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Differential Expression and Biological Effects of Insulin-like Growth Factor-binding Protein-4 and -5 in Vascular Smooth Muscle Cells*

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Insulin-like growth factor-I (IGF-I) plays an important role in regulating vascular smooth muscle cell (VSMC) proliferation, migration, and apoptosis. The bioactivity of IGF-I is modulated by a group of high affinity, specific binding proteins (IGF-binding proteins; IGFBPs) that are present in the interstitial fluid. Previously, we have reported that porcine VSMCs synthesize and secrete IGF-I and several forms of IGFBPs, including IGFBP-2, IGFBP-4, and IGFBP-5. In this study, we examined the role of autocrine/paracrine secreted IGF-I in controlling the expression of IGFBP-4 and IGFBP-5 as well as the effects of these IGFBPs in modulating the cellular replication response to IGF-I. The concentrations of IGFBP-4 in the conditioned medium increased significantly from <50 ng/ml to 742 ± 105 ng/ml. This increase was associated with a decrease in the activity of an IGF-I-regulated IGFBP-4 protease. In contrast, the synthesis of IGFBP-5 was inversely correlated with culture density, and its concentration decreased from 792 ± 91 to 44 ± 14 ng/ml. IGFBP-5 mRNA in sparse cultures was 3-fold higher compared with those in confluent cultures. This culture density-dependent change in IGFBP-5 mRNA correlated closely with endogenous IGF-I levels. Since treatment of VSMC with exogenous IGF-I increased IGFBP-5 mRNA levels, we neutralized the effect of endogenously secreted IGF-I with an anti-IGF-I antibody to determine if it would alter IGFBP-5 mRNA abundance. This resulted in a 4.4-fold decrease in IGFBP-5 mRNA levels. When added together with IGF-I, exogenous IGFBP-4 inhibited IGF-I-induced DNA synthesis in a concentration-dependent manner. IGFBP-5, on the other hand, potentiated the effect of IGF-I. Therefore, IGFBP-4 and IGFBP-5 appear to be differentially regulated by autocrine/paracrine IGF-I through distinct mechanisms. These two proteins, in turn, play opposing roles in modulating IGF-I action in stimulating VSMC proliferation.

Vascular smooth muscle cell (VSMC)[1] proliferation, apoptosis, and directed migration from arterial media into the intima are critical events for the development of fibromuscular lesions commonly associated with atherosclerosis. Several lines of evidence suggest that insulin-like growth factor-I (IGF-I) plays an important role in these processes. IGF-I mRNA and peptide are detected in intimal lesions that develop after angioplasty, and both their levels increase significantly after balloon denudation injury (1–3). These increases temporally precede an increase in VSMC proliferation. *In vitro*, IGF-I acts synergistically with other growth factors to stimulate DNA synthesis (4). Cultured VSMCs have been shown to express IGF-I mRNA and secrete IGF-I (5–7). Specific inhibition of the endogenously produced IGF-I, using a neutralizing antibody, resulted in decreased cell proliferation, which indicates that IGF-I acts as an autocrine growth regulator in VSMCs (4). The IGF-I receptor, which mediates the biological actions of the IGFs, is expressed in VSMCs. Selective inhibition of the receptors by antisense targeting results in marked reduction of VSMC proliferation (8). Recent studies have demonstrated that IGF-I is a very potent regulator of VSMC migration, and this action is mediated by the IGF-I receptor (9, 10). It has been reported that IGF-I also plays a crucial role in regulating VSMC apoptosis (11, 12). These findings provide strong evidence to support the concept that IGF-I is an autocrine/paracrine factor that regulates VSMC proliferation, apoptosis, and migration.

The pericellular bioactivity of IGF-I in a defined tissue is not only dependent on IGF-I and its receptors but is also influenced by a group of high affinity specific binding proteins (IGFBPs) that are present in the local environment. We have previously shown that porcine VSMCs secrete three IGFBPs: IGFBP-2, IGFBP-4, and IGFBP-5, with IGFBP-2 being the predominant form (13, 14). Similarly, IGFBP-2, IGFBP-4, and IGFBP-5 have been reported to be synthesized in rat and human VSMCs (14–17). A recent study indicated that IGFBP-6 is secreted in human VSMCs (18). Although these IGFBPs are believed to play a role in modulating the biological actions of IGF-I in VSMCs, their specific role and their interactions with the autocrine/paracrine secreted IGF-I remain to be defined. To further elucidate the roles of specific forms of IGFBPs that are synthesized by VSMCs, we studied the expression and biological effects of IGFBP-4 and IGFBP-5 in porcine VSMCs. The results of the present study indicate that the extracellular fluid concentrations of these two molecules are differentially regulated by the autocrine/paracrine IGF-I through distinct mechanisms. Furthermore, IGFBP-4 and IGFBP-5 exhibited opposing effects in modulating the IGF-I-actions in this cell type.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum, Dulbecco's minimum essential medium (DMEM) with high glucose, and penicillin-streptomycin were purchased from Life Technologies, Inc. Trypsin was obtained from Boehringer Mannheim. Recombinant human IGF-I and rat IGF-II were purchased from Bachem, Inc. (Torrance, CA). Human IGFBP-4 and IGFBP-5 were purified as described previously (19).

