Neointima Formed by Arterial Smooth Muscle Cells Expressing Versican Variant V3 Is Resistant to Lipid and Macrophage Accumulation

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Objective—Extracellular matrix (ECM) of neointima formed following angioplasty contains elevated levels of versican, loosely arranged collagen, and fragmented deposits of elastin, features associated with lipid and macrophage accumulation. ECM with a low versican content, compact structure, and increased elastic fiber content can be achieved by expression of versican variant V3, which lacks chondroitin sulfate glycosaminoglycans. We hypothesized that V3-expressing arterial smooth muscle cells (ASMC) can be used to form a neointima resistant to lipid and macrophage accumulation associated with hypercholesterolemia.

Methods and Results—ASMC transduced with V3 cDNA were seeded into ballooned rabbit carotid arteries, and animals were fed a chow diet for 4 weeks, followed by a cholesterol-enriched diet for 4 weeks, achieving plasma cholesterol levels of 20 to 25 mmol/L. V3 neointimae at 8 weeks were compact, multilayered, and elastin enriched. They were significantly thinner (57%) than control neointimae; contained significantly more elastin (118%), less collagen (22%), and less lipid (76%); and showed significantly reduced macrophage infiltration (85%). Mechanistic studies demonstrated that oxidized low-density lipoprotein stimulated the formation of this monocyte-binding ECM, which was inhibited in the presence of V3 expressing ASMC.

Conclusion—These results demonstrate that expression of V3 in vessel wall creates an elastin-rich neointimal matrix that in the presence of hyperlipidemia is resistant to lipid deposition and macrophage accumulation. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: lipids ■ macrophages ■ elastin ■ hypercholesterolemia ■ versican

Neointima that develops following angioplasty is characterized by extracellular matrix (ECM) containing elevated levels of the chondroitin sulfate proteoglycan (CSPG) versican, loosely arranged collagen, and scattered deposits of elastin, features that predispose to lipid and macrophage accumulation.1,2 In both humans and animal models of atherosclerosis, versican is a prominent ECM component that accumulates in the arterial-intima and plays important roles in atherogenesis, including trapping of low-density lipoproteins and promotion of inflammation.3 The chondroitin sulfate (CS)–rich glycosaminoglycan chains of versican, which are present in the 3 principal variants (V0, V1, and V2), are integral to these roles. For example, the negatively charged CS glycosaminoglycans bind cationic-rich sequences of apolipoprotein B-100 of low-density lipoprotein4 which leads to monocyte ingress, differentiation to macrophages, uptake of lipid-proteoglycan (PG) complexes, and formation of foam cells.5,6 Recent evidence indicates that versican also plays a direct role in the inflammatory response. V1 stimulates expression of the proinflammatory cytokines tumor necrosis factor-α and interleukin-6 by macrophages through Toll-like receptor family members.7 In addition, in the Athsq1 mouse model of atherosclerosis, which exhibits large macrophage-rich lesions, versican accumulation is mediated by macrophages.8 Versican also mediates leukocyte aggregation through binding to P-selectin glycoprotein ligand-1,9 and more recently it has been demonstrated that versican plays a critical role in hyaluronan-dependent binding of monocytes to ECM.10,11 Accumulation of versican in the cardiovascular system, such as in neointimae formed following angioplasty12 and CS accumulation in patients with the genetic diseases Costello syndrome and Hurler disease,13 is associated with impaired elastogenesis and aberrant remodeling of arterial walls. Chondroitin sulfate glycosaminoglycans interfere with the assem-
by tropoelastin into elastic fibers. Notably, however, the versican variant V3, which lacks CS, does not interfere with elastic fiber assembly; rather, when overexpressed, it markedly enhances tropoelastin expression and its assembly into elastic fibers, both in vitro and in vivo.

Collectively, these data predict that expression of V3 by arterial smooth muscle cells (ASMC) could potentially have several beneficial effects on vessel wall, such as increased elastin content, reduced accumulation of atherogenic lipids, and, importantly, a reduced macrophage-mediated inflammatory response. We provide evidence in support of this hypothesis, using an established angioplasty model and a hypercholesterolemic stimulus, and demonstrate that expression of V3 provides a novel approach for inhibiting atherogenic changes.

Methods
A full description of methods can be found in the Supplemental Material (available online at http://atvb.ahajournals.org).

Cell Culture
Primary ASMC were obtained from rabbit aortae by explant outgrowth and cultured as previously described. Cells were used between passages 3 and 10.

