Extracellular Matrix Glycoprotein Biglycan Enhances Vascular Smooth Muscle Cell Proliferation and Migration

Ryoko Shimizu-Hirota, Hiroyuki Sasamura, Mari Kuroda, Emi Kobayashi, Matsuhiko Hayashi, Takao Saruta

Abstract—Proteoglycans are produced and secreted by vascular smooth muscle cells, but the pathophysiological role of these glycoproteins in the vasculature is an enigma. Because the small leucine-rich proteoglycan (SLRP) biglycan is overexpressed in arteriosclerotic lesions, we produced mice constitutively overexpressing biglycan in the vascular smooth muscle, in order to examine the effects on vascular pathology. In the aorta and renal vasculature, increased vascular proliferation was seen both in the basal state and after infusion of angiotensin II (Ang II) in the transgenic mice compared with wild-type controls. In addition, the combination of biglycan overexpression and Ang II infusion resulted in marked increases in vascular smooth muscle cell proliferation and migration in the coronary arteries, as well as increases in fibrosis surrounding the vessels. In vitro, biglycan caused an increase in thymidine incorporation and migration of vascular smooth muscle cells, whereas these parameters were unchanged or reduced in endothelial cells. Moreover, addition of biglycan resulted in an increase in cdk2 expression and decrease in p27 levels in the vascular smooth muscle cells. These results suggest that this extracellular matrix SLRP may be involved in the regulation of vascular smooth muscle growth and migration through cdk2- and p27-dependent pathways. Furthermore, changes in biglycan expression could be a factor influencing the susceptibility of arteries to vascular injury, and may play a direct role in the pathogenesis of vascular lesions. (Circ Res. 2004;94:1111-1121.)

Key Words: proteoglycan • vascular injury • angiotensin

The cells in the vascular wall are held together by a complex network of macromolecules that collectively form the extracellular matrix. The species of glycoproteins known as proteoglycans are an important component of this matrix. These glycoproteins consist of a core protein covalently bound to one or more glycosaminoglycan (GAG) side chains.1 Based on the composition of the GAG moiety, the proteoglycans may be classified into chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate proteoglycans. In the vascular wall, a major proteoglycan synthesized by endothelial cells and vascular smooth muscle cells is the proteoglycan biglycan, which consists of a core protein bound to two chondroitin sulfate/dermatan sulfate side chains.

Biglycan is a member of the small leucine-rich proteoglycan (SLRP) family of proteoglycans, which are characterized by the presence of repeated sequences containing a high proportion of leucine residues.2 Recent studies have suggested that these SLRPs are not simply an inert structural component of the extracellular matrix, but may be actively involved in the control of collagen deposition, and the activation and inactivation of cytokines and growth factors.1,3

Studies from our and other laboratories have shown that biglycan expression is markedly altered in disease states, and that therapeutic intervention with hormones or antihypertensive agents can alter biglycan expression in vivo and in vitro.4-6 Of importance, biglycan expression in the blood vessel wall has been shown to be increased in atherosclerotic lesions and restenotic lesions both in animal models7,8 and in human samples.9,10

At present, the functions of biglycan in the blood vessel wall are still unclear. In this study, we used a vascular smooth muscle cell-specific promoter to amplify biglycan expression in the vasculature, and examined the susceptibility of these vessels to vascular injury mediated by angiotensin II infusion. We also examined if biglycan can affect growth and migratory properties in vascular smooth muscle cells in vitro.

Materials and Methods

Production of Transgenic Mice Overexpressing Biglycan in the Vasculature

A transgenic construct was produced by ligating the human smooth muscle α-actin promoter11 (pBS-HSMA-EA4.7, generously provided by Dr Miwa, Osaka University, Osaka, Japan) upstream of the human biglycan cDNA12 (P16, generously provided by Dr Fisher, National Institute of Dental and Craniofacial Research, Bethesda, Md). Purified DNA was microinjected into fertilized ova of C56BL/6J mice using standard techniques. Transgenic mice were identified by PCR and Southern blotting, and further experiments...
were performed on two independent transgenic lines. All animal experiments were performed in accordance with institutional guidelines.

