Expression and Insulin-like Growth Factor–Dependent Proteolysis of Insulin-like Growth Factor–Binding Protein-4 Are Regulated by Cell Confluence in Vascular Smooth Muscle Cells


Abstract Insulin-like growth factor (IGF)-I is markedly induced after balloon injury in the rat aorta, where it may serve to mediate vascular remodeling. Because the bioavailability of IGF-I is modulated by IGF-binding proteins (IGFBPs), we examined the regulation of IGFBPs by IGFs in primary cultures of rat aortic smooth muscle cells (SMCs). Serum-deprived SMC-conditioned medium contains IGFBPs of 38 to 45 kD (only in confluent cultures), 30 kD (possibly IGFBP-2), 28 kD, and 24 kD (IGFBP-4), the latter being the most abundant. IGF-I and IGF-II but not insulin evoked a marked decrease of IGFBP-4 as early as 4 hours after treatment. IGFBP-4 mRNA abundance, however, was entirely unaffected by IGF-I for up to 48 hours. IGF-I analogues with high affinity for the IGF-I receptor and weak affinity for IGF-II paradoxically evoked a small increase in IGFBP-4, probably through a general increase in protein synthesis. IGF-I only minimally decreased IGFBP-4 content in medium of sparse cultures, whereas it completely abolished IGFBP-4 content in conditioned medium of superconfluent SMCs. IGF-I also evoked a concentration-dependent increase in the abundance of IGFBP-3 in confluent, but not sparse, SMCs without affecting IGFBP-3 mRNA. Addition of IGF-I to cell-free medium conditioned by confluent, but not by sparsely cultured, SMCs led to rapid degradation of IGFBP-4. Interestingly, IGFBP-4 mRNA was markedly induced in confluent relative to sparsely grown SMCs in an IGF-I independent fashion. Thus, both biosynthesis and IGF-dependent proteolysis of IGFBP-4 are increased in confluent SMCs. Proteolysis was maximal at 37°C and was abrogated by EDTA and by benzamidine. Phenylmethylsulfonyl fluoride and the plasmin inhibitor bdellin had minor inhibitory activity, whereas aprotinin, angiotensin-converting enzyme inhibitors, and N-ethylmaleimide were without effect. The protease does not affect the structure of IGF-I as determined by reverse-phase high-performance liquid chromatography and size-exclusion chromatography of 125I-IGF-I incubated for up to 24 hours with SMC-conditioned medium containing IGFBP-4. In summary, SMCs elaborate a cation-dependent protease in a confluence-dependent fashion, which degrades bound IGFBP-4 and likely releases free structurally intact IGF-I, presumably to interact with the cell surface receptor and/or other IGFBPs. (Circ Res. 1994;74:576-585)

Key Words • insulin-like growth factor • smooth muscle cells • protease • insulin-like growth factor binding proteins
tioned media from a variety of cell types. The expression of IGFBPs tends to be tissue and cell specific and is developmentally regulated. We and others have reported that rat aortic SMCs elaborate IGFBPs of 37 to 42, 30, and 24 kD. The 24-kD IGFBP is the most abundant form in subconfluent SMC cultures; it comigrates, is probably identical to IGFBP-4, and is induced by PDGF. By stimulating the expression of the inhibitory protein IGFBP-4, PDGF may set in motion mechanisms to limit the extent of the mitogenic response. Specifically, proteolytic activities have been identified in the conditioned medium of fibroblasts of different species that cleave IGFBP-4 after exposure to IGF-I. In the present study, we examined the effects of IGFs on the production of their carrier proteins in SMCs, with a focus on IGFBP-4. We demonstrate that IGF-I regulates the abundance of IGFBP-4 by evoking its proteolysis by cation-dependent protease(s), thus releasing free structurally intact IGF-I. Furthermore, both biosynthesis and the IGF-I-dependent degradation of IGFBP-4 are highly sensitive to cell density. These data predict that an intricate set of events control access of IGF-I to its cognate receptor in vascular SMCs.

