Insulin-like growth factor binding proteins-2 to -6 are expressed by human vascular smooth muscle cells

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Abstract

We have investigated the expression and secretion of insulin-like growth factor binding proteins (IGFBPs-1 to -6) in human vascular smooth muscle cells (hVSMCs) cultured from human renal arteries. Solution hybridization was used to determine IGFBP mRNA levels and Western immunoblot to detect the corresponding peptides. The hVSMCs expressed mRNAs for IGFBPs-2 to -6; IGFBP-1 mRNA was not detected. IGFBPs-3, -4 and -6 mRNAs were the most abundant, IGFBP-5 was also highly expressed, whereas the IGFBP-2 mRNA was just above the limit of detection. Serum starvation for 48 h significantly decreased the mRNA levels of IGFBPs-2 to -5 and tended to decrease IGFBP-6 mRNA also. IGFBPs-2, -4, -5 and -6 peptides could be detected in conditioned medium, but IGFBP-3 peptide was not detected. IGFBP-4 was the only peptide detected without any concentration step. Low-molecular-mass immunoreactive degradation products were found for IGFBPs-2 and -4. Exogenous IGFBPs-1, -3 and -4 in concentrations of 50 ng/ml inhibited DNA synthesis induced by 1 nM IGF-I, whereas IGFBPs-2, -5 and -6 had no significant inhibitory effects at this concentration. We conclude from these results that all IGFBPs except IGFBP-1 are expressed in hVSMC. Our results indicate that locally produced, in addition to circulating, IGFBPs may have an important role in the regulation of hVSMC.

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Introduction

Vascular smooth muscle cells (VSMCs) are important structural components of the arterial wall. Although they are normally quiescent, VSMCs proliferate during development of atherosclerotic lesions, and proliferation of VSMC is the cause of restenosis after angioplasty (Schwartz *et al.* 1990, Ross 1993). Insulin-like growth factor (IGF)-I is a growth factor for VSMCs both *in vitro* and *in vivo* (Bornfeldt *et al.* 1991, Arnqvist *et al.* 1995) and also stimulates their migration (Bornfeldt *et al.* 1995).

The bioavailability of IGF-I is regulated by at least six high-affinity IGF binding proteins (IGFBPs-1 to -6) (Jones & Clemmons 1995, Kelley *et al.* 1996). IGFBPs regulate the availability of IGFs-I and -II by serving as transporter proteins and as storage pools. Furthermore, they are important modulators of IGF action at the cellular level. Recent data suggest that they also may have biological activities independent of the IGFs, possibly acting through receptors of their own (Liu *et al.* 1992, Jones *et al.* 1993*a*, Oh *et al.* 1993*a*). The IGFBPs exhibit different properties, and their gene expressions are differently regulated by hormones, growth factors and cytokines (Kamyar *et al.* 1994, McCusker & Clemmons 1994, Fan *et al.* 1996, Zhou *et al.* 1996). The affinities of the IGFBPs for IGF-I differ, showing at the most a one-hundredfold variation (Oh *et al.* 1993*b*). Structurally, IGFBPs contain two globular, well-conserved domains, with an intermediate non-conserved domain connecting the other two (Spencer & Chan 1995). IGFBPs-3 to -6 are glycosylated at certain amino acid residues, and IGFBPs-1, -3 and -5 can be phosphorylated to different degrees (Kelley *et al.* 1996). IGFBPs-1 and -2 contain RGD sequences, whereas all IGFBPs except IGFBP-4 contain heparin-binding sequences (Bourner *et al.* 1992, Jones *et al.* 1993*b*, Hodgkinson *et al.* 1994, Gockerman *et al.* 1995). The discovery of specific proteases for IGFBPs has led to new insights as to how IGFBPs regulate IGF-action. To date, proteases for IGFBPs-2 to -5 have been described (Collett-Solberg & Cohen 1996).

