Expression and Activation of $\alpha_v\beta_3$ Integrins by SDF-1/CXCL12 Increases the Aggressiveness of Prostate Cancer Cells

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BACKGROUND. Stromal cell-derived factor-1 (SDF-1 or CXCL12) and CXCR4 are key elements in the metastasis of prostate cancer cells to bone—but the mechanisms as to how it localizes to the marrow remains unclear.

METHODS. Prostate cancer cell lines were stimulated with SDF-1 and evaluated for alterations in the expression of adhesion molecules using microarrays, FACs, and Western blotting to identify $\alpha_v\beta_3$ receptors. Cell–cell adhesion and invasion assays were used to verify that activation of the receptor is responsive to SDF-1.

RESULTS. We demonstrate that SDF-1 transiently regulates the number and affinity of $\alpha_v\beta_3$ receptors by prostate cancer cells to enhance their metastatic behavior by increasing adhesiveness and invasiveness. SDF-1 transiently increased the expression of $\beta_3$ receptor subunit and increased its phosphorylation in metastatic but not nonmetastatic cells.

CONCLUSIONS. The transition from a locally invasive phenotype to a metastatic phenotype may be primed by the elevated expression of $\alpha_v\beta_3$ receptors. Activation and increased expression of $\alpha_v\beta_3$ within SDF-1-rich organs may participate in metastatic localization. Prostate 67: 61–73, 2007. © 2006 Wiley-Liss, Inc.

KEY WORDS: SDF-1; prostate cancer; integrin $\alpha_v\beta_3$; $\beta_3$ phosphorylation; metastasis; bone

INTRODUCTION

Bone metastasis is one of the most severe complications of prostate cancer and is a leading cause of death. Understanding the molecular mechanisms that regulate metastasis will facilitate the identification of novel therapeutic strategies and targets to reduce metastatic disease. Recent studies indicated that tumor cell migration and metastasis are not random processes; rather, chemokines and chemokine receptors play a central role in determining the metastatic destination of tumor cells [1–12]. Malignant cells from different types of tumors express a variety of chemokine and chemokine receptor expression profiles, but CXCR4 is expressed by all tumors that metastasize to bone [1]. CXCR4 belongs to a unique class of chemokine receptors...
that appears to have only one ligand, namely stromal cell-derived factor-1 (SDF-1 or CXCL12). SDF-1 is produced by the bone marrow stroma and by stromal cells of mesenchymal origin including osteoblasts and endothelial cells [13–15]. SDF-1 and CXCR4 play key roles in the metastasis of hematopoietic cells to bone marrow [16–18]. Several groups (including ours) have demonstrated that the SDF-1/CXCR4 axis plays a crucial role in the metastasis of malignant hematopoietic and nonhematopoietic cells to marrow [12,13,19,20]. Evidence that supports the aforementioned conclusion includes the fact that native prostate cancer cells and prostate cancer cell lines express CXCR4, adhere to endothelium, and migrate in response to SDF-1 [13,21]. In animal models, blockade of SDF-1 and CXCR4 in prostate cancer cells prevents both metastasis and tumor growth [13], but how the SDF-1/CXCR4 axis determines the invasion patterns of prostate cancer cells is unclear.

Cancer cell adhesion to the vascular endothelium is a crucial component of the metastatic cascade; the adhesion process is regulated largely by selectins and integrins [22,23]. Tissue-selective trafficking of tumor cells to different organs is thought to be mediated by adherence of metastatic cells to particular types of endothelial cells. For example, prostate cancer cells adhere preferentially to human bone marrow endothelial (HBME) cells versus other types of vascular endothelial cells [24]. Recent studies revealed that the heterodimeric transmembrane integrin αvβ3 receptor may be involved in adhesion, migration, invasion, growth, and angiogenesis in many types of tumors [25,26]. Furthermore, fewer αvβ3 receptors are expressed in primary lesions and relatively poorly invasive lines of cancer cells, such as LNCaP cells [27], than in more aggressive cell types [28,29].

