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Role of abnormal integrin-cytoskeletal interactions in impaired β1 integrin function in chronic myelogenous leukemia hematopoietic progenitors

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Abnormal circulation and unregulated proliferation of chronic myelogenous leukemia (CML) progenitors is related, at least in part, to BCR/ABL induced abnormalities in β1 integrin-mediated adhesion and signaling. The BCR/ABL oncogene has several potential interactions with cytoskeletal elements that are important for normal integrin signaling. In the present study, we evaluated whether abnormalities in β1 integrin-cytoskeletal interactions were present in primary CML progenitors and contributed to defective integrin function. β1 integrin-cytoskeletal interactions were studied in CML and normal $CD34^+$ primary hematopoietic progenitors as well as BCR/ABL-transfected or mock-transfected M07e cells. In normal $CD34^+$ progenitors, antibody-mediated cross-linking of β1 integrins resulted in their redistribution into caps via a process requiring receptor-cytoskeletal interactions. CML $CD34^+$ cells demonstrated significantly impaired β1 integrin capping. This defect was related to the presence of the BCR/ABL gene, because capping also was impaired in BCR/ABL-transfected M07e cells. Defective receptor capping was not seen for non-integrin receptors. In addition, CML $CD34^+$ and $M07e^{BCR/ABL}$ cells also demonstrated increased actin polymerization and altered actin cytoskeletal organization. Further studies suggested that impaired β1 integrin capping and defective integrin-mediated adhesion and proliferation inhibition in CML cells were related to abnormally enhanced integrin-cytoskeletal association and restricted receptor mobility. Finally, interferon α, which restores integrin-mediated adhesion and signaling in CML progenitors, also enhanced integrin capping in $CD34^+$ cells. These studies suggest that $p210^{BCR/ABL}$ induces abnormal association of integrin receptors with the cytoskeleton and restricted receptor mobility and provide new insights into mechanisms underlying abnormal integrin function in CML progenitors.

Keywords: **Chronic myelogenous leukemia—BCR/ABL—Integrin—Hematopoietic progenitors—Cytoskeleton**

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Introduction

α4β1 and α5β1 integrin receptors play important roles in normal hematopoietic progenitor growth and development. β1 Integrins, in addition to contributing to hematopoietic progenitor adhesion and homing to the marrow microenvironment [1–3], also play a role in microenvironmental regulation of normal hematopoietic progenitor proliferation and differentiation [4,5]. Chronic myelogenous leukemia (CML), a malignancy of the hematopoietic stem cell, is characterized by unregulated, continuous proliferation of malignant hematopoietic progenitors and their premature release from the bone marrow cavity. Abnormalities in β1 integrin-dependent interactions between CML progenitors and the marrow microenvironment may contribute to the abnormal circulation and unregulated proliferation of CML progenitors [6–9]. Although CML progenitors express normal levels of β1 integrins on their cell surface, they demonstrate significantly reduced β1 integrin-dependent adhesion to stroma and fibronectin (FN) and are unresponsive to β1 integrin-mediated inhibition of proliferation indicating that these receptors function abnormally [8,9].

Integrins are heterodimers of various α and β subunits, which can pair to form more than 20 different receptors [10]. Integrins have a large ligand-binding extracellular domain formed by both the α and β subunits, a transmembrane segment, and short cytoplasmic C-terminal tails [10]. Integrin function can be regulated by signals received from other receptors that lead to modulation of their ligand binding ability (”inside-out” signaling). In addition to mediating cell adhesion and mobility, integrins can transduce signals into cells that lead to changes in cell shape, gene expression, proliferation, survival, and differentiation (”outside-in” signaling) [10]. Interactions between receptors and the cytoskeleton play a critical role in both signaling through β1 integrin receptors as well as modulation of integrin activity in response to signals from other receptors [11,12]. Following engagement and clustering of β1 integrin receptors by multivalent extracellular matrix ligands, their cytoplasmic tails

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associate directly and indirectly with several cytoskeletal proteins including α-actinin, talin, vinculin, and tensin, to form multimolecular complexes known as focal adhesions [11–14]. These interactions result in linkage of the extracellular matrix with F-actin fibers and lead to rearrangement of the actin cytoskeleton. In addition, signal transduction proteins, such as paxillin, and kinases, such as the focal adhesion kinase, RAFTK/PYK2, and the phosphoinositol-3 kinase (PI-3 kinase) [11,15,16], are recruited to focal adhesion complexes leading to activation of downstream signaling pathways.

The characteristic cytogenetic abnormality in CML, a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)], results in the fusion of the c-ABL gene on chromosome 9 with the BCR gene on chromosome 22 and formation of the BCR/ABL fusion gene [17,18]. It is widely accepted that the BCR/ABL gene plays a critical role in the pathogenesis of CML [19,20]. In contrast to its protooncogene counterpart $p145^{c\text{-}ABL}$, the $p210^{BCR/ABL}$ protein product of the BCR/ABL gene demonstrates enhanced binding to the actin cytoskeleton via its COOH-terminus actin binding domain and predominantly cytoplasmic localization [21, 22]. Enhanced cytoskeletal binding appears to contribute to the transforming properties of BCR/ABL [23]. Binding of $p210^{BCR/ABL}$ to F-actin may alter the cytoskeleton directly. Alternatively, it may allow colocalization of the $p210^{BCR/ABL}$ tyrosine kinase with focal adhesion proteins, including paxillin, vinculin, talin, tensin, and focal adhesion kinase, which become substrates for $p210^{BCR/ABL}$-induced phosphorylation [24–27]. We have shown that suppression of BCR-ABL expression with BCR/ABL breakpoint-specific antisense oligodeoxynucleotides restores β1 integrin-mediated adhesion and growth regulation in CML progenitors [28]. In addition, inhibition of BCR/ABL tyrosine kinase activity with Tyrphostin AG957, which reduces tyrosine phosphorylation of proteins such as paxillin, crkl, cbl, and the p85 subunit of PI-3 kinase, also restores β1 integrin function in CML progenitors [29]. These observations led us to hypothesize that the abnormal β1 integrin function in CML may result from abnormal integrin-cytoskeletal interactions secondary to $p210^{BCR/ABL}$ interactions with cytoskeletal elements important for normal β1 integrin signaling.

In the present study we directly evaluated integrin-cytoskeletal interactions in CML progenitors. We demonstrate that β1 integrin capping, which is an indicator of receptor-cytoskeletal interactions, is impaired in CML progenitors. This defect is related to the presence of the BCR/ABL oncogene. Our studies indicate that there is enhanced cytoskeletal binding of integrin receptors in CML cells. Treatment with IFN partially reverses defective integrin-cytoskeletal interactions in CML progenitors. These studies provide new insights into the mechanisms underlying BCR/ABL induced abnormalities in integrin-mediated microenvironmental regulation of hematopoiesis in CML.

Methods

Patients

Twenty-eight patients with CML and 26 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. Of the CML patients studied, 2 had not received any prior treatment, 19 had been treated with hydroxyurea alone, 5 had previously received interferon α (IFN) in addition to hydroxyurea, and 1 had previously received All-trans-Retinoic Acid (ATRA). In all patients who had received prior IFN treatment, IFN had been stopped at least 1 month prior to study.

Selection of $CD34^+$ progenitor subpopulations

Heparinized bone marrow samples were obtained from CML patients or normal individuals by aspiration from the posterior iliac crest. Bone marrow mononuclear cells were isolated by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) density gradient separation and $CD34^+$ cell-enriched populations selected from bone marrow mononuclear cells using sequential avidin-biotin immunoadsorbtion columns (CellPro Inc., Bothell, WA) or immunomagnetic column separation (Miltenyi Biotech Inc., Auburn, CA) as previously described [9].

