Transforming Growth Factor β1 Induces αvβ3 Integrin Expression in Human Lung Fibroblasts via a β3 Integrin-, c-Src-, and p38 MAPK-dependent Pathway*§

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In response to transforming growth factor β1 (TGFβ) stimulation, fibroblasts modify their integrin repertoire and adhesive capabilities to certain extracellular matrix proteins. Although TGFβ has been shown to increase the expression of specific αv integrins, the mechanisms underlying this are unknown. In this study we demonstrate that TGFβ1 increased both β3 integrin subunit mRNA and protein levels as well as surface expression of αvβ3 in human lung fibroblasts. TGFβ1-induced αvβ3 expression was strongly adhesion-dependent and associated with increased focal adhesion kinase and c-Src kinase phosphorylation. Inhibition of β3 integrin activation by the Arg-Gly-Asp tripeptide motif-specific disintegrin echistatin or αvβ3 blocking antibody prevented the increase in β3 but not β5 integrin expression. In addition, echistatin inhibited TGFβ1-induced p38 MAPK but not Smad3 activation. Furthermore, inhibition of the Src family kinases, but not focal adhesion kinase, completely abrogated TGFβ1-induced expression of αvβ3 and p38 MAPK phosphorylation but not β5 integrin expression and Smad3 activation. The TGFβ1-induced αvβ3 expression was blocked by pharmacologic and genetic inhibition of p38 MAPK but not Smad2/3, Sp1, ERK, phosphatidylinositol 3-kinase, and NF-κB-dependent pathways. Our results demonstrate that TGFβ1 induces αvβ3 integrin expression via a β3 integrin-, c-Src-, and p38 MAPK-dependent pathway. These data identify a novel mechanism for TGFβ1 signaling in human lung fibroblasts by which they may contribute to normal and pathological wound healing.

One of the key events in wound repair is the infiltration of fibroblasts from surrounding tissue to the extracellular matrix (ECM) in which they proliferate and differentiate into myofibroblasts. Under normal conditions myofibroblasts play a crucial role in ECM deposition and subsequent wound contraction and then disappear as the fibrotic response diminishes and normal structure and function are achieved (1). However, their retention, uncontrolled proliferation, and excessive synthesis of ECM proteins represents a pathologic process that ultimately results in fibrosis (2). Both fibroblast proliferation and differentiation, as well as ECM protein synthesis, are profoundly influenced by growth factors such as TGFβ as well as cell adhesion (3–6). Adhesion of cells to ECM is mediated by a family of transmembrane proteins known as integrins that are expressed on the cell surface as αβ heterodimers (7, 8). Importantly, integrins not only support cell attachment but also act in concert with receptors for several growth factors, including TGFβ3, to regulate survival, migration, proliferation, and differentiation of fibroblastic, epithelial, and endothelial cells (reviewed in Refs. 7, 8). Over the past few years a close relationship between αv integrins (recognizing RGD motif) and TGFβ signaling pathways has been identified (9). These include activation of latent TGFβ complexes by αvβ6 and αvβ8 integrins in airway epithelium (8, 10), augmented TGFβ signaling by αvβ3 and αvβ5 integrins in scleroderma fibroblasts (11), and TGFβ receptor type II (TGFβRII)-αvβ3 integrin interaction-dependent proliferation and differentiation of human lung fibroblasts (4). Previous studies have also identified several molecules as inducers of αv integrin expression in various tissue culture systems (12–14). It was also reported that growth factors are able to activate integrins and that this activation provided an additional mechanism for a growth factor to induce a broad spectrum of cellular responses (15–17). Recently we demonstrated that TGFβ1 not only synergistically interacts with αvβ3 integrins but also induces their gene transcription in human

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2 The abbreviations used are: ECM, extracellular matrix; TGFβ1, transforming growth factor β1; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; GFP, green fluorescent protein; SPK, Src family kinase; EGF, epidermal growth factor; RGD, Arg-Gly-Asp tripeptide motif; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PDCD, pyrroldine dithiocarbamate; mAb, monoclonal antibody; sRNA, short interfering RNA; AB, antibody; FN, fibronectin; WB, Western blot; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MFI, mean fluorescence intensity.
lung fibroblasts (4). However, the mechanisms involved in this process are still unknown. Therefore, this study was undertaken to delineate the signaling mechanisms that mediate αvβ3 up-regulation in response to TGFβ1 stimulation. The results of this study demonstrate that TGFβ1-dependent induction of β3 integrin expression does not involve Smad2/3 or Sp1 transcription factors, but it is mediated by selective and specific activation of the integrin itself and c-Src and p38 MAPK pathways.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Pharmacological Inhibitors**—The pcDNA3-Smad7 plasmid was provided by Dr. Steven Mutsaers (University of Western Australia), and the pcDNA3-p38-KM (kinase mutant) plasmid was provided by Dr. Kun Liang Guan (University of Michigan). The GFP-FRNK (FAK-related non-kinase)-expressing adenovirus (Adv-GFP-FRNK) and adenovirus expressing GFP alone (Adv-GFP) were kindly provided by Dr. Allen M. Samarel (the Cardiovascular Institute, Loyola University Medical Center). The pharmacological inhibitors SB203580, U0126, the Src inhibitor, PP2, and its inactive isomer PP3 were purchased from Calbiochem, and SB202190, wortmannin, the β3 selective disintegrin echistatin from Echis carinatus, Src inhibitor SU6656, Sp1 inhibitor mithramycin A from Streptomyces plicatus, and the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma.

