Oxidative Stress and Lipid Retention in Vascular Grafts
Comparison Between Venous and Arterial Conduits

Yi Shi, MD, PhD; Sachin Patel, MD; Kelly L. Davenpeck, PhD; Rodica Niculescu, DVM; Evelio Rodriguez, MD; Michael G. Magno, PhD; Michael L. Ormont, MD; John D. Mannion, MD; Andrew Zalewski, MD

Background—Because saphenous vein grafts (SVGs) exhibit greater cellular heterogeneity and worse clinical outcomes than arterial grafts (AGs), we examined oxidative stress and lipid retention in different vascular conduits.

Methods and Results—In a porcine model of graft interposition into carotid artery, superoxide anion (O$_2^-$) was measured at 2 weeks after surgery. SVGs demonstrated increased O$_2^-$ production compared with AGs (SOD-inhibitable nitro blue tetrazolium reduction, $P<0.01$). The NAD(P)H oxidase inhibitor diphenyleneiodonium ($P<0.01$) abolished SVG-derived O$_2^-$, whereas the inhibitors of other pro-oxidant enzymes were ineffective. The change in oxidative stress was also reflected by lower activity of the endogenous antioxidant superoxide dismutase in SVGs than in AGs ($P<0.001$). SVG remodeling was associated with increased synthesis of sulfated glycosaminoglycans and augmented expression of a core protein, versican. These changes were accompanied by SVGs retaining significantly more $^{125}$I-labeled LDL than AGs ex vivo ($P<0.001$). In hyperlipemic animals, lipid accumulation and oxidized epitopes were preferentially noted in the intima of SVGs at 1 month after surgery.

Conclusions—This study demonstrated significant differences in the biology of SVGs and AGs. SVGs exhibited higher oxidative stress, LDL accumulation, and the presence of oxidized epitopes. These findings suggest that proatherogenic changes in SVGs may commence early after surgical revascularization. (Circulation. 2001;103:2408-2413.)

Key Words: atherosclerosis ■ bypass ■ grafting ■ remodeling

It is well recognized that saphenous vein grafts (SVGs) demonstrate lower patency rates than arterial grafts (AGs) in patients who undergo surgical coronary revascularization (reviewed by Motwani and Topol). Early attrition of SVGs has been attributed to thrombosis and rapid neointimal formation within the first year after surgery. After a period of clinical quiescence, the loss of SVG patency resumes because of graft atherosclerosis, manifested by occlusive lesions beginning at 3 to 5 years after revascularization. Neither better preoperative patient selection nor improved intraoperative handling of vascular conduits has been sufficient to eliminate the disparity between SVGs and AGs. Recent studies have increasingly focused on biological differences between venous and arterial conduits that may affect their response to surgery. In addition to structural differences (eg, less-developed elastic tissues), the cellular composition of veins is dissimilar to that of arteries. “Nonmuscle” fibroblasts, which are typically present in the adventitia of normal vessels, are common in the media of saphenous veins. These poorly differentiated cells are highly proliferative, and their population is further augmented by migration of adventitial and perivascular fibroblasts through the injured media. In contrast, smooth muscle cells (SMCs) of the arterial conduit exhibit less cellular activation, and adventitial fibroblasts are prevented from transmural migration by usually intact elastic tissues in AGs.

Oxidative stress is an important modulator of vascular cell functions. Increased generation of reactive oxygen species has been implicated in vascular cell proliferation, apoptosis, and the induction of transcriptional factors (eg, nuclear factor-$kB$), as well as oxidative modifications of retained lipoproteins. Under normal conditions, superoxide anion (O$_2^-$) is rapidly inactivated by superoxide dismutase (SOD) stored in the extracellular matrix of the tunica media. Interestingly, fibroblast-rich adventitia generates more O$_2^-$ than medial SMCs, although the importance of this phenomenon has not been fully explained. The differences in cellular composition between SVGs and AGs raise the possibility that the activation of fibroblasts or the loss of SOD activity results in the increase in oxidative stress in the media of SVGs. In this study, we demonstrate that SVGs and AGs exhibit dissimilar oxidative stress, lipoprotein accumulation, and oxidative modification of retained LDLs. These findings illustrate that early changes during SVG remodeling may...
contribute to their attrition because of accelerated atherogenesis.

