BCR-Tyrosine 177 Plays an Essential Role in Ras and Akt Activation and in Human Hematopoietic Progenitor Transformation in Chronic Myelogenous Leukemia

Su Chu, Liang Li, Harjeet Singh, and Ravi Bhatia

Department of Hematopoietic Stem Cell and Leukemia Research, Division of Hematology and Hematopoietic Cell Transplantation, City of Hope National Medical Center, Duarte, California

Abstract
Chronic myelogenous leukemia (CML) results from the transformation of a primitive hematopoietic cell by the BCR/ABL gene. BCR/ABL signaling has been studied in cell lines and murine models, but the transforming effects of BCR/ABL are highly dependent on cellular context, and mechanisms responsible for the transformation of primitive human hematopoietic cells remain poorly understood. Current targeted therapies fail to eliminate malignant CML progenitors, and improved understanding of crucial molecular mechanisms of progenitor transformation may facilitate the development of improved therapeutic approaches. We investigated the role of BCR/ABL tyrosine 177 (BCR/ABL-Y177) in CML progenitor transformation by comparing the effects of expression of Y177-mutated BCR/ABL, wild-type BCR/ABL, or green fluorescent protein alone on normal CD34+ cells. We show that BCR/ABL-Y177 plays a critical role in CML progenitor expansion, proliferation, and survival. BCR/ABL expression results in enhanced Ras and Akt activity but reduced mitogen-activated protein kinase activity in human hematopoietic cells, which is reversed by BCR/ABL-Y177 mutation. Blocking BCR/ABL-Y177–mediated signaling enhances targeting of CML progenitors by imatinib mesylate. Our studies indicate that BCR/ABL-Y177 plays an essential role in Ras and Akt activation and in human hematopoietic progenitor transformation in CML. [Cancer Res 2007;67(14):7045–53]

Introduction
Chronic myelogenous leukemia (CML) results from the transformation of a primitive hematopoietic cell by the BCR/ABL oncogene (1). The enhanced, unregulated tyrosine kinase (TK) activity of BCR/ABL plays a critical role in hematopoietic cell transformation in CML (2–4). The small-molecule kinase inhibitor imatinib mesylate (IM) is highly effective in the treatment of CML (2–4). The small-molecule kinase inhibitor imatinib mesylate (IM) is highly effective in the treatment of CML (2–4). However, clinical resistance to IM can occur (7, 8). Even in patients responsive to IM, elimination of malignant stem and progenitor cells is usually incomplete (9). Development of additional approaches to target leukemogenic cells is required, but requires improved understanding of mechanisms crucial for primitive progenitor transformation in CML.

Studies using transformed fibroblasts and cell lines have provided useful information regarding BCR/ABL signaling pathways (10). However, BCR/ABL expression results in variable effects, and the contribution of different signaling mechanisms to transformation varies from one cell type to the other (10, 11). Proteins that interact with BCR/ABL are differentially expressed in different cell types. Cell lines are derived from blast crisis CML patients and have an acute leukemia phenotype. Expression of BCR/ABL in murine hematopoietic cells using retroviral transduction and transplantation to irradiated hosts results in efficient induction of a myeloproliferative disorder (12) and has been useful for investigating mechanisms underlying BCR/ABL transformation of primary hematopoietic cells (13–15). However, murine disease differs from clinical CML in being very aggressive and fulminant and progressing to acute T-cell leukemia. These differences are likely related to interspecies variations between human and murine hematopoietic cells. Study of primary progenitor cells from CML patients is highly relevant to clinical disease, but studies of molecular mechanisms are restricted by limited cell numbers, interpatient variability, presence of Ph-cell populations, and difficulty in performing mechanistic studies. As a result, signaling mechanisms responsible for the transformation of primitive human hematopoietic cells in CML are not well understood.

A CML model based on ectopic expression of the BCR/ABL gene in human CD34+ progenitor cells reproduces abnormalities in proliferation, adhesion, and migration seen in primary CML progenitors and facilitates the study of molecular mechanisms of hematopoietic transformation in CML (16–18). We have used this model to show that abnormal kinase activity plays an essential role in increased proliferation, and both kinase-dependent and independent mechanisms contribute to altered adhesion and migration in CML progenitors (17). An autophosphorylation site at BCR/ABL Tyrosine 177 (BCR/ABL-Y177) can bind the adapter protein growth factor receptor binding protein 2 (Grb2), induce Grb2-Sos complex formation, and activate Ras signaling in cell lines (19). The importance of BCR/ABL-Y177 is highly dependent on the cell type studied (11, 20, 21). Mutation of BCR/ABL-Y177 diminishes Ras activation and transformation in fibroblasts but does not reverse growth-factor independence in BCR/ABL-transformed hematopoietic cell lines or murine bone marrow cells in vitro. This has been attributed to continued activation of Ras through alternate mechanisms in addition to BCR/ABL-Y177. On the other hand, mutation of BCR/ABL-Y177 prevented the induction of a myeloproliferative disorder in a murine CML transduction-transplantation model (13, 22). Given the variable importance of Y177 signaling in BCR/ABL-mediated transformation in different cellular contexts, it is unclear whether this motif is crucial for the transformation of primary CML progenitors. We therefore investigated the effect of Y177 mutation in BCR/ABL-induced abnormalities in proliferation.
and adhesion in human CD34+ progenitor cells and its contribution to BCR/ABL-mediated activation of important downstream signaling pathways.