Previous studies have demonstrated that rat and porcine VSMC express and secrete IGFBPs-2, -4 and -5 mRNAs and proteins respectively (Giannella-Neto *et al.* 1992, Cohick *et al.* 1993, Kamyar *et al.* 1994, Duan *et al.* 1996) and bovine VSMC IGFBPs-3 and -4 mRNAs (Boes *et al.* 1996). To our knowledge, there has been no systematic study of which IGFBPs are expressed and secreted by VSMCs from different species, and there is only one

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published study of human VSMCs (Boes *et al.* 1996). The purpose of the present study was to investigate the basal expression and secretion of the different IGFBPs in human VSMCs. In addition, we studied the effects of recombinant IGFBPs-1 to -6 on IGF-I-induced DNA synthesis.

Materials and Methods

Cell culture technique

Human vascular smooth muscle cells (hVSMCs) were cultured from renal artery, using an explant technique (Ross 1971). The hVSMCs were allowed to migrate from the primary explants and were subsequently passaged at confluence. Cells were maintained in F12-medium containing 15% fetal bovine serum, 0·05 mg/ml ascorbic acid, 2 μg/ml fungizone and 200 IU/ml penicillin. Culturing was performed at a temperature of 37 °C, humidity of 85% and at a CO_2 concentration of 5% in air. The medium was changed twice a week and the cells were harvested at passages 2–8 using a solution of trypsin (0·25%) and EDTA (0·02%). The cells were characterized as smooth muscle cells both by morphological criteria and by a human α-actin antibody that recognizes a unique epitope of α-actin (Skalli *et al.* 1986). In control experiments, the α-actin antibody did not bind to cells from a breast cancer cell line, MCF-7 (data not shown). On the basis of morphology and our experiments with the α-actin antibody, the cells were identified as a uniform population of smooth muscle cells. The viability of the cells was good, and the cells survived serum starvation for at least 48 h without detaching or changing morphology.

[^{3}H]Thymidine incorporation into DNA

DNA synthesis was quantified by measuring incorporation of [^{3}H]thymidine into DNA. The cells were grown in 24-well plates and incubated for 24 h with the addition of 1 μCi/ml [^{3}H]thymidine and in the presence or absence of IGF-I and the different IGFBPs at indicated concentrations. The cells were washed with F12-medium and DNA was precipitated with 5% ice-cold trichloroacetic acid (TCA). DNA was solubilized in 0·1 M KOH, 500 μl of the solution in each well was added to scintillation liquid and the radioactivity was counted in a liquid scintillation counter. Data were expressed as % of control cells, incubated without reagents.

Solution hybridization/RNase protection assay

The near-confluent cells from three Petri dishes (diameter 100 mm) were harvested either directly or after 48 h of serum starvation in 1 × SET buffer (1% SDS, 10 mM EDTA, 20 mM Tris, pH 7·5) and homogenized with a polytron. Proteinase K was added and the samples were extracted with phenol and chloroform according to the method of Durnam & Palmiter (1983). Total nucleic acids were precipitated in 95% ethanol. The DNA content was measured as described by Labarca *et al.* (1980). The mRNA levels for IGFBPs-1 to -6 were analysed with a solution hybridization assay using [^{35}S]UTP-labelled RNA-probes. The IGFBP-1 probe was synthesized from 350 bp of a human cDNA (Brinkman *et al.* 1988), the IGFBP-2 probe from 446 bp of a human cDNA (Binkert *et al.* 1989), the IGFBP-3 probe from 475 bp of a human cDNA (Wood *et al.* 1988), the IGFBP-4 probe from 505 bp of a human cDNA (Shimasaki *et al.* 1990), the IGFBP-5 probe from 317 bp of a human cDNA (Shimasaki *et al.* 1991*b*), and the IGFBP-6 probe from 267 bp of a human cDNA (Shimasaki *et al.* 1991*a*). The probes were prepared as described earlier by Melton *et al.* (1984). The samples were hybridized with their probe for 18 h, at 70 °C. The hybridization solution contained a total volume of 40 μl 0·6 M NaCl, 20 mM Tris, 4 mM EDTA, 0·75 mM dithiothreitol, 25% formamide and 0·1% SDS. At least 10 000 c.p.m. [^{35}S]UTP-labelled probe was used per hybridization. RNases were then added and the double-stranded RNA was precipitated in 6 M TCA. The hybrids were collected on a filter and the radioactivity was counted in a liquid scintillation counter.

