Inhibition of BCR-ABL Expression With Antisense Oligodeoxynucleotides Restores β1 Integrin-Mediated Adhesion and Proliferation Inhibition in Chronic Myelogenous Leukemia Hematopoietic Progenitors

By Ravi Bhatia and Catherine M. Verfaillie

Chronic myelogenous leukemia (CML) results from the malignant transformation of a hematopoietic stem cell and is characterized by a massive expansion of hematopoietic progenitors, as well as more differentiated hematopoietic cells, originating from the malignant clone. In addition, large numbers of circulating progenitors are seen in CML, reflecting their enhanced release from the bone marrow cavity.1 Retention and homing of progenitors to the marrow microenvironment is in large part dependent on members of the β1 integrin family of adhesion receptors, in particular the α4β1 receptor.2-4 Signaling through β1 integrin receptors also plays an important role in the regulation of proliferation,5,6 differentiation,7 and survival8 of a variety of cell types. We have shown that signals transduced from the extracellular matrix by β1 integrins play an important role in microenvironmental regulation of normal hematopoietic progenitor proliferation.9,10 In contrast to normal hematopoietic progenitors, CML progenitors show deficient adhesion to the microenvironment.11-13 Although malignant CML CD34+ HLA-DR+ (DR+) cells with breakpoint-specific AS-ODNs significantly increased adhesion of CML progenitors to stroma and fibronectin (FN), pretreatment with breakpoint-specific ODNs also resulted in significant inhibition of CML progenitor proliferation after ligand or antibody-mediated β1 integrin engagement. Breakpoint-specific ODNs were significantly more effective in restoring CML progenitor adhesion and proliferation inhibition than control ODNs. BCR-ABL mRNA and p210 BCR-ABL levels in CML CD34+ cells were significantly reduced after incubation with breakpoint-specific AS-ODN. These studies indicate a role for BCR-ABL in abnormal circulation and defective integrin-dependent microenvironmental regulation of proliferation of CML hematopoietic progenitors.

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evaluated the effect of inhibition of BCR-ABL expression by AS-ODNs directed against the BCR-ABL breakpoint on β1 integrin-mediated adhesion and proliferation inhibition of CML progenitors. Our studies indicate that inhibition of BCR-ABL expression in primary malignant CML CD34+HLA-DR+ cells with breakpoint-specific AS-ODNs restores integrin-mediated adhesion of CML colony-forming cells (CFC) to stroma and FN substrates and restores inhibition of CML CFC proliferation after contact with stroma and FN substrates or after antibody-mediated integrin stimulation. These results indicate a role for p210BCR-ABL in abnormal β1 integrin function in CML.

MATERIALS AND METHODS

Seventeen patients with CML and eight normal healthy volunteers were evaluated after informed consent was obtained using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota and the Institutional Review Board at the City of Hope National Medical Center. Fifteen patients were in the chronic phase of CML, whereas two were in accelerated phase. Nine patients were receiving treatment with interferon (IFN)-α, whereas six were treated with hydroxyurea alone, which was discontinued at least 4 days before study. Eight patients had been previously treated with interferon (IFN)-α, which had been discontinued for at least 3 months before study. The b2a2 BCR-ABL breakpoint was present in six patients, whereas the b2a2 breakpoint was present in 11 patients. Heparinized bone marrow samples were obtained by aspiration from the posterior iliac crest. Bone marrow mononuclear cells (BMMNC) were isolated by Ficoll-Hypaque (Sigma Diagnostics, St Louis, MO) density gradient separation (specific gravity, 1.077) for 30 minutes at 37°C and 400g.