Evaluation of receptor capping and clustering

Capping of β1 integrins and other receptors following antibody-mediated receptor stimulation was evaluated using previously reported techniques, with minor modification [30]. Capping studies were performed on (1) CML and normal $CD34^+$ cells and (2) M07e cells transfected with BCR/ABL (MBA-4) or control cells transfected with the Neomycin resistance gene alone (MYN) [31] (a kind gift of Dr John Dick, Toronto, Canada). Cells (50,000–100,000) were suspended in Iscove's modified Dulbecco's medium (IMDM) with 0.3% bovine serum albumin (BSA) and incubated at 4°C for 30 minutes with control mouse IgG or primary antibodies directed against the β1 integrin (P4C10), α5 integrin (P1D6), α4 integrin (P4C2) (all from Gibco-BRL, Gaithersburg, MD), CD11b (LPM19c) (Dako, Carpenteria, CA), CD9 (ALB6) (Coulter Westbrook, ME), CD44 (L178), CD38 (HB7), and HLA-DR (L243) (all from Becton-Dickinson San Jose, CA). Cells were washed with cold IMDM + 0.3% BSA and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated secondary goat anti-mouse antibodies for 30 minutes at 4°C. Warm IMDM 0.5 mL with 20% fetal bovine serum (at 37°C) was added and the cells incubated at 37°C for 30 to 210 minutes, washed with cold phosphate-buffered saline (PBS) with 0.3% BSA, and fixed with 0.4% paraformaldehyde. Cytospins were prepared, coverslips mounted with Antifade (Oncor Inc., Gaithersburg, MD), and sealed and stored at −20°C until they were evaluated. Cell surface distribution of receptors was determined using immunofluorescence microscopy using a Olympus BX50 microscope with fluorescent attachments. Capping was defined as the aggregation of all receptors into a single compact area on the cell surface (<1/4 of the total surface area).

In other experiments, cells were fixed either directly after sequential incubation for 30 minutes at 4°C with anti-β1 integrin antibodies (P4C10) and FITC-conjugated secondary goat anti-mouse antibodies or after further incubation at 37°C for 30 to 210 minutes. Receptor distribution at the cell surface was photographed using a

Zeiss 310 laser scanning confocal microscope (Zeiss Optical systems, Thornwood, NY) [32].

Evaluation of F-actin content and distribution

CML and normal CD34$^+$ cells and MBA-4 and MYN cells were labeled with rhodamine-conjugated phalloidin. F-actin content and distribution of labeled cells were analyzed by FACS and immunofluorescence microscopy, respectively. Cells (100,000–200,000) were suspended in IMDM + 0.3% BSA. Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed with PBS, and incubated for 30 minutes in the dark at room temperature with 0.165 nM rhodamine phalloidin (Molecular Probes, Eugene, OR) and 500 μg/mL palmitoyl D,L-α-lysophosphatidyl choline, (16:0) (Sigma) in PBS [33]. For fluorescent microscopy or laser scanning confocal microscopy, cytospin preparations of the stained cells were made, coverslips mounted with Antifade, and sealed and stored at −20°C until they were evaluated. For FACS analysis, cells were analyzed within 1 hour of staining. The mean channel fluorescence for rhodamine was used as a measure of total cellular F-actin content.

Cytochalasin D treatment

Cells were preincubated with 0.2–5.0 μM cytochalasin D (Sigma) at 37°C for 30 minutes prior to performing adhesion, proliferation, and capping assays. For normal samples, 83.0 ± 20.5 colony-forming cells (CFC) were generated from 2000 CD34$^+$ cells after cytochalasin D (1.0 μM) pretreatment compared with 73.4 ± 14.4 CFC after preincubation without cytochalasin D. For CML samples 106.0 ± 25.2 CFC were generated from 2000 CD34$^+$ cells with cytochalasin D (1.0 μM) compared with 117 ± 27.8 CFC after preincubation without cytochalasin D.

Evaluation of adhesion

Proteolytic fragments of FN (120-kDa) containing the cell-binding sequence RGDS (60 μg/mL) (Gibco-BRL) were adsorbed overnight in 96-well plate as previously described [1,9]. Control wells were adsorbed with 5 mg/dL BSA (>99% pure, fatty acid free, Sigma). CML 5,000 cells or normal CD34$^+$ cells were used in adhesion assays on FN-coated plates as previously described. Both adherent and nonadherent fractions were plated in methylcellulose assay for 14–18 days and assessed for the presence of colony-forming unit granulocyte-macrophage, burst-forming unit-erythroid, and colony-forming unit-mix colonies as previously described. The percent adherent CFC was calculated as [adherent CFC ÷ (adherent CFC + nonadherent CFC)] × 100%.

Evaluation of proliferation

The proliferation of CML and normal CFC was evaluated using thymidine suicide assays. To induce proliferation, CML and normal CD34$^+$ cells were cultured in stroma-conditioned long-term bone marrow culture medium (SCM), prepared as previously described, for 96 hours [34,35]. Cultured CD34$^+$ cells (5000) were resuspended in IMDM + 0.3% BSA and incubated with monoclonal anti-β1 integrin antibody [P4C10 (1:1000), Gibco-BRL], or control mouse IgG (Jackson Immunoresearch, West Grove, PA) for 30 minutes followed by goat anti-mouse antibody (Jackson Immunoresearch) for 4 hours. The proliferation of CFC following culture with or without anti-β1 integrin antibodies was evaluated in thymidine suicide assays as previously described [9].

Interferon treatment

Cells were incubated with or without interferon α2b (IFN) (Schering), 10,000 U/mL, in SCM for 48 hours at 37°C in a humidified atmosphere with 5% CO_2, as previously described [34], prior to evaluating its effect on capping, adhesion, and proliferation. CFC 91.1 ± 16.9 were generated in 2000 CML CD34$^+$ cells after IFN pretreatment compared with 106.7 ± 22.6 CFC after preincubation without IFN.

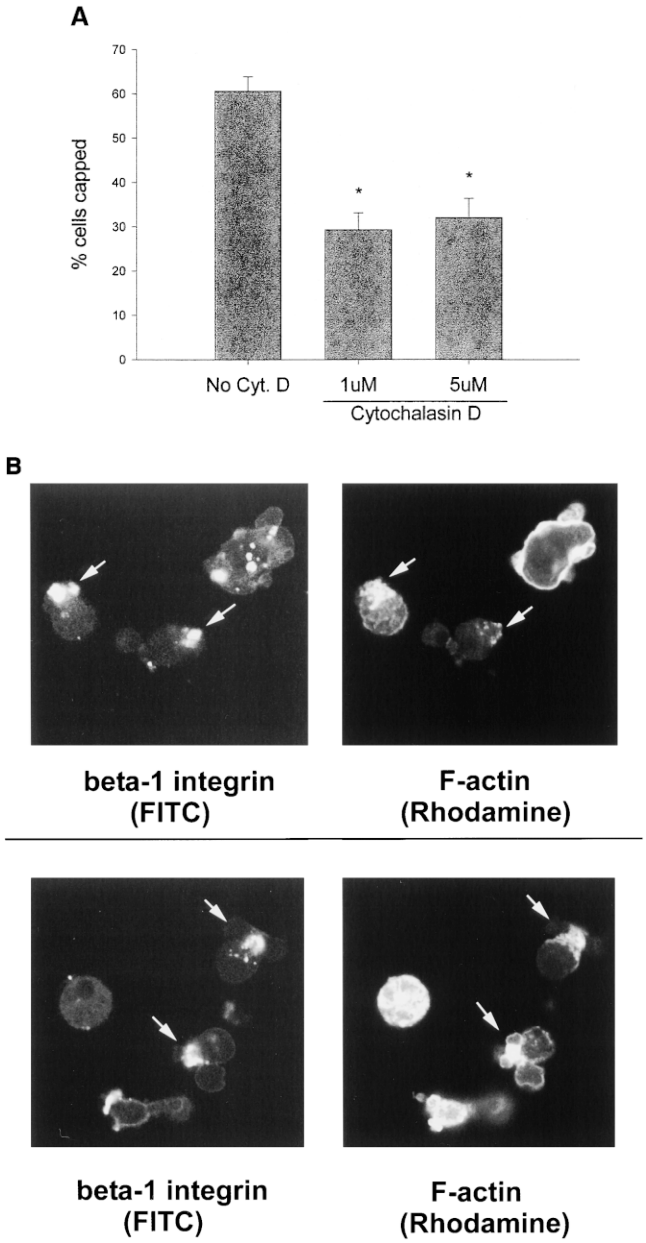

Figure 1. β1 Integrin capping requires cytoskeletal rearrangement. **(A)** β1 Integrin capping was evaluated in normal CD34$^+$ cells after preincubation with cytochalasin D (1 μM, n = 6; or 5 μM, n = 4) or without cytochalasin D (n = 6) as described in the Methods. Results are given as mean ± SEM of separate experiments. Significance levels: $*p < 0.005$ compared with capping without cytochalasin D. **(B)** F-actin distribution following induction of capping in normal CD34$^+$ cells with anti-β1 integrin antibodies and secondary FITC-conjugated goat anti-mouse antibody was evaluated by fixing cells with paraformaldehyde and labeling with rhodamine phalloidin. Colocalization (shown by the arrows) of F-actin staining with the β1 integrin cap is seen.