**Cell Culture**—Normal human diploid lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C and 5% CO₂ in F-12K Nutrient Mixture medium (F-12K) supplemented with 10% FBS (Invitrogen) and antibiotics before they reached 90% confluence. Prior to the experiments cells were washed extensively in PBS and quiesced in serum-free F-12K for 24 h.

**Flow Cytometry**—HFL-1 cells were cultured to 90% confluence and stimulated with recombinant human TGFβ1 (PeproTech, Rocky Hill, NJ) or epidermal growth factor (EGF) (Sigma) in serum-free conditions for the indicated time periods. For experiments examining signaling pathways, cells were pretreated with pharmacological inhibitors of specific signaling molecules for 40 min prior to the addition of TGFβ1. Adherent cells were collected after trypsin/EDTA immersion, and surface expression of integrins was allowed to recover for 30 min in PBS with 10% FBS at room temperature. This step also served to block nonspecific antibody binding. Cells were then fixed with 2% paraformaldehyde for 10 min on ice. Cell surface expression of αvβ3 integrin was analyzed using an anti-human αvβ3 integrin antibody (mouse IgG1, clone LM609, Chemicon, Temecula, CA) and normal mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) as an isotype control, followed by incubation with phycoerythrin-conjugated goat anti-mouse F(ab’)2 (Cedarlane Laboratories, Ontario, Canada). Cell-associated fluorescence was acquired by a Coulter EPICS XL flow cytometer (Beckman Coulter, Ontario, Canada) and analyzed using WinMDI 2.8 software.

**RNA Extraction and Real Time Reverse Transcription-PCR**—See on-line supplemental material for details.

**Transfection**—HFL-1 were seeded at 1 × 10⁵ cells per well in 6-well tissue culture plates for 24 h prior to transfection. The cells were transiently transfected with 2 µg of mouse Smad7 DNA using Lipofectamine Plus (Invitrogen) for 24 h according to the manufacturer’s instructions. Confirmation of function was determined by Western blot (WB) analysis of α-smooth muscle actin expression in TGFβ1-stimulated cells (see supplemental Fig. 1SA). Human Smad3-specific chimera-RNA interference (Smad3 siRNA) (Abnova Corp, Taipei City, Taiwan) and nonsilencing control siRNA (Qiagen, Ontario, Canada) were transfected into HFL-1 cells by using HiPerFect transfection reagent (Qiagen) as instructed by the manufacturer. Forty eight hours after transfection cells were washed with fresh culture medium and further stimulated with TGFβ1 for 24 h. siRNA transfection efficiency was determined by WB (supplemental Fig. 1SB). The mammalian expression plasmids, pcDNA3-p38-KM, pcDNA3 (empty vector control), and pcDNA3-GFP (transfection efficiency control) were transfected into lung fibroblasts using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. The optimal ratio of FuGENE 6 (µl) to DNA (µg) was determined to be 6:1 for HFL-1 cells (supplemental Fig. 2SA). Expression of plasmids was monitored by WB for p38 MAPK expression and by fluorescence microscopy for GFP (data not shown). Following transfection, cells were incubated with 10% FBS in media for 24 h and then with 1% FBS for the next 24 h. Medium was changed, and cells were cultured for an additional 18 h in the presence or absence of TGFβ1. Replication-defective adenoviruses encoding GFP-FRNK fusion protein and GFP alone were amplified and purified using HEK-293 cells as described (18). Preliminary experiments determined that a concentration of 50–100 particles of Adv-GFP-FRNK and Adv-GFP per cell strongly induced the expression of these proteins (supplemental Fig. 2SB) and infected virtually every fibroblast (~90% of GFP positive cells by flow cytometry) after 48 h of exposure (data not shown). After infection with Adv-GFP-FRNK and Adv-GFP, cells were cultured in the presence of serum for 24 h, then serum-starved for additional 24 h, and stimulated with TGFβ1 for the indicated time periods.