Selection of CD34+DR+ progenitor populations. CD34+ cell enriched populations were selected from CML or normal BMMNC using sequential avidin-biotin immunoadsorption columns (CellPro Inc, Bothell, WA) or immunomagnetic column separation (Miltenyi Biotec Inc, Auburn, CA). CD34+HLA-DR+ (DR+) cells were then selected by fluorescence-activated cell sorting (FACS) as previously described. Adhesive substrates. Bone marrow stromal layers were established from normal BMMNC in T-75 or T-150 flasks as previously described, irradiated at 1,250 cGy using a Cesium irradiator to eliminate hematopoietic cells, and subcultured in 24-well plates (Costar, Cambridge, MA). Human plasma fibronectin was a kind gift from Dr James B. McCarthy (University of Minnesota, Minneapolis, MN). FN was purified as a byproduct of factor VIII production by sequential ion exchange and gelatin chromatography and 75-kD tryptic fragments containing the cell binding sequence RGD and 33- and 66-kD COOH-terminal heparin binding fragments were purified as previously described. AS-ODNs. 18 or 24 mer phosphodiester AS-ODNs to BCR-ABL junctional regions were added to CML or normal DR+ cells as indicated in the Results (Table 1). In addition to AS-ODNs specific to the breakpoint present in the cells being studied, cells were incubated with missense ODNs and ODNs to the alternate breakpoint, eg, b2a2 if the tested cells contained the b2a2 breakpoint, as controls (control ODNs).

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IMDM (pH 7.3) and divided into two equal fractions. They were then suspended in 200 µL serum-free IMDM and incubated at 37°C with or without 5 mM [3H]-thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA). After 20 minutes, 5 mL cold thymidine (500 mg/mL in IMDM with 20% fetal calf serum; Sigma) was added to all samples to competitively inhibit further [3H]-thymidine uptake. Cells were washed in IMDM and plated in methylcellulose progenitor culture for 14 to 18 days. The percentage of CFC in S-phase was calculated as the percentage of CFC killed in the presence of [3H]-thymidine [% CFC in S-phase = [(CFC present in absence of [3H]-thymidine − CFC present in presence of [3H]-thymidine) × 100] divided by CFC present in the absence of [3H]-thymidine].

**Methylcellulose progenitor culture.** DR cells recovered from adhesion and thymidine suicide assays were plated in methylcellulose culture as previously described. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO₂ for 14 to 18 days and assessed for the presence of colony-forming-unit-granulocyte macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-MIX colonies.

**Polymerase chain reaction (PCR) analysis.** After incubation with AS-ODNs, CML DR cells were counted and frozen at −70°C in PBS. Total cellular RNA was extracted from thawed samples using the method of Chomczynski and Saachi. After reverse transcription, amplification of BCR-ABL mRNA was performed with the primers shown in Table 2, using 30 to 40 cycles of single-step PCR. Amplified samples were size separated by gel electrophoresis, ethidium bromide stained, and photographed. β-Actin controls were run for each sample. The ratio of BCR-ABL to β-actin was determined by densitometry.

**Western blotting.** After incubation with AS-ODNs, CML DR cells were counted and protein extracts obtained by incubating with lysis buffer (1% Triton-X 100; 1 mMol/L EDTA; 150 mMol/L NaCl; 50 mMol/L Tris-HCl; 10 mMol/L sodium orthovanadate; 10 mMol/L sodium fluoride; 10 mMol/L phenylmethyl sulfonyl fluoride [PMSF]; 0.15 U/mL Aprotinin; 10 U/mL leupeptin, pH 7.5). Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a nitrocellulose filter. p145-ABL and p210-BCR-ABL were visualized with an anti-c-ABL monoclonal antibody (antibody 24-11; Santa Cruz Biotechnology Inc, Santa Cruz, CA) or antibody Ab-3; Oncogene Science Inc, Manhasset, NY), peroxidase-conjugated goat antimouse IgG (Jackson Immunoresearch Laboratories Inc, West Grove, PA) and a Western blot chemiluminescence reagent (Amersham Life Sciences, Arlington Heights, IL). The ratio of BCR-ABL to c-ABL was determined by densitometry.

**Statistical analysis.** Results of experimental points obtained from multiple experiments were reported as mean ± standard error of mean (SEM). Significance levels for differences between different samples was determined using two-tailed Student’s t-test.

**RESULTS**

**Anti–BCR-ABL AS-ODNs restore CML CFC adhesion to stroma or FN.** In initial experiments, CML DR cells were incubated with 18-mer AS-ODNs specific for the BCR-ABL breakpoint (b_{a2} or b_{a3}) present in the cells that were being evaluated. Negative controls included mismatch sense AS-ODNs, eg, b_{a2} mismatch if the b_{a2} breakpoint was present, and AS-ODNs to the alternate breakpoint, eg, b_{a3}; if the tested cells contained the b_{a3} breakpoint. Preincubation with breakpoint-specific AS-ODNs or control ODNs after which their adhesion to BSA (A); stromal-adherent layers (B); FN 75-kD (C); and FN 33-66-kD (D) coated wells was evaluated as described in the text. Results shown represent the mean ± SEM for six (A, C, and D) or nine (B) separate experiments.

