Inhibition of Accelerated Atherosclerosis in Vein Grafts by Placement of External Stent in ApoE*3-Leiden Transgenic Mice


Objective—Vein grafts fail because of the development of intimal hyperplasia and accelerated atherosclerosis. Placement of an external stent around vein grafts resulted in an inhibition of intimal hyperplasia in several animal studies. Here, we assess the effects of external stenting on accelerated atherosclerosis in early vein grafts in carotid arteries in hypercholesterolemic apolipoprotein E*3-Leiden transgenic mice.

Methods and Results—Venous interposition grafting was performed in apolipoprotein E*3-Leiden mice fed standard chow or a highly cholesterol-rich diet for 4 weeks. After engraftment, external stents with different inner diameters (0.4 or 0.8 mm) were placed. In unstented vein grafts in hypercholesterolemic mice, thickening up to 50 times the original thickness, with foam cell–rich lesions, calcification, and necrosis, was observed within 28 days. The atherosclerotic lesions observed showed high morphological resemblance to atherosclerotic lesions observed in human vein grafts. In stented vein grafts in hypercholesterolemic mice, no foam cell accumulation or accelerated atherosclerosis was observed. Compared with unstented vein grafts, stenting of vein grafts in a hypercholesterolemic environment resulted in a 94% reduction of vessel wall thickening. These effects were independent of stent size.

Conclusions—Extravascular stent placement results in strong inhibition of accelerated vein graft atherosclerosis in hypercholesterolemic transgenic mice and thereby provides a perspective for therapeutic intervention in vein graft diseases. (Arterioscler Thromb Vasc Biol. 2002;22:1433-1438.)

Key Words: atherosclerosis ■ vein grafting ■ external stent ■ transgenic mice

Vein bypass grafting remains the most common method of vascular reconstruction for bypassing obstructive arterial lesions.1 However, aorta-coronary and peripheral vein grafts are known to have a high failure rate: 10% to 40% after 1 year and 50% to 60% after 10 years.2 These vein grafts undergo early intimal thickening and accelerated atherosclerosis,3–6 both of which may contribute to eventual graft failure.

In patients with angiographic evidence of occlusive disease after vein grafting, atherosclerotic lesions within the graft have been demonstrated as early as 6 to 12 months after surgery.3,4,6,7 Vein graft atherosclerotic lesions are more diffuse, concentric, and friable with a poorly developed or absent fibrous cap, whereas native vessel atheroma are proximal, focally eccentric, and nonfriable with a well-developed fibrous cap.8,9 Also, compared with native atherosclerotic lesions, accelerated atherosclerotic lesions in vein grafts contain more foam cells with varying degrees of lipid accumulation and macrophage/mononuclear and inflammatory cell infiltration.10 Compared with atherosclerosis in native arteries, this accelerated atherosclerosis in vein grafts progresses more rapidly.11,12

Recently, Dietrich et al13 described the fast initiation of accelerated atherosclerosis in vein grafts in hypercholesterolemic transgenic mice with the use of the very elegant, recently described technique of placing a venous interposition in the mouse carotid artery.14 The atherosclerotic lesions observed in these murine grafts show high morphological resemblance to lesions observed in human vein grafts.

A reduction of vein graft thickening or, more precisely, a reduction of neointimal thickening in vein grafts in various animal models was achieved by external stenting of grafted veins,15–18 yielding decreased smooth muscle cell (SMC) proliferation. However, in none of these studies was the effect of external stenting on accelerated atherosclerosis in grafts determined.

In the present study, we investigate the effect of external stenting on accelerated atherosclerosis, monitored as accumulation of foam cells, in vein grafts in apoE*3-Leiden transgenic mice.

ApoE*3-Leiden transgenic mice19 develop diet-dependent hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. This animal model is currently considered to...
be 1 of the animal models of atherosclerosis that is closest to that occurring in humans.20

In the present study, this animal model of cholesterol-induced atherosclerosis has been used to evaluate the effect of placement of an external polyethylene stent around vein grafts in the carotid artery in hypercholesterolemic mice. We show that compared with no stenting of grafts, stent placement around vein grafts in hypercholesterolemic ApoE*3-Leiden mice resulted in a strong inhibition of vein graft thickening as well as accelerated atherosclerosis. This inhibition of vein graft atheroma may contribute to further understanding of the mechanisms of accelerated vein graft atherosclerosis.