Retroviral Transduction and In Vitro Analyses
Full-length sense rat V3 cDNA was inserted into the BamHI site of the empty retroviral vector (LXSN) (courtesy of D.A. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) to produce the V3-containing vector (LV3SN) using methodology described previously. Transduction was confirmed by in situ hybridization using a tagged DNA primer, Texas Red–X-TCC CGC TTC AGT GAC AAC GTC GAG CAC AGC (Invitrogen), to detect mRNA for the selectable marker neomycin phosphotransferase II. Induction of the AAC GTC GAG CAC AGC (Invitrogen), to detect mRNA for the

Experimental Protocol and Cholesterol Feeding
All animals were fed a normal chow diet for 4 weeks to allow them to develop a neointima in the absence of cholesterol feeding. At the beginning of week 5, the normal chow was replaced with cholesterol-coated pellets, 0.15% by weight, increased to 0.3% by weight at the beginning of week 7. Blood samples were taken in weeks 4, 5, 7, and at the end of week 8 for total cholesterol analysis by LabPLUS, Auckland Hospital, Auckland, New Zealand.

Histochemistry
Ten-micrometer frozen tissue sections, cut from O.C.T.-mounted fixed vessel segments, were stained with Oil Red O for lipid and neutral triglycerides and with orcein for elastin. Oil Red O provided the necessary depth of color and definition for morphometric analysis. Thin (1 μm) sections cut from epon-embedded vessel segments were stained in 1% toluidine blue.

Immunodetection of CSPG and Macrophages
CSPG and macrophages were detected in paraformaldehyde-fixed sections using mouse monoclonal antibody 9BA12 (DSHB, University of Iowa) and mouse anti-rabbit RAM11 (Dako) respectively. RAM11 recognizes all tissue macrophages in rabbits, particularly those residing in vascular wall in animals on both normal and hypercholesterolemic (Western) diets and does not cross-react with human, rat or monkey macrophages.

Morphometric Analyses
Vessel parameters and lipid and macrophage content were determined by morphometric analysis of multiple images using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/).

Electron Microscopy
Structure and organization of neointima were assessed by electron microscopy. Morphometric analysis of multiple images was used to determine changes in content and distribution of constituent extracellular components of elastin, collagen, and matrix, as previously described. Morphometric analysis of multiple images was used to determine changes in content and distribution of constituent extracellular components of elastin, collagen, and matrix, as previously described. Morphometric analysis of multiple images was used to determine changes in content and distribution of constituent extracellular components of elastin, collagen, and matrix, as previously described.

Statistics
Data were analyzed by 2-tailed Student t test. A value of P<0.05 was taken as significant. Data are presented as mean±SEM.

Results
Plasma Cholesterol Levels
Following balloon angioplasty and cell seeding, all animals were placed on a regular chow diet for 4 weeks. Mean total plasma cholesterol level for all animals in week 4 was 1.8 mmol/L, with no difference between vector-alone and V3 animals (Figure 1). All animals were placed on a 0.15% cholesterol diet for weeks 5 and 6 and a 0.3% cholesterol diet for weeks 7 and 8. Final cholesterol levels were 20 to 25 mmol/L, with no significant difference between groups (Figure 1).

Neointimal Structure and Composition
Neointimae of carotids seeded with V3-expressing ASMC were significantly thinner than carotids seeded with vector-alone ASMC (57%) (P<0.03) (Figure 2A). Medial widths were not significantly different, and neointimal-medial width ratios reflected the thinner neointimae of the V3 animals (data not shown). The ECM of control neointimae showed a typical myxoid structure, characteristic of neointimae that develop following balloononing, of loosely arranged collagen bundles and
scattered deposits of elastin, usually more prominent in the mid to deep musculoelastic layer than the subendothelial zone (Figures 2B and 3A). In contrast, the ECM of V3 neointimae was significantly more compact with a layered structure, reflected by circumferentially arranged multiple bands of elastin between elongated ASMC (Figures 2C and 3D). These bands of elastin were strongly autofluorescent (Figure 2E), indicative of cross-links and maturation \(^{30}\) and were especially prominent in the subendothelial zone. On the other hand, the scattered small elastin deposits in the neointimae of the controls showed minimal autofluorescence and only in the mid to deep neointimal layers (Figure 2D; yellow fluorescence in Figure 2D is lipid) \(^{31}\). To evaluate CSPG content, sections were stained with 9BA12, which specifically recognizes CS. Control neointimae stained strongly with 9BA12, notably in the lipid and macrophage-rich areas (Figure 2F). In contrast, compact V3 neointimae stained lightly or not at all for CS (Figure 2G).

Electron microscopy of control neointima revealed small scattered deposits of elastin and small-diameter, randomly oriented collagen fibrils in the subendothelial zone (Figure 3B) and more prominent but discrete deposits of elastin in an open matrix containing bundles of woven collagen fibrils in the midintimal (Figure 3C) and deeper regions. On the other hand, neointimae of V3-seeded vessels contained prominent, multiple, circumferentially arranged bands of elastin throughout the full width of neointimae in a matrix with reduced collagen and between elongated, differentiated ASMC (Figure 3E and 3F). The bands of elastin between adjacent ASMC were frequently bilamina (Figure 3F and 3G), indicative of a dual origin from opposing parallel ASMC, a feature reported previously for formation of medial elastic lamellae \(^{32}\).