Materials and Methods

Constructs
The MIG R1 and MIG 210 vectors were provided by Dr. Warren Pear (University of Pennsylvania, Philadelphia, PA; ref. 12). MIG R1 has a 5′ murine stem cell virus-based long terminal repeat and an enhanced green fluorescent protein (GFP) gene downstream of an internal ribosome entry site (IRES). The MIG 210 vector has the p210 BCR/ABL oncogene (7.1 kb) inserted into an EcoRI site upstream of the IRES. The MIG 210-Y177F vector was generated by excising a BCR/ABL gene with a point mutation at Tyr77 (BCR/ABL-Y177F) vector (a kind gift from Dr. Ruibao Ren, Brandeis University, Waltham, MA) and inserting into the EcoRI site of the MIG vector.

Samples
Human cord blood samples and CML bone marrow samples were obtained using protocols approved by the Institutional Review Board of the City of Hope National Medical Center.

Retroviral Transduction of CD34+ Cells
Mononuclear cells were isolated from cord blood samples using Ficoll-Hypaque separation. CD34+ cells were selected using immunomagnetic separation (Miltenyi Biotec). Infectious virus particles were produced by transfection of 293 cells with retroviral and packaging plasmids (pCL-amphi, provided by Dr. Martin Haas, University of California San Diego, San Diego, CA) as described (17). Supernatants were collected 24 to 48 h after transfection, filtered, and stored. CD34+ cell transduction was done as previously described (17). Cells were cultured for 48 h in serum-free medium (SFM, Stem Cell Technologies) with 100 ng/ml Flt-3 ligand (FL), 50 ng/ml stem cell factor (SCF), 10 ng/ml thrombopoietin, 10 ng/ml interleukin-6 (IL-6), and 25 ng/ml IL-3 on Fibronectin CH-296 (Retronectin; Pan Vera)—coated plates. Cells were resuspended in virus supernatant (multiplicity of infection = 10) with growth factors (GF) and replated on Retronectin-coated plates. This procedure was repeated after 24 h. Forty-eight hours later, cells were labeled with anti-CD34-APC antibodies (Becton Dickinson), and CD34+ GFP+ cells were collected by flow cytometry sorting (MoFlo, Cytometry Inc.).

Evaluation of Progenitor Growth

Colony-forming cell assays. CD34+ GFP+ cells were plated in methylcellulose progenitor culture and assessed for the presence of colony-forming cells (CFC) as described previously (23). Cell expansion in liquid culture with GFs. CD34+ GFP+ cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) with 30% fetal bovine serum (FBS) and GF as used in CFC culture (erythropoietin added to 48 h after transfection, filtered, and stored. CD34+ cell transduction was done as previously described (17). Cells were cultured for 48 h in serum-free medium (SFM, Stem Cell Technologies) with 100 ng/ml Flt-3 ligand (FL), 50 ng/ml stem cell factor (SCF), 10 ng/ml thrombopoietin, 10 ng/ml interleukin-6 (IL-6), and 25 ng/ml IL-3 on Fibronectin CH-296 (Retronectin; Pan Vera)—coated plates. Cells were resuspended in virus supernatant (multiplicity of infection = 10) with growth factors (GF) and replated on Retronectin-coated plates. This procedure was repeated after 24 h. Forty-eight hours later, cells were labeled with anti-CD34-APC antibodies (Becton Dickinson), and CD34+ GFP+ cells were collected by flow cytometry sorting (MoFlo, Cytometry Inc.).

GF dose response. CD34+ GFP+ cells were plated in SFM with graded concentrations of GF, ranging from 50× (the concentrations used in CFC culture) to 0.1×. The number of viable cells after 72 h was assayed using an MTS assay (Promega) as recommended by the manufacturer.

Cell proliferation. CD34+ GFP+ cells were labeled with SNARF-1 SE (20 μmol/L, Invitrogen Molecular Probes) at 37°C for 30 min, followed by the addition of FBS and washing with IMDM with 20% FBS. Cells were incubated overnight to release unbond dye and cultured in low concentrations of GF similar to those present in stroma-conditioned medium (GM-CSF 200 pg/ml, G-CSF 1 ng/ml, SCF 200 pg/ml, leukemia inhibitory factor 50 pg/ml, macrophage inhibitory protein 1 1× (200 pg/ml), and IL-6 1 ng/ml; ref. 24). SNARF fluorescence intensity, which reduces with each cell division, was assessed by flow cytometry after 1 to 3 days of culture. The parent generation was determined based on fluorescence of cells fixed with paraformaldehyde after SNARF labeling and overnight incubation.

Apopotosis. CD34+ GFP+ cells were cultured overnight in SFM supplemented with low concentrations of GF as described for cell proliferation, washed with IMDM, and cultured in SFM without GF for 3 days. Apoptosis was assessed on days 1, 2, and 3 by flow cytometry after labeling with Annexin V-Cy5 and 7-AAD (BD PharMingen).