**Western Blotting**—After TGFβ1 stimulation, control or cell signaling inhibitor-prevented cell monolayers were lysed in protein extraction buffer with protease and phosphatase inhibitor cocktails (Sigma). Equal concentrations of protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with a mixture of primary antibodies. p38 MAPK phosphorylation was determined using mouse mAb against human phosphorylated p38 MAPK (Thr180/Tyr182) and rabbit polyclonal antibodies against human p38 MAPK (both from Cell Signaling Technology, Danvers, MA). FAK phosphorylation was determined using mouse mAb against human phosphorylated FAK (pY397) (BD Biosciences) and rabbit polyclonal Ab against C-terminal region of human FAK (pp125FAK, Sigma). c-Src and Smad3 phosphorylation was determined using rabbit mAb against FAK (Y416) (Cell Signaling Technology), rabbit polyclonal Ab against c-Src (Santa Cruz Biotechnology), and rabbit mAb against phospho-Smad3 (Ser423/425) (Epitomics, Burlingame, CA), respectively, and anti-β-tubulin mAb (Upstate Biotechnology Inc., Lake Placid, NY) to control equal protein loading. Expression of β3 integrin chain, β5 integrin chain, and fibronectin was detected with
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mouse mAb (BD Biosciences) or rabbit polyclonal Ab (Cell Signaling Technology) against human β3 integrin, polyclonal rabbit Ab against human β5 integrin (Abcam, Cambridge, MA), and cellular fibronectin (Chemicon), respectively. Detection was performed with IR700 and IR800 anti-mouse and anti-rabbit antibodies (Cell Signaling Technology) and the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE) using the manufacturer’s protocol. Density of the bands was analyzed with Odyssey software 1.1 (LI-COR Biotechnology) using two infrared channels independently. The results are expressed as a phosphorylated protein/nonphosphorylated protein density ratio or protein/β-tubulin density ratio.

**Statistical Analysis**—Data are expressed as mean ± S.E. of at least three independent experiments. Statistical comparisons were performed using ANOVA with post hoc Fisher’s protected least significant difference. Probability values were considered significant if they were less than 0.05. All tests were done using StatView 5.0 software (SAS Institute Inc., Cary, NC).

**RESULTS**

**TGFβ1 Increases β3 Protein Expression and Enhances Cell Surface Expression of αvβ3 Integrin on Human Lung Fibroblasts**—

Recently we showed that TGFβ1 increased β3 steady-state mRNA expression (4). Consistent with the increased β3 transcription, fibroblasts also increased β3 subunit and αvβ3 integrin expression on the cell surface after exposure to TGFβ1 at a concentration of 10 ng/ml and higher. As can be seen in Fig. 1A, β3 protein levels significantly increased after 24 h of exposure to TGFβ1 at a concentration of 10 ng/ml. As shown in Fig. 1B, surface expression of αvβ3 heterodimer was also significantly elevated after cell stimulation with TGFβ1 (60% increase in MFI and 2-fold increase in the percentage of αvβ3-positive cells). Increased integrin expression on the cell surface was observed for up to 48 h, although the magnitude of expression was not significantly different from that seen at 24 h (data not shown). In contrast, EGF treatment did not significantly modify β3 protein production or surface expression of αvβ3 after 24 h of exposure (Fig. 1, A and B). Moreover, when EGF was simultaneously added with TGFβ1, it abrogated both TGFβ1-induced β3 subunit and αvβ3 cell surface expression (Fig. 1, A and B). In parallel we examined the effect of TGFβ1 on another αv partner, the β5 integrin. We found that incubation of HFL-1 with TGFβ1 for 18 h induced robust expression of αvβ3 (supplemental Fig. 3B) by increasing β5 protein production (Fig. 1C) similar to the effects on αvβ3 expression. In addition, both removal of exogenously added TGFβ1 (washing) and neutralizing of endogenously produced TGFβ1 with a pan-TGFβ-blocking antibody (clone 1D11), dramatically attenuated the effect of TGFβ1 on β3 and β5 integrin expression (Fig. 1C). These results demonstrate that the TGFβ1 effects on αvβ3 and αvβ5 integrin expression are: 1) associated with up-regulation of corresponding β integrin chain expression, and 2) highly specific and not mediated by a secondary mediator.

**TGFβ1-induced Expression of αvβ3 Integrin by Human Lung Fibroblasts Does Not Require Smad and Sp1**—In our previous studies we have shown that both Smad2 and p38 MAPK were activated in human lung fibroblasts within 10 min of TGFβ1 stimulation, and peak activation was reached at 1 h (19). In pilot experiments we found that both TGFβ1-induced Smad3 and p38 MAPK phosphorylation was still detectable after 18 h and correlated with the increased levels of β3 and β5 integrins (supplemental Fig. 4B and Fig. 1C).