In the present study, we demonstrate that SDF-1 stimulates an increase in the expression of activated αvβ3 receptors in two lines of metastatic prostate cancer, namely PC3 and LNCaP C4-2B, but does not affect αvβ3 receptor expression in nonmetastatic LNCaP cells. This in turn enhances adhesion and invasion to extracellular matrix by prostate cancer cells in vitro. We speculate that during the transformation of a locally invasive phenotype into a metastatic phenotype, an increase in the expression of αvβ3 receptors may be central to the metastatic cascade; specifically, we hypothesize that once cells enter the vasculature and reach tissue within which SDF-1 is expressed abundantly, expression and activation of αvβ3 receptors by SDF-1 promotes metastasis.

**MATERIALS AND METHODS**

**Cell Lines**

Prostate cancer cell lines (CaP) (PC3, LNCaP C4-2B, and LNCaP), and the bone marrow endothelial cells (HBME) were cultured in RPMI medium 1640, supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% l-glutamine (Invitrogen Corp., Carlsbad, CA). The human PC3 cells were originally isolated from a vertebral metastasis and were obtained from American Type Culture Collection (Rockville, MD). LNCaP cells were isolated from a lymph node of a patient with disseminated bony and lymph node involvement. The LNCaP sub lines (C4-2B cells) were derived from the parental LNCaP cell lines that were serially passaged in mice to obtain a more metastatic cell line [30]. The HBME cells were isolated from a normal Caucasian male and immortalized with SV40 large T-antigen [24].

**Reagents and Antibodies**

Recombinant human SDF-1 and the isotype IgG1 were purchased from R&D Systems (Minneapolis, MN); the anti-αvβ3 (LM609), anti-β3 (MAB2008), and anti-αv antibodies were obtained from Chemicon Corp. (Temecula, CA); the monovalent ligand-mimetic antibody WOW-1 Fab was the kind gift of Dr. Sanford Shattil (Scripps Research Institute, La Jolla, CA); the anti-β3(1A2) antibody was the gift of Dr. Scott D. Blystone (SUNY Upstate Medical University, Syracuse, NY), the PE-conjugated goat anti-mouse IgG1 was purchased from BD Biosciences (Franklin Lakes, NJ), the Alexa Fluor® 488F(ab’)2 fragment of goat anti-mouse IgG (H + L) and Vybrant™ CFDA-SE cell tracer kits (V-12883) were obtained from Molecular Probes (Eugene, OR). The C3 exoenzyme was purchased from Calbiochem (San Diego, CA). The ProtOnTM Fluorescein labeling kit (PLK-1201) was obtained from by Vector Laboratories (Burlingame, CA). Cytochalasin D, cyclohexamide, brefeldin A, human thrombin, purified human vitronectin, pertussis toxin (PTX), anti-phospho-β3 integrin [pTyr773], anti-α-v-tubulin antibody, and the in vitro toxicology assay kits using 23-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from Sigma Chemical Corp. (St. Louis, MO).

**Adhesion to HBME Cells**

CaP cell lines were labeled for 30 min with 10 μM carboxy-fluorescein diacetate, succinimidyl ester (CFD-SE, Molecular Probes), washed and rested for an additional 30 min. Labeled cells were stimulated with 0–200 ng/ml SDF-1 at 37°C for 45 min and 1 × 10^5 cells were deposited directly onto HBME monolayers, spun at 500 rpm for 5 min, and binding performed for 15 min at 4°C. Total fluorescent counts and specific binding were quantified on a 96-well fluorescent plate reader (IDEXX Research Products, Westbrook, ME) after extensive washing with Ca^{2+}/Mg^{2+} PBS.
Integrin Adhesion Assay

Cell adhesion kits for αβ3 and β1 (Chemicon) were used to assess the affinity of αβ3 and β1 integrins on CaP cells. For these studies, the cells were detached from culture with PBS/EDTA, rested for 30 min, and seeded on to the anti-integrin antibody-coated plates according to the manufacturer’s instructions. SDF-1 (0–200 ng/ml) or thrombin (1 IU/ml) as a positive control [31] were used to stimulate the cells for 2 hr at 37°C. Adhesion was quantified using a multi-well scanning spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) at 540 nm.