Morphometric analysis of electron micrographs confirmed that the ECM compartment of V3 neointimae contained a significantly increased content of elastin (118%, \(P<0.01\)) and small but significant reductions of collagen (22%, \(P<0.05\)) and matrix space (20%, \(P<0.02\)) compared with control neointima (Figure 3I). Analysis of the neointimal subregions showed further differences in the content of elastin (Figure 3J). In vector control neointimae, elastin content was higher in the mid and deeper zones compared with the subendothelial zone. In contrast, the elastin content of V3 neointimae was higher overall and similar across all zones of the neointimae, with the subendothelial region showing the largest increase in elastin (196% increase, \(P<0.02\)) (Figure 3J). Analysis of total neointima showed that cell and ECM volumes fractions were not significantly different between the 2 groups, although there was a trend toward reduced ECM in the V3 neointimae (Figure 3H), indicating that the increase in elastin largely compensated for the decrease in collagen and matrix space.

**Lipid Accumulation**

Lipid accumulation was assessed by morphometric analysis of Oil Red O–stained sections. V3 neointimae had a significantly reduced total lipid content (80% reduction, \(P<0.03\)) and a reduced lipid content expressed as a percentage of...
neointimal area (76% reduction, \(P<0.01\)) compared with control neointimae, as determined by morphometric analysis of Oil Red O staining (Figure 4A and 4B). Thickened vector control neointimae often contained extensive deposits of lipid (Figure 4C and 4D). Some control neointimae were minimally thickened but also positive for lipid (Supplemental Figure 1VC). Overall, lipid occupied 20% of neointimal area of control vessels. In contrast, lipid occupied 5% of neointimal area of V3-seeded vessels, with the thin neointimae frequently completely devoid of lipid (Figure 4E and 4F). Lipid deposits, where present, were generally more diffuse and often restricted to small focal lesions and with the staining predominantly subendothelial in location. (For additional images see Supplemental Figure IV.)

**Macrophage Accumulation**

Macrophage accumulation was assessed by morphometric analysis of RAM11 immunostaining. Control neointimae stained strongly for RAM11-positive macrophages, which were largely distributed and colocated to the lipid-positive regions, and notably in the subendothelial zone (Figure 5A to 5C). Macrophages were also present in the deep neointima and less frequently in the inner media (Figure 5C). Lipid-negative V3 neointimae did not stain for macrophages (Fig-
ure 5D to 5F). In some V3 vessels or vessel segments, small, discrete, raised subendothelial lesions contained accumulation of macrophages (Figure 5G and 5H). Rarely were macrophages seen in the media. (For additional images, see Supplemental Figure V.) Morphometric analysis confirmed the reduced accumulation of macrophages in the neointima and media of V3 compared with control animals (85% reduction, \( P<0.008 \)) (Figure 5I).

**In Vitro Mechanistic Studies**

There are a number of different mechanisms that may explain the in vivo observations. First, we addressed whether exposure of ASMC to modified low-density lipoprotein in the form of oxLDL stimulates formation of an ECM that binds monocytes in a hyaluronan-dependent manner. Previous studies in our laboratory have shown that oxLDL alters production of PGs by ASMC.29 Because hypercholesterolemia was the in vivo stimulus used in the current study, we asked whether lipids induce ASMC to produce an ECM that binds monocytes. We treated the ASMC with oxLDL, or, as a positive control, tunicamycin, which is known to promote the formation of an ECM by ASMC that binds monocytes in a hyaluronan-dependent manner,33 and we found that oxLDL promoted formation of an ECM that bound monocytes in a hyaluronan-dependent manner (Figure 6A). V3-generated ECM, however, was significantly less adherent for human monocytes when stimulated with either oxLDL or tunicamycin (\( P<0.005 \)) (Figure 6A).

To determine whether the elastin-rich ECM generated by V3-expressing ASMC resists monocyte adhesion, we compared the ability of collagen versus elastin to support monocyte adhesion using an in vitro assay. Artificial matrices containing elastin were significantly less adherent for monocytes compared with gels containing collagen (\( P<0.01 \); Figure 6B). These data, coupled to our in vivo observations in this current study, support a protective role for elastic fibers as antiinflammatory elements in the ECM.

**Discussion**

Transduction and seeding of ASMC expressing versican variant V3 into ballooned rabbit carotid arteries resulted in formation of highly structured and elastin-rich neointimae resistant to lipid deposition and to macrophage ingress. Our previous investigations in the rat similarly demonstrated that V3-expressing ASMC form structurally compact neointimae with multiple layers of elastin.16 This present study demonstrates that such neointimae have the significant additional advantage of resistance to lipid deposition and to inflammatory changes stimulated by hypercholesterolemia.
elastin compared with collagen. 

