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The Effect of Extracellular Matrix Proteins on Porcine Smooth Muscle Cell Insulin-like Growth Factor (IGF) Binding Protein-5 Synthesis and Responsiveness to IGF-I*

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The aim of this study was to determine if cultured porcine vascular smooth muscle cells (pSMCs) that had been maintained on different extracellular matrix proteins had an alteration in their expression of insulin-like growth factor binding protein-5 (IGFBP-5) and their responsiveness to insulin-like growth factor-I (IGF-I). When pSMCs were plated on fibronectin, they synthesized 6.0 ± 1.2-fold more IGFBP-5 than did cells maintained on laminin and type IV collagen. IGF-I increased IGFBP-5 gene expression 3-fold in the cells plated on fibronectin. The addition of an RGD peptide and echistatin to pSMC cultures that had been plated on fibronectin inhibited IGFBP-5 mRNA expression. The addition of an antibody against $\alpha_2\beta_1$ integrin partially reversed the inhibitory effects of laminin and type IV collagen on IGFBP-5 expression. Cells maintained on fibronectin had a 5.0 ± 1.1-fold greater DNA synthesis response to IGF-I compared with those maintained on laminin/type IV collagen, and echistatin significantly inhibited the DNA synthesis response of the fibronectin-maintained cells to IGF-I. The anti-$\alpha_2\beta_1$ antibody partially reversed the inhibitory effect of laminin and type IV collagen on IGF-I-stimulated DNA synthesis. The addition of IGFBP-5 to cultures plated on laminin and type IV collagen significantly increased their response to IGF-I. Atherosclerotic plaques from pig aorta contained abundant fibronectin and had increased IGFBP-5 mRNA (4.5 ± 1.5-fold) compared with tissue from the normal vessel wall that had a low fibronectin content. These results indicate that fibronectin, laminin, and type IV collagen have major effects on IGFBP-5 expression and on IGF-I-stimulated pSMC responses and that these effects are mediated by their respective integrins.

Vascular smooth muscle cells (SMCs)[1] in the media of normal blood vessels are surrounded by a basement membrane that contains type IV collagen and laminin and maintain a differentiated, contractile state characterized by abundant myofilaments and expression of contractile protein isoforms such as α-actin (1). They are quiescent, and the proliferative index is low (1). In contrast, SMCs in the neointima seem to have dedifferentiated. This dedifferentiation includes a marked structural reorganization of the cells, with loss of myofilaments and the formation of an extensive rough endoplasmic reticulum and large Golgi complex (2–4). Their abundance of smooth muscle α-actin decreases, whereas the expression of nonmuscle β- and γ-actin increases (2–4). They synthesize increased amounts of fibronectin and deposit it into their extracellular matrix (ECM). This is termed the synthetic phenotype, and these cells have acquired the ability to proliferate, migrate, and synthesize ECM in response to stimulation by growth factors and cytokines (5). Additionally, cells in the synthetic phenotype constitutively synthesize peptide growth factors, and they have the ability to replicate in the absence of exogenously added mitogens (1).

Extracellular matrix has been shown to play an important role in the modulation of the SMC phenotype in culture (6). Aortic SMCs assume the synthetic phenotype when they are cultured on a fibronectin substratum and will proliferate in response to stimulation by appropriate growth factors. In contrast, if the cells are cultured on laminin and type IV collagen, they will remain in the contractile phenotype. These ECM proteins function by binding to their integrin receptors on the cell surface. Cultured SMCs express the $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins, which bind fibronectin, and the $\alpha_2\beta_1$ integrin, which binds to laminin and several forms of collagen (7).

The growth and differentiation of SMCs are modulated by a number of peptide growth factors, including the IGFs. SMCs in culture have been shown to synthesize IGF-I, and IGF-I stimulates SMC proliferation in an autocrine fashion (8–10). *In vivo*, IGF-I mRNA and immunoreactive IGF-I are detected in atherosclerotic lesions that develop after injury (11), and the increase in IGF-I mRNA and immunoreactive peptide after balloon denudation injury precedes an associated increase in SMC proliferation (12, 13). IGF-I has also been shown to stimulate SMC migration (14), and this response is mediated by the IGF-I receptors but also requires ligand occupancy of the $\alpha_V\beta_3$ integrin. This ligand occupancy of integrins may play a role in controlling IGF-I responsiveness.

The bioactivities of IGFs are modulated by a group of specific, high affinity IGF binding proteins (IGFBPs) (15). Previous studies have shown that porcine aortic SMCs secrete IGFBP-2, -4, and -5 (16, 17) and that IGFBP-5 can modulate IGF-I-stimulated cell proliferation and migration (18). Because modulation of their phenotypic state alters pSMC responses to peptide growth factors, we determined the ability of specific EMC components to regulate IGFBP-5 expression and the responsiveness of these cells to IGF-I.

EXPERIMENTAL PROCEDURES

Materials—Human fibronectin, type IV collagen, laminin, and echistatin were purchased from Sigma. The peptides GRGESP and

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[1] The abbreviations used are: SMC, smooth muscle cell; IGFBP-5, insulin-like growth factor binding protein-5; IGF-I, insulin-like growth factor I; pSMC, porcine vascular smooth muscle cells; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; SM, smooth muscle.

GRGDSP were purchased from Telios Pharmaceuticals Inc. (San Diego, CA). Recombinant human IGF-I was obtained from Bachem Inc. (Torrance, CA). Polyvinylidene difluoride filters were supplied by Millipore Corp. Autoradiographic film was purchased from Eastman Kodak (Rochester, NY). Fetal bovine serum, Dulbecco's modified Eagle's medium, and penicillin-streptomycin were purchased from Life Technologies, Inc. P1E6, a monoclonal antibody against the $\alpha_2\beta_1$ integrin, was a gift from Dr. Leslie Parise (Department of Pharmacology at UNC-Chapel Hill). Trypsin was obtained from Boehringer Mannheim. [^{3}H]Thymidine was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). [^{32}P]dCTP was purchased from Amersham Corp. Polyclonal antisera against IGFBP-2 and -5 were prepared as described previously (19, 20, 39). A monoclonal antibody that was specific for smooth muscle α-actin and rhodamine-labeled phalloidin were purchased from Sigma.

