrated cells in response to SDF-1α or medium alone/number of input cells.

Real-time Quantitative PCR

RNA isolation was done using SV total RNA isolation system (Promega Co., Madison, WI). Reverse transcription was done with SuperScript II RNase H reverse transcriptase (Invitrogen, Cergy Pontoise, France). Primers for CXCR4 mRNA and intronic CXCR4 mRNA were as follows: CXCR4 mRNA sense 5′-CTGGCTCCCTCTGCTGAT-3′, CXCR4 mRNA antisense 5′-GCGCAACTGATGTTGCTGAA-3′, probe 5′-TTTATCCTTGCGCGAAGCTAGTGACTTT-3′; intronic CXCR4 sense 5′-CCCTCCCGGCTCTTAAAATT-3′, intronic CXCR4 antisense 5′-CCACCGGCTTCCTCTCGGGG-3′, probe 5′-ACTGCCTAACAGACATCCCGGCTTC-3′. PCRs were carried out in the ABI Prism GeneAmp 5700 Sequence Detection System (Perkin-Elmer, Warrington, United Kingdom) using Taqman Universal PCR Master Mix (ABI). For each fraction, the level of expression of CXCR4 was expressed relatively to the actin or 18S-microglobulin housekeeping gene (PerkinElmer). For calculation of fold augmentation, RNA amounts were normalized to actin mRNA.

Western blot analysis. Protein levels of BCR-ABL and phosphotyrosine were determined by immunoblotting equal amounts of proteins (50 μg) with anti c-abl antibody (Ab-3, Calbiochem, Merck, Darmstadt, Germany) and anti-phosphotyrosine antibody (4G10, Euromedex, Mundolsheim, France). The bands were developed with an enhanced chemiluminescence system (ECL kit, Amersham, Buckinghamshire, United Kingdom). For analysis of p42/44 mitogen-activated protein kinase (MAPK) activation, cells were washed and deprived from serum and GM-CSF for 12 hours. Cells were stimulated with 300 ng/mL of SDF-1α for various lengths of time or GM-CSF for 5 minutes. Endogenous MAPK activity was detected with an antibody specific for the Thr202 and Tyr204 phosphorylated forms of Erk1 and Erk2 (phospho-p44/42 MAPK, Cell Signaling Technologies, Beverly, MA). Membranes were reprobed with a total p44/42 MAP antibody (Cell Signaling Technologies, Beverly, MA). The relative activity of MAP kinase in stimulated versus unstimulated cells was determined using the MacBass2 software.

Statistics. Results of experimental points obtained from multiple experiments were reported as the mean ± SD.

Results

CXCR4 function and expression is decreased in patient in blastic but not chronic phase. We investigated the ability of SDF-1 to induce in vitro migration of CD34+ cells prepared from peripheral blood of patients at different stages of the CML disease. The data (Fig. 1A) show that CD34+ cells derived from patients in the chronic phase and those from normal MPB exerted efficient

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migration in response to SDF-1 with no significant difference between these two groups \((P = 0.5)\). In contrast, CD34+ cells from patients in blast phase migrated poorly. CD34+ cells from accelerated phase had about a 50% decrease in their migratory response compared with MPB \((P < 0.05)\).

To determine whether CXCR4 expression was altered in cells from CML patients, CD34+ cells were dually labeled with a PE-anti

CD34 mAb and APC-12G5 mAb and analyzed by multivariable flow cytometry. To compare exactly the expression between samples, we defined the mean channel fluorescence ratio as the ratio between mean channel fluorescence for CXCR4 and their respective negative control. Although, the reactivity of CXCR4 was variable among samples, there was no significant difference between chronic-phase CML, accelerated phase, and MPB CD34+ cells \((P < 0.1)\). In contrast, level of CXCR4 was constantly lower in blastic-phase CML CD34+ cells compared with MPB CD34+ cells \((P < 0.05\); Fig. 1B). cDNA samples from healthy individuals and patients with CML in chronic-phase and blastic-phase stages were then analyzed for CXCR4 mRNA expression by real-time reverse transcription-PCR. Patients cells with CML in blast phase showed a significantly lower level of CXCR4 expression as compared with healthy individuals or patients with CML in chronic phase, whereas no significant difference between the latter two groups could be detected (Fig. 1C). These data indicate that CXCR4 is underexpressed in patients with CML at the blastic phase compared with the chronic phase of the disease.