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[1] The abbreviations used are: VSMC, vascular smooth muscle cell; DMEM Dulbecco's minimum essential medium; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein.

Cell Culture—Porcine aortic smooth muscle cells (VSMCs) were isolated from thoracic aorta of 3-week-old piglets. The cells were grown in 10-cm dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in DMEM supplemented with glutamine (4 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) plus 10% fetal bovine serum. The medium was changed every fourth day until the cells became confluent.

Western Ligand Blot and Immunoblot Analysis—In order to identify the forms of IGFBPs secreted by VSMCs, samples were concentrated 20 times by ultrafiltration through a Centricon-10 microconcentrator (Amicon, Berkeley, MA). The volume of conditioned medium that was analyzed was adjusted for difference in cell number. The proteins were separated by SDS-polyacrylamide gel electrophoresis using 12.5% polyacrylamide gels under nonreducing conditions. After transfer to filters (Immobilon P, 0.45-μm pore size, Millipore Corp., Bedford, MA), the filters were probed with ^{125}I-IGF-I, and autoradiographs were obtained as described previously (14).

Immunoprecipitation—Cells were grown in 10-cm dishes (Falcon) and rinsed three times with serum-free DMEM before being incubated in serum-free DMEM containing 100 μg/ml heparin for 8 h. Media were collected, and IGFBP-5 was immunoprecipitated from the media by the addition of an anti-human IGFBP-5 antibody (prepared in guinea pig, 1:1000 dilution) or normal guinea pig serum (control). IGFBP-2 was immunoprecipitated from the same media by the addition of an anti-bovine IGFBP-2 antiserum. The volume of conditioned medium used was normalized by cell number. The immune complexes were precipitated by adding protein A-Sepharose (Sigma) and analyzed by 12.5% SDS-polyacrylamide gel electrophoresis gels followed by Western ligand blotting.

To quantify the IGFBP-5 concentrations, known amounts of pure human IGFBP-5 (2–200 ng) were added to parallel lanes, and the band intensities of the unknown samples were compared with those obtained using the pure human IGFBP-5 standard. The lower limit of detection was 25 ng/ml. To quantify IGFBP-4, the media samples were concentrated (20-fold) and then analyzed by SDS-polyacrylamide gel electrophoresis with immunoblotting as described previously (13). The band intensities were quantified by PhosphorImager image analysis using ImageQuant (Molecular Dynamics, Inc.). Image intensities were compared with the known pure human IGFBP-4 standards that were analyzed on the same gel to calculate the IGFBP-4 concentrations. The lower limit of detection was 50 ng/ml. To determine IGF-I concentrations, the conditioned media were extracted using a Sep-Pak C-18 cartridge (20) (Millipore) to remove all of the IGFBPs. The extracts were then analyzed by a specific IGF-I radioimmunoassay.

RNA Isolation and Northern Blot Analysis—RNA was isolated from cell cultures using TriReagent following the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH) and was quantified by measuring UV absorption at an OD of 260 nm. RNA samples were size-fractionated on a 1.2% agarose-formaldehyde gel, blotted, and fixed onto a nylon membrane (ICN Biochemical, Inc., Irvine, CA). They were hybridized with the [^{32}P]dCTP-labeled human IGFBP cDNAs (14). A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX) was used to determine that the same amount of RNA was loaded. The band densities were quantified by exposing the filters to phosphor screens, which were scanned on PhosphorImager SF followed by image analysis using ImageQuant software (Molecular Dynamics).

Protease Assays—To study the change in IGFBP-4 and IGFBP-5 protease activity, VSMC-conditioned media were collected and stored at −20 °C until used. The conditioned media were incubated with 50 ng of pure human IGFBP-4 or IGFBP-5 for 14 h at 37 °C in 60 μl of Tris (50 mM) containing 50 mM NaCl and 2.0 mM $CaCl_2$ (pH 7.4). For IGFBP-4 protease assays, 50 ng/ml IGF-I was included in the incubation mixture. The digestion products were analyzed by immunoblotting as described above.

[^{3}H]Thymidine Incorporation Assay—To determine the rate of DNA synthesis, porcine VSMCs were plated onto 96-well plates (Falcon) at 10,000 cells/well in DMEM supplemented with 10% fetal bovine serum and incubated for 3 days without a medium change. After being rinsed three times with DMEM, the cultures were exposed to DMEM that contained 1 μCi of [^{3}H]thymidine (ICN Biochemicals, Inc., CA), 0.2% human platelet poor plasma, and the stated concentration of IGF-I and/or IGFBP in a final volume of 200 μl. Each treatment was added to triplicate cultures. After 24–48 h, media were collected for ligand and immunoblotting analysis. Cells were then washed twice with phosphate-buffered saline and twice with cold 5% trichloroacetic acid for 10 min at 4 °C and solublized in 200 μl of 0.1 M NaOH and 1% SDS at room temperature for 1 h. The solublized DNA was harvested for liquid scintillation counting. The results are expressed as the percentage change from the IGF-I alone controls.

