Insulin-Like Growth Factor Binding Protein-4 Protease Produced by Smooth Muscle Cells Increases in the Coronary Artery After Angioplasty

Antoni Bayes-Genis, Robert S. Schwartz, Debra A. Lewis, Michael T. Overgaard, Michael Christiansen, Claus Oxvig, Khalid Ashai, David R. Holmes, Jr, Cheryl A. Conover

Abstract—Insulin-like growth factor (IGF)-I stimulates vascular smooth muscle cell (VSMC) migration and proliferation, which are fundamental to neointimal hyperplasia in postangioplasty restenosis. IGF-I action is modulated by several high-affinity IGF binding proteins (IGFBPs). IGFBP-4 is the predominant IGFBP produced by VSMCs and is a potent inhibitor of IGF-I action. However, specific IGFBP-4 proteases can cleave IGFBP-4 and liberate active IGF-I. In this study, we document IGFBP-4 protease produced by human and porcine coronary artery VSMCs in culture as pregnancy-associated plasma protein-A (PAPP-A). This was shown by a distinctive IGFBP-4 cleavage pattern, specific inhibition of IGFBP-4 protease activity with PAPP-A polyclonal antibodies, and immunorecognition of PAPP-A by monoclonal antibodies. Furthermore, we found a 2-fold increase in IGFBP-4 protease activity in injured porcine VSMC cultures in vitro (P<0.05). We also evaluated IGFBP-4 protease/PAPP-A expression in vivo after coronary artery balloon injury. Twenty-five immature female pigs underwent coronary overstretch balloon injury, and vessels were examined at defined time points after the procedure. Abundant PAPP-A expression was observed in the cytoplasm of medial and neointimal cells 7, 14, and 28 days after angioplasty (P<0.01 vs control). The highest PAPP-A labeling indices were located in the neointima (36.1±2.1%) and the media (31.7±1.2%) 28 days after injury. Western blot analysis confirmed increased PAPP-A in injured vessels. PAPP-A, a regulator of IGF-I bioavailability through proteolysis of IGFBP-4, is thus expressed by VSMCs in vitro and in restenotic lesions in vivo. These results suggest a possible role for PAPP-A in neointimal hyperplasia. (Arterioscler Thromb Vasc Biol. 2001;21:335-341.)

Key Words: insulin-like growth factor binding protein proteases restenosis

Coronary restenosis remains the major complication of modern percutaneous revascularization procedures and is characterized by neointimal hyperplasia. Neointima forms after coronary angioplasty and is characterized by proliferation, migration, and matrix protein synthesis by activated vascular smooth muscle cells (VSMCs). It is modulated by several growth factors, including insulin-like growth factor (IGF)-I. Studies performed in several animal models of restenosis show increased IGF-I expression in the media and neointima after balloon injury. IGF-I is also abundantly expressed in restenotic human atherectomy specimens. In vitro studies further support the role of IGF-I in VSMC proliferation and migration. The predominant IGF-I production within the vessel wall after balloon injury suggests that autocrine or paracrine growth factor production may be important in vascular repair. However, the ultimate cellular response to IGF-I depends on the context of 6 high-affinity IGF binding proteins (IGFBPs) and the IGFBP proteases. IGFBP-4, the major IGFBP produced by VSMCs, binds and inhibits IGF-I action. A recent study shows that paracrine overproduction of IGFBP-4 in vivo results in vascular smooth muscle hypoplasia due to IGF-I blockade. VSMC proteolytic activity is present in IGFBP-2, IGFBP-4, and IGFBP-5. The resulting IGFBP fragments have greatly reduced affinity for IGF-I. These proteases may function to release IGF-I, making it available to bind and activate receptors.

We recently identified a novel IGFBP-4 protease in human fibroblast- and osteoblast-conditioned medium and in human ovarian follicular fluid as pregnancy-associated plasma protein-A (PAPP-A). PAPP-A is 1 of 4 proteins originally isolated in 1974 from human pregnancy serum. The biological function of PAPP-A was unclear for many years, but particular interest in PAPP-A developed with its clinical utility as an index of placental function and a first-trimester screen for Down’s syndrome. The new and unsuspected
function of PAPP-A as a specific protease that cleaves IGFBP-4, which liberates IGF-I, suggests that it may also play a role in local proliferative responses.

In the present study, we found that PAPP-A is a biologically active IGF-dependent IGFBP-4 protease produced by human and porcine coronary artery VSMCs in vitro. Furthermore, we demonstrate that PAPP-A expression is markedly upregulated in coronary arteries in vivo after balloon angioplasty, supporting a role for the action of PAPP-A in vascular injury and repair.