Materials and Methods

Cell Culture

SMCs were isolated from rat thoracic aorta and cultured by a modification of the method of Chamley-Campbell et al. with minor modifications. Briefly, 90-day-old male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind) weighing 300 g were anesthetized with CO2. A section of the aorta, from the aortic arch to the level of the renal arteries, was aseptically excised and placed in cold Hanks’ balanced salt solution (HBSS, Gibco, Grand Island, NY). Adhering fat and connective tissue from the adventitia were stripped off by blunt dissection. Three or four vessels were extensively washed with fresh HBSS before being longitudinally opened. The opened aortas were dissociated in an enzymatic solution containing 0.1% collagenase (type CLS 2, 130 U/mg, Worthington Biochemical Corp, Freehold, NJ), 0.05% elastase (Sigma Chemical Co, St Louis, Mo), 100 U/mL penicillin, and 100 μg/mL streptomycin (Irvine Scientific, Santa Ana, Calif) for 15 to 20 minutes at 37°C in 95% air/5% CO2. The rest of the external connective tissue was then carefully removed using magnification, and the endothelial layer was scraped with a forceps. After dissection, aortas were placed in a fresh enzyme solution, minced into 1- to 2-mm pieces, and incubated in the above-mentioned conditions for an additional 1.5 to 2.0 hours. The suspension was triturated at 30-minute intervals. After the enzymatic disaggregation, the suspension was filtered through a nylon mesh and pelleted by centrifugation at 110g for 10 minutes. Cells were resuspended with 8 mL of Dulbecco’s modified Eagle’s medium (DMEM, Irvine Scientific). An aliquot of 100 μL was withdrawn, and cell viability was evaluated by trypan blue dye exclusion before inactivating the enzymes by addition of 1.98 mL of fetal calf serum (FCS, Hyclone Laboratories, Logan, Utah). Flasks (75 cm2, Costar Corp, Cambridge, Mass) were inoculated at a density of 2x106 viable cells per square centimeter.

After 2 to 7 days, the cells from the primary culture were harvested with a solution of trypsin (0.01%, Sigma) and collagenase (0.02%, Sigma) and plated at 2x104 cells per square centimeter. Subcultures were grown in DMEM supplemented with 10% FCS, 200 mM/L l-glutamine (Irvine Scientific), and antibiotics, with media changes three times weekly.

Cells were used between the third and seventh passage. After being rinsed twice, cells were incubated with serum-free DMEM (Irvine Scientific) supplemented with l-glutamine (Irvine Scientific), 5 μg/mL transferrin, 0.2 mM/L ascorbate (Sigma), and antibiotics for 3 days. After this period of cellular synchronization, the medium was replaced with or without addition of the indicated concentrations of the mitogens for varying time intervals. Recombinant human IGF-I, IGF-II, or insulin (Bachem Inc, Torrance, Calif) was dissolved in a vehicle of 10 mM/L acetic acid and 0.2% bovine serum albumin (BSA, Sigma). For the studies measuring IGFBP by Western-ligand blotting, cells were plated at 2x104 cells per square centimeter onto six-well clusters and grown in DMEM with 10% FCS. After 3 days in serum-free medium, cells were incubated with the indicated reagents. At the appropriate times, medium was collected, immediately frozen, and stored until assayed, and cells were electronically counted (Counter counter, Coulter Electronics, Inc, Hialeah, Fla).

Ligand Blots

Western-ligand blots were performed as previously described. Briefly, SMC-conditioned medium was collected and immediately stored at −20°C until assayed. Two milliliters of conditioned medium was concentrated by ultracentrifugation through a Centrifuge-10 microcentrator (Amicon Division, W.R. Grace & Co). A 75-μL aliquot of the retentate was mixed with 25 μL of 4-fold sample buffer (62.5 mM/L Tris, 10% glycerol, 2.3% sodium dodecyl sulfate [SDS], and 10 mM/L dithiothreitol, pH 6.8) and heated to 60°C for 10 minutes. Forty microliters of this mixture was loaded onto an 11% discontinuous SDS-polyacrylamide gel in nonreducing conditions according to the method of Hossenlopp et al. The samples were electrophoresed at a constant current of 15 MA for 3 to 4 hours or until 15 minutes after the dye front reached the bottom of the gel. Proteins were transferred in solution onto a 0.45 μM/L nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad Laboratories, Richmond, Calif) in a transfer cell at 200 MA for 40 minutes. The membrane was washed with 0.1% Tween 20 in saline for 1 hour and then with 1% BSA (IGF-I free) in 0.9% NaCl for 1 hour. The membrane was then incubated with [125I]IGF-I (200 000 cpm) in saline with 1% BSA and 0.1% Tween for 24 hours at 4°C. After hybridization, the membrane was washed with 0.9% NaCl with 1% BSA and 0.1% Tween for the time of 20 minutes each. Binding protein bands were visualized by autoradiography. The molecular weights were estimated by comparisons with [14C]labeled protein standards (Amersham Corp, Arlington Heights, IL), and densities of the bands were determined by scanning densitometry.