Cell differentiation. CD34+ GFP+ cells were cultured in conditions used for cell expansion with or without EPO. On day 4, 7, and 11, cells were labeled with antibodies [CD34, CD14, glycophorin A, CD 45 and CD71 (Becton Dickinson)] and analyzed by flow cytometry.

Adhesion and Migration Assays

CD34+ GFP+ cells were incubated for 24 h in SFM with low GF as described for cell proliferation, washed, resuspended in IMDM with bovine serum albumin (BSA), and plated on Retronectin or BSA (Sigma)-coated wells for 2 h. Nonadherent and adherent fractions were plated in CFC culture, and the percentage of adherent CFC was calculated (25). Progenitor migration was measured using a transwell assay. Transwell filters (6.5-mm, 5-μm pores) were coated with Retronectin. CD34+ GFP+ cells incubated in SFM with low GF concentrations for 24 h were plated in the upper transwell chamber, with or without SF−1α (100 ng/ml) addition to the lower chamber. Cells migrating to the lower chamber over 6 h were assayed for CFC. The percentage of migrating CFC was calculated (26).

Western Blotting

CD34+ GFP+ cells were cultured as for cell expansion cultures for 10 days to obtain sufficient cell numbers for signaling studies. Protein extracts were prepared as described (27). Protein were resolved on 4% to 20% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 10% nonfat milk in PBS and 0.1% Tween 20 and labeled with primary antibodies [anti-Abl (Ab-5; Oncogene Science), anti-actin (AC-15; Sigma), antiphosphotyrosine (4G10; a kind gift from Dr. Brian Druker, Oregon Health Sciences University, Portland, OR), anti-Akt and anti-P-Akt (cell signaling), anti-Erk and anti-P-Erk (Santa Cruz Biotechnology), anti-P-Raf (Ser259), anti-P-Raf (Ser338), anti-mitogen-activated protein (MAP)/extracellular signal-regulated kinase (MEK), anti–P-MEK, anti–STAT5 (Santa Cruz), and anti-P-STAT5 (BD Biosciences)], followed by horseradish peroxidase–conjugated anti-mouse and anti-rabbit antibodies (1:8000; Jackson Laboratories). Membranes were sequentially reprobed with antibodies to phosphospecific and total proteins and to actin. Antibody detection was done using enhanced chemiluminescence (SuperFemto kit, Pierce Biotechnology).

Immunoprecipitation

Cells were lysed in 50 mmol/L sodium chloride, 50 mmol/L Tris-HCl (pH, 7.4), 5 mmol/L EDTA and 0.5% NP-40, supplemented with proteinase and phosphatase inhibitors. Protein extract were cleared using Protein A beads (Pierce Chemical Company) at 4°C for 1 h. Primary antibodies (antiphosphotyrosine, anti-STAT5) were added to protein extracts and incubated overnight at 4°C, followed by incubation with 30 μl True Blot beads (eBioscience) for 2 h. Beads were isolated by centrifugation, washed with PBS plus 1% NP-40, and boiled with 2× sample loading buffer and resolved by SDS-PAGE followed by Western blotting.

Ras, Mitogen-Activated Protein Kinase, and AKT Kinase Assays

A Ras activation assay kit (Pierce) was used following the manufacturer’s instructions. Cell lysates were prepared, and aliquots were set aside to allow quantitation of total Ras. The remaining lysate was mixed with 10 μl 0.5 mmol/L EDTA (pH, 8.0) and 5 μl of 10 mmol/L GTPγS and incubated at 30°C for 15 min. The mixture was added to a column containing immobilized GST-Raf1-BDD fusion protein and incubated on ice for 1 h. Bound protein was eluted with 2× SDS sample buffer. Glutathione S-transferase (GST)-bound Ras (active Ras) and total Ras were detected by Western blotting with an anti-Ras antibody. AKT kinase or p44/42 MAP kinase (MAPK) assay kits (Cell Signaling Technology) were used following the manufacturer’s instructions. AKT or p44/42 MAPK proteins were immunoprecipitated, and in vitro kinase reactions were done using glycogen synthesis kinase-3 (GSK-3) and ELK-1 fusion proteins as
substrates, respectively. Reaction products were subjected to Western blotting with antibodies to phospho-GSK-3α/β or phospho-ELK-1. One third of the lysate was retained for Western blotting for actin to check loading.

**Nuclear Extraction and Electrophoretic Mobility Shift Assays**

CD34+/GFP+ cells were lysed in hypotonic buffer [20 mmol/L HEPES (pH, 7.9), 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L sodium fluoride, 1 mmol/L sodium vanadate, 1 mmol/L sodium PPI, 1 mmol/L DTT] with protease inhibitors and 0.2% NP40 and centrifuged at 13,000 rpm for 1 min at 4°C. Nuclear pellets were resuspended in hypertonic buffer [20 mmol/L HEPES (pH, 7.9), 420 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L EGTA, 20% glycerol, 20 mmol/L sodium fluoride, 1 mmol/L sodium vanadate, 1 mmol/L sodium PPI, 1 mmol/L DTT] with protease inhibitors, rocked for 30 min at 4°C, and centrifuged. Nuclear-cytoplasmic separation was confirmed by Western blotting with anti-Sp1 and anti-tubulin antibodies. Complementary oligonucleotides containing a STAT5 consensus binding site from the β-casein promoter (5′-AGATTTCAGGAATTC-AATCC-3′) were annealed and end labeled with [γ-³²P] ATP using T4 polynucleotide kinase (Gel shift assay systems, Promega). Nuclear extracts (10 μg) were incubated with the [³²P]-labeled oligonucleotide probe. The binding reaction mixtures were resolved on a 4% nondenaturing polyacrylamide gel and analyzed by autoradiography. For competitive binding reaction mixtures were resolved on a 4% nondenaturing polyacrylamide gel and analyzed by autoradiography. For competitive binding reaction mixtures were resolved on a 4% nondenaturing polyacrylamide gel and analyzed by autoradiography. For competitive binding reaction mixtures were resolved on a 4% nondenaturing polyacrylamide gel and analyzed by autoradiography.