We used two approaches to evaluate the role of Smad signaling in regulating the effect of TGFβ1 on integrin expression. In the first set of experiments, a murine Smad7-expressing con-
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FIGURE 2. Effect of TGFβ1 on αvβ3 expression is Smad2/3- and Sp1-independent. A, fibroblasts transiently transfected with Smad7 cDNA or transfection reagent alone were stimulated with TGFβ1 (10 ng/ml) for 24 h, and αvβ3 integrin cell surface expression was analyzed by flow cytometry as described under "Experimental Procedures." Values are expressed as a percentage above control MFI of three independent experiments ± S.E. B, HFL-1 cells were transfected with human Smad3 inhibiting chimera-RNA interference or control siRNA, and stimulated with TGFβ1 (10 ng/ml) for 24 h. Treatment with Smad3 siRNA abrogated TGFβ1-induced β3 integrin but did not change expression of β5 integrin in HFL-1 cells. Protein levels were determined by densitometry, and data are expressed as a percent of protein compared with transfection reagent control (TrR) (mean ± S.E., n = 3; *, p < 0.05 compared with control siRNA and 1 nM Smad3 siRNA). C, HFL-1 cells were treated with different concentrations of the Sp1 inhibitor mithramycin A for 40 min and then stimulated with TGFβ1 (10 ng/ml) for 18 h. β3 integrin subunit, fibronectin, and β-tubulin expression were determined by immunoblotting. One representative immunoblot of two independent experiments demonstrates that mithramycin A in a concentration-dependent manner attenuates up-regulatory effect of TGFβ1 on fibronectin production, whereas it has no effects on spontaneous and TGFβ1-induced β3 integrin production of human lung fibroblasts.

with control (supplemental Fig. 1S B). In keeping with the results from Smad7 overexpression, inhibition of Smad3 did not influence the expression of β3 integrin induced by TGFβ1. In contrast, Smad3 siRNA significantly attenuated β5 integrin expression induced by TGFβ1 (Fig. 2B).

Cooperation between the Smad proteins and the transcription factor Sp1 may represent a general mechanism for conferring TGFβ1 inducibility of several genes, including integrins (13, 20). We sought to determine whether Sp1 was involved in the up-regulation of αvβ3 integrin induced by TGFβ1. As shown in Fig. 2C, the Sp1 inhibitor mithramycin had no effect on β3 subunit, but it efficiently prevented the increase in fibronectin expression induced by TGFβ1. Together these results indicate that the Smad/Sp1 pathway does not play a major role in TGFβ1-induced up-regulation of αvβ3 integrins on human lung fibroblasts.

**TGFβ1-induced β3 Expression Requires Cell Adhesion and Is Enhanced by Integrin Activation with ECM Proteins—**Our previous studies have demonstrated that several TGFβ-mediated effects in human lung fibroblasts are adhesion- and integrin-dependent (3, 4, 6). In preliminary experiments we found that TGFβ1 was unable to increase αvβ3 integrin expression in cells in suspension but did so in cells adherent on a plastic surface (data not shown). Therefore, we determined whether integrin activity following adhesion to different ECM proteins influences the ability of TGFβ1 to induce αvβ3 expression. Fig. 3 demonstrates that cell adhesion to either fibronectin (FN) or collagen did not significantly modify basal β3 expression; however, FN strongly potentiated the effect of TGFβ1. These findings suggest that signals mediated by integrin activation following cell adhesion are necessary for TGFβ1 to elevate β3 integrin expression.

**TGFβ1-induced β3 Integrin Expression Is Dependent on αvβ3 Activation—**To evaluate the role of integrin activation on enhanced αvβ3 expression, we treated adherent fibroblasts with the disintegrin echistatin, which has been shown to inhibit the activation of RGD-binding integrins in several cell types (17, 21, 22). Echistatin had no effect on cell attachment but dose-dependently impaired the ability of fibroblasts to spread...
To demonstrate that echistatin inhibits shown in Fig. 4 apart from show that echistatin prevents activation of fibronectin (1 HFL-1 cells were placed on the wells of a tissue culture plate pre-coated with fibronectin (1 μg/ml) or collagen type I (COL, 1 μg/ml) and stimulated with 10 ng/ml of TGFβ for 24 h. β3 integrin expression was determined by immunoblotting. Bars are mean ± S.E. (n = 3); *, p < 0.05 compared with FN without TGFβ stimulation (Non-stim).

and support monolayer integrity (supplemental Fig. 5S). As shown in Fig. 4A, exposure of cells to echistatin abrogated the effect of TGFβ1 on β3, but not on β5 integrin expression. To show that echistatin prevents activation of αvβ3 and downstream integrin-mediated signaling, we determined levels of Tyr397-FAK and Tyr416-Src phosphorylation following exposure to the disintegrin. Echistatin dramatically inhibited TGFβ1-induced c-Src kinase (Fig. 4B) and FAK phosphorylation (data not shown) in a concentration-dependent manner. To demonstrate that echistatin inhibits β3 integrin expression by blocking integrin, but not TGFβ1 signaling, FN expression in response to TGFβ1 exposure was determined. In contrast to β3, FN expression induced by TGFβ1 was enhanced in the presence of echistatin indicating that TGFβ1 up-regulates β3 integrin expression directly by activation of the integrin on the cell surface rather than indirectly through enhanced production of ECM proteins. Echistatin also had no effect on Smad3 activation per se (Fig. 4B), but it inhibited TGFβ1-induced p38 MAPK in parallel with β3 integrin expression (Fig. 4, A and B).

