Protease-Resistant Insulin-Like Growth Factor (IGF)-Binding Protein-4 Inhibits IGF-I Actions and Neointimal Expansion in a Porcine Model of Neointimal Hyperplasia

T. C. Nichols, W. H. Busby, Jr., E. Merricks, J. Sipos, M. Rowland, K. Sitko, and D. R. Clemmons

Department of Medicine, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina 27599

IGF-I has been shown to play a role in the progression of atherosclerosis in experimental animal models. IGF-binding protein-4 (IGFBP-4) binds to IGF-I and prevents its association with receptors. Overexpression of a protease-resistant form of IGFBP-4 has been shown to inhibit the ability of IGF-I to stimulate normal smooth muscle cell growth in mice. Based on these observations, we prepared a protease-resistant form of IGFBP-4 and infused it into hypercholesterolemic pigs. Infusion of the protease-resistant mutant inhibited lesion development by 53.3 ± 6.1% (n = 6; P < 0.01). Control vessels that received an equimolar concentration of IGF-I and the protease-resistant IGFBP-4 showed no reduction in lesion size compared with control lesions that were infused with vehicle. Infusion of a nonmutated form of IGFBP-4 did not significantly inhibit lesion development. Proliferating cell nuclear antigen analysis showed that the mutant IGFBP-4 appeared to inhibit cell proliferation. The area occupied by extracellular matrix was also reduced proportionally compared with total lesion area. Immunoblots revealed that the mutant IGFBP-4 remained intact, whereas the wild-type IGFBP-4 that was infused was proteolytically cleaved. Further analysis of the lesions revealed that a marker protein, IGFBP-5, whose synthesis is stimulated by IGF-I, was decreased in the lesions that received the protease-resistant, IGFBP-4 mutant, whereas there was no change in lesions that received wild-type IGFBP-4 or the mutant protein plus IGF-I. These findings clearly illustrate that infusion of protease-resistant IGFBP-4 into the perilesion environment results in inhibition of cell proliferation and attenuation of the development of neointima. The findings support the hypothesis that inhibiting IGFBP-4 proteolysis in the lesion microenvironment could be an effective means for regulating neointimal expansion. (Endocrinology 148: 5002–5010, 2007)

Increased expression of IGF-I has been shown to be a component of the vascular response to injury (1, 2). Arterial wall cell types such as smooth muscle cells (SMC) and endothelial cells possess IGF-I receptors (3). When these cells are grown in culture, they respond to IGF-I with increases in cell proliferation and migration (4). Studies in whole animals have indicated that IGF-I synthesis by vascular cell types is significantly increased after injury. After rat carotid artery injury, there is a major increase in IGF-I synthesis which occurs at the time of the maximal increase in SMC division (1, 5, 6). Other types of injuries, such as exposure to hyperglycemia or high cholesterol, stimulate activated macrophages to synthesize IGF-I (7, 8) Zhu et al. (9) demonstrated that targeted overexpression of IGF-I in vascular SMC in mice resulted in a hyperproliferative response to injury and increased neointimal formation. That this response is due to locally produced IGF-I has been suggested by studies in which hypophysectomized animals that have been injured show increased IGF-I gene expression in the vessel wall area of injury but no change circulating IGF-I (6). IGF-binding protein (IGFBP)-4 is one member of a family of IGFBP that binds IGF-I with high affinity (10). Unlike several other members of the IGFBP family, IGFBP-4 does not attach to extracellular matrix or to cell surfaces, and it has been shown to inhibit the binding of IGF-I to cell surface receptors (11). To further determine the role of locally secreted IGF-I, Zhang et al. (12) prepared a protease-resistant mutant of IGFBP-4 and overexpressed it in vascular, bladder, and intestinal SMC. This resulted in SMC hypoplasia in the developing mice, whereas expression of nonmutated IGFBP-4 did not inhibit smooth muscle growth. Those results suggested that IGF-I is an important growth factor for fetal SMC proliferation.