**Assessment of IM Sensitivity**

CD34+/GFP+ cells were incubated in SFM supplemented with GF at low concentrations at 37°C in a humidified atmosphere with 5% CO₂ for 24 h, followed by exposure to IM (0–1.0 μmol/L) in SFM containing the above GF for 72 h. The number of viable cells remaining was assayed using an MTS assay.

**Statistics**

Data obtained from multiple experiments were reported as the mean ± SE. Significance levels were determined by Student’s t test and ANOVA analysis.

**Results**

Ectopic expression of the BCR/ABL gene in CD34+ cells results in abnormalities in progenitor growth characteristic of primary CML progenitors (17). We used this CML progenitor model to determine the role of BCR/ABL-Y177 in BCR/ABL-mediated transformation of primitive human hematopoietic cells. CD34+ cells transduced with retrovirus vectors expressing the wild-type BCR/ABL (BCR/ABL) or BCR/ABL-Y177F (Y177F) transgene upstream of an IRES and the GFP gene were selected by flow cytometry sorting of CD34+GFP+BCR/ABL-Y177F (Y177F) transgene upstream of an IRES and the retrovirus vectors expressing the wild-type BCR/ABL (BCR/ABL) or CD34+ cells (Fig. 2A). Colony frequency was reduced in BCR/ABL-Y177F–expressing progenitor culture compared with controls expressing GFP alone. A modestly increased number of colonies in methylcellulose culture was observed in progenitor cultures expressing BCR/ABL-Y177F–transduced cells (Fig. 2A). Importantly, BCR/ABL-expressing CD34+ cells generated vastly increased numbers of colonies in liquid culture using the same GF as in methylcellulose progenitor culture compared with controls, whereas BCR/ABL-Y177F–expressing CD34+ cells generated significantly reduced cell numbers compared with BCR/ABL-expressing cells (Fig. 2A). Increased proliferation of BCR/ABL-expressing cells and markedly reduced growth of BCR/ABL-Y177F–expressing cells was seen across a wide range of GF concentrations (Fig. 2B). These results indicate a critical role for Y177 signaling in the vast expansion of BCR/ABL-expressing CD34+ cells.

To directly evaluate cell division, CD34+ cells were labeled with the dye SNARF-1 SE and cultured in GF-containing media, and cell division was analyzed based on the reduction in SNARF-1 fluorescence as detected by flow cytometry. Proliferation indices were calculated using ModFit software (28). BCR/ABL transduction resulted in a marked increase in CD34+ cell proliferation compared with controls, which was significantly reduced in BCR/ABL-Y177F–expressing CD34+ cells (Fig. 2C). To evaluate the contribution of BCR/ABL-Y177 to the antipapoptotic signaling by BCR/ABL, transduced cells were cultured in serum- and GF-free conditions. BCR/ABL-expressing cells showed significantly reduced apoptosis following GF deprivation, whereas BCR/ABL-Y177F–expressing cells showed significantly increased apoptosis compared with BCR/ABL-expressing cells (Fig. 2D).

BCR/ABL expression resulted in significant expansion of both myeloid (CD45+/CD14+ and CD14+) and erythroid cells [CD45+/CD71+ and glycophorin A (GPA)+] compared with controls.

![Figure 1](image-url)

**Figure 1.** Expression of p210BCR/ABL in transduced CD34+GFP+ cells. A, BCR/ABL expression in retrovirus-transduced human hematopoietic cells was done using Western blotting as described in Materials and Methods. Protein extracts from cells expressing GFP alone (R1), BCR/ABL (BA), and BCR/ABL-Y177F (Y177F) were analyzed. The same membrane was sequentially probed with anti-Abl, antiphosphotyrosine, and anti-actin antibodies. Blots were incubated with anti-ABL antibodies to detect BCR/ABL proteins. B, tyrosine-phosphorylated proteins were detected by Western blotting with antiphosphotyrosine antibodies. Blots were reprobed with anti-actin antibodies to assess protein loading.
BCR/ABL-Y177F–expressing cells showed significantly reduced expansion of both these populations. BCR/ABL expression also resulted in abnormal expansion of CD33+GPA+ (Fig. 3C) immature erythroid progenitor cells. Erythroid cell expansion was observed without the addition of erythropoietin (Epo) to the cultures (Fig. 3D). These results indicate a crucial role for BCR/ABL-Y177 signaling in abnormal myeloid and erythroid cell expansion in CML and in Epo-independent cell growth.