Cell Adhesion to Matrix Proteins

Microtiter plates were coated overnight with vitronectin or osteopontin (1 μg/ml, R&D Systems), washed twice with PBS, and nonspecific binding blocked with 1% BSA in PBS for 1 hr at 37°C. CaP cell lines were detached using a cell dissociation solution (Sigma), and washed twice with PBS. The cells were suspended in RPMI 1640 medium and incubated in the presence or absence of anti-αβ3 (LM609) antibody or an isotype-matched control (R&D systems) for 15 min on ice to a final dose of 10 μg/ml. Thereafter the cells were seeded at 1 x 10⁵ cells/well in the presence or absence of SDF-1 at 37°C for 1–2 hr. After the removal of the nonadherent cells, the adherent cells were quantified using XTT.

Invasion Assays

BD BioCoatTM matrigel™ invasion chambers (Chemicon) were used to assess the invasive activities of CaP cells. After pretreatment of the cells with 15 μg/ml anti-αβ3 antibody or an IgG1 isotype-matched control for 15 min, the cells were placed in the upper well (3 x 10⁵ cells/well) of the invasion chamber. SDF-1 (200 ng/ml) or PBS vehicle were added to the lower chambers also in RPMI containing 1% FBS. After 24 hr at 37°C, 40 or 80 μl of 5 mg/ml MTT (Sigma) were added to the upper/lower chambers, respectively, for an additional 4 hr. The nonmigrating cells were removed with cotton swabs and the purple residues representing the migrated cells were solubilized in 0.5 ml isopropanol. The plate was rocked for 30 min at a medium speed, and 100 μl was transferred from each well into a 96-well plate and read at 595 nm.

Real Time RT-PCR

Confluent CaP cell lines (LNCaP, LNCaP C4-2B, and PC3) were treated with 0–200 ng/ml SDF-1 for 2 hr at 37°C. The cells were lysed using an RNaseasy Mini Kit (Qiagen, Valencia, CA). RNA integrity and purity was checked by electrophoresis with ethidium bromide and absorbance at A₂₆₀/A₂₈₀. First strand cDNA synthesis was generated with 1 μg of total RNA using random hexamers and SuperScript™ II RT Kit (Invitrogen). Amplification primers were designed using PrimerExpress™ software (Applied Biosystems, Foster City, CA) to cross intron/exon boundaries and were validated by sequencing the resulting product by the University of Michigan DNA Sequencing Core Facilities. The sequences of the forward and reverse primers of αv were 5’-GAAAAGAATGACACGGTTCG and 5’-AGTGATGAGATGGTTCGCC, respectively. The sequences of the forward and reverse primers of β3 were 5’-AAGCCCTGTTGTGACTCCGACT and 5’-CGC GTGGTACAGTTCGATGT, respectively. Quantitative RT-PCR was performed by using an ABI PRISM 7700 instrument (Applied Biosystems). PCR was performed with 12.5 μl of a SYBR® PCR master mixture (Applied Biosystems), each of the primers at a concentration of 80 nM, and 1 μl of the RT product in a total volume of 25 μl. The two-step PCR reaction (95°C for 15 sec, 60°C for 60 sec) was run for 40 cycles after an initial single cycle of 95°C for 10 min to activate the Taq polymerase. The mRNA levels were expressed in relative copy numbers (% control) normalized against GAPDH. The sequences of the forward and reverse primers of GAPDH were 5’-AGCCACATCGCTCAGACACC and 5’-CCAATACGCAAATCCGGTG. Standard curves were constructed from serial dilutions of GAPDH, αv and β3 cdnas generated cloned derived from PCR products.