Previous studies that have attempted to inhibit IGF-I actions in blood vessels to determine its role in neointimal expansion have had mixed results. When a synthetic peptide that inhibited IGF-I binding to its receptor and IGF-I-stimulated [3H]thymidine incorporation in vitro was administered to rats after carotid artery injury, it had no effect on inhibiting neointimal thickening (13). In contrast, when hypercholesterolemic pigs who had arterial lesions induced by placement of Goldblatt clamp received an infusion of an αvβ3 inhibitor that had been shown to block IGF-I actions, they had attenuation of neointimal expansion (14). However, that study did not exclude the possibility that the αvβ3 inhibitor had effects on neointimal expansion that were independent of inhibiting IGF-I ac-
tions. Therefore this study was undertaken to determine whether infusion of a protease-resistant mutant of IGFBP-4, a very specific inhibitor of IGF-I actions, could inhibit the effects of locally secreted IGF-I on the development and expansion of neointima in pigs.

Materials and Methods

Animal preparation

Male and female pigs, spotted Poland/China cross, age 12 months, were treated according to the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23) using an approved protocol. The animals were fed a diet containing 1% cholesterol, 20% beef tallow, and 0.75% cholic acid for 4 wk before the initiation of the study (14). This diet has been shown to result in the appearance of cholesterol deposition and foam cells after 4 wk (15–19). Under anesthesia, a 0.5-cm Goldblatt steel clamp was applied to both common carotid and to both femoral arteries in each of seven animals. The clamps were closed to produce an 80% stenosis. The clamp technique results in the formation of neointimal lesions within 3 wk that are composed of predominantly SMC (14, 20–23). However, because the animals have also been placed on a high-fat diet, these lesions also contain macrophages (14, 20, 23). Alzet pumps (2.0 ml; Alzet Corp., Cupertino, CA) were placed in the tissue adjacent to the artery, and infusion catheters were placed within the vessel wall under the Goldblatt clamp (14). The pump reservoirs were filled with 2.0 ml PBS that contained the specific proteins that were used in each treatment group. Four vessels from each animal (e.g., two carotids and two femorals) were randomized so that each received one of the four treatments. That is, each of the four treatments was administered to each animal. One set of vessels (n = 7) received the mutant form of IGFBP-4 (48 µg/mL) alone. The IGFBP-4 mutant protein had been shown to inhibit SMC migration in vitro (24). It had also been shown to be resistant to proteolytic cleavage in vitro (12). A second set of vessels received an identical concentration of nonmutated IGFBP-4. A third set of vessels received the mutant IGFBP-4 protein plus an equimolar concentration of IGF-I (10 µg/mL). The recombiant human IGF-I was a gift from Genentech Inc. (South San Francisco, CA). The fourth set received PBS alone. The infusion pumps delivered charged microscopic slides, deparaffinized in xylene, and hydrated in a series of graded alcohol solutions. Endogenous peroxidases were quenched by exposure of the tissue sections to a 0.3% hydrogen peroxide in absolute methanol before antigen retrieval was performed. Trypsin (1.5 mg/mL; LabVision, Fremont, CA) was used for antigen retrieval (10 min at 37°C) for macrophage and PCNA detection but not for detection of mucopolysaccharides or collagen. Nonspecific binding was blocked by exposure of all sections to 2% horse serum.

To detect macrophages, the tissue sections were then incubated with MAC387 (NeoMarkers, Fremont, CA), a monoclonal antibody that detects pig macrophages using a 1:200 dilution for 1 h at room temperature (29). This was followed by incubation with a horse antimouse secondary antibody and Vectastain R.T.U. Elite ABC reagents supplied in the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA). The stain was visualized using NovaRed (Vector) and counterstained with hematoxylin for enhanced contrast. An average of five sections were counted per pig. The average number of positively stained cells in the neointima was determined.

