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Transforming Growth Factor β_1 Inhibits Fetal Lamb Ductus Arteriosus Smooth Muscle Cell Migration

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ABSTRACT

Anatomical closure of the ductus arteriosus (DA) requires normally quiescent smooth muscle cells (SMC) to migrate out of the muscle media into the subendothelial space, forming intimal mounds that eventually coalesce to occlude the vessel's lumen. Transforming growth factor-β_1 (TGFβ_1), a potent modulator of vascular SMC migration, is found in the wall of the closing DA. We examined the effect of TGFβ_1 on the migration of fetal lamb DA-SMC. Although TGFβ_1 has been shown to be a chemoattractant for other mesenchymal cells, it had no chemotactic effect on DA-SMC; furthermore, TGFβ_1 did not enhance the migration of DA-SMC (as has been reported for aortic SMC). Rather, incubating DA-SMC with TGFβ_1 for 22 h decreased the rate of migration of SMC on extracellular matrix substrata composed of fibronectin, vitronectin, laminin, and collagen I and IV. Exposure of DA-SMC to TGFβ_1 was associated with an increase in the formation of focal adhesion plaques (tight associations between the cells' surface and extracellular matrix). DA-SMC use integrin receptors to attach to and migrate on extracellular matrix components. The decrease in DA-SMC migration was not associated with a significant change in the profile of integrin receptors expressed by the cell. TGFβ_1 had little effect on overall DA-SMC integrin expression, except for a modest increase in the fibronectin receptor ($\alpha_5\beta_1$ integrin). Rather, the decrease in migration and changes in cell morphology were associated with an increased ability of integrin receptors to associate with the cytoskeleton. TGFβ_1 appears to anchor the cell's cytoskeleton to the extracellular matrix, making the cells more adherent and less capable of migrating. (***Pediatr Res* 37: 561–570, 1995**)

Abbreviations

DA, ductus arteriosus
SMC, smooth muscle cell
ECM, extracellular matrix
FN, fibronectin
IDME, Iscove's modified Dulbecco's medium
LN, laminin
PMSF, phenylmethylsulfonyl fluoride
TGFβ_1, transforming growth factor-β_1
VN, vitronectin

Closure of the DA is a necessary adaptation to extrauterine life. Term infants functionally close their DA within the first several hours of life, followed by vessel wall reorganization and permanent anatomical closure. Preterm infants, however, frequently show initial constriction of the muscular media (functional closure), but fail to initiate vessel wall remodeling, leading to patency of the DA and its associated morbidity.

DA remodeling is dependent upon the migration of SMC from the muscle media into the subendothelial space; this forms the intimal mounds necessary for obliteration of the vessel's lumen and permanent anatomical closure. For migration to occur, DA-SMC must recognize and interact with molecules of the ECM (*e.g.* FN, LN, VN, and collagen types I and IV). We have recently identified a group of integrin receptors on the surface of DA-SMC that mediate cellular adhesion to and migration on the ECM (1). Each integrin receptor is a heterodimer in which one of several α subunits associates noncovalently with a β subunit. When isolated cells make an integrin-mediated contact with the ECM, the integrins typically cluster into groups of focal contacts or plaques in the plane of the lipid bilayer. The creation of focal plaques depends on the ability of integrins to associate with the actin cytoskeleton (2). The formation of focal contacts leads to changes in cell shape and biologic responsiveness.

Vascular SMC migration is also modulated by growth factors such as TGFβ_1 (3, 4), which has been found in the wall of the fetal DA (5) and which increases during postnatal closure

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of the vessel (Tannenbaum J, Waleh N, Perkett E, Gold L, Clyman RI, manuscript in preparation). TGFβ_1 is secreted by both endothelial cells and vascular SMC (6) and has been implicated in the remodeling of blood vessels during hypertension (7), atherosclerosis (8), and angioplasty (9). TGFβ_1 has been reported to regulate the expression of integrin receptors in a variety of SMC derived from blood vessels other than the DA (10, 11). The effects of TGFβ_1 on cell motility have varied according to the type of cells that have been examined: TGFβ_1 stimulates chemotaxis of fibroblasts (12), macrophages (13), and SMC (9, 14), but inhibits migration of endothelial cells (15–17).

Because TGFβ_1 has been shown to affect vascular SMC migration in a variety of physiologic and pathologic circumstances, we examined the effects of TGFβ_1 on DA-SMC migration. We found that DA-SMC behaved differently from aortic SMC in their response to TGFβ_1. Aortic SMC have been reported to respond to TGFβ_1 with an increase in integrin synthesis and an increase in cell migration (9–11, 14); in contrast, we found that DA-SMC responded to TGFβ_1 with a decrease in cell migration and only minimal changes in integrin expression. TGFβ_1 increased DA-SMC focal plaque formation by increasing the association of the cell's integrins with the cytoskeleton. TGFβ_1 appears to anchor the DA-SMC to the surrounding ECM, making them less capable of migrating.

METHODS

Cell culture

Vascular SMC were isolated from medial explants of DA obtained from fetal lambs (106-d gestation; term = 145 d) and characterized by their "hill and valley" morphology at confluence and their ability to be recognized by a MAb against smooth muscle actin (1). The SMC were grown in monolayer culture in IDME supplemented with 4.5 g/L (25 mM) glucose, 0.29 g/L (2 mM) glutamine, 10% FCS, 100 U/mL penicillin, 100 μg/mL (0.17 mM) streptomycin, and 1.25 μg/mL (1.35 μm) Fungizone. Cells were passaged at confluence with trypsin-EDTA and were used between passages 4 and 9.

Antibodies

Rat. The following rat MAb were used: anti-integrin β_1 subunit ($A_{II}B_2$) and anti-human α_5 subunit (B_IE_5) (provided as ascites fluid by Dr. C. Damsky, University of California, San Francisco).

Mouse. The following mouse MAb were used: anti-integrin β_3 subunit (E8, provided by Dr. Larry Fitzgerald, University of California, San Francisco), anti-human integrin α_v subunit (LM 142) (provided by Dr. D. Cheresh, Research Institute of Scripps Clinic), and anti-vinculin (clone VIN-II-5, ICN Immunobiologicals, Lisle, IL).

Rabbit. The following polyclonal antibodies were used: anti-integrin β_3 subunit (poly β_3) from platelet II_bIII_a complex (provided by Dr. K. Knudsen, Lankenau Hospital Research Center, Philadelphia, PA), anti-integrin $\alpha_v\beta_3$ complex (Telios Pharmaceuticals, San Diego, CA), anti-integrin α_v (anti-α_v) derived from the cytoplasmic domain of the integrin α_v subunit (characterized by competition ELISA and by Western immunoblot, and provided by Dr. L. Reichardt, University of California, San Francisco), anti-human FN (anti-FN) (Cappel, Westchester, PA), and anti-integrin α_5 (anti-α_5) and anti-integrin α_3 (anti-α_3) derived from the cytoplasmic domain of the integrin subunits α_5 and α_3, respectively (provided by Dr. R. Hynes, Harvard University, Cambridge, MA).

