Inhibition of Versican Synthesis by Antisense Alters Smooth Muscle Cell Phenotype and Induces Elastic Fiber Formation In Vitro and in Neointima After Vessel Injury

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Abstract—The proteoglycan versican is implicated in several atherogenic events, including stimulation of vascular smooth muscle cell (VSMC) growth and migration, retention of lipoproteins, and promotion of thrombogenesis. A high content of intimal versican also correlates with a low content of elastin, suggesting an inhibitory role for versican in elastogenesis. To determine whether reduced production of versican can be used to enhance elastogenesis, we transduced Fischer rat VSMC (FRSMC) with a versican antisense sequence using the retroviral vector LXSN. Stable expression of versican antisense (LVaSN) significantly reduced versican production, induced a flattened morphology, reduced cell proliferation and migration, increased tropoelastin synthesis, increased elastin binding protein (S-Gal/EBP), and increased deposition of elastic fibers in long-term cultures. Add-back of chondroitin sulfate chains, or versican, decreased S-Gal/EBP and elastic fiber formation. LVaSN cells seeded into balloon catheter-injured rat carotid arteries formed neointimae containing low levels versican, increased amounts of S-Gal/EBP, and increased elastin deposits 7 days postinjury. At 4 weeks, neointimae formed from LVaSN cells were highly structured and contained multiple layers of elastic fibers and lamellae. These results indicate a central role for versican and its constituent chondroitin sulfate chains in controlling cell phenotype, elastogenesis, and intimal structure. (Circ Res. 2006;98:370-377.)

Key Words: versican antisense ■ elastogenesis ■ vascular injury ■ remodeling

Versican, a chondroitin sulfate (CS) proteoglycan, is a major determinant for the structural and physiological properties of arterial wall extracellular matrix.1,2 The amino-terminal globular domain (G1) of versican core protein binds to hyaluronan (HA) and affects cell adhesion, proliferation, and migration.3-5 The carboxy-terminal domain (G3) mediates changes in morphology and adhesiveness by interacting with various other matrix molecules such as tenascin-R, fibrillin-1, and fibulins.6-9 Flanked between G1 and G3 is the glycosaminoglycan (GAG) binding region, which is encoded by 2 exons (α and β), which may be differentially spliced to give rise to 4 versican variants: V0 (α and β exons), V1 (β exon only), V2 (α exon only), and V3 (neither exon).10 These GAG subdomains, in addition to conferring tissues with viscoelastic properties, can differentially affect growth and apoptosis11 and interact with other extracellular matrix components.12 Furthermore, CS inhibits assembly of elastic fibers, a process controlled by the 67-kDa cell-surface elastin binding protein (EBP), which is identical to the alternatively spliced catalytically inactive variant of β-galactosidase (Gal), S-Gal.13,14 In the presence of excess galactosugars, such as chondroitin and dermatan sulfates, S-Gal/EBP releases tropoelastin and is shed from the cell surface, leading to impaired elastogenesis.15,16 In culture, removal of excess CS-containing GAG by chondroitin ABC lyase digestion reverses this impaired elastogenesis.17,18 These findings suggest that it may be possible to increase the elastin content of vessel wall by decreasing the content of versican. Recently, it has been demonstrated that overexpression by vascular smooth muscle cells (VSMC) of the versican variant V3, which lacks GAG chains, induces elastin synthesis and fiber deposition in vitro and in neointimae formed from V3-expressing cells seeded into balloon-damaged rat carotid arteries.19 Although the mechanism by which V3 induces elastogenesis is not known, these and the above findings suggest that CS content may be a critical factor.

In this study, we reduced versican production by Fischer rat VSMC (FRSMC) through retroviral transduction with an antisense sequence that recognizes the coding domains for G1 and G3 common to all versican variants. We then examined the transduced cells in vitro and investigated the structure of neointima formed by seeding the antisense-expressing cells...
into balloon-injured artery. The results support a central role for CS-containing versican in controlling cell phenotype, elastogenesis, and intimal structure.