Flow Cytometry

In some cases CaP cells were pretreated with cytochalasin D (10 μM, 1 hr), cyclohexamide (30 μg/ml, 2 hr), and brefeldin A (10 μg/ml, 4 hr) prior to removal from culture. CaP cells were then incubated with 0–200 ng/ml SDF-1 in Hanks Buffered Saline Solution (HBSS, Life Technologies) at 37°C for 15–45 min. Total αvβ3 was determined by staining the cells with mAb1960H (LM609) PE (1:200) in HBSS for 30 min on ice. PE-conjugated goat anti-mouse IgG1 (clone MOPC21, Sigma) served as negative control. Activated αvβ3 levels were identified with the ligand mimetic antibody Fab WOW-1 (10 μg/ml) [32] in HBSS for 30 min at 22°C. Detection of the WOW-1 reagent was performed with the Alexa Fluor® 488F(ab’)2 fragment of goat anti-mouse IgG (H + L) antibody at a 1:200 dilution on ice for 30 min. Binding of cell matrix components to SDF-1-treated (10 min at 25°C) cells was performed using fluorescein-labeled human vitronectin (ProtOn™ Fluorescein labeling kit (PLK-1201) (Vector Laboratories)) followed by fixation in 1% paraformaldehyde in PBS. Stained cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA).
Western Blot and Immunoprecipitation

CaP cells were pretreated with 500 ng/ml pertussis toxin (PTX, Sigma) for 1.5 hr at 37°C and washed with PBS. After stimulation with SDF-1 (0–200 ng/ml), the cells were lysed (25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM beta-glycerophosphate, 0.1 mM Na₃VO₄, and 2% protease inhibitor cocktail (Sigma)). The lysates were precleared by centrifugation and separated on 8% SDS–PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 0.1% TBST and 5% nonfat dry milk, and incubated with anti-phospho-integrin β₃ [pTyr⁷⁷³] (Sigma). Total α₅ and β₃ integrin subunits were detected with monoclonal antibodies (SAP) (Chemicon). Secondary anti-mouse HRP antibody was used in conjunction with ECL for band detection (Amersham Biosciences, UK). Normalization of the membranes was performed using an anti-α-tubulin antibody.

Cells treated with the exoenzyme C3 (1 × 10⁶ cells/10 µg/ml for 1.5 hr at 37°C) were stimulated with SDF-1 (200 ng/ml or vehicle) and lysed in 100 µl of the buffer described above. The lysates were precleared for 1 hr at 4°C with gelatin-sepharose and immunoprecipitated with goat anti-mouse sepharose beads coated with anti-β₃ monoclonal antibody (1A2) for 2 hr at 4°C. Samples were separated on 8% SDS–PAGE gels, and probed with the anti-phospho-integrin β₃ [pTyr⁷⁷³] (Sigma) antibody. One-tenth of the total lysates were probed for total β₃ using the anti-β₃ monoclonal antibody (SAP) (Chemicon).

Statistical Analysis

Each experiment was repeated a minimum of three times. Numerical data are expressed as mean ± the standard deviation. Statistical analysis of the results was performed with STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK) using ANOVA followed by post hoc Newman–Keuls test for comparison between groups, with the level of significance at P < 0.05.

RESULTS

SDF-1 Enhances Prostate Cancer Cell Adhesion via α₅β₃ Receptors

To determine whether SDF-1 regulates the adhesiveness of prostate cancer cells to HBME cells, we used cell–cell adhesion assays. Treatment of two lines of prostate cancer cell that metastize to bone marrow (PC3 and LNCaP C4-2B cells) with SDF-1 increased the adhesiveness of these cells (Fig. 1A). By contrast, the adhesiveness of nonmetastatic LNCaP cells was not altered even at the highest dose of SDF-1 tested (Fig. 1A).