The characterization of the antibodies used in this investigation was referenced in earlier publications (1, 18, 19). Neither the mouse MAb against human α_v (LM 142) nor the rat MAb against human α_5 (B_IE_5) bound to the corresponding ovine α subunits; they were used as control antibodies when other mouse and rat antibodies were tested. Heat-inactivated normal rabbit serum was used as a control for rabbit antisera.

ECM

Type I collagen from bovine skin was purchased from Celtrix, Palo Alto, CA (Vitrogen 100). FN was purified from outdated human plasma by using gelatin Sepharose affinity chromatography (20). VN was purified from plasma by glass bead column chromatography and heparin Sepharose chromatography (21). Both LN and type IV collagen were isolated from Englebreth Holm Swarm tumors grown in C57 BL/6 mice (22). The LN used in these experiments was found to be free of type IV collagen and entactin (18).

Growth Factor

TGFβ_1 (from human platelets) was obtained from R and D Systems (Minneapolis, MN).

Adhesion

Adhesion of DA-SMC to protein-coated microtiter plates was assayed as described previously (18). Confluent cell monolayers were washed with medium without FCS and were incubated with or without TGFβ_1 (250 pM) for an additional 22 h. Cells were removed from the tissue culture plates by brief incubation (2 min) at room temperature with trypsin-EDTA. After inactivation of the trypsin with soybean trypsin inhibitor (Worthington Biochemical, Freehold, NJ), the cell pellet was washed twice with cold IDME and resuspended in cold IDME with BSA [1 mg/mL (16.6 μM)].

The wells of polystyrene 96-well microtiter plates (Serocluster Costar Corp., Cambridge, MA) were precoated with either FN [10 μg/mL (25 nM)], LN [25 μg/mL (31 nM]), VN [5 μg/mL (71 nM)], I [5 μg/mL (17 nM)], IV [1 μg/mL (1.8 nM)], or BSA [1 mg/mL (16.6 μM)] dissolved in sterile PBS for 1 h at 37°C. The amount of ligand that adsorbed to the polystyrene plate has been shown to be directly related to the concentration of ligand used to coat the plate (23–25). The concentration of ligand used to precoat the wells was the concentration that allowed 60–90% of the maximal cell attachment to that particular ligand (data not shown). The wells were then washed with PBS, and nonspecific adherence to the coated wells was blocked with 1 mg/mL (16.6 μM) BSA in IDME for 1 h at 37°C. Cells were added (2×10^4 cells/well) and allowed to attach to the wells at 37°C for 30 min when assessing the

initial attachment characteristics of the cells (1), or for 1.75 h when assessing the total number of cells that attach to the substrates before the migration assay (see below). During the assay, TGFβ_1 (250 pM) was added to the wells of the TGFβ_1-treated cells. After washing unattached cells from the wells, adherent cells were quantified by a colorimetric assay for hexosaminidase, a lysosomal enzyme, and the data from each experiment were expressed as the mean of triplicate wells (26).

Migration

To examine SMC migration, we used the circular outgrowth assay described previously (1). The 96-well upper chamber of a Minifold filtration apparatus (Schleicher & Schuell, Keene, NH) was used to coat FN, LN, VN, and collagens I and IV onto an uncharged polystyrene sheet by filling the individual wells with the same concentrations described above under "Adhesion." The nonspecific binding sites on the polystyrene sheet subsequently were blocked by incubation with 1 mg/mL (16.6 μM) BSA in PBS. A stainless steel screen with 940-μm diameter circular perforations was then inserted as a barrier between the substrate-coated polystyrene sheet and the upper 96-well template. DA-SMC (2×10^4 cells/well), pretreated with or without TGFβ_1 (as described above under "Adhesion"), were placed into the upper wells and allowed to attach to the substrate-coated sheet for 1.75 h at 37°C. The metal screen prevented the cells from attaching to the coated sheet except at the perforated areas. Once the cells had attached, the screen was removed, leaving discrete circular areas of cells attached to the coated sheet. The upper chamber was filled with 1 mg/mL (16.6 μM) BSA in IDME [with appropriate concentrations of TGFβ_1 and with anti-FN (1/40 dilution) when used]. The cells then were allowed to migrate out, on top of the substrate-coated sheet, from their original 940-μm diameter circular area, to form an enlarging circle. After 7 h the cells were fixed with 70% ethanol and stained with hematoxylin. The migration rate was calculated as the increase in diameter of the circular area covered by the cells between 0 and 7 h. The data from each experiment were expressed as the mean of eight wells.

Chemotaxis

Smooth muscle cell movement to a chemoattractant was assayed as previously described (27) using modified Boyden chambers (Transwell, Costar, Cambridge, MA) that had polystyrene membranes (6.5-mm diameter; 10-μm thickness; 8-μm pore size) separating the upper and lower chambers. The upper and lower surfaces of the membrane were precoated with collagen I [5 μg/mL (17 nM)], and nonspecific adherence was blocked with 1 mg/mL (16.6 μM) BSA. Confluent cells were removed from the tissue culture plates, as described above, and resuspended in IDME with BSA.

Cells (8×10^4) were added in 100 μL to the upper chamber and allowed to attach to the substrate-coated membrane for 1.75 h at 37°C. Once the cells had attached, the upper chamber was washed with IDME with BSA, and the lower chamber was filled with IDME with BSA (500 μL) containing TGFβ_1 (0.025–250 pM). The plates were incubated for 4 h at 37°C. The assay was terminated by fixing and staining the wells with Leukostat (Fisher Diagnostics, Pittsburgh, PA). In some experiments, DA-SMC were pretreated with or without TGFβ_1 (250 pM) for 22 h before the experiment (see "Adhesion" above); when this occurred, both the upper and lower chambers of the chemotaxis wells were filled with TGFβ_1 during the 4-h assay.

After the upper surface of the membrane was wiped clean of cells, cell migration to the lower surface was expressed as the mean number of cell nuclei per 400× high-power field on the lower surface. The value for each membrane was obtained by counting nine predetermined fields per membrane. Each assay well was performed in duplicate, and the cell numbers differed by less than 10%.