The area of neointimal extracellular matrix staining positive for mucopolysaccharides and collagen was measured using the modified Movat’s procedure (30, 31) according to the manufacturer’s specifications (Movat’s Staining kit; Electron Microscopy Sciences, Hatfield, PA). This staining procedure stains mucopolysaccharides blue-green and collagen yellow. Images were captured with the computer-driven photomicroscope system described above. The total neointimal area was measured as described above and NIH program Image J was used with the threshold color plug-in to measure the area of the neointima that stained for mucopolysaccharides or collagen. The results for all three individual areas (neointimal, mucopolysaccharides, and collagen) are expressed as square micrometers. An average of three to five sections per vessel were analyzed.

To detect PCNA, an index of cell proliferation (14, 23, 32), tissue sections were incubated with a 1:100 dilution of PC-10, a polyclonal antibody to PCNA (Dako Corp. Carpinteria, CA) for 1 h at 22°C and then exposed to the ABC Vectastain secondary horse antirabbit antibody and the chromogen diaminobenzidine. An average of three to five sections were counted per vessel. The artery was divided into four quadrants, and at least 100 neointimal and 100 medial cells were counted per quadrant. The number of cells that stained for PCNA in the neointima or media was then determined by direct counting and the result divided by the total number of cells counted in the respective regions of each
FIG. 2. Carotid arteries from the four treatment groups. Arterial cross-sections from the four treatment groups were stained with Verhoff van Gieson. The portions of the neointima outlined by the black box inserted on the left panels are shown at higher magnification in the right panels. A and B, IGFBP-4 wild-type; C and D, IGFBP-4 mutant; E and F, IGFBP-4 mutant plus IGF-I; G and H, vehicle. Magnification bars apply to all of the panels in each respective column. The degree of magnification was identical from panels A, C, E, and G and for panels B, D, F, and H. The neointimal areas for this specific figure are as follows (in μm²): A, 722,000; C, 47,850; E, 586,125; G, 257,450.
TABLE 1. Changes in neointimal area

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neointimal area ± SD (µm²)</th>
<th>P value compared with vehicle</th>
<th>P value compared with wild type</th>
<th>P value compared mutant plus IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-BP-4 (wild type) (n = 6)</td>
<td>211.371 ± 46.670</td>
<td>0.862</td>
<td>0.224</td>
<td></td>
</tr>
<tr>
<td>IGF-BP-4 (mutant) (n = 6)</td>
<td>100.618 ± 42.604</td>
<td>0.032</td>
<td>0.046</td>
<td>0.002</td>
</tr>
<tr>
<td>IGF-BP-4 (mutant) + IGF-I (n = 7)</td>
<td>279.522 ± 39.887</td>
<td>0.296</td>
<td>0.224</td>
<td>0.296</td>
</tr>
<tr>
<td>Vehicle (n = 7)</td>
<td>209.593 ± 41.143</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n, The number of vessels that were analyzed in each treatment group.

arterial cross-section to calculate the percentage of cells that were labeled (13, 14, 23, 32). Endothelial cells were excluded from this analysis.

For immunohistochemical methods, detecting macrophages or PCNA, positive controls consisted of detection of staining of the respective antigens in paraffin-embedded intestine and pig coronary and aortic atherosclerotic plaques that had been obtained in other studies, (14, 25, 33). These tissues were also used to confirm that the Movat’s staining reagents were functioning properly. Negative controls were processed as described above except that the primary antibody was not used. The analyses were evaluated by two blinded observers who had a 5% inter-observer variability.