Immunoprecipitation/Western-Ligand Blotting

To confirm the identity of the 24-kD IGFBP, 300 μL of an 8-fold concentrate of SMC-conditioned medium was incubated with 20 μL of one of the following polyclonal antibodies: α4, αδ (to rat and human IGFBP-4, respectively; kind gifts from P. Fielder and R. Rosenfeld, Stanford University), anti-IGFBP-4 (human) (UBI, Lake Placid, NY), or nonimmune rabbit serum in the presence of 30 μL of protein A (Repligen, Cambridge, Mass) for 16 hours at 4°C, with shaking. After centrifugation, the pellets were washed, dried, and resuspended in 1X sample buffer, without β-mercaptoethanol, and Western-ligand–blotted as described.

Protease Assays

SMC-conditioned media were collected and immediately stored at −20°C until assayed. To study degradation of IGFBP-4, the conditioned medium was exposed to varying temperatures or incubated with the various protease inhibitors for the indicated times before Western-ligand blotting. To further study IGFBP-4 protolysis, 80 000 cpm of purified human 1-3IGFBP-4 (100 μCi/μg) was incubated with 50 μL of an 8-fold concentrate of SMC-conditioned medium for 6 hours in the presence or absence of 50 ng/mL IGF-I, des (1-3) IGF-I,
or R3 IGF-I (Gro-Pep, Adelaide, Australia), with or without the indicated concentrations of protease inhibitors. Unreduced samples were fractionated by SDS-polyacrylamide gel electrophoresis using a 10% to 20% linear gradient.

RNA Extraction

RNA was extracted from cells in culture by the method of Chirgwin et al. Briefly, the medium was removed from each flask and stored at ~70°C for further use. The cells were washed twice with 4°C sterile 0.01 mol/L phosphate-buffered saline, pH 7.5. Five milliliters of a lysis solution containing 4 mol/L guanidine isothiocyanate, 3 mol/L sodium acetate (pH 6.0), 0.5% sodium N-lauroylsarcosine, and 0.83% β-mercaptoethanol was added to each culture flask, and the cells were scraped with a rubber policeman. The suspension was homogenized with a sonicator for 30 seconds before being layered onto 4 mL of 5.7 mol/L cesium chloride and centrifuged for 18 to 20 hours at 170 000g at 20°C. The supernatant was carefully removed, and the clear RNA pellet was resuspended with 0.3 mol/L sodium acetate by repeated pipetting. The solubilized RNA was precipitated overnight with 2.5 vol of ethanol at ~20°C. The RNA was then pelleted, washed with 80% ethanol, dried, and resuspended in 10 mmol/L Tris-HCl (pH 7.5) and 1 mmol/L EDTA. UV spectrophotometry at 260 mmol/L was used for quantification of total RNA. The integrity and accuracy of the RNA quantification were confirmed by submitting 5.0-μg aliquots of each sample to 1% agarose/formaldehyde gel electrophoresis and ethidium bromide staining. Only undegraded samples with intact 18S and 28S ribosomal RNA and optical absorbance A260/280 ratios of >1.9 were processed.

Northern Blots

For Northern blot analysis, gel electrophoresis of 20 μg total RNA was performed on 1% agarose gels containing 2.2 mol/L formaldehyde, as previously described. The filters were hybridized in a buffer containing 50% formamide, 5× SSPE (43.8 g/L NaCl, 6.9 g/L NaH₂PO₄·H₂O, and 1.85 g/L EDTA), 5× Denhardt’s solution (1 g/L polyvinylpyrrolidone, 1 g/L BSA, and 1 g/L Ficoll 400), 0.1% SDS, and 200 μg/mL salmon sperm DNA for 16 hours at 32°C with 32P-labeled probes. Probing filters were labeled with [α-32P]dCTP using the random-primer technique following the manufacturer’s protocol (Stratagene, La Jolla, Calif). The following rat probes were used: IGFBP-1, pRBP, and EcoRI-EcoRI fragment (29); IGFBP-2, pRBP-2, and pEcoRI-HindIII fragment (30); IGFBP-3, pRFI507, and Apa I-Rso I fragment (30); IGFBP-4, pRBPl-501, and Sma I-HindIII fragment (30); and cyclin C, pCD15.8.1, and BamHI fragment (30). Chromatography of 125I-IGF-I in SMC-Conditioned Medium

