Retrovirally Mediated Overexpression of Versican V3 by Arterial Smooth Muscle Cells Induces Tropoelastin Synthesis and Elastic Fiber Formation In Vitro and In Neointima After Vascular Injury

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Abstract—Versican is an extracellular matrix (ECM) proteoglycan that is synthesized as multiple splice variants. In a recent study, we demonstrated that retroviral-mediated overexpression of the variant V3, which lacks chondroitin sulfate (CS) chains, altered arterial smooth muscle cell (ASMC) phenotype in short-term cell culture. We now report that V3-overexpressing ASMCs exhibit significantly increased expression of tropoelastin and increased formation of elastic fibers in long-term cell cultures. In addition, V3-overexpressing ASMCs seeded into ballooned rat carotid arteries continued to overexpress V3 and, at 4 weeks after seeding, produced a highly structured neointima significantly enriched in elastic fiber lamellae. In contrast to the hydrated, myxoid neointima produced by rounded or stellate vector-alone–transduced cells, V3-expressing cells produced a compact and highly ordered neointima, which contained elongated ASMCs that were arranged in parallel arrays and separated by densely packed collagen bundles and elastic fibers. These results indicate that a variant of versican is involved in elastic fiber assembly and may represent a novel therapeutic approach to facilitate the formation of elastic fibers. (Circ Res. 2002;90:481-487.)

Key Words: versican • elastin • smooth muscle cells • vascular injury • artery

The extracellular matrix (ECM) proteoglycan versican, the major chondroitin sulfate proteoglycan of vessel wall,1 is distinguished by a central extended region with attached glycosaminoglycan (GAG) chains by an amino-terminal globular domain (G1) that binds hyaluronan (HA) and by a carboxy-terminal selectin-like domain (G3) that binds to other matrix components including tenascin-R and fibulin-1.2–6 The mRNA that codes the GAG attachment region, which in the full-length form has 2 domains, can undergo differential splicing to produce 4 variants: V0, with 2 (α and β) GAG binding regions; V1 (with the β GAG exon); V2 (with the α GAG exon); and V3 with neither GAG exon and formed from the G1 and G3 domains only.7–9 V3 is thus predicted to be a glycoprotein and not a proteoglycan. V3 mRNA has been demonstrated in aortic tissue as well as cultures of ASMCs by Northern blot10 and has been detected, by polymerase chain reaction (PCR), from cDNA libraries for various tissues including brain, stomach, and liver.9

Recently, we reported that retroviral insertion and overexpression of the gene for V3 into cultured Fischer rat ASMCs induces a number of phenotypic changes.11 Compared with vector-alone–transduced cells, V3 ASMCs are more flattened and spread, show a large increase in area of close contacts and in the size of peripheral focal contacts, increased resistance to trypsin detachment, reduced pericellular coats, and decreased rates of growth and migration. The mechanism by which V3 effects these changes is not clear, although the morphological features, the reduced growth and migration, and the reduced cell coat, raise the possibility that V3 may affect the accumulation of components of the extracellular matrix. For example, Evanko et al12 showed recently that the V1 isoform of versican in the pericellular coat interacts with hyaluronan and is necessary for both proliferation and migration of ASMCs. The carboxy-terminal G3 region of versican may also interact with other matrix components to facilitate changes in phenotype in ways not yet understood. For example, the lectin-like domain of G3 binds fibulin-1, a component of elastic fibers.4 The GAG chains may also play a role. Chondroitin sulfate (CS) impairs elastic fiber assembly by binding to and causing shedding of an elastin binding protein at the cell surface.13

These possibilities led us to ask whether the production of specific matrix components is altered in V3-overexpressing ASMCs. We have found that overexpression of V3 by rat
ASMCs has a profound effect on tropoelastin production and elastic fiber formation in vitro and on elastic fiber deposition in neointima of balloon-injured vessels.

Materials and Methods

Retroviral Transduction

Cloning of rat V3 cDNA and transduction of Fischer rat ASMCs with V3 has been described previously. Briefly, the rat V3 cDNA was inserted into the BamHI site of the retroviral vector LXSN (courtesy of Dr A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, Wash.). Orientation was confirmed by PCR. The retroviral vector containing the V3 gene (LV3SN), as well as the empty control vector (LXSN), was used to infect cultured ASMCs from Fischer rats using PA317 packaging cells as previously described.