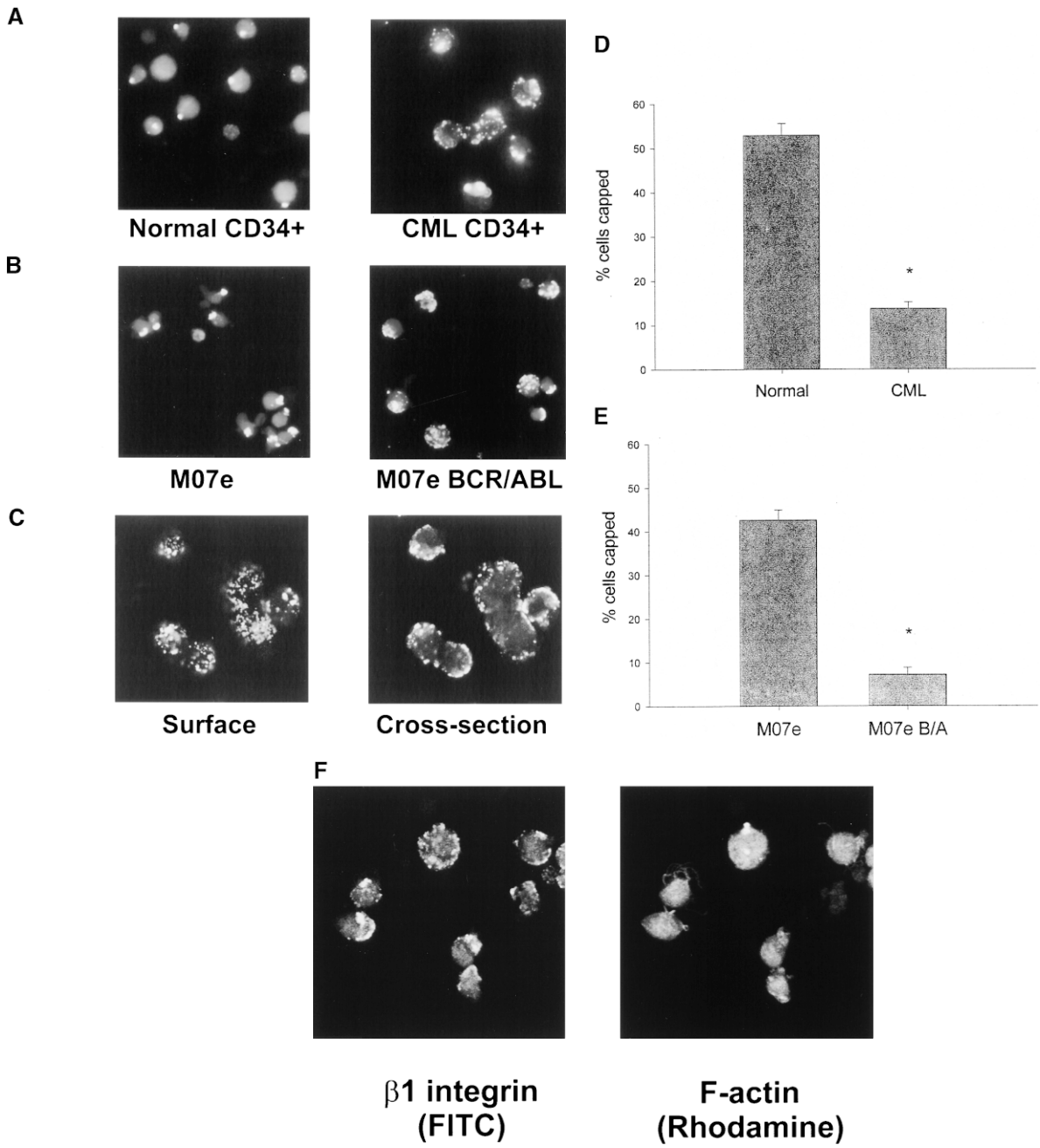

Figure 2. β1 Integrin capping is impaired in CML CD34$^+$ cells and BCR/ABL-containing M07e cells. CML and normal CD34$^+$ cells and BCR/ABL-transfected M07e cells (M07e$^{BCR/ABL}$) and mock-transfected M07e cells (M07e) were incubated with anti-β1 integrin antibodies and secondary FITC-conjugated goat anti-mouse antibody as described in the Methods, after which capping of β1 integrins was evaluated. **(A)** Results of representative experiments with CML and normal CD34$^+$ cells. **(B)** Results of a representative experiment with M07e$^{BCR/ABL}$ and mock-transfected M07e cells. **(C)** Confocal microscopic images of integrin distribution at the level of the lower surface and through the center of a M07e$^{BCR/ABL}$ cell demonstrating localization of the integrin receptor at the cell membrane. **(D)** Mean ± SEM of results of separate experiments with CML (n = 15) and normal (n = 13). CD34$^+$ cells. Significance levels: $*p < 0.0001$ compared with normal CD34$^+$ cells. **(E)** Mean ± SEM of five separate experiments with M07e$^{BCR/ABL}$ and mock-transfected M07e cells. Significance levels: $*p < 0.0001$ compared with mock-transduced M07e cells. **(F)** F-actin distribution following incubation of M07e$^{BCR/ABL}$ cells with anti-β1 integrin antibodies and secondary FITC-conjugated goat anti-mouse antibody. Lack of redistribution of F-actin staining following integrin cross-linking in these cells is seen.

Statistical analysis

Results of experimental points obtained from multiple experiments were reported as mean ± SEM. Significance levels for differences between different samples were determined using two-tailed Student's *t*-test.

Results

β1 Integrin capping in normal CD34$^+$ cells and M07e cells

Normal CD34$^+$ cells were incubated with blocking anti-β1 integrin antibodies at 4°C for 30 minutes and then labeled

with secondary FITC-conjugated goat anti-mouse antibody at 4°C for 30 minutes followed by incubation at 37°C for 30–210 minutes. Cytospin preparations were evaluated for receptor redistribution by immunofluorescence microscopy. Receptor redistribution into caps was apparent following 30 minutes incubation and reached a maximum after 90–210 minutes incubation at 37°C (data not presented). Similar results were seen in the myeloid cell line M07e. Subsequent evaluations were done following incubation at 37°C for 210 minutes. β1 Integrin capping is an active, energy-dependent process. Capping was not observed when cells were incubated at 4°C instead of 37°C. Preincubation of cells with the metabolic inhibitors sodium azide (which blocks electron transport) and 2-deoxyglucose (a competitive inhibitor of glucose metabolism) significantly inhibited β1 integrin capping. Inhibition of capping was more pronounced when the two metabolic inhibitors were used in combination (data not presented).

β1 Integrin capping was significantly inhibited when normal $CD34^+$ cells or M07e cells were pretreated with 1–5 μM cytochalasin D, an inhibitor of actin polymerization, prior to receptor cross-linking (Fig. 1A). In contrast, the microtubule inhibitor nocodazole (2–20 μM) did not affect β1 integrin capping (data not presented). Using rhodamine-conjugated phalloidin to label F-actin, we also showed that F-actin staining colocalized with the β1 integrin cap in several cells (Fig. 1B). Thus, β1 integrin receptor capping following antibody-mediated engagement and cross-linking of β1 integrin receptors requires interaction of the β1 integrin receptor with the actin cytoskeleton and is associated with F-actin reorganization.

Impaired integrin capping in CML $CD34^+$ cells and BCR/ABL-transfected M07e cells

In contrast to normal $CD34^+$ cells, β1 integrin receptor capping was significantly impaired in CML $CD34^+$ cells. Instead of capping, receptor "patching," i.e., redistribution into several patchy areas of fluorescence, was observed (Figs. 2A and 2D). As we showed previously, there was no significant difference in β1 integrin expression between CML and normal $CD34^+$ cells (data not shown) [8]. Likewise, capping of the α4 and α5 integrin receptors was significantly reduced in CML $CD34^+$ cells (Table 1). Cytochalasin D at high concentrations (1–5 μM) did not affect integrin capping in CML $CD34^+$ cells (cytochalasin D = 0 mM: 15.2% ± 3.9% capping; cytochalasin D = 1 mM: 15.8% ± 4.7% capping; cytochalasin D = 5 mM: 14.6% ± .3% capping, n = 5).