For quantitative analysis of mRNA, a standard curve was included in each assay. The curve was created by hybridizing known amounts of *in vitro*-synthesized standard (sense) RNA with probe (antisense). By comparing the sample values with the values of the curve, a quantitative measure of mRNA could be given. The amount of mRNA was then related to the amount of DNA measured in the extract of total nucleic acid as described above and expressed as amol/μg DNA.

To control the specificity of the probes for IGFBPs-1 to -6, each probe was hybridized to every sense strand of IGFBPs-1 to -6. No hybrids could be detected, indicating that no cross-reactivity between the probes took place. The results thus showed that although the IGFBPs are structurally closely related, each probe bound only to its complementary sense strand, indicating that the IGFBP probes were specific for their sense RNA.

Immunodetection of IGFBPs in hVSMC conditioned medium

Conditioned medium from three Petri dishes (diameter 100 mm), was collected after 48 h of serum deprivation. EDTA at a final concentration at 5 μM was added in order to prevent protease activity. The conditioned medium was concentrated with a final concentration of 5% TCA and the proteins were co-precipitated with 50 μl 0·1% BSA and kept on ice over night. Finally, the conditioned medium was centrifuged at 11 000 r.p.m. for 30 min at 4 °C and the pellets were dissolved in electrophoresis sample buffer, obtaining a 20-fold concentration.

The concentrated samples were heated at 95 °C for 3 min before samples were applied to a 15% SDS–polyacrylamide gel. The size-fractionated proteins were electroblotted onto polyvinylidene difluoride membranes for 1 h at 200 mA. The membranes were blocked in a buffer containing polyvinylalcohol (0·2%) low molecular range in PBS pH 7·5 for 1 h at 20 °C or over night in a refrigator. The membranes were washed once for 15 min and twice for 5 min with Tris-buffered saline with Tween (TBS-T) (0·1%) and then incubated with specific antibodies for 1 h at 20 °C (with gentle shaking), IGFBP-2 and IGFBP-4 antisera with a 1 : 2000 dilution, IGFBP-5 antiserum with a 1 : 1500 dilution and IGFBP-3 and IGFBP-6 antisera with a 1 : 1000 dilution. The membranes were then washed as above in TBS-T (0·1%). After incubation with a second anti-immunoglobulin G antibody coupled to horseradish peroxidase, the membranes were washed as above. Finally the proteins were visualized by chemiluminescence using the enhanced chemiluminescence Western blotting detection system (Amersham Int., Amersham, Bucks, UK).

Materials

Proteinase K was from Merck (Darmstadt, Germany), [^{35}S]UTP, [^{3}H]leucine and [^{3}H]thymidine were from Amersham Int. and the chemicals for antisense (probe) and sense (standard) synthesis from Promega (Madison, WI, USA). RNase A, RNase T1 and herring sperm DNA were obtained from Boehringer (Mannheim, Germany). Phenol was from Fisher Scientific (Fair Lawn, NJ, USA), trypsin from Difco Labs (Detroit, MI, USA) and collagenase type I from Sigma (St Louis, MO, USA). Scintillation liquid (Ultima Gold) was obtained from Packard Instrument Company (Meriden, CT, USA). The monoclonal antibody against α-smooth muscle actin was from Sigma Immuno Chemicals (La Jolla, CA, USA). Chemicals and solutions for cell culture were from Gibco Brl Life Technologies (Täby, Sweden). Recombinant human IGFBPs-2, -4, -5 and -6 peptides were purchased from Austral Biologicals (San Ramon, CA, USA). Recombinant IGFBP-3 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Antibodies for IGFBPs-2, -3 and -4 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Antibodies for IGFBPs-5 and -6 were purchased from Austral Biologicals (San Ramon, CA, USA). Recombinant IGF-I was a gift from Pharmacia Upjohn (Stockholm, Sweden).

Statistical analysis

Values are reported as means ± S.E.M. Data were analysed using standard statistical methods including ANOVA (Scheffé's test), Student's *t*-test and linear regression analysis. A value of $P \leq 0{\cdot}05$ was considered to be significant.