Figure 6. Monocyte adherence in vitro. A, Hyaluronidase-
sensitive monocyte binding to 21-day cultures of vector control 
and V3 ASM in untreated cultures, and cultures stimulated 
with 5 μg/mL tunicamycin or 5 μg/mL oxLDL for 20 hours, 
showing reduced binding to matrix generated by V3-expressing 
ASM. B, Monocyte binding to polyacrylamide gels treated with 
collagen and elastin, showing significantly reduced binding to 
elastin compared with collagen.

Our finding that oxLDL promotes formation of a matrix 
that binds monocytes in a hyaluronan-dependent manner is of 
particular interest. Previous studies have shown that agents 
that promote ER stress in ASM, such as tunicamycin and 
poly(I.C), stimulate the formation of a hyaluronan-dependent 
proadhesive ECM for monocytes. It is clear that oxLDL is 
capable of promoting ER stress in vascular cells, but 
whether this pathway is responsible for the ECM inductive 
effect of oxLDL in the present study is not yet certain. What 
is remarkable, however, is that V3 expressed by ASM 
reduces the capacity of monocytes to bind to the ECM 
induced by classical stimulators of ER stress, such as tunicamycin 
and proatherogenic oxLDL. Such findings highlight a 
potential therapeutic application for V3.

The inverse relationship between elastin distribution and 
regions of lipid deposition and inflammatory changes has 
been recognized previously and is reflected in the classification 
of human lesions. In the early stages of lesion development, 
intimal thickenings have 2 distinct zones, a subendothelial 
PG-rich zone, depleted in elastin and where lipid is 
first deposited and where monocytes first ingress, and a 
deeper lipid-free musculoelastic or musculofibrous zone where 
the elastin and collagen content is higher and PG 
content lower. These regional structural differences are present 
also in neointima induced by balloon catheter injury, 
including the rabbit in which synthetic ASM in the 
subendothelial zone synthesize and secrete higher amounts of 
matrix PGs than differentiated contractile ASM in the 
musculoelastic layer.

In addition to increased elastin content, V3 neointima in 
this study were also characterized by a reduced content of CS. 

Other studies have reported that V3 expression leads to 
reduced CSPG, including decreased accumulation of aggregan 
by chondrocytes. The importance of CSPGs for trapping 
of lipoproteins has been demonstrated experimentally, and it has been shown that susceptibility of coronary bypass 
vessels to atherosclerosis relates to CSPG content.

The enhanced formation of elastic fibers in V3 neointima is 
likely directly related to reduced versican. The precise 
signaling mechanism by which V3 overexpression induces 
formation of elastic fibers is not yet clear. Elastic fibers are 
assumed in cell surfaces and versican, as well as free CS 
chains, prevent fiber assembly by causing premature shedding 
of the elastin binding receptor (spliced variant of β-galactosidase) from the cell surface, preventing transfer of 
tropeoelastin to the microfibrillar scaffold. Notably, V3-expressing cells are characterized by reduced pericellular matrix, known to be enriched in versican. We postulate that reduction in cell surface versican permits a longer residence time for elastin binding receptor and enhanced assembly of elastin into fibers. Reduction of versican at the cell surface by overexpression of versican antisense similarly enhances elastogenesis in neointima. Notably, subendothelial ASM of normal neointima actively synthesize and secrete tropoelastin, but assembly into elastic fibers does not take place in this region of high versican content.

Versican also forms hydrated matrices that favor cell 
proliferation. Moreover, a reciprocal relationship between 
heightened cellular proliferation and lack of mature elastic 
fiber deposition has been documented in cultures of ASM and fibroblasts derived from patients with supravalvular 
aortic stenosis. This relationship has also been noted in Williams-Beuren syndrome, where there is haploinsufficiency 
of the elastin gene, and in elastin transgenic mice. 

Thus, reducing CS and maintaining existing elastic fibers, as well as stimulating formation of new fibers in a CS-depleted environment, may be an effective strategy for preventing cell proliferation and restenosis associated with angioplasty. Our finding of reduced levels of immunodeectable CS and the absence of lipid in the neointima of the V3 hypercholesterolemic rabbits suggests that V3-mediated reduction of CS in the vessel wall may constitute an effective strategy for preventing development of atherosclerotic lesions.

This study demonstrates that expression of V3 produces a 
stable vessel wall that exhibits antiinflammatory properties. 
Our finding of reduced monocyte adherence to matrix 
generated by V3 ASM, and to elastin-containing gels compared 
with collagen gels, indicates a possible mechanism that 
explains these antiinflammatory properties. In vessel wall, 
intact elastic fibers are considered to reduce ongoing invasion 
of monocytes that normally respond chemotactically to elastin 
degradation products resulting from elastolysis by macrophage-derived matrix metalloproteinases. Decellularized 
arterial elastic lamellae prevent monocyte adhesion and 
transmigration by activating signal regulatory protein α and Src homology 2 domain containing protein-tyrosine phosphatase-1 in monocytes. Furthermore, ASM phenotype is affected by the mechanical properties and rigidity of the ECM, which effects cell adhesion, migration, and proliferation. Lipid content, however, may also affect inflam-
matory changes. Observations on early human atherosclerosis show that lipid accumulation consistently precedes macrophage ingress, and the reduced ingress of macrophage in the V3 animals is consistent with this model of matrix-driven lipid influence on macrophage ingress and accumulation. Notably, in our in vitro assay, monocyte adherence to matrix was significantly reduced in V3 cell cultures stimulated by oxLDL, indicating that V3 matrix has antiinflammatory properties under hypercholesterolemic conditions.