As will be shown below, confluent SMCs release a protease that cleaves IGFBP-4 only after it is bound to IGFs. To determine how this proteolytic activity affects IGF-I itself, 0.1 μCi 125I-IGF-I was incubated with 50 μL cell-free conditioned medium from confluently plated SMCs for up to 48 hours. At the indicated times, the integrity of the labeled IGF-I was examined by gel permeation chromatography and reverse-phase high-performance liquid chromatography (HPLC). Gel permeation was performed in the presence or absence of 10% β-mercaptoethanol, using a Sphero gel TSK 3000 PW column. The mobile phase was 0.3 mol/L triethylamine/PO₄·H₂O, pH 7.0, and 20% propanol. For reverse-phase HPLC, the mobile phase was made up to 500 μL with 1% trifluoroacetic acid (phase A). The mobile phase was a gradient of 85% to 100% methanol in 30 minutes (phase B), at a rate of 1 mL/min. Samples were injected into a Beckman HPLC apparatus consisting of 110B solvent delivery modules, a System Gold programmable detector controlled by a 406 analog interface, and a 25-cm Vydac C18 column. One-milliliter fractions were collected and counted.

Results

As shown in Fig 1, IGF-I evoked a rapid concentration-dependent decrease in the abundance of the 24-kD protein, previously shown to comigrate with IGFBP-4. In contrast, there was a modest increase in the abundance of the 28- and 30-kD IGFBPs at 24 hours. Interestingly, the rapid inhibition of IGFBP-4 by IGF-I occurs in the absence of changes in the steady-state levels of the 2.6-kb IGFBP-4 mRNA (Fig 1B).

To confirm the identity of the 24-kD IGFBP in SMC-conditioned medium, immunoprecipitation/Western-ligand blotting experiments were performed using three different polyclonal antibodies to IGFBP-4 (Fig 2). As shown in panel A, the two antibodies directed against human IGFBP-4 recognized the corresponding purified protein (lanes 4 and 5), revealing a band that comigrated with human IGFBP-4, which had not been subjected to immunoprecipitation. Panel B shows an almost identical immunoprecipitation/Western-ligand blot with a concentrate of rat SMC–conditioned medium, which in addition was also faintly recognized by the low-affinity rat-specific IGFBP-4 antiserum α-4.

A comparison of the effects of the major members of the insulin/IGF family on IGFBP-2 mRNA in SMCs is shown in Fig 3. IGF-I and IGF-II were equipotent at decreasing the abundance of the 24-kD IGFBP in SMC-conditioned medium. Concentrations as low as 0.1 mmol/L of IGF-I and IGF-II were sufficient to markedly decrease 24-kD IGFBP-4 content at 24 hours. In contrast, insulin lacked this effect, even at concentrations in which cross-reactivity with the IGF-I receptor would be expected (i.e., 100 nmol/L). Neither IGF-I, II, nor insulin modulated the content of IGFBP-4 or the 1.8-kb IGFBP-2 mRNA transcript at either 4 hours (data not shown) or 24 hours.

These data suggest that IGFs are exerting an inhibitory effect on IGFBP-4 in a receptor-independent fashion. To further explore this possibility, SMCs were treated with IGF-I analogues, which retain high affinity for the IGF-I receptor but bind with much lower affinity to IGFBP. As shown in Fig 4, long R3 IGF-I and des (1-3) IGF-I failed to significantly decrease 24-kD IGFBP abundance (if anything, they evoked a small paradoxical stimulation).