Materials and Methods

Cell Culture

Aortic smooth muscle cells from male Fischer 344 rats (FRSMC) (Simenson Co, Gilroy, Calif) were obtained and cultured as described previously.20 Cells between 3 and 10 passages were used for the experiments.

Retroviral Transduction

Full-length antisense and sense V3 sequences were used to construct retroviral vectors using methodology described previously for V3.21 Briefly, rat V3 cDNA and the complementary product were each inserted into the BamHI site of empty retroviral vector (LXSN) to produce V3 and versican antisense-containing vectors (LV3SN and LVA9SN), respectively. Orientation of the inserts was confirmed by PCR. LXSN, LV3SN, and LVA9SN retroviruses, produced by transfection of PA317 packaging cells, were used to transduce cultured FRSMC as described previously.22–24 Three LXSN, 1 LV3SN, and 3 LVA9SN FRSMC clones between 3 and 10 passages after initial transductions were used for the following experiments.

Detection of Versican Antisense

RT-PCR was performed with ImProm-II Reverse Transcription System (Promega A3800) in accordance with the instructions of the manufacturer. Total RNA from 48-hour cultures of LXSN, LV3SN, and clones 1 to 3 of LVA9SN was reverse transcribed with forward primer LSXNF (5'-CCTGAAACTGCTCTGGTGCA3') and reverse primer JL27 (5'-GACTATGGCTGGCAAC3').

Versican and Tropoelastin mRNA Levels

Total RNA was isolated and Northern hybridization performed using a pool of V3, versican antisense, and β-gag (V25) probes as described previously.25 Denatured double-stranded V3 cDNA served to detect both sense and antisense versican sequences, whereas V25 identified versicans V0 and V1. Tropoelastin mRNA was detected by a human probe generously provided by Dr C. D. Boyd (University of Hawaii).26

Versican Production

Cells were seeded at a density of 5×10^5 cells/100-mm culture dish, maintained in 10 mL of 10% FBS growth medium for 24 hours, starved for 24 hours in 10 mL of growth medium with 0.1% FBS, and incubated in 10 mL of 10% growth medium containing 100 μCi/mL [35S]-sulfate for a further 24 hours. Labeled medium proteoglycans were isolated as described previously27 and prepared for SDS-PAGE.28 Samples, including a parallel set digested with chondroitin ABC lyase (ICN, Costa Mesa, Calif) were run on a 4% to 12% gradient polyacrylamide-SDS resolving gel with a 3.5% polyacrylamide stacking gel. Loading volumes reflected the amount of proteoglycan produced by an equal number of cells. The gel was processed as described previously,28 and versican production determined from band intensities of undigested and chondroitin ABC lyase digested samples.

Versican Core Proteins

Core proteins of chondroitin ABC lyase-digested medium samples were separated on SDS-PAGE and Western blotted as described previously.28 V0 and V1 core proteins were detected with versican antibody LF99 (rabbit anti-human, kindly provided by Dr Larry Fisher, National Institute of Dental and Craniofacial Research, NIH, Bethesda, Md), diluted 1:5000 in 5 mL of Tris-buffered saline with 2% FBS.29 Following exposure to Kodak XAR-2 film, band intensities were analyzed by NIH Imagej.

Growth and Migration

Cell growth and migration rates were determined as described previously.21 Migration was determined by measurement of movement of cells into a cell-free zone created by scraping confluent cultures.

Immunodetection of Versican, CS, Tropoelastin, and S-Gal/EBP

Versican

Cells seeded at 5000 cells/well on chamber glass slides were cultured in standard medium for 24 hours, fixed in 0.9% formaldehyde, washed in PBS, incubated for 1 hour with primary versican antibody LF99, washed in PBS, and incubated for 1 hour in Texas Red-conjugated goat anti-rabbit secondary antibody (1:100; Invitrogen T2767). Slides were rinsed in PBS and mounted with Dako Fluorescent Mounting Medium (S3023). Fluorescent micrographs were converted to gray scale in Adobe Photoshop 7.0 and staining intensities measured by NIH Imagej analysis and expressed as optical density (OD).