**Methods**

**Mice**
The Netherlands Organization for Applied Scientific research animal welfare committee approved all experiments. ApoE*3-Leiden mice19 were crossbred for 18 generations with C57BL/6 mice. Male animals, aged 8 to 10 weeks, were allocated randomly to 1 of the 2 experimental diets on the basis of age and litter. For analysis of the time course, 3 mice were used at each time point. For all other experiments, groups of 6 mice per condition were analyzed.

**Diet**
During the experimental period, animals were fed a chow diet or a cholesterol-enriched high-fat diet containing 0.5% cholate (referred to as the HFC-0.5% diet, which also contained 0.5% casein, 1% choline chloride, 0.2% methionine, 15% cocoa butter, 0.5% cholate, 1% cholesterol, 40.5% sucrose, 10% cornstarch, 1% corn oil, 5.1% cellulose, and 5.1% mineral mixture) 4 weeks after the chow or HFC-0.5% diet, mice were anesthetized with Hypnorm/Dormicum. The thorax was opened longitudinally, were placed around the mid region of the artery in hypercholesterolemic mice. We show that compared with no stenting of grafts, stent placement around vein grafts in hypercholesterolemic ApoE*3-Leiden mice resulted in a strong inhibition of vein graft thickening as well as accelerated atherosclerosis. This inhibition of vein graft atheroma may contribute to further understanding of the mechanisms of accelerated vein graft atherosclerosis.

**Vein Graft Procedure**
After 4 weeks of the chow or HFC-0.5% diet, mice were anesthetized with Hypnorm (25 mg/kg IP, Bayer) and Dormicum (25 mg/kg IP, Roche). Atropine sulfate (1.7 mg/kg, Brocacef BV) was administered to keep the respiratory tract in good condition. The procedure used for vein grafts, a caval vein interposition in the carotid artery, has been described by Zou et al.14 In brief, the common carotid artery was dissected free from the bifurcation at the distal end toward the proximal end. The artery was cut in the middle, and cuffs were placed at the end on both sides. Next, both ends of the artery were everted over the cuffs and ligated with an 8-0 silk ligature. The vena cava was harvested and grafted between the 2 ends of the artery by means of a 3.7% formaldehyde (vol/vol) solution for 5 minutes. After perfusion, vein grafts were harvested, fixed overnight in 3.7% formaldehyde (vol/vol) in PBS, and paraffin-embedded.

Serial cross sections (5 μm thick) were used throughout the entire length of the graft for histological analysis. Cryosections were made from 1 mouse in each condition. All samples were routinely stained with hematoxylin-phloxine-saffron. SMCs were visualized with α-smooth muscle actin (α-SM-actin) staining (Dako), and Mac-3 (Pharmingen) macrophage staining was used to detect monocytes/macrophages. Anti-intercellular adhesion molecule (ICAM)-1 antibodies (R&D Systems) were used as markers of endothelial cells. Lipid deposition was visualized with oil red O (Gurr, Searle Diagnostics) staining on cryosections. The stainings were performed as described previously.21,22 von Kossa staining was used to detect calcification. Apoptotic cells were identified by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining corrected for false-positive RNA synthesis and splicing.23

**Quantification of Intimal Lesions**
To quantitate the effect of external stenting on intimal thickening in murine vein grafts, mice on either a chow diet or an HFC-0.5% diet (with stented [0.4- or 0.8-mm inner diameter] or unstented grafts) were euthanized 28 days after surgery. For each mouse, 6 equally spaced cross sections of the (un)stented graft were used to quantify the lesion area. By use of image analysis software (Qwin, Leica), total cross-sectional area was measured between the lumen and adventitia to determine vein thickening. Because only very few layers of cells are in the media of mouse veins and because there is no morphological border between the neointima and media (such as the internal elastic lamina in arteries), vein graft thickening (ie, the region between the lumen and adventitia) was used to define the lesion area.

