Brief Communication

Induction of Insulin-Like Growth Factor I Messenger RNA in Rat Aorta After Balloon Denudation

Bojan Cercek, Michael C. Fishbein, James S. Forrester, Richard H. Helfant, and James A. Fagin

Insulin-like growth factor I (IGF-I) is a widely distributed mitogen that mediates the growth-promoting effects of platelet-derived growth factor in mesenchymal cells. We show that rat aortic IGF-I messenger RNA (mRNA) is induced 24 hours after deendothelialization, at a time when smooth muscle cell proliferation within the intima is still not apparent. After 7 days, IGF-I mRNA induction peaks at about ninefold control levels and then falls to about threefold 14 days after denudation when smooth muscle cell proliferation is at its peak. We also show that, of the 5' untranslated IGF-I mRNA transcripts, only the class C transcript is expressed and regulated in aortic tissue. In contrast, treatment of rats with supraphysiological doses of growth hormone, the major endocrine regulator of IGF-I gene expression, elicits only twofold induction of aortic IGF-I mRNA. Our findings suggest that IGF-I may be an important autocrine or paracrine regulator of smooth muscle cell proliferation and that it may be significant in determining the cellular response to arterial wall injury. (Circulation Research 1990;66:1755–1760)

Intimal smooth muscle cell (SMC) proliferation is the principal cellular response to arterial wall injury and the hallmark of the process of restenosis after transluminal angioplasty and atherosclerosis. The primary stimulus for SMC migration and proliferation after endothelial disruption is believed to be platelet-derived growth factor (PDGF) released from platelets after adhesion to the injured vessel wall.1,2 Local PDGF production, possibly by SMC within the injured vessel wall, may also play a role in initiating and perpetuating the response to endothelial injury.3 However, it is apparent from in vitro studies that PDGF requires the presence of other growth factors, such as insulin-like growth factor I (IGF-I), to fully induce DNA synthesis and cell division in mesenchymal cells.4 Furthermore, PDGF stimulates the production of an immunoreactive IGF-I–like material in human fibroblasts.

PDGF-stimulated [3H]thymidine incorporation into porcine aortic SMCs grown in IGF-I-depleted media is blunted by coincubation with a monoclonal antibody that binds to IGF-I and blocks its action.5 These in vitro data strongly suggest that IGF-I is an important autocrine or paracrine regulator of arterial wall response to injury.

The purpose of this study was to investigate the possible role of IGF-I in the arterial wall response to injury by examining aortic IGF-I gene expression after balloon denudation in rats. We report that IGF-I messenger RNA (mRNA) content from balloon-denuded vessels is induced approximately ninefold in a time-dependent fashion. In contrast, treatment of rats with supraphysiological doses of growth hormone (GH) elicited a relatively minor twofold induction of aortic IGF-I mRNA. Marked changes of aortic IGF-I gene expression in response to paracrine stimulation suggest that this growth factor may play a cardinal role in the events occurring after arterial injury.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (400–500 g) were anesthetized with sodium pentobarbital (20 mg/kg i.p.). The left iliac artery was exposed, and an F2 embolectomy catheter was advanced to the aortic arch. The balloon was inflated with 0.7 ml saline and...
withdrawn into the abdominal aorta at least three times. A separate study had shown that inflation with 0.7 ml saline consistently removed all the endothelium without medial necrosis. The decendothelialization was not consistently complete when the balloon was inflated with less than 0.7 ml saline, whereas inflation with 1.0 ml occasionally resulted in extensive medial necrosis. The iliac artery was subsequently ligated. Rats were killed in groups of four at 1, 3, 7, and 14 days after balloon denudation or after a sham operation. Another group of animals was killed without prior intervention. The effects of GH on aortic IGF-I gene expression was examined in rats that had not undergone balloon denudation. Two dosages of human recombinant GH (1.88 and 35 mg/kg/week s.c.) were used; five rats were used for each dosage regimen. After the rats were killed, their aortas were removed, stripped of adventitia, weighed, and immediately frozen in liquid nitrogen until assayed. The aortic tissues of four rats were pooled for RNA extraction at each data point.

To assess the extent of endothelial regrowth and neointimal proliferation after denudation, rats were killed 1, 3, 7, and 14 days after denudation or sham operation (four rats at each time point). Evans blue (60 mg/kg) injection was followed by perfusion fixation with 1% glutaraldehyde in 0.1 M cacodylate buffer before the rats were killed. By using a rear projection theater, photographic transparencies of the denuded aortic segment were projected at a magnification of ×10 on a lattice with cross sections for point counting. These test points were spaced 2.0 mm apart. The number of test points falling on blue (non-reendothelialized) areas and nonblue (reendothelialized) areas were counted. The reendothelialized area (number of nonblue points) was then expressed as the percentage of total points.