Because multiple genetic changes are frequently observed at blast crisis, we determined whether the low CXCR4 expression found in blastic-phase CML CD34+ cells could be reversed by inhibition of BCR-ABL activity. Recent reports have indicated that the BCR-ABL tyrosine kinase inhibitor STI-571 can suppress growth and induce apoptosis of BCR-ABL expressing cells. CD34+ cells from three
patients with CML in myeloid blast phase and MPB CD34+ cells were cultured in the presence or absence of 1 μmol/L of STI-571 and CXCR4 expression was analyzed after 6 or 24 hours. As shown in Fig. 2A, an important up-regulation of CXCR4 surface expression was detected upon STI-571 treatment. Moreover, in vitro exposure to STI-571 resulted in a 1.5- to 10-fold up-regulation of CXCR4 mRNA levels (Fig. 2C). Remarkably, no significant modulation of CXCR4 expression was documented in the control MPB CD34+ cells cultured with the same concentration of STI-571 (Fig. 2B and C). Taken together, these results suggest that CXCR4 expression in blast-phase CML cells can be increased when the BCR-ABL tyrosine kinase activity is inhibited.

**BCR-ABL oncogene alters SDF-1α signaling in a dose-dependent manner in Mo7e cells.** Previous studies have indicated that the effect of p210\(^{\text{BCR-ABL}}\) on cytokine independence depended on its expression levels (29, 30). To precisely determine the dose-effect relationship of p210\(^{\text{BCR-ABL}}\) on CXCR4 signaling, the cytokine-dependent Mo7e cell line was infected with MSCV-Neo-p210\(^{\text{BCR-ABL}}\) retrovirus encoding p210\(^{\text{BCR-ABL}}\) and the Neo resistance gene. Retroviruses encoding Neo resistance gene alone (Neo) were used as control. Three polyclonal populations, expressing different amounts of p210\(^{\text{BCR-ABL}}\), were generated through infection with increasing MOI (MOI of 0.2, 2, and 10 particles per cell). After G418 selection, the expression level of p210\(^{\text{BCR-ABL}}\) was determined by Western blot. The three polyclonal populations Mo7e-Neo, Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I, II, and III expressed increasing levels of p210\(^{\text{BCR-ABL}}\) kinase (Fig. 3A). The response to SDF-1α was inversely correlated with the level of p210\(^{\text{BCR-ABL}}\) (Fig. 3B). The average decrease in migration ranged from 28% in the Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I population, to nearly 100% in the Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) III cells compared with control.

We also investigated if a signaling defect was present by using a MAPK activation assay. We used immunoblotting with antibodies against phosphorylated Erk1/Erk2 to analyze basal and SDF-1α induced phosphorylation of Erk1/2 in Mo7e cell lines expressing different levels of p210\(^{\text{BCR-ABL}}\). Mo7e-Neo cells were used as a control in these experiments. In Mo7e-Neo cells, Erk1 and Erk2 phosphorylation was induced by SDF-1α, with a rapid and transient induction which peaks between 2 and 5 minutes (Fig. 3C). Erk1 and Erk2 were phosphorylated with the same time course (30 minutes, 2, and 5 hours) in Mo7e-Neo and Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I (low p210\(^{\text{BCR-ABL}}\) expression) with very few quantitative differences. In contrast, Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) III cells (high p210\(^{\text{BCR-ABL}}\) expression) exhibited a constitutive activation of Erk1/Erk2. This phosphorylation was not increased by SDF-1α stimulation. These results strongly suggest a dose-dependent effect of BCR-ABL on SDF-1α signaling.