Maintenance of Porcine Aortic Smooth Muscle Cells (pSMCs) in Culture—pSMCs were obtained from thoracic aortas of pigs using a previously described method (21). They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5/liter glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 10% fetal bovine serum (complete medium) in 10-cm tissue culture plates (Falcon 3001). The cells were used between passages 2 and 5. To culture the cells on specific ECM proteins, the cells were detached with trypsin/EDTA, trypsin was neutralized with 10% fetal bovine serum, and the cells were rinsed twice with serum-free DMEM and suspended in the same medium. They were then plated on tissue culture plates or glass coverslips that had been exposed to specific ECM proteins at a density of 2×10^4 cell/cm^2. The surfaces of culture dishes or glass coverslips were precoated with fibronectin (1 μg/cm^2) or type IV collagen (2 μg/cm^2) plus laminin (1 μg/cm^2) by incubating the mixture at room temperature for 4–5 h as recommended by the manufacturer. The liquid was then aspirated, and the dishes were rinsed twice with PBS and maintained in DMEM-H/0.01% bovine serum albumin for 15 min before use. To attach IGFBP-5 to matrix proteins, the plates that contained the ECM proteins were rinsed twice with binding buffer (Eagle's minimal essential medium without bicarbonate, supplemented with 20 mM Hepes, pH 7.4, and 0.01% bovine serum albumin) and incubated overnight at 4 °C with the same buffer and 100 ng/ml of IGFBP-5 or with buffer alone. The wells were then rinsed twice with PBS before use. In some experiments, the cells were incubated with an RGD peptide, echistatin, or anti-$\alpha_2\beta_1$ antibody (P1E6) at the indicated concentration for 15 min before the cells were plated on culture dishes.

Immunocytochemistry—The cells were plated on glass coverslips precoated with ECM substrates for 2 days before they were fixed in 2% formaldehyde in PBS for 20 min and then permeabilized with 0.2% Triton X-100 in PBS for 3 min. To determine the expression of smooth muscle α-actin, the cells were exposed to a 1:200 dilution of a monoclonal antibody prepared against smooth muscle α-actin for 2 h followed by 1:500 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG for 30 min. To determine the expression of total actin, the cells were exposed to 1:500 dilution of rhodamine-labeled phalloidin. They were rinsed with PBS, mounted in 90% glycerol with 0.1% paraphenylenediamine, and then analyzed using a Nikon Labophot microscope with epifluorescence optics.

RNA Isolation and Northern Blotting—RNA was isolated from cell cultures by Tri-Reagent following the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH) and then quantified by measuring UV absorbance at 260 nm. To isolate RNA from pig aortas, the tissue specimens were obtained from atherosclerotic lesions and from normal aortic subendothelium from six pigs who had been fed a high fat diet for 4 months (22). These are early proliferative phase lesions that contain abundant monocytes, macrophages, and smooth muscle cells. The lesion tissue was immediately frozen in liquid nitrogen and pulverized at −80 °C using pre-cooled mortar and pestle. The pulverized tissues were then homogenized for 1 min with Polytron homogenizer in the presence of Tri-Reagent. RNA was isolated as described previously (17). The RNA samples were size fractionated on a 1.2% agarose/formaldehyde gel, blotted, fixed onto nylon membranes (Biotrans, ICN Biochemical Inc.), and then hybridized with either a [^{32}P]dCTP-labeled human 627-base pair IGFBP-5 cDNA probe (17) or a 542-base pair IGFBP-2 cDNA probe (15). The probes were labeled to specific activities of 200 μCi/μg by random priming (15). The relative abundance of the radiolabeled bands was determined by PhosphorImage analysis using Image Quant SF (Molecular Dynamics).

Western Immunoblotting Analysis—The pSMC cultures were rinsed with PBS and scraped into SDS sample buffer containing 100 mM dithiothreitol. The protein concentration of each sample was determined by the BCA protein assay (Pierce), and equal amounts of protein (5 μg) per lane were loaded onto 12.5% SDS-polyacrylamide gels. The separated proteins were then transferred to filters (Immobilon PSQ, 0.45-mm pore size, Millipore). The filters were exposed to a monoclonal antibody against smooth muscle α-actin (diluted at 1:1,000 in TBS containing 3% bovine serum albumin) for 14 h at 4 °C. The immunoblots were developed with the enhanced chemiluminescence detection system according to the manufacturer's recommendation (Amersham). To determine the fibronectin content in the aortic tissue specimens, the tissue was immediately frozen in liquid nitrogen and then pulverized at −80 °C. The pulverized tissues were transferred into tubes to which lysis buffer containing 20 mM Tris-HCl, pH 7.4, 2% Triton X-100, 10 mM EDTA had been added. The suspension was homogenized for 1 min at 4 °C followed by centrifugation for 5 min at 12,000 $\times$ *g* at 4 °C. The protein concentration in the supernatant was measured and adjusted to 1 mg/ml by adding 2 $\times$ Laemmli sample buffer with 0.1 M dithiothreitol. 30 μg of protein was loaded per gel lane, and the proteins in the mixture were separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1 M dithiothreitol. The separated proteins were transferred to Immobilon PSQ filters. Fibronectin was detected with anti-fibronectin polyclonal antibody using a 1:1000 dilution, and the immune complexes were detected by chemiluminescence according to the procedure cited above.

Immunoprecipitation of IGFBPs—To analyze IGFBP synthesis and secretion into conditioned medium, pSMC monolayers were exposed to 50 μCi/ml [^{35}S]methionine (56 Ci/mmol) for 8 h in methionine-deficient (10^{-6} M) DMEM. The medium was incubated with a 1:1000 dilution of anti-IGFBP-5 or 1:1000 dilution of anti-IGFBP-2 antisera, and the immune complexes were precipitated with protein A-Sepharose as described previously (17). The precipitates were analyzed by SDS-PAGE with fluorography and autoradiography (17).