Immunofluorescent Staining

Glass coverslips were coated with 100 μL of FN [10 μg/mL (25 nM)], collagen I [5 μg/mL (17 nM)], or VN [5 μg/mL (71 nM)] as described above. DA-SMC, preincubated with or without TGFβ_1 (250 pM) were removed from the tissue culture plates, suspended in IDME with 1 mg/mL (16.6 μM) BSA, and overlaid (2×10^4 cells) onto the coverslips (see "Adhesion" above). After 4 h the cells were fixed and permeabilized (1). The samples were incubated with either anti-integrin β_1 subunit ($A_{II}B_2$) (1:100), anti-integrin β_3 subunit (E8) (1:10), or anti-FN (1:50), followed by goat secondary antisera coupled to FITC. The coverslips were mounted in Fluoromount (Fisher Scientific Co., Santa Clara, CA) and viewed on a Nikon microscope equipped with epiluminescence optics (1).

In some experiments, after the cells adhered to the coverslips for 4 h, they were solubilized at 4°C with either the "Triton extraction buffer" for 5 min or the "deoxycholate buffer" for 1 h (see "Differential Integrin Extractions" below) before fixation, permeabilization, and staining with anti-integrin β_1 subunit ($A_{II}B_2$) or rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR) (1:20).

Cell Surface Radioiodination

The cell monolayers from two confluent 10-cm dishes were washed with medium without FCS and were incubated with or without TGFβ_1 (250 pM) for an additional 22 h. The cells were lifted from the plate by a 1.5-min exposure to trypsin-EDTA. The trypsin was inactivated by soybean trypsin inhibitor (Worthington Biochemical, Freehold, NJ), and the cells were washed in iodination buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MnSO_4$). The cells were suspended in 1 mL of iodination buffer, containing 200 mU/mL glucose oxidase and 200 μg/mL (20 U/mL) lactoperoxidase, and were surface-labeled with 1 mCi/mL $Na^{125}I$ as previously described (18). An aliquot of the cell suspension was used to determine cell number. There was no difference in the number of cells obtained from the TGFβ_1-treated cultures compared with the control cultures (ratio of cell number from TGFβ_1 treatment *versus* control = 1.08 ± 0.15, mean ± SD).

^{35}S-Metabolic Labeling

The cell monolayers from three confluent 10-cm dishes were washed with medium free of methionine, cysteine, and FCS

and were incubated with or without TGFβ_1 (250 pM) for an additional 22 h. Ten hours before the experiment, 30 μCi/mL [^{35}S]methionine/cysteine (DuPont NEN, Boston, MA) was added to the medium. At 22 h the cells were scraped from the plate into solubilization buffer (see below).

Solubilization and Immunoprecipitation of Integrins

Membrane proteins were solubilized by extracting the ^{125}I- or ^{35}S-radiolabeled cells for 1 h at 4°C with a 2-mL solution of 200 mM octyl-β-glucopyranoside, 50 mM Tris-HCl, pH 7.4, and 1 mM $MnSO_4$. Protease inhibitors (1 mM PMSF, 2 μg/mL (300 nM) aprotinin, and 10 mM *N*-ethylmaleimide) were added during solubilization and throughout the subsequent procedures. An aliquot of the solubilized membrane extract was subjected to trichloracetic acid precipitation. In each experiment, there was no difference in the trichloracetic acid-precipitable radioactivity in equal volumes of solubilized extracts from control or TGFβ_1-treated cells (ratio of count/min from TGFβ_1 treatment *versus* control = 1.02 ± 0.13, n = 7). Equal volumes of solubilized whole-cell extracts from control and TGFβ_1-treated cells were immunoprecipitated with excess primary antibody and then analyzed by SDS-PAGE as previously described (18). The relative intensity of the immunoprecipitated integrins from each experimental condition was determined by either cutting out the appropriate band of the gel and counting it in a γ or β counter or by densitometric scanning of the autoradiograms made from the gels. Parallel immunoprecipitations with control irrelevant antibodies were performed. Negligible radioactivity was recovered in control precipitates (not shown).

Extraction of Integrins into "Soluble" and "Cytoskeleton-Associated" Fractions

In some experiments, SMC were exposed to TGFβ_1 or control conditions, and were metabolically labeled with [^{35}S]methionine/cysteine as described above. After the 22-h incubation, with or without TGFβ_1, labeled cells were removed from the tissue culture plates with trypsin-EDTA. The trypsin was inactivated by soybean trypsin inhibitor, and the cells were washed twice and resuspended in IDME with 1 mg/mL (16.6 μM) BSA.

Ten-centimeter dishes were precoated with either FN [10 μg/mL (25 nM)] or VN [5 μg/mL (71 nM)] in sterile PBS overnight at 4°C. The plates were then washed with PBS, and nonspecific adherence to the coated dishes was blocked with 1 mg/mL BSA in IDME for 1 h at 37°C.

Equal numbers of control or TGFβ_1-treated cells (8×10^6 cells) were added to the substrate-coated dishes and were allowed to attach for 3 h at 37°C. TGFβ_1 (250 pM) was added to the medium of the cells that had been pretreated with TGFβ_1. The plates were then washed with ice-cold PBS and placed in ice.

Adherent cells were sequentially extracted using a protocol that differentially removes soluble proteins and phospholipids while leaving the cytoskeleton intact (28). Adherent cells were solubilized initially at 4°C in 2 mL of 10 mM Pipes buffer, pH 6.8, containing 0.5% (vol/vol) Triton X-100, 300 mM sucrose, 100 mM KCl, 3 mM $MgCl_2$, 10 mM EGTA, 2 mM PMSF, 20 μg/mL aprotinin, and 10 mM *N*-ethylmaleimide. After 1.5 min, this Triton-containing extraction buffer was removed and transferred to a separate tube on ice. The cells were washed with 0.5 mL of the same buffer for 1 min. This was collected and pooled with the initial 2 mL and referred to as the "soluble" fraction. The Triton-resistant fraction that remained on the dishes was removed by mechanical scraping into 2.5 mL of deoxycholate buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mg/mL (35 mM) sodium deoxycholate, 1% (vol/vol) Triton X-100, 1 mg/mL (3.5 mM) SDS plus PMSF, aprotinin, and *N*-ethylmaleimide]. Tubes containing this fraction were incubated for 1 h at 4°C on a rocking platform and were referred to as the "cytoskeleton-associated" fraction. Tubes containing the "soluble" and "cytoskeleton-associated" fractions were centrifuged for 10 min at 2000 × g followed by 15 min at 15,000 × g. Aliquots of the supernatants were used to determine the protein concentration (BCA protein assay, Pierce, Rockville, IL) and trichloracetic acid-precipitable radioactivity in each fraction. The pH of both radiolabeled supernatant fractions was adjusted to 7.4 before immunoprecipitation. Anti-integrin antibodies were added to extracts of the "soluble" and "cytoskeleton-associated" fractions containing equivalent amounts of protein, and the tubes were incubated overnight at 4°C. The samples were immunoprecipitated and analyzed by SDS-PAGE as described above. Simultaneous studies found similar results (see below) if the fraction extracts were adjusted for equivalent amounts of trichloracetic acid-precipitable radioactivity, instead of protein, before immunoprecipitation (data not shown).