Preparation and purification of protease-resistant IGF-BP-4

Because proteolysis of IGF-BP-4 results in a marked reduction in its affinity for IGF-I (24), we expressed a mutant form of IGFBP-4 that had been shown to be resistant to proteolytic cleavage (12). The molecular cloning and mutagenesis methods necessary to prepare the IGFBP-4 mutant and cDNA vector have been described previously (12). The vector containing this mutant was provided by Dr. James Fagin, University of Cincinnati, Cincinnati, OH. This was transfected into B104 cells using a previously described method (27). Cells were then grown to confluency in medium containing G-418 (500 µg/ml), and conditioned medium was collected from confluent cultures after a 48-h incubation. Approximately 2 liters of conditioned medium was collected and then purified as described previously for IGFBP using phenyl-Sepharose hydrophobic interaction chromatography, IGF affinity chromatography, and reverse-phase HPLC, C4 column (24). The purified fractions were analyzed by SDS-PAGE with immunoblotting for c-myc. To remove residual nonmutated IGFBP-4, the mutant protein was passed over a reverse-phase C4 HPLC column and eluted with an acetonitrile gradient from 20–40% over 30 min. The final preparation was concentrated and then reconstituted in 17.5 ml PBS. Control lesions received the same treatment % labeling in neointima ± SD (% labeling in media ± SD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% labeling in neointima ± SD</th>
<th>% labeling in media ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-BP-4 (wild type) (n = 6)</td>
<td>17.5 ± 3.8</td>
<td>1.8 ± 1.1</td>
</tr>
<tr>
<td>IGF-BP-4 (mutant) (n = 6)</td>
<td>11.2 ± 2.7a</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td>IGF-BP-4 (mutant) + IGF-I (n = 7)</td>
<td>17.8 ± 3.7</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>Vehicle (n = 7)</td>
<td>15.7 ± 3.7</td>
<td>2.2 ± 1.6</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with vehicle.

HCl (pH 7.2) that contained 5 mM EGTA, 2% Triton X-100, 10 µg/ml aprotinin, 2 mM phenylmethylsulfonfluoride, and 1 mM pepstatin for 3 min at room temperature. The extract was centrifuged at 14,000 × g for 20 min. IGF-BP-4 was quantified by direct immunoblotting of the supernatant. Twenty-five microliters of the extract (40 µg protein) were mixed 1:1 with 2× Laemmli sample buffer, and the proteins were separated by SDS-PAGE, 9% gel. Protein concentrations were determined by bicinchoninic acid analysis (24). After transfer to Immobilon membranes, the membranes were washed as described previously and then incubated with a 1:5000 dilution of anti-IGFBP-4 antisem (34). The immune complexes were visualized using enhanced chemiluminescence. After autoradiographic analysis, the gels were scanned by scanning densitometry (AGFA Scanner, Ridgefield Park, NJ), and the data were analyzed using NIH Image version 1.6. Each lesion extract was analyzed three times, and the results represent the mean of three separate analyses. Intact IGF-BP-4 was run as a control, and the intensity of that band was used to standardize the measurements of band intensity. In some experiments, direct immunoblotting of the myc-labeled IGFBP-4 mutant was undertaken to determine whether the mutated protein remained intact. After electrophoresis, the labeled proteins were transferred to Immobilon membranes and immunoblotted using a 1:1000 dilution of antihuman myc antisem. The immune complexes were visualized by enhanced chemiluminescence. The myc IGFBP-4 was run in a parallel lane as a standard for comparison.

IGFBP-5 analysis

To determine the effect of inhibiting IGF-I actions in SMC, the amount of IGFBP-5 in the lesion microenvironment was quantified by immunoblotting for human IGFBP-5. IGFBP-5 synthesis has been shown to be directly stimulated in this cell type by IGF-I (35); therefore, induction of IGFBP-5 serves as a marker of IGF-I action. Porcine IGFBP-5 reacts well with the antihuman IGFBP-5 antisem, and therefore, this antisem was used to detect the porcine form of the protein. Approximately 50 µg arterial protein extract was separated by SDS-PAGE (7.5% gel) and transferred to Immobilon filters. The filters were exposed to a 1:2000 dilution of antihuman IGFBP-5. Pure human IGFBP-5 was run as a standard in a parallel lane. Estimates of intact IGFBP-5 (30 kDa) band intensity were determined by scanning densitometry and analyzed using NIH Image. The results are the mean ± SEM of three separate determinations of each lesion.