Cell Culture

ASMCs from male Fischer 344 rats (Simensons Co, Gilroy, Calif) were obtained and cultured as described previously. Several transductions were carried out on cells of different passages, and for each transduction, parallel pools of LXSN and LV3SN cells were selected by means of the neomycin analogue G418 (800 μg/mL) and passaged together in DME-high glucose medium (Irvine Scientific, Santa Ana, Calif) supplemented with 10% FBS (Atlantic Biologicals catalog No. S11150), sodium pyruvate (IS No. 9334), nonessential amino acids (IS No. 9304), and glutamine pen-strep (IS No. 9316). Cells were used for experiments between 5 and 9 passages after initial transductions.

Immunocytochemistry In Vitro

ASMCs that had been transduced either with control vector (LXSN) or with LV3SN were plated near confluent density on glass coverslips and grown for 10 to 11 days in medium with 10% FBS. Cultures were rinsed in PBS and then fixed in 3% paraformaldehyde in PBS for 30 minutes at 4°C. Coverslips were preincubated for 4 hours in PBS with 10% calf serum and 1% normal goat serum and then incubated for 2 hours at ambient temperature with a rabbit polyclonal antibody against recombinant bovine tropoelastin (1:200), which also recognizes rat elastin (a kind gift of Dr Robert Mecham, Washington University, St. Louis, Mo). After rinsing with PBS, cells were incubated with TRITC-tagged goat anti-rabbit antiserum (1:300, Jackson Immunoresearch Laboratories, Inc) for 1 hour, rinsed again in PBS, and mounted in Gel-Mount (Biomedca Corp). Fluorescent-tagged antibodies were localized by a fluorescent microscopy-equipped Zeiss photomicroscope II and digital micrographs were captured during 0.5- to 2-second exposures with a Nikon Coolscan digital camera.

Northern Analysis of V3 and Tropoelastin

RNA was isolated and Northern analysis was performed as previously described. The rat V3 sequence, rVe, was excised from the pBSM13+ vector using Xhol and EcoRI and used as a DNA probe. To confirm the direction of insertion of the V3 sequence in rat cells transduced with LV3SN, an identical blot was probed with an antisense probe. Antisense RNA probe was prepared by linearizing the rVe plasmid with Xhol and transcribing in vitro with T3 RNA polymerase, in the presence of α-[32P]UTP, using a kit from Ampliscribe. Tropoelastin mRNA was detected with a human probe generously provided by Dr C.D. Boyd (University of Hawaii, Manoa, Honolulu, Hawaii). Blots were hybridized, washed 3 times for 5 minutes in 2×SSPE 0.1% SDS at room temperature, twice for 15 minutes in 0.3×SSPE 0.1% SDS, and twice for 15 minutes in 0.1×SSPE 0.1% SDS. SDS was washed from the blots and single stranded probe was digested from the blots with 40 μg/mL RNase A in (in mmol/L) 300 NaCl, 10 Tris, 5 EDTA, pH 7.4, for 30 minutes and washed with 0.1×SSPE 0.1% SDS for 15 minutes.

Balloon Injury and Cell Seeding

Balloon injury and cell seeding in Fisher 344 rats were performed as described previously in studies examining the effects of retrovirally introduced genes, . The distal half of the left common carotid artery was surgically exposed and isolated and a 2F balloon catheter introduced through an arteriotomy in the external branch. The balloon was passaged 3 times through the left common carotid artery in order to remove the endothelium. The transduced ASMCs were trypsinized and approximately 1×10⁶ of either LXSN or LV3SN ASMCs in 0.04 mL of culture medium infused into the isolated carotid segment. Three animals received LXSN cells and 6 animals the LV3SN cells. The seeded ASMCs were allowed to adhere for 15 minutes. Subsequently, the catheter was removed, the external carotid ligated, blood flow restored, and the wound closed.

Animals were maintained on a normal diet for 4 weeks and the animals euthanized with an overdose of pentobarbital. All surgical procedures were performed according to the Principles of Laboratory Animal Care and the Guild for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 86-23, revised 1985).