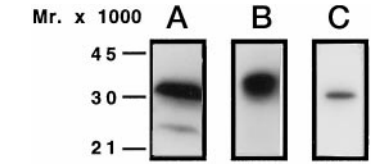

FIG. 1. **IGFBPs secreted by cultured porcine VSMCs.** *A*, ligand blot analysis of the medium conditioned by porcine VSMCs. *B*, immunoprecipitation and ligand blotting analysis of IGFBP-2. The same conditioned medium samples shown in *A* were immunoprecipitated with an anti-bovine IGFBP-2 antibody prior to ligand blot analysis. *C*, immunoprecipitation and ligand blotting analysis of IGFBP-5. The same conditioned medium samples shown in *A* were immunoprecipitated with an anti-human IGFBP-5 antibody prior to ligand blot analysis.

Statistical Analysis—Student's *t* test was used to compare the difference between the control and test groups. Values are means ± S.E. $p < 0.05$ is considered significant.

RESULTS

IGFBPs Secreted by Porcine VSMCs—The ligand blotting analysis of conditioned, serum-free medium of subconfluent porcine VSMCs revealed two major bands of approximately 24 and 30–32 kDa (Fig. 1*A*). The 24-kDa protein has previously been identified as IGFBP-4 (13). The 30–32-kDa doublet band is a mixture of IGFBP-2 and IGFBP-5. Since these two proteins are similar in size (*e.g.* 32 and 31 kDa) and indistinguishable by ligand blot analysis, they were further separated by immunoprecipitation and analyzed by ligand blot analysis (Fig. 1, *B* and *C*). Northern blotting analysis revealed the expression of IGFBP-2, IGFBP-4, and IGFBP-5 mRNA (data not shown), a finding that is consistent with the protein results. These results indicate that porcine VSMCs synthesize and secrete three IGFBPs, namely IGFBP-2, IGFBP-4, and IGFBP-5.

Culture Density-dependent Expression of IGFBPs—Conditioned media were collected from porcine VSMCs growing at three different densities and subjected to immunoprecipitation and ligand blot analysis. As shown in Fig. 2, the accumulated levels of IGFBP-4 and IGFBP-5 varied substantially, depending on culture density. While the IGFBP-2 levels remained the same under these conditions, the IGFBP-4 levels increased significantly with increasing culture density (Fig. 2). When IGFBP-4 in the medium was quantified by comparing its band intensity to known standards, the concentration increased from <50 ng/ml to 742 ± 105 ng/ml (mean ± S.D. of three separate experiments). In contrast, the IGFBP-5 levels decreased dramatically with the increase in culture density. When IGFBP-5 was quantified, it decreased from 792 ± 91 to 44 ± 14 ng/ml in media from the high density cultures. IGF-I also decreased from 94 ± 14 ng/ml in the sparse cultures to 18 ± 9 ng/ml in the high density cultures.

To determine if these changes in IGFBP levels were regulated at the level of mRNA abundance, total RNA was isolated and then subjected to Northern blot analysis. No significant change in IGFBP-2 mRNA was observed (Fig. 3). The IGFBP-4 mRNA levels showed a small decrease (<30%). This result was not in agreement with the substantial increase in the accumulated IGFBP-4 levels in the conditioned medium (Fig. 2). On the other hand, the cell density-dependent increase in accumulated IGFBP-5 was accompanied by a comparable increase in IGFBP-5 mRNA levels. The steady-state levels of IGFBP-5 mRNA levels decreased rapidly with the increasing culture density (Fig. 3). The IGFBP-5 mRNA levels in confluent cultures were 2.6-fold higher in sparse cultures as compared with those of confluent cultures, suggesting that this change in

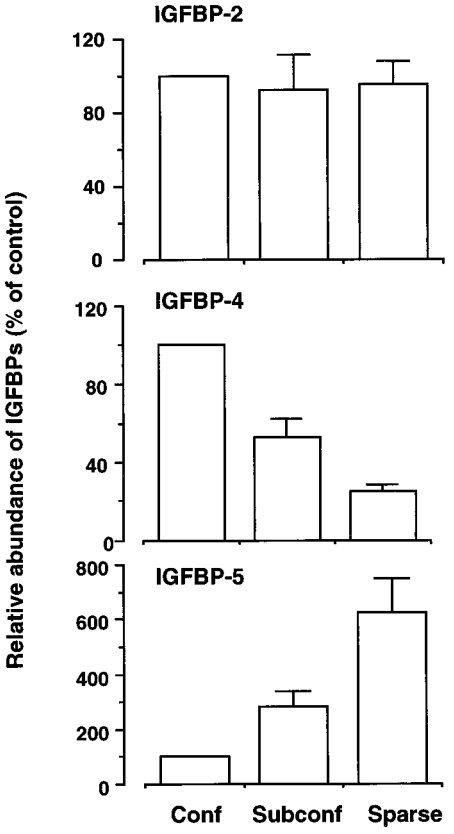

FIG. 2. **Effects of cell density/confluence on IGFBP levels in porcine VSMC cultures.** Serum-free medium was conditioned by confluent (*Conf*), subconfluent (*Subconf*), and sparse porcine VSMCs for 24 h and subjected to ligand blot analysis (IGFBP-4) or immunoprecipitation followed by ligand blot analysis (IGFBP-2 and IGFBP-5) as described under "Experimental Procedures." The band intensities were quantified by PhosphorImager analysis. Values are means ± S.E. (n = 3) expressed as a percentage of the confluent cultures.