To explore which adhesion molecules are involved in the SDF-1-induced increase in adhesiveness, DNA microassays were performed to compare LNCaP C4-2B cells stimulated with SDF-1 (200 ng/ml for 2 hr) to unstimulated LNCaP C4-2B cells. The β₃ integrin subunit and CD164 were among the adhesion molecules that were most responsive to SDF-1 (data not presented). Because expression of the β₃ integrin subunit (in affiliation with the α₅ subunit) is elevated in metastatic prostate tumors [26,33,34], we further...
explored the role of $\alpha_{v}\beta_{3}$ receptors in the response to SDF-1. The relationship of CD164 to prostate cancer will be presented in a companion manuscript [52].

Adhesion assays were used to assess the affinity of $\alpha_{v}\beta_{3}$ receptors expressed at the cell surface of prostate cancer cell lines that were exposed to the SDF-1. As a positive control, thrombin was used to activate $\alpha_{v}\beta_{3}$ receptors (Fig. 1B) [31]. SDF-1 significantly increased the binding of PC3 and LNCaP C4-2B cells to anti-$\alpha_{v}\beta_{3}$ receptor-coated plates. The enhanced binding of LNCaP cells decreased after 2 hr (Fig. 1B). As a control for specificity, we also examined the effects of SDF-1 on $\beta_{1}$ integrin. Treatment of prostate cancer cells with SDF-1 had no effect on binding to plates coated with anti-$\beta_{1}$ integrin antibodies (Fig. 1C).

**SDF-1 Enhances $\alpha_{v}\beta_{3}$ Receptor-Mediated Prostate Cancer Cell Adhesion and Invasion**

To further explore how SDF-1 regulates adhesion, the adhesion of SDF-1-stimulated prostate cancer cells to the $\alpha_{v}\beta_{3}$ receptor ligands vitronectin and osteopontin was examined. SDF-1 treatment dramatically increased the adhesion of metastatic PC3 and LNCaP C4-2B cells to vitronectin (Fig. 2A,B) and osteopontin (data not presented). Inclusion of the anti-$\alpha_{v}\beta_{3}$ receptor antibody, LM609, reduced the basal binding of PC3 cells and inhibited the SDF-1-induced enhancement of binding (Fig. 2A). Similar results were obtained for LNCaP C4-2B cells (Fig. 2B), although the anti-$\alpha_{v}\beta_{3}$ receptor antibody did not alter the basal level of binding. The binding of nonmetastatic LNCaP cells to vitronectin was not altered by SDF-1 (Fig. 2C), while inclusion of the anti-$\alpha_{3}\beta_{3}$ receptor antibody reduced binding to below the baseline level in the presence of SDF-1 (Fig. 2C).

To investigate whether $\alpha_{v}\beta_{3}$ receptors regulate SDF-1-induced invasion of extracellular matrix by prostate cancer cells, we used an in vitro assay of invasion. SDF-1 stimulated invasion of an artificial extracellular matrix (Matrigel) by PC3 and LNCaP C4-2B cells but not LNCaP cells (Fig. 3). The addition of the anti-$\alpha_{v}\beta_{3}$ receptor antibody LM609 blocked the SDF-1-induced augmentation of invasion, whereas an isotype-matched control antibody had no such effect (Fig. 3A–C). Collectively, these observations demonstrate that the SDF-1/CXCR4 axis mediates the activation of $\alpha_{v}\beta_{3}$ receptor by SDF-1 to modulate adhesion and invasion of extracellular matrix by prostate cancer cells.