Methods

Animal Model

A porcine model of a graft interposition in the common carotid artery was used as described. Briefly, saphenous veins were harvested without distension. Both carotid arteries were dissected free, an 2- to 3-cm section of the carotid artery was excised, and reversed vein interposition grafting was performed. The excised carotid artery was then grafted into the contralateral carotid artery. The animals were given aspirin 650 mg/d PO. At the times indicated, animals were euthanized and tissues harvested. A separate group of animals (n = 14) was placed on the atherogenic diet modified from Weiner et al17 at 1 week before surgery and continued until graft harvest. These animals demonstrated an increase in serum cholesterol from 79 \pm 6 to 250 mg/dL at baseline to >250 mg/dL within 3 to 5 days of the atherogenic diet.

Measurement of \( \text{O}_2^- \)

Superoxide anion (\( \text{O}_2^- \)) production was measured by SOD-inhibitable conversion of nitro blue tetrazolium (NBT) to formazan.15,16 Normal saphenous veins, arteries, SVGs, and AGs were harvested at 2 weeks after surgery. Vascular media was cut into strips and balanced in phenol-free DMEM at 37°C in CO\(_2\) for 30 minutes. NBT (0.1 mg/mL in phenol-free DMEM) was added for 3 hours with or without addition of SOD (1000 U/mL). The reaction was terminated by addition of 0.5 N HCl. To extract formazan, tissues were pulverized in liquid nitrogen and dissolved in 100% pyridine at 80°C for 30 minutes. Supernatants were read at 540 nm, and NBT reduction was calculated as follows: NBT reduction = A×V/(T×E×L), where A is absorbance, V, volume of solubilizing solution; T, time of incubation with NBT (minutes); E, extinction coefficient = 0.72 mmol/mg/mm; and L, length of light travel through the solution (10 mm). The SOD-inhibitable NBT reduction was calculated as a measure of \( \text{O}_2^- \) production (pmol \( \cdot \) mg wet wt\(^{-1} \cdot \) min\(^{-1} \)). In some experiments, tissues were incubated with enzyme inhibitors to determine the source of \( \text{O}_2^- \). The following inhibitors were used: diphenyleneiodonium [DPI, an inhibitor of NAD(P)H oxidase, 100 \( \mu \)mol/L], oxypurinol (an inhibitor of xanthine oxidase, 300 \( \mu \)mol/L), rotenone (an inhibitor of mitochondrial respiration, 50 \( \mu \)mol/L), and \( N^\bullet \)-nitro-L-arginine methyl ester (L-NAME; an inhibitor of NO synthase, 1 \( \mu \)mol/L).

SOD Activity

SOD activity was measured by SOD-dependent inhibition of cytochrome c reduction catalyzed by xanthine/xanthine oxidase.18,19 Vascular media was homogenized in 10 vol 50 mmol/L potassium phosphate (pH 7.4) containing 0.3 mol/L KBr and a cocktail of protease inhibitors (0.5 mmol/L PMSF, 90 mg/L aprotinin, 10 mg/L pepstatin, 10 mg/L leupeptin) followed by sonication (10 seconds) and extraction at 4°C for 30 minutes. The extracts were centrifuged at 20,000g for 30 minutes. The supernatants were added to the reaction mixture, consisting of 0.1 mmol/L EDTA, 0.090 mmol/L xanthine, and 0.018 mmol/L cytochrome c (pH 7.4). SOD activity was assessed by monitoring the inhibition of xanthine oxidase–mediated cytochrome c reduction, with the absorbance measured at 550 nm over 3 minutes, as described.19