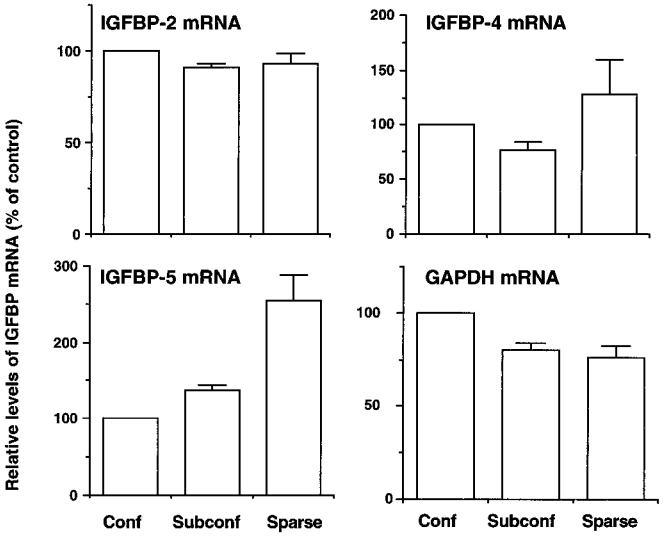

FIG. 3. **Effects of cell density/confluence on the steady-state levels of IGFBP mRNA in porcine VSMC cultures.** Confluent (*Conf*), subconfluent (*Subconf*), and sparse porcine VSMC cultures were exposed to serum-free medium for 24 h. Total RNA were isolated and subjected to Northern blot analysis using cDNA probes for IGFBP-2, IGFBP-4, IGFBP-5, and GAPDH. PhosphorImager analysis of three experiments was shown. Values are means ± S.E. (n = 3) expressed as a percentage of mRNA levels in the confluent cultures.

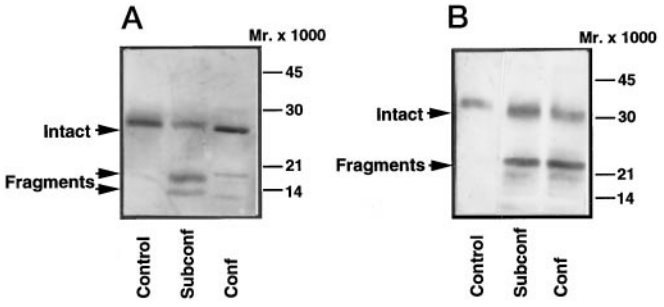

FIG. 4. **Effects of cell density/confluence on IGFBP-4 (*A*) and IGFBP-5 (*B*) protease activity in porcine VSMC cultures.** Exogenous human IGFBP-4 or IGFBP-5 (50 ng) was incubated with media conditioned by subconfluent (*Subconf*) and confluent (*Conf*) porcine VSMCs in the presence of IGF-I for 14 h. Samples without conditioned medium were incubated for 14 h as controls. Intact IGFBP as well as proteolytic fragments were detected by immunoblot analysis.

IGFBP-5 is attributable at least in part to altered synthesis.

Since porcine VSMCs are known to secrete proteases for IGFBP-4 and IGFBP-5, the conditioned media were analyzed to determine if changes in culture density were associated with potential changes in IGFBP-4 and IGFBP-5 protease activity. Analysis of the IGFBP-4 protease activity *in vitro* showed that it was greatly decreased when cells reached confluence, as evidenced by the decreased proteolytic fragment formation (Fig. 4*A*). In comparison, only a small increase in the IGFBP-5 protease activity was detected (Fig. 4*B*), and this change was not sufficient to explain the dramatic decrease in intact IGFBP-5 in the media from confluent cultures.

IGF-I is an Autocrine/paracrine Regulator of IGFBPs in VSMCs—As shown in Fig. 3, the steady-state levels of IGFBP-5 mRNA were significantly lower in confluent cultures. The culture density-dependent decrease in IGFBP-5 mRNA levels correlated well with a culture density-dependent decrease in the endogenous IGF-I levels that had been previously shown in this culture system (5). To examine the potential role of IGF-I as an autocrine/paracrine regulator of IGFBP-5 synthesis, IGF-I was added exogenously to confluent and sparse cultures. In the confluent cultures where the endogenous IGF-I is low, the IGFBP-5 mRNA levels were lower (40% of those in sparsely growing cultures). Adding exogenous IGF-I increased the steady-state levels of IGFBP-5 mRNA to levels comparable with those in the sparsely growing cultures (Fig. 5). We next neutralized the endogenously secreted IGF-I in sparsely growing cultures using an IGF-I-neutralizing antibody (Sm 1.2) and examined its effect on IGFBP-5 gene expression. As shown in Fig. 6, the IGFBP-5 mRNA levels were significantly higher in the sparsely grown cells compared with confluent control (*lanes 1* and *2*). When the monoclonal IGF-I antibody, Sm 1.2, was added to the cultures at the dilution rates of 1:2000 and 1:500, the IGFBP-5 mRNA levels were significantly decreased (*lanes 3* and *4*). Normal mouse IgG at the same concentration had no effect. These results indicate that the IGFBP-5 synthesis is regulated by the endogenously produced IGF-I.