**BCR-ABL reduces CXCR4 cell surface expression in Mo7e cells in a dose-dependent manner.** To investigate the mechanisms responsible for the regulation of CXCR4 function by BCR-ABL, we choose to transduce Mo7e cells with a retrovirus encoding both p210\(^{\text{BCR-ABL}}\) and GFP protein (Migr-p210\(^{\text{BCR-ABL}}\)-GFP). In this bicistronic retroviral vector, the two cDNA are separated by an ECMV1 IRES; thus, the GFP intensity is a direct reflect of the expression of the p210\(^{\text{BCR-ABL}}\) gene. We also expect that the p210\(^{\text{BCR-ABL}}\) and GFP protein (Migr-p210\(^{\text{BCR-ABL}}\)-GFP) are expressed in the same vector and in the same cell (Fig. 3D). The response to SDF-1α was inversely correlated with the level of p210\(^{\text{BCR-ABL}}\) (Fig. 3E and F). The average decrease in migration ranged from 28% in the Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I population, to nearly 100% in the Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) III cells compared with control.

Three polyclonal populations, expressing different amounts of p210\(^{\text{BCR-ABL}}\), were generated through infection with increasing MOI (MOI of 0.2, 2, and 10 particles per cell). After G418 selection, the expression level of p210\(^{\text{BCR-ABL}}\) was determined by Western blot. The three polyclonal populations Mo7e-Neo, Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I, II, and III expressed increasing levels of p210\(^{\text{BCR-ABL}}\) kinase (Fig. 3A). The response to SDF-1α was inversely correlated with the level of p210\(^{\text{BCR-ABL}}\) (Fig. 3B). The average decrease in migration ranged from 28% in the Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I population, to nearly 100% in the Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) III cells compared with control.

CXCR4 Expression in BCR-ABL-Expressing Cells and CML

Figure 3. Dose-dependent effect of p210\(^{\text{BCR-ABL}}\) on CXCR4 signaling. Three polyclonal populations, expressing different amounts of p210\(^{\text{BCR-ABL}}\), were generated through infection with increasing MOI (MOI of 0.2, 2, and 10 particles per cell). After G418 selection, the expression level of p210\(^{\text{BCR-ABL}}\) was determined by Western blot using an anti-c-Ab antibody (top). The membrane was then stripped and reprobed with an anti-phosphotyrosine antibody (lower panel). Total cell lysates from Mo7e-Neo cells was used as negative control. The position of p210\(^{\text{BCR-ABL}}\) and the endogenous p145 c-Abl (endogenous loading control) are indicated (A). Selected populations were subjected to chemotaxis to SDF-1α. Columns, mean % input cells (y axis) of three independent experiments performed in duplicate; bars, SD (±). Time course of Erk1/Erk2 activation (top) after SDF-1α stimulation (30 seconds, 2, 5, and 10 minutes) in Mo7e-Neo, Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) I, and Mo7e-Neo-p210\(^{\text{BCR-ABL}}\) III cells (low and high BCR-ABL expression). GM-CSF stimulation for 5 minutes was used as a positive control. C, membrane was then stripped and reprobed with an anti-Erk1/2 antibody (bottom).
sorted according to the gate R5 (Fig. 4A). Total lysates from these cells were then analyzed by Western blotting. As shown in Fig. 4C, the amount of p210BCR-ABL protein levels correlated with GFP intensity being low in gate R2 and higher in gate R3.

We also evaluate the migration responses of both transduced and untransduced cells in these cultures. The data (Fig. 4D) show that MO7e cells infected with the control Migr-GFP retrovirus exhibited an efficient response to SDF-1. Furthermore, when migrated cells were analyzed for GFP expression, a similar percentage of uninfected (GFP−, R4) and infected (GFP+, R5) cells was found indicating that expression of GFP per se did not alter the migration properties of the cells. In contrast, when MO7e cells were infected with the Migr-p210BCR-ABL−GFP retrovirus, the response to SDF-1α was inversely correlated with the level of p210BCR-ABL (Fig. 4D).