On repeated experimentation, a degree of variability in basal and IGF-I–dependent changes in IGFBP content of SMC-conditioned medium was observed, an effect that appeared to be dependent on cell density. The influence of cell confluence on IGF-I regulation of IGFBP was therefore systematically explored (Figs 5 and 6). SMCs were seeded at three different densities and grown in 10% FCS until sparse, subconfluent, and confluent conditions were attained (cell counts at the time of harvest were 6.2, 9.8, and 11.2×10⁴ cells per square centimeter, respectively). As shown in Fig 5, basal levels as well as the IGF-I–dependent increase of the 36- to 45-kD IGFBP (IGFBP-3) were barely detectable in sparsely plated cells, whereas confluent cells showed a clear concentration-dependent increase in this protein. Cell confluence had no effect on IGFBP-3 mRNA abundance of basal or IGF-I–treated SMCs (Fig 6). Basal levels of the 24-kD IGFBP were unaffected by confluence; the IGF-I–mediated decrease, however, was clearly cell-density dependent (Fig 5). In marked
contrast, confluent SMCs showed a marked increase of basal IGFBP-4 mRNA as compared with sparse cells but remained unresponsive to IGF-I (Fig 6). Finally, the content of both the 30-kD IGFBP and the IGFBP-2 mRNA (Figs 5 and 6, respectively) decreased as a function of cell confluence but remained largely unchanged by IGF-I treatment (there is no expression of IGFBP-1 or IGFBP-5 mRNA in these cells).

The evidence shown so far indicates that the IGFBP-4 is decreased in SMC-conditioned medium after incubation with IGF-I, but not with IGF-I analogues with impaired ability to bind IGFBP, and that this effect takes place at a translational or posttranslational level, because steady-state IGFBP-4 mRNA abundance is not affected by IGF-I. To examine the possibility that IGF-I acts by promoting degradation of IGFBP-4, cell-free conditioned media from sparsely plated or confluent SMC cultures were incubated with or without 50 ng/mL IGF-I at 37°C for the indicated times, and the abundance of the 24-kD IGFBP was examined by Western-ligand blotting. As shown in Fig 7, IGFBP-4 levels in conditioned medium are essentially stable in the absence of added IGF-I over the 4-hour incubation period (the rate of degradation in the ab-

![Image](https://via.placeholder.com/150)

Fig 1. Effects of insulin-like growth factor (IGF)-I on IGF-binding protein (BP) levels of rat aortic smooth muscle cells (SMCs). SMCs were serum-deprived for 72 hours and then treated with the indicated concentration of IGF-I for the times shown. A, Western-ligand blot of SMC-conditioned medium blotted with ¹²⁵I-IGF-I is shown. Three major BP bands are observed: 30, 28, and 24 kD. Arrows on the left indicate position of molecular weight markers. B through E, Northern blots show 30 µg RNA of SMCs derived from the same experiment shown in panel A and sequentially hybridized with the following rat cDNA probes: BP 4 (B), BP 2 (C), and cyclophilin (D).

![Image](https://via.placeholder.com/150)

Fig 2. Immunoprecipitation/Western-ligand blotting of insulin-like growth factor-binding protein-4 (IGFBP-4) from rat aortic smooth muscle cell (SMC)-conditioned medium. A, Purified human IGFBP-4 was immunoprecipitated with nonimmune rabbit serum (lane 2), anti-rat IGFBP-4 antibody α-r-4 (lane 3), anti-human IGFBP-4 antibody α-h-4 (lane 4), or anti-IGFBP-4 antibody from UBI (lane 5). Lane 1 shows purified human IGFBP-4 not subjected to immunoprecipitation before Western-ligand blotting. B, Conditioned medium from SMCs was immunoprecipitated with the same antibodies as shown in panel A (lanes 2 through 5) before Western-ligand blotting. Lane 1 (positive control) was not subjected to immunoprecipitation before Western blotting.
Concentration of IGF-I is greater in the confluent than in the sparsely plated cells). IGF-I promotes a rapid degradation of IGFBP-4, particularly in medium from confluent SMCs. The proteolysis of IGFBP-4 in the presence of IGF-I was absent at 4°C, slightly greater at 20°C, and maximal at 37°C (data not shown) and completely abrogated by EDTA and by high concentrations of benzamidine. Phenylmethylsulfonyl fluoride (PMSF) and the plasmin inhibitor bdellin were relatively ineffective (Fig 8).

Proteolysis of IGFBP-4 was further explored by incubating purified human 125I-IGFBP-4 with SMC-conditioned medium (Fig 9). The labeled binding protein was stable in the absence of conditioned medium for 6 hours, even when incubated with 50 ng/mL IGF-I. Conditioned medium from confluent SMCs contained proteolytic activity, which generated fragments of ~18 and 14 kD. Generation of these products was markedly enhanced by the addition of IGF-I. Proteolysis was inhibited by EDTA and, to a lesser degree, by PMSF. There was no enhancement of basal proteolysis by addition of the IGF-I analogues long R3 or des (1-3) IGF-I. Finally, both basal proteolysis and IGF-I-stimulated proteolysis were less marked when the 125I-IGFBP-4 was incubated with conditioned medium from sparsely plated SMCs.