**Analysis of Transgene Expression by RT-PCR-RFLP and Immunohistochemistry**

Total RNA was purified from aorta, kidney, heart, lung, testis, and skeletal muscle by the acid guanidine-phenol-chloroform method, and quantified by absorbance at 260 nm in a spectrophotometer. Semiquantitative RT-PCR was performed using protocols described by us previously. The biglycan primers used corresponded to an area of complete homology in the nucleotide sequences of human and mouse biglycan cDNA and spanned several introns in the genomic sequence. Moreover, the amplified cDNA products could be distinguished by the digestion with the restriction enzyme SacI because the human cDNA contains an internal SacI site, resulting in two DNA fragments (228 bp and 177 bp) after digestion of amplified DNA derived from transgenic transcription products, whereas the native biglycan transcript yielded one fragment of size 405 bp. Thus, comparison of the abundance of the DNA of these sizes allows an estimate of the relative levels of mRNA expression derived from the native biglycan gene and transgenic biglycan cDNA, respectively.

RT-PCR of transforming growth factor-β (TGF-β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as described. Immunohistochemistry of biglycan expression in the aorta was performed using an anti-human biglycan antibody (LF-51) kindly provided by Dr Fisher (National Institute of Dental and Craniofacial Research, Bethesda, Md). Deparaffinized samples were pretreated with chondroitinase ABC for 1 hour, then subjected to immunohistochemistry as described later.

**Animal Treatments and Assays**

Eight-week-old male heterozygous transgenic mice were infused with Ang II (1 μg/kg/min) or vehicle (saline) for 2 weeks using osmotic minipumps (Alzet). Wild-type littersmates were used as controls. Blood pressures were measured by tail-cuff plethysmography. Mice were euthanized by ether anesthesia and tissue samples of aorta, heart, and kidneys were fixed by rapid immersion in 4% paraformaldehyde (PFA), before embedding in paraffin. Preliminary experiments were performed to compare the results seen in mice perfusion-fixed at blood pressure before euthanasia versus mice that had not been perfusion-fixed. These experiments confirmed that similar differences between the groups were obtained with or without perfusion-fixation at blood pressure. Aortic sections were stained with Azan, whereas heart and kidney sections were stained with the Masson trichrome stain. Plasma renin activity was measured by standard techniques.

**Measurement of Collagen Content**

Cardiac collagen content was assessed by estimation of hydroxyproline content. Cardiac sections were hydrolyzed by treatment with 6 mol/L HCl at 115°C for 24 hours, then hydroxyproline content in the lyophilizes was estimated by reaction with chloramine T and p-dimethylaminobenzaldehyde.

**Morphometric and Immunohistochemical Analyses**

Media thickness and lumen diameters in the vasculature were measured using NIH image computer software. The lumen diameter was calculated from the lumen circumference, assuming that the vessel is circular, and the media/lumen ratio calculated by dividing the media thickness by the lumen diameter. For the assessment of perivascular fibrosis, the area of fibrosis (collagen deposition stained with aniline blue) immediately surrounding the coronary arterial wall was measured, and the fibrotic index was determined as the area of fibrosis divided by total vessel area. To estimate the incidence of coronary stenosis/occlusion, the three main coronary arteries were examined, and the heart was defined to have a coronary stenotic lesion if at least one neointimal lesion occupying >30% of the lumen area was seen. Immunohistochemical analyses were performed on PFA-fixed paraffin-embedded 5-μm sections by the streptavidin-biotin-peroxidase method. Briefly, after deparaffination and quenching of endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 minutes, serial sections were incubated with 2% normal goat serum to reduce nonspecific background staining, then incubated with polyclonal antibodies directed against proliferating cell nuclear antigen (PCNA), α-smooth muscle actin, CD68, or CD31 at a dilution of 1:100. Negative control experiments were performed by replacing the primary antibodies with normal rabbit serum. Subsequently, biotinylated secondary antibody and then streptavidin conjugate were applied. Positive staining was visualized with either DAB or tetra-methylbenzidine using a commercially available kit (Toyobo).

**In Vitro Studies**

In vitro studies were performed on rat VSMCs and bovine aortic endothelial cells. Assessment of cell proliferation, collagen synthesis, cell migration, and Western blot analysis of cell cycle proteins was performed based on previously described protocols, which are presented in the expanded Materials and Methods section of the online data supplement (available at http://circres.ahajournals.org).