Distinct Biological Effects of IGFBP-4 and IGFBP-5 in VSMCs—To determine the role that each IGFBP plays in regulating the action of IGF-I on VSMC proliferation, increasing concentrations of IGFBP-4 and IGFBP-5 were added to quiescent cultures in the presence of 50 ng/ml of IGF-I. IGF-I alone caused a 200 ± 32% increase in [^{3}H]thymidine incorporation (mean of seven experiments). Co-incubation of IGFBP-4 for 48 h inhibited the effect of IGF-I in a dose-dependent manner (Fig. 7*A*). This inhibition was significant at the higher concentrations ($p < 0.05$). At lower concentrations, nearly all of the IGFBP-4 was degraded (Fig. 7*B*), and the inhibition was

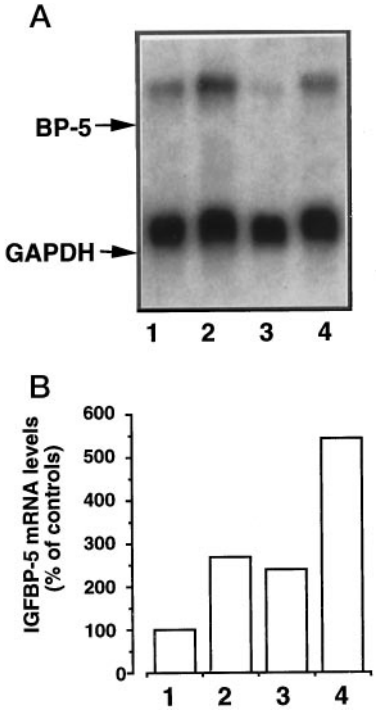

FIG. 5. **IGF-I treatment increases IGFBP-5 gene expression in confluent porcine VSMC cultures.** *A*, autoradiogram showing the effects of exogenously added IGF-I to confluent (*lanes 1* and *2*) cultures and sparsely growing cultures (*lanes 3* and *4*). Porcine VSMCs were incubated in serum-free medium without (*lanes 1* and *3*) or with IGF-I (100 ng/ml, *lanes 2* and *4*) for 24 h. Total RNA was isolated and subjected to Northern blotting with cDNA probes for IGFBP-5 and GAPDH. *B*, PhosphorImager analyses. Values are the means of two experiments. They are expressed as percentages of mRNA levels in the confluent, untreated control cultures.

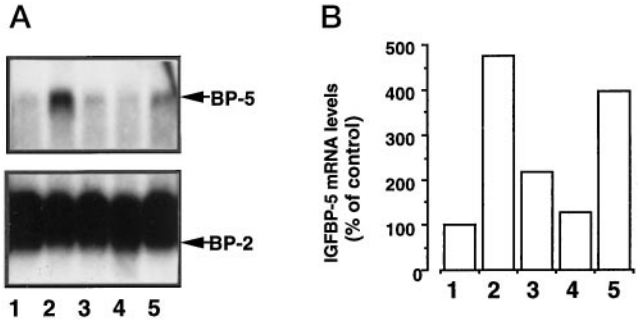

FIG. 6. **Neutralizing endogenous IGF-I decreases IGFBP-5 gene expression in sparsely growing porcine VSMCs.** *A*, autoradiogram showing the effect of neutralizing endogenous IGF-I. Confluent (*lane 1*) and sparse VSMC cultures (*lanes 2–5*) were exposed to serum-free medium for 24 h without (*lanes 1* and *2*) or with the 1:2000 (*lane 3*) or 1:500 (*lane 4*) dilutions of the monoclonal IGF-I antibody Sm 1.2 or mouse IgG (*lane 5*). Total RNA was isolated from VSMC cultures and subjected to Northern blotting with cDNA probes for IGFBP-5 and GAPDH. *B*, PhosphorImager analyses. Values are the means of two experiments. They are expressed as a percentage of mRNA levels in the confluent, untreated control cultures.