Chondroitin Sulfate

Cells cultured for 7 days were fixed for 30 minutes in cold 100% ethanol and immunostained with 5 μg/mL of a monoclonal antibody to CS (C-8035, Sigma) and a fluorescein-conjugated secondary antibody (FITC).19

Tropoelastin

Seven-day confluent cultures were fixed for 30 minutes in cold 100% ethanol, immunostained with 10 μg/mL polyclonal bovine tropoelastin antibody (Elastin Products Co Inc, Owensville, Mo), and detected by FITC. Three-week cultures, with and without daily CS add-back (200 μg/mL), were fixed in 0.9% formaldehyde and immunostained with the tropoelastin antibody and Texas Red-conjugated secondary antibody.

S-Gal/EBP

Two-day subconfluent cultures were immunostained with 20 μg/mL anti-S-Gal that recognizes the sequence that binds elastin,13 and with anti-EBP (Elastin Products Co Inc), using FITC and Texas Red secondary antibody detection respectively. The latter antibody was also used to assess the effect of 24 hour add-back of versican (200 μg/mL) on EBP staining intensity, which was quantified by NIH Imagej analysis.

S-Gal/EBP and β-Gal-related proteins were also isolated from cell layers and detected by Western blotting with 3 different antibodies, anti-P-Gal, raised to the β-Gal precursor and recognizes all forms of β-Gal,13 anti-S-Gal, and anti-EBP, as previously described.13 Horse-radish peroxidase secondary antibody signals were detected by enhanced chemiluminescence.

Effect of Versican and CS Add-Back on S-Gal/EBP and Elastin Deposition

Cells were seeded at 5000 cells/well on 8-well Laboratory-Tek Chamber Glass Slides (NUNC 177399) and maintained in medium containing 200 μg/mL versican30 or chondroitin sulfate A (Sigma C-8529). The concentration was predetermined by a dose-range experiment of CS (10, 100, 200, and 400 μg/mL) to achieve optimal effect (results not shown). Cells cultured for 24 hours in the versican-containing medium were immunostained with anti-EBP. Cells cultured for 3 weeks in CS-containing medium were immunostained for tropoelastin. Controls were maintained in medium only. Slides were fixed in 0.9% formaldehyde for 10 minutes for the immunocytochemistry. For Western blot detection of P-Gal, S-Gal, and EBP, cells were cultured for 7 days with and without CS (400 μg/mL).

Insoluble Elastin

Quadruple cultures of LXSN and LVA9SN (plated at 5×10^5 cells) were grown to confluence in the 6-well culture dishes. Twenty microcuries of [3H]-valine (New England Nuclear, Boston, Mass)
Balloon Catheter Injury and Cell Seeding of Rat Carotid Arteries

Balloon injury and cell seeding in Fischer 344 rats were performed in accordance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication, revised 1985) as described previously. Control or antisense-expressing cells (3.75 to 10^6) were suspended in 150 μL of serum-free growth medium and injected into each ballooned and isolated common carotid artery. Rats was inverted for 2 minutes and then returned to the original position for 13 minutes to allow for even-cell settlement. Following restoration of blood flow and wound closure, rats were maintained on a normal diet and euthanized on days 7 (6 rats from each control and antisense group) and 28 (8 rats from each control and antisense group) as described previously. A 50-mg tablet of bromodeoxyuridine (BrdUrd) was subcutaneously implanted in each rat 24 hours before euthanasia for the purpose of measuring cell proliferation.

Histochemistry and Immunohistochemistry on Seeded Vessels

Seeded vessels were harvested, fixed in 4% paraformaldehyde, and processed for paraffin embedding as described previously. Five-micrometer sections were stained with Toliuidine Blue or orcein to display general tissue morphology and elastin organization. In addition, sections were immunostained for versican or EBP using DAKO Envision rabbit anti-human horseradish peroxidase (DAB). BrdUrd-positive cells were detected using a monoclonal antibody to BrdUrd (Boehringer-Mannheim).