Because echistatin may also influence other RGD integrins apart from αvβ3, we next aimed to confirm the identity of the integrin involved in TGFβ1-induced β3 expression. To do this, we incubated fibroblasts with 10 μg/ml monoclonal blocking antibodies to human αvβ3 integrin (clone LM609) and αvβ5 integrin (clone P5H9) 1 h before addition of TGFβ1. Similar to the effect seen with echistatin, LM609, but not P5H9 or control IgG, completely abrogated the effect of TGFβ1 on β3 integrin expression (Fig. 4C). These results provide further support for the concept that the effect of TGFβ1 on β3 subunit expression is dependent on the activation of αvβ3, but not αvβ5 integrin, on the cell surface and recruitment of nonreceptor protein tyrosine kinases, including FAK and c-Src kinase.

Adenovirally Mediated Overexpression of FRNK Inhibits TGFβ1-induced FAK Activation but Not αvβ3 Expression—Based on the observation that integrin activation and clustering results in activation of FAK, we determined the effect of TGFβ1 on FAK phosphorylation. Fig. 5A demonstrates that TGFβ1 induced time-dependent phosphorylation of FAK on Tyr397, its major autophosphorylation site. The enhanced Tyr397 phosphorylation of FAK was somewhat delayed, being first observed 3 h following TGFβ1 exposure and further increased at later time points (Fig. 5A, p < 0.05) coincidentally with the induction of β3 expression. To confirm whether FAK activation was involved in TGFβ1-induced αvβ3 expression, fibroblasts were infected by a replication-defective adenovirus encoding a GFP-FRNK fusion protein (Adv-GFP-FRNK) and exposed to

FIGURE 3. TGFβ1-induced αvβ3 expression is dependent on integrin-mediated cell adhesion. Bar graph shows the synergistic positive effect of fibronectin on TGFβ1-induced β3 integrin expression in lung fibroblasts. HFL-1 cells were placed on the wells of a tissue culture plate pre-coated with fibronectin (1 μg/ml) or collagen type I (COL, 1 μg/ml) and stimulated with 10 ng/ml of TGFβ1 for 24 h. β3 integrin expression was determined by immuno-blotting. Bars are mean ± S.E. (n = 3); *, p < 0.05 compared with FN without TGFβ1 stimulation (Non-stim).

FIGURE 4. Blocking of αvβ3 integrin activation by disintegrin echistatin or LM609 monoclonal antibodies completely abrogates up-regulatory effects of TGFβ1 on β3 integrin expression in lung fibroblasts. A, echistatin dose-dependently inhibits TGFβ1-induced β3 integrin but not β5 integrin expression (n = 3; *, p < 0.05 compared with TGFβ1 alone). B, echistatin dose-dependently inhibits c-Src phosphorylation at Tyr416 but promotes production of fibronectin (left panel, representative immunoblot for two independent experiments). Echistatin (2 μg/ml) did not change Smad3 phosphorylation but inhibited TGFβ1-induced p38 MAPK activation (right panel, representative immunoblot for two independent experiments). C, αvβ3 blocking antibody (clone LM609, PBS solution without preservatives and carrier proteins, 10 μg/ml), but not αvβ5 blocking antibody (clone P5H9, carrier proteins-free solution) and mouse IgG isotype control applied at the same concentration 1 h before stimulation with TGFβ1, prevents the increase in β3 protein induced by cytokine in human lung fibroblasts. Data are means ± S.E. of three independent experiments (*, p < 0.05 compared with controls and αvβ3 blocking antibody). Ctrl, control.
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**FIGURE 5.** Blocking of TGFβ1-induced FAK autophosphorylation does not prevent up-regulatory effect on αvβ3 integrin expression. A, TGFβ1-induced FAK activation was determined by immunoblotting. Following stimulation with TGFβ1 for the indicated times, H1 cells were lysed, and cell-extracted proteins were separated by SDS-PAGE. Immunoblots were performed with specific monoclonal antibody against C-terminal region of human FAK protein (FAK). Immunoblots were scanned for the band densities, and pFAK/FAK ratio was calculated for each individual experiment. Data are expressed as percent increase in comparison with nonstimulated cells. Ctrl, control; B, two overlaid histogram plots demonstrating MFI and percentage of αvβ3 expressing cells in Adv-GFP- and Adv-GFP-FRNK-infected populations of fibroblasts either nonstimulated (shaded histograms, M1 marked area) or TGFβ1-stimulated (unshaded histograms, M2 marked area) are representative data of three independent experiments (dashed line histogram demonstrates MFI of cells stained with isotype control antibody). C, graph bars demonstrate a percentage of αvβ3 expressing cells. Data are mean ± S.E. of three independent experiments (*, p < 0.05 compared with nonstimulated cells).