GAG Synthesis

Dry defatted tissue (DDT) was digested with papain (7 U/mL) in 100 mmol/L sodium acetate, 5 mmol/L cysteine, 5 mmol/L EDTA at 60°C for 24 hours. After precipitation with 0.1% cetylpyridinium chloride in 0.1 mol/L sodium citrate (pH 4.8) for 2 hours at 37°C, the pellets were washed with ethanol, air-dried, and dissolved in distilled water (100 mg/mL). Sulfated GAG was measured by dye-binding assay (Blyscan, Biocolor Ltd). Briefly, dye reagent (1,9-dimethylmethylen blue), which was added to the samples, bound to sulfated GAG and forms the insoluble complex.20 GAG-bound dye...
was recovered with a dissociation reagent, and the absorbance of the recovered dye was measured in a spectrophotometer at 656 nm. Sulfated GAG (μg) in vascular tissues was calculated from the calibration curve by use of the GAG standard. The values were normalized per mg of DDT.

**LDL Retention Ex Vivo**

To assess LDL retention in vascular tissues, normal saphenous veins, normal arteries, SVGs, and AGs were harvested at 14 days after surgery. After the removal of the adventitia and endothelium, vessels were cut into ~5-mm fragments and placed in 24-well plates. They were then incubated with 125I-labeled LDL (1 mg/mL, 30 cpn/ng) in DMEM (0.5 mL/well) for 24 hours with gentle rocking at 37°C. Tissues were rinsed 5 times (15 min/wash) and blotted dry. Samples were counted in a gamma counter, and values derived from empty wells with 125I-labeled LDL were subtracted. LDL retention was expressed per wet weight (mg), DDT (mg), surface area (mm²), and protein content (ng).

**Immunohistochemistry**

The Vectastain Elite ABC system (Vector Laboratories) was used as previously described. Tissues were fixed in HistoChoice (Amresco) and processed for paraffin-embedded or frozen sections. They were incubated with primary antibodies for 1 hour, followed by biotinylated secondary horse anti-mouse antibodies (1:2000, Vector Laboratories), and finally counterstaining with hematoxylin. Monoclonal antibodies against the hyaluronate-binding region of human versican (1:200, Developmental Studies Hybridoma Bank), apolipoprotein B (apoB) (1:50, Biodesign), and oxidized epitopes (1:50, Biodesign) were used. Negative controls included nonimmune serum instead of primary antibody.

**Statistical Analyses**

Data were expressed as mean±SEM. The statistical significance regarding multigroup comparisons was determined by ANOVA. A value of P<0.05 was considered significant.

**Results**

**Oxidative Stress in Vascular Grafts**

Reactive oxygen species modulate several cellular functions important in vascular remodeling. Accordingly, we have examined oxidative stress in venous and arterial grafts measuring intragraft pro-oxidant and antioxidant properties. As shown in Figure 1, basal production of O₂⁻ (SOD-inhibitable NBT reduction) was higher in saphenous veins (n=6, P<0.01) than in normal arteries (n=6) before grafting. Importantly, vein arterialization further upregulated levels of O₂⁻ in SVGs (n=5, P<0.01) compared with AGs (n=7) at 2 weeks after surgery. To determine the source of O₂⁻ in SVGs, several inhibitors of known oxidant enzymes were used. The incubation of SVGs with DPI [100 μmol/L, NAD(P)H oxidase inhibitor] almost entirely abolished O₂⁻ (reduction by >95%), whereas the inhibitors of mitochondrial dehydrogenase, xanthine oxidase, or nitric oxide synthase showed no effects (Figure 2).

Because the graft oxidative stress depends not only on the generation of O₂⁻ but also on antioxidant properties of the tissue, we examined whether the arterialization of saphenous veins affects SOD activity. As illustrated in Figure 3, normal veins (n=5) and arteries (n=5) demonstrated comparable SOD activity (cytochrome c reduction assay). Nonetheless, SVGs (n=8, P<0.001) exhibited a significant loss of SOD activity, whereas AGs (n=7) showed no changes at 2 weeks after surgery.