Transmission Electron Microscopy and Morphometric Analyses

Confluent cultures and segments of the seeded carotid arteries were fixed in 3% glutaraldehyde, postfixed in 1% OsO4, stained in 2% uranyl acetate, and embedded in Epon as described previously. Sections were mounted on formvar-coated grids (Pro Sci Tech GCu300), stained in 2% uranyl acetate and lead, and viewed on a JEOL 1200 EXII microscope. Volume fractions for cell, elastin, collagen, and matrix space were determined by point counting as described previously.

Statistics

Data were analyzed by Student’s t test and ANOVA, and a value of P<0.05 was taken as significant.

Results

Expression of Versican Antisense

LVaSN clones 1 to 3 expressed the predicted 973-bp product of RT-PCR, with clone 2 showing the highest expression. Expression level, however, did not correlate with the degree of versican knockdown or other changes as presented below; all clones were effective at reducing versican. Neither LXSN nor LV3SN cells expressed detectable levels of antisense (Figure 1A).

Versican Antisense Reduced Versican mRNA and Versican Production

Total RNA extractions from 14-day cultures of LXSN, LV3SN, and LVaSN were probed for versican expression (Figure 1B). LVaSN cells were negative for all versican transcripts. Similar to previous results, LXSN and LV3SN cells showed low levels of V0 at 13 to 11 kb, V1 at 9 to ∼7.7 kb, and in V3, V3/neo at 6 kb. The level of V1 mRNA in LV3SN cells was increased compared with LXSN. A previ-
ous study, however, showed V1 mRNA levels in LV3SN to be variable.\textsuperscript{21}

[\textsuperscript{35}S]-Labeled versican secreted by LXSN, LV3SN, and LVaSN cells was separated on SDS-PAGE as previously described.\textsuperscript{27,28} Versican production, measured as the difference in band densities of chondroitin ABC lyase digested and nondigested samples, was reduced by 95% (\(P<0.001\)) in the 3 LVaSN clones (Figure 1C). Versican V1 and V0 core proteins, analyzed by Western hybridization, showed a significant reduction of 85% (\(P<0.005\)) in band density of the LVaSN clones compared with LXSN clones (Figure 1D).

Low-density cultures of LVaSN cells showed reduced immunostaining for versican compared with LXSN cells (Figure 1E). The mean percentage decrease in staining intensity, calculated from OD measurements for 4 independent experiments, was 53.3\(\pm\)2.3\% (\(P<0.001\)). Similarly, confluent 7-day cultures of LVaSN cells stained less intensely for CS than LXSN cultures (Figure 1F). Based on these results, and those from Northern, PAGE, and Western analyses, we concluded that the versican antisense significantly reduced versican production by FRSMC in vitro.

Versican Antisense Induced Changes in Cell Phenotype
LVaSN cells at both low and high density were more flattened and spread than LXSN cells (Figure 2A through 2D). LVaSN cells proliferated at a rate half that of LXSN cells (Figure 2E), and, in a scrape wound assay, showed a significantly slower rate of migration during the first 12 hours compared with LXSN cells (Figure 2F). At later time periods, the migration rate was similar but the initial difference was maintained. The flattened LVaSN cells were also more adhesive, as measured by a trypsin resistance assay,\textsuperscript{21} and had reduced cell coats, as measured by a particle exclusion assay\textsuperscript{21} (data not shown). These morphological and physiological features were similar to those found previously for LV3SN.\textsuperscript{21}