Subsequently, the aortas were immersed in 3% glutaraldehyde for a minimum of 16 hours, serially sectioned, processed routinely, embedded in Epon 812, and stained with toluidine blue. Neointimal proliferation was measured in full cross sections, 2 μm thick, from five subserial sections taken from the length of the denuded segment. Each section was photographed at ×14 magnification. The transparencies were projected at a magnification of ×10, and a planimeter (Numonics, Montgomeryville, Pennsylvania) was used to measure the neointimal and medial area. The extent of neointimal proliferation was expressed as the ratio of intimal to medial area for each denuded aorta.

RNA Extraction

RNA extraction was performed according to the methods of Chirgwin et al. Tissues were ground to a fine powder under liquid nitrogen; homogenized in 4 M guanidium thiocyanate, 25 mM EDTA (pH 7.5), 0.5% sodium N-lauroylsarcosine, and 0.13% antifoam A; and centrifuged at 8,000 rpm for 10 minutes. The supernatant was centrifuged for 20 hours at 36,000 rpm at 20°C on a 5.7 M/2.4 M CsCl step gradient. The pellet was solubilized in 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 5% sodium N-lauroylsarcosine, 5% phenol, and 0.1% M NaCl and extracted with phenol:chloroform:isoamyl alcohol (50:49:1, vol/vol/vol). Sodium acetate (pH 5.5) was added to the aqueous phase to a final concentration of 0.2 M, and the RNA was subsequently precipitated overnight with 2.5 vol ethanol at -20°C. The RNA was then pelleted, dried, and resuspended in diethyl pyrocarbonate-treated water, and RNA was quantified by ultraviolet spectrophotometry. To test the integrity of the RNA, aliquots of each extract were electrophoresed in 1% agarose minigels that contained formaldehyde and were stained with ethidium bromide. Only samples with intact 18S and 28S ribosomal subunit bands and 28S/18S ribosomal RNA ratios of about 2 were further tested.

Riboprobes

IGF-I mRNA content was determined with a solution hybridization–RNase protection assay with specific riboprobes designed to allow the relative quantification of the 5′ and 3′ posttranscriptional alternative processing variants of the primary IGF-I mRNA transcript. Riboprobes specific to the 5′ and 3′ portions of the rat IGF-I gene were originally provided by C. Roberts Jr. and D. LeRoith (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md.). Three different IGF-I mRNAs arise through alternative splicing at the 5′ untranslated region of the primary transcript. For detection of 5′ untranslated variant IGF-I mRNA transcripts, a 322-base EcoRI-Sau3 fragment from clone pIGF-Ib-42 ligated into a pGEM-2 vector was linearized with EcoRI and antisense RNA was synthesized with T7 polymerase. A 404-base transcript containing 82 bases of pGEM plasmid and 322 bases complementary to class A IGF-I mRNA was generated. Hybridization with rat liver RNA was expected to result in three protected bands of 322, 297, and 241 bases corresponding to class A, B, and C untranslated regions of IGF-I mRNA, respectively.

For detection of the 3′ variant IGF-I mRNA transcripts, IGF-Ia and IGF-Ib, an EcoRI-Sau3 fragment from the 3′ end of pIGF-Ib-42 clone complementary to IGF-Ib was ligated into the pGEM-2 vector. IGF-Ib and IGF-Ia mRNA differ according to the presence or absence, respectively, of a 52-base insert in the region that encodes the carboxy terminal E-peptide. After hybridization with liver RNA, this probe yields a protected band of 168 bases corresponding to IGF-Ib and a 116-base band corresponding to IGF-Ia mRNA.

For the generation of the riboprobes, the constructs were linearized and the gel was purified. The DNA template (50 μg/ml) was transcribed and labeled in vitro with [α-32P]UTP and T7 RNA polymerase (Promega, Madison, Wisconsin) as described elsewhere.
RNase Protection Assay

The RNase protection assay was performed according to methods described previously. Forty micrograms of tissue total RNA was resuspended in a hybridization buffer containing 20 mM Tris HCl (pH 7.6), 1 mM EDTA, 0.4 M NaCl, 0.1% sodium dodecyl sulfate, 75% deionized formamide, and 200,000 cpm of labeled riboprobe. The mixture was heated to 85°C for 5 minutes and then incubated at 45°C for 16 hours. RNase A (40 μg/ml) and RNase T1 (2 μg/ml) were added for 1 hour at 30°C. The mixture was then incubated with sodium dodecyl sulfate (final concentration, 0.6%) and 150 μg/ml proteinase K for 15 minutes at 37°C, and then extracted with phenol-chloroform. After the addition of 20 μg transfer RNA, the RNA was precipitated in 2 vol ethanol at −20°C. Pellets containing the labeled RNA hybrids were resuspended in a gel loading buffer containing 80% deionized formamide and were separated by size on an 8% polyacrylamide–8 M urea denaturing gel. After autoradiography, protected RNA bands were quantified by scanning densitometry. Five separate solution hybridization–RNase protection assays were performed. In each experiment, total RNA pooled from four rat aortas wasblotted at each time point.