Transcriptional mechanisms are involved in CXCR4 down-regulation by BCR-ABL. Our results presented thus far show that p210BCR-ABL down-regulates CXCR4 at the membrane expression level. Moreover, higher CXCR4 membrane expression and mRNA levels were detected upon STI-571 treatment of CD34+ cells from CML patients suggesting that p210BCR-ABL may act on CXCR4 transcription or mRNA stability. To analyze how p210BCR-ABL can affect CXCR4 expression, we investigated the level of CXCR4 transcripts by real-time RT-PCR in MO7e cells. The MO7e-p210BCR-ABL+++ cells (high GFP expression, gate R3, Fig. 4B), the MO7e-p210BCR-ABL+ cells (low GFP expression, gate R2, Fig. 4B) and MO7e-GFP cells (gate R5, Fig. 4A) were sorted and their CXCR4 mRNA levels were compared (Fig. 5A). Expression of CXCR4 mRNA was normalized to actin mRNA. The MO7e-p210BCR-ABL+++ cells showed a 4- to 7-fold reduction of CXCR4 mRNA as compared with parental MO7e cells or to control MO7e-Migr-GFP cells. In contrast, the MO7e-p210BCR-ABL+ cells (low p210BCR-ABL expression) showed no alterations of CXCR4 mRNA compared with parental MO7e cells or to control MO7e-Migr-GFP cells.

To determine whether the decrease in CXCR4 transcripts was the result of a reduced transcription, RT-PCR was done with DNase I-treated RNA, using pairs of primers located in the CXCR4 intron to reveal CXCR4 pre-mRNA. Compared with MO7e-Migr-GFP+ cells, levels of CXCR4 pre-mRNA were markedly reduced in MO7e-p210BCR-ABL+++ cells (Fig. 5B). In addition, cells were exposed to actinomycin D, a DNA-primed RNA polymerase inhibitor that blocks transcription, for different lengths of time and total RNA was subjected to real-time RT-PCR (Fig. 5C and D). The half-life of CXCR4 transcripts was estimated to be about 2 hours (2 hours 12 minutes) in MO7e-Migr-GFP cells. Although CXCR4 transcripts were considerably decreased, its half-life was not significantly changed in MO7e-Migr-p210BCR-ABL−GFP cells (about 2 hours). Together, these data indicate that CXCR4 down-regulation occurs at the transcriptional level.

Down-regulation of CXCR4 by p210BCR-ABL requires its tyrosine kinase activity. p210BCR-ABL is a chimeric oncoprotein that phosphorylates various targets through a deregulated tyrosine kinase activity, carried by the Abl portion. This tyrosine kinase activity is essential for transformation of cells in culture indicating that protein phosphorylation is crucial for the oncogenic pathways. To determine if BCR-ABL tyrosine kinase activity is necessary for its effects on CXCR4 receptor, we used a kinase-dead protein (K1172R; ref. 33). Retroviral expression of the kinase-deficient p210BCR-ABL mutant in Mo7e cells failed to down-regulate CXCR4 expression, even at high level of expression (Fig. 6A and C). Moreover, this mutant did not reduce the chemotactic response of the cells to SDF-1α (Fig. 6B). This observation was further confirmed by the addition of the tyrosine kinase inhibitor STI-571 (10 μmol/L) before Mo7e infection with the Migr-p210BCR-ABL−GFP retroviral vector. In this condition, Mo7e-p210BCR-ABL−GFP+ expressed a normal level of CXCR4 (Fig. 6D). These results show that tyrosine kinase activity of p210BCR-ABL is necessary to down-regulate the expression and function of CXCR4 receptor.

Figure 4. Dose-dependent effect of p210BCR-ABL on CXCR4 membrane expression. MO7e cells were transduced with Migr-GFP (A, A’) or Migr-p210BCR-ABL− retroviruses at an MOI = 0.2 (B, B’). After 48 hours, CXCR4 membrane expression was assayed by fluorescence-activated cell sorting. A, CXCR4 membrane expression on cells in R4 (thin line) and R5 (dashed line). B, CXCR4 membrane expression on cells in R1 (bold line), R2 (dashed line), and R3 (thin line). Solid histograms, controls PE-IgG2a. C, after cell sorting according to GFP levels (R5, R2, and R3), the expression levels of p210BCR-ABL was determined by Western blot using an anti-c-Abl antibody (top). The membrane was then stripped and reprobed with an anti-phosphotyrosine antibody (bottom). Position of p210BCR-ABL, and the endogenous p145c-Abl (endogenous loading control). D, in vitro transwell migration assays on MO7e cells according to the gates in Fig. 3A and B. Columns, mean ± input cells (y axis) of three independent experiments performed in duplicate; bars, SD.
Discussion