Cell Replication—To measure [^{3}H]thymidine incorporation into porcine smooth muscle cells, pSMCs were plated at a density of 2×10^4/cm^2 in 48-well culture plates (Costar 3548) that had been precoated with fibronectin or type IV collagen and laminin with or without IGFBP-5, as described previously. In some experiments, pSMCs were preincubated with echistatin 10^{-7} M or the P1E6 antibody at a dilution of 1:500 for 15 min before they were plated on the fibronectin or laminin plus type IV collagen. The cultures were maintained for 24 h in serum-free DMEM (with or without the various treatments); then, fresh DMEM (0.1 ml) supplemented with 0.2% human platelet-poor plasma (21), 1 μCi/well [^{3}H]thymidine (specific activity, 35 mCi/mmol), and IGF-I (0–20 ng/ml) were added. After 24 h, the plates were placed on ice, washed with ice-cold phosphate-buffered saline twice, and incubated with ice-cold 5% trichloroacetic acid for 10 min. The trichloroacetic acid precipitates were solubilized by adding 0.1 ml of 1% SDS, 0.1 N NaOH overnight, and radioactivity was quantified using a Beckman scintillation counter using ScintiSafe Econo 2 (Fisher Scientific) as a scintillant. In some experiments, the cell number was determined in a particle data counter (model ZBI, Coulter Electronics, Hialeah, FL). For these experiments, the cells were plated at a density of 2×10^4 cells/cm^2 in 24-well plates (Costar 3424). After 24 h, the treatments were added, and the incubation continued for 36 h. The cells were detached with 0.2% trypsin, and the cell number was determined.

RESULTS

Expression of Smooth Muscle (SM) α-Actin—SM α-actin was used as a marker for SMC differentiation. Indirect immunofluorescence microscopy showed that the percentage of cells with microfilament bundles that stained intensely for smooth muscle α-actin was greater when the cultures were plated on laminin plus type IV collagen compared with cultures plated on fibronectin (Fig. 1*A*). Immunoblotting analysis with a monoclonal antibody against smooth muscle α-actin was also used to examine the effect of the different substrata on the level of smooth muscle α-actin expression. A single band of 42 kDa was detected. A direct comparison by immunoblotting showed that pSMCs cultured on fibronectin contain 4.7-fold less smooth muscle α-actin than the cells plated on laminin plus type IV collagen (Fig. 1*B*). Thus, the cells that were plated on laminin/type IV collagen maintained this important property of differentiated pSMC. In contrast, the expression of total actin was not decreased in pSMCs plated on fibronectin, as demonstrated using rhodamine-labeled phalloidin (Fig. 1*C*).

Extracellular Matrix Proteins Regulate IGFBP-5 Synthesis and Secretion—Cultures that were plated on fibronectin or

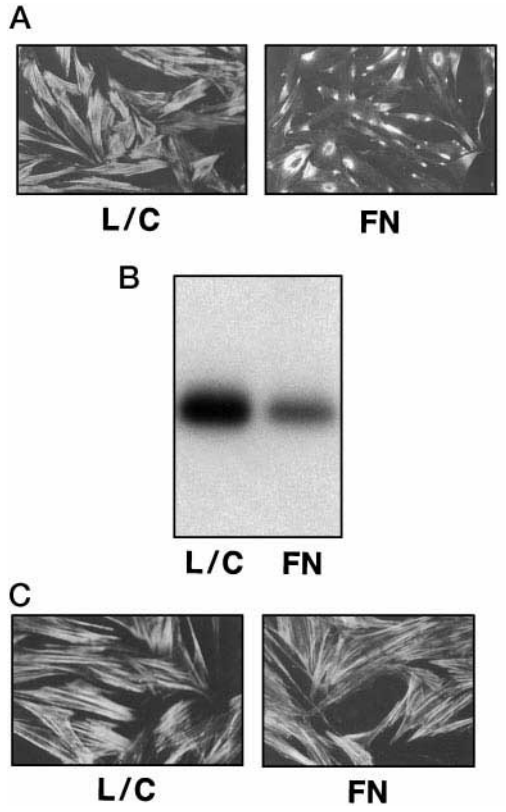

FIG. 1. *A*, immunohistochemical staining of smooth muscle α-actin in pSMCs cultured on fibronectin (FN) or laminin plus type IV collagen (L/C) for 48 h. The cells were exposed to monoclonal anti-SM α-actin for 2 h followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG. SM α-actin was demonstrated by indirect immunofluorescence microscopy. *B*, immunoblot analysis of smooth muscle α-actin. pSMCs were maintained on fibronectin or laminin plus type IV collagen. Cell lysates were prepared by solubilizing the cells with 2 × Laemmli sample buffer, analyzed by SDS-PAGE, transferred to Immobilon-P, and immunoblotted using a 1:1000 dilution of a monoclonal antibody that had been raised against SM α-actin. SM α-actin was detected by chemiluminescence. *C*, immunohistochemical staining of total actin in pSMCs cultured on fibronectin or laminin plus type IV collagen for 48 h. The cells were exposed to rhodamine-labeled phalloidin for 30 min. Total actin was demonstrated by indirect immunofluorescence microscopy.