These data suggest that IGFBP-4 is inactivated by a cation-dependent protease in the presence of IGF-I or IGF-II. To examine whether this process affects the integrity of IGF-I itself, 125I-IGF-I was incubated with
IGF-I is an attractive candidate to serve as one of the significant mediators of the response to vascular injury. IGF-I is the most abundant circulating polypeptide growth factor, and it is also elaborated by SMCs within the vessel wall, where it may exert its effects in a paracrine fashion. IGF-I is additive in its effects with both PDGF and FGF, widely regarded as powerful mitogens for arterial SMCs. The abundance of IGF-I may ultimately be a major determinant of the magnitude of the mitogenic response after injury. The biologic activity of IGF-I is determined in part by its association with IGF-binding proteins in the extracellular space, which can modulate its interaction with the cell membrane receptor. The major form elaborated by vascular SMCs in vitro is a 24-kD IGFBP, thought to be IGFBP-4, which has been shown to inhibit IGF-I action in a variety of systems. It is not known whether IGFBP-4 is also inhibitory in vivo.

In the present study, we present evidence that IGFs regulate the 24-kD IGFBP-4 in rat aortic SMCs by promoting its degradation in a confluence-dependent manner. In addition, the elaboration and IGF stimulation of 38- to 42-kD IGFBP-3 in these cells is also observed more readily in highly confluent SMCs, without concomitant changes in IGFBP-3 mRNA abundance, suggesting that this binding protein may also be regulated posttranslationally by IGF-I. There have been several reports recently describing a decrease of IGFBP-4 levels by IGF-1 in conditioned medium of fibroblasts, osteoblasts, decidual cells, and epidermal cells, as well as tumor-derived cell lines. Inhibition of IGFBP-4 by IGFs has been reported to occur without a decrease in IGFBP-4 mRNA levels in human fibroblasts and in a rat neuroblastoma cell line. Our

**Discussion**

**Fig 5.** Effects of cell confluence on insulin-like growth factor (IGF)-binding protein content of smooth muscle cell (SMC)-conditioned medium: effects of IGF-I. Sparse, subconfluent, and highly confluent SMCs were serum-deprived for 72 hours and then treated with the indicated concentration of IGF-I for 24 hours. Western-ligand blot with [125I]-IGF-I shows IGF-binding proteins of 38 to 45, 30, and 24 kD.

**Fig 6.** Effects of cell confluence on insulin-like growth factor (IGF)-binding protein (BP) mRNA levels of rat aortic smooth muscle cells. Sparse and confluent plated smooth muscle cells were serum-deprived for 72 hours and treated with the indicated concentration of IGF-I for 24 hours before RNA extraction. Northern blot of 30 μg total RNA was sequentially hybridized with BP-3, BP-2, BP-4, and cyclophilin cDNA probes.
data also indicate that neither insulin, IGF-I, nor IGF-II alters IGFBP-4 mRNA abundance in SMCs. Evidence that the effects of IGF on IGFBP-4 are not mediated through the IGF-I receptor in SMCs is supported by the fact that IGF-I analogues that bind with normal affinity to the IGF-I receptor but not to IGF-binding proteins [des (1-3) and long R3 IGF-I] failed to decrease IGFBP-4 levels. Neely and Rosenfeld27 have also shown that the inhibitory effects of IGF-I on IGFBP-4 in a human fibroblast cell line are not blocked by the IGF-I receptor blocking antibody α-IR-3. Although most cell lines and primary cultures of mesenchymal cells express IGFBPs and many of them produce IGFBP-4, the response of these to IGF-I has been variable. For instance, insulin and IGF-I have been reported to increase production of 24-kD IGFBP in bovine fibroblasts and in the squamous cell carcinoma cell line SLC-1.27,48 The cell specificity of IGF effects on IGFBP production and posttranslational processing is therefore of significant interest and relevant to the understanding of IGF-I action. In the present study, we report that in SMCs, IGF-I regulation of IGFBP-4 is largely posttranslational. We demonstrate that IGF-I inhibition of IGFBP-4 content in conditioned medium

---

**Fig 7.** Effect of cell confluence on degradation of 24-kD insulin-like growth factor (IGF)-binding protein (BP)-4 in cell-free conditioned medium in the absence or presence of 50 ng/mL IGF-I. Top, Western ligand blot with 125I-IGF-I is shown. Cell-free conditioned medium from sparse and confluent smooth muscle cells was incubated with vehicle or IGF-I at 37°C for the indicated times before Western blotting. Bottom, Time course of IGF BP-4 disappearance from conditioned medium as measured by Western ligand blotting is shown. Data are plotted as percent of control levels (IGF BP-4 before incubation).