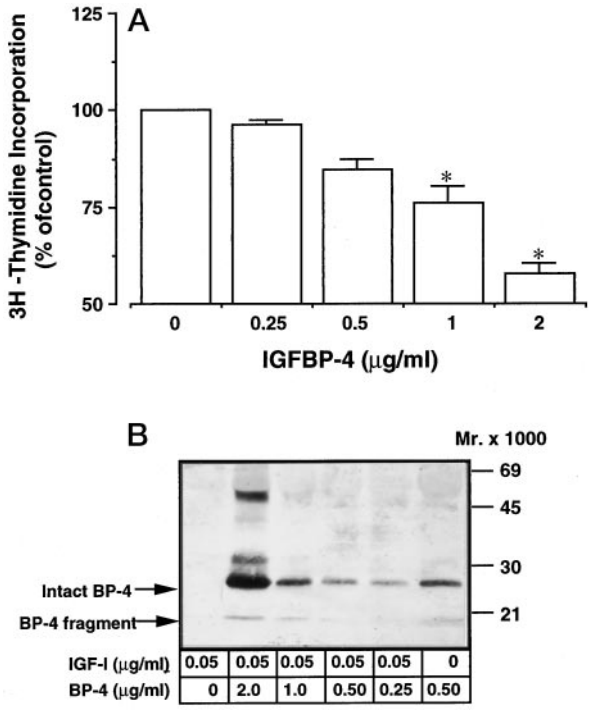

FIG. 7. *A*, effect of human IGFBP-4 on IGF-I-stimulated DNA synthesis in porcine VSMCs. Cells were plated as described under "Experimental Procedures" and incubated 3–5 days without a medium change. Cultures were then exposed to serum-free medium containing [^{3}H]thymidine, 0.2% human platelet-poor plasma, IGF-I (50 ng/ml), and the concentrations of IGFBP that are shown. After 48 h, [^{3}H]thymidine incorporation into DNA was determined as described under "Experimental Procedures." The results are expressed as the percentage increase over control cultures exposed to IGF-I and platelet-poor plasma alone. Values are the means ± S.E. of four experiments. Each experiment point was performed in triplicate for each experiment. *, $p < 0.05$. *B*, immunoblot analysis of the added IGFBP-4 in the medium at the termination of the thymidine incorporation experiments. Intact human IGFBP-4 as well as proteolytic fragments were detected by an anti-human IGFBP-4 antiserum and are indicated by *arrows*.

moderate.

In contrast to IGFBP-4, IGFBP-5 increased the IGF-I-stimulated thymidine incorporation when incubated together with IGF-I for 48 h (Fig. 8*A*). This potentiating effect was statistically significant at 0.5 μg/ml ($p < 0.05$). The potentiating effect of IGFBP-5 on the IGF-I-stimulated DNA synthesis appeared to require a long incubation period. When incubated with IGF-I for 24 h, IGFBP-5 had no potentiating effect (data not shown).

We further examined the degradation of the exogenous IGFBP-5 under these assay conditions. As shown in Fig. 8*B*, with the exception of the highest concentration (1 μg/ml), the exogenously added IGFBP-5 was completely degraded after a 48-h incubation. The disappearance of intact IGFBP-5 appeared to be associated with the potentiation effect of IGFBP-5 in porcine VSMCs.

DISCUSSION

Porcine VSMCs have been previously shown to synthesize and secrete three forms of IGFBPs including IGFBP-2, IGFBP-4, and IGFBP-5 (13, 14). The results of this study indicate that IGFBP-4 and IGFBP-5 are differentially regulated by the autocrine/paracrine secretion of IGF-I. IGF-I decreases IGFBP-4 concentrations by activating its proteolytic cleavage, whereas it increases IGFBP-5 by stimulating gene expression. IGFBP-4 and IGFBP-5, in turn, play opposing roles in modulating IGF-I action. IGFBP-4 inhibits IGF-I-stimulated DNA synthesis, whereas IGFBP-5 has potentiating effects. These results indicate an IGF-I-regulated autocrine loop is present and plays an important role in regulating cell proliferation in porcine VSMCs.

In rat aortic VSMCs, the basal IGFBP-3 levels were higher, while IGFBP-2 levels were lower in confluent cultures as compared with subconfluent cultures (17). The biosynthesis and proteolytic cleavage of IGFBP-4 were higher in confluent as compared with sparse cultures. As a result, the accumulated

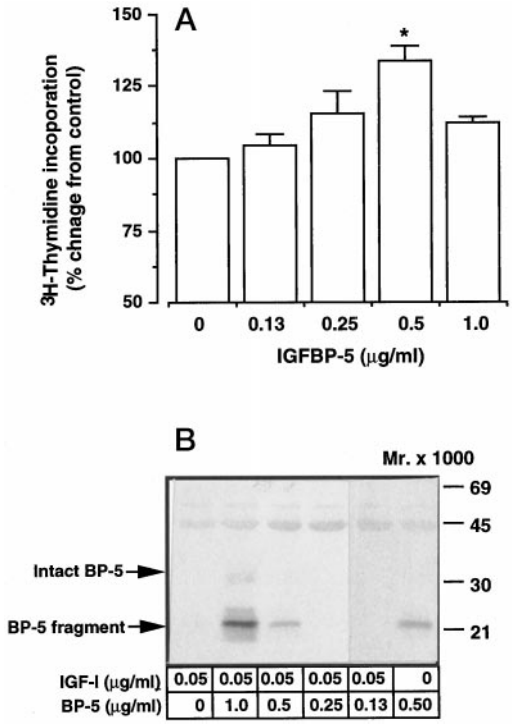

FIG. 8. *A*, effect of human IGFBP-5 on IGF-I-stimulated DNA synthesis in porcine VSMCs. Cells were plated as described under "Experimental Procedures" and incubated 3–5 days without a medium change. Cultures were then exposed to serum-free medium containing [^{3}H]thymidine, 0.2% platelet-poor plasma, IGF-I (50 ng/ml), and the concentrations of IGFBP-5 that are shown. After 48 h, [^{3}H]thymidine incorporation into DNA was determined as described under "Experimental Procedures." The results are expressed as the percentage increase over control cultures exposed to IGF-I and human platelet-poor plasma alone. Values are the means ± S.E. of three experiments. Each experiment point was performed in triplicate for each experiment. *, $p < 0.05$. *B*, immunoblot analysis of the added IGFBP-5 in the medium at the termination of the thymidine incorporation experiments. Intact human IGFBP-5 as well as proteolytic fragments were detected by an anti human IGFBP-5 antiserum and are indicated by *arrows*.