Determination of IGF-I

IGF-I was quantified by a RIA using reagents supplied by ALPCO (22 IGF-I R20; American Laboratory Products Co., Windham, NH). Briefly, the samples were solubilized by homogenizing the tissue (100 mg) in the buffer listed previously and adding 13 µl of the homogenate (20 µg protein) to 0.5 ml assay buffer that contained 0.05 mM Na acetate (pH 3.1). An excess of IGF-II (0.2 µg/ml) was added to neutralize binding protein interference (36). Cross-reactivity of the antibody for IGF-II is less than 0.05%. After IGF-II addition, the pH of the assay buffer was neutralized to pH 7.0, and [125I]IGF-I (20,000 cpm/tube, specific activity 130 mCi/mg) plus a 1:1000 dilution of anti-IGF-I antisera were added. After a 48-h incubation at 4°C, the immune complexes were precipitated after the addition of goat antirabbit antisem. The results were read against a known standard curve using human IGF-I as a standard. The lower limit of detection is 0.2 ng/ml. The interassay coefficient of variation is 5.5%.
were compared using Student’s t test. As an additional control, an equimolar concentration of IGF-I was infused with the protease-resistant mutant form of IGFBP-4. These lesions also showed no change in neointimal area compared with the PBS control. Therefore, only the vessels that received the protease-resistant mutant of IGFBP-4 showed a significant decrease in neointimal area (Table 1). These findings strongly suggest that IGFBP-4 is binding to IGF-I in the extracellular fluid and preventing it from interacting with receptors and thus reducing neointimal thickening. They further suggest that proteolysis of IGFBP-4 is reducing its capacity to inhibit the effect of IGF-I.

To further define the mechanism by which IGFBP-4 was functioning to reduce neointimal area, PCNA labeling was determined. As shown in Table 2, the IGFBP-4 mutant resulted in a significant decrease in the number of PCNA-positive cells within the neointima. In contrast, there was no significant change in the number of positive cells in the media. This finding suggests that the reduction in lesion size is due to inhibition of cell proliferation, although an additional effect on cell migration or on cell size cannot be excluded. To determine the degree to which lesion size might have been altered by changes in extracellular matrix, the lesions were stained for mucopolysaccharides and collagen. The results in Table 3 show that the mutant IGFBP-4 reduced mucopolysaccharides by 63% and collagen by 58% compared with vehicle. These changes were comparable to the changes in neointimal area. In contrast, lesions that received the combined treatment had a 24% reduction in total extracellular matrix staining that was not significant. The lesions that received nonmutated IGFBP-4

![Fig. 3. Immunohistochemical detection of macrophages in neointima using the monoclonal antibody MAC387. A, Portion of the artery with neointima; B, area enclosed by the black rectangle in A shown at higher magnification. Macrophages stain deep red with this detection method.](https://academic.oup.com/endo/article-abstract/148/10/5002/2501857)
had a 41% reduction in mucopolysaccharides but a 108% increase in collagen; therefore, the total extracellular matrix staining was increased minimally (e.g. 12%), and this change was not significant. To further characterize the lesions, they were stained for the presence of macrophages. As shown in Fig. 3, macrophages could be detected at the base of the neointima. When the number of macrophages that were present in the neointimal areas that received each of the four treatments was determined, there were no significant differences.