**Effect of BCR/ABL-Y177F on cell adhesion and migration.**

CD34+ cells expressing BCR/ABL showed reduced adhesion to FN compared with controls consistent with reduced β1 integrin-mediated adhesion of CML progenitors to fibronectin (29, 30). Adhesion of BCR/ABL-Y177F cells did not differ from that of BCR/ABL-expressing cells, indicating that Y177 does not significantly contribute to adhesion abnormalities in CML (Fig. 4A). CML progenitors also show reduced chemotaxis to the chemokine SDF-1 (31). BCR/ABL-expressing progenitors showed significantly reduced migration towards an SDF-1 gradient compared with controls. BCR/ABL-Y177F–expressing cells showed a modest but significant increase in directed migration to SDF-1 compared with BCR/ABL-expressing cells, but not to the same extent as controls. Partial correction of the chemotactic defect in BCR/ABL-Y177F–expressing cells indicates that BCR/ABL-Y177 contributes to abnormal CML progenitor migration, but that other BCR/ABL signaling mechanisms also play a role (Fig. 4B).

**Effect of BCR/ABL-Y177F on downstream growth signaling pathways in BCR/ABL-transformed human hematopoietic cells.** We examined the role of Y177 in potential signaling activities downstream of BCR/ABL. BCR/ABL-Y177F interaction with Grb2 potentially promotes association of Grb2SH3 domains with the guanine nucleotide exchange factor SOS, which can activate Ras by stimulating exchange of GDP for GTP (32). Ras activation plays a central role in mitogenic signaling, and abnormal Ras activation is frequently observed in neoplastic cells (33). We observed increased levels of active GTP-bound Ras in BCR/ABL and reduced Ras activity in BCR/ABL-Y177F–expressing cells (Fig. 5A). These results indicate a critical role for Y177-mediated interactions in BCR/ABL-induced activation of Ras in CML hematopoietic cells. Signaling through the phosphoinositide-3-kinase (PI3K) pathway may contribute to protection from enhanced proliferation and survival of BCR/ABL-expressing cells. PI3K consists of a regulatory 85-kDa subunit and a catalytic 110-kDa subunit (33). AKT or protein kinase B is a key downstream effector of PI3K signaling. Western blotting with anti–phospho-AKT (Ser 473) antibodies showed increased AKT phosphorylation, indicating increased activation, in BCR/ABL-expressing cells, which was markedly diminished in

![Figure 2. Effect of BCR/ABL-Y177F on abnormal cell expansion, proliferation and apoptosis of human hematopoietic progenitors.](image-url)
BCR/ABL-Y177F–expressing cells (Fig. 5C). These results were confirmed by direct assessment of AKT activity in a substrate-phosphorylation assay (Fig. 5C). These results indicate a critical role for BCR/ABL-Y177 in enhanced PI3K/AKT activity in BCR/ABL-transformed hematopoietic cells.

The Raf/MEK/MAPK signaling pathway is activated downstream of Ras (34). However, Western blotting with phospho-MAPK antibodies [anti-Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204)] revealed that P-MAPK levels were reduced in BCR/ABL-expressing cells (Fig. 5B), but increased in BCR/ABL-Y177F compared with BCR/ABL-expressing cells. These results were confirmed by direct assessment of MAPK activity using a substrate phosphorylation assay (Fig. 5B). We further show that CD34+ cells obtained from CML patients also showed reduced P-MAPK levels compared with cord blood CD34+ cells. Because reduced MAPK activity contrasted with increased Ras activity in BCR/ABL-expressing cells, we investigated Raf and MEK activity by Western blotting with antibodies recognizing active Raf [anti-Phospho-c-Raf (Ser 338)] and MEK [anti-phospho-MEK1/2 (Ser217/221)] (Fig. 5B). Raf Ser338 phosphorylation was enhanced in BCR/ABL but not in BCR/ABL-Y177F–expressing cells. Phosphorylation of Raf Ser259 by AKT can regulate and inhibit Raf activity. Raf Ser259 phosphorylation was found to be increased in BCR/ABL and reduced in BCR/ABL-Y177F–expressing cells, consistent with AKT activity levels in these cells. However, MEK-1/2 Ser217/221 phosphorylation was increased in BCR/ABL-expressing cells, indicating increased Raf activity. These results suggest that diminished MAPK activity in BCR/ABL-expressing cells result from regulatory mechanisms acting at the level of MAPK itself.

Enhanced STAT5 activity has been described in BCR/ABL-transformed cell lines and murine hematopoietic cells (35, 36). Western blotting revealed increased phospho-STAT5 (Tyr694) levels in BCR/ABL-expressing hematopoietic cells (Fig. 5D). Increased STAT5 tyrosine phosphorylation was also detected by immunoprecipitation with antiphosphotyrosine antibodies and Western blotting with anti-STAT5 antibodies (data not shown). BCR/ABL-Y177F–expressing cells showed partial reduction in phosphorylated STAT5. STAT5 DNA-binding activity assessed in an electrophoretic mobility shift assay using a STAT5 consensus binding site from the h-casein promoter was increased in BCR/ABL-expressing cells and partially reduced in BCR/ABL-Y177F cells (Fig. 5D). Binding was inhibited by excess unlabeled probe, and addition of anti-STAT5 antibody resulted in supershift of the protein-DNA complex, confirming specificity of binding. We conclude that BCR/ABL-Y177 contributes to enhanced STAT5 activity in CML cells, but that other signaling mechanisms are also involved.