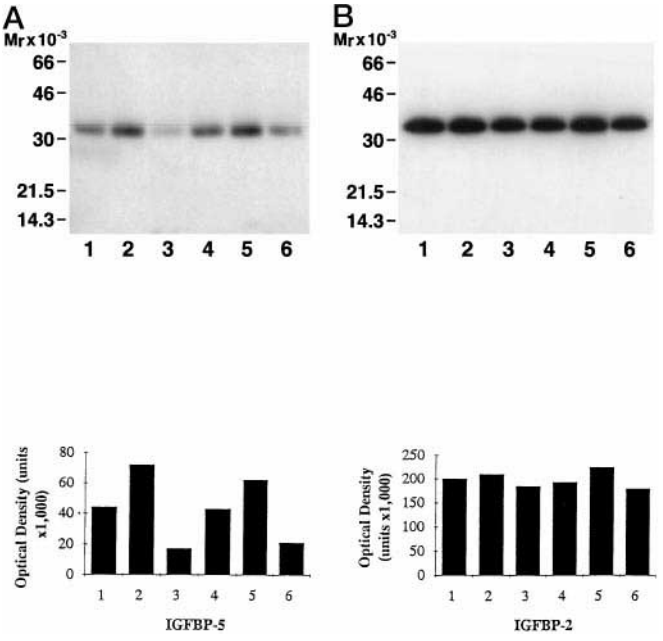

FIG. 2. **Effects of ECM substratum on the synthesis of IGFBP-5 (*A*) and IGFBP-2 (*B*).** SMCs were cultured on plastic dishes (*lanes 1* and *4*) on fibronectin (*lanes 2* and *5*) or laminin plus type IV collagen (*lanes 3* and *6*) for 48 h (*lanes 1–3*) and 96 h (*lanes 4–6*). During the last 8 h of incubation, the cells were exposed to methionine-free medium containing 50 μCi of [^{35}S]methionine and 50 μg/ml heparin (added to inhibit IGFBP-5 proteolysis). Culture media were collected and immunoprecipitated using antiserum against IGFBP-5 (*A*) or IGFBP-2 (*B*). The immunoprecipitated proteins were analyzed by SDS-PAGE with autoradiography. *Upper panels*, autoradiographs; *lower panels*, PhosphorImage analyses. This experiment was repeated three times with similar results.

laminin/type IV collagen had significant differences in the amount of IGFBP-5 that was synthesized and secreted. As shown in Fig. 2*A*, media obtained from the cells grown on fibronectin had a 1.7-fold ± 0.3 ($p < 0.05$) increase in intact IGFBP-5 compared with cultures maintained on plastic. More important, the amount of IGFBP-5 was 4.0 ± 1.0-fold ($p < 0.01$) greater than the cells that were plated on laminin/type IV collagen. In contrast, the synthesis of IGFBP-2 was unchanged when cells that were cultured using these conditions were compared (Fig. 2*B*).

To determine if the change of IGFBP-5 synthesis was accompanied by a corresponding change in mRNA, total RNA was isolated from the cells cultured on laminin plus type IV collagen or fibronectin and analyzed by Northern blotting. Compared with cultures maintained on plastic, plating on fibronectin increased IGFBP-5 mRNA levels by 1.5 ± 0.3-fold ($p < 0.05$) (Fig. 3). In contrast, in the cultures plated on laminin and type IV collagen, the IGFBP-5 mRNA abundance was decreased by 85 ± 6% ($p < 0.01$) compared with control cultures. The expression of IGFBP-2 mRNA showed no significant changes (Fig. 3).

Influence of ECM on the Ability of IGF-I to Regulate IGFBP-5 mRNA Abundance—As IGFBP-5 gene expression has been shown to be stimulated by IGF-I (17), we examined whether maintaining the cultures on different ECM proteins would alter this cellular response. As shown in Fig. 4, IGF-I increased IGFBP-5 mRNA abundance in the pSMCs that had been plated on either fibronectin or laminin plus type IV collagen. Although cells maintained in laminin/type IV collagen responded to IGF-I with an increase in IGFBP-5 expression (6.1 ± 1.0 to 41 ± 5.0, arbitrary units $p < 0.01$), the absolute level of IGFBP-5 mRNA was substantially below that detected in cultures that had been maintained on fibronectin (54 ± 4.0 to 140 ± 15, arbitrary units $p < 0.01$).

Cell Replication Assays of pSMCs Cultured on Different ECM Substrata—pSMCs cultured on different substrata were also analyzed for their ability to synthesize DNA in response to IGF-I. The results showed that the response to IGF-I was significantly greater (560 ± 84% compared with 112 ± 12%, $p < 0.01$) for cells plated on fibronectin compared with cells plated on laminin plus type IV collagen (Fig. 5). These data are consistent with the responses in Fig. 4 showing that cells plated on type IV collagen retain some capacity to respond to IGF-I, but the absolute level that can be attained is decreased significantly. The addition of IGFBP-5 to cultures that were plated on laminin and type IV collagen resulted in marked potentiation of the cellular response to IGF-I. In contrast, the addition of IGFBP-5 to cultures plated on fibronectin did not alter the cellular response to IGF-I significantly.

The Effect of Different ECM Proteins Is Mediated by Their Respective Integrins—As pSMCs have been reported to express $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_2\beta_1$ integrins, we examined whether blocking ligand binding to these integrins would influence IGFBP-5 expression. Following a 30-min exposure to synthetic peptides or to anti-integrin antibodies, the cells were plated on fibronectin or laminin/type IV collagen dishes. The adherence of pSMC to the substratum was unaffected by preincubating with the