The structural changes seen in V3 neointima, particularly as revealed by electron microscopy, demonstrate a major and significant change in the balance and organization of ECM components, to an extent not previously reported. The formation of multiple layers of elastin throughout the full width of V3 neointima, including the subendothelial zone, where lipid deposition and inflammatory changes normally first occur, demonstrates that overexpression of V3 effects a major structural reorganization. Although a number of mechanisms may be responsible for the V3 effects, the capacity for V3 to enrich the ECM with elastin and to stimulate a matrix with a reduced CSPG content and reduced adherence for monocytes are beneficial effects that can be used to engineer a neointima resistant to early lipid deposition and macrophage infiltration. We note also that the ability of V3 to confer improved structural characteristics and stability to vessels could have wider application for the repair of damaged and aneurysmal vessels or aortic dissection.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Methods

Cell Culture. Primary ASMC were obtained from rabbit aortae by explant outgrowth and cultured as previously described\(^1\) in DME-high glucose medium (Irvine Scientific, No. 9024) supplemented with 10% FBS (Atlantic Biologicals, Catalogue No. S11150), sodium pyruvate (IS, No. 9334), nonessential amino acids (IS, No. 9304), and glutamine pen-strep (IS, No. 9316). Cells were used between passages 3 and 10.

Retroviral Transduction. Full-length sense rat V3 cDNA was inserted into the BamHI site of the empty retroviral vector (LXSN) (courtesy of D.A. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) to produce the V3-containing vector (LV3SN) using methodology described previously.\(^2\) Orientation of the insert was confirmed by PCR and LXSN and LV3SN retroviruses, produced by transfection of PA317 packaging cells, used to transduce ASMC as previously described.\(^2\)

In situ Hybridization and In Vitro Analyses. V3-transduced cells were detected \textit{in vitro} and \textit{in vivo} by standard\(^3\) in situ hybridization procedures using a tagged DNA primer, Texas Red-X-TCC CGC TTC AGT GAC AAC GTC GAG CAC AGC (Invitrogen, CA), to detect mRNA for the selectable marker neomycin phosphotransferase II. Cells and tissue sections, fixed in 4% paraformaldehyde, were washed several times in PBS made up in DEPEC water (used for all solutions), incubated in 0.1% tween/triton X in PBS for 10 minutes, washed in PBS, prehybridized at room temperature for 1 hour in pre-hybridization buffer (5x Denhardt’s solution, 4xSSP, 50% formamide, 0.25% herring sperm DNA, and 10% dextran sulfate), hybridized overnight at 42°C in hybridization buffer containing the
tagged probe (0.5 pmoles/μl), washed at room temperature. Transduction of ASMC with the retroviral vector LXSN was confirmed by in situ hybridization of cultured cells for the selectable marker neomycin phosphotransferase II using a Texas red tagged DNA probe (Supplemental Figure IA,B). Consistent with previous studies, V3-transduced ASMC in culture displayed a flattened, elastogenic phenotype and produced increased amounts of immunostainable elastin and a 50% increase (P<0.001) in insoluble (cross-linked) elastin compared with empty vector controls (Supplemental Figure IC-F).

**Immunodetection of Elastin In Vitro.** Confluent 10-day cultures of vector-alone and V3 transduced cells were fixed for 10 minutes in 2% paraformaldehyde, washed 3x5 minutes in PBS, blocked for 10 minutes in FBS, incubated overnight at room temperature with mouse monoclonal BA-4 (Abcam) at 1:400, washed 3x5 minutes in PBS, incubated for 1 hour at room temperature with goat anti-mouse secondary antibody conjugated with Alexa Fluor 954 (Invitrogen, Ca) at 1:700, washed in PBS and mounted in Citifluor (Agar Scientific, Essex, England).

**Insoluble Elastin.** Insoluble elastin in cell layers of cultures was measured as previously described. Quadruple cultures of LXSN and LV3SN (plated at 5 x 10^5 cells/well) were grown to confluence in 6-well culture plates. Twenty μCi of [3H]-valine (New England Nuclear, Boston, MA) were added to each well at day 4, along with fresh media, and cells cultured for a further 3 days. At day 7, media were removed and cell layers scrapped in 0.1N NaOH, sedimented by centrifugation, and boiled in 0.5 ml of 0.1N NaOH for 45 minutes to solubilize all matrix components except elastin. The resulting pellets containing insoluble elastin were solubilized by boiling in 200μl of 5.7 N HCL for 1 hour. Aliquots were mixed with scintillation fluid and counted. Aliquots from each culture were also taken for DNA determination.
using DNeasy Tissue System from Qiagen. Results were normalised to DNA content and expressed as cpm/μg DNA.