**Statistical Analyses**

Results are expressed as the mean ± SEM. Statistical comparisons were made by ANOVA followed by Fisher’s post hoc test. Growth curves were compared using repeated measures ANOVA. Values of P<0.05 were considered statistically significant.

**Results**

**Expression of Human Biglycan in Biglycan Transgenic Mice**

The transgene construct contained the mouse α-smooth muscle actin promoter ligated to the coding sequence of human biglycan cDNA. This promoter effects a pattern of transgene expression similar to that of the endogenous α-smooth muscle actin, which is the dominant actin isoform in vascular tissue. Six founder mice containing the human biglycan transgene were identified by PCR and Southern blot analysis, and two lines were propagated and used for subsequent experiments. Figure 1 shows the results of a representative experiment to examine the expression of biglycan in the aorta of transgenic mice and their nontransgenic littermates. The presence of the biglycan transgene DNA in tail genomic DNA was assessed using PCR primers spanning intron 2, which yielded a 263-bp PCR product in the case of the intronless transgene, and 550-bp PCR product in the case of the native biglycan gene. Expression of biglycan mRNA in the aorta was assessed by RT-PCR. We have shown that RT-PCR performed in the linear phase of the amplification reaction enables a comparison of total biglycan mRNA expression between different animals. Moreover, because the primers correspond to identical sequences of the human and mouse biglycan cDNA sequences, and only human biglycan cDNA sequence contains a SacI site, relative levels of transgene and native biglycan mRNA expression in the same animal could also be directly assessed by comparison of the amplified bands after the addition of a SacI digestion step after RT-PCR. These methods suggested that biglycan mRNA was increased (2- to 4-fold) in the transgenic mice aorta. In contrast, biglycan mRNA from the transgene was below detectable levels in other tissues such as kidney, heart, lung, testis, and skeletal muscle (Figure 1C). To further confirm overexpression of human biglycan in the vasculature,
immunohistochemical staining was performed. Positive staining for biglycan protein was substantially increased in the medial layer of biglycan-transgenic mice compared with their wild-type littermates (Figure 1D).

**Effects of Biglycan Overexpression on the Aorta, Coronary Arteries, and Renal Arterioles**

The effects of biglycan overexpression on the structure of large (aorta), medium-sized (coronary arteries), and small (renal arterioles) arteries were examined. Preliminary experiments suggested that proliferating cells in the vascular media stained by PCNA were increased compared with controls, resulting in increased medial thickness. To confirm these findings, mice were divided into 4 groups: groups 1 and 2 were wild-type mice, and groups 3 and 4 were transgenic mice. Groups 1 and 3 were treated with vehicle, whereas groups 2 and 4 were infused with a pressor dose of Ang II to accentuate vascular hypertrophy and hyperplasia. Online Table 1 (available in the online data supplement at http://circres.ahajournals.org) shows the results of changes in heart and body weight, blood pressure, and plasma renin activity in the groups with and without Ang II infusion. The initial body and heart weights were similar in the transgenic mice and wild-type mice, and the heart/body weight ratios were significantly increased after Ang II infusion in groups 2 and 4. Baseline systolic blood pressure did not differ significantly between the 4 groups. Ang II increased systolic blood pressure from 100±3 to 132±7 mm Hg in wild-type mice (group 2) and from 103±4 to 136±2 mm Hg in transgenic mice (group 4). We also measured PRA from blood samples of the 4 groups at the end of the study. PRA was below the detection level in the Ang II–treated groups (2 and 4). No significant change in PRA was found between the untreated wild-type and transgenic mice. The changes in native and transgenic biglycan mRNA in the Ang II–treated and –untreated groups are shown in Figure 2A. Consistent with our previous report, Ang II treatment resulted in an increase in the native biglycan, but did not cause a significant change in the transgenic biglycan mRNA. Aortic and cardiac TGF-β1 mRNA was also increased by Ang II treatment, but no major differences were observed between wild-type and transgenic mice (Figures 2B and 2C).