**SDF-1 Transiently Increases $\beta_{3}$ mRNA Expression in Metastatic Prostate Cancer Cell Lines**

We examined the effects of SDF-1 on $\alpha_{v}$ and $\beta_{3}$ mRNA expression in prostate cancer cell lines using real-time reverse transcription-polymerase reaction (RT-PCR). The relative level of expression of $\alpha_{v}$ and $\beta_{3}$ mRNA was high in PC3 cells, while LNCaP C4-2B cells expressed high levels of $\alpha_{v}$ mRNA but considerably less $\beta_{3}$ mRNA compared to PC3 cells, and the nonmetastatic LNCaP cells expressed low levels of both transcripts (Fig. 4A,B). SDF-1 stimulation for 2 hr (Fig. 4C) or longer (4, 8, and 24 hr; data not presented) did not alter the expression of $\alpha_{v}$ mRNA in any of the cell lines examined. SDF-1 significantly increased $\beta_{3}$ mRNA expression in PC3 and LNCaP C4-2B cells but had no effect on LNCaP cells (Fig. 4D).

**SDF-1 Enhances the Expression and Activation of $\alpha_{v}\beta_{3}$ Receptors in Cancer Prostate Cells**

Flow cytometry was used to examine the effects of SDF-1 on $\alpha_{v}\beta_{3}$ receptor protein expression in prostate cancer cell lines. In the absence of SDF-1, the highly metastatic PC3 cells expressed the highest amount of $\alpha_{v}\beta_{3}$ receptor protein, LNCaP C4-2B cells expressed...
moderate amounts of αvβ3 receptor protein, and LNCaP cells expressed the lowest levels of αvβ3 receptor protein among the cell lines examined (Fig. 5A). SDF-1 increased the expression of αvβ3 receptor protein in PC3 and LNCaP C4-2B cells, whereas SDF-1 decreased αvβ3 receptor protein expression in LNCaP cells (Fig. 5B).

To determine whether SDF-1 altered the affinity of the αvβ3 receptor for ligand, prostate cancer cells were pretreated with SDF-1 for 15–30 min. The ability of these cells to bind αvβ3 receptor ligands was probed using fluorescein isothiocyanate-labeled vitronectin. SDF-1 significantly increased the binding of vitronectin by PC3 and LNCaP C4-2B cells, but not by LNCaP cells (Fig. 5C). To distinguish whether the aforementioned effect was due to an increase in the number of αvβ3 receptors expressed or change in receptor affinity, the effect of SDF-1 on αvβ3 receptor activation in prostate cancer cells was evaluated using a monovalent ligand-mimetic antibody, WOW-1 Fab, which detects only the activated state of this receptor [32]. SDF-1 treatment for 15 min increased the activation of αvβ3 receptors.
Fig. 5. SDF-1 Regulates $\alpha_v\beta_3$ Integrins Expressed by CaP Cells

A

B

C

D

Fig. 5.
receptors by 30% and 23% in PC3 and LNCaP C4-2B cells, respectively, relative to untreated cells (Fig. 5D). SDF-1 failed to alter the activation state of αvβ3 receptors in LNCaP cells (Fig. 5D).

To determine whether SDF-1 enhanced binding of vitronectin and the WOW-1 antibody by increasing the number of αvβ3 receptors expressed or by enhancing the activation of pre-existing receptors, we examined the effects of SDF-1 on PC3 cells that were pretreated with the protein synthesis inhibitor, cyclohexamide (30 μg/ml, 2 hr). Cyclohexamide decreased the basal level of vitronectin binding, but SDF-1-stimulated binding was elevated as early as 15 min after the start of the addition of cyclohexamide and returned to the basal level by 30 min (Fig. 6A,D). An increase in the number of activated αvβ3 receptors was detected 15 min after the addition of cyclohexamide, but this increase began to decline towards the baseline level by 30 min (Fig. 6B,E). Under the same conditions, total αvβ3 receptor expression detected using the LM609 antibody was not altered until 30 min after stimulation with SDF-1 (Fig. 6C,F). These results indicate that the number of αvβ3 receptors expressed on the cell surface increases over a 30-min period, but that activation of the receptor in response to SDF-1 is transient and requires the active recruitment of receptors from intracellular stores.