**Fig 8.** Bar graph showing the effect of protease inhibitors on degradation of 24-kD insulin-like growth factor (IGF)-binding protein (BP)-4. Conditioned medium from confluent smooth muscle cells was incubated for 4 hours at 37°C with 50 ng/mL IGF-I in the presence or absence of the following protease inhibitors: EDTA (10 mmol/L), benzamidine (50 mmol/L), phenylmethylsulfonyl fluoride (PMSF, 1 mmol/L), aprotinin (2 μg/mL), N-ethylmaleimide (10 mmol/L), bdeflin (1 to 100 μg/mL), captopril (1 μmol/L), or the angiotensin-converting enzyme (ACE) inhibitor pGlu-Trp-Pro-Arg-Pro-GLH-le-Pro-Pro (10 μmol/L). Data are plotted as the percent inhibition of degradation of IGF BP-4 observed in the absence of protein inhibitors (only 2% of the initial IGF BP-4 levels were detected after a 4-hour incubation in the presence of IGF-I).
of SMCs can be replicated in a cell-free system and that it is maximal at 37°C and inhibited at 4°C. These data are compatible with the presence of a protease that recognizes IGFBP-4 only in its bound conformation. Studies with purified human 125I-IGFBP-4 demonstrate that the conditioned medium of confluent SMCs degrades the labeled protein into two discrete fragments even in the absence of added IGF-I. We25 and others21 have reported that cultured rat aortic SMCs express IGF-I mRNA and secrete immunoreactive IGF-I. Because concentrations as low as 0.1 nmol/L IGF-I or IGF-II decrease IGFBP-4 abundance in SMC-conditioned medium (Fig 3), it is reasonable to postulate that basal degradation is due in part to the presence of endogenously secreted IGF-I. IGF-I-mediated degradation of IGFBP-4 was abrogated completely by EDTA and partially by the serine protease inhibitors benzamidine and PMSF. These data are compatible with the findings of Fowlkes and Freemark,28 who reported the presence of a cation-dependent serine protease for IGFBP-4 in dermal fibroblasts. Plasmin has recently been shown to dissociate IGF-I from IGFBPs present in osteosarcoma cell-conditioned medium.99 However, plasmin does not appear to be the physiologically active IGFBP-4 protease in SMCs, because the IGF-I–dependent degradation of IGFBP-4 was only partially prevented by the plasmin inhibitor bdellin. The fact that both metalloprotease inhibitors and, to a lesser degree, serine protease inhibitors interfere with IGFBP-4 degradation is puzzling and suggests that more than one enzyme is involved, perhaps in some kind of activation cascade.

A novel aspect of these observations is the remarkable dependence on cell confluence for the proteolysis of IGFBP-4. Whether confluence is associated with increased expression of the IGFBP-4 protease or with its activation is unclear, because at this point these conclusions are based on a qualitative assay. It should be stated that the assessment of total secreted proteins revealed no appreciable difference between sparse and confluent cultures (as determined by Coomassie blue staining of conditioned medium). In spite of the increase in IGFBP-4 proteolytic activity in media from confluent cells, basal IGFBP-4 content was not different in conditioned medium from sparse, subconfluent, or highly confluent SMCs. This is possibly explained by the marked increase of IGFBP-4 mRNA abundance observed in confluent SMCs. Thus, in confluent SMCs, both biosynthesis and proteolysis of IGFBP-4 are increased. The apparent coordination between IGFBP-4

Fig. 9. Proteolysis of human 125I–insulin-like growth factor (IGF)-binding protein (BP)-4 in smooth muscle cell (SMC)-conditioned medium. 125I–IGFBP-4 (80 000 cpm) was incubated at 37°C for 6 hours under the following conditions before gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis: 125I–IGFBP-4 alone (lane 1) or with 50 ng/mL IGF-I (lane 2), confluent SMC-conditioned medium without (lane 3) or with 50 ng/mL IGF-I (lane 4), conditions same as for lane 4 with 10 mM EDTA (lane 5), conditions same as lane 4 with 5 mM phenylmethylsulfonyl fluoride (lane 6), confluent SMC-conditioned medium with des(1-3) IGF-I (lane 7) or long R3 IGF-I (lane 8), and conditioned medium from sparse SMCs without (lane 9) or with 50 ng/mL IGF-I (lane 10).