To determine that the infused mutant form of IGFBP-4 remained intact, both immunoblotting of intact IGFBP-4 as well as immunoblotting for myc-labeled IGFBP-4 were undertaken. Immunoblotting of IGFBP-4 showed that the lesions that received the mutant form of IGFBP-4 had easily detectible intact IGFBP-4 bands (Fig. 4, left side, lanes 1–4, A–C). In contrast, the lesions that received native, nonmutated IGFBP-4 or PBS showed minimal intact IGFBP-4. Scanning densitometry revealed that the arteries that received nonmutated IGFBP-4 had a 78% reduction in mucopolysaccharides but a 108% increase in IGFBP-4 and IGFBP-5 band intensities compared with the arteries that received the IGFBP-4 mutant (Table 4). The coinfusion of IGF-I did not result in any

![Image](https://academic.oup.com/endo/article-abstract/148/10/5002/2501857/12x27)

**FIG. 4.** A–C. IGFBP-4 immunoblot (left panel) and myc-tagged proteins (right panel). The vessels that received various treatments were extracted, and the extracts immunoblotted for IGFBP-4 (lanes 1–4, left panel) or the same extracts were immunoblotted for the myc tag (lanes 1–4, right panel). The treatments were as follows: lane 1, PBS control; lane 2, nonmutated IGFBP-4; lane 3, IGFBP-4 mutant plus IGF-I; lane 4, IGFBP-4 mutant alone. The arrow denotes the position of intact IGFBP-4. The lower band detected in the lanes in the right panel is a nonspecific band detected by the myc antibody that does not react with the IGFBP-4 antibody. Lane 5 in panel A shows the myc-tagged protein standard that was added to a lesion extract in vitro before immunoblotting. Panels B and C show the same four treatments from two different animals. To control for loading differences, the blots were stripped and reprobed for a-smooth muscle actin, which is shown as the lower band in the left panel. The results show that there were no significant loading differences in the 12 samples that were immunoblotted for IGFBP-4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Scanning units ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-4 (wild type) (n = 6)</td>
<td>3,079 ± 487</td>
</tr>
<tr>
<td>IGFBP-4 (mutant) (n = 6)</td>
<td>14,022 ± 1,399*</td>
</tr>
<tr>
<td>IGFBP-4 mutant + IGF-I (n = 7)</td>
<td>13,337 ± 1,524*</td>
</tr>
<tr>
<td>Vehicle (n = 7)</td>
<td>2,664 ± 505</td>
</tr>
</tbody>
</table>

n = the number of vessel areas in each treatment group that were analyzed.

*P < 0.01 compared with vehicle.

b P < 0.05 compared with vehicle.

change in the amount of intact IGFBP-4. The results were further confirmed by immunoblotting for the myc tag (Fig. 3, right side, lanes 1–4, A–C). These results showed that there was an easily detectible, intact IGFBP-4 band in lesions that received the mutant form of IGFBP-4 and that any myc-containing protein was detected in the lanes that received vehicle or IGF-I alone. These results also confirm that the infused mutant IGFBP-4 remained in an intact form. The results strongly support the conclusion that mutation of IGFBP-4 prevented its degradation in porcine arteries as had been shown previously in cultured cells and that despite the duration of the infusion, most of the mutant IGFBP-4 that was infused that was present in the vessels at the conclusion of the experiment was in an intact form and thus capable of binding to IGF-I.

To further confirm that the infused mutant form of IGFBP-4 could inhibit IGF-I actions, the abundance of the marker protein IGFBP-5, whose synthesis is directly stimulated in SMC by IGF-I (35), was quantified by immunoblotting. The results showed almost no detectible IGFBP-5 in the lesions that received the mutant form of IGFBP-4 (Fig. 5). Quantification of the IGFBP-5 band intensities showed that there was an 82% decrease in the abundance of IGFBP-5 in lesions that received mutant IGFBP-4 compared with PBS control (Table 4). Importantly, the lesions that received the nonmutated form of IGFBP-4 showed a 17% decrease in IGFBP-5 abundance compared with lesions that received saline, and this change was not significant. The lesions that received the mutant IGFBP-4 plus IGF-I showed a 21% (P < 0.05) increase in IGFBP-5. Therefore, using this marker protein, we conclude that IGF-I action was markedly inhibited in the lesions that received the IGFBP-4 mutant compared