In some experiments the ^{35}S-labeled suspended cells were not placed on substrate-coated plates but were extracted while in suspension to determine the integrin distribution between the "soluble" and "cytoskeleton-associated" fractions in nonadherent cells. The suspended cells were pelleted at 180 × g for 4 min and taken up in Triton extraction buffer for 1 min at 4°C. They were then centrifuged again for 4 min, and the cell pellet was taken up in deoxycholate buffer and rocked for 1 h as described above.

Northern Blotting

Confluent monolayers of DA-SMC were washed with medium without FCS and then were incubated with or without TGFβ_1 (250 pM) for varying time periods. RNA was isolated by the guanidinium-cesium chloride method (29). From each culture condition, 15 μg of total RNA were electrophoresed in 1% agarose gel containing 6% formaldehyde (30). After electrophoresis, gels were stained with ethidium bromide to visualize the positions of 28S and 18S RNA. The RNA was then transferred to nylon membranes (Hybond N, Amersham Corp., Arlington Height, IL) by capillary blotting and fixed to the filter by exposure to UV light. Hybridizations were carried out at 50°C in 50% formamide, 5 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution, 0.1% (3.5 mM) SDS, and 0.3 mg/mL salmon sperm DNA with the appropriate ^{32}P-labeled cDNA probe (30). Filters were washed twice at room temperature for 15 min in 1 × SSC, 0.1% SDS, and once at 55°C in 0.1 × SSC, 0.1% (3.5 mM) SDS for 1 h.

Filters were exposed to x-ray film at −70°C using an intensifying screen.

Integrin subunit cDNA probes (290–360 bp), homologous to the extracellular domain of the respective molecule for α_1 (ovine), α_2 (ovine), α_3 (ovine), α_4 (ovine), α_5 (human), α_6 (ovine), α_8 (ovine), α_v (human), β_1 (ovine), β_3 (ovine), and β_5 (human) were generated in our laboratories, and their identities confirmed by sequencing (31).

The relative intensities of the hybridized radiolabeled bands and the ethidium bromide-stained 28S RNA band of the same gels were determined with a video densitometer (Applied Imaging, Santa Clara, CA). The intensities of the hybridized bands were normalized to the amounts of 28S RNA for each experimental condition and are presented as the ratio of intensity of hybridization from TGFβ_1-treated cells compared with that from control cells.

RESULTS

TGFβ_1 does not affect DA SMC adhesion. DA-SMC adhere to ECM components FN, collagen I, collagen IV, LN, and VN (1). Exposing DA-SMC to TGFβ_1 (250 pM) for 22 h before the adhesion assay had no effect on either initial adhesion to the ECM proteins (30 min) or on the total number of cells that adhered to the ECM after 1.75 h (Fig. 1).

TGFβ_1 has different effects on DA-SMC chemotaxis and migration. TGFβ_1 has a chemoattractant effect for cells of mesenchymal origin including rat aortic vascular SMC (9, 12–14). However, in our experiments, TGFβ_1 had no chemotactic effect for DA-SMC (Fig. 2). In addition, when DA-SMC were incubated with TGFβ_1 for 22 h before the experiment, TGFβ_1-treated cells were much less migratory than control cells (Figs. 3 and 4). The inhibitory effects of TGFβ_1 were seen on all ECM proteins tested and were dependent upon both the length of time that the cells were exposed to TGFβ_1 and the concentration of TGFβ_1 used. The inhibitory effects of TGFβ_1 were seen at 250 and 25 pM but not at 2.5 pM or lower concentrations (data not shown).

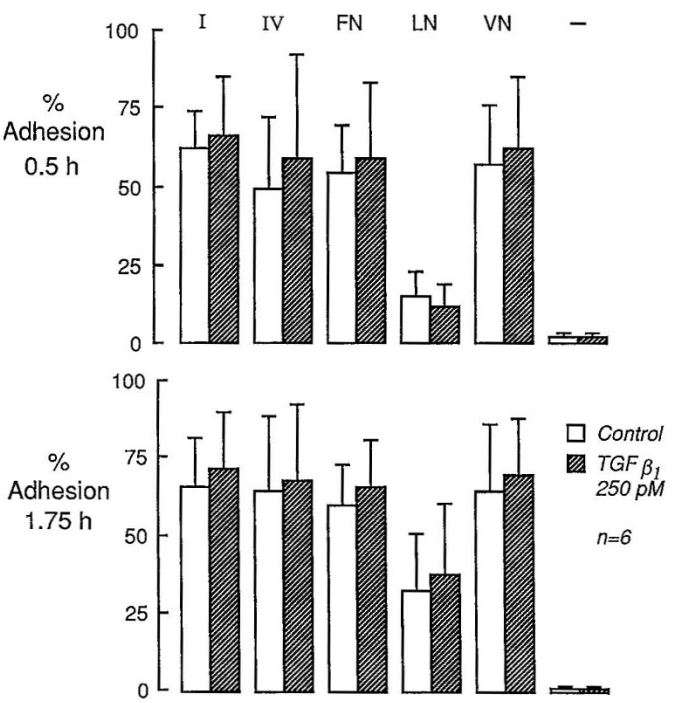

Figure 1. TGFβ_1 does not affect DA-SMC adhesion to collagen I (*I*), collagen IV (*IV*), FN, LN, VN, or BSA (−). SMC, preincubated with or without TGFβ_1 (250 pM) for 22 h, were allowed to attach to the substrate-coated wells for 30 min or 1.75 h. Values represent the mean percentage (±SD) of hexosaminidase activity that remained in the wells in six separate experiments. Data from each experiment were expressed as the mean of three wells.

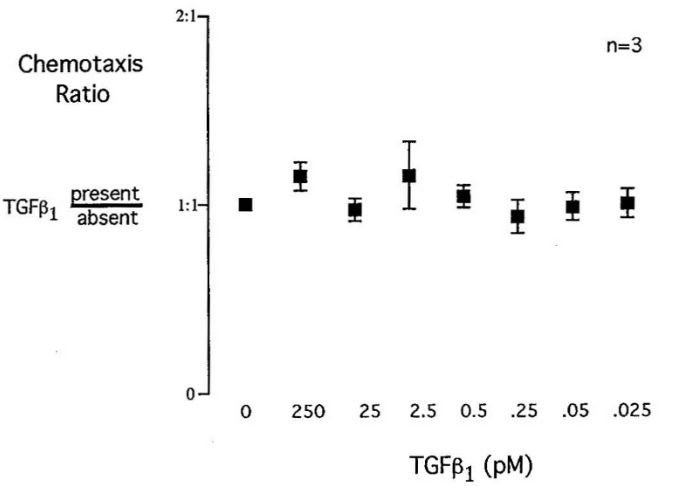

Figure 2. TGFβ_1 is not a chemoattractant for DA-SMC. SMC were placed in the upper well of a modified Boyden chamber and tested for their ability to migrate toward TGFβ_1 in the lower chamber. Data from each experiment were expressed as the ratio of the number of cells migrating to the lower surface of the membrane in the presence of TGFβ_1 (*present*) *vs* the number migrating in its absence (*absent*). The *squares* represent the average ratio (±SD) for three experiments. Chemotaxis ratios >1 indicate increased migration due to the presence of TGFβ_1. At all concentrations of TGFβ_1 tested, the chemotaxis ratio was essentially 1.