Morphological changes in the aorta, coronary arteries, and renal arterioles were assessed. As shown in Figure 3, an increase in PCNA-positive cell counts was seen in the aorta of the transgenic mice compared with wild-type littermates. Moreover, increased media/lumen ratios were also seen in these vessels. Similar results were seen in the renal arterioles (Figure 4). In the case of the coronary arteries, more pronounced changes were seen (Figures 5 and 6). In the Masson-trichrome–stained sections, the effect of Ang II to cause cellular proliferation was accentuated in the biglycan transgenic mice, resulting in a dramatic proliferation of cells with areas of partial stenosis/occlusion of the coronary arteries in mice of group 4 (transgenic mice treated with Ang II) (Figure 5A). The incidence of coronary artery stenosis (defined as the presence of at least one lesion occupying >30% of the lumen) in the four groups of mice were as follows: wild-type Ang II (–) 0/15 (0%); wild-type Ang II (++) 2/15 (13%); transgenic Ang II (–) 0/18 (0%); and transgenic Ang II (++) 16/26 (62%).

The prominent lesions seen in the transgenic mice treated with Ang II were examined immunohistochemically. Quantitation of PCNA-positive cell counts revealed significant elevation in the transgenic mice with or without Ang II infusion compared with wild-type mice (Figure 5B). To characterize the cell types responsible for the stenotic lesions, staining was also performed using specific markers. It was found that the intimal cells stained strongly for α-smooth muscle cell actin, suggesting that the cells were derived from proliferating vascular smooth muscle cells (Figure 6).

We also assessed perivascular fibrosis in the coronary arteries by measurement of the fibrotic index. Both untreated
and Ang II–treated transgenic mice showed increased fibrotic index compared with the wild-type mice (Figure 5C). Assessment of cardiac collagen content was also performed by measuring hydroxyproline content in cardiac sections. Although baseline cardiac collagen content did not differ significantly between the transgenic and the wild-type mice, Ang II treatment resulted in a greater increase (1.7-fold change from 1.13 ± 0.14 to 1.90 ± 0.20 μmol/g; P < 0.05) in the transgenic mice compared with wild-type mice (1.2-fold change from 1.30 ± 0.29 to 1.55 ± 0.28 μmol/g; P = NS).

**Effects of Biglycan on VSMC Proliferation, Collagen Synthesis, and Migration In Vitro**

To further examine the effects of biglycan on the vasculature, experiments were performed on cultured vascular cells (VSMCs and endothelial cells) in vitro. Figure 7 shows the effects of biglycan on VSMC growth. Biglycan caused an increase in VSMC numbers, and a dose-dependent increase in thymidine incorporation by VSMCs, whereas an opposite trend was found in thymidine incorporation in the case of endothelial cells. The effects of biglycan were additive with

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**Figure 2.** A, RT-PCR-RFLP analysis of biglycan mRNA from the aorta of wild-type (WT) and transgenic (Tg) mice with and without Ang II treatment. All samples were digested with SacI before electrophoresis to yield 2 fragments (228 and 177 bp) in the case of biglycan mRNA derived from the transgene, whereas the native biglycan mRNA is undigested (405 bp). Top, Representative result of RT-PCR analysis. Bottom, Results of densitometric analysis; **P < 0.01 (n = 5 per group). B, RT-PCR analysis of TGF-β1 mRNA from the aorta of wild-type (WT) and transgenic (Tg) mice with and without Ang II treatment; *P < 0.05 (n = 5 per group). C, Western blot analysis of TGF-β1 in the hearts of wild-type (WT) and transgenic (Tg) mice with and without Ang II treatment.

**Figure 3.** Vascular morphology of aorta of wild-type (WT) and transgenic (Tg) mice with and without Ang II treatment. A, Representative cross sections of aortic wall. Top photomicrograph, PCNA immunostaining; bottom photomicrograph, Azan stain. Original magnification ×100. B, Quantitation of PCNA-positive cell counts in aorta of wild-type and transgenic mice. C, Quantitation of media thickness and media/lumen ratios in aorta of wild-type and transgenic mice. *P < 0.05, **P < 0.01 vs wild-type [Ang II (−)]; +P < 0.05, ++P < 0.01 vs Tg [Ang II (−)]; #P < 0.05, ##P < 0.01 vs wild-type [Ang II (+)] (n = 8 to 16 per group).
the effects of Ang II. Biglycan also caused a significant increase in collagenase-sensitive proline incorporation in Ang II–treated VSMCs but not in endothelial cells.

We next examined the migratory response to biglycan in VSMC and endothelial cells. As shown in Figure 7, biglycan treatment elicited a robust migratory response in VSMCs, and further enhanced the Ang II–induced migratory response. In contrast, no apparent change was seen in endothelial cell migration.