As was observed for β1, α4, and α5 integrins, capping of the β2 integrin, CD11b, occurred in normal $CD34^+$ cells but was reduced in CML $CD34^+$ cells. Capping of CD9, an integrin-associated protein, also was significantly reduced on CML compared with normal $CD34^+$ cells. In contrast, capping of other cytoskeleton-associated receptors such as CD44, CD38, and HLA-DR receptors occurred to the same extent on CML $CD34^+$ cells as on normal $CD34^+$ cells (Table 1).

Table 1. Capping of integrin and nonintegrin receptors in CML and normal $CD34^+$ cells

Receptor	Normal	CML	*p*
β1 integrin	50.8 ± 4.5	15.2 ± 3.9	0.0003
α4 integrin	50.4 ± 3.6	15.2 ± 3.0	0.00007
α5 integrin	48.6 ± 3.9	14.8 ± 2.0	0.0003
β2 integrin (CD11b)	45.4 ± 5.9	17.6 ± 2.5	0.007
CD9	39.4 ± 3.0	19.6 ± 4.0	0.005
CD44	15.3 ± 2.8	11.0 ± 4.0	NS
HLA-DR	29.6 ± 5.3	32.0 ± 5.6	NS
CD38	40.8 ± 2.0	42.8 ± 3.8	NS

CML and normal $CD34^+$ cells were incubated with antibodies to the various receptors and FITC- or PE-conjugated secondary antibodies. Capping was evaluated by immunofluorescence microscopy as described in the Methods. Results are given as mean ± SEM of five separate experiments.

We also evaluated the effect of β1 integrin receptor engagement and cross-linking on receptor redistribution in BCR/ABL-transfected M07e myeloid cells [31]. As was seen with CML $CD34^+$ cells, β1 integrin receptor capping was significantly reduced in BCR/ABL-transfected M07e cells and instead receptor "patching" was seen (Figs. 2B and 2E). Confocal microscopy was performed to confirm that the patched receptors had not been internalized (Fig. 2C). Redistribution of F-actin staining was not observed following incubation of $M07e^{BCR/ABL}$ cells with anti-β1 integrin antibodies and secondary goat anti-mouse antibody (Fig. 2F). These studies show that there are abnormalities in integrin receptor redistribution in CML cells that are related to the presence of the BCR/ABL oncogene.

Alterations in F-actin content and distribution in CML cells

To evaluate further the mechanism of impaired redistribution of β1 integrin receptors in CML cells, we quantitated the total cellular F-actin content of CML and normal cells by labeling with rhodamine-conjugated phalloidin followed by FACS analysis (Fig. 3A). CML $CD34^+$ cells were found to have significantly more F-actin than normal $CD34^+$ cells. Similarly, BCR/ABL-containing M07e cells had significantly higher levels of total cellular F-actin than mock-transfected cells (Fig. 3B). Thus, the presence of the BCR/ABL gene is associated with significantly enhanced basal actin polymerization. Immunofluorescence microscopic evaluation demonstrated that CML $CD34^+$ cells were more irregular in shape, with increased F-actin–containing cytoplasmic projections compared with normal $CD34^+$ cells (Fig. 3C). Similar differences were seen between $M07e^{BCR/ABL}$ cells and mock-transfected cells. Using confocal microscopy we also saw alterations in the distribution of F-actin at the submembranous level in BCR/ABL positive cells, with formation of abnormal aggregates, as well as increased cor-

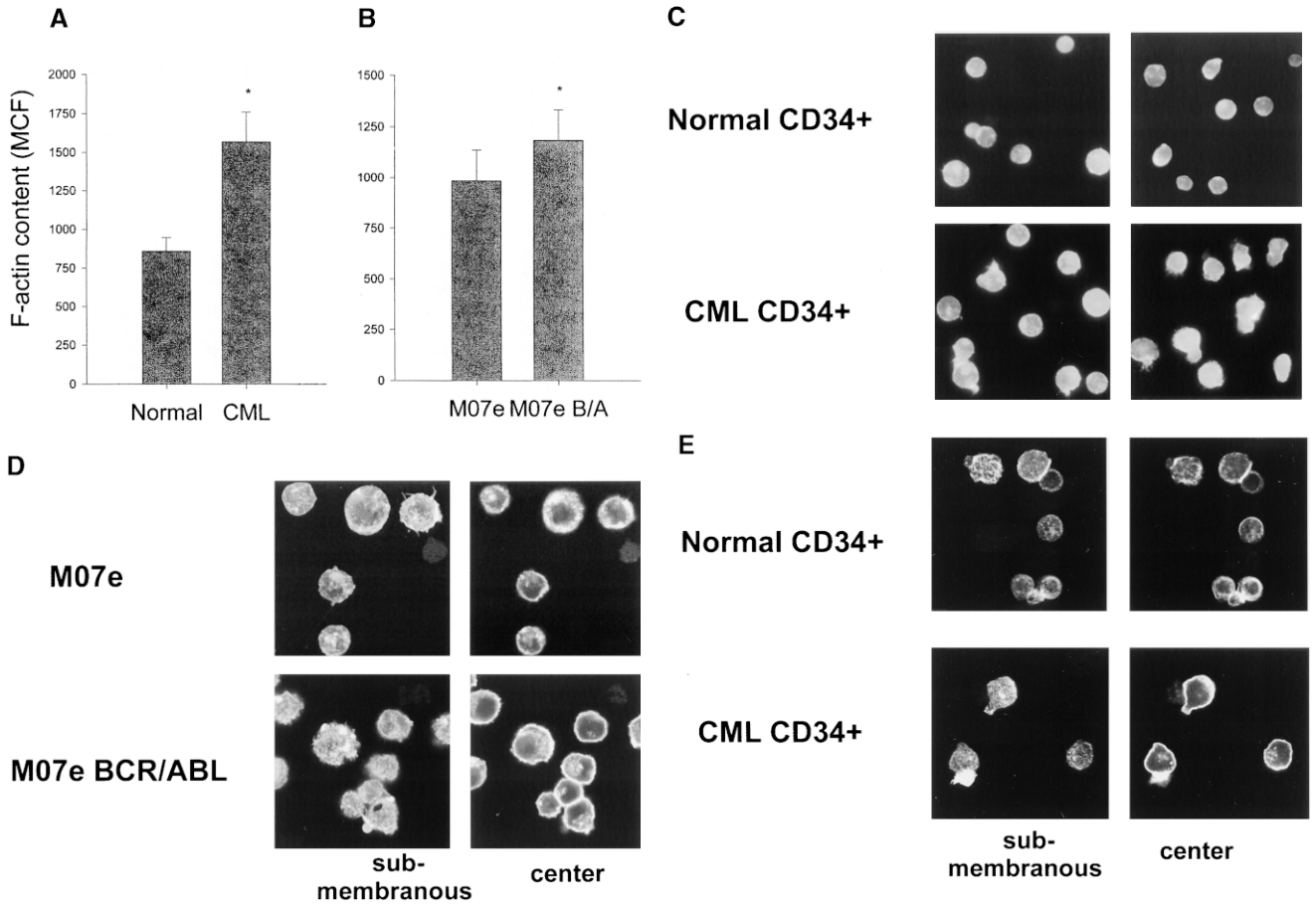

Figure 3. Increased actin polymerization and altered cytoskeletal organization in CML CD34$^+$ cells and BCR/ABL-transfected M07e cells. CML and normal CD34$^+$ cells were labeled with rhodamine phalloidin as described in the Methods. **(A)** F-actin content of cells labeled with rhodamine phalloidin analyzed by FACS as described in the Methods. Results are given as mean ± SEM of results from 7 CML and 6 normal samples. Significance levels: $*p < 0.01$ compared with normal CD34$^+$ cells. **(B)** BCR/ABL-transfected M07e cells (M07e$^{BCR/ABL}$) and mock-transfected M07e cells (M07e) were labeled with rhodamine-conjugated phalloidin and F-actin content analyzed by FACS. Results are given as mean ± SEM of 11 experiments. Significance levels: $*p < 0.01$ compared with mock-transduced M07e cells. **(C)** Intensity and distribution of F-actin staining in cytospin preparations of rhodamine phalloidin labeled CML (n = 7) and normal (n = 5) CD34$^+$ cells evaluated with fluorescence microscopy. Results from representative experiments are shown. Confocal microscopic images of F-actin distribution in sections taken at the submembranous level and through the center of the cell in M07e$^{BCR/ABL}$ and mock-transfected M07e cells **(D)** and CML and normal CD34$^+$ cells **(E)**.

tical F-actin in cross-sectional views (Fig. 3D). Abnormal F-actin distribution and increased cortical F-actin were similarly observed in CML CD34$^+$ cells compared with normal CD34$^+$ cells (Fig. 3E).