Expression of p210(BCR-ABL) in human and murine cell lines blocks CXCR4 signaling. However, it has not been established at this time if these abnormalities have any relevance with regard to human CML in which only subtle or no abnormalities in SDF-1-induced migration were reported. We analyzed the expression of CXCR4 and SDF-1 induced chemotaxis in CML patients at different stage of the disease. We found that the migratory properties of normal and CML CD34+ cells are nearly identical. In contrast, CD34+ cells derived from CML blast crisis exhibited profound diminution of chemotaxis. This low chemotactic response is correlated with a low CXCR4 expression. This was shown by the lower CXCR4 expression at protein and mRNA level in CD34+ cells from blastic-phase CML compared with CD34+ cells from chronic-phase CML or CD34+ cells from healthy mobilized individuals. Many studies showed that blast crisis cells express more BCR-ABL than chronic-phase progenitors. This suggests that BCR-ABL is capable to modulate the expression of CXCR4 depending on its expression level.

To determine the dose-effect relationship of BCR-ABL on CXCR4 protein, we used the bicistronic retroviral vector Migr in which the GFP cDNA and p210 cDNA are separated by an ECMV1 IRES; thus, the GFP intensity is a direct reflect of the p210(BCR-ABL) expression. This approach allows us to avoid any selection as the effect on CXCR4 expression and function can be analyzed shortly after infection. Using different MOI, we generated three polyclonal populations of MO7e-p210BCR-ABL cells expressing different amounts of p210BCR-ABL. This model distinctly shows that p210BCR-ABL expression can alter CXCR4 protein through at least two different mechanisms depending on its expression level. At low expression, a signaling defect was detected without any modification of CXCR4 expression. However, higher p210BCR-ABL expression induced a marked down-regulation of CXCR4 expression. This was shown by comparing CXCR4 membrane expression according to GFP expression in the same culture. This comparison shows an inverse correlation between the level of BCR-ABL expression and CXCR4 membrane expression. Higher BCR-ABL expression resulted in a markedly decreased CXCR4 expression. Other workers have found that expression of BCR-ABL in cytokine-dependent cell lines, including Mo7e cells, has reduced or abrogated requirements for exogenous growth factors. Consistent with these data, we found that high p210BCR-ABL expression resulted in growth factor independence of Mo7e cells, whereas at low p210BCR-ABL expression, the cells remained GM-CSF dependent.
The down-regulation of CXCR4 expression is essentially related to its decreased transcription. In favor of this assumption are the demonstrations that (i) the CXCR4 mRNA was markedly decreased, (ii) the half-life of the mRNA was identical in wild-type and p210BCR-ABL expressing MO7e cells, (iii) and the quantity of CXCR4 pre-mRNA was reduced in MO7e-p210BCR-ABL. This transcriptional regulation was dependent on the tyrosine kinase activity of p210BCR-ABL because a tyrosine kinase dead mutant did not modify CXCR4 expression. In addition, pretreatment of cells with STI-571 prevents CXCR4 down-regulation by p210BCR-ABL. Together, these data strongly suggest that the signaling pathway induced by p210BCR-ABL is able to suppress or activate some transcription or repressor factors involved in the regulation of CXCR4 expression. Similar transcriptional silencing by p210BCR-ABL was reported for the G-CSF receptor in 32Dcl3 cell line and in CML patients in blast crisis (34). Perrotti et al. have shown that p210BCR-ABL was able to down-regulate C/EBPδ by inhibiting its translation through hnRNP2 leading to transcriptional inhibition of G-CSFR. As for our experiments on CXCR4, G-CSFR requires high level of p210BCR-ABL as well as the presence of its tyrosine kinase activity. However, in contrast to CXCR4, this effect is observed only after a prolonged culture. Further studies will be required to determine if C/EBPδ is involved in the transcriptional silencing of CXCR4 by p210BCR-ABL.