Results

Reendothelialization and Neointimal Proliferation

We used Evans blue stain to determine the extent of deendothelialization. In rats killed 1 and 3 days after denudation, deendothelialization was found to be complete, with the entire luminal surface blue. Seven days after denudation, 42±13% of the luminal surface was reendothelialized, and 14 days after denudation 76±16% of luminal area did not stain with Evans blue. There was no neointimal proliferation in rats killed 1 and 3 days after denudation. The ratio of intimal to medial area was 0.033±0.021 and 0.306±0.061 (mean±SEM) 7 and 14 days after denudation, respectively. There was no staining with Evans blue or neointimal proliferation in sham-operated animals or animals treated with GH.

Aortic IGF-I mRNA Content

IGF-I mRNA content was determined at various intervals after balloon denudation. Figure 1 shows a representative RNase protection assay of total aortic RNA hybridized with the 3′ riboprobe complementary to IGF-Ib mRNA. IGF-Ia and IGF-Ib bands are expressed at a ratio of approximately 10:1 in both the liver (positive control) and the aorta. After denudation, IGF-I mRNA content is greatly increased in a time-dependent fashion. The IGF-Ia/IGF-Ib ratio was unchanged in the aortas before and at 1, 3, 7, and 14 days postdenudation. We have previously reported that the approximately 123-base protected fragment shown in Figure 1 probably represents incompletely digested probe hybridized to IGF-Ia mRNA rather than an additional splicing variant.5

![Figure 1. Regulation of alternative 3′ insulin-like growth factor I (IGF-I) messenger RNA (mRNA) transcripts in the rat aorta after balloon denudation. A solution hybridization–RNase protection assay with an IGF-I riboprobe complementary to IGF-Ib mRNA is shown. Lanes: 1, labeled riboprobe (not treated with RNase); 2, hybridization with 40 μg transfer RNA served as a negative control; 3, hybridization with 40 μg total RNA from normal rat liver served as a positive control; 4–7, 40 μg total RNA from aortas pooled from four rats before and 1, 3, and 7 days after balloon denudation, respectively. Arrows indicate protected fragments of 168 (IGF-Ib) and 116 bases (IGF-Ia). The intermediate-sized band is an aberrant digestion product of IGF-Ia.](http://circres.ahajournals.org/content/1757)
FIGURE 2. Regulation of alternative 5' insulin-like growth factor I (IGF-I) messenger RNA (mRNA) transcripts in the aorta after balloon denudation. A solution hybridization–RNase protection assay with an IGF-I riboprobe complementary to class A IGF-I mRNA is shown. All samples were hybridized with the riboprobe as described in “Materials and Methods.” Arrows indicate protected segments of 322 (class A) and 241 (class C) bases. Upper panel: Hybridization with 40 µg transfer RNA (lane 1); 40 µg total RNA from liver of hypophysectomized rats (lane 2), rats treated with growth hormone (GH, 35 mg/kg/week s.c.) (lane 3), and normal rats (lane 4); and 40 µg total RNA from aortas pooled from four rats before (lane 5), and 3 days (lane 6) and 7 days (lane 7) after balloon denudation. Lower panel: Total RNA (40 µg) from normal rat liver (lane 1) and from aortas from four rats before (lane 2), and 3 days (lane 3), 7 days (lane 4), and 14 days (lane 5) after balloon denudation. Also shown is hybridization with 40 µg aortic total RNA from four rats treated with GH (1.88 mg/kg/week [lane 6] and 35 mg/kg/week [lane 7]).

then progressively increased to fivefold and ninefold control levels at 3 and 7 days after denudation, respectively, decreasing to about twofold 14 days after denudation. IGF-I mRNA content in sham-operated animals was unchanged. Induction of aortic IGF-I mRNA after denudation was also present in tissues that had been mechanically stripped of endothelium before RNA extraction (data not shown).

**Discussion**

The finding of this study is that after balloon denudation, there is a marked induction of aortic IGF-I mRNA preceding neointimal proliferation. Although the histological events that follow endothelial injury with balloon denudation are well characterized, the cell signals that drive the process are not. It has been suggested that SMC migration and proliferation are initiated by adhering platelets with the release of PDGF. Adhering platelets disappear within days of injury, and luminal platelets are reportedly not required for rat carotid artery SMC proliferation after deendothelialization. Because SMC proliferation persists for weeks after deendothelialization, growth factors from within the vessel wall also may be involved in the initiation and perpetuation of the process. PDGF-A chain and PDGF-B chain mRNAs are expressed by cultured synthetic phenotype arterial SMCs, which also secrete a PDGF-like molecule, suggesting that this growth factor may be operating in an autocrine fashion to promote SMC growth. The amount of secreted PDGF-like molecules is increased several-fold in cultures of SMCs isolated from injured arteries. However, the significance of paracrine vascular PDGF expression remains unresolved, because neointimal proliferation after balloon denudation of thrombocytopenic animals is reduced even though there is induction of PDGF-A chain mRNA.