Impaired β1 integrin capping as well as integrin-mediated adhesion and proliferation inhibition in CML cells are related to enhanced integrin-cytoskeletal association. We next evaluated whether decreased capping and deficient integrin signaling in CML progenitors were related to increased basal cytoskeletal activation and increased receptor-cytoskeletal binding. As cytochalasin D at low concentrations partially disrupts F-actin fibers and releases integrin receptors from cytoskeletal constraints [36], we evaluated the effects of low dose (0.2–0.5 μM) cytochalasin D on integrin capping and integrin-mediated adhesion and proliferation inhibition. Pretreatment with 0.2–0.5 μM cytochalasin D partially restored β1 integrin capping in CML CD34$^+$ cells (Fig. 4), but did not significantly increase integrin capping in normal CD34$^+$ cells (Fig. 5). Higher concentrations of cytochalasin D inhibited capping in normal CD34$^+$ cells but did not affect capping in CML CD34$^+$ cells. Likewise, partial restoration of integrin capping was seen with 0.2–0.5 μM cytochalasin D in BCR/ABL-transfected M07e cells but not mock-transfected M07e cells [in M07e$^{BCR/ABL}$ cells, 7.2% ± 1.6% capping was seen without cytochalasin D and 19.2% ± 3.6% capping was seen with 0.2 μM cytochalasin D ($p < 0.03$, n = 5)]; in mock-transfected M07e cells, 42.6% ± 2.3% capping was seen without cytochalasin D and 46.4% ± 4.6% capping was seen with 0.2 μM cytochalasin D (p = NS, n = 5). Pretreatment with 0.2–0.5 μM cytochalasin D also partially restored CML CFC adhesion to FN (Fig. 5) but did not significantly affect adhesion of nor-

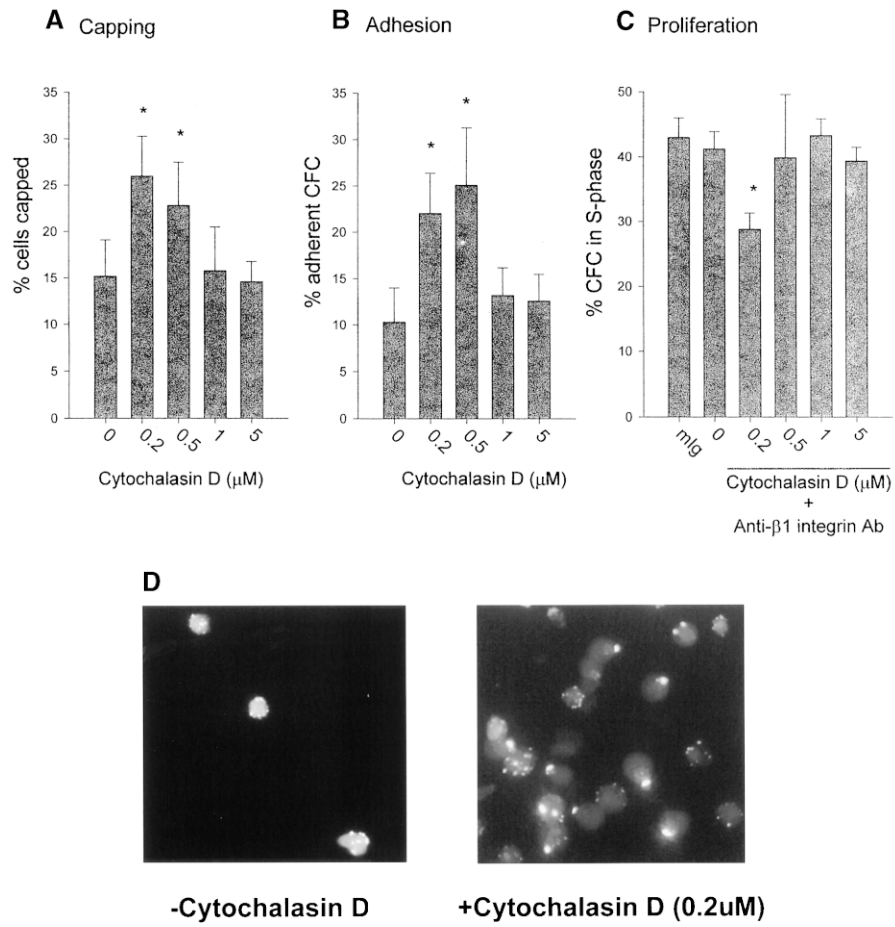

Figure 4. Partial cytoskeletal inhibition with low-dose cytochalasin D is associated with partial restoration of integrin capping as well as integrin-mediated adhesion and proliferation inhibition in CML CD34$^+$ cells. CML CD34$^+$ cells were preincubated with or without 0.2–5.0 μM cytochalasin D at 37°C for 30 minutes prior to evaluating β1 integrin capping **(A)**, adhesion to FN 120-kD **(B)**, and proliferation following antibody-mediated β1 integrin receptor cross-linking **(C)** as described in the Methods. For proliferation studies, cells were incubated in SCM for 72–96 hours at 37°C in a humidified atmosphere with 5% CO_2 prior to cytochalasin D preincubation. Results are given as mean ± SEM of five separate experiments. Significance levels: *$p < 0.05$ compared with cells incubated without cytochalasin D. **(D)** Results for capping with and without cytochalasin D from a representative experiment.

mal progenitors to FN. Higher concentrations of cytochalasin D inhibited normal CFC adhesion but did not alter CML CFC adhesion. Pretreatment with 0.2 μM cytochalasin D also partially restored inhibition of CML CFC proliferation following β1 integrin cross-linking (Fig. 4) but did not alter integrin-mediated inhibition of normal CFC proliferation (Fig. 5). However, 0.5 μM and higher concentrations of cytochalasin D inhibited integrin-mediated inhibitory signaling in normal CFC and did not alter integrin-mediated proliferation inhibition in CML CFC. These studies strongly suggest that abnormal cytoskeletal activation and integrin-cytoskeletal association may contribute to impaired integrin capping as well as deficient integrin-mediated adhesion and proliferation inhibition in CML progenitors.

To assess further whether decreased receptor redistribution was related to increased integrin-cytoskeletal association, we examined β1 integrin receptor distribution at the cell surface in BCR/ABL-transformed and control M07e cells labeled sequentially for 30 minutes each at 4°C with anti-integrin antibodies and FITC-conjugated secondary anti-mouse antibodies using laser scanning confocal microscopy. In control M07e cells, punctate, evenly distributed clusters of integrin receptors were seen, whereas in BCR/ABL-transformed cells, larger, nonuniformly distributed clumps were observed (Fig. 6A). Similar abnormalities in β1 integrin distribution were observed in primary CML CD34$^+$ cells compared with normal CD34$^+$ cells (Fig. 6B). When cells were subsequently incubated at 37°C, β1 integrin receptor clusters in control cells progressively polarized and aggregated into cell surface caps over time. However, β1 integrins in BCR/ABL containing cells maintained their abnormally clumped organization at 30, 90, and 210 minutes, although they became more discrete. Distinct clumps remained visible even in cells in which some polarization of receptor distribution was seen (Fig. 7). These studies indicate that integrin receptors are abnormally ag-