**Methods**

Animal studies were performed with the approval of the Institutional Animal Care and Use Committee of the Mayo Foundation.

**Cell Culture**

Primary VSMC cultures were derived from adult human coronary arteries (hVSMCs, from donors aged 33 to 39 years, Clonetics). These cells were cultured in smooth muscle basal medium (modified MCDB 131) containing 5% FBS and antibiotics (50 mg/mL gentamycin and 50 μg/mL amphotericin B). Porcine coronary artery VSMCs (pVSMCs) were obtained from domestic crossbred pigs by enzymatic digestion with 1% collagenase. The arteries were excised aseptically and placed in serum-free medium 199. After removal of periadventitial fat, the vessels were cut longitudinally, opened flat, and incubated in medium containing collagenase for 20 minutes. The endothelial and adventitial surfaces were scraped off with a cell scraper. The digested vessels were cut into small rings and placed in 60-mm-diameter dishes in medium 199 containing 10% heat-inactivated FBS, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL), Earle’s salts, and NaHCO3 (2.2 g/L). The medium was changed every 3 days. When cells reached 70% confluence, they were trypsinized and plated in 100-mm-diameter dishes.

**IGFBP-4 Protease (PAPP-A) Activity Assay**

Cell-free IGFBP-4 proteolysis was assayed in VSMC CM, as previously described.18 VSMC CM (25 μL) was incubated at 37°C for 6 hours with [125I]IGFBP-4 in the absence and presence of 5 nmol/L IGF-II. Reaction products were separated by 7.5% to 15% SDS-PAGE under nonreducing conditions and were visualized by autoradiography. In some experiments, an inhibitory PAPP-A polyclonal antibody in the absence of IGF-II. Reaction products were analyzed by autoradiography.

**IGFBP-5 Protease Activity Assay**

IGFBP-5 proteolysis was assayed in VSMC CM as previously described.21 The conditioned medium (25 μL) was incubated at 37°C with [125I]IGFBP-5 for 3 hours with and without PAPP-A polyclonal antibodies in the absence of IGF-II. Reaction products were analyzed as for IGFBP-4 proteolysis.

**Enzyme-Linked Immunosorbent Assay**

A sandwich bixin-tinuyamide-amplified ELISA (sensitivity 0.03 mU/mL, units from World Health Organization’s International Reference Standard 78/610) was performed with the use of PAPP-A polyclonal antibodies for capturing and PAPP-A monoclonal antibodies for detection.

**Cell Scraping of VSMCs (In Vitro Mechanical Injury)**

Cultured pVSMCs were mechanically injured by the method of Crowley et al.26 Briefly, confluent cultures grown in 100-mm-diameter dishes were injured by scraping the cell monolayer with a cell scraper (Fisher Scientific). Each culture dish was scraped in a systematic manner, with 2 circumferential and 3 horizontal and vertical scrapes. By direct visualization, ~80% of the cell monolayer surface area was injured and detached from the surface after injury. This degree of mechanical injury resulted in 20% cell death, which was determined by trypsin blue staining. CM was collected at 24 hours from injured and control VSMCs.

**Balloon Angioplasty of Coronary Arteries**

Oversized coronary balloon angioplasty was performed in 25 immature female pigs (Sus scrofa, weight 29±3 kg), and neointimal hyperplasia was evaluated as described previously.27 Three days before the procedure, pigs began oral aspirin (325 mg), which was continued for the remainder of the course. General anesthesia was achieved with ketamine (3 mg/kg IM) and xylazine (30 mg/kg IM). Additional medication at the time of induction included atropine (1 mg IM) and antibiotic (1g IM flocculin). During the angioplasty procedure, an intra-arterial bolus of heparin (10 000 U) was administered. Briefly, an 8F sheath was inserted into the left carotid artery, and a JL3.5 (Cordis) 8F guide catheter was advanced to the ostium of the desired coronary artery under fluoroscopic guidance. Two arteries of each animal were randomly injured with clinical percutaneous transluminal coronary angioplasty catheters (Scimed) that were sized so that the ratio of the inflated balloon to artery was ~1.3:1. The balloon was inflated to 8 atm for 30 seconds. The catheters were withdrawn, the cutdown site was sutured, and the animals were allowed to recover from the procedure. Five groups with 5 animals in each group were selected for survival intervals of 1, 7, 14, 28, and 90 days after angioplasty.