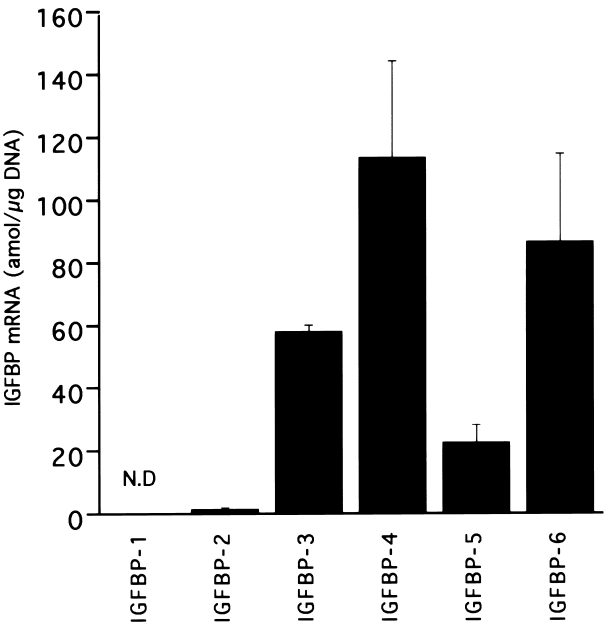

Figure 1 Gene expression of IGFBPs-1 to -6 in hVSMCs. hVSMCs were seeded out in Petri dishes (150 000 cells/dish) and harvested after 4 days, when reaching near-confluency. The mRNA levels were determined by solution hybridization as described in Materials and Methods. Samples were analysed in triplicate. Values are given as means ± S.E.M., $n=4$.

Results

IGFBP mRNA levels in hVSMCs

The mRNAs for IGFBPs-1 to -6 were measured by solution hybridization in hVSMCs. We were able to show that hVSMCs express mRNAs for IGFBPs-2 to -6 (Fig. 1). IGFBP-1 mRNA was not detected with our assay (limit of detection 0·1 amol/μg DNA). IGFBPs-4, IGFBP-6 and IGFBP-3 mRNAs were the most highly expressed, IGFBP-5 was highly expressed, and IGFBP-2 mRNA was just above the limit of detection (Fig. 1). To test if the expression of IGFBPs is regulated by serum factors, we measured mRNA levels after 48 h of serum deprivation. The mRNA levels of IGFBPs-2 to -5 were decreased significantly when serum was omitted, and that of IGFBP-6 tended to be decreased also (Fig. 2).

IGFBPs in conditioned medium

To determine if IGFBP mRNA expression is associated with the release of the corresponding IGFBPs, conditioned medium from the hVSMCs was analysed by Western immunoblot. IGFBPs-2, -4, -5 and -6 could be detected in the conditioned medium (Fig. 3). We were not able to detect IGFBP-3, despite the fact that the peptide could be detected in human serum with the antibody used (data not shown). IGFBP-4 was the only protein that could be

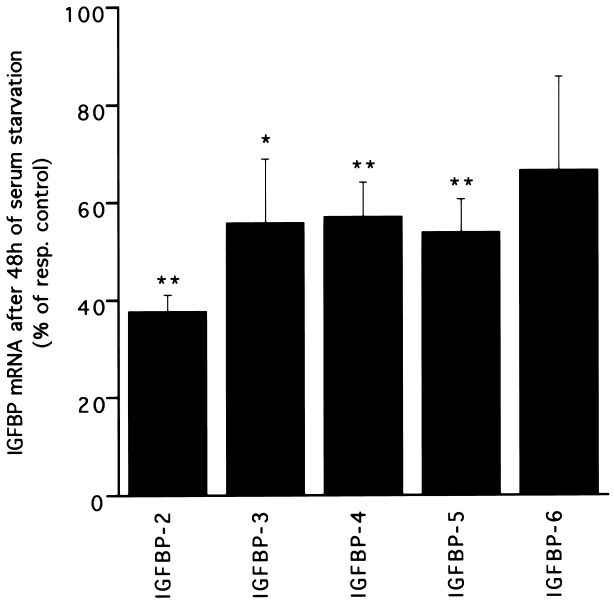

Figure 2 Effect of serum starvation on expression of IGFBPs-2 to -6 mRNAs in hVSMCs. The cells were seeded out in Petri dishes (150 000 cells/dish) and cultured for 4 days until near-confluency. They were then serum-starved for 48 h. The cells were harvested and the mRNA levels were determined by solution hybridization as described in Materials and Methods. Data are given as % of respective (resp.) control (not serum-deprived hVSMCs) and presented as means ± S.E.M. Statistical comparisons were made according to Student's one way *t*-test. **P*<0·05, ***P*<0·01 compared with respective control.