The signaling defect of CXCR4 observed in our experiment at low expression level of p210BCR-ABL is consistent with previous studies from Salgia et al. (25) who showed that p210BCR-ABL in MO7e cells blocks CXCR4 signaling without altering its expression. However, in the above study, the p210BCR-ABL dosage response effect on CXCR4 expression was not determined. Therefore, it is likely that a low p210BCR-ABL expression was present in the cells studied by Salgia et al. (25). The mechanisms involved in this migration defect are not understood, although it has been suggested that p210BCR-ABL may disrupt CXCR4 signaling by strongly activating Lyn and PI3-kinase (26).

The major hallmark of CML in chronic phase is an early release of myeloid cells and progenitors from the marrow and their accumulation in the blood. We observed that CXCR4 expression was low and very similar on freshly isolated normal MPB CD34+ and chronic-phase CML CD34+ cells in contrast to bone marrow CD34+ cells (data not shown) indicating that the low CXCR4 expression is a common feature of circulating normal MPB and circulating chronic-phase CML CD34+ cells. Altogether, these results suggest a parallel between the effects of hematopoietic growth factors and of the CML disease on CXCR4 expression and function. At present, the mechanisms that lead to the circulation of normal and chronic-phase CML CD34+ cells are not completely understood. There are increasing evidence that circulation of

**Figure 6. Requirement of the tyrosine kinase activity of p210BCR-ABL for down-regulation of CXCR4.** A, representative fluorescence-activated cell sorting analysis of CXCR4 expression on sorted GFP+ after transduction of MO7e with Migr-GFP, Migr-p210BCR-ABL, and Migr-K1172Rp210BCR-ABL retroviruses. MO7e-K1172R mutation in p210BCR-ABL protein suppresses kinase activity of the oncoprotein. MO7e-Migr-GFP (sparse tracing), MO7e-Migr-p210BCR-ABL+++ (light tracing), MO7e-Migr-K1172Rp210BCR-ABL (dark tracing). Background staining determined using a nonspecific isotype matched IgG (solid histogram). B, in vitro transwell migration assays on GFP+ cells sorted from cultures of MO7e cells transduced with Migr-GFP, Migr-GFP-p210BCR-ABL, and Migr-K1172Rp210BCR-ABL retroviruses. Columns, mean % input cells (y axis) of three independent experiments performed in duplicate; bars, SD. C, level and phosphorylation states of p210BCR-ABL and K1172Rp210BCR-ABL. The whole cell extracts of MO7e-Migr, MO7e-Migr-p210BCR-ABL, and MO7e-Migr-K1172Rp210BCR-ABL cells were immunoprecipitated with an anti-c-Abl and blotted with an anti-c-Abl (top) or an anti-phosphotyrosine (bottom). D, effect of the tyrosine kinase inhibitor STI-571 on p210BCR-ABL-induced down-regulation of CXCR4 membrane expression. MO7e cell culture medium was either supplemented with 10 μmol/L STI-571 (right) or control diluent (left) by the time they were infected with Migr-p210BCR-ABL retroviruses. Forty-eight hours later, CXCR4 cell surface expression was analyzed by fluorescence-activated cell sorting.
CD34+ cells after administration of mobilizing agents is related to both CXCR4 and V-CAM cleavage by neutrophil proteases (35, 36). Thus, a similar mechanism may operate in chronic-phase CML to release hematopoietic precursors. However, the possibility that leukemic progenitors can be mobilized with greater efficiency cannot be excluded. Indeed, leukemic progenitor cells have been shown to have altered adhesive properties and lower chemotaxis to SDF-1α depending on the patient. Moreover, primitive CML progenitors are insensitive to the suppressive effect of SDF-1α in contrast to normal progenitors (37) suggesting that BCR-ABL may have subtle effects on CXCR4 signaling.

In conclusion, we provide evidence that p210BCR-ABL expression to CXCR4 is a dose-dependent event requiring a certain threshold of p210BCR-ABL expression to take place. At high level, p210BCR-ABL can induce a transcriptional silencing of CXCR4, leading to abnormal chemotaxis. Further studies are required to understand the precise molecular mechanism responsible for this transcriptional silencing of CXCR4. We also show that CXCR4 expression was greatly decreased during CML blast crisis. The decrease of CXCR4 expression may lead to a loss of CXCR4 signaling which may favor an altered response to growth factors, the release of blast cells in the blood and the colonization of nonhematopoietic tissues.

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