TGFβ1. FRNK is the C-terminal noncatalytic domain of FAK and acts as a negative regulator of FAK autophosphorylation (23, 24). Western Blot for GFP-FRNK revealed abundant expression in Adv-GFP-FRNK-infected cells but not in control Adv-GFP-infected cells, and infection of fibroblasts with Adv-GFP-FRNK completely abrogated spontaneous and TGFβ1-induced β3 expression and suggest that c-Src kinase and p38 MAPK activation, but not Smad3, is a key element in the process.

p38 MAPK Inhibition Blocks TGFβ1-induced αvβ3 Integrin Expression—Besides Smad-mediated transcription, TGFβ1 activates other signaling cascades, including MAPK and NF-κB...
TGFβ1 induces αvβ3 integrin expression

![Graph](image)

FIGURE 6. TGFβ1-induced and αvβ3 integrin-mediated c-Src activation is required for the increase in β3 integrin and αvβ3 cell surface expression. Quiescent, adherent fibroblasts were stimulated with TGFβ1 (10 ng/ml) in the absence/presence of the Src inhibitor PP2 (10 μM) for 18 h. Cell surface expression of αvβ3 was assessed by flow cytometry (A) and β3 subunit mRNA expression by real time reverse transcription-PCR (B). Graph bars are the mean ± S.E. of three independent experiments (*, p < 0.05 compared with TGFβ1-stimulated cells without inhibitor). C, PP2 and the more specific Src inhibitor SU6656 almost completely abrogated TGFβ1-induced β3 integrin expression in human lung fibroblasts. PP3, pharmacologically inactive form of PP2, used at the same concentrations and experimental conditions as PP2 did not influence TGFβ1-induced β3 protein (*, p < 0.05 compared with PP2 and SU6656 inhibitors, n = 3). D, HFL-1 cells treated with the Src inhibitor SU6656 for 45 min and then stimulated with TGFβ1 for additional 45 min. Phosphorylation of p38 MAPK and Smad3 was determined by immunoblotting and quantified by densitometry. The Src inhibitor SU6656 abrogated TGFβ1-induced p38 MAPK phosphorylation but increased phosphorylation of Smad3 in HFL-1 cells (*, p < 0.05 compared with controls (Ctrl); #, p < 0.05 compared with TGFβ1 alone, n = 3).

pathways (reviewed in Ref. 26). Importantly, Src kinases can interfere with these pathways directly or indirectly, and our results demonstrate that p38 MAPK activation by TGFβ1 is dependent on integrin activation and SFK recruitment. Therefore, we assessed whether inhibition of MAPK, including p38 and p42/p44, NF-κB, and PI3K affected TGFβ1-induced αvβ3 integrin expression. As seen in Fig. 7A, no changes in the cell surface expression of αvβ3 integrin were observed after treatment with wortmannin. Similar effects were seen for TGFβ1-induced β3 protein (data not shown). However, the p38 MAPK inhibitor SB203580 (10 μM), and the more potent inhibitor SB202129 at a concentration of 0.2 μM and higher, decreased β3 subunit and αvβ3 integrin expression induced by TGFβ1 (Fig. 7, A and B).

To further validate the role of p38 MAPK, we transiently overexpressed a kinase mutant p38 MAPK (p38-KM) construct in fibroblasts (20). As expected, an increased level of p38 MAPK protein was seen in p38-KM-transfected cells but not in cells transfected with empty pcDNA3 vector (supplemental Fig. 2S). As seen in Fig. 7C, TGFβ1 failed to induce β3 integrin expression in p38-KM-transfected cells. Importantly, the Smad signaling pathway was completely preserved and TGFβ1 efficiently induced β3 integrin expression in p38-KM-transfected cells (supplemental Fig. 2A and Fig. 7C). Induction of p38 MAPK phosphorylation by treatment with recombinant tumor necrosis factor-α did not up-regulate β3 integrin in HFL-1 cells (data not shown). Thus, limiting TGFβ1-induced p38 MAPK activity did not alter Smad-dependent induction of β3 integrin, but it substantially inhibited β3 protein and αvβ3 cell surface expression.

DISCUSSION

Adhesion of fibroblasts to the ECM via specific integrins can alter cell responses to growth factors, including TGFβ (3–6), which are often produced in excess and may promote specific cell-ECM interactions giving a rise to several pathological cycles and abnormal behavior of the cells in the settings of fibrotic diseases (2, 11). In this study, we demonstrate that TGFβ1 increases β3 integrin expression and subsequent αvβ3 surface expression on
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human lung fibroblasts via an autocrine pathway involving c-Src- and p38 MAPK-dependent mechanisms. Significantly, this effect is independent of the conventional Smad/Sp1 signaling pathway. Similarly, although the effect was dependent on cell adhesion and FAK was phosphorylated by TGFβ1, inhibiting FAK activation did not influence the effect of TGFβ1 on αvβ3 expression. We show that the effect of TGFβ1 is dependent on αvβ3-mediated cell adhesion and activation of c-Src kinase and p38 MAPK pathway. Blockade of c-Src activity also completely abrogated the stimulatory effect of TGFβ1 on activation of p38 MAPK, suggesting that c-Src is upstream of this signaling kinase.