![Fig 1. Anti–BCR-ABL AS-ODNs restore CML CFC adhesion to stroma or fibronectin. A total of 10,000 DR cells were incubated in serum-free medium with or without breakpoint-specific and control ODNs after which their adhesion to BSA (A); stromal-adherent layers (B); FN 75-kD (C); and FN 33-66-kD (D) coated wells was evaluated as described in the text. Results shown represent the mean ± SEM for six (A, C, and D) or nine (B) separate experiments.](image)

**Table 2. PCR Primers**

**PCR Primer**  
BCR-ABL  
5'-CTC-CAG-ACT-GTC-CAC-AGC-ATT-CGG-3'  
5'-CTA-GAC-CCT-GAG-GCT-CAA-AGT-C-3'  
β-Actin  
5'-TAC-CTC-ATG-AAG-ATC-CTC-A-3'  
5'-TTC-GTG-GAT-GCC-ACA-GGA-C-3'
stroma-conditioned LTBMC medium (SFM-LC). After 4 days, approximately 50% of CML DR CFC were in S-phase as evaluated by thymidine suicide assays. Proliferating CML DR cells were exposed to AS and control ODNs during the last 36 hours of culture and then cocultured with stroma and FN substrates for 4 hours, and the percentage of CFC in S-phase was evaluated using thymidine suicide assays. Consistent with previous studies, proliferation of untreated CML CFC was similar whether cells were kept in suspension or were cocultured with stroma or FN fragments. Preincubation with either breakpoint-specific AS-ODNs or control ODNs did not alter proliferation of CML CFC kept in suspension. However, significant reduction in proliferation of CML CFC preincubated with breakpoint-specific AS-ODNs was seen after coculture with stroma (Fig 2), primarily as a result of inhibition of proliferation of adherent CFC. CML CFC proliferation was also significantly reduced when DR cells pretreated with AS-ODNs were cocultured with FN 75-kD and 33-66-kD substrates (Fig 2). In contrast to breakpoint-specific AS-ODNs, control ODNs did not significantly reduce proliferation of CML CFC cocultured with either stroma or FN (Fig 2). Inhibition of CML CFC proliferation was not a nonspecific effect of adhesion because proliferation of CML CFC treated with AS-ODNs was not reduced after coculture with the adhesogen poly-L-lysine (Fig 2). In additional experiments, we examined the effect of antibody-mediated stimulation of β1 integrins on CML CFC proliferation. Proliferation of untreated CML CFC was not significantly altered by cross-linking of β1 integrin receptors with blocking anti-β1 integrin mouse monoclonal antibodies and secondary antimouse antibodies (Fig 3). However, proliferation of CML CFC pretreated with anti–BCR-ABL AS-ODNs, but not control ODNs, was significantly inhibited after antibody-mediated cross-linking of β1 integrin receptors (Fig 3). These observations indicate that breakpoint-specific BCR-ABL AS-ODNs can restore β1 integrin-mediated inhibition of CML CFC proliferation.

The effect of anti–BCR-ABL AS-ODNs on CML progenitor adhesion and proliferation is specific. The specificity of the observed effects of breakpoint-specific AS-ODNs on CML CFC adhesion and proliferation was further evaluated in experiments in which CML DR+ cells were incubated with additional breakpoint-specific and control AS-ODNs. These included 24-mer b2a2 and b3a2 breakpoint-specific AS-ODNs and a control 18-mer missense anti-b3a2-ODN containing a TAT sequence 5’ from the breakpoint, which is associated with nonspecific effects in BCR-ABL–containing cell lines. Both 18- and 24-mer breakpoint-specific AS-ODNs restored CML CFC adhesion to FN and proliferation inhibition after contact with FN, significantly more than any of the control ODNs (Fig 4).