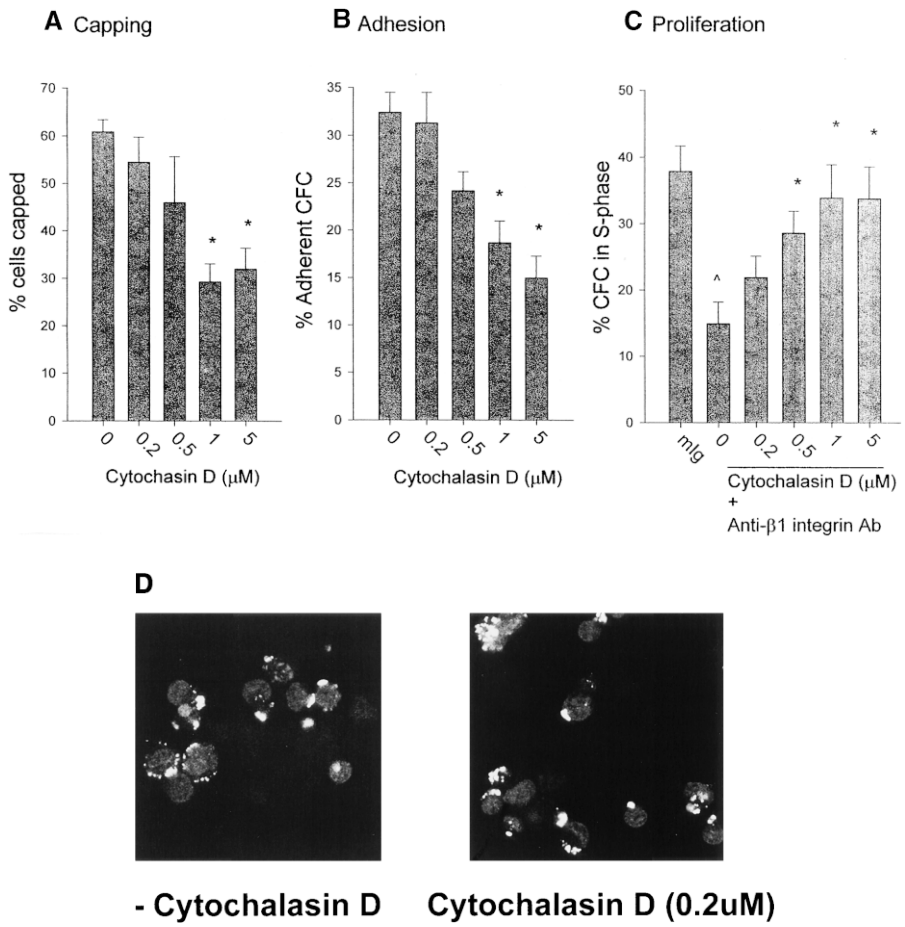

Figure 5. Low-dose cytochalasin D does not affect integrin capping or integrin-mediated adhesion and proliferation inhibition in normal $CD34^+$ cells. Normal $CD34^+$ cells were preincubated with or without 0.2–5.0 μM cytochalasin D at 37°C for 30 minutes prior to evaluating β1 integrin capping **(A)**, adhesion to FN 120-kDa **(B)**, and proliferation following antibody-mediated β1 integrin receptor cross-linking **(C)** as described in the Methods. For proliferation studies, cells were incubated in SCM for 72–96 hours at 37°C in a humidified atmosphere with 5% CO_2 prior to cytochalasin D preincubation. Results are given as mean ± SEM of five separate experiments. Significance levels: $*p < 0.05$ compared with cells incubated without cytochalasin D. **(D)** Results for capping with and without cytochalasin D from a representative experiment.

gregated in the basal state (at 4°C) in BCR/ABL positive cells and further suggest the presence of abnormal integrin-cytoskeletal association.

Increased β1 integrin capping in CML $CD34^+$ cells following IFN treatment

We previously showed that treatment of CML $CD34^+$ cells with IFN enhances CML CFC adhesion to FN and restores integrin-mediated inhibition of CFC proliferation following FN or anti-β1 integrin antibody-mediated receptor ligation [9,34]. We hypothesized that restored integrin function following IFN treatment is associated with restoration of integrin-cytoskeletal interactions. We therefore studied the effect of IFN pretreatment on β1 integrin capping in CML $CD34^+$ cells. Incubation of CML $CD34^+$ cells for 48 hours with 10,000 U/mL IFN significantly enhanced β1 integrin capping compared with untreated cells (Fig. 8). IFN also significantly enhanced capping of the α4 and α5 integrin but did not affect capping of CD11b or CD9 (Table 2). Of the nonintegrin receptors studied, CD38 capping increased following IFN treatment, whereas HLA-DR and CD44 capping was not affected. Therefore, IFN selectively alters receptor-cytoskeletal interactions for some but not all cytoskeletally associated receptors, including β1 integrins receptors. IFN had no effect on β1 integrin receptor capping in normal $CD34^+$ cells (48.3% ± 1.9% capping without IFN and 46.8% ± 3.1% capping with IFN pretreatment; n = 4). Increased β1 integrin receptor capping on CML $CD34^+$ cells correlated with enhanced anti-β1 integrin antibody-mediated proliferation inhibition of CML CFC following IFN treatment ($r^2 = 0.6$). We studied the effect of cytochalasin D on β1 integrin capping in IFN-treated CML $CD34^+$ cells. As was seen with normal $CD34^+$ cells but in contrast to untreated CML $CD34^+$ cells, β1 integrin capping, as well as integrin-mediated adhesion and proliferation-inhibitory signaling, in IFN-treated CML $CD34^+$ cells

was significantly inhibited by high concentrations (1–5 μM) of cytochalasin D (Fig. 9). Finally, low concentrations of cytochalasin D (0.2 μM), which partially restore β1 integrin capping and integrin-mediated adhesion and signaling in CML $CD34^+$ cells, did not alter capping, adhesion, or signaling in IFN-treated CML $CD34^+$ cells (Fig. 9). These studies suggest that IFN-induced enhancement of integrin-mediated adhesion and proliferation inhibition is associated with partial restoration of normal integrin-cytoskeletal interactions in CML $CD34^+$ cells.

Discussion

Deficient β1 integrin-mediated adhesion to the stromal microenvironment and lack of responsiveness to normal β1 integrin-mediated microenvironmental inhibition of proliferation may contribute to the growth advantage of malignant CML progenitors [8,9]. Integrin-dependent adhesion and signaling can be regulated independently of changes in integrin expression levels by alteration in receptor conformation and affinity for ligand as well as by changes in interactions between integrin cytoplasmic domains and the cytoskeleton [10,37,38]. Integrin-cytoskeletal interactions can modulate integrin function both by affecting receptor conformation and ligand binding affinity as well as by modulating receptor mobility and clustering [14,38]. In the present study we evaluated whether alterations in integrin-receptor interactions with the cytoskeleton contributed to abnormal β1 integrin function in CML.

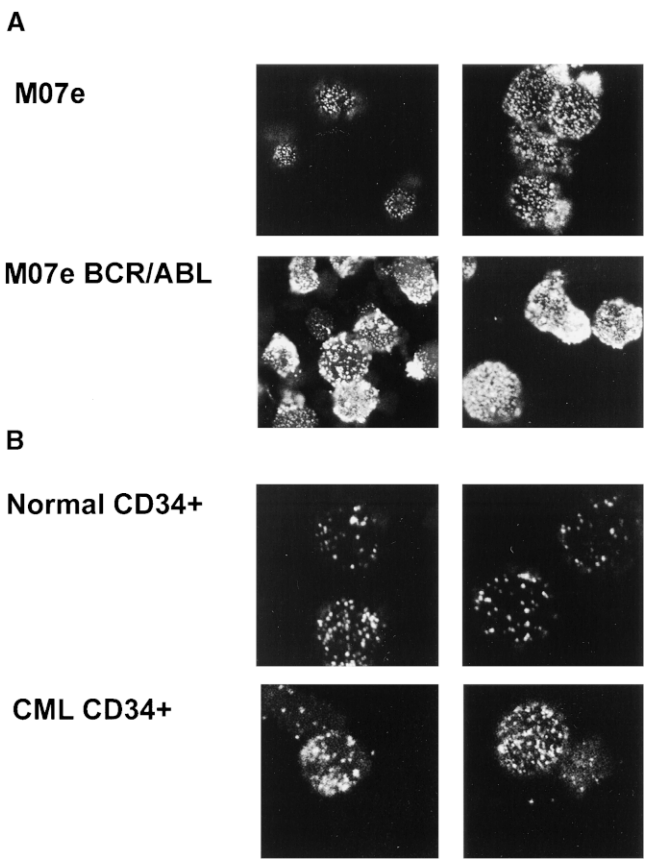

Figure 6. Altered β1 integrin distribution at the cell surface in BCR/ABL-transformed cells as evaluated by confocal microscopy. $M07e^{BCR/ABL}$ and control M07e cells (n = 5) **(A)** and CML and normal $CD34^+$ cells (n = 3) **(B)** were labeled with anti-β1 integrin antibodies for 30 minutes at 4°C followed by FITC-conjugated secondary anti-mouse antibodies for 30 minutes at 4°C and fixed with 4% paraformaldehyde. β1 Integrin receptor distribution was evaluated using laser scanning confocal microscopy. Representative images of surface distribution of integrin receptors are shown.