To date there have been several reports investigating the effects of TGFβ1 in modulating expression and function of αvβ3 integrins on different cell types. For instance, TGFβ1 has been shown to have no effect on αvβ3 expression in human airway epithelial cells (27). In contrast, in human vascular smooth muscle cells (28), human WI-38 fibroblasts (12), and a variety of malignant cell lines, TGFβ1 increases αvβ3 expression. Although these studies focused on how the temporal expression of αvβ3 integrins is influenced by exposure to TGFβ1, they highlight that the effects of TGFβ1 on integrin expression may be cell type-specific, and little is known about the underlying signal transduction pathways involved. Binding of TGFβ1 to TGFβRII causes the recruitment and activation of TGFβIR and subsequently Smad2/3 phosphorylation, which then associates with Smad4. This complex then translocates to the nucleus where it modulates transcription of a large number of genes. In contrast, Smad7 inhibits the TGFβ1-mediated phosphorylation of Smad2/3 through competition for binding to the TGFβ1 receptor (26, 29). Using several independent approaches, we determined that the Smad2/3 pathway is not required for the TGFβ stimulation of αvβ3 integrin. Specifically, we have shown that in contrast to β5 expression, which was strongly dependent on Smad3 activation, TGFβ1-induced β3 integrin was maintained in the cells transfected with either Smad3 siRNA or a dominant Smad7 expression construct. Interestingly, in \textit{in silico} analysis of the mouse and human β3 integrin gene promoters (30, 31) (GenBank accession numbers AF026510 and AF02055, respectively) revealed considerable sequence homology across a 1.3-kb region upstream of the transcription start site and several conserved binding elements for Sp1 but not for Smad proteins. Recently, it has been demonstrated that cooperation between Sp1 and Smad proteins may represent a general regulatory mechanism for conferring the TGFβ1 inducibility of several genes, including β5, α5, and α11 integrins (13, 20, 32) and different types of collagen (33, 34). In these studies the antibiotic mithramycin A selectively and efficiently reduced TGFβ3-induced collagen and α11 integrin expression in mesenchymal cells via inhibition of Sp1 binding to the gene promoter. In this study, we also demonstrated that mithramycin A significantly inhibited TGFβ1-induced expression of cellular FN, but it had no effect on β3 integrin expression. These paradoxical observations indicate that the effects of TGFβ1 on integrin expression are dependent on cell type and specific regulatory elements of the individual integrin gene promoters. Significantly, our data also suggest that TGFβ1 is able to activate signaling pathways in human lung fibroblasts to promote the expression of integrin complexes independently of Smad2/3 and Sp1.

In addition to Smad-mediated transcription, it has been shown that TGFβ also activates several specific signaling cascades, including MAPK, NF-κB, and PI3K (19, 35, 36). However, in this study pharmacological inhibition of NF-κB, MEK-ERK, and PI3K pathways did not significantly influence the induction of αvβ3 by TGFβ1. In contrast, blocking of p38 MAPK activation by either selective inhibitors or overexpres-
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FIGURE 8. Schematic model of the proposed synergistic interaction between αvβ3-mediated c-Src and TGFβ1-induced p38 MAPK activation in the β3 subunit expression by human lung fibroblasts. αvβ3 is activated upon interaction with the TGFβ type II receptor and its ligand TGFβ1 (4, 37). Following αvβ3 activation c-Src, associated with the β3 integrin subunit, is phosphorylated at Tyr397 and then recruited to the kinase domain of TGFβRII, which results in phosphorylation of the receptor at Tyr684 (37). In addition, αvβ3 binding to ECM proteins also promotes the activation of SFK. Once activated c-Src coordinates the activation of TGFβ1-induced p38 MAPK signaling pathway and thereby the transcriptional regulation of β3 integrin subunit expression. We propose the rapid increase in β3 integrin leads to high αvβ3 cell surface expression giving rise to a positive feedback loop affecting several fibroblast functions such as adhesion, migration, ECM remodeling, and autocrine TGFβ expression and signaling (zigzag indicates phosphorylation sites).

...function in organizing the signaling pathways associated with the attachment of integrins to ECM. For example, vascular endothelial growth factor is able to enhance the affinity of αvβ1 and αvβ3 integrins in human endothelial cells (16). In addition, platelet-derived growth factor or EGF-induced activation of FAK through integrins was proposed as an important proximal link between growth factor receptor and integrin signaling pathways (24). In agreement with these findings, we showed that TGFβ1 significantly increases affinity of αv integrins on lung fibroblasts to their respective ligands and also activates FAK.