**Statistical Analysis**
All data are presented as mean±SEM. Overall comparisons between groups were performed with the Kruskal-Wallis test. If a significant difference was found, groups were compared with their control by using Mann-Whitney rank sum tests. Values of P<0.05 were regarded significant.

**Results**
Body weights were monitored at surgery and euthanasia. No significant changes in body weights were registered for any of the animals. Compared with the chow diet, the HFC-0.5% diet increased plasma cholesterol concentrations significantly (27.6±3.4 versus 2.3±0.6 mmol/L for the chow diet, P<0.05), whereas serum triglyceride concentrations were decreased as described earlier.21

**Time Course of Development of Intimal Lesions in Stented and Unstented Vein Grafts**
To study the effect of external stenting (stent size 0.8-mm inner diameter, 2.0-mm length) on foam cell accumulation (as a parameter for accelerated atherosclerosis) and vessel wall thickening in vein grafts over time, mice were randomized into 2 groups: the HFC-0.5% diet group with unstented grafts and the HFC-0.5% diet group with stented grafts. In each group, mice (n=3) were killed at 0, 1, 7, 14, and 28 days after surgery. Abundant plaque formation was observed in unstented vein grafts, whereas in stented grafts, no plaque formation was observed.

One day after surgery, in the HFC-0.5% diet group with unstented grafts, endothelial staining with the use of anti–ICAM-1 demonstrated a disturbed endothelial cell lining of the vein grafts (Figure 1). Also, 1 day after surgery, numerous
platelets and polymorphonuclear leukocytes (PMNs) were observed adherent to the endothelial surface in this group. In the stented group, however, fewer PMNs and platelets adhered to the endothelial surface. Seven days after surgery, in the unstented group, a marked decrease of \( \alpha \)–SM-actin–positive cells was observed. This phenomenon is in accordance with previous reports of SMC loss in human saphenous vein bypass grafts in the first week after surgery.\(^{10}\) However, in the stented group, no apparent SMC loss was observed. Also, after 7 days, foam cell accumulation was observed in unstented grafts of mice on the HFC-0.5% diet. This early deposition of lipid-loaded macrophages and signs of accelerated atherosclerosis were never observed in the stented vein grafts. In late vein grafts (14 and 28 days), intimal thickening progressed in the unstented group. In vein grafts of mice on the HFC-0.5% diet, thickening up to 50 times the original thickness was observed (Figure 1). In stented grafts, however, Mac-3 and oil red O staining revealed no lipid deposition in the stented grafts. Moreover, calcifications frequently observed in the unstented grafts were never seen in stented graft segments. Anti–ICAM-1 staining revealed no

To quantify the effect of external stent placement on vein graft wall thickness over time, graft thickness in the stented and unstented grafts was determined at 0, 1, 7, 14, and 28 days after surgery. In the unstented grafts, a gradual increase in graft thickness in time was observed, whereas in the stented grafts, hardly any change in wall thickness was observed (please see online Figure II, available at http://www.atvb.ahajournals.org).

Characterization and Quantification of Accelerated Atherosclerosis in Stented and Unstented Vein Grafts

ApoE*3-Leiden mice on an HFC-0.5% diet were randomized into 2 groups. One group (\( n=6 \)) underwent vein grafting without placement of an external stent. In the other group (\( n=6 \)), an external stent (0.8-mm inner diameter, 2.0-mm length) was placed after vein grafting. Microscopic analysis of unstented vein grafts after 28 days (Figure 2) revealed that numerous macrophages and foam cells were present in the subendothelial space and interspersed between the SMCs. Also, abundant foam cell deposition was observed on the adventitial side of the unstented grafts. In addition, oil red O staining revealed excessive lipid accumulation in the unstented grafts. Furthermore, calcification with signs of necrosis was frequently observed in these grafts. In the stented grafts, however, Mac-3 and oil red O staining revealed no lipid deposition in the stented grafts. Moreover, calcifications frequently observed in the unstented grafts were never seen in stented graft segments. Anti–ICAM-1 staining revealed no
macrophage and foam cell accumulation within the vessel wall. Also, ICAM-1 staining revealed an apparently intact endothelial cell lining in stented grafts, whereas in unstented grafts, evidence of endothelial damage was apparent.