**Western Blot Analysis of Intracellular Signaling Pathways of Biglycan in Vascular Cells**

The effects of biglycan on the expression of genes affecting VSMC proliferation were examined by Western blot analysis. As shown in Figure 8, stimulation with biglycan resulted in an enhancement of cdk2 expression within 6 hours in VSMCs. In contrast, levels of p27 were reduced at 24 hours, whereas p21 expression remained at low levels throughout the experiment.

**Discussion**

Biglycan is a major glycoprotein of the extracellular matrix. Biochemically, it belongs to the SLRP family of proteoglycans and is composed of a distinct core protein covalently bound to two chondroitin sulfate/dermatan sulfate-containing glycosaminoglycan side chains.

The functions of biglycan are only beginning to be understood. Similar to decorin, biglycan has been shown to bind
TGF-β in vitro; however, there is controversy whether biglycan exerts TGF-β inhibitory effects in vivo. Biglycan has also been shown to associate with type I and type VI collagens in vitro, suggesting a role in the control of collagen fibrillogenesis. Indeed, targeted disruption of the biglycan gene results in a phenotype characterized by abnormal collagen fibrils in tendons, together with abnormal bone structure and reduced bone mass.

Biglycan is known to be expressed in the arterial wall and has been purified from the normal human aorta. However, the role of biglycan in the vasculature is unclear. Of note is the fact that expression of biglycan is markedly upregulated in diseased arteries, including atherosclerotic lesions, arteriosclerotic lesions, areas of restenosis postangioplasty, and in transplant coronary arteriopathy. Of interest, it has been reported that biglycan colocalizes with apolipoprotein E in atherosclerotic plaques, suggesting the hypothesis that biglycan, by virtue of its ability to bind apolipoproteins, may be directly involved in the retention of these atherogenic molecules in the diseased vessel wall.

Because the upregulation of biglycan in diseased arteries suggested a role in vascular disease, our aim in this study was to examine the direct consequences of increasing biglycan content in the vessel wall. To this end, transgenic mice that overexpressed biglycan in the vasculature were produced using a transgene construct consisting of the human biglycan cDNA ligated downstream of the smooth muscle cell α-actin promoter. Experiments were performed on two transgenic lines that yielded similar results.

No gross changes in the development or behavior of the transgenic mice were observed compared with their nontransgenic littermates. Moreover, cardiac echocardiography did not

Figure 6. Immunohistochemical assessment of coronary lesions in Ang II–treated transgenic mice. A, Results of immunostaining of coronary arteries from Ang II–infused transgenic mice with antibodies to α-smooth muscle cell actin (αSMA), CD68 (marker for macrophages), CD31 (marker for endothelial cells), and PCNA. Original magnification ×200.

Figure 7. Effects of biglycan and Ang II on cell numbers (A), thymidine incorporation (B), collagen synthesis (C), and cell migration (D) in VSMCs and endothelial cells. *P < 0.05, **P < 0.01 vs biglycan (−), Ang II (−); +P < 0.05, ++P < 0.01 vs biglycan (−), Ang II (−); #P < 0.05, ##P < 0.01 vs corresponding values for biglycan (+), Ang II (−) (n = 4).
reveal a major difference in ejection fraction between untreated transgenic and wild-type mice (data not shown). However, increased proliferation of the aortic wall and renal arterioles was seen, which was evidenced by increased numbers of PCNA-positive cells in these vessels, accompanied by increases in the medial thickness of the vessels.

In this study, we examined the effects of infusion of Ang II on the vasculature in wild-type and transgenic mice, because Ang II is a peptide hormone that is thought to play a major role in the progression of vascular disease, a concept which has been underscored by the efficacy of Ang receptor blockade in attenuating the progression of hypertensive vascular disease. Ang II infusion resulted in an increase in vascular hypertrophy and proliferation of vascular cells, and these effects were enhanced in the aorta of the transgenic mice. Another notable finding were the lesions seen in the coronary arteries of the Ang II-infused transgenic mice, which were reminiscent of vascular lesions in coronary disease, with marked increases in the vascular intima, resulting in some cases in partial occlusion of coronary arteries, together with increases in perivascular fibrosis.