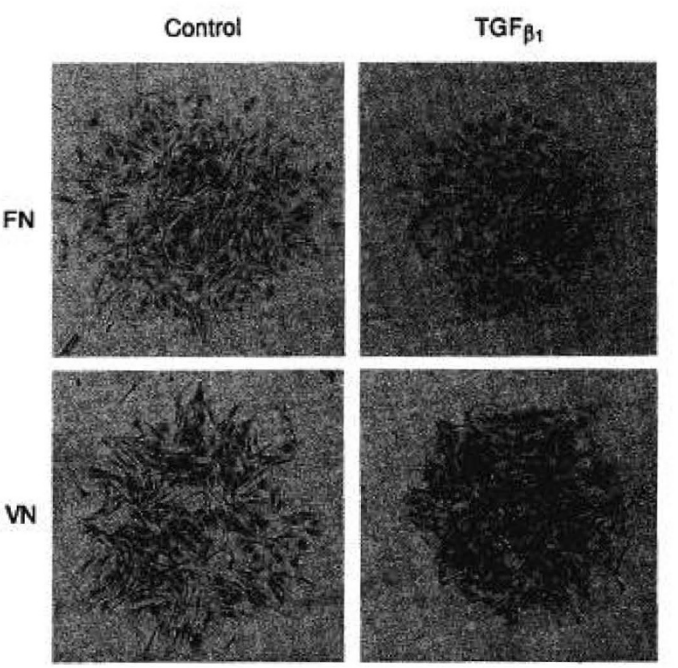

Figure 3. Appearance of DA-SMC, treated with or without TGFβ_1 (250 pM) after a 7-h migration assay on FN [10 μg/mL (25 nM)] or VN [5 μg/mL (71 nM)].

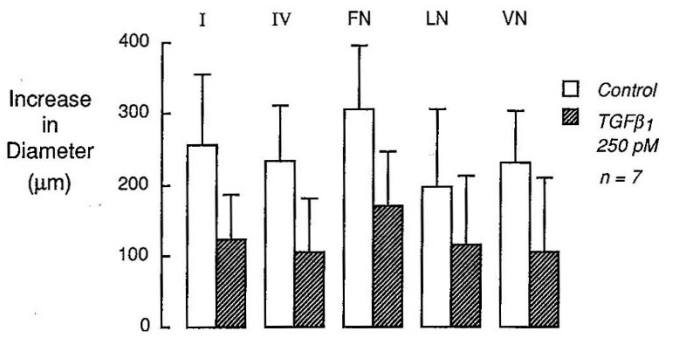

Figure 4. TGFβ_1 inhibited DA-SMC migration on collagen I [*I*, 5 μg/mL (17 nM)], collagen IV [*IV*, 1 μg/mL (1.8 nM)], FN [10 μg/mL (25 nM)], LN [25 μg/mL (31 nM)], and VN [5 μg/mL (71 nM)]. SMC, preincubated for 22 h with or without TGFβ_1 (250 pM), were allowed to out-migrate on polystyrene sheets coated with different substrates. TGFβ_1 was added to the media of appropriate wells. The initial diameter at 0 h was 940 ± 5 μm. Values represent the mean ± SD of the increase in diameter of the cellular area over 7 h in seven experiments. Data from each experiment were expressed as the mean of eight wells. By using a paired *t* test, we found that TGFβ_1-treated cells migrated at a significantly ($p < 0.05$) slower rate on collagens I and IV, FN, LN, and VN.

TGFβ_1 alters SMC interactions with the ECM. TGFβ_1-treated cells appeared more spread out and rectangular than the spindle-shaped control cells (Fig. 3). As TGFβ_1-treated cells spread on the ECM substratum (FN, VN, and collagens I and IV), they formed an increased number of focal adhesion plaques compared with control cells (Fig. 5). Focal adhesion plaques are regions of the plasma membrane where bundles of actin filaments terminate and associate with clusters of integrin receptors. To see whether this increased plaque formation was due to an increased expression of cell surface integrin receptors, SMC were grown in the presence of TGFβ_1 for up to 22 h and were analyzed for changes in mRNA expression of individual integrin subunits. DA-SMC previously have been shown to express specific integrin receptors that bind to unique ECM molecules: $\alpha_1\beta_1$ (collagens I and IV, LN), $\alpha_2\beta_1$ (collagens I and IV), $\alpha_3\beta_1$ (FN), $\alpha_5\beta_1$ (FN), $\alpha_6\beta_1$ (LN), $\alpha_v\beta_1$ (FN, VN), $\alpha_v\beta_3$ (VN, LN, FN, collagens I and IV) (1). In the current experiments, using Northern analysis, we detected low to moderate amounts of α_1, α_2, α_5, α_6, α_v, β_1, β_3, and β_5 mRNAs. We detected no α_3, α_4, α_7, or α_8 transcripts. TGFβ_1 had only a small effect on the overall mRNA expression of integrin subunits in DA-SMC (Fig. 6). There was only a modest increase in the mRNA expression of α_5 and a slight decrease in the expression of β_3. There was no change in the mRNA expression of any of the other integrin subunits examined (Fig. 6). Similarly, we found that except for a modest increase in the amount of the α_5 subunit (immunoprecipitated as the $\alpha_5\beta_1$-FN receptor) (Fig. 6), there was no change in the presence of either surface (^{125}I) or metabolically (^{35}S) labeled integrins. There-

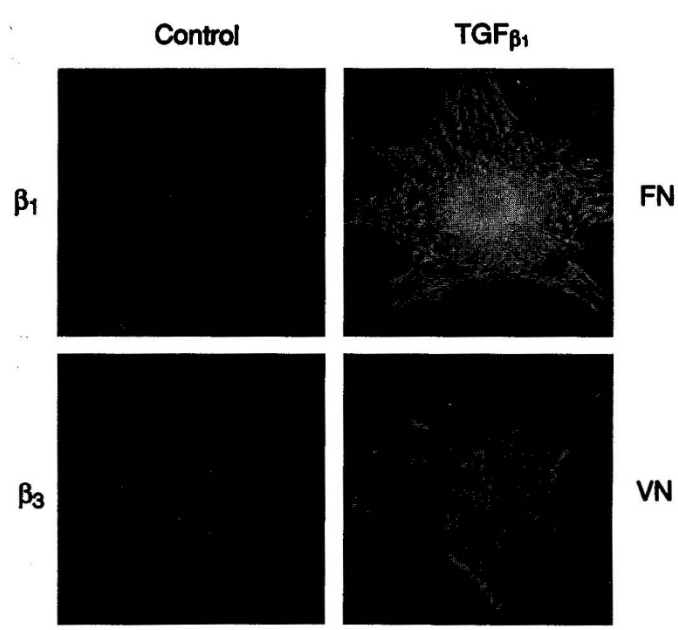

Figure 5. Increased staining of β_1 and β_3 integrin receptors in focal adhesion plaques of TGFβ_1-treated cells. DA-SMC, preincubated for 22 h with or without TGFβ_1 (250 pM), adhered to coverslips coated with FN or VN for 4 h as described in "Methods." After the samples were fixed and permeabilized, they were stained with antibodies to β_1 ($A_{II}B_2$) or β_3 (E8).