**Figure 3.** Role of BCR/ABL-Y177 in abnormal myeloid and erythroid cell proliferation of human hematopoietic progenitors CD34+GFP+ cells expressing GFP alone (R1), BCR/ABL (BA), and BCR/ABL-Y177F (Y177F) were cultured in IMDM with GF and harvested on day 4, 7, and 11 as described in Materials and Methods. Cells lineage was analyzed by labeling with CD33, CD14, CD45, CD71 and glycophorin A antibodies. A, myeloid lineage, CD45+CD71- (left) or CD33+CD14+ (right). B, erythroid lineage, CD45-CD71+ (left) or glycophorin A+ (right). C, CD33+Glycophorin A+ double-positive cells following culture in the presence of EPO. D, erythroid lineage (Glycophorin A+) cell generation in the absence of EPO. Points, mean of the indicated number of experiments; bars, SE. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 BCR/ABL compared with R1 and Y177F (n = 4).
Transduced CD34+ cells were cultured in low GF-containing medium in transwells absorbed with FN. SDF-1 (100 ng/mL) was added to the lower chamber. BCR/ABL-Y177F-expressing cells were plated in FN-coated wells for 2 h, following which nonadherent and adherent fractions were separated and plated in methylcellulose progenitor culture, and the percent adherent CFC was calculated. Cell migration was measured in a transwell assay by evaluating the movement of CD34+GFP+-transduced cells from the upper to lower chamber of transwells absorbed with FN. SDF-1 was added to the lower chamber. Results indicate that the BCR/ABL-Y177F mutation significantly reduced both myeloid cell expansion and erythroid cell expansion from BCR/ABL-transformed CD34+ cells. These results are in contrast to previous reports that BCR/ABL-Y177F could rescue erythropoiesis in murine EpoR−/− fetal liver cells (37, 38).

In addition to enhanced proliferation, CML progenitors show altered adhesion to fibronectin and impaired chemotaxis to the chemokine SDF-1. In contrast to its important role in hematopoietic proliferation, BCR/ABL-Y177F made a modest contribution to abnormal chemotaxis to SDF-1 and did not contribute to reduced adhesion to fibronectin. Both kinase-dependent and kinase-independent mechanisms contribute to BCR/ABL-induced abnormalities in progenitor adhesion and migration (17). BCR/ABL kinase-independent effects may be mediated by the ABL COOH-terminal actin-binding domain and proline-rich domains. Our results indicate that the BCR/ABL-Y177F autophosphorylation site contributes to kinase-dependent signals leading to migration defects, but that other kinase-dependent mechanisms also play a role in abnormal adhesion and migration.

Discussion

CML results from transformation of a primitive hematopoietic cell by the BCR/ABL gene. Although the role of BCR/ABL TK activity in CML progenitor transformation is well accepted, the other BCR/ABL signaling mechanisms that are critical for human hematopoietic cell transformation in CML are not clear. Here, we have used a CML progenitor model based on ectopic expression of BCR/ABL in primary human hematopoietic cells to show that the BCR/ABL-Y177 Grb2 binding motif plays a critical role in hematopoietic progenitor transformation in CML, including abnormal proliferation, protection from apoptosis, and altered differentiation. We further show that BCR/ABL-Y177 mediates enhanced Ras and Akt, but not MAPK, signaling downstream of the BCR/ABL gene in human hematopoietic cells. These studies therefore elucidate key downstream pathways responsible for BCR/ABL-mediated transformation of primitive human hematopoietic cells in CML.

Expression of the BCR/ABL gene in normal CD34+ cells induces abnormal hematopoietic cell expansion as well as adhesion and chemotaxis defects characteristic of CML progenitors. We observed that BCR/ABL expression resulted in enhanced sensitivity to GF and vastly increased hematopoietic cell expansion in culture, which was significantly reduced by BCR/ABL-Y177 mutation. These results indicate a critical role for BCR/ABL-Y177 in abnormal myeloproliferation in CML. BCR/ABL-Y177 contributes to hematopoietic cell expansion by both increasing cell division and reducing apoptosis. BCR/ABL-transformed progenitors generated vastly increased numbers of both myeloid and erythroid cell populations. EPO-independent erythroid cell growth was observed, consistent with observations that CML erythroid progenitors can proliferate and differentiate in the absence of EPO, and that BCR/ABL can complement EPO signals required for erythropoiesis (37, 38). Expansion of a population coexpressing the early myeloid and erythroid marker (CD33+Glycophorin A+) was seen. BCR/ABL-Y177 mutation significantly reduced both myeloid cell expansion and erythroid cell expansion from BCR/ABL-transformed CD34+ cells. These results are in contrast to previous reports that BCR/ABL-Y177F could rescue erythropoiesis in murine EpoR−/− fetal liver cells (37, 38).