In another set of experiments, incubation of normal DR+ cells with anti–BCR-ABL ODNs did not alter the adhesion of normal CFC to FN (Fig 5). In addition, inhibition of normal
CFC proliferation usually seen after coculture with FN substrates was unaltered after preincubation with BCR-ABL AS-ODNs. These results indicate that the effects of BCR-ABL AS-ODNs on CML CFC adhesion and proliferation are not the result of nonspecific effects of ODNs on adhesion or proliferation.40

Anti–BCR-ABL AS-ODNs inhibit BCR-ABL expression in CML DR<sup>+</sup> cells. DR<sup>+</sup> cells incubated with breakpoint-specific AS-ODNs or control ODNs were evaluated by reverse transcriptase (RT)-PCR for BCR-ABL mRNA levels and by Western blotting for p210<sub>BCR-ABL</sub> levels to determine the effect of BCR-ABL AS-ODNs on BCR-ABL expression. We show that exposure of CML DR<sup>+</sup> cells to breakpoint-specific AS-ODNs, but not control ODNs, results in significant reduction of BCR-ABL mRNA levels (Table 3 and Fig 6). Similarly, significant reduction in p210<sub>BCR-ABL</sub> levels was observed after incubation with breakpoint-specific AS-ODNs resulted in CML DR<sup>+</sup> cells, but not control AS-ODNs (Table 4 and Fig 7). These studies indicate that, under the conditions used, incubation with breakpoint-specific AS-ODNs can, at least partially, inhibit BCR-ABL expression.

DISCUSSION

Adhesion receptor-mediated interactions with the extracellular matrix play an important role in the regulation of cellular proliferation, differentiation, and survival.5-8 Neoplastic transformation is often associated with altered cell-matrix interactions and perturbation of normal microenvironmental regulation of...
cell growth and function. The abnormal circulation and unregulated expansion of malignant hematopoietic cells in CML may be related to defective function of β1 integrin adhesion receptors. We hypothesized that the BCR-ABL oncogene may induce abnormal β1 integrin function in CML progenitors through its interactions with cytoskeletal proteins critical for normal integrin function. We show here that downregulation of BCR-ABL expression with breakpoint-specific AS-ODNs results in restoration of β1 integrin function in CML progenitors. These studies suggest a possible mechanism by which the BCR-ABL oncogene may contribute to abnormal unregulated proliferation and abnormal circulation of malignant hematopoietic progenitors in CML.

In previous studies, BCR-ABL AS-ODNs have inhibited clonogenic growth of CML-blast crisis progenitors but have failed to consistently inhibit growth of CML-chronic phase progenitors. In the present studies, the growth of chronic phase CML CFC was not altered after 48 hours of exposure to BCR-ABL AS-ODNs. In addition, BCR-ABL AS-ODNs did not by themselves alter the percentage of CML progenitors in S-phase. However, the present studies indicate that downregulation of BCR-ABL expression with BCR-ABL breakpoint-specific AS-ODNs is associated with restoration of progenitor-microenvironmental interactions in CML. Malignant CML progenitors show deficient adhesion to stroma and FN through α4β1 and α5β1 integrins. Treatment with breakpoint-specific AS-ODNs resulted in enhanced CML progenitor adhesion to stroma and FN, and engagement of the integrin receptors with anti-β1 antibodies, leads to inhibition of progenitor proliferation. CML hematopoietic progenitors, in contrast, are unresponsive to β1 integrin-mediated inhibition of proliferation. Breakpoint-specific AS-ODNs, besides restoring CML CFC adhesion, also restore inhibition of CML progenitor proliferation after coculture with stroma, FN, or anti-β1 integrin antibodies. Reduced BCR-ABL expression does not seem to represent increased numbers of normal cells given the lack of reduction in CFC numbers after culture, the tiny fraction of BCR-ABL–negative cells in the starting CD34+ population, and the lack of significant expansion in normal CFC number after 36 hours of culture in medium with low cytokine concentrations. These observations support the hypothesis that p210BCR-ABL is responsible for abnormal β1 integrin-mediated adhesion and inhibition of proliferation in CML progenitors.