**Morphometric Analysis**

Animals were euthanized with an overdose of a commercial intravenous barbiturate (Sleepaway [Fort Dodge Laboratories, Fort Dodge, Iowa] 10 mL by ear vein) at the selected time points. The hearts were then immediately removed, and the coronary arteries were fixed by pressure perfusion (100 mm Hg) with 10% neutral buffered formalin for 24 hours. After fixation, injured and control coronary sections were excised and were subsequently paraffin-embedded and stained with hematoxylin-eosin and elastic van Gieson. Some sections were kept frozen for Western blot analysis. Digital images of van Gieson’s elastin stains were used to perform histomorphometric analysis (Diagnostic Instruments, Inc). Morphometric measurements to define neointimal area were performed as previously described. The degree of arterial injury induced by angioplasty was assessed according to the injury score of Schwartz et al28: 0, endothelial denudation; 1, internal elastic lamina (IEL) laceration; 2, IEL and media laceration; and 3, external elastic lamina laceration. Neointimal area and thickness, mean injury score, and percent stenosis were calculated for each coronary section. Only those vessels with distinct IEL laceration (injury score >1) were analyzed. This eliminated from the study those vessels in which the balloon catheter failed to break the IEL and/or media (12 vessels).

**Immunohistochemistry**

Immunohistochemical staining was performed on 5-μm-thick paraffin sections with the use of a peroxidase-labeled streptavidin-biotin method. The slides were developed with 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Monoclonal human PAPP-A antibody (234-520)23 was used at a concentration of 20 μg/mL. This antibody cross-reacts with porcine PAPP-A.80 IGFBP-4 polyclonal antibody (kind gift from Diagnostic Systems Laboratories, Inc, Webster, Tex) was used at a dilution of 1:100, and IGF-I polyclonal antibody (Serotec Ltd) was used at a concentration of 20 μg/mL. The staining patterns were evaluated by independent observations of 2 investigators, with an interobserver variation <5%. PAPP-A expression was detected in medial smooth muscle cells and neointimal cells.
Total cell count and PAPP-A-positive cells were analyzed at $\times$100 magnification in oil immersion for 5 regions in each vessel. A PAPP-A labeling index was defined as the total number of PAPP-A–labeled cells in the media or neointima divided by the total number of hematoxylin-labeled cells in the same vessel compartment times 100. Positive controls consisted of porcine placental tissue. Negative controls were stained by omitting the primary antibody.

**Western Blot Analysis**

Coronary artery lysates from injured and control arteries were electrophoresed under reducing conditions and immunoblotted for PAPP-A with the use of polyclonal antibodies.24

**Statistical Analysis**

Results are presented as mean±SD. One-way ANOVA was used to assess differences among angioplasty groups. A 2-tailed unpaired Student $t$ test was used for comparisons. A value of $P<0.05$ was considered statistically significant.

**Results**

**Identification of IGFBP-4 Protease in VSMCs as PAPP-A**

Figure 1 shows IGF-dependent IGFBP-4 protease activity in hVSMC CM. [125I]IGFBP-4 remained intact in cell-free CM in the absence of IGF-II. Addition of IGF-II initiated the proteolysis of [125I]IGFBP-4 into radiolabeled fragments of 18 and 14 kDa (please see Figure II, which can be accessed online at http://atvb.ahajournals.org). This inhibition was specific for PAPP-A antibodies (data not shown).

![Image](https://example.com/image1.png)

**Figure 1.** IGF-dependent IGFBP-4 protease (PAPP-A) activity in hVSMC CM. Medium from 3 separate cultures was incubated with [125I]IGFBP-4 in the absence (−) and presence (+) of 5 nmol/L IGF-II as described in Methods. Arrows indicate intact IGFBP-4 and 18- and 14-kDa cleavage products.

**Increased IGFBP-4 Protease/PAPP-A After Injury**

To determine whether mechanical injury altered IGFBP-4 protease/PAPP-A, we assayed IGFBP-4 protease activity in CM from injured pVSMCs in vitro. Cell injury resulted in a 2-fold increase in IGFBP-4 proteolytic activity at 24 hours. Under control conditions, IGF-II cleaved 34±3% of intact IGFBP-4, whereas in CM from injured pVSMCs, 69±5% of intact IGFBP-4 was cleaved ($P<0.05$; please see Figure II). This result was replicated in 2 separate experiments.

These in vitro findings prompted PAPP-A assessment after injury in vivo. We performed oversized balloon injury of porcine coronary arteries and evaluated PAPP-A expression after 1, 7, 14, 28, and 90 days. The Table shows the morphometric data of injured arteries at the different time points. No differences in injury score or in neointimal area and thickness were observed between groups for days 7, 14, 28, and 90 ($P=NS$). The extent of lumen reduction (percent stenosis) was higher 28 and 90 days after injury compared with day 7 after injury ($P<0.05$). No neointimal hyperplasia was present 1 day after injury or in control uninjured arteries.