In addition to enhanced proliferation, CML progenitors show altered adhesion to fibronectin and impaired chemotaxis to the chemokine SDF-1. In contrast to its important role in hematopoietic proliferation, BCR/ABL-Y177F made a modest contribution to abnormal chemotaxis to SDF-1 and did not contribute to reduced adhesion to fibronectin. Both kinase-dependent and kinase-independent mechanisms contribute to BCR/ABL-induced abnormalities in progenitor adhesion and migration (17). BCR/ABL kinase-independent effects may be mediated by the ABL COOH-terminal actin-binding domain and proline-rich domains. Our results indicate that the BCR/ABL-Y177F autophosphorylation site contributes to kinase-dependent signals leading to migration defects, but that other kinase-dependent mechanisms also play a role in abnormal adhesion and migration.

Ras activity was increased in BCR/ABL-transformed human hematopoietic cells and reversed by mutation of BCR/ABL-Y177, indicating that Ras activation in CML progenitors is dependent by BCR/ABL-Y177 and plays an important role in BCR/ABL-mediated hematopoietic cell expansion. Ras activation may result from BCR/ABL-Y177-Grb2 association with SOS, an activator of Ras, as well as through Shp2 activation by association with Gab2, which binds to...
BCR/ABL-Grb2 complexes. These results are consistent with studies in mice indicating that Ras complements BCR/ABL-Y177 function, and that coexpression of oncogenic Ras with BCR/ABL-Y177F rapidly and efficiently induces CML-like myeloproliferative disorder (10).

Our studies suggest that signaling through the PI3K/Akt pathway is a primary effector of Ras in BCR/ABL-transformed human hematopoietic cells. Increased Akt activity in BCR/ABL-expressing cells was reversed in BCR/ABL-Y177F–expressing cells. In contrast MAPK activity was reduced in BCR/ABL-expressing hematopoietic cells, but increased following mutation of BCR/ABL-Y177. Enhanced PI3K signaling in BCR/ABL-expressing human hematopoietic cells likely results from BCR/ABL-Y177 and Grb2-dependent activation of the p110 catalytic subunit by activated Ras (39). Modulation of the PI3K p85 regulatory subunit by Gab2 may also contribute to PI3K activation. Activation of Akt potentially triggers several signaling networks regulating cell survival, proliferation, and growth. Akt mediates survival through phosphorylation and inactivation of proapoptotic proteins, phosphorylation, and cytosolic export of forkhead transcription factors, activation of nuclear factor κB and transcription of apoptosis inhibiting proteins (40). Akt signaling may enhance cell cycle progression through inactivation of GSK3-b, increased cyclin D1 and p27Kip1 phosphorylation (41). PI3K-Akt also signals through the mammalian target of rapamycin which phosphorylates p70S6-kinase and eukaryotic initiation factor 4E-binding protein-1, which regulate mRNA translation (33). Reduced Akt signaling may therefore explain reduced cell expansion and proliferation and increased apoptosis in BCR/ABL-Y177F–expressing cells.

Figure 5. Effect of BCR/ABL-Y177F on BCR/ABL signaling in human hematopoietic cells. Protein extracts from transduced cells expressing GFP alone (R1), BCR/ABL (BA), and BCR/ABL-Y177F (Y177F) were analyzed. A, Ras. Ras activity assays were done using GST-Raf-1-RBD agarose-conjugated beads to bind Ras-GTP, which was detected by Western blotting with an anti-Ras antibody, and compared with total Ras. Blots were reprobed with an anti-GST antibody to assess loading of GST-fusion protein. B, MAPK. Western blot analysis of phospho-MAPK and total MAPK levels, as well as of phosphorylated and total Raf and Mek levels in transduced cells was done. Blots were reprobed for actin to assess protein loading. MAPK kinase activity assays were done using MAPK immunoprecipitated from transduced cells and in vitro kinase assays using an ELK-1 substrate followed by Western blotting with an anti-phospho-ELK-1 antibody. A fraction of the lysates used for these assays was reserved for Western blotting to detect input levels of MAPK in the samples. Western blot analysis of phospho-MAPK, total MAPK, and actin levels was also done on CD34+ cells isolated from two cord blood (CB1, CB2) and two CML bone marrow (CML1, CML2) samples. C, Akt. Western blot analysis of phosphorylated (Ser473) Akt and total Akt levels in transduced cells was done. Akt kinase assays were done using Akt immunoprecipitated from transduced cells and in vitro kinase assays using a GSK-3 substrate followed by Western blotting with an anti-phospho-GSK-3 antibody. A fraction of the lysates used for these assays was reserved for Western blotting to detect input levels of Akt in the samples. D, STAT5. Western blot analysis of phospho-STAT5 (Tyr694), total STAT5, and actin levels was done. Electrophoretic mobility shift assays were done to measure STAT5-DNA binding to the β-casein promoter STAT5 binding site (lanes 1–3). Specificity of binding was shown by addition of 100-fold excess unlabeled probe (lanes 4–6). Supershift assays using anti-STAT5 antibodies are shown in lanes 7–9.
The observation of reduced MAPK activity despite increased Ras activity in BCR/ABL-expressing cells and in CD34+ cells from CP CML patients suggests that negative regulation of MAPK signaling may occur in the context of activation of complex signaling networks activated downstream of BCR/ABL. MAPK signaling is subject to several levels of feedback regulation and cross-regulation (34, 42, 43). Negative regulation of MAPK may be a response to cross-signaling from enhanced Akt and STAT5 signaling. Akt phosphorylation of Raf at Ser259 leads to inhibition of Raf activity (44). Although we observed that Raf Ser259 phosphorylation was increased, Raf kinase activity did not seem to be reduced in BCR/ABL-expressing cells because Mek phosphorylation was increased. Raf and MEK activity were increased in BCR/ABL-expressing cells compared with controls, suggesting that negative regulation of MAPK signaling may occur at the level of MAPK itself. The strong suppression of MAPK signaling observed implies that the activation of mechanisms involving dephosphorylation and inactivation of MAPK may be involved. Our own observations do not support a role for the dual-specificity protein phosphatase MKP-1 in MAPK deactivation (data not shown), and the precise mechanisms that regulate signaling through MAPK in BCR/ABL-expressing cells requires further elucidation. The observation that proliferation and survival of BCR/ABL-Y177F–expressing cells are markedly reduced compared with wild-type BCR/ABL-expressing cells despite showing strong MAPK activity indicates that enhanced MAPK signaling may not be a critical mechanism for proliferation of early phase CML progenitors. On the other hand, increased MAPK activity is associated with progression from CP to BC CML and seems to contribute to altered expression of genes such as c-Myc and CAAT/enhancer binding protein α that may play important roles in abnormal proliferation, survival, and differentiation in BC CML progenitors (45, 46). Therefore, increased activation of MAPK signaling may be a feature of BC CML progenitors and play a critical role in disease progression in early-phase disease.