**Balloon Catheter Injury and Cell Seeding.** Balloon injury and cell seeding experiments were approved by the University of Auckland Animal Ethics Committee (approval R435). Right common carotid arteries of young adult NZ white male rabbits (average weight 2.3 kg) were de-endothelialised using the method of Manderson et al.⁵ Anaesthesia was induced with propofol (Diprivan™) and maintained with isoflurane. Prior to surgery, animals received a subcutaneous injection of Baytril (5mg/kg). Following surgical exposure, the carotid was injured with three passes of a 2F balloon catheter introduced via a sub-branch of the salivary artery. Flow was halted using microclips and transduced ASMC (60 x 10⁶/ 0.1 ml of serum free growth medium) introduced via a syringe and flexible catheter and allowed to dwell for 15 minutes. Following seeding, the vessel was flushed with heparin in fresh culture medium to remove non-adherent cells, microclips removed, and flow re-established before closure of the wound. Animals were injected subcutaneously with Rimadyl (Carproten) to control post operative pain. At eight weeks, animals were euthanized with sodium pentobarbitone and right and left carotids exposed and perfusion-fixed for 10 minutes with 2.5% paraformaldehyde in PBS, administered via left ventricular outflow tract at a pressure of 100 mm Hg. Both carotid arteries were removed, with the left (uninjured contralateral) serving as an additional control, and post-fixed for a further 24 hours in 4% paraformaldehyde before storage in PBS. Nine rabbits were seeded with LXSN ASMC and nine with LV3SN ASMC. Initial histological analysis showed that 3 control and 2 V3-seeded vessels were completely occluded, with degenerate media; these vessels were excluded from subsequent analyses. The presence of seeded control and V3-transduced ASMC in 8-week neointimae was
confirmed by in situ hybridization on tissue sections using the same Texas red tagged DNA probe. Transduced cells were present in control (Supplemental Figure II A,C) and V3 neointimae (Supplemental Figure II B,D), dispersed among host cells, and restricted to neointimae, a pattern consistent with previous studies.⁶

**Experimental Protocol and Cholesterol Feeding.** All animals were fed a normal chow diet for four weeks, with blood samples taken in week four for total cholesterol analysis by LabPLUS, Auckland Hospital, Auckland. At the beginning of week five, the normal chow was replaced with cholesterol-coated pellets (0.15% by weight),⁷ prepared by immersing in cholesterol (Sigma C8503) dissolved in ether and allowing the ether to evaporate in a fume hood overnight, and blood samples for cholesterol analysis taken at the end of week five. At the beginning of week seven, the cholesterol content was increased to 0.3% and blood samples taken midway through week seven and at the end of week eight.

**Histochemistry.** *Oil Red O staining:* Fixed vessel segments were mounted on stubs with Tissue-tek® O.C.T. and frozen to -18°C. 10μm frozen sections were cut and collected on ‘Superfrost plus’ microscope slides (Biolab Scientific NZ) and air dried. Slides were washed for 1 minute in warm tap water then stained with 0.3% Oil Red O (Raymond A. Lamb, London) in 60% isopropyl alcohol for 10 minutes, washed briefly in 60% isopropyl alcohol, washed in tap water for 1 minute, then mounted in glycerine jelly and cover-slipped. *Orcein staining:* O.C.T.-mounted sections were washed in tap water, stained in 1% orcein (Sigma) in 70% acid alcohol for 15 minutes, dedifferentiated in 1% acid alcohol, washed in tap water and mounted in DEPEC. *Toluidine blue:* Epon embedded thin (1μm) sections were stained for 1 minute in 1% toluidine blue, rinsed in water, air dried and mounted in DEPEC.
Immunodetection of CSPG and Macrophages In Vivo. CSPG: Paraformaldehyde-fixed sections mounted in Tissue-tek® O.C.T. (Sakura, The Netherlands) were rinsed in PBS, blocked in peroxidase for 20 minutes, washed 5 x 2 minutes in PBS, incubated in 0.5% FBS for 10 minutes, incubated overnight at 37°C in the supernatant of primary mouse monoclonal antibody 9BA12 for chondroitin sulfate (DSHB, University of Iowa), washed 5 x 2 minutes in PBS, and incubated in secondary antibody polymer HRP (mouse, DAKO) for 30 minutes at 24°C. Following washing in PBS, and incubation in DAB for 7 minutes, sections were washed in tap water, counter stained in Gill’s hematoxylin, dehydrated, and mounted in DEPEX.