Concerning the mechanisms of these changes, we found that stimulation of VSMCs with biglycan resulted in an increase in thymidine incorporation, an effect that was additive with Ang II. These results were consistent with the in vivo findings in the transgenic mice and provided further evidence that biglycan has a proliferative effect on VSMCs.

It is well known that VSMC proliferation is coordinately regulated by cell cycle proteins. Activation of a cdk2-cyclin complex during the G1 phase is involved in the G1 to S phase transition, and this complex is inhibited by the cdk inhibitor p27. Therefore, our findings that biglycan enhances cdk2 kinase expression while diminishing p27 levels is consistent with the proliferative effects of biglycan that we found both in vivo and in vitro. It has been suggested that cell cycle proteins may also regulate VSMC migration. Taken together, our results suggest that the observed phenotype in the biglycan transgenic mice could be explained by the effects on these intracellular proteins.

It should be noted that the proliferative effects of biglycan appeared to depend on the cell type, because biglycan caused a decrease in thymidine incorporation in cultured endothelial cells in clear contrast to the effects on VSMCs. Similarly, biglycan increased migration in VSMCs but not in endothelial cells. With regards to the effects of biglycan on other cells, biglycan has been suggested to stimulate growth and differentiation of monocydic lineage cells and brain microglial cells, whereas in pancreatic cancer cells proliferation was suppressed by biglycan, and in mesangial cells biglycan had no significant effect alone, but inhibited the proliferative effects of PDGF-BB. Of further interest is the fact that the related SLRP decorin has been shown to induce p21, p27, and growth arrest in many cell types including both VSMCs.

Figure 8. Effect of biglycan (50 nmol/L) on cdk2 kinase (A), p27 (B), p21 (C), and β-tubulin (D) levels in VSMCs. Left, Representative Western blot. Right, Results of densitometric analysis. Densitometric data were normalized to β-tubulin and expressed as relative levels compared with control (0 hour). *P<0.05, **P<0.01 vs control (n=4).
and endothelial cells.\textsuperscript{30,31} Taken together, these results suggest that biglycan and decorin found in the extracellular matrix may be involved in the cell-specific regulation of cell proliferation and growth. It remains to be determined whether the differences between biglycan and decorin exist because of differences in the core protein structure, or the number of glycosaminoglycan side-chains associated with these two related molecules.

We did not differentiate between the influence of Ang II per se on the vasculature, versus the indirect effects of the increased blood pressure mediated by Ang II. Because comparable data were obtained using cultured VSMCs in vitro, it is probable that the effects on cell growth and perivascular fibrosis in vivo may be attributed at least in part to biglycan modulating a direct (blood pressure–independent) effect of Ang II on the vasculature in the transgenic mice. However, the possibility that a blood pressure–mediated mechanism could also contribute to changes cannot be ruled out. Another possibility is that changes in growth factor expression could be involved as an intermediary mechanism. The fact that major differences in TGF–β1 mRNA were not found between the wild-type and transgenic mice suggest that changes in the expression of this growth factor do not play a major role in the observed changes.

The results of this study have several implications for the understanding of vascular biology and the pathogenesis of vascular injury. In designing the study, our initial hypothesis was that biglycan overexpression would result in an attenuation of vascular injury, because the related SLRP decorin is a potential candidate for the gene therapy of diseases such as postangioplasty restenosis, pulmonary fibrosis, and renal glomerulosclerosis.\textsuperscript{32–34} In fact, the results of this study suggest that the upregulation of biglycan reported in diseased arteries may contribute directly to the pathogenesis of vascular disease, instead of being solely a secondary response to vascular injury. It is well known that susceptibility to vascular stenosis or atherosclerosis differs markedly between individuals and between different arteries within the same individual. The hypothesis that differences in biglycan expression may account for some of these differences requires investigation. Similarly, our observation that the effects of biglycan on cell growth depends on the cell type may be useful not only for understanding the mechanisms of growth regulation of vascular cells in the presence of different types of extracellular matrix, but also may be of future benefit for designing strategies for accelerating the proliferation of one type of cell over another.

In summary, we have shown that overexpression of the extracellular matrix proteoglycan biglycan in the blood vessel to levels similar to those found in diseased arteries results in an increase in vascular hypertrophy, proliferation, and a heightened susceptibility of the coronary arteries to Ang II–induced vascular injury and perivascular fibrosis. These effects may be mediated at least in part by changes in cell cycle–regulatory proteins. These results reinforce the concept that molecules in the extracellular matrix can affect the behavior of the surrounding smooth muscle cells and may have important implications for our understanding of the processes influencing vascular injury.