**Influence of External Stent Size on Accelerated Atherosclerosis in Vein Grafts**

To assess the influence of stent size on foam cell accumulation and accelerated atherosclerosis in vein grafts, mice were randomized in 6 groups: unstented and stented grafts (0.4 or 0.8-mm inner diameter) in groups fed the chow diet or the HFC-0.5% diet.

In unstented vein grafts in mice on the HFC-0.5% diet, the total vessel wall thickening (expressed as vein graft area) was increased after 28 days from 0.18±0.01 to 0.80±0.08 mm² compared with total vessel wall thickening in unstented grafts in mice on the chow diet (Figure 3). Also, the total circumference of unstented vein grafts of hypercholesterolemic mice was significantly increased from 0.64±0.03 to 1.18±0.08 mm compared with the total circumference of the unstented vein graft in normocholesterolemic mice, indicating outward remodeling in hypercholesterolemic animals. Vessel wall thickening in stented grafts of mice on the chow diet or on the HFC-0.5% diet (0.01±0.001 and 0.03±0.002 mm², respectively) was significantly less compared with vessel wall thickening in the unstented control group on the chow diet or on the HFC-0.5% diet (0.05±0.002 and 0.05±0.004 mm², respectively). In the 0.4- and 0.8-mm stented vein grafts, the luminal diameter of the vein grafts was somewhat constricted because of the inner diameter of the stents. In an attempt to quantify the accelerated atherosclerosis in stented and unstented grafts, the macrophage/foam cell accumulation in the vein grafts was determined. For this determination, the total Mac-3–positive area was determined in cross sections of stented or unstented vein grafts in mice on the HFC-0.5% diet. The total Mac-3–positive area in the unstented grafts was 11.2±1.4% of the total vein graft area compared with 1.28±0.16% in the stented grafts with 0.4-mm inner diameters and 0.69±0.15% in the stented grafts with 0.8-mm inner diameters (Figure 4).

**Discussion**

In the present study, we determined that external stent placement around vein grafts in hypercholesterolemic transgenic apoE*3-Leiden mice prevents the development of accelerated atherosclerosis and vein graft thickening. The vessel wall thickening and subsequent atherosclerotic changes in vein grafts are regarded as intrinsic adaptations to increased wall tension, cyclic stretching, and shear stress, which are due to higher pressure and flow velocities in the
arterial circulation. Recent experimental studies have reported that application of an external stent to the outside of vein grafts reduces intimal hyperplasia to varying degrees. In all these studies, performed in normolipidemic animals, external stenting of vein grafts resulted in a decrease of vessel wall thickening predominantly consisting of SMCs. However, the effect of external stenting on lipid deposition and accelerated atherosclerosis was not determined. The extremely fast initiation of accelerated atherosclerosis in vein grafts in hypercholesterolemic transgenic mice enabled us to assess the effect of external stenting on atheroma formation in vein grafts. The structural changes observed in the unstented vein grafts of hypercholesterolemic mice, such as early disturbance of the endothelial lining, aggregation of PMNs, and reendothelialization within the first week after grafting, are morphological features reported for vein-to-artery grafts in humans. In stented grafts in normolipidemic and in hyperlipidemic mice, however, circumferential distension was prevented by the stent, resulting in minimized endothelial injury and less PMN adherence. This has been described previously in a pig vein graft model in which an external biodegradable supporting conduit protected the endothelium in vein grafts.

In unstented vein grafts, SMC loss was observed during the first week after surgery. This is in accordance with SMC loss described in specimens of early vein graft in patients and in various experimental studies. In stented murine grafts, however, no evident cellular loss was observed 7 days after engraftment. This significant decrease in disruption of SMC layers in stented grafts compared with unstented grafts may be explained by decreased production of enzymes, such as elastase and collagenase, by activated PMNs and, most likely, by factors that induce SMC apoptosis, as observed 7 days after surgery.