Other growth factors, such as basic fibroblast growth factor, endothelial cell growth factor, transforming growth factors α and β, and the IGFs, are also produced within the vessel wall. The role of local production of vascular growth factors other than PDGF and their interaction in the arterial response to endothelial denudation have not been reported. We have shown that after balloon denudation in rats, IGF-I mRNA content is induced at 24 hours, a time when SMC proliferation within the intima is still not apparent. After 7 days, IGF-I mRNA induction peaks to about a ninefold increase of the control level in denuded vessels and falls to about threefold 14 days after denudation when SMC...
proliferation is at its peak. Autocrine or paracrine production of IGF-I is believed to be necessary for PDGF to exert its mitogenic effects on mesenchymal cells in vitro.4 Fibroblast growth factor purified from brain is also a powerful stimulus for IGF-I production in cultured human fibroblasts.18 Basic fibroblast growth factor is produced by endothelial cells and may be secreted with other extracellular matrix components upon endothelial disruption. Therefore, local IGF-I gene expression could be induced as a response to either PDGF or fibroblast growth factor and could also be instrumental in mediating their effects. The induction of IGF-I mRNA was also seen in balloon-denuded aorta that had been stripped of the endothelial layer before RNA extraction, suggesting that the predominant source of IGF-I production is within the medial layer. IGF-I immunoreactivity was shown by immunohistochemistry to be present in both regrowing endothelial cells as well as in the media after arterial wall injury.19 However, Kern et al20 showed that endothelial cells produce IGF binding protein, IGF-II, and very little, if any, IGF-I. Because immunostaining for IGF-I may be due to cross-reactivity with IGF binding protein or to binding of the ligand to the receptor, definitive localization studies with in situ hybridization must be performed to clarify the cellular source of IGF-I gene expression.

Although IGF-I is a single-copy gene, multiple mRNA transcripts are generated through alternative processing of both the 5' and 3' portions of the primary transcript.21 These alternative transcripts give rise to a heterogeneous family of prepro IGF-I molecules. The biological role of these alternative transcripts is unclear, since all are eventually processed to an identical mature form of IGF-I. At the 3' end of the coding sequence, alternative E domains in rat prepro IGF-I are generated according to whether a 52-base exon is spliced either out (IGF-1a) or into (IGF-1b) the primary transcript. These 3' alternative splicing variants of IGF-I mRNAs were coordinately regulated in our model of arterial wall injury. Recently, rat IGF-I mRNAs with heterogeneous 5' untranslated regions (classes A, B, and C) have been demonstrated. It has been suggested that the 5' untranslated transcripts could exert translational control of IGF-I gene expression.21 The three alternative 5' untranslated regions are expressed in a tissue-specific manner. Class A is abundant in the liver, although it is also present to some extent in other tissues. Class B is found exclusively in the liver. Both of these appear to be particularly sensitive to GH regulation.21 We found only class C 5' untranslated IGF-I mRNA in rat aorta, which was induced ninefold over control levels after balloon denudation and only twofold after treatment with GH. These results are compatible with a model of IGF-I gene expression whereby tissues that are GH responsive and produce IGF-I destined to act in an endocrine fashion, such as the liver, process the primary transcript into the three 5' untranslated variant IGF-I mRNAs. In contrast, tissues in which IGF-I is produced in response to paracrine stimuli, and where IGF-I is destined to act locally, process the IGF-I mRNA to the class C variant. Whether class C 5' untranslated IGF-I mRNA is subject to a different set of tissue-specific transcriptional and/or translational regulatory mechanisms is an important question that needs to be addressed.

One of the cardinal features of the arterial wall response to injury has been the migration and localization of proliferating SMCs to the intima. The finding that a marked induction of IGF-I gene expression precedes neointimal proliferation after balloon denudation suggests that IGF-I may play an important role in initiation and perpetuation of this process.

Acknowledgments

We thank Elaine Tang, Mehran Khorsandi, Reza Salavati, and Monica Correa for technical assistance and D. LeRoith and C. Roberts for providing the rat IGF-I complementary DNA.

References


**KEY WORDS** • insulin-like growth factor I • arterial wall injury • neointimal proliferation • smooth muscle cells
Induction of insulin-like growth factor I messenger RNA in rat aorta after balloon denudation.

B Cercek, M C Fishbein, J S Forrester, R H Helfant and J A Fagin

doi: 10.1161/01.RES.66.6.1755

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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/66/6/1755

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/