Versican Antisense Increased Tropoelastin mRNA and Elastic Fiber Deposition In Vitro
Tropoelastin mRNA levels of LVaSN cells were significantly increased compared with LXSN cells (Figure 3A), and 7 day LVaSN cultures stained more intensely for elastin than LXSN cultures (Figure 3B). Morphometric analysis confirmed significantly (\(P<0.001\)) increased immunostaining of elastin, and the reciprocal finding of decreased immunostaining of CS (Figure 3C). Metabolic labeling with \([\textsuperscript{3}H]\)-valine showed that insoluble elastin in 7day LVaSN cultures was significantly increased (Figure 4A), and immunostaining of 3-week cultures of LVaSN cells showed increased deposits of elastin and elastic fibers (Figure 4B, top). CS add-back decreased both insoluble and immunostained elastin (Figure 4A and 4B,
respectively). The increase in extracellular elastin in LVaSN cultures was confirmed by transmission electron microscopy (TEM) of 3-week cultures. Elastin volume fraction, measured by point counting of extracellular elastin in the cell layer, was 10-fold higher in LVaSN (4.3%) compared with LXSN (0.4%) (P < 0.005).

Versican Antisense Increased S-Gal/EBP
LVaSN cells cultured for 48 hours stained more intensely with anti–S-Gal (Figure 5A) and with anti-EBP (Figure 5B, upper panels) than did LXSN cells. The mean percentage increase for anti-EBP staining intensity, measured as OD, was 50.4 ± 3.9% (P < 0.001). Addition of 200 μg/mL versican for 24 hours reversed this increase (Figure 3C); EBP OD decreased 37.5 ± 2.9% (P < 0.001). Add-back of 200 μg/mL of CS resulted in a similar reduction in anti-EBP immunostaining of LVaSN cells (data not shown).

Western blot analysis of cell lysates with anti–P-Gal showed that LXSN and LVaSN had similar amounts of the 88-kDa β-Gal precursor and the mature 64-kDa form of the active enzyme. In contrast, the 67-kDa catalytically inactive spliced variant of β-Gal (S-Gal), detected by anti–P-Gal, anti–S-Gal, and by anti-EBP, was increased in LVaSN cell lysates compared with LXSN (Figure 5D). Add-back of CS decreased the 67kDa S-Gal/EBP in both LVaSN and LXSN cells but had no effect on precursor or active β-Gal (Figure 5D).

Versican Antisense–Expressing Cells Formed a Versican-Depleted and Elastic Fiber–Rich Neointima
Seven-day neointima formed from LXSN cells was characterized by stellate or rounded FRSMC embedded in a matrix...
that was high in versican (Figure 6A), relatively depleted of EBP (Figure 6C), and with few elastin deposits (Figure 6E). Seven-day neointima formed from LVaSN cells was characterized by reduced versican staining (Figure 6B), increased EBP staining (Figure 6D), and numerous small deposits of elastin preferentially distributed at cell surfaces (Figure 6F). Morphometric analysis showed a nonsignificant trend toward reduced thickness of neointima formed from the LVaSN cells (data not shown). Cell proliferation indices, determined from BrdUrd labeling, were not significantly different in the 2 groups (data not shown).

Sections of 28-day LXSN neointima, stained with Tolu- idine Blue or orcein, showed punctate elastin deposits in a myxoid matrix surrounding stellate cells (Figure 7A, 7C, and 7E). Twenty-eight-day neointima formed by LVaSN cells was more compact with significantly more elastin (Figure 7B, 7D, and 7F), which, in regions of highest content, was organized into wavy circumferential fibers and lamellae between elongated cells (Figure 7B and 7D), resembling a developing media. Similar to 7-day neointima, there was a nonsignificant trend toward decreased intimal thickness of neointima formed from LVaSN cells (data not shown).

These structural and morphological differences were confirmed by analysis of TEM micrographs of 28-day neointima (Figure 8A and 8B). Point-counting of cells and extracellular matrix components (Figure 8C) showed that neointima formed by LVaSN cells had a significantly ($P<0.001$) higher elastin volume fraction (36.5%), and a significantly ($P<0.01$) lower matrix space volume fraction (12.1%), compared neointima formed from LXSN cells (19.4% and 18.2%, respectively). Cell and collagen volume fractions were not significantly different.