We further show that BCR/ABL-Y177 contributes to STAT5 phosphorylation, nuclear translocation, and binding to target sequences. Mutation of BCR/ABL-Y177 did not fully reverse STAT5 activation, suggesting that additional BCR/ABL signaling mechanisms also contribute to STAT5 activation. Janus-activated kinase 2 and Src kinases have been observed to be activated in BCR-ABL expressing erythroid cells and could contribute to STAT5 activation by BCR/ABL (47). STAT5 plays a key role in EPO signaling, promoting erythroid differentiation and expansion, and controls a rate-determining step regulating early erythroblast survival (48). Residual STAT5-dependent erythroid cell growth may account for incomplete reversal of BCR/ABL-mediated erythroid cell expansion in cells expressing Y177-mutated BCR/ABL genes.

Although the BCR/ABL kinase inhibitor IM has been highly successful in the treatment of CML, clinical resistance can occur. One approach to treating imatinib-resistant cells in CML is through inhibition of critical signaling mechanisms downstream from the BCR/ABL kinase. Interestingly, it was recently shown that primary CML CD34+ CFC display a higher sensitivity to silencing of SHP2, STAT5, and Gab2 as compared with normal CFC (49). We observed that inhibition of signaling through BCR/ABL-Y177 in combination with kinase inhibition with IM-enhanced suppression of progenitor growth. However, because Y177 phosphorylation and signaling activity are downstream of TK activity in BCR/ABL-mediated signaling, the effect of combining imatinib with inhibition of signaling through BCR/ABL-Y177 is more likely to be additive rather than synergistic. This is consistent with our observations that BCR/ABL-Y177F–expressing progenitors do not show enhanced sensitivity to imatinib compared with wild-type BCR/ABL-expressing cells. These results support further exploration of the therapeutic value of targeting BCR/ABL-Y177 or downstream effectors in imatinib-resistant CML.

We conclude that BCR/ABL-Y177 plays a crucial role in abnormal CML hematopoietic progenitor growth by mediating activation of the Ras and PI3K/Akt pathways. Our results support further exploration of blockage of BCR/ABL-Y177–activated signals to enhance targeting of CML stem cells.

Acknowledgments


Grant support: R01 HL77847 and R01 CA95684 (R. Bhatia), and the General Clinical Research Center Grant 5M01 RR00043. R. Bhatia is a Scholar in Clinical Research of the Leukemia and Lymphoma Society.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Ning Niu, Tinisha McDonald, and Jessica Ma for their technical assistance, and Lucy Brown, Claudio Spalla, and Alex Spalla, Analytical Cytometry Core, for performing cell sorting. We thank Dr. Richard Jove and Dr. Sangkil Nam for assistance with STAT5 assays. We are grateful to StemCyte for their generous gift of cord blood samples.

Figure 6. Effect of IM treatment on BCR/ABL-Y177F–expressing cells. The number of viable cells after culture of CD34+ GFP+ cells expressing GFP alone (R1), BCR/ABL (BA), and BCR/ABL-Y177F (Y177F) for 72 h with increasing concentrations of IM was determined using an MTS assay. Points, mean of six replicates obtained from two independent experiments; bars, SE. A, percent inhibition of growth relative to untreated controls. B, relative number of viable cells present with or without treatment as determined by absolute MTS values.

A

B

References

16. CML Progenitor Transformation by BCR/ABL-Y177
BCR-Tyrosine 177 Plays an Essential Role in Ras and Akt Activation and in Human Hematopoietic Progenitor Transformation in Chronic Myelogenous Leukemia

Su Chu, Liang Li, Harjeet Singh, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/14/7045

Cited articles
This article cites 48 articles, 31 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/14/7045.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/67/14/7045.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.