Traditionally FAK activation has been associated with integrin signaling, because it is recruited and phosphorylated at sites of focal adhesions upon integrin aggregation, and it serves as a bridge between growth factor receptors and β integrin cytoplasmic tails (24). In this study we found that TGFβ1 induced FAK autophosphorylation on Tyr397 (Tyr(P)397-FAK), and FAK protein paralleled the time course of β3 integrin mRNA expression (4). Critically, TGFβ1 did not influence αvβ3 expression on fibroblasts in suspension, but adhesion of cells on ECM proteins significantly enhanced the effect of TGFβ1 on β3 integrin expression. However, although overexpression of the FAK inhibitor, FRNK, completely inhibited basal and TGFβ1-induced Tyr(P)397-FAK, it did not significantly influence TGFβ1-induced αvβ3 expression. In contrast, inhibition of c-Src kinase, which also has been shown to play an important role in downstream signaling of integrin receptors, dramatically blunted TGFβ1-induced αvβ3 expression and Tyr(P)397-FAK.

Although c-Src kinase activity is clearly required for rapid and full phosphorylation of Tyr(P)397-FAK (39), c-Src kinase activity is not affected in FAK−/− cells (40), suggesting that c-Src can be activated independently of FAK. Furthermore, activated Src, unlike FAK, is concentrated in perinuclear and plasma membrane but not in focal adhesions after integrin ligation (39), suggesting some divergence in their signaling. These findings corroborate our results showing that activation of c-Src kinase, but not FAK autophosphorylation, is the critical downstream pathway in TGFβ1-induced αvβ3 expression.

Recently, it has been shown that Src associates with αvβ3 and is activated following integrin ligation (39, 41). Moreover, the cytoplasmic tail of the β3 integrin, but not other integrins, directly interacts with Src kinase through the β3-specific domain (39, 41, 42). These findings may explain the specificity of αvβ3 integrins for TGFβ1-induced β3 subunit expression in human lung fibroblasts. Indeed, our data demonstrate that...
either disintegrin or monoclonal antibody-mediated blockade of αβ3 activation (but not αβ5), prior to TGFβ1 stimulation, efficiently abrogated the increases of both c-Src activation and β3 integrin expression.

How then does TGFβ1 activate or promote activation and signaling of αβ3 integrins? Our data suggest through a process of inside-out signaling whereby activated TGFβRI or TGFβRII directly interacts with the β3 cytoplasmic domain, which in turn induces a conformational change in the integrin resulting in activation of the downstream SFKs (4, 37). A similar process of inside-out activation of αβ3 integrin by ligation of insulin-like growth factor I receptor in the murine preadipocyte 3T3-L1 cell line has been described recently (17).

TGFβ1 could also induce synthesis of several αβ3 ligands, including FN, vitronectin, and tenasin (3, 34, 43), and by enhancement of ligand-integrin interactions activate integrin-mediated signaling. Indeed, we demonstrated that seeding of fibroblasts on FN significantly enhanced the effect of TGFβ1 on β3 subunit expression. However, these exogenously added ECM proteins did not induce or up-regulate β3 expression in the absence of TGFβ1. Furthermore, inhibition of FN production by mithramycin did not prevent TGFβ1-induced expression of the β3 integrin.

Finally, TGFβ1-induced activation of αβ3 integrin could be modified by the expression level of the αv subunit. We showed that TGFβ1-induced expression of both β3 and β5 subunit is sufficient to up-regulate the cell surface of αβ3 and αβ5, respectively. This is consistent with the notion that the αv subunit is constitutively and excessively expressed in the cytoplasm of fibroblasts as a monomer, and the cell surface expression level of αv-containing integrins is controlled by the levels of β subunits, which are exclusively involved in the integrin-mediated signaling mechanisms (11, 14).

Given that TGFβ1 up-regulates β3 expression in a delayed manner, another possibility is the involvement of other factors induced by TGFβ1. EGF is a well known and potent activator of integrins (16, 24, 36). However, in this study, although EGF induced a robust FAK autophosphorylation it did not influence αβ3 expression, either alone or in combination with TGFβ1. Moreover, TGFβ neutralization with blocking mAb and removal of recombinant cytokine after a short time exposure almost completely abrogated β3 integrin expression suggesting that activation of integrin/c-Src signaling as well as production of autocrine TGFβ is exclusively dependent on the initial TGFβRI ligation by active TGFβ1. Altogether these data also indicate that the effect on αβ3 expression is highly specific for TGFβ1.

In summary, our results suggest a model where TGFβ1 induces activation of αβ3 integrin, which in turn leads to c-Src phosphorylation and the initiation of p38 MAPK signaling cascade that is essential for β3 chain expression (Fig. 8). Our study for the first time describes a mechanism for the regulation of the integrin β3 subunit expression in human fibroblasts, which involves TGFβ1 and the αβ3 integrin itself. Significantly, this appears to be a Smad-independent process. Our data support the concept of a functional synergy between TGFβ1-dependent signaling pathways and αβ3-mediated adhesion processes in normal wound healing and pathological phenomena such as fibrosis.

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