**FIG. 5.** IGFBP-5 immunoblot. The extracts obtained from four arterial samples from two pigs are shown. Lane 1, IGFBP-5 standard added to PBS control extract in vitro. Lanes 2–5 were obtained from the same animal, and lanes 6–9 were from a different animal. Lanes 2 and 6, Mutant IGFBP-4; lanes 3 and 7, wild-type IGFBP-4; lanes 4 and 8, mutant IGFBP-4 plus IGF-I; lanes 5 and 9, PBS. The arrow denotes the position of IGFBP-5. The lower panel shows the SMC actin control.
with PBS control and that the lesions that received mutant plus IGF-I had a significant stimulation of IGFBP-5.

As an additional control to confirm that the differences were not simply due to differences in the total IGF-I concentration, total IGF-I was quantified after removal of binding protein interference (36). The lesions that received IGF-I plus mutant IGFBP-4 showed a 43 ± 9% increase in the total IGF-I concentration (93 ± 30 ng/50 mg tissue) compared with saline control lesions (65 ± 18 ng/50 mg tissue) (\(P < 0.05\)). The lesions that received only the mutant IGFBP-4 (63 ± 18 ng/50 mg tissue) or wild-type IGFBP-4 (62 ± 17 ng/50 mg tissue) showed no difference compared with saline control (\(P\) value not significant). Therefore, the effect of IGFBP-4 mutant was not due to reduction in total IGF-I concentration but rather to a reduction in IGF-I action as assessed by the decrease in IGFBP-5.

**Discussion**

IGF-I interacts cooperatively with other growth factors to stimulate SMC proliferation and migration *in vitro*, both processes that are believed to contribute to cellular accumulation within lesions (37, 38). In addition, targeted overexpression of IGF-I in arteries of transgenic mice increased neointimal area (9). Studies that have investigated the role of IGFBP in regulating IGF-I action both in arterial wall cell types and in other tissues have shown that these proteins can either inhibit or stimulate IGF-I actions depending upon the particular protein that is being synthesized, the cellular location of the protein, whether or not it has been proteolytically cleaved, and whether or not it is associated with the cell surface or extracellular matrix (10, 11, 39–42). Most *in vitro* and a few *in vivo* studies have shown that if a large molar excess of IGFBP is added to extracellular fluids and their affinity is high enough to inhibit IGF-I binding to receptors, they will inhibit IGF-I actions (11, 39).

Pertinent to this study, overexpression of a protease-resistant form of IGFBP-4 during postnatal development in mice resulted in SMC hypoplasia (12, 43). Expression of native IGFBP-4 that was proteolytically cleaved did not result in growth inhibition, and ligand-blotting analysis of tissues showed that the animals that overexpressed the protease-resistant mutant form of IGFBP-4 had substantially higher intact IGFBP-4 concentrations, leading the authors to conclude that inhibition of IGF-I actions resulted in inhibition of SMC growth. More recently, mice that did not express pregnancy-associated plasma protein A (a protease that has been shown to cleave IGFBP-4) were shown to have decreased neointima formation after mechanical injury (44). However, in that study, no controls were provided to show that the effect of eliminating pregnancy-associated plasma protein A resulted in increased intact IGFBP-4 in blood vessels; therefore, it is difficult to conclude that the result obtained was specific for inhibition of IGF-I actions.