Fig. 10. Chromatographic profile of 125I–insulin-like growth factor-I (125I-IGF-I) incubated with smooth muscle cell-conditioned medium for the indicated times. Top, Bar graph shows results of reverse-phase high-performance liquid chromatography. Middle, Bar graph shows results of gel permeation chromatography. Bottom, Bar graph shows results of gel permeation in the presence of 10% β-mercaptoethanol.
production and degradation is unique and suggests that the regulation of IGF-I bioavailability may be under far more subtle and complex controls than previously thought. Of note is that abundance of the 30-kD IGFBP decreased as a function of cell confluence, as did IGFBP-2 mRNA levels. These data further emphasize the concept that cell density plays a major role in the regulation of IGFBP content of SMC-conditioned medium and may be critical in determining IGF-I action. There are several examples of genes whose expression is altered by cell confluence in vascular SMCs. Density-arrested vascular SMCs show increased expression of extracellular matrix mRNAs for type III collagen and fibronectin.\textsuperscript{50} The vascular smooth muscle \( \alpha \)-actin gene is maximally induced in postconfluent mouse BC3H1 cells, a line derived from cerebrovascular SMCs.\textsuperscript{61} Indeed, Min et al\textsuperscript{52} have identified a “cell density responsive element” between positions –1074 and –372 of the \( \alpha \)-actin 5' flanking region. Therefore, the expression of both differentiated genes and of proteins regulating cell attachment and growth is confluence dependent in vascular SMCs.

Recently, Martin et al\textsuperscript{53} reported that IGF-I can evoke release of preformed IGFBP-3 present on the cell surface of neonatal skin fibroblasts and that this effect does not require interaction of IGF-I with its specific receptor. An increase of IGFBP-3 in conditioned medium was also observed after treatment of cells with heparin, suggesting that IGFBP-3 may be attached to glycosaminoglycans on the cell surface. We were unable to demonstrate a similar effect of heparin on IGFBP-3 release in vascular SMCs (data not shown). The mechanism for IGF-induced increase of IGFBP-3 is at this point unclear, although in all likelihood it is due to posttranslational events, because no induction of IGFBP-3 mRNA was observed.

Finally, our data clearly demonstrate that, even after prolonged incubation, the proteolytic activity in conditioned medium from confluent cultures does not affect the integrity of IGF-I itself. This strengthens the contention that the cation-dependent protease is involved in increasing the availability of free IGF-I for association with its cell membrane receptor or other binding proteins. However, bioactivity of IGF-I released from its binding site on IGFBP-4 needs to be formally tested. We have previously reported that IGF-I gene expression increases in the rat aorta after balloon injury.\textsuperscript{12,13,42} The biologic effects of IGF-I are likely to be dependent not only on the abundance of the growth factor itself but also on the presence of IGFBPs in the extracellular environment and on the activation of specific proteases that ultimately release IGF-I for interaction with vascular SMCs.

Acknowledgments

This study was supported in part by National Institutes of Health grant CA-50706 and a grant from the United Hostesses Foundation. Dr Fagin is the recipient of an Established Investigator Award of the American Heart Association and Bristol-Myers-Squibb. We are grateful to Drs P. Fielder and R. Rosenfeld (Stanford University) for the anti-rat IGFBP-4 antibody and to Drs N. Ling and S. Shimasaki (University of California, San Diego) for IGFBP-4 cDNA constructs.

References


38. Shimasaki S, Koza A, Mercado M, Shimonaka M, Ling N. Conserved DNA sequence and expression of a cDNA clone encoding a fetal rat binding protein (IGF-BP3) and tissue distribution of its mRNA. Biochem Biophys Res Commun. 1989;165:907-912.


Expression and insulin-like growth factor-dependent proteolysis of insulin-like growth factor-binding protein-4 are regulated by cell confluence in vascular smooth muscle cells.

A Kamyar, C J Pirola, H M Wang, B Sharifi, S Mohan, J S Forrester and J A Fagin

Circ Res. 1994;74:576-585
doi: 10.1161/01.RES.74.4.576

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/4/576