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Online data supplement: Expanded Materials and Methods

Cell cultures

Rat VSMC were prepared from the thoracic aortae of 6-week-old male Wistar rats by collagenase digestion and cultured in DMEM supplemented with 10% fetal calf serum. Bovine aortic endothelial cells were obtained from Cell Applications Inc. (San Diego, CA, USA), and cultured in bovine endothelial cell medium with growth supplements as recommended by the manufacturer.

Assessment of cell proliferation and collagen synthesis

For the assessment of cell numbers, VSMC in 24-well plates were cultured in DMEM + 0.1% FCS in the presence or absence of biglycan (50 nmol/L), then cells were removed by trypsinization and counted in a Coulter counter. To assess changes in $^3$H thymidine incorporation, subconfluent VSMC were made quiescent by changing to serum-free media for 48 h then stimulated with biglycan for a further 48 h. $^3$H thymidine (1 uCi/ml) was added during the last 24 h of the experiment, then the cells were harvested for the quantitation of $^3$H thymidine incorporation into TCA-precipitable material. Collagen synthesis was determined by measurement of collagenase-sensitive $^3$H proline incorporation.

Cell migration assay

Cell migration assays were performed using a modified Boyden’s chamber method. In brief, VSMC suspension (5 x 10$^5$ cells/ml, 200 ul) was added to the upper chambers containing a 8 um-pore polycarbonate filter (Chemotaxicel, Kurabo, Japan) in serum-free DMEM+0.1% BSA, and various concentrations of biglycan with or without 10$^{-7}$ mol/L AngII were added to the lower chamber in DMEM + 0.1% BSA. The chamber was incubated at 37 °C for 12 h, then the filters were removed, fixed in methanol, and stained with Mayer’s solution. Cells from 4 randomly chosen high-power fields (magnification×20) on the lower surface of the filter were counted.
Western blot analysis of cdk2 kinase, p27 and p21 and TGF-β1

VSMC were rinsed with PBS and lysed in ice-cold RIPA buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10ug/ml PMSF, 30 ug/ml aprotinin, 1 mmol/L sodium orthovanadate). Lysates were centrifuged at at 15,000 g at 4 °C for 15 min, and the supernatant was taken for protein assay and Western blot analysis. 30 ug of protein was separated on 4-12% SDS-polyacrylamide gels and then blotted onto nitrocellulose membranes by wet electroblotting. Blots were blocked with 5% non-fat dry milk for 1 h at room temperature in TBS-T (20 mmol/L Tris-HCl pH 7.6, 137 mmol/L NaCl, 0.1% Tween) and then incubated with anti-cdk2, anti-p27, anti-p21, or anti-β-tubulin antibody (Santa-Cruz Biotechnology Inc, 1:200) for 1 h at room temperature. Specific proteins were detected by enhanced chemiluminescence (ECL, Amersham Life Sciences, Little Chalfont, UK) according to the manufacturer’s instructions. In the case of cardiac TGF-β1, 50 ug of heart homogenate in RIPA buffer was subjected to Western blot analysis using anti- TGF-β1 antibody (Santa-Cruz Biotechnology Inc, 1:200).

Materials

Cell culture reagents, PCR, and electrophoresis reagents were obtained from Life Technologies-BRL (Grand Island, NY, USA), Perkin Elmer (Branchburg, NJ, USA), and Biorad (Hercules, CA, USA) respectively. Radiochemicals were obtained from NEN (Boston, MA, USA). Other chemicals, including biglycan purified from bovine articular cartilage, were obtained from Sigma (St Louis, MO, USA), unless otherwise stated.
### Online data supplement Table 1

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<td>nd</td>
<td>7.6±1.0</td>
<td>nd</td>
</tr>
</tbody>
</table>

Results shown are the mean±SEM.

*, **: p<0.05, p<0.01 vs Wild-type (Ang II (-));  +, ++: p<0.05, p<0.01 vs Tg (Ang II (-)) nd: not detectable (< 0.1 ng/ml/h)

Table 1. Effects of Ang II on blood pressure, heart weight, and PRA in wild-type and transgenic mice