To determine whether protein trafficking is required for the stimulatory effects of SDF-1, we tested the effects of brefeldin A (a metabolite of the fungus Especillium brefeldianum that specifically and reversibly blocks protein transport from the endoplasmic reticulum to the Golgi apparatus [35]) and cytochalasin D (which inhibits actin polymerization and disrupts receptor internalization [36]). The binding of vitronectin and the WOW-1 antibody to αvβ3 receptors activated by SDF-1 was inhibited by brefeldin A (Fig. 6G,H), whereas cytochalasin D had no such effect (Fig. 6I and data not shown). Collectively, these findings suggest that rapid expression and activation of pre-existing αvβ3 receptors likely occurs after SDF-1 binds αvβ3 receptors and that this process is independent of actin polymerization and protein synthesis.

**SDF-1 Increases Phosphorylation of β3**

Because phosphorylation of β3 is required for αvβ3 receptor-mediated adhesion to vitronectin [37], we determined whether SDF-1 altered phosphorylation of the β3 subunit. As shown in Figure 7A, B, SDF-1 rapidly increased the phosphorylation of β3 in PC3 and LNCaP C4-2B cells, but this response was considerably weaker in LNCaP cells (Fig. 7C).

As SDF-1 binding to CXCR4 activates Gz3 proteins, we used a specific inhibitor of Gz3 proteins, namely PTX to determine whether the Gz3 signaling pathway is required for phosphorylation of β3 [37]. As shown in Figure 7A, B, pretreatment of SDF-1-stimulated prostate cancer cells with PTX significantly inhibited phosphorylation of β3. To verify these results, we used C3 transferase, which is an exoenzyme that specifically ADP-ribosylates Rho proteins and inhibits Rho activation [38]. The enhanced phosphorylation of β3 induced by SDF-1 was diminished by pretreatment of cells with C3 transferase (Fig. 7D,E). These data suggest that Gz3 proteins and small GTP-binding proteins (the Rho family) are involved in SDF-1-induced enhancement of β3 phosphorylation.

**DISCUSSION**

We reported previously that the SDF-1/CXCR4 axis plays a crucial role in directing tumor metastasis to bone marrow by modulating metastatic cell adhesion and invasion. However, the molecular mechanisms that underlie this process are unknown. In the present study, we focused on the role of the αvβ3 receptor in the SDF-1α/CXCR4 axis because this molecule is known to play a major role in metastasis [25]. We observed that SDF-1 transiently increased the adhesiveness and invasiveness of the metastatic prostate cancer cell lines, PC3, and LNCaP C4-2B (both of which metastasize to bone), whereas the nonmetastatic prostate cancer cell line, LNCaP-C4-2B, was unaffected. The affinity of αvβ3 receptors in the metastatic cell lines increased rapidly in response to exposure of cells to SDF-1. In addition, we found that SDF-1-stimulated adhesion and invasion were inhibited by an anti-αvβ3 receptor antibody. The basal level of αvβ3 receptor mRNA and protein expression were not markedly different between LNCaP and LNCaP C4-2B cells, even though these cell lines exhibit vastly different responses in in vivo metastatic assays [39]. This prompted us to further explore the mechanism by which SDF-1 might activate αvβ3 receptors. Our results suggest that when cells that are capable of metastasis encounter an environment within which there is abundant expression of SDF-1 (e.g., lymph nodes, liver, or bone marrow), SDF-1 dramatically upregulates β3 mRNA expression and activates the αvβ3 receptor. This differential regulation of the αvβ3 receptor, which depends on the location of receptors as well as other intrinsic factors, may explain in part why PC3 and LNCaP C4-2B cells preferentially metastasize to bone.