Engagement of integrin receptors with adhesion-blocking antibodies and cross-linking with secondary antibodies, which mimics binding and clustering of integrin receptor by multivalent ligands [12], resulted in redistribution of β1 integrins into cell surface caps in normal hematopoietic progenitors [30]. The ability of receptors to cap is an indicator of receptor-cytoskeleton interactions and receptor mobility. We confirmed that integrin redistribution into caps in normal $CD34^+$ cells does not simply reflect passive diffusion of integrin receptors but rather is an active process requiring receptor-cytoskeletal interaction and associated with F-actin reorganization. We observed that α4β1 and α5β1 integrin capping is significantly impaired in CML progenitors, indicating that integrin-cytoskeletal interactions required for normal receptor mobility are abnormal in CML. Abnormalities in integrin-cytoskeletal interactions in CML $CD34^+$ cells appear to be related to the presence of the BCR/ABL gene. Similar defects in capping were observed in M07e cells following introduction of the BCR/ABL gene as are seen in CML $CD34^+$ cells. These observations suggest that abnormal β1 integrin function in CML progenitors may result from $p210^{BCR/ABL}$-induced abnormalities in integrin-cytoskeleton interactions.

We observed that primary CML $CD34^+$ cells and BCR/ABL-transformed M07e cells had increased total F-actin content, altered actin distribution, and increased F-actin–containing cytoplasmic projections in an unstimulated baseline state compared with normal $CD34^+$ cells and mock-transfected M07e cells. These results extend the observations made by Salgia et al. [39] and others [21,40] that cytoskeletal abnormalities are present in BCR/ABL-transformed cell lines, to primary CML progenitor cells. However, impaired integrin capping and adhesion in CML appears to be related to alteration in the interactions of integrin receptor with the actin cytoskeleton and impaired integrin receptor mobility rather than to increased actin polymerization alone. It is known that cytoskeletal proteins can limit lateral movement of integral membrane glycoproteins [41]. Cytoskeletal tethering can restrict integrin mobility and prevent receptor clustering at adhesion sites and prevent strengthening of adhesion. Release of integrin receptor from cytoskeletal constraint by low doses of cytochalasin D or by agonists such as Phorbol Myristate Acetate (PMA) can enhance integrin-mediated adhesion [36,32,42]. In the present study, cyto-

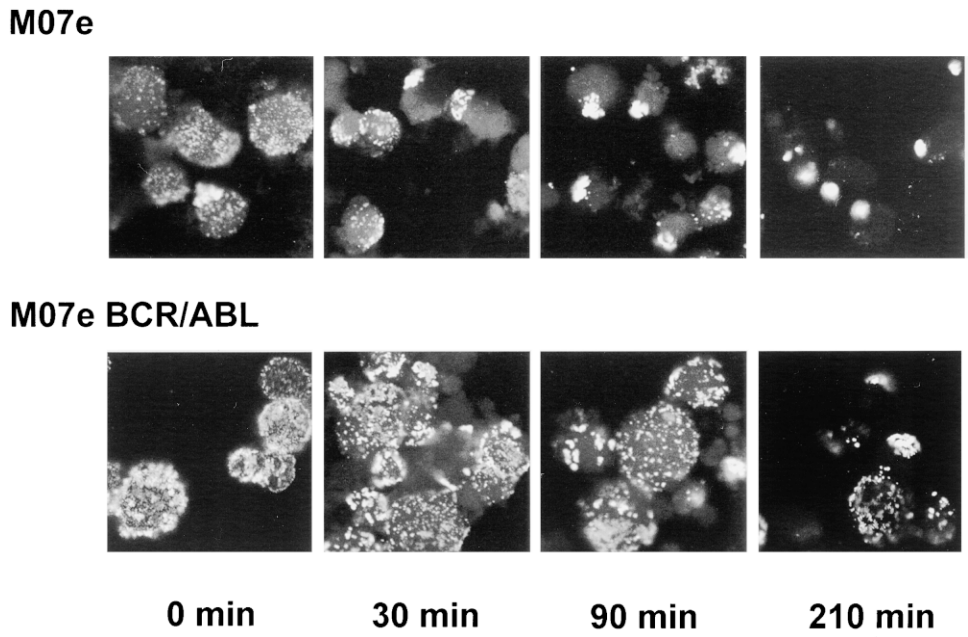

Figure 7. Impaired redistribution of β1 integrin clusters on the cell surface of BCR/ABL-transformed cells. M07e$^{BCR/ABL}$ and control M07e cells were labeled with anti-integrin antibodies and FITC-conjugated secondary anti-mouse antibodies for 30 minutes each at 4°C and then further incubated for 30–210 minutes at 37°C and β1 integrin receptor distribution evaluated using laser scanning confocal microscopy. Representative images of surface distribution of integrin receptors at various time points are shown. Data are representative of four individual experiments.

chalasin D, at low concentrations that partially disrupt the actin cytoskeleton, enhanced integrin capping in CML CD34$^+$ cells and BCR/ABL positive cell lines and partially restored integrin-mediated adhesion and signaling in CML progenitors. This observation suggests that BCR/ABL-dependent cytoskeletal alterations with increased cytoskeletal restraint of integrin mobility may underlie abnormal integrin-mediated capping, adhesion, and signaling in CML progenitors. Abnormal integrin-cytoskeletal association is suggested further by the altered β1 integrin receptor distribution at the cell surface in BCR/ABL-containing cells and increased aggregation of receptors into abnormal clumps. In normal cells, β1 integrin receptor clusters progressively aggregate into caps over time, whereas in CML cells β1 integrins demonstrate restricted mobility and maintain an abnormally clumped organization over time. These observations are consistent with the hypothesis that integrin receptors have abnormal association with cytoskeletal elements and restricted mobility in CML cells, which contributes to their abnormal adhesion and signaling function. However, be-

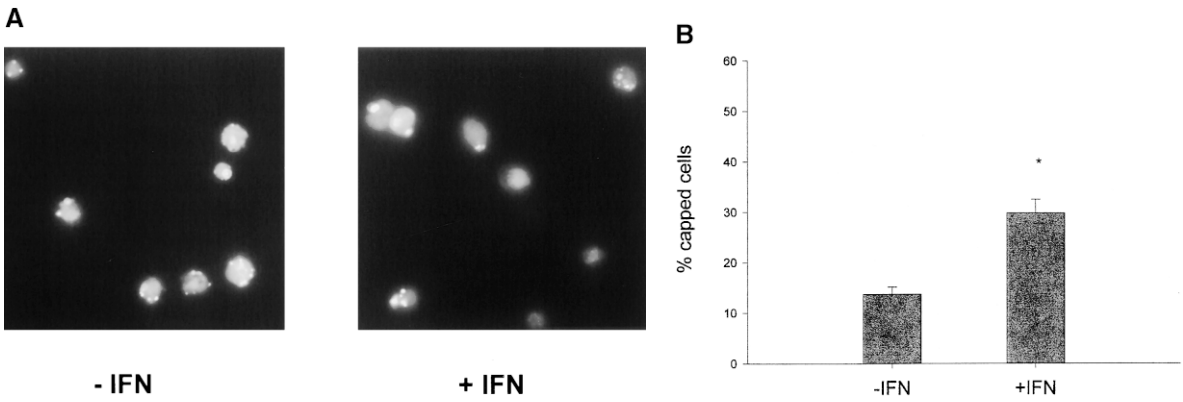

Figure 8. Increased β1 integrin capping in CML CD34$^+$ cells following IFN treatment. CD34$^+$ cells were incubated with or without IFN (10,000 U/mL) in SCM at 37°C in a humidified atmosphere with 5% CO_2 for 48 hours, after which β1 integrin capping was evaluated as described in the Methods. **(A)** Results from a representative experiment. (B) Mean ± SEM of 15 separate experiments. Significance levels: *$p < 0.0001$ compared with cells incubated without IFN.