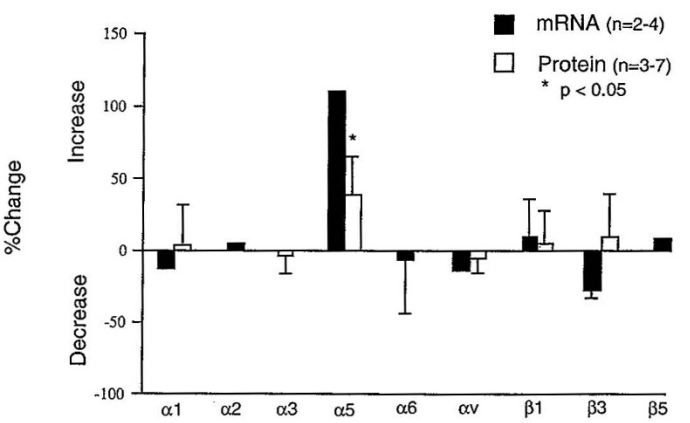

Figure 6. Effects of TGFβ_1 on integrin expression in DA-SMC. mRNA: SMC were treated with or without TGFβ_1 (250 pM) for 22 h before RNA isolation and Northern blotting (see "Methods"). The relative intensity of hybridization of a specific probe with mRNA from control and TGFβ_1-treated cells was determined for each of two experiments (for α_1, α_2, α_3, α_4, α_5, α_8, α_v, and β_5), three experiments (for α_6 and β_1), or four experiments (for β_3). *Closed bars* represent the mean (± SD, when $n > 2$) percent change in hybridization of individual probes with mRNA from TGFβ_1-treated cells compared with control cells. In these experiments, there was no hybridization of α_4 or α_8 and minimal hybridization of α_3 with mRNA from both control and TGFβ_1-treated cells; no percent change in hybridization was calculated for these integrin subunits. Similar results were obtained for cells incubated with or without TGFβ_1 for 6 h (data not shown). Immunoprecipitation: SMC were treated with or without TGFβ_1 (250 pM) for 22 h and then were either surface-labeled with ^{125}I (four experiments) or metabolically labeled with ^{35}S (three experiments). Equal aliquots of detergent extracts were processed for immunoprecipitation, SDS-PAGE, and autoradiography as described in "Methods" using the following antibodies and antisera: α_3 (anti-α_3), α_5 (anti-α_5), α_v (anti-α_v), β_1 ($A_{II}B_2$), and β_3 (E8). The amount of radiolabeled α_1 subunit was determined from the α_1 band obtained from the proteins immunoprecipitated with the $A_{II}B_2$ antibody (see Ref. 1). The gels were run under nonreducing conditions. The relative intensity of individual integrin subunit radiolabeling for control and TGFβ_1-treated cells was determined for each experiment (as described in "Methods"). The *open bars* represent the mean (±SD) percent change in intensity in TGFβ_1-treated cells compared with control cells from seven experiments (α_1, α_5, α_v, β_1, and β_3) or three experiments (α_3). The percent change in intensity was calculated as: [(intensity$_{\text{TGF}\beta1\text{ cells}}$ − intensity$_{\text{control cells}}$) ÷ intensity$_{\text{control cells}}$] × 100. The horizontal bars are the average percent changes for each integrin subunit. Only α_5 was consistently increased in each individual experiment ($p < 0.05$, *t* test) in TGFβ_1-treated cells.

fore, the increased condensation of β_1 and β_3 integrins into focal adhesion plaques when TGFβ_1-treated cells attached to ECM molecules did not appear to be due to increased synthesis of integrins specific for these ECM molecules.

The increased presence of the $\alpha_5\beta_1$-FN receptor in TGFβ_1-treated SMC was consistent with the observation that these cells remodeled existing FN substrata into a fibrillar matrix to a much greater extent than untreated cells (Fig. 7). We considered the possibility that the increased number of $\alpha_5\beta_1$-FN receptors might make the TGFβ_1-treated cells less mobile by increasing their adhesion to FN (32). Several observations disagree with this hypothesis: Boudreau *et al.* (33) found that a pentapeptide containing the amino acid sequence Arg-Gly-Asp, which inhibits $\alpha_5\beta_1$ from interacting with FN, inhibited rather than stimulated DA-SMC migration. Similarly, in our experiments, antibodies to FN, which prevent the cells from adhering to FN, inhibited rather than stimulated DA-SMC migration (Fig. 8). In addition, antibodies against FN had no stimulatory effect on the migration of TGFβ_1-treated cells; rather, they limited the migration of TGFβ_1-treated cells even further (Fig. 8). Therefore, it is unlikely that TGFβ_1 inhibits DA-SMC migration by specifically increasing the cells' adhesiveness to FN; rather, it appears that DA-SMC use FN to migrate on other ECM substrata even when treated with TGFβ_1.

We next considered the possibility that the increased coalescence of integrin receptors into focal plaques was due to an increased association of integrins with the actin cytoskeleton. SMC integrins were labeled with ^{35}S and extracted according to a protocol that preferentially removed plasma membrane proteins that were not tightly associated with the cytoskeleton. Initial brief exposure of the cells to Triton X-100 buffer left their cortical cytoskeleton intact. Similarly, Triton buffer extraction did not alter the distribution of integrins in the focal plaques on the cells' basal surface. On the other hand, exposure of the cells to deoxycholate buffer caused disruption of the cells' actin cytoskeleton and loss of integrin-associated focal plaques (28) (data not shown).