IGFBP-4 protease/PAPP-A was assessed immunohistochemically by using PAPP-A monoclonal antibodies (Figure 3). The PAPP-A labeling index was slightly higher in arteries 1 day after injury than in control arteries, although this was not statistically significant ($P=NS$). Abundant PAPP-A expression was observed in the cytoplasm of medial and neointimal cells 7, 14, and 28 days after angioplasty ($P<0.01$ versus control). The highest labeling indices were located in the neointima (36.1±2.1%) and the media (31.7±1.2%) 28 days after injury (Figure 4). A 200-kDa band in Western blotting of coronary artery lysates confirmed increased PAPP-A protein expression in injured arteries with neointimal hyperplasia (data not shown). Areas of medial laceration also showed substantial PAPP-A upregulation 14 days after injury (media 20.2±2%, neointima 25.6±1.5%; $P<0.01$ versus control; please see Figure III, which can be accessed online at http://atvb.ahajournals.org). This is the period of active cell migration, proliferation, and protein synthesis in the porcine restenosis model. At 90 days, a time point at which neointimal hyperplasia after balloon injury is complete, PAPP-A labeling in the media (1.7±0.8%) and in the neointima (1.2±0.4%) was similar to that in controls ($P=NS$; please see Figure IV, which can be accessed online at http://atvb.ahajournals.org). IGFBP-4 showed low and diffuse staining of all vascular cell types in balloon-injured sections but not in uninjured sections. No IGF-I staining was observed in either injured or uninjured sections (data not shown).

**Figure 2.** Inhibition of IGFBP-4 protease activity from hVSMC CM with PAPP-A antibody (Ab). CM was assayed for IGFBP-4 protease activity as described in Figure 1 and in Methods, except that PAPP-A antibody (1:100) or nonspecific IgG was included during the incubation.
Morphometric Data for Injured Arteries at Different Time Points

<table>
<thead>
<tr>
<th>Injury Score</th>
<th>Neo Area, mm²</th>
<th>Neo Thickness, mm</th>
<th>Stenosis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.57±0.21</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Day 7</td>
<td>1.44±0.37</td>
<td>1.7±0.3</td>
<td>0.27±1</td>
</tr>
<tr>
<td>Day 14</td>
<td>1.9±0.22</td>
<td>1.9±0.2</td>
<td>0.28±0.12</td>
</tr>
<tr>
<td>Day 28</td>
<td>1.67±0.17</td>
<td>2.0±0.3</td>
<td>0.31±0.14</td>
</tr>
<tr>
<td>Day 90</td>
<td>1.6±0.19</td>
<td>2.0±0.5</td>
<td>0.30±0.17</td>
</tr>
</tbody>
</table>

Values are mean±SD. No differences were found for injury score, neointimal (Neo) area, or Neo thickness between groups (P=NS). *P<0.05 vs day 7.

Discussion

The present study shows that the IGFBP-4 protease PAPP-A is expressed in human and porcine VSMCs. Moreover, PAPP-A is markedly upregulated in vivo in neointimal hyperplasia after coronary angioplasty. This suggests that IGFBP-4 protease/PAPP-A may act in an autocrine or paracrine fashion during vascular repair after balloon injury.

VSMCs, human and porcine, constitutively secreted PAPP-A in vitro and exhibited distinctive IGF-dependent IGFBP-4 protease activity. In the absence of IGF-II, we found no significant cleavage of IGFBP-4, whereas the addition of IGF-II resulted in 2 IGFBP-4 cleavage fragments (18 and 14 kDa). We previously showed that IGFBP-4 cleavage fragments do not bind to IGF-I and that IGF-II–induced IGFBP-4 proteolysis is associated with enhanced cellular response to IGF-I. In the present study, activation of PAPP-A proteolytic activity in vitro was induced with exogenous IGF-II; in vivo IGF-II is likely to come from platelet α granules and/or myoblasts.