Macrophages: Paraformaldehyde-fixed sections mounted in Tissue-tek® O.C.T. were rinsed in PBS, blocked in 5% NGS 1% BSA in PBS for 1 hour at room temperature, incubated in primary antibody mouse anti-rabbit RAM11 (Dako) 1:100 in 0.1%BSA-PBS or 3 μg/ml Mouse IgG (negative control) overnight at 4°C, incubated in secondary biotinylated goat-anti-mouse for 1 hour at room temperature, followed by Vector ABC elite for 30 minutes at room temperature, and then chromagen Nova Red (Vector) for 10 minutes. Sections were washed in tap water, counterstained in Gill’s hematoxylin, and mounted in DEPEX.

Morphometric Analysis of Lipid and Macrophage Content. 10 Oil Red O stained sections from each animal, sampled at 1 mm intervals distal (5 sections) and proximal (5 sections) to the central 4 mm portion of each carotid (taken for electron microscopy), were analysed. Digital photographs, taken on a Leica DMR light microscope with a 4x objective lens, were analysed using public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). Original images were converted to gray scale, imported into NIH Image at a screen magnification of 145x, and lipid quantified by dividing the gray scale range of 256 into 10 bins and thresholding;
values exceeding 75 were recorded as positive staining for lipid. Intimal area was
determined and results expressed as % lipid/total neointimal area and as area (sq mm)
of lipid/neointima, with mean values calculated for each animal and a mean of means
(± SEM) for each group. Neointimal and medial depths (μm) were measured at four
random points on each section, determined by clock face positions of 12, 3, 6 and 9,
and mean values for animals and groups (± SEM) determined. Area of neointima and
media occupied by RAM11-positive macrophages was determined by point counting8
of immunostained sections in both the distal and proximal regions of each vessel of
animal. A minimum of four sections were analysed for each animal with four sites
sampled for each section, with sites determined by clock face positions, as done for
wall thicknesses.

**Electron Microscopy.** Central segments of 3 LXSN- and 3 LV3SN-seeded carotid
arteries, fixed in 4% paraformaldehyde, were post-fixed in 1% OsO₄, stained in 2%
uranyl acetate, and embedded in Epon. One micron sections were stained with
toluidine Blue. Ultrathin sections were examined on a JEOL 1200 EXII microscope.
For each animal, a minimum of 12 digital photographs were taken at a magnification
of 9,700x, with a minimum of 4 images in each of the subendothelial, mid-intimal and
deep-intimal regions. Volume fractions for cells, ECM, and constituent extracellular
components of elastin, collagen and matrix were determined by point counting8 prints
(magnification 15,000x) as previously described9 (see Supplemental Figure III). Mean
volume fractions (%) of components were calculated for total neointimae of each
group and separately for each of the three neointimal regions.

**Monocyte Adhesion Assays.** Monocyte adhesion to rabbit ASMC: Monocyte
adhesion assay was performed as previously described.10 Briefly, ASMC were seeded
at 1.7 X 10⁴/cm² on 96-well plates in DMEM with 10% FBS and cultured for 17 days
to allow elastin accumulation. ASMC were treated with SMGM-2 culture media (Lonza, Basel, Switzerland) for 48 hours growth-arrested by using DMEM with 0.1% FBS, and stimulated with 5μg/ml tunicamycin or 5μg/ml oxidized LDL in SMGM-2 media. Oxidized LDL was prepared as previously described. Human monocytic U937 cells labelled with calcein-AM (Invitrogen) were then added to SMC at 1 x 10^5/well and allowed to adhere for 30 minutes at 37°C. Some SMC were treated with Streptomyces hyaluronidase (0.66 U/ml) for 20–30 minutes before the adhesion assay. Plates were washed gently three times, and measured in a Fusion Series Universal Microplate Analyzer (Packard Bioscience Co., Meriden, CT), with excitation and detection wavelengths of 485 and 535 nm, respectively. *Monocyte adhesion on collagen- or elastin- conjugated polyacrylamide gels:* Polyacrylamide gels were prepared as previously described. Briefly, acrylamide (3% and 7.5%) and bis acrylamide (0.05 - 0.15%) were polymerized on activated 12mm coverslips using ammoniumpersulfate and N,N,N9,N9- tetramethylethlenediamine, as described previously. Polymerized gels were washed with 0.1M MES (2-(N-morpholino)ethanesulfonic acid, pH 6.0) three time and treated with 26mg/ml EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride, Pierce, Rockford, IL) and 0.6mg/ml N-hydroxysuccinimide in 0.1M MES acrylamide solution for 2 hours at room temperature. The gels were then washed with 0.1M MES three times and treated with 0.1 mg/ml rat tail collagen type I (BD Bioscience, Franklin Lakes, NJ) or soluble elastin from human aorta (Sigma Aldrich, St. Louis, MO) for overnight at 4°C. These gels were washed with 0.1M MES three times and immobilized on 24-well tissue culture plates with cyanoacrylate. Human monocytic U937 cells labelled with calcein-AM (Invitrogen) were then added to these gels at 1 x 10^5/well and
allowed to adhere for 90 minutes at 4°C. Adherent monocytes were quantified with a Fusion Microplate Analyzer as described above.