detected without any concentration step. Low-molecular-mass immunoreactive bands appeared for IGFBP-2 at 20 kDa and for IGFBP-4 at 16 kDa, suggesting degradation products. IGFBPs-4 and -6 were identified with double bands, indicating a possible glycosylation of these IGFBPs.

Effects of IGFBPs on IGF-I-induced DNA synthesis

IGF-I induced DNA synthesis in a dose-dependent way (Fig. 4). EC_{50} was calculated as 0·1 nM and an IGF-I concentration of 1 nM gave a maximal effect. IGF-I (1 nM) was subsequently added together with 50 ng/ml of the respective IGFBP. In a concentration of 50 ng/ml, IGFBPs-1, -3 and -4 significantly inhibited IGF-I-induced DNA synthesis (Fig. 5). IGFBP-2 tended to inhibit IGF-I action, whereas IGFBPs-5 and -6 had no effect (Fig. 5). IGFBPs-1 to -6 added alone had no significant effects compared with control (Fig. 5).

Discussion

Expression of IGFBP mRNAs in human VSMCs

In this study, we have shown that VSMC derived from human renal artery express IGFBPs-2 to -6, and that the corresponding proteins could be demonstrated for IGFBPs-2, -4, -5 and -6. Boes *et al.* (1996) have recently shown that human VSMC cultured from aorta, coronary and pulmonary arteries express IGFBPs-3, -4 and -6, according to standard Northern blot analysis. These IGFBPs were also the most highly expressed binding proteins in our study, with IGFBP-4 mRNA being most abundant. However, we were also able to detect considerable amounts of IGFBP-5 mRNA. In addition, low amounts of IGFBP-2 mRNA (just above the limit of detection of our assay) were detected. Boes *et al.* (1996) were able to detect robust bands for IGFBP-2 mRNA in human pulmonary SMC using reverse transcription PCR, whereas faint bands were shown with IGFBPs-1 and -5 primers. A difference between our study and that of Boes *et al.* (1996) is our detection of high levels of IGFBP-5 mRNA. To our knowledge, these two reports are the only studies investigating IGFBP gene expression in human VSMCs.

Bovine aortic SMCs have been shown to express predominantly IGFBPs-3 and -4 (Boes *et al.* 1996). Porcine VSMCs have previously been shown to express IGFBPs-2, -4 and -5 mRNAs (Cohick *et al.* 1993, Duan *et al.* 1996), and rat VSMCs seem to express predominantly IGFBPs-2 and -4 (Kamyar *et al.* 1994). According to the available data, there seem to be species differences in IGFBP gene expression in VSMCs. In all species, IGFBP-1 is either not or only very faintly expressed in VSMCs.

IGFBP protein levels in hVSMC conditioned medium

We were able to detect IGFBPs-4 and -6 peptides in large amounts in the hVSMC conditioned medium after 48 h of serum-free incubation. IGFBP-3 peptide could not be detected, and IGFBP-5 peptide was detected only with a faint band, despite their high mRNA levels. In contrast to this, IGFBP-2 peptide was detected in about the same concentrations as those shown for IGFBPs-4 or -6. Boes *et al.* (1996) have described that human aortic SMCs, in common with coronary and pulmonary artery SMCs, secrete IGFBPs-3 and -4, and a 30 kDa IGFBP, identified as IGFBP-6. In comparison with their study, our results differed with respect to our detection of high concentrations of IGFBP-2 peptide, our failure to demonstrate IGFBP-3, and our detection of IGFBP-5. In another study (van der Ven *et al.* 1996), IGFBP-3 was determined by RIA in concentrations of 13–288 ng/ml in the conditioned medium of human uterine SMC. Interestingly, high intensity immunostaining was located on SMC membranes and in the cytoplasm. A high degree of IGFBP-3 binding to the SMC could be one explanation of why we fail to detect IGFBP-3; another reason could be high protease activity, as demonstrated by Boes *et al.* (1996), although no immunoreactive degradational products were found in our Western blots. However, the