Reverse Transcriptase–PCR for Vector and Endogenous V3

RNA was isolated from frozen and homogenized pooled carotids by the method of Chomczynski and Sacchi. One microgram of total RNA was reverse transcribed and amplified using random hexamer primers and the Superscript Preamplification System Kit (Gibco/ BRL). To detect LV3SN sequences, the forward primer 5'-AGTGCCAAAAGACCTCC-3' in the LXSN vector sequences and the reverse primer (5'-GGTTGAAAATGCAAGGTAGG-3') in the versican immunoglobulin-like domain were used. To amplify all known versican splice variants, a forward primer in the lectin-like domain (5'-GACATGGCTGGCCACCA-3') and a reverse primer (5'-GTCCTTGGATGTCAGA-3') in the “tail”, which follows the complement regulatory-like domain, were used. The same reverse primer was used with a primer (5'-CTTTGGACAGTGGCA-TTAGC-3') in the second proteoglycan tandem repeat for the specific detection of the V3 isoform. For the detection of LV3SN sequences and total versican sequences, 35 PCR cycles were performed.
Sections from paraffin-embedded carotids were stained with H&E, Masson's Trichrome, and orcein to show general morphology, collagen, and elastin, respectively.

Electron Microscopy and Morphometrics
Confluent 3 week old cultures of LXSN- and LV3SN-transduced ASMCs were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, secondarily fixed in 1% OsO4, processed, and sectioned at right angles to the vessel axis. Thin sections of cells mounted on formvar-coated grids and vessels were stained with uranyl acetate and lead citrate and viewed on a JEOL 1200 EXII microscope.

Volume fractions for cell and matrix components of the cultured material and the carotids were determined by point counting. A transparent point-counting grid (100 points) was overlaid on each micrograph and mean volume fractions calculated for individual components.

Statistics
Data were analyzed by Student’s t test and a value of \( P < 0.05 \) taken as significant.

Results

Morphology of Long-Term Cultures
Dense multilayered long-term (3 week) cultures of LXSN and LV3SN ASMCs retained their respective distinctive morphological features seen in short-term cultures and reported previously.11 Compared with vector-alone cells, V3 cells were more spread, flatter, and less spindle-shaped (Figure 1). The most notable difference, however, and one not seen in short-term cultures, was the presence of an irregular network of fibers, especially prominent around the periphery of cells adherent to the culture dish (Figure 1). Immunocytochemistry of cultured cells showed the fibers to be tropoelastin positive with fibers forming in the LV3SN cultures by 10 days (Figure 1, bottom panels). Staining in the LXSN cultures was light with some patches of tropoelastin-positive material.

Electron microscopy of these cultures also demonstrated that the network was made of elastic fibers (Figure 2, middle panel) deposited in the extracellular compartment between the layered cells and with especially prominent deposits between the substratum and the adjacent cells. Notably, many of the elastin deposits were closely associated with cell surfaces (Figure 2, lower panel). The vector-alone cultures also contained elastic fibers but these were smaller and fewer in number (Figure 2, top panel).

These subjective assessments on elastin content were confirmed by morphometric analysis (Figure 3). Volume fractions, determined by point counting, for cells, matrix, matrix space, and elastin, showed that the primary difference in components between the vector-alone and the V3 cultures was the fraction occupied by elastin. Analysis of the T2 and the T3 lines separately (Figure 3b) showed that the V3 cells of both lines had a significantly higher volume fraction than their corresponding vector-alone control. It was also noted that the vector-alone T3 cell line had a higher volume fraction of elastin than the T2 line.

V3 Expression and Tropoelastin mRNA Levels

As previously shown,11 V3-transduced ASMC lines expressed V3 mRNA. Expression of V3 in 3 representative cell lines is shown in Figure 4 (top panel) with strong expression in 2 of the
lines (T2 and T3). All vector-alone LXSN lines, established from the same cell pools as their corresponding LV3SN-transduced line, were negative for V3 expression. The expression of the V3 mRNA message in the sense direction was confirmed by hybridization to antisense V3 RNA probe, which also hybridized to the V1 mRNA message (data not shown).

Northern blots of mRNA prepared from confluent cultures of LXSN and LV3SN were probed for tropoelastin (Figure 5). The vector-alone control cells showed small but variable amounts of tropoelastin mRNA. In contrast, V3-transduced cells showed marked upregulation. Importantly, this increased expression of tropoelastin expression was found in mRNA isolated from confluent week-old cultures, which is before the elastin fiber network was visible by light microscopy, indicating early upregulation of mRNA for tropoelastin.