ASMC were maintained in Dulbecco’s modified Eagle medium (DMEM) high-glucose medium supplemented with 10% FBS (HyClone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.43 mg/ml GlutaMAX-1, and penicillin–streptomycin (penicillin G sodium, 100 U/ml, and streptomycin sulfate, 0.10 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) at 37°C in 5%CO₂.

**Statistics.** Data were analyzed by two tailed Student’s *t* test and where indicated by paired *t* test. A value of $P<0.05$ was taken as significant. Data are presented as mean ± SEM.

**References (Supplemental Material)**


proteoglycans that have enhanced native low density lipoprotein binding properties. *J Biol Chem.* 2000;275:4766-4773.


Supplemental Figure Legends

**Supplemental Figure I. Transduction and in vitro morphology, elastin immunostaining and production of insoluble elastin.** A, B, Fluorescent in situ hybridisation, with Texas Red-tagged DNA probe for selectable marker neomycin phosphotransferase II, showing viral incorporation in V3-transduced (B) compared with non-transduced (A) cultured ASMC (DAPI-stained). C and D, Cultured ASMC showing flattened and spread morphology of V3-transduced cells (V3) compared with spindle shaped vector-alone (vector) cells. E and F, Elastin immunostaining of 10-day ASMC cultures showing increased deposition of elastin (Alexa Fluor 954) in V3 cell layer compared with vector alone cells. Scale bars for all panels 20μm. G, Insoluble elastin, extracted from cell layers of vector-alone and V3 cultures labelled with [3H]-valine, showing increased deposition of elastin (mean ± SEM) in V3 cultures.

**Supplemental Figure II. Transduction and carotid seeding of ASMC.** A-D, Fluorescent in situ hybridization, with the Texas Red-tagged DNA probe, of 8-week neointimae formed after seeding ballooned carotids with retrovirally-transduced ASMC, showing presence of vector control (A and C) and V3 (B and D) transduced cells. Texas Red fluorescence is merged with autofluorescence (green) of elastin to show location of the internal elastic laminae (IEL) and neomycin-negative media below the IEL. Panels C and D show the boxed areas of panels A and B at higher magnification respectively. Low level of diffuse elastin autofluorescence surrounding neomycin-positive vector control cells (C) contrasts with strong autofluorescence between layered V3 cells (D), indicating presence of mature cross-linked elastin. Scale bars 20μm.
Supplemental Figure III. Point counting of electron micrographic images.

Electron micrographic images of the mid-intimal zone of vector control (LXSN) and V3 neointimae, each overlain with a 100-point grid. The ends of each of the fifty (white) lines provide the 100 points at which the underlying tissue components are sampled. Note that for the layered structure of components in the V3 neointima, the grid alignment of the grid is off-set to the alignment of the tissue structures which is necessary to obtain an accurate determination of volume %. Measurements were performed on printed images measuring 20 x 25cm (magnification of 15,000x).

Supplemental Figure IV. Oil Red O stained cross-sections of seeded carotid arteries. Additional (to panels C and E in Figure 4 of printed paper) representative cross-sectional images of carotid arteries from three animals seeded with vector-alone ASMC (A-C) and three animals with V3-expressing ASMC (D-E), perfusion fixed 8 weeks after seeding and following 4 weeks of the cholesterol supplemented diet, and stained with Oil Red O. Animals seeded with vector-alone ASMC show eccentric thickenings (A and B) and a range of lipid staining patterns including one vessel segment (C) with minimal neointimal thickening, but medial staining for lipid. Neointimae of V3-seeded vessels show thinner neointimal thickenings and minimal Oil Red O staining and where present (F) mostly sub-endothelial (arrow). Arrow heads indicate position of internal elastic lamina. Scale bar in panel A (200μm) applies to all panels.

Supplemental Figure V. Macrophage immunostaining (RAM11) of seeded carotids. Additional (to Figure 5 of print version) representative cross-sectional-
images of three animals seeded with vector-alone ASMC (A-C) and three animals with V3-expressing ASMC (D-E). Vector-alone vessels demonstrate the range of patterns of macrophage accumulations. These are: extensive subendothelial accumulations (arrow in A), predominance of deep neointimal accumulation (arrows in B), and focal subendothelial accumulations (C). V3 panels D and E show typical macrophage-free neointimae; panel F shows maximum macrophage staining seen in V3 animals. Arrow heads indicate position of internal elastic laminae. Scale bar in panel A Figure IV (20μm) applies to all panels.
Supplemental Figure I
Supplemental Figure II