IGFBP-4 levels remained unchanged (17). In the porcine VSMC cultures, the accumulated IGFBP-4 protein levels increased significantly when cultures reached confluence. This increase in IGFBP-4 protein levels is not associated with any increase in IGFBP-4 mRNA levels, suggesting that it is not due to alteration in IGFBP-4 biosynthesis. Porcine VSMCs have been shown to secrete a protease(s) that degrades IGFBP-4 (13, 21). This enzyme(s) is a calcium-dependent serine protease with an estimated molecular size of 48 kDa (21). Although minimal proteolytic activity can be detected in the absence of IGF-I or IGF-II, when either of these growth factors is present the rate of proteolysis is greatly accelerated (13, 21). A major decrease in the IGFBP-4 protease activity was detected when cells reached confluence, suggesting that the increased IGFBP-4 level associated with confluence is probably due to the decreased IGF-I-dependent proteolysis.

A novel observation made in this study is the culture density-dependent expression of IGFBP-5. In contrast to IGFBP-4, the accumulated IGFBP-5 levels were higher in sparse, proliferating cultures. When cells reached confluence, the IGFBP-5 levels declined to nearly undetectable levels. Because porcine VSMCs secrete abundant protease activity that degrades IGFBP-5 (14), we examined whether the change in IGFBP-5 might be regulated by variations in the IGFBP-5 protease. This appears unlikely, since there was no substantial change in IGFBP-5 protease activity when low and high density cultures were compared. Instead, an increase in IGFBP-5 mRNA levels was detected by Northern blot analysis, suggesting that the change in IGFBP-5 is probably due to an increase in the synthesis of this protein.

The inverse correlation between IGFBP-5 abundance and culture density mimics that of the endogenous IGF-I reported previously (5). Previously we have shown that the addition of IGF-I to VSMC cultures significantly increases the IGFBP-5 concentrations. This increase in IGFBP-5 is primarily due to an increase in its synthesis (14). In light of the close correlation between endogenous IGF-I and IGFBP-5 concentrations and the fact that exogenously added IGF-I stimulates IGFBP-5 synthesis, we investigated whether the change in IGFBP-5 might be regulated by variations in the endogenous IGF-I. Neutralization of the endogenous IGF-I with an anti-IGF-I antibody clearly suppressed the IGFBP-5 mRNA expression to the levels of the confluent cultures. Therefore, the endogenous IGF-I, whose expression is also inversely correlated with the culture density (5), may play an important role in determining the relative abundance of IGFBP-4 and IGFBP-5.

The underlying molecular mechanism(s) that regulates culture density/confluence-dependent expression of the IGF-I gene is unclear. There are several examples of cell density-dependent expression of other proteins/genes in VSMCs. The levels of type III collagen and fibronectin mRNA have been shown to be increased in confluent, quiescent VSMCs (22). In mouse BC3H1 cells, a cell line derived from cerebrovascular VSMCs, the smooth muscle cell α-actin gene is maximally induced in confluent cultures (23). A "cell density-responsive element" has been identified in the promoter region of this gene (24). Further effort is needed to determine if a similar cell density-responsive element is present and functional in the IGF-1 gene promoter.

The results of this study demonstrate that IGFBP-4 inhibits, while IGFBP-5 potentiates, IGF-I-stimulated VSMC proliferation under identical culture conditions and that both effects could be demonstrated using concentrations of IGFBP-4 and -5 that were within the range of those endogenously produced by sparse (IGFBP-5) or confluent (IGFBP-4) VSMC cultures. The inhibitory effect of IGFBP-4 on IGF-stimulated cell proliferation is not unique to VSMCs. IGFBP-4 has been consistently shown to be an inhibitory IGFBP in many other cell types (25). A previous study, however, failed to show such an effect in cultured porcine VSMCs. Identical concentrations of IGFBP-4, however, inhibited the effect of IGF-I on fibroblast growth. This is probably due to the presence of IGFBP-4 protease activity in VSMC cultures. When a limited amount of IGFBP-4 is added, it is degraded rapidly, and this probably explains why low concentrations of IGFBP-4 did not inhibit IGF-I action (13). When higher concentrations of IGFBP-4 were tested (*e.g.* >0.5 μg/ml), some intact protein could be detected, and an inhibitory effect was noted.

In contrast to the inhibitory effect of IGFBP-4, IGFBP-5 has been shown to be capable of both inhibiting and potentiating the IGF-I actions in other cell types (26–29). In porcine VSMCs, IGFBP-5 potentiated the IGF-I-induced increase of DNA synthesis. Relatively high concentrations (0.5 μg/ml) and long incubation times (48 h) were required for its potentiation effect. When incubated for a relatively short time (24 h), we found that high concentrations of IGFBP-5 had a moderate inhibitory effect (data not shown). We examined the degradation of the exogenously added IGFBP-5 under these conditions. Almost all of the added IGFBP-5 was degraded after more than 48 h of incubation, but a substantial proportion of IGFBP-5 remained intact within the first 24 h of incubation. Therefore, the difference in the degree of IGFBP-5 proteolysis may explain the different results. Intact IGFBP-5 in the culture medium