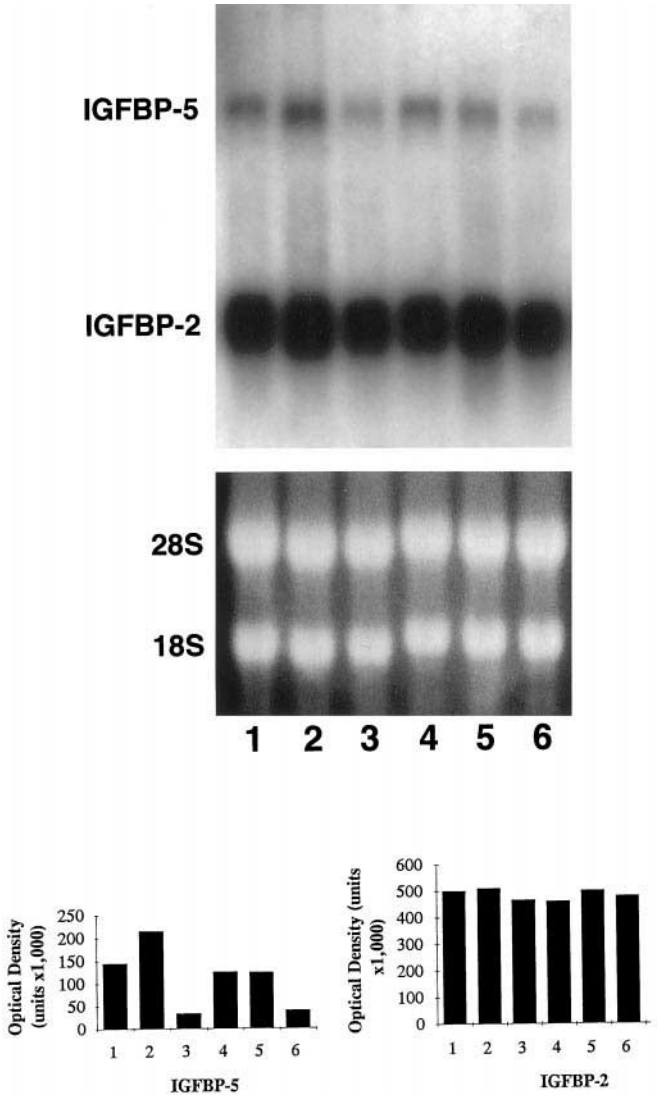

FIG. 3. **Effects of ECM substratum on the steady-state levels of IGFBP-5 and IGFBP-2 mRNA.** *A*, total RNA was isolated from SMCs that had been maintained on plastic dishes (*lanes 1* and *4*), on fibronectin (*lanes 2* and *5*), or on laminin plus type IV collagen (*lanes 3* and *6*) for 48 (*lanes 1–3*) or 96 h (*lanes 4–6*). Total RNA was isolated and subjected to Northern blotting. RNAs were detected with specific cDNA probes for human IGFBP-5 and -2. *Upper panel*, autoradiography of IGFBP-5 and -2 mRNAs; *middle panel*, 28 S and 18 S RNA ethidium bromide staining; *lower panels*, PhosphorImage analyses. This experiment was repeated three times with similar results.

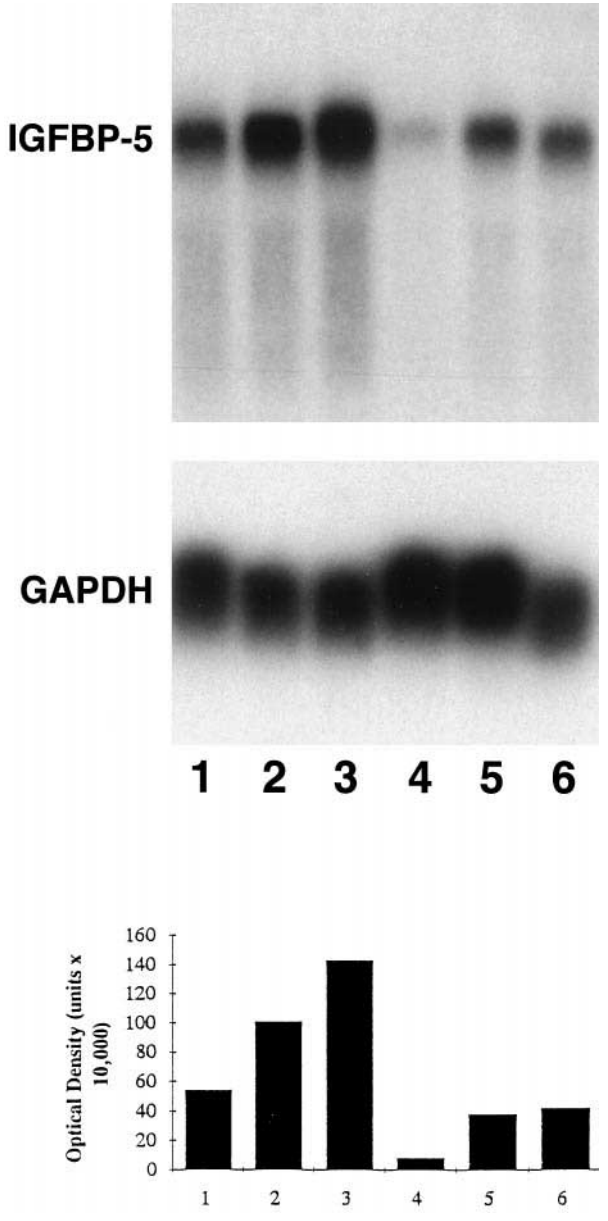

FIG. 4. **IGF-I regulates the mRNA expression of IGFBP-5 in pSMCs cultured on fibronectin (*lanes 1–3*) or laminin plus type IV collagen (*lanes 4–6*).** pSMCs were plated on these ECM proteins and maintained for 24 h; then, IGF-I was added, and the incubation was continued for another 24 h. Total RNA was isolated from the cells and subjected to Northern blotting using the cDNA probe for human IGFBP-5. *Lanes 1* and *4*, no IGF-I; *lanes 2* and *5*, IGF-I (50 ng/ml); *lanes 3* and *6*, IGF-I (100 ng/ml). *Upper panel*, autoradiograph of IGFBP-5 mRNA; *middle panel*, autoradiograph of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA; *lower panel*, PhosphorImage analysis of IGFBP-5 mRNA. Three experiments were conducted with similar results.

these reagents at the given concentrations. After a 24-h incubation, IGFBP-5 mRNA abundance was quantified. The synthetic RGD peptide that binds $\alpha_5\beta_1$ and echistatin blocked the fibronectin-induced change in IGFBP-5 expression (Fig. 6). A control RGE peptide had no effect. The inhibition of IGFBP-5 expression that was induced by laminin and type IV collagen was partially reversed by the antibody against $\alpha_2\beta_1$ integrin. These data indicate that binding of these matrix proteins to their respective integrins is an important step in their ability to modulate IGFBP-5 expression. To determine if these integrins were also involved in modulating the DNA synthesis response to IGF-I, a similar experiment was conducted using [^{3}H]thymidine incorporation as an index of IGF-I action. As shown in Fig. 7, exposure of pSMC plated on fibronectin to echistatin attenuated the cellular DNA synthesis response to IGF-I. In contrast, the P1E6 antibody partially reversed the inhibitory effect of ligand occupancy of $\alpha_2\beta_1$ on IGF-I-stimulated replication. To determine if altering ligand occupancy of these integrins would alter the cellular growth response to IGF-I, cell number was determined using a similar experimental design. Quiescent pSMC cultures that were plated on fibronectin increased their final cell number by 61 ± 7% in response to 20 ng/ml of IGF-I. The addition of echistatin (10^{-7} M) decreased this response to 32 ± 4%. The cells plated on laminin/type IV collagen increased 11 ± 2.1%, and those that were exposed to the P1E6 antibody increased 18 ± 2.1%.