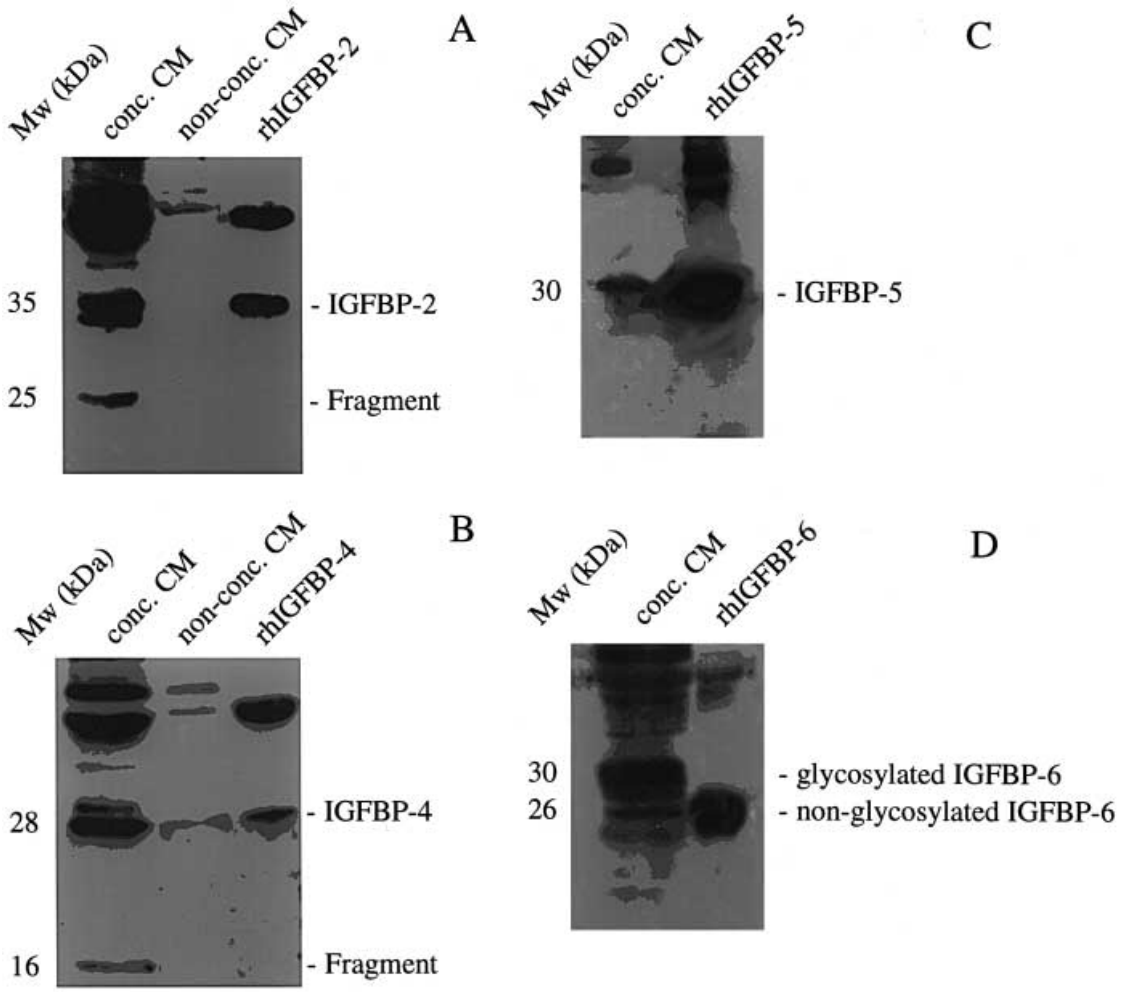

Figure 3 Levels of (A) IGFBP-2, (B) IGFBP-4, (C) IGFBP-5 and (D) IGFBP-6 peptides in conditioned medium (CM). Near-confluent hVSMCs were serum-starved and after 48 h the conditioned medium was collected, concentrated (1 : 20) and subjected to Western immunoblot analysis as described in Materials and Methods. Major bands at higher molecular masses (Mw) in all blots at approximately 65 kDa are bovine serum albumin. The figures show representative data ($n=3$).