It is intuitive that tumors that metastasize to bone likely target components of the bone matrix for adhesion; these targets might include protein sequences that contain the arginine-glycine-aspartate (RGD) motif that are present in numerous bone matrix proteins, many of which bind the αvβ3 receptor (e.g., collagens, fibronectin, vitronectin, and osteopontin).
Fig. 6. Expression and activation of $\alpha_\text{v}\beta_3$ receptors do not require protein synthesis. PC3 cells were pretreated with vehicle (A–C), cycloheximide (D–F), brefeldin A (G, H), or cytochalasin D (I) before being treated SDF-1$\alpha$ (200 ng/ml) for 0, 15, or 30 min. Binding was detected using labeled vitronectin (A, D, G, I), the WOW-1 Fab monovalent ligand-mimetic antibody or a Fab antibody control and an FITC-labeled goat anti-mouse IgG (H + L) secondary antibody (B, E, H), or the anti-$\alpha_\text{v}\beta_3$ receptor antibody LM609 (C, F) followed by FACS analysis.
These observations are based upon reports that (a) invasive prostate cancer cell lines (PC3 and DU145) express a\textsubscript{v}b\textsubscript{3} receptors and (b) antibodies raised against a\textsubscript{v}b\textsubscript{3} receptors or the RGD motif inhibit the binding of extracellular matrix in bone by prostate cancer cells [27]. Not surprisingly, a\textsubscript{v}b\textsubscript{3} receptor expression has been reported to be correlated with metastatic potential [40,41]. In line with this finding, overexpression of the b\textsubscript{3} subunit in LNCaP cells induces a more aggressive metastatic phenotype [27]. Moreover, a\textsubscript{v}b\textsubscript{3} receptors bind metargidin, an adhesion molecule that is also upregulated in metastatic prostate cancer cells [42,43]. Collectively, these findings suggest that a\textsubscript{v}b\textsubscript{3} receptors play a central role in the establishment of metastatic prostate cancer within bone [44].

SDF-1 increases the expression and activation of a\textsubscript{v}b\textsubscript{3} receptors, but the mechanisms that underlie this effect are unknown. Phosphorylation of the b\textsubscript{3} subunit at Y747 is essential for the activation of small GTP-binding proteins (the Rho family) [45], and G\textsubscript{q}i and Rho proteins are involved in CXCR4 signaling pathways [46–48]. Activation of Rho is necessary for invasion and migration in a wide variety of cell types [49]. We found in the present study that specific inhibition of G\textsubscript{q}i proteins with PTX or inhibition of Rho proteins with C3 transferase reduced the SDF-1-induced phosphorylation of b\textsubscript{3}. These findings suggest that CXCR4 activates multiple signaling pathways to alter a\textsubscript{v}b\textsubscript{3} receptor expression and activation.

Enhancement of integrin affinity, avidity, and integrin–cytoskeleton interactions are all necessary to permit firm adhesion [45]. Recent studies suggested that activation of integrin a\textsubscript{v}b\textsubscript{3} receptors is crucial for the arrest and attachment of tumor cells under dynamic flow conditions [53,54]. The fact that phosphorylation of b\textsubscript{3} is required for a\textsubscript{v}b\textsubscript{3} receptor-mediated adhesion to vitronectin suggests that phosphorylation of b\textsubscript{3} permits
the assembly of a signaling complex at the adhesion site that may be independent of actin stress fiber generation [45,50]. Nevertheless, it is unlikely that phosphorylation of β3 alone is sufficient to activate the receptor; activation likely requires that a minimum threshold of receptor turnover and trafficking may also contribute to SDF-1-induced enhancement of binding, because it is likely very important that cells mobilize intracellular stores of preformed αvβ3 receptors and relocate these receptors to the cell surface to establish firm adhesive interactions under conditions of flow. Further studies are required to distinguish between the aforementioned possibilities.

In summary, SDF-1 enhances the expression and activation of αvβ3 receptors in metastatic prostate cancer cell lines in vitro but does not affect nonmetastatic prostate cancer cells. The aforementioned effect of SDF-1 involves an increase in αvβ3 receptor mRNA and protein expression and conformational changes to αvβ3 receptors that enhance receptor affinity. SDF-1 also increases the phosphorylation of β3, which may be a key downstream event that results in the activation of αvβ3 receptors.

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