Atherosclerotic Lesions Contain Abundant Fibronectin and Have Greater IGFBP-5 mRNA Expression Compared with Normal Aorta—To determine if the SMCs in lesions express more IGFBP-5 than cells in the normal arterial wall, atherosclerotic

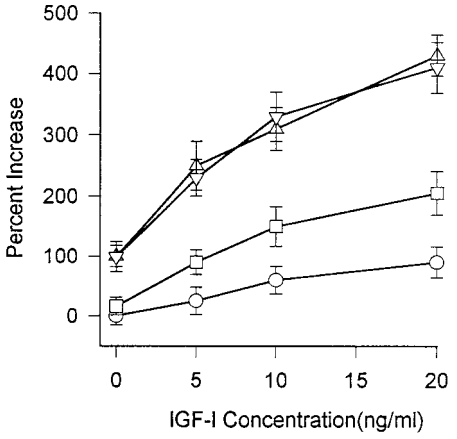

FIG. 5. **[^{3}H]Thymidine incorporation responses to IGF-I of pSMC plated on fibronectin or laminin plus type IV collagen.** pSMCs were plated and incubated for 24 h and then exposed to [^{3}H]thymidine (1.0 μCi/well) and IGF-I at varying concentrations another 24 h. Purified IGFBP-5 was either omitted from the assay or preincubated with the substrata prior to plating the cells. The amount of [^{3}H]thymidine incorporated into the cells was measured by scintillation counting. ▽, cells plated on fibronectin; ○, cells plated on laminin and type IV collagen; △, cells plated on fibronectin preincubated with IGFBP-5; □, cells plated on laminin and type IV collagen preincubated with IGFBP-5. The results were expressed as percent of increase over that of the cells plated on laminin and type IV collagen and incubated with DMEM plus 0.2% platelet-poor plasma only.

lesions that were diet induced were extracted, and their RNA was analyzed by Northern blotting. The results showed that lesions contain 4.5 ± 1.5-fold ($p < 0.05$) ($n = 4$ separate animals) more IGFBP-5 mRNA compared with normal subendothelial tissue. A representative experiment is shown in Fig. 8*A*. The fibronectin content in these tissue specimens was also determined. The results show that a 5.9-fold greater amount of fibronectin was detected in the lesion tissue compared with normal aorta (Fig. 8*B*).

DISCUSSION

In normal blood vessels, SMCs are attached to a basement membrane that contains type IV collagen and laminin. Within the arterial wall, most SMCs are maintained in a quiescent state and express contractile protein isoforms. However, in response to vascular injury, SMCs alter their phenotype and are stimulated to proliferate and migrate. This phenotypic transition from the contractile to the synthetic state is accompanied by enhanced expression of specific matrix proteins such as fibronectin (9). The phenotypic transition can be modulated *in vitro* by changing the composition of ECM substratum (6). Aortic SMCs have been reported to convert to the synthetic phenotype when cultured on a fibronectin substratum, and, if maintained on fibronectin, they will proliferate if they are also exposed to appropriate growth factors. In contrast, if the cells are cultured on laminin plus type IV collagen, early passage (*e.g.* <p5) cells can be maintained in a nonproliferative state for 1 week (6). This was confirmed in our study by showing abundant expression of smooth muscle α-actin and an increased number of microfilament bundles that stained for α-actin. In contrast, cells that were plated and maintained on fibronectin had much lower α-actin content. Based on these results, we believe that the *in vitro* modulation of the pSMC phenotype was similar to that previously reported by others (6).

A major finding in the present study is that, compared with cells cultured on laminin and type IV collagen, pSMCs cultured on fibronectin synthesize and release more IGFBP-5. The increase in IGFBP-5 synthesis is associated with increased IGFBP-5 mRNA expression. The effect becomes manifest between the 2nd and 4th days after changing the ECM substratum. The effect is specific for IGFBP-5 as IGFBP-2 protein and mRNA levels were unaltered by the ECM proteins. This suggests that expression of this gene may be an important component of phenotype transition. Because IGFBP-5 has been shown to potentiate the cellular growth-promoting effect of IGF-I (23), this may be relevant to the observation noted by several groups that synthetic phenotype cells are more responsive to growth factors (9, 24–26). This conclusion is strengthened by our observation that the addition of IGFBP-5 to cultures plated on laminin/type IV collagen enhanced their DNA synthesis response to IGF-I, suggesting that the enhanced expression of IGFBP-5 in the fibronectin-exposed cultures may