Table 2. Effect of IFN on capping of integrin and nonintegrin receptors in CML $CD34^+$ cells

Receptor	CML	CML + IFN	*p*
β1 integrin	16.9 ± 4.9	28.3 ± 4.5	0.003
α4 integrin	15.5 ± 3.9	27.5 ± 3.9	0.003
α5 integrin	15.0 ± 2.6	26.5 ± 0.6	0.01
β2 integrin (CD11b)	17.3 ± 3.2	18.3 ± 2.8	NS
CD9	22.5 ± 3.6	24.8 ± 3.0	NS
CD44	11.0 ± 4.08	0.3 ± 2.8	NS
HLA-DR	37.3 ± 2.7	37.0 ± 3.0	NS
CD38	44.5 ± 4.4	54.3 ± 3.1	0.05

CML $CD34^+$ cells were preincubated with and without IFN (10,000 U/mL) for 48 hours. Cells were then incubated with antibodies to the various receptors and FITC- or PE-conjugated secondary antibodies. Capping was evaluated by immunofluorescence microscopy as described in the Methods. Results are given as mean ± SEM of four separate experiments.

cause low-dose cytochalasin D only partially restored integrin capping, adhesion, and signaling, it is possible that additional defects beyond enhanced cytoskeletal binding may contribute to abnormal integrin function in CML cells.

Our studies suggest that abnormalities in integrin-cytoskeletal interactions in CML $CD34^+$ cells are induced by the BCR/ABL gene, because similar defects in integrin-cytoskeletal interactions seen in CML $CD34^+$ cells, such as impaired capping and abnormal clustering and redistribution, improved capping with low-dose cytochalasin D, as well as increased F-actin, also were seen in M07e cells following introduction of the BCR/ABL gene. There are several potential mechanisms by which BCR/ABL could induce cytoskeletal abnormalities and altered integrin-cytoskeletal interactions in CML cells. These include direct association of BCR/ABL with the actin cytoskeleton [21,22,23], activation of Rho [40,43] and/or PI-3 kinase [44–46] leading to increased actin polymerization and altered integrin-cytoskeletal interactions [47], and association with and phosphorylation of focal adhesion proteins such as paxillin and tensin [27,48] and docking proteins such as p110HEF-1/CAS-L and p130CAS [49–51], which are important for normal integrin signaling. Future studies need to assess the role of these different mechanisms, which are active in BCR/ABL-transduced cells lines, in abnormal integrin-cytoskeletal interactions and abnormal integrin-mediated adhesion and growth regulation in CML cells.

In addition to abnormal capping of β1 integrins, capping of the β2 integrin receptor, CD11b, and the integrin-associated membrane-tetraspan protein, CD9, also was deficient on CML progenitors. However, capping of other receptors known to associate with the cytoskeleton, such as the MHC class II [52] and CD44 adhesion receptors [53], as well as the type II transmembrane glycoprotein, CD38, was not impaired in CML progenitors. Therefore, cytoskeletal alterations in CML progenitors do not affect all cytoskeletally associated receptors. This suggests that abnormal integrin function in CML reflects a selective defect in integrin-cytoskeletal interactions and/or the mechanisms that normally regulate these interactions rather than just increased F-actin content and a global cytoskeletal defect. These may include alterations in focal adhesion proteins and the PI-3 kinase, which are important for normal integrin function but may not be important for interactions of other receptors with the cytoskeleton. For example, CD44-cytoskeletal in-

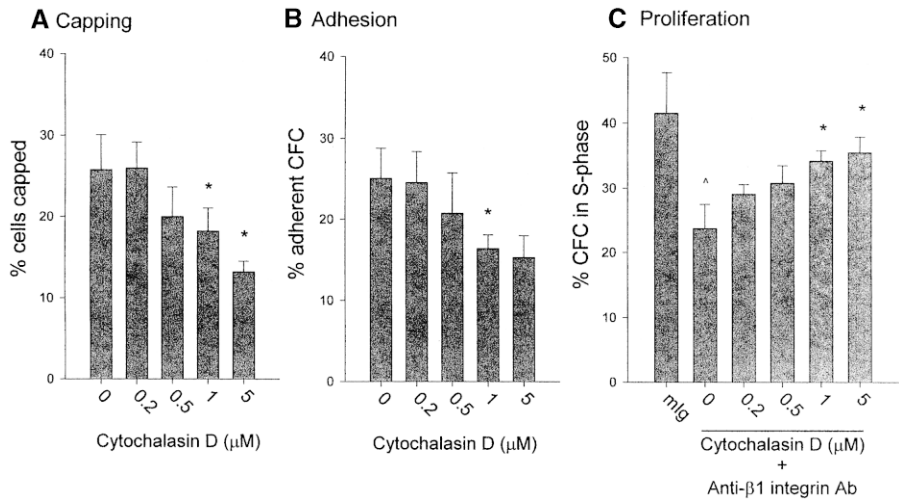

Figure 9. Low concentrations of cytochalasin D do not alter integrin capping or integrin-mediated adhesion and signaling in IFN-treated CML $CD34^+$ cells. CML $CD34^+$ cells, following incubation with IFN (10,000 U/mL) in SCM at 37°C in a humidified atmosphere with 5% CO_2 for 48 hours, were incubated with or without 0.2–5.0 μM cytochalasin D at 37°C for 30 minutes. Subsequently, β1 integrin capping **(A)**, adhesion to FN 120-kDa **(B)**, and proliferation following antibody-mediated β1 integrin receptor cross-linking **(C)** were evaluated as described in the Methods. For proliferation studies, cells were incubated in SCM for 72–96 hours, and IFN was added for the last 48 hours prior to cytochalasin D preincubation. Results are given as mean ± SEM of four separate experiments. Significance levels: $*p < 0.05$ compared with cells incubated without cytochalasin D.

teractions involve linker proteins such as ankyrin that may not be affected by BCR/ABL [53].

IFN, which has considerable efficacy in the treatment of CML, can restore β1 integrin-mediated adhesion of CML progenitors to FN as well as integrin-mediated microenvironmental inhibition of proliferation in CML [9,34]. We now show that IFN also significantly enhances β1 integrin receptor capping in CML progenitors. Induction of enhanced capping by IFN correlated with restoration of integrin-mediated proliferation inhibition following antibody-mediated receptor ligation. This suggests that IFN may enhance integrin function in CML progenitors, at least in part, by restoring normal cytoskeletal interactions with β1 integrin receptors. Consistent with this, unlike untreated CML $CD34^+$ cells but like normal $CD34^+$ cells, low concentrations of cytochalasin D did not further enhance capping, adhesion, and proliferation in IFN-treated CML progenitors, whereas high concentrations of cytochalasin D prevented capping and integrin-mediated adhesion and signaling. The mechanisms by which IFN restores integrin-cytoskeletal interactions is not clear, but may be related to its ability to suppress BCR/ABL expression in CML hematopoietic progenitors, therefore reversing $p210^{BCR/ABL}$ effects on integrin-cytoskeletal interactions, or through direct effects on receptor-cytoskeletal interactions [54]. The ability of IFN to restore β1 but not β2 integrin capping suggests that IFN may selectively modulate receptor-cytoskeletal interactions specifically involving the β1 integrin receptor.

The results of the present study indicate that impaired integrin-mediated adhesion and growth regulation in CML primary progenitors is related to BCR/ABL-induced abnormalities in integrin-cytoskeletal interactions, which include abnormal receptor-cytoskeletal association and impaired receptor mobility. These studies suggest that abnormal endogenous activation of formation of integrin-cytoskeletal complexes by BCR/ABL may interfere with propagation of normal adhesion and signal transduction following ligand binding. Future studies will be directed toward understanding the specific pathways and molecules involved in BCR/ABL-induced alteration of integrin-cytoskeletal interaction in CML progenitors, as well as the mechanism by which these may be partially reversed by IFN.

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