antibody may not have affinity for the degradation products. Experience from other species suggests that IGFBPs-2, -4 and -5 are secreted from porcine VSMCs (Cohick *et al.* 1993, Duan *et al.* 1996) and predominantly IGFBPs-2 and -4 from rat VSMCs (Kamyar *et al.* 1994); bovine VSMCs have been found to secrete IGFBP-4 and barely detectable levels of IGFBP-3 (Boes *et al.* 1996).

Regulation of IGFBP mRNAs and peptides

A trend observed in our study was a downregulation of IGFBP mRNA in hVSMCs that were serum-deprived for 48 h, compared with cells grown in serum-containing medium. IGFBPs-3, -4 and -5 mRNAs were significantly decreased after 48 h of serum deprivation, and IGFBPs-2 and -6 tended to be decreased.

Grant *et al.* (1996) were unable to detect IGFBPs-1 to -5 in smooth muscle layers of normal human coronary artery when using immunocytochemistry. Furthermore, IGF-I and the type I IGF receptor were not found. However, all the IGFBPs, in addition to IGF-I and its receptor, were localized in the cytoplasm of synthetic SMC in atherectomy plaques. This suggests that expression of the IGF-system in VSMCs is a reflection of the phenotype of the cells.

We found low-molecular-mass immunoreactive bands for IGFBPs-2 and -4 in the conditioned medium of the hVSMCs. This has previously been described in porcine VSMCs by Gockerman & Clemmons (1995) and by Parker *et al.* (1995), indicating the presence of fragments of these IGFBPs. Proteases secreted by rat and porcine VSMCs have also been reported for these IGFBPs

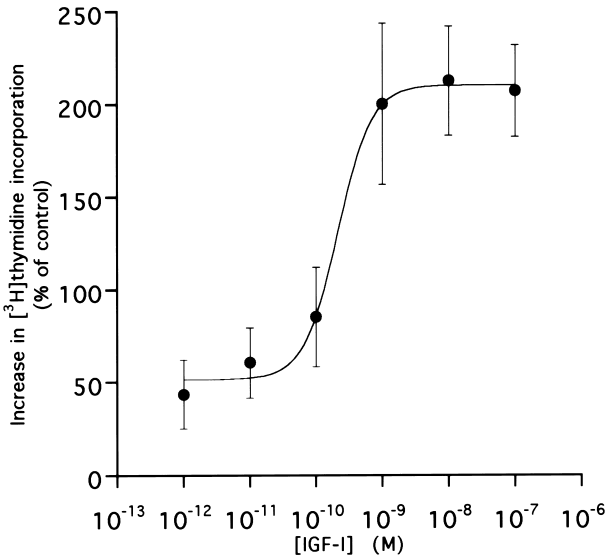

Figure 4 Dose–response curve for IGF-I-stimulated DNA synthesis in cultures of hVSMCs. Values represent % of control values. Near-confluent cell cultures were starved for 24 h and subsequently exposed to IGF-I (10^{-6}-10^{-12} M) for 24 h, after which [^{3}H]thymidine incorporation was determined. Values are given as means ± S.E.M., $n=3$; samples were measured in triplicate.