When nonadherent cells (*i.e.*, cells maintained in suspension) were solubilized according to the above schema, the

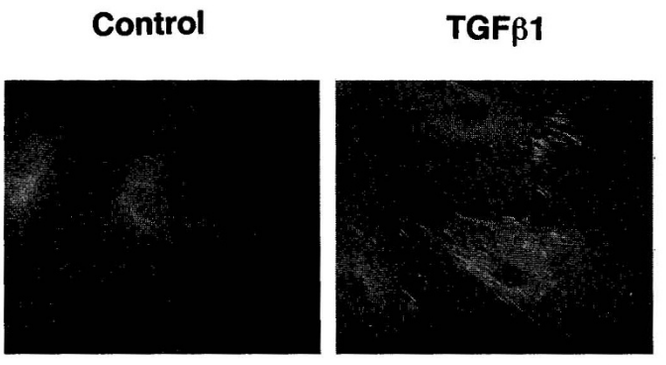

Figure 7. TGFβ_1-treated cells have increased remodeling of a preexisting FN matrix. DA-SMC, preincubated for 22 h with or without TGFβ_1 (250 pM), adhered to coverslips coated with FN for 4 h as described in "Methods." Staining the samples with anti-FN antiserum demonstrated increased clearing (*black areas*) of the FN substratum (*black arrows*) by TGFβ_1-treated cells.

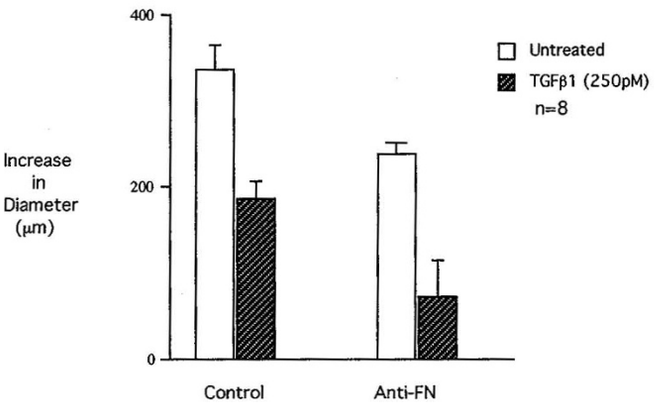

Figure 8. Antiserum against FN inhibited migration of both TGFβ_1-treated and untreated cells. DA-SMC, pretreated for 22 h with TGFβ_1 (250 pM), were assayed for out-migration on collagen I substratum as described in Figure 4. Antiserum against FN (1:40) was added to the media of some wells. In other experiments, this concentration of anti-FN antibody blocked SMC adhesion to FN [10 μg/mL (25 nM)] by 78 ± 23% (n = 4) without affecting cell adhesion to LN [50 μg/mL (62 nM)] or collagen I [2.5 μg/mL (8.5 nM)]. Values represent the means ± SD of the increase in diameter over 7 h in eight experiments. Data from each experiment were expressed as the mean of eight wells. By using a paired t test, we found that anti-FN inhibited the migration of both TGFβ_1-treated and untreated cells ($p < 0.05$).

$\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrin heterodimers were completely extracted into the Triton X-100 soluble fraction. Pretreatment of the cells with TGFβ_1 did not promote cytoskeletal association of the integrins when the cells were in suspension (Fig. 9). When SMC adhered to a substratum of VN or FN, there was an increase in the association of the integrins with the cytoskeleton. When cells were adherent to VN, most (75%) of the $\alpha_v\beta_3$ (VN receptor) was extracted with the cytoskeletal framework (Fig. 9). In contrast, the $\alpha_1\beta_1$ (collagen/LN receptor) and the $\alpha_5\beta_1$ (FN receptor) integrins had a looser association with the cytoskeleton. When SMC were pretreated with TGFβ_1 and allowed to adhere to VN, there was increased anchoring of $\alpha_v\beta_3$ (VN receptor) to the cytoskeleton (Fig. 9). At the same time, TGFβ_1-treated cells that adhered to VN did not increase the cytoskeletal anchoring of either the $\alpha_1\beta_1$ or $\alpha_5\beta_1$ integrins.

To test the specificity of this observation, we examined the behavior of integrins when SMC adhered to FN. In DA-SMC, $\alpha_v\beta_3$ binds weakly to FN (1). When cells adhered to FN, the $\alpha_5\beta_1$ (FN receptor) integrin was tightly coupled (71%), whereas the $\alpha_1\beta_1$ and $\alpha_v\beta_3$ integrins were loosely associated with the cytoskeleton (Fig. 9). Treatment of SMC with TGFβ_1 produced an even tighter association of the $\alpha_5\beta_1$ receptor with the cytoskeleton. In contrast, the association of $\alpha_1\beta_1$ and $\alpha_v\beta_3$ with the cytoskeleton did not change when TGFβ_1-treated cells adhered to FN. Thus, TGFβ_1 did not seem to cause a generalized increase in anchoring of the cytoskeleton to all of the cell's integrin receptors; rather, increased anchoring of integrins to the cytoskeleton was specific only for those integrins that were used in binding to the particular substratum that the cell was encountering (*i.e.* increased anchoring of the VN receptor ($\alpha_v\beta_3$) when cells were plated on VN, and of the FN receptor ($\alpha_5\beta_1$) on FN).

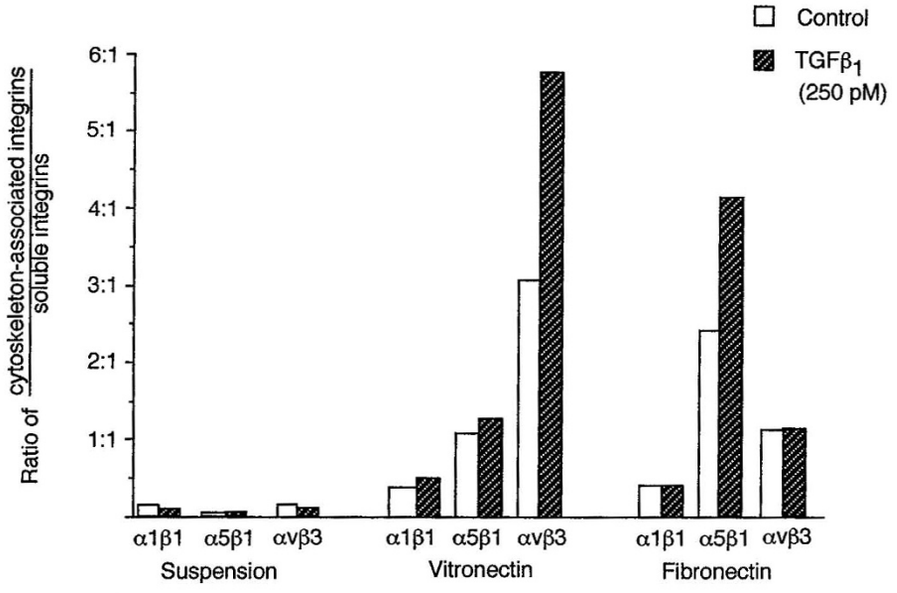

Figure 9. TGFβ_1 increases the cytoskeletal association of the specific integrins used to adhere to the substratum. DA-SMC that had been pretreated for 22 h with or without TGFβ_1 and metabolically labeled with ^{35}S (see "Methods") were maintained in suspension or allowed to attach to either a VN or a FN substratum. SMC were then extracted with Triton X-100-containing buffer to obtain the "soluble" fraction, followed by deoxycholate buffer to obtain the "cytoskeleton-associated" fraction. Aliquots of the two separate cell extracts, containing equal amounts of protein, were immunoprecipitated with anti-α_5, poly-β_3 (or E8), and $A_{II}B_2$ to determine the amount of radiolabeled $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_1\beta_1$ (Fig. 6) associated with each fraction, respectively. The samples were resolved by SDS-PAGE under nonreducing conditions, and the relative amount of integrin subunits in each fraction was determined as described in "Methods." Values expressed in the figure represent the ratio of intensity of radiolabeling in the "cytoskeleton-associated" fraction compared with the "soluble" fraction.