V3 Expression In Vivo

To determine whether carotids seeded with LV3SN-transduced cells expressed V3 mRNA, RNA was isolated and subjected to nonquantitative reverse transcriptase-PCR analysis (Figure 5). When amplified with a primer in the retroviral sequence and a primer in the V3 sequence, a band of the appropriate size was amplified from the LV3SN plasmid (Figure 5A, lane 3) and from RNA isolated from carotids

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**Figure 3.** a, Volume fractions, determined from electron micrographs, for cells, matrix (including collagen), matrix space (areas devoid of staining), and elastin in 3-week cultures of LXSN and V3 ASMCs. Values are means and standard errors for transductions 2 and 3 combined. b, LXSN and LV3SN volume fractions of elastin for transductions 2 (T2) and 3 (T3) graphed separately and combined. The numbers above the standard errors are the numbers of electron micrographs analyzed by point counting. Note the higher volume fraction for T3 LXSN compared with T2 LXSN cells (see Figure 5).

**Figure 4.** Northern blots of mRNA from vector-alone (LXSN)– and V3 (LVSN)-transduced ASMC lines T1, T2, and T3 probed for V3 expression, with a versican sequence recognizing the recombinant V3 (V3), and for rat tropoelastin. The 24-hour exposure period shows clear upregulation of tropoelastin in V3 cells of the T1 and T2 compared with their respective LXSN controls. The 2-hour exposure period shows the increased expression of tropoelastin in V3 cells compared with their respective LXSN controls, which had a higher level of expression than the T1 and T2 control lines, a feature consistent with the higher volume fraction of elastin in T3 LX cultures (see Figure 4). Bottom panel shows loading densities.

**Figure 5.** Analysis of versican expression in carotids seeded with ASMCs transduced with retrovirus. Carotids were removed and RNA isolated, reverse transcribed, and amplified by PCR. A, Lanes 1 to 3, amplified with primers designed to detect LV3SN plasmid; lanes 4 to 6, amplified with primers to detect all versican isoforms; lanes 1 and 5, carotids seeded with LXSN control-transduced ASMCs; lanes 2 and 6, carotids seeded with LV3SN-transduced ASMCs; lane 4, uninjured and unseeded contralateral carotids from LXSN animals; and lane 3, PCR amplification of the LV3SN plasmid DNA. B, Amplification with primers designed to recognize the V3 isoform only. Lane 1, control reverse transcription without RNA; lane 2, uninjured contralateral carotids from LXSN animal; lane 3, carotids seeded with LXSN control-transduced ASMCs; and lane 4, carotids seeded with LV3SN-transduced ASMCs.
seeded with LV3SN-transduced ASMCs (Figure 5A, lane 2), but not from carotids seeded with the control vector (Figure 5A, lane 1). Amplification with a pair of primers designed to recognize all versican isoforms showed that versican is expressed by both LV3SN- and LXSN-seeded carotids. For each animal, only 1 of the carotids was seeded with ASMCs. RNA isolated from the uninjured, unseeded, contralateral carotid of the LXSN animals also expressed versican RNA (Figure 5A, lane 4). A pair of primers designed to amplify the V3 isoform specifically showed that endogenous V3 mRNA is present in LV3SN-seeded, LXSN-seeded, and control uninjured, unseeded rat carotid.

**Neointima Formed From V3-Transduced ASMCs**

Neointima formed from V3 cells seeded into ballooned rat carotid arteries differed significantly from neointima formed by vector-alone cells (Figure 6). Control neointima was characterized by typical stellate or rounded ASMCs (Figures 6a and 6c) embedded in a myxoid matrix containing mostly randomly oriented bundles of collagen, proteoglycans, and elastin (arrow heads). In contrast, V3 ASMCs are elongated and sandwiched between layers of dense extracellular matrix with prominent deposits of elastin (arrow heads), often organized into lamellae (b). Magnifications: a and b ×1500; c and d ×2500; e and f ×7500.

Electron microscopy further demonstrated differences between neointima formed from V3 and vector-alone cells (Figure 7). Compared with the control ASMCs (Figures 7a and 7c), V3 ASMCs (Figures 7b and 7d) were greatly attenuated and aligned in parallel arrays between a dense matrix of collagen and elastin. The elastin was often...
arranged in bi-laminar fibers between adjacent ASMCs (Figure 7d), a feature described previously for developing media.24 In some regions, the elastin was less organized and was arranged as bundles of fibers (Figure 7f) similar to that seen for vector-alone neointima (Figure 7e). The total volume fraction of elastin, determined by point counting from electron micrographs, was significantly higher in neointima formed by V3-transduced cells (Figure 8).

Discussion
The results presented in this study show that overexpression of the small versican variant V3 by rat ASMCs causes significantly increased expression of tropoelastin and increased formation of elastic fibers in vitro and in vivo. V3-transduced cells seeded into balloon-injured arteries produced a compact and highly structured neointima enriched in elastin. These results highlight the importance of different ECM molecules in dictating the organization and remodeling of the complex mixture of matrix components that make up the blood vessel wall.