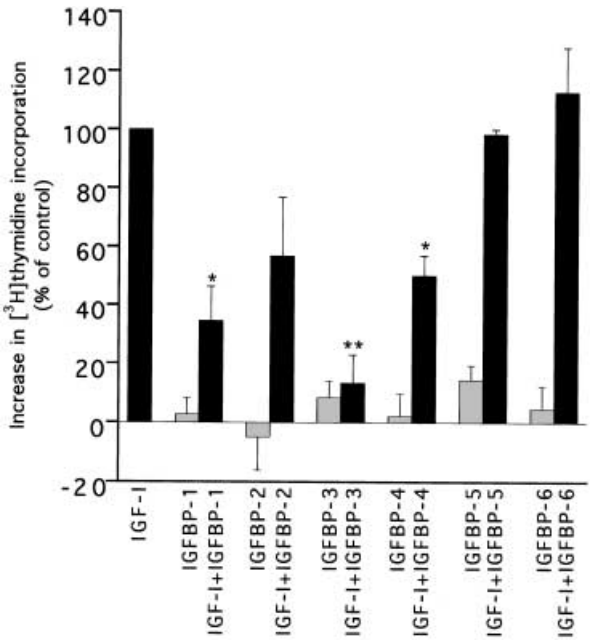

Figure 5 Modulation by IGFBPs-1 to -6 of the response of hVSMCs to IGF-I. Effect of IGF-I (10^{-9} M) added together with IGFBPs-1 to -6 (50 ng/ml) on [^{3}H]thymidine incorporation. Values are given as means ± S.E.M., $n=4$; samples were measured in triplicate. ANOVA, Scheffé's method, was used for comparing more than two groups. *P<0·05, **P<0·01, $n=4$.

(Kamyar *et al.* 1994, Gockerman & Clemmons 1995, Parker *et al.* 1995). The specific proteases secreted from human VSMCs need to be characterized.

Modulatory actions of IGFBPs on IGF-I-induced DNA synthesis

The stimulatory action of IGF-I on DNA synthesis in human VSMCs reached a maximal effect at a concentration of 1 nM, which was subsequently used in the experiments performed with IGFBPs. Each of the IGFBPs was added in a concentration of 50 ng/ml (1·4 nM–2·0 nM), which is about equimolar to the concentration of IGF-I (1nM) used. In the present study, IGFBPs-1, -3 and -4 were potent inhibitors of IGF-I (1 nM)-induced DNA synthesis, whereas IGFBPs-2, -5 and -6 had no significant effects. IGFBPs-1, -3, -4 and -5 have about the same K_a value (20×10^{-9} M^{-1}) (Martin & Baxter 1986, Jones *et al.* 1991, Kiefer *et al.* 1992) for IGF-I, whereas IGFBPs-2 and -6 have a lower K_a value (1×10^{-9} M^{-1}) (Roghani *et al.* 1991, Kiefer *et al.* 1992). The inhibitory actions of the IGFBPs are in agreement with their affinity for IGF-I, with the exception for IGFBP-5. IGFBP-5 is rapidly degraded, and this could be one explanation for its lack of effect (Imai *et al.* 1997). We have previously shown that IGFBPs-1, -2 and -4 all dose-dependently inhibit IGF-I-induced DNA and protein synthesis in rat VSMCs (Gustafsson *et al.* 1999), IGFBPs-1 and -4 being more potent than IGFBP-2. The effects of IGFBPs-3 and -6 have been less extensively studied to date. The physiological concentrations of IGFBPs-2, -4 and -6 in the circulation of healthy individuals are about 250 ng/ml, whereas those of IGFBP-1 fluctuate in accordance with the metabolic state (0–500 ng/ml) (Collett-Solberg & Cohen 1996); the concentration of IGFBP-3 in the circulation is about 4000 ng/ml and that of IGFBP-5 approximately 600 ng/ml (Collett-Solberg & Cohen 1996). The contribution to the pericellular concentration of IGFBPs that is attributable to IGFBPs produced by VSMCs is not easily measured. However, if IGFBPs in the circulating concentrations gain access to the pericellular space, this will have an inhibitory effect on IGF-I-stimulated processes.

In conclusion, this study demonstrates that hVSMCs express mRNAs for IGFBPs-2 to -6 and secrete IGFBPs-2, -4, -5 and -6. IGFBPs-1, -3 and -4 have inhibitory actions on IGF-I-induced DNA synthesis in concentrations less than those in the circulation. This suggests that locally produced and circulating IGFBPs are of importance in vascular physiology and in the response to endothelial injury.

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