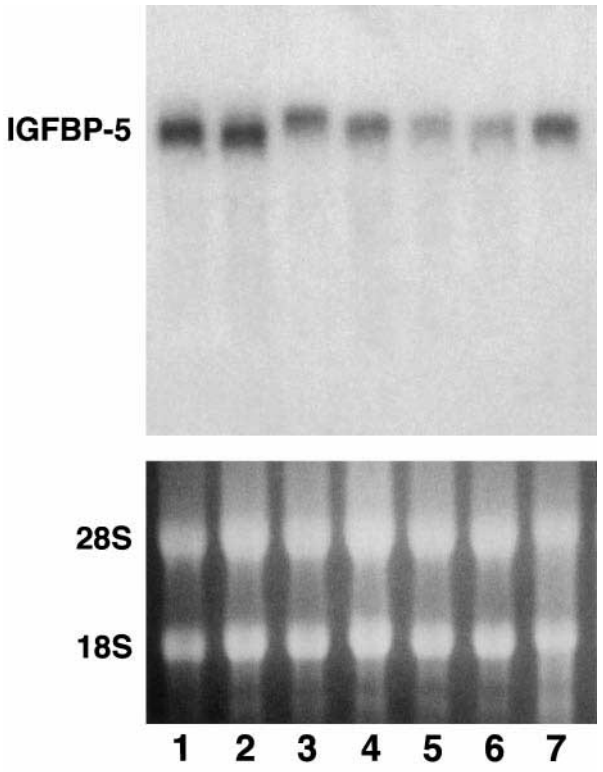

FIG. 6. **Effects of integrin-blocking peptides or antibodies on IGFBP-5 mRNA expression.** pSMCs were preincubated with no treatment (*lane 1*), a control RGE peptide 10^{-7} M (*lane 2*), RGD peptide 10^{-7} M (*lane 3*), or echistatin 10^{-7} M (*lane 4*) for 30 min and then plated on fibronectin. pSMCs were incubated with no treatment (*lane 5*), 1:500 dilution of mouse IgG (*lane 6*), or 1:500 dilution of the anti-$\alpha_2\beta_1$ antibody (*lane 7*) before plating on laminin plus type IV collagen. After 24 h of incubation, the RNA was isolated from the cells and analyzed by Northern blotting using the cDNA probe for human IGFBP-5. *Upper panel*, autoradiograph; *middle panel*, ethidium bromide staining for 28 S and 18 S RNA; *lower panel*, PhosphorImage analysis.

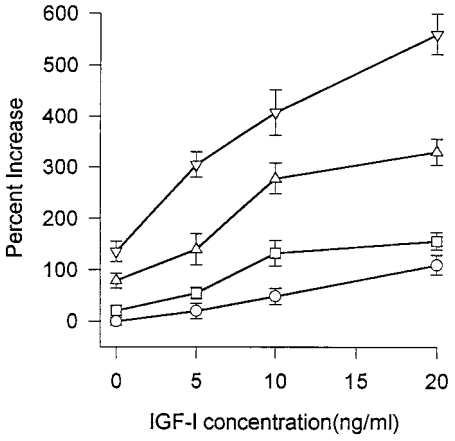

FIG. 7. **[^{3}H]Thymidine incorporation responses to IGF-I of pSMC cultured on fibronectin or laminin plus type IV collagen and exposed to echistatin or P1E6 antibody.** pSMCs were plated on the two substrata, incubated for 24 h, and then exposed to [^{3}H]thymidine (1.0 μCi/well) and IGF-I at varying concentrations for another 24 h. Treatments included the following: ▽, cells plated on fibronectin; △, cells plated on fibronectin in the presence of 10^{-7} M echistatin; □, cells plated on laminin and type IV collagen in the presence of P1E6; ○, cells plated on laminin and type IV collagen. The results were expressed as percent of increase over that of the cells plated on laminin and type IV collagen and incubated with DMEM plus 0.2% platelet-poor plasma only. The results represent the mean ± S.E. of three separate experiments.

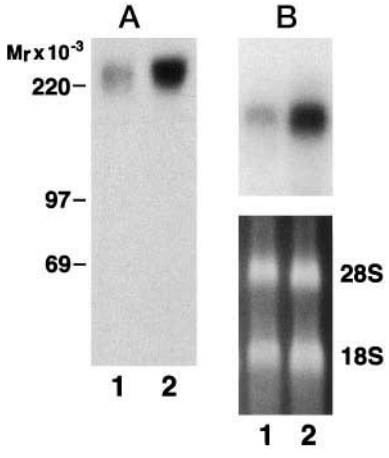

FIG. 8. **Fibronectin content (*A*) and IGFBP-5 mRNA abundance (*B*) in cellular extracts obtained from atherosclerotic lesion and normal vessel wall of porcine aorta.** *A*, proteins were extracted from tissue lysates, and fibronectin was detected by immunoblotting as described under "Experimental Procedures." *B*, total RNA was isolated from a representative atherosclerotic lesion and normal vessel wall (from which the endothelial and advential cells had been removed) and analyzed by Northern blotting. The RNA was detected with a cDNA probe for human IGFBP-5. *Lane 1*, normal aorta; *lane 2*, atherosclerotic lesion. RNA samples were also obtained from four separate animals and analyzed as stated above. The results were not different from the representation example that is shown.

be an important component of this enhanced replication response to IGF-I.

These changes were also reflected in the IGFBP-5 synthesis response to IGF-I. We had previously shown that IGF-I induced a 6-fold increase in IGFBP-5 mRNA (17); therefore, we analyzed the responses of pSMC that expressed each phenotype. Although pSMC plated on fibronectin had a greater increase in IGFBP-5 expression in response to IGF-I, cultures maintained in the nonproliferative phenotype still showed some IGF-I responsiveness. There are two possible interpretations of this result. First, fibronectin is acting to directly stimulate basal (non-IGF-I dependent) IGFBP-5 expression and to enhance the response to IGF-I. This conclusion is supported by our data showing that peptide antagonists of ligand binding to $\alpha_V\beta_3$ and $\alpha_5\beta_1$ can inhibit IGFBP-5 expression. Second, it is also possible that a low percentage of cells have not been maintained in the nonproliferative phenotype and, therefore, have acquired IGF-I responsiveness. In either case, it seems that the synthetic phenotype cells are more responsive to IGF-I. In addition to modulating the IGFBP-5 synthesis response to IGF-I, plating pSMC on different substrata modulated the DNA synthesis response. This effect also seemed to be ECM protein-mediated as cells that were plated on laminin/type IV collagen had a reduced DNA synthesis response to IGF-I compared with the response of cells plated on fibronectin.