When interpreting the results of experiments using AS-ODNs, it is important to recognize that cellular responses observed after exposure to AS-ODNs may not necessarily be the result of specific inhibition of the mRNA targeted by that ODN. In previous studies, missense ODNs, and AS-ODNs to the breakpoint not present in the cells being evaluated, have had nonspecific effects on BCR-ABL–transduced cell lines. In the present studies, however, significant inhibition of BCR-ABL expression at the protein and mRNA level in CML CD34+ cells was observed with breakpoint-specific BCR-ABL AS-ODNs, but not control ODNs. In addition, 18- and 24-mer breakpoint-specific AS-ODNs restored β1 integrin-mediated adhesion and proliferation inhibition of CML CFC significantly more than control missense ODNs, control AS-ODNs to the breakpoint not present in the cells being studied, and a control b2a2 missense ODNs containing the TAT sequence present in anti-b3a2 AS-ODNs, which is particularly associated with nonspecific effects. Our studies indicate that even partial inhibition of BCR-ABL expression in CML primary progenitors is sufficient to allow restoration of integrin-dependent microenvironmental regulation. Although AS-ODNs can nonspecifically alter cell adhesion, this does not appear to be the case here because normal CFC adhesion or adhesion-mediated proliferation inhibition was not affected by BCR-ABL AS-ODNs.

BCR-ABL–expressing cell lines express several-fold more p210BCR-ABL than primary CML hematopoietic cells. As a result, in previous studies using CML cell lines, high concentrations of phosphorothioate AS-ODNs, which are more likely to have nonspecific effects, have been required to achieve inhibition of BCR-ABL expression. The selective effects of breakpoint-specific BCR-ABL AS-ODNs on BCR-ABL expression and

![Figure 6](image-url)
CML CFC adhesion and proliferation in our studies may thus be related to the use of phosphodiester AS-ODNs, the use of serum-free medium to prevent phosphodiester AS-ODN degradation, and the use of relatively low concentrations of AS-ODNs, all of which reduce the likelihood of nonspecific effects. Higher concentrations or prolonged exposure to AS-ODNs, or the use of phosphorothioate AS-ODNs, may possibly have resulted in more complete inhibition of BCR-ABL expression in our studies, but would probably have caused increased nonspecific effects.

p210BCR-ABL may induce abnormal integrin function in CML by associating with and/or abnormally phosphorylating cytoskeletal proteins or cytoskeleton-associated signaling proteins important for normal integrin function. We have recently shown that β1 integrin receptor capping after antibody-mediated receptor engagement and cross-linking, which is an indicator of the ability of the integrin receptor to interact with the cytoskeleton, is significantly impaired in BCR-ABL–transformed cells, indicating abnormal receptor cytoskeletal interactions.

Inhibition of BCR-ABL mRNA and protein expression by insertion of antisense gene constructs restores β1 integrin capping in these cells, supporting a role for BCR-ABL in defective capping. Integrin cytoplasmic domains do not have intrinsic enzymatic activity, but mediate “inside-out” and “outside-in” signaling through interactions with cytoskeletal elements such as α-actin, talin, vinculin, paxillin, and tensin in focal adhesion complexes. These proteins, besides connecting integrin receptors to actin microfilaments, also serve as a framework for the association of other signaling proteins, including protein tyrosine kinases such as FAK and SRC and adapter proteins such as GRB-2 and CRK, thereby linking integrin receptor stimulation to various intracellular signaling pathways. Integrin stimulation is also associated with cytoskeletal translocation and activation of the phosphoinositide 3-kinase (PI-3 kinase), which may also play an important role in modulation of integrin function. The focal adhesion-associated proteins paxillin, FAK, vinculin, talin, and tensin are constitutively phosphorylated in BCR-ABL–transfected cell lines. Recent studies suggest that the adapter proteins CRKL and CBL are prominent p210BCR-ABL substrates in primary CML cells and may form a complex with p210BCR-ABL and link it with paxillin and the p85 regulatory subunit of PI-3 kinase.

Finally, other studies have shown that the COOH terminus of c-ABL has F- and G-actin binding domains with actin bundling activity, that p210BCR-ABL shows enhanced actin binding through this COOH-terminal domain, and that this interaction may be important for the transforming ability of BCR-ABL. Some or all of these interactions of p210BCR-ABL with integrin-related proteins may contribute to abnormal integrin function in CML.

In conclusion, we show that inhibition of BCR-ABL expression with breakpoint-specific AS-ODNs restores β1 integrin-mediated adhesion and proliferation inhibition in CML CFC. Our studies link the BCR-ABL oncogene with altered integrin-mediated adhesion and proliferation inhibition in CML CFC.
dependent microenvironmental regulation and abnormal circulation of hematopoietic progenitor proliferation in CML. Future studies will be directed towards understanding the specific molecular mechanisms underlying p21BCR-ABL-induced disruption of integrin function in CML progenitors.

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