DISCUSSION

Processes required for SMC migration play a key role in the ability of the DA to undergo permanent anatomical closure. SMC use integrin receptors, as well as the ECM and growth factors, to modulate the phenotype and migratory properties of the cells (1, 9, 34, 35). DA-SMC express several integrin receptors of the β_1 and β_3 integrin families that enable the cells to adhere to and migrate on ECM components like FN, LN, and collagens I and IV. While SMC adhesion to these substrata is entirely dependent on the presence of functioning β_1 integrins, cell migration depends on both β_1 and β_3 integrins (1).

In the present study, we examined the effects of TGFβ_1, an important growth factor involved in tissue remodeling and repair, on the migration and integrin expression of SMC cultured from the fetal sheep DA. TGFβ_1 has been found in the fetal DA (5) and observed to increase dramatically during postnatal ductus closure (Tannenbaum JE, Waleh NS, Gold L, Perkett E, Clyman RI, manuscript in preparation). Aortic SMC exposed to TGFβ_1 show an increased expression of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ integrin receptors (10, 11). In the present study, TGFβ_1 increased both expression of the α_5 subunit mRNA and $\alpha_5\beta_1$ receptor protein in SMC derived from the DA (Fig. 6). However, TGFβ_1 did not appear to affect the expression of any of the other integrin subunits that have been isolated from DA-SMC. In particular, there was no change in the presence of the $\alpha_v\beta_3$ integrin and, if anything, there was a consistent modest decrease in β_3 mRNA in three experiments (Fig. 6). The observed increase in the $\alpha_5\beta_1$ FN receptor in DA-SMC treated with TGFβ_1 is consistent with the finding that these cells remodel preexisting FN substratum much more extensively than cells not treated with TGFβ_1 (Fig. 7).

The effects of TGFβ_1 on cell migration depend on the origin of the cells studied: stimulatory for monocytes (13) and fibroblasts (12), inhibitory for endothelial cells (3). In general, TGFβ_1 appears to enhance the migration and act as a chemoattractant for vascular SMC derived from the aorta (3, 11, 14). However, TGFβ_1 also has been shown to suppress the chemotactic effects of other chemoattractants (14, 36) and to inhibit the migration of saphenous vein SMC (37). The stimulatory effects of TGFβ_1 on aortic SMC migration appear to be due to increased FN production and increased $\alpha_5\beta_1$ FN receptor expression by SMC (38). The exact role of the $\alpha_5\beta_1$ integrin in cell migration is unclear. In one study, overexpression of the $\alpha_5\beta_1$ integrin in Chinese hamster ovary cells increased their migratory behavior (39); in contrast, other investigators found that expression of the $\alpha_5\beta_1$ integrin in Chinese hamster ovary cells suppressed their migratory, transformed phenotype (32). FN appears to facilitate the migration of DA-SMC through a collagen gel (33). Our results support the facilitating role of FN in DA-SMC migration. We previously (19) observed that DA-SMC make FN and LN during their migration over a two-dimensional collagen substratum. In the current experiments, we found that antibodies against FN (and against LN; data not shown) inhibit the cells' ability to migrate on collagen; similarly, antibodies against FN (and LN; data not shown) further inhibit the migration of DA-SMC that have been treated with TGFβ_1 (Fig. 8).

Despite its stimulatory effects on $\alpha_5\beta_1$ integrin expression, TGFβ_1 did not increase DA-SMC migration. In contrast with aortic SMC (14), DA-SMC, exposed to TGFβ_1 for short intervals, displayed no evidence of chemotaxis (Fig. 2) (or chemokinesis; data not shown). Rather than stimulating migra-

tion, long-term exposure to TGFβ_1 inhibited overall DA-SMC migratory behavior (Figs. 3 and 4).

The mechanism(s) by which TGFβ_1 inhibits DA-SMC migration is unclear. Agents that promote strong substrate attachment appear to be antithetical to motility (40–44). Stationary cells seem to use their focal contacts as localized sites of very strong adhesion, ill-suited for migration (45). Migrating cells have fewer focal contacts and diminished stress fiber organization (46–48). In addition, the integrin receptors on locomoting cells have high lateral motility, whereas those on stationary cells are relatively immobile (48). We found that decreased migration after treatment of DA-SMC with TGFβ_1 is associated with increased cytoskeletal anchoring of integrin receptors, but only after they adhered to the surrounding ECM (Fig. 9). This, in turn, is associated with an increased organization of integrin receptors into focal adhesion plaques (Fig. 5) and a more rectangular appearance of the cells. The mechanisms by which TGFβ_1 enhances the integrin-cytoskeletal association in DA-SMC are unknown. Phosphorylation and dephosphorylation events may be crucial to this process because protein kinase C, cAMP-dependent kinases, tyrosine kinases, and myosin light chain kinase are all known to phosphorylate cytoskeletal and membrane components involved in adhesion plaque formation (49). The increased plaque formation and decreased cell migration after TGFβ_1 treatment also could be due to the release of yet another ECM component (other than FN or LN) that might inhibit SMC migration (10, 50–53). However, our observations do not support this hypothesis. We found that after TGFβ_1 treatment, the increased anchoring of integrins to the cytoskeleton is limited to only those specific integrins that are involved with binding the original substratum. Therefore, TGFβ_1 appears to impede migration by promoting cytoskeletal organization and/or focal plaque stabilization of those integrin receptors that are already engaged by the surrounding ECM.

Thus DA-SMC have a unique and complex migratory response to TGFβ_1. TGFβ_1 increases the cell surface integrin $\alpha_5\beta_1$, which facilitates DA-SMC migration; however, this effect is outweighed by the ability of TGFβ_1 to inhibit cell movement by increasing integrin anchoring to the cytoskeleton. Through its multiple effects on DA-SMC, TGFβ_1 may play an important role in modulating SMC migration in the closing DA.

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