To test the hypothesis that the effects of these ECM molecules were mediated by their respective integrins, pSMCs were incubated with an antibody or peptides that bind to and block integrin ligand occupancy. The adherence of pSMCs to matrix-coated plates was unaffected by preincubating the cells with either RGD peptide or the antibody at the indicated concentrations. These results indicate that the effect of fibronectin on IGFBP-5 expression is mediated by $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins, as both the RGD peptide and echistatin, a disintegrin that has been shown to block fibronectin binding to $\alpha_V\beta_3$ integrin, inhibited the induction of IGFBP-5 by fibronectin. Because we have previously shown that vitronectin binding to $\alpha_V\beta_3$ is required for pSMC to migrate in response to IGF-I and because echistatin also blocked the IGFBP-5 response to IGF-I, it is likely that $\alpha_V\beta_3$ occupancy by fibronectin is also augmenting this response. In contrast to fibronectin, exposure to type IV collagen/laminin resulted in maintenance of IGFBP-5 expression at a low level. When occupancy of the $\alpha_2\beta_1$ integrin was blocked with the P1E6 antibody, this inhibition of IGFBP-5 expression could be partially reversed. This strongly suggests that $\alpha_2\beta_1$ integrin ligand occupancy is acting to inhibit IGFBP-5 expression. Additionally, the effects of these ECM proteins on IGF-I-stimulated DNA synthesis seem to be integrin mediated as blocking binding to $\alpha_2\beta_1$ or $\alpha_V\beta_3$ partially reversed the effects of these ligands on IGF-I-stimulated replication. Taken together, these results support the conclusion that the effects of the ECM proteins used in this study are integrin mediated and that integrin ligand occupancy modulates IGF-I responsiveness. Therefore, the capacity of this growth factor to stimulate pSMC replication is directly related to the context in which the growth factor signal is presented to its cognate receptor.

IGFs exert multiple effects on target cells that are mediated by the IGF-I receptor on the cell surface. IGF-I has been shown to stimulate proliferation as well as migration of pSMC (14). In addition to the direct influences on the cells, IGF-I exposure is also required for the full activity of several other growth factors or cytokines. IGF-I has been shown to enhance the effects of platelet-derived growth factor (27), thrombin (28), and angiotensin II (29) on cultured SMCs. Selective inhibition of IGF-I binding to its receptor with anti-IGF-I antibody results in marked reduction in the mitogenic response to thrombin (28) and angiotensin II (29). *In vivo*, IGF-I mRNA and immunoreactive IGF-I are detected in intimal lesions that develop in humans (11). IGF-I mRNA and immunoreactive IGF-I levels both increase severalfold after balloon denudation injury in rats, and these increases are temporally preceded by an increase in SMC proliferation (12, 13). Thus, IGF-I may be important for SMC proliferation and migration *in vivo* and may play an important role in the development of atherosclerotic lesions.

Our findings suggest that variables such as enhanced binding of fibronectin to $\alpha_V\beta_3$ or increased binding of type IV colla-

gen to $\alpha_2\beta_1$ may also modulate responsiveness of pSMC to other growth factors. Recent studies have shown that ligand occupancy of the $\alpha_V\beta_3$ integrin functions to regulate growth factor actions. Platelet-derived growth factor (30) and IGF-I (14) have a diminished capacity to stimulate cell migration if ligand occupancy of this integrin is attenuated. Similarly blocking integrin or growth factor receptor-associated signaling pathways, such as induction of phosphatidylinositol-3 kinase and inositol phosphate-3 formation, results in inhibition of migration (30). The mechanistic explanations that have been proposed to explain these observations include coactivation of signal transduction elements by growth factor tyrosine kinase receptors and integrins such as insulin receptor substrate-1 (31) or calmodulin-II kinase (32, 33) and, in the case of fibroblast growth factor, direct activation of the receptor kinase activity by integrin occupancy (34). Our findings suggest that the $\alpha_V\beta_3$ occupancy is activating IGF-I signaling, resulting in not only enhanced migration but also in enhanced replication. In contrast, no studies have been reported to date that blocking ligand occupancy of $\alpha_2\beta_1$ will enhance growth factor actions. In the case of platelet-derived growth factor, occupancy of this integrin was stated to enhance the SMC migration response (35). Therefore, further studies that are directed toward elucidating the mechanism by which $\alpha_2\beta_1$ occupancy is inhibiting IGF-I actions are warranted.

IGF bioactivities are modified by a family of six high affinity IGFBPs. Among them, IGFBP-5 has the unique property of adhering tightly to ECM (23). When associated with ECM, the affinity of IGFBP-5 is lowered 8-fold. This affinity shift results in a more favorable equilibrium between IGF-I bound to ECM-associated IGFBP-5 and to the IGF-I receptor, and this is associated with an enhanced cellular DNA synthesis response to IGF-I. Additionally, the amount of ECM-associated IGFBP-5 has been shown to be related to the degree of enhancement of the cellular growth response (23). This hypothesis was confirmed by our results showing that the addition of IGFBP-5 to cultures that were plated on laminin and type IV collagen significantly increased the cellular response to IGF-I. Therefore, factors such as the induction of IGFBP-5 synthesis by fibronectin could result in further enhancement of the cellular responses to IGF-I. Similarly, an increase in the affinity of $\alpha_V\beta_3$ for its ligands, which occurs following IGF-I binding to its receptor (14), may contribute to this response.

These observations may have some relevance to the development of atherosclerotic lesions *in vivo*. This study demonstrated that atherosclerotic lesions contain more IGFBP-5 mRNA compared with comparable amounts of normal vessel wall. Although several cell types exist in atherosclerotic plaques, SMCs are the predominant cell type (5). Similarly, as the SMCs within lesions have assumed the synthetic phenotype and because fibronectin is much more abundant, it is possible that the increase that we observed in IGFBP-5 mRNA content in lesions reflects the differences that we observed in pSMC cultures *in vitro* and that this increase could be related to lesion enlargement. Because the $\alpha_V\beta_3$ integrin is not expressed in normal vessel wall but is abundant in atherosclerotic lesions (36), it is possible that this change also contributes to enhanced IGFBP-5 expression. Furthermore, because other ECM proteins that bind to $\alpha_V\beta_3$, such as thrombospondin and osteopontin, are abundant in lesions and have the potential to act through $\alpha_V\beta_3$ to alter the smooth muscle cell phenotype and responsiveness (37, 38), it will be of interest to determine their effects on IGF-I and IGFBP-5 actions.

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