**Discussion**

The results of this study demonstrate the central importance of versican in controlling vascular cell phenotype and composition of the extracellular matrix, in vitro and in vivo. Retroviral transduction of cultured FRSMC with a versican antisense sequence decreased production and deposition of versican, induced a flattened morphology, slowed proliferation and migration, increased tropoelastin and its chaperone S-Gal/EBP, and increased elastin deposition and fiber formation. Seeding of versican antisense expressing cells into balloon injured rat carotid arteries resulted in neointima with a highly ordered structure, reduced versican content, and increased S-Gal/EBP and elastic fiber content.

A number of studies have reported an inverse relationship between versican and elastogenesis. In versican-rich tissues,
such as in restenotic lesions and the ductus arteriosus, elastic fibers are scarce.\textsuperscript{17,33} Conversely, neonatal SMC that produce little CS, show a high level of tropoelastin synthesis.\textsuperscript{34,35} Impaired elastogenesis also occurs in Hurler disease and Costello syndrome, in which galactosugar-containing GAGs accumulate around cells in the skin and other organs, including vessels. Accumulation of galactosugars has been demonstrated to negatively interfere with the function of S-Gal/EBP.\textsuperscript{16,18} Chondroitin ABC lyase digestion and exposure of fibroblasts to high levels of CS results in demonstration increased elastin and fiber formation in VSMC and LVaSN cells indicates that the change in S-Gal/EBP in the neointima formed from LXSN and LVaSN cells was accompanied by increased tropoelastin expression, including any endogenous V3. Our findings support the first hypothesis, namely that it is the depletion of versican that is permissive for elastic fiber assembly. We cannot discount, however, that versican influences elastin stability and turnover through modulation of protease activity.

The increased deposition of elastin in cultures of versican-depleted cells was accompanied by increased tropoelastin mRNA. A high level of tropoelastin expression also occurs in neonatal SMC that produce little or no versican.\textsuperscript{34} The mechanism responsible for this upregulation is not known but could be attributable to, either or both, an increase in the transcription rate of the elastin gene or increased stability of tropoelastin message. Current studies from our laboratories, including those suggesting that forces generated during changes in the cell shape may induce signals stimulating elastin gene expression, will likely resolve this question.

Interestingly, and in contrast to the elastic fibers seen in vitro, the neoimal elastic fibers were organized in layers between circumferentially arranged and generally elongated SMC. We postulate that this regular organization may be attributable to mechanical forces associated with systole and diastole. These fibers may in turn have regulatory functions of maintaining a stable and highly structured neointima. In this regard, it is notable that disruption of elastin assembly, by elastin gene knockout, induces subendothelial VSMC proliferation and obstruction of the lumen.\textsuperscript{37}

Finally, the experimental model used in this study to form a versican-depleted neointima may be especially valuable for other studies, including testing the role of versican in binding atherosclerotic lesions and other factors associated with the development of atherosclerosis. Modulation of versican levels by antisense or other approaches, including V3, may also

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**Figure 8.** Top, TEM micrographs of 28-day neointimae formed by LXSN (A) and LVaSN (B) cells seeded into balloon-catheter injured rat carotid arteries. Neointima formed by LVaSN cells contained significantly more elastin (dark deposits indicated by arrow heads) than the LXSN neointima. Magnification, $\times 10,000$. Bottom, Volume fractions (%) for cells (C), elastin (E), collagen (Co), and matrix space (M), determined from TEM micrographs of 28-day neointimae formed from LXSN and LVaSN cells. Neointima formed by LVaSN cells contained a significantly higher ($^*P<0.001$) elastin volume fraction (36.5%) and a significantly lower ($^{**}P<0.01$) matrix space volume fraction (12.1%) compared with neointima formed by LXSN cells (19.4% and 18.5%, respectively).
offer novel therapeutic approaches for remodeling other elastin-deficient tissues.

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