**Expression of Sulfated GAG and Core Protein Proteoglycans**

Vascular graft adaptation includes the changes in the extracellular matrix, which may influence the properties of the conduits. To this end, we examined the accumulation of sulfated GAG (dye-binding assay) in grafts harvested at 2 weeks after surgery. Not surprisingly, normal saphenous veins (n=8, 2.4±0.8 μg/mg DDT) and arteries (n=4, 6.3±0.8 μg/mg DDT) differed in the amount of sulfated GAGs before surgery. Importantly, however, vein arterialization (n=8) was accompanied by a significant accumula-

<table>
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<th>Tissue</th>
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<th>Dry Weight, mg</th>
<th>Protein, mg</th>
<th>Surface Area, mm²</th>
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<td>122±8</td>
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<td>56±3</td>
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<td>2.9±0.2*</td>
<td>31±3*</td>
<td>671±51*</td>
</tr>
<tr>
<td>Arterial graft</td>
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<td>78±7</td>
<td>0.6±0.1</td>
<td>8±2</td>
<td>82±11</td>
</tr>
</tbody>
</table>

Normal vessels and vascular grafts (2 weeks after surgery) were incubated with 125I-LDL (1 mg/mL) as described in Methods. Values represent mean±SE.

*P<0.01 vs normal vein, artery, and arterial graft (ANOVA).
tion of sulfated GAG (3.6±0.8-fold increase, \(P<0.01\) versus normal vein). In contrast, AGs (\(n=4\)) showed no increase in the amount of GAG (0.68±0.4-fold increase, \(P=\text{NS}\) versus normal artery). Because sulfated GAGs constitute side chains of proteoglycans, we further verified the above results examining the expression and localization of a representative core protein (versican). As shown in Figure 4, versican immunoreactivity was elevated in the neointima at 2 weeks. In contrast, AGs showed no apparent changes in versican expression (not shown).

**Lipid Retention and Its Modification After Grafting**

The differences in vessel permeability and composition (eg, sulfated GAG content) may increase lipid retention. To address this issue, normal saphenous veins, arteries, SVGs, and AGs were harvested, and \(^{125}\)I-LDL retention was examined ex vivo. As illustrated in the Table, intact saphenous veins retained more LDL than arteries, which most likely reflected their dissimilar permeabilities, although the difference did not reach statistical significance. At 2 weeks after surgery, SVGs trapped even more radiolabeled LDL over the 24-hour period than normal saphenous vein, normal artery, or AG (\(P<0.001\)) regardless of several methods used for data normalization. In contrast, no changes in LDL accumulation were seen in AGs. Because the differences in LDL retention in SVGs and AGs ex vivo do not include hemodynamic factors present in vivo, we verified the intragraft accumulation of lipid in hyperlipemic animals (serum cholesterol 545±49 mg/dL, \(n=14\)). As shown in Figure 5, AGs showed

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**Figure 5.** Lipid retention in vascular grafts from hyperlipemic animals at 1 month after surgery. A, AGs showed preserved elastic tissues and no significant intima (Verhoeff's stain). Rectangular box identifies area shown in B (serial sections); B, no significant lipid accumulation is noted in AGs (oil red O stain); C, SVGs with prominent neointima (\(n\)) and remodeled media (\(m\)) (Verhoeff's stain). Rectangular box identifies area shown in D through H (serial sections); D, focal lipid retention in neointima (oil red O stain); E through G, lipid-positive regions contain apoB, oxidized epitopes, and versican; H, negative control (N/C) stained without primary antibody. Magnification: A and C, \(\times 20\); B and D through H, \(\times 200\).
no apparent lipid accumulation, whereas SVGs contained both extracellular and intracellular deposits of lipid (oil red O stain). Focal accumulation of apo B and oxidized epitopes was localized in the regions of the neointima containing versican (Figure 5).

Discussion

Major findings of this study point to several biological characteristics that distinguish SVGs from AGs. The arteri- alization of saphenous veins is marked by a significant shift in the oxidative stress, an accumulation of sulfated proteo- glycans, and early lipid retention.