The basis of the V3-induced changes in smooth muscle cell phenotype and in vessel wall structure is not understood, and the mechanism by which V3 promotes elastin is open to speculation. Several studies and observations, however, point to an important relationship between the larger parent chondroitin sulfate containing versican (V1) and elastin production and assembly. Neonatal ASMCs, which express little or no chondroitin sulfate-containing versican,25 have been shown to express high levels of tropoelastin26 and to form elastic fibers and lamellae in long-term cultures.27 Conversely, elastic fibers are depleted in tissues where versican or chondroitin sulfate levels are elevated, such as in the restenotic lesions28 or in the intimal cushions of the ductus arteriosus.13

The basis for this reciprocal relationship is not clear but may involve different forms of versican and their role in interference with assembly of elastic fibers at the cell surface. Interestingly, the chondroitin sulfate-rich GAG side chains of versican appear to be implicated in this process, and their absence in V3 may be of particular significance in the enhanced elastogenesis seen in this study. Notably, addition of exogenous chondroitin sulfate to cultures has been shown to displace a 67-kDa elastin binding protein (EBP) that mediates assembly of tropoelastin into mature elastic fibers.13 This chondroitin sulfate-induced shedding of the EBP results in impaired elastic fiber assembly and also promotes detachment and migration of cells.29 Recently, Hinek et al30 reported that cultures of skin fibroblasts from patients with Costello syndrome show excess accumulation of chondroitin sulfate-bearing proteoglycans, an associated loss of EBP, and impaired production of elastic fibers. Treatment with chondroitin ABC lyase restored normal production of elastic fibers. It will be important to determine if V3-producing cells have altered EBP patterns.

Thus, a working hypothesis is that V3 promotes elastogenesis through competitive removal of V1, with its constituent chondroitin sulfate chains, from the pericellular coat, thus counteracting the effects of high concentrations of galactosugar-bearing moieties at or near the cell surface. In our previous study,11 we reported on the reduced pericellular coat in cultured V3-transduced ASMCs and hypothesized that this phenotypic feature might arise through properties associated with the G1 domain of V3 that contains the HA-binding region. The larger versican variants V0 and V1, with their attached chondroitin sulfate chains, bind to HA to form pericellular coats that are antiadhesive and necessary for both proliferation and migration.12 Thus, we reasoned that V3, through the G1 domain, may compete with and displace the larger antiadhesive versican isoforms in the pericellular coats, thereby increasing adhesion and decreasing proliferation and migration.

V3-induced elastogenesis, however, could also be due to specific interactions of V3 with elastic fiber components. For example, ligands for the lectin-like domain of G3 may be particularly important. One of those ligands, fibulin-1, is a component of elastic fibers and binds to the lectin-like domain of G3 of both aggrecan and versican.4,31 In vivo, versican is closely associated with elastic networks.32 Our recent work has shown that overexpressed V3 is secreted by ASMCs and deposited in the extracellular matrix in close association with elastin (M.G. Kinsella, K.R. Braun, M.J. Merrilees, T.N. Wight, unpublished data, 2001).

The link between ASMC phenotype and elastin production is further strengthened by studies on mice lacking elastin.33 Disruption of elastin synthesis by targeting the promoter and exon 1 of the elastin gene induces subendothelial proliferation of ASMCs followed by obstructive disease and eventual obliteration of the lumen. Thus, although chondroitin sulfate chains of proteoglycans may affect the formation of elastic fibers, elastin in turn may also have a regulatory function, controlling ASMC proliferation and stabilizing arterial structure. Our findings of an association between a highly structured neointima and increased elastic fiber deposition support this view.
Finally, the creation of an elastin-rich and highly structured neointima from seeded V3-transduced ASCs is a novel finding that not only has implications for understanding atherogenesis but for other diseases where there is impaired elastic fiber formation. A number of studies have shown that tropoelastin synthesis can be upregulated in tissues. Of particular relevance to the present study, tropoelastin mRNA levels are markedly increased in neointima formed after balloon injury.\textsuperscript{34,35} Elastin also accumulates in this injury model, but in contrast to our findings for neointima formed by V3-overexpressing ASCs, these deposits are not organized as defined elastic fibers. Further studies on the mechanisms by which V3 promotes a structured neointima may offer a novel therapy for remodeling tissues where there is a deficiency of elastin.

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