Our findings suggest that IGFBP-4 is functioning primarily by inhibiting IGF-I binding to its receptor rather than a direct inhibitory effect that is mediated through a non-IGF-dependent mechanism. Because a high concentration of the infused mutant form of IGFBP-4 remained intact, it is likely that it prevented IGF-I binding and thus inhibited receptor activation. Because infusion of mutant IGFBP-4 was associated with no change in the total IGF-I concentration compared with lesions that received vehicle alone, the findings strongly suggest that there was a reduction in the concentration of IGF-I that was available to receptors, thus resulting in decreased SMC stimulation. Although several binding proteins, principally IGFBP-3 and IGFBP-5, have been shown to have non-IGF-dependent actions in cell culture models, direct addition of IGFBP-4 to SMC cultures was shown to inhibit IGF-I-stimulated DNA synthesis, but it had no effect that was independent of IGF-I (24). Some studies have shown that fragments of IGFBP-4 can have direct effects (45). However, some of these fragments have been shown to bind IGF-I, suggesting that part of their ability to inhibit IGF-I action is through inhibiting IGF-I binding to its receptor (46). In this study, we included a group of vessels that were treated with an IGFBP-4 plus an equimolar concentration of IGF-I. Vessels that received this combination had no reduction in lesion area and no attenuation of the ability of IGF-I to stimulate IGFBP-5. Therefore, we conclude that IGFBP-4 in this model system is working primarily by inhibiting IGF-I association with its receptor and thus inhibiting the ability of IGF-I to stimulate SMC growth and migration. It is also possible that IGFBP-4 is acting to inhibit the actions of IGF-II. Although we did not measure changes in IGF-II expression in lesions, others have shown that targeted overexpression of IGF-II in mice leads to focal intimal thickening that was attributed to an increase in SMC proliferation (47). Because IGFBP-4 binds IGF-II with high affinity, its ability to inhibit neointimal formation in animal models of atherosclerosis may also be due to inhibition of IGF-II actions as well as IGF-I.

Although our studies did not definitively identify the mechanism by which IGF-I is stimulating neointimal expansion, they suggest that cell proliferation is a major component of the process because administration of protease-resistant IGFBP-4 resulted in a decrease in PCNA labeling. This finding does not exclude the possibility that IGF-I also is increasing neointimal area by stimulating SMC migration or by increasing SMC size. Additionally, there was no change in macrophage abundance, suggesting that IGF-I does not function by altering macrophage recruitment. Our analysis of changes in extracellular matrix was consistent with these results. Specifically, infusion of IGFBP-4 was associated with a reduction in extracellular matrix area that stained for mucopolysaccharides and collagen, and this reduction was similar to the change in total neointimal area. This is consistent with the changes obtained in neointimal area, but it does not completely exclude the possibility that there was a preferential decrease in extracellular matrix synthesis per cell because this was not measured directly. Because IGF-I is a known stimulant of extracellular matrix protein synthesis, these results are also consistent with the conclusion that mutant IGFBP-4 inhibited IGF-I actions.

These studies confirm the findings in the transgenic mice that a protease-resistant form of IGFBP-4 inhibits IGF-I actions in arterial smooth muscle and extend them...
to a model of neointimal hyperplasia. Infusion of the protease-resistant mutant form of IGFBP-4 resulted in reduction in neointimal expansion and inhibition of IGF-I action as determined by assessing the abundance of IGFBP-5 in the arterial tissues. The findings are consistent with a role for IGF-I in stimulating neointimal expansion and support the conclusion that attempts to target IGF-I action during the proliferative phase of atherosclerosis may result in a decrease in neointimal thickening.

Acknowledgments

We gratefully acknowledge Ms. Laura Lindsey for her help in preparing the manuscript. We thank Dr. James Fagin for his gift of the plasmid containing the mutant IGFBP-4 c-DNA.

Received April 30, 2007. Accepted July 11, 2007.

Address all correspondence and requests for reprints to: David R. Clemmons, M.D., CB 7170, 8024 Burnett-Womack, Division of Endocrinology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7170. E-mail: endo@med.unc.edu.

This work was supported by National Institutes of Health Grant HL-56850.


References

27. Motov KZ 1955 Demonstration of all connective tissue elements in a single section: pentachrome stains. AMA Arch Pathol 60:289–295
34. Blum WE, Breier BH 1994 Radioimmunoassays for IGFBPs. Growth Regul 1:11–19


Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.