The identification of IGFBP-4 protease as PAPP-A was based on the distinctive IGFBP-4 cleavage pattern produced by incubation of IGFBP-4 with purified PAPP-A, inhibition of IGFBP-4 protease activity with PAPP-A polyclonal antibodies, and immunorecognition of PAPP-A by monoclonal PAPP-A antibodies. The IGFBP-4 protease identified in the present study as PAPP-A is a high molecular mass (>220-kDa) zinc-binding metalloprotease. Although in the present study we inhibited IGFBP-4 proteolytic activity from VSMC CM with a PAPP-A antibody, we cannot rule out other proteases that use IGFBP-4 as a substrate. Parker et al described another IGF-dependent IGFBP-4 protease characterized as a 55-kDa serine protease. The relationship between these 2 proteases is unknown but may be due to the different vessels of origin (the coronary artery in the present study versus the aorta in the study of Parker et al). PAPP-A antibodies directed against specific IGFBP-4 proteolysis did not inhibit the proteolytic activity of IGFBP-5 in VSMC CM. Previous reports in porcine VSMCs demonstrate that IGFBP-5 undergoes limited proteolysis by serine proteases.

The second objective of the present study was to determine whether mechanical injury modulates IGFBP-4 protease/PAPP-A activity and expression. We used cell scraping to induce mechanical injury of cultured pVSMCs and demonstrated increased IGFBP-4 protease activity 24 hours after injury. These in vitro results suggest that pVSMCs may contain preformed PAPP-A, which is released into the pericellular environment on mechanical injury. A similar response was seen for the release of platelet-derived growth factor and basic fibroblast growth factor with the use of the same in vitro injury model. However, it is most likely that the increase in PAPP-A activity after injury also involves gene transcription.

The in vivo mechanical injury model of coronary angioplasty demonstrates that IGFBP-4 protease/PAPP-A is present in VSMC cytosol from the media and neointima of porcine lesions beginning 1 day after injury, with abundant PAPP-A expression after the first week. PAPP-A labeling was highest between days 14 and 28 after injury. This makes it unlikely that increased vascular PAPP-A content would result from tissue infiltration by plasma, in which case a rapid increase after injury would have been observed. These observations suggest that injured VSMCs by mechanical means are likely to induce protein translation leading to the visible PAPP-A expression detected by immunohistochemistry and Western blot analysis. Regardless, the molecular regulation of PAPP-A expression is unclear, and in situ hybridization will help to document the time course of PAPP-A message and gene expression after injury. Newly synthesized PAPP-A in the vascular environment may modulate IGF-I bioactivity in an autocrine/paracrine fashion in the process of vascular repair after balloon injury. The association of neointimal hyperplasia with local PAPP-A expression suggests that local and blood-derived growth factors regulate the vascular re-
response to injury and that these act at specific times and places in the vessel wall.

IGFBP-4, the substrate for PAPP-A, showed diffuse low-level staining over the time course of restenosis, a likely means for tonic inhibition of IGF-I action in the vessel microenvironment that could be liberated by proteolysis. Immunohistochemistry of IGF-I has a major technical caveat that hampers interpretation of the data. Immunoavailable

Figure 4. Porcine coronary arteries 4 weeks (28 days) after angioplasty. A, PAPP-A staining (brown) in medial smooth muscle cells and neointimal cells. B and C, Hematoxylin-eosin (B) and elastic van Gieson (C) stains of the same artery showing the presence of neointima and cellular disorganization in the luminal side of the media.
IGF-I within the vessel wall in restenotic lesions is most likely below our detection method. Furthermore, IGFBPs have a high affinity for IGF-I and markedly interfere with the interaction between IGF-I and its antibody, thus explaining the absence of a specific localization pattern. Other groups have found increased IGF-I expression in restenotic lesions with the use of colloidal gold immunohistochemistry and increased IGF-I mRNA levels with the use of reverse transcription–polymerase chain reaction analysis.8

The functional significance of IGFBPs after vascular injury has been addressed primarily through in vitro studies of the modulation of the action of IGF-I in cultured VSMCs by exogenous IGFBP. IGFBP-1 can inhibit or enhance IGF-I stimulation of VSMC proliferation depending on its phosphorylation status.34 IGFBP-1 also stimulates VSMC migration, independent of IGF-I binding, through interaction with α5β1 integrin receptors.35 These differential effects of IGFBP-1 may help explain why intravenous administration of IGFBP-1 after angioplasty to rat carotid arteries did not inhibit neointimal thickening.36 IGFBP-2 generally inhibits IGF-I activity and biological effects of IGF-I. PAPP-A proteolytic activity releases local IGF-I and may increase the available fraction of IGF-I. Free IGF-I appears important in several aspects of human reproduction, eg, bone remodeling and wound healing34,17,20,21; hence, it is likely that PAPP-A has growth-promoting effects in a highly specialized injury response such as restenosis. It may also be a marker of neointimal formation and a target for future restenosis therapies. Further investigation will more fully define the regulation and functional role of PAPP-A in VSMCs and the vascular response to injury.

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References


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