Foam cell accumulation within 7 days after surgery demonstrated the extremely fast initiation of accelerated atherosclerosis in unstented grafts of hypercholesterolemic mice. In stented grafts of hypercholesterolemic mice, no foam cell accumulation could be detected within the experimental period. This important observation indicates that external stent placement around a vein graft in a hypercholesterolemic environment not only inhibits SMC intimal hyperplasia but also prevents accelerated atherosclerosis. This finding is of major clinical interest because both features contribute to vein graft failure in humans.

Previous reports have shown that elevated circumferential stress results in increased wall thickness. Placing an external stent around the grafted vein reduces circumferential stress, resulting in decreased vein graft thickening. In addition, shear stress regulates vessel wall caliber and structure in arteries. Stent placement increases shear stress on the endothelial cell lining and thereby inhibits vein graft thickening. Furthermore, high blood flow velocity is correlated with reduction of neointimal formation and vessel wall thickening. Opposed to unstented vein grafts in which laminar flow is disturbed and blood flow velocity is rather low compared with arterial flow, stented vein grafts display blood flow velocity that is believed to contribute highly to the reduction of vessel wall thickening.

The exact mechanism by which stent placement prevents accelerated atherosclerosis is still unclear. Earlier studies have shown that an increase of circumferential and shear stresses in in vitro models results in altered expression of adhesion molecules. This altered expression of adhesion molecules was prevented when external stents were applied around the saphenous vein segments. In the present study, we observed a disturbed endothelial cell layer in the unstented vein graft 1 day after surgery, whereas in the stented vessel, an apparently intact endothelial layer was observed. This disturbance of endothelial cell layer may act as an inducing factor of vascular remodeling and accelerated atherosclerosis. A reduction in circumferential stress has also been shown to inhibit atherosclerosis in arteries. Furthermore, in the present study, the reduced circumferential stress in vein grafts caused by extravascular stenting resulted in reduced apoptosis, as demonstrated by reduced TUNEL-positive staining in stented vein grafts opposed to unstented vein grafts at various time points. Because apoptosis is thought to be a trigger for the onset of vascular remodeling, external stenting may prevent the initial trigger for the onset of vein graft remodeling, macrophage accumulation, and, thus, accelerated atherosclerosis. This stress-induced apoptosis in unstented grafts has been described previously by Mayr et al, who used the same murine interposition graft model. Moreover, the increased thickness and fibrosis of vein grafts, induced by vascular remodeling, enhances the accumulation of LDLs in the grafted vein and, thus, favors atherosclerosis.

In the present study, all stented groups showed a profound reduction in vein graft atheroma independent of stent size. Consistent with this observation is the fact that vein graft thickening, predominantly consisting of SMCs, was reduced independent of stent size in a pig saphenous vein bypass graft model. This indicates that precise size matching between graft and stent is not a prerequisite, which will facilitate external stent procedures in clinical practice.

In conclusion, the present study has demonstrated that placement of an external stent around a vein graft in hypercholesterolemic transgenic mice results in a profound, highly reproducible reduction of accelerated atherosclerosis within the graft that is independent of stent size; therefore, the
present study provides valuable information regarding therapeu
tic interventions in vascular diseases.

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References
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Legends to Figures

Figure I

Panel A:
TUNEL-staining for apoptosis in cross sections of unstented and stented vein grafts 7d after surgery. In unstented vein grafts, apoptotic cells were observed (arrow), whereas in stented grafts hardly any apoptotic cells were present. TUNEL-staining was corrected for false positive cells due to RNA synthesis and splicing. Magnification 20x-40x.

Panel B:
Quantification of TUNEL-positive cells in stented and unstented vein grafts on 7 and 14 days after surgery. A significant difference in percentage positive cells per segment between stented and unstented grafts were observed in both timepoints. (n=3, p<0.05).

Figure II
Quantification of vein graft thickening in mice on HFC-0.5% diet at 0,1,7,14 and 28d after vein grafting. Thickening was measured by software analysis calculating total vein graft area in histological sections (6 sections per mouse) per time point. Expressed are the stented versus unstented grafts (mean±SD, N=3).
vein graft area (mm²)

- unstented
- stented

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