The balance between reactive oxygen species and endog- enous antioxidants is an important homeostatic mechanism in vascular tissues. Studies of the arterial system have underscored a preferential generation of \(-\text{O}_2^\cdot\) by adventitial fibroblasts compared with medial SMCs. Although cellular heterogeneity of the venous media, which contains fibroblasts, could contribute to the higher levels of \(-\text{O}_2^\cdot\), additional studies are necessary to confirm cell-dependent generation of oxidative stress in SVGs. The upregulation in \(-\text{O}_2^\cdot\) in arterialized veins could also be attributed to several additional factors. They include pulsatile stretch and medial injury sustained by SVGs. In fact, focal vascular trauma has been shown to upregulate \(-\text{O}_2^\cdot\) in the arterial system, although studies of vascular grafts have been limited. Numerous growth factors released at the site of tissue injury, including thrombin, are known to increase NAD(P)H oxidase activity. As shown in the present study, an inhibitor of NAD(P)H oxidase almost entirely abolished \(-\text{O}_2^\cdot\) production in SVGs. These findings are consistent with an emerging role of NAD(P)H oxidase as a primary source of \(-\text{O}_2^\cdot\) in the vasculature.

Two mechanisms aimed at removing \(-\text{O}_2^\cdot\) also appear to be impaired in venous grafts. First, venous endothelial cells are less effective than arterial cells in the synthesis of nitric oxide, which interacts with \(-\text{O}_2^\cdot\). Second, as shown in the present study, the overall activity of SOD, a major antioxidant enzyme, is attenuated in SVGs. It remains to be determined which form of SOD is reduced, although its extracellular form is less abundant in the veins. The observed oxidative stress could explain the higher cell proliferation seen early after vein arterialization but generally absent in AGs. Furthermore, redox-sensitive transcriptional factors (eg, nuclear factor-\(\kappa\)B) may induce the expression of adhesive molecules, such as vascular cell adhesion molecule-1, which in turn promote the influx of blood-borne inflammatory cells into the healing SVGs. These mechanisms often lead to excessive neointimal formation and early occlusive lesions in SVGs.

Although vein graft atherosclerosis is clinically manifested several years after surgery, the results of the present study suggest that this process begins much earlier. Normal saphenous veins retained more LDL ex vivo owing to less developed elastic laminae and probably higher permeability. Importantly, LDL retention significantly increased after vein arterialization (2 weeks), in contrast to AGs. In hypercholes- terolemic animals, lipid accumulated in the neointima of SVGs. In addition to vessel permeability and hemodynamic factors, lipid retention is influenced by extracellular matrix components. In particular, sulfated GAG proteoglycans derived from proliferating cells have higher binding affinity to LDL. Our previous studies have shown that vascular tissues rich in proliferating fibroblasts produced higher amounts of sulfated GAG and exhibited avid lipid retention compared with differentiated SMCs. Thus, oxidative stress and the synthesis of matrix proteins, which retain LDL, may promote oxidative lipid modifications and create conditions promoting early onset of SVG atherosclerosis. This study has important clinical implications, providing biological rationale for the use of arterial conduits for coronary revascularization.

In those patients in whom SVGs cannot be avoided, it remains to be determined whether the inhibition of vascular NAD(P)H oxidase (eg, with ACE inhibitors) reduces SVG attrition and cardiovascular events. Furthermore, early lipid retention in SVGs confirms the need for aggressive correction of lipid abnormalities in postbypass patients.

In summary, this study demonstrated significant differences in the biology of SVGs and AGs. Early changes in SVGs are characterized by oxidative stress due to higher production of \(-\text{O}_2^\cdot\) and lower activity of SOD. Furthermore, SVGs increase the synthesis of sulfated GAG proteoglycans, which is associated with LDL retention. These findings also suggest that although SVG atherosclerosis is clinically manifested 3 to 5 years after surgery, proatherogenic changes may commence early after surgical revascularization.

Acknowledgments

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References


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