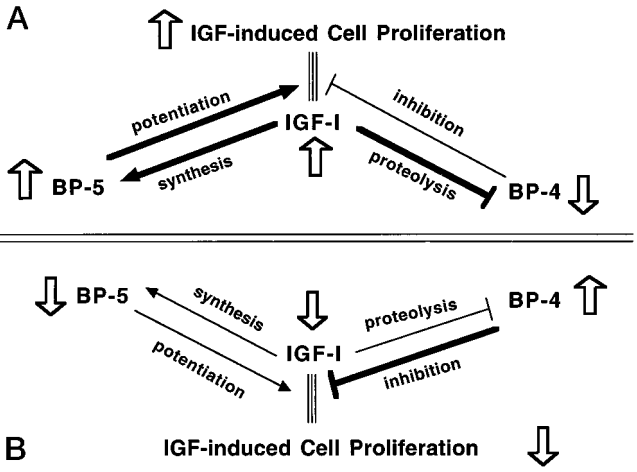

FIG. 9. **Proposed model for IGF-I and IGFBP interactions in VSMCs.** *A*, in the sparse/proliferating cells, the IGF-I expression is high. IGF-I, secreted by VSMCs, up-regulates IGFBP-5 levels through transcriptional activation of the IGFBP-5 gene expression and down-regulates IGFBP-4 levels by activating the IGFBP-4 proteolysis. The relatively higher IGFBP-5 and lower IGFBP-4 levels in turn result in a greater mitogenic response to IGF-I. *B*, in confluent/differentiated cells, the expression of IGF-I and IGFBP-5 is low, whereas the inhibitory IGFBP-4 level is significantly higher due to the decreased IGF-dependent proteolysis. As a result, an attenuation of cellular response to IGF-I is seen under this condition.

binds to IGF-I with a high affinity that is at least 10-fold greater than the IGF-I receptor (25). Consequently, when it is added to the culture medium it limits receptor association (30). Under conditions in which long incubation times are used, most, if not all, of the added IGFBP-5 is degraded into a 21-kDa fragment. This fragment has very low affinity for IGF-I (14). This switch in binding affinity results in the equilibrium favoring binding of IGF-I to its receptor. This hypothesis is supported by the recent observation that high concentrations of a mutant form of IGFBP-5 that is protease-resistant inhibits IGF-I actions in porcine VSMCs (30). Alternatively, this potentiating effect of IGFBP-5 may be related to its capacity to bind to extracellular matrix (ECM) proteins (31). Jones *et al.* (26) have shown that ECM-bound IGFBP-5 potentiates, while soluble IGFBP-5 added to the cultures inhibits, IGF-I-induced cell proliferation in human fibroblasts. The ECM-bound IGFBP-5 has an affinity for IGF-I that is 8-fold lower than the IGF-I receptor, thus permitting better equilibration. The ECM-associated IGFBP-5 is also resistant to proteolysis (31). Therefore, the major function of the protease may be to limit the amount of intact IGFBP-5 in the conditioned medium while allowing the ECM-associated IGFBP-5 to accumulate.

We propose, based on the findings of this and previous studies, a model to explain the potential mechanism by which members of the IGF family interact with each other to determine the cellular response in VSMCs (Fig. 9). The endogenous IGF-I, whose expression is inversely correlated with culture density, plays an central role in controlling IGFBP-4 and IGFBP-5 concentrations. In the sparse/proliferating cells, the IGF-I expression is high, and it increases IGFBP-5 concentrations by stimulating gene expression. It decreases IGFBP-4 concentrations through activating proteolysis. The relatively higher IGFBP-5 and lower IGFBP-4 levels in turn result in a greater mitogenic response to IGF-I. In the confluent/differentiated cells, the expression of IGF-I and therefore IGFBP-5 is low, and the intact IGFBP-4 level is increased due to the decreased IGF-dependent proteolysis. As a result, there is an attenuation of cellular response to IGF-I.

Other investigators have reported a relationship between the confluence or differentiation state of various cell types and the relative abundance of IGFBPs. In human colon cancer CaCo2 cells, which express and secrete IGF-II and IGFBP-2 and IGFBP-4, proliferating cells express abundant IGF-II and low quantities of inhibitory IGFBP-4, while differentiated cells secrete abundant IGFBP-4 and virtually undetectable IGF-II (32). In MC3T3-E1 murine osteoblasts, IGFBP-2 and IGFBP-4 are produced at their highest concentrations in differentiated cells, while IGFBP-5 production is higher in proliferating cells (33). Similar elevated expression associated with confluency was also noted for another inhibitor of IGFs, IGFBP-6, in keratinocytes and myoblasts (34, 35). During the differentiation of mouse C2 myoblasts in culture, proliferating cells express negligible amounts of IGFBP-5 (36, 37). In response to switching to a differentiation medium, myoblasts exit the cell cycle and commence along a differentiation pathway that is accompanied by an extensive induction of IGFBP-5. In this system, however, the levels of endogenous IGFs also increased during the differentiation. It would appear, therefore, the expression of IGFs and IGFBPs is coordinately regulated in response to changes in culture confluence/cell differentiation state in many cell types. Analysis of the mechanisms that control culture density/confluence-dependent changes in IGF-I and IGFBP expression may have important implications for understanding the molecular mechanisms underlying cell growth and differentiation in VSMCs.

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