Vein adaptation to arterialization in an experimental model

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Purpose: **The events preceding myointimal thickening in vein grafts after vascular reconstructions are not well characterized. Indeed, the injury response associated with vein graft arterialization may be different than that observed in the balloon angioplasty model. Therefore, we used a rat model to study the early cellular response after arterialization of vein grafts.**

Methods: **Epigastric veins were placed as femoral artery interposition grafts in 37 male Lewis rats (weight range, 350-400 g). Vein grafts and contralateral epigastric veins were harvested at different time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 21 days, 30 days, and 70 days). Tissue specimens were processed for histology and immunohistochemistry with antibodies for the proliferating cell nuclear antigen (PCNA) and for different cell types. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was used as a means of determining the presence of apoptosis. Electron microscopy was used as means of assessing the integrity of the endothelial cell surface (SEM) and confirming the presence of apoptosis (TEM). Specimens were also snap frozen in liquid nitrogen for RNA isolation and molecular analysis.**

Results: **At 1 day, endothelial denudation with platelet deposition on the surface was shown by means of SEM. Both apoptosis and necrosis of smooth muscle cells (SMCs) were present in the media, along with monocyte infiltration. Cellular proliferation and apoptosis were most intense within the first week of implantation. PCNA staining was first seen in the adventitial fibroblasts and microvessels, then in the medial SMCs at 3 days. With reverse transcriptase polymerase chain reaction, upregulation of vascular endothelial growth factor (VEGF) messenger RNA (mRNA) was noted at 1 day. Myointimal thickening progressively developed, with no apparent diminution of the luminal area as long as 70 days after implantation. By means of the analysis of the transforming growth factor β1, mRNA showed expression during intimal thickening and accumulation of extracellular matrix. Reendothelialization was complete at 30 days.**

Conclusions: **These observations indicate that the cellular composition in our vein graft model is similar to human stenotic explants. Endothelial denudation is observed in rat vein grafts with complete regeneration by 30 days. VEGF mRNA is upregulated at 1 day, followed by proliferation of microvessel endothelial cells in the adventitia. Cellular proliferation and apoptosis are minimal after 21 days, with progressive intimal thickening likely to be the result of matrix accumulation. (J Vasc Surg 2001;33:561-9.)**

Human autogenous vein grafts develop myointimal thickening after transplantation into the arterial circulation.[1,2] Vascular stenosis often results from an exaggeration of this hyperplastic process through mechanisms that remain unknown. Controversy remains about the degree of proliferative response after angioplasty or after open revascularization procedures in arteries. An average proliferative index of 1.34% has been shown by means of our analysis of human vein graft stenotic lesions retrieved between 3 and 18 months after primary bypass grafting procedures.[3] Although a few lesions exhibited evidence of hypercellularity within the intima, most lesions displayed areas of hypocellularity with presence of apoptotic cells and excessive accumulation of extracellular matrix (unpublished data). Apoptosis is a physiologic mechanism of cell death that regulates normal embryonic development and remodeling, tissue mass, and cellular homeostasis.[4] It has been implicated in the regulation of intimal thickening in experimental models of intimal hyperplasia.[5] However, the early profiles of cellular proliferation versus apoptosis after arterialization of vein grafts have not been characterized. Our previous observations have also indicated a relatively greater proliferative activity in smooth muscle cells (SMCs) of the media and microvascular endothelial cells of the adventitia.[6] Whether neovascularization promotes the growth of the neointima, as observed in some neoplastic processes, or appears secondarily as part of an adaptive response to changing metabolic demands remains unknown.[7] To determine the role of cellular proliferation and apoptosis in neointima formation and the time course of neovascularization in this process, we expanded the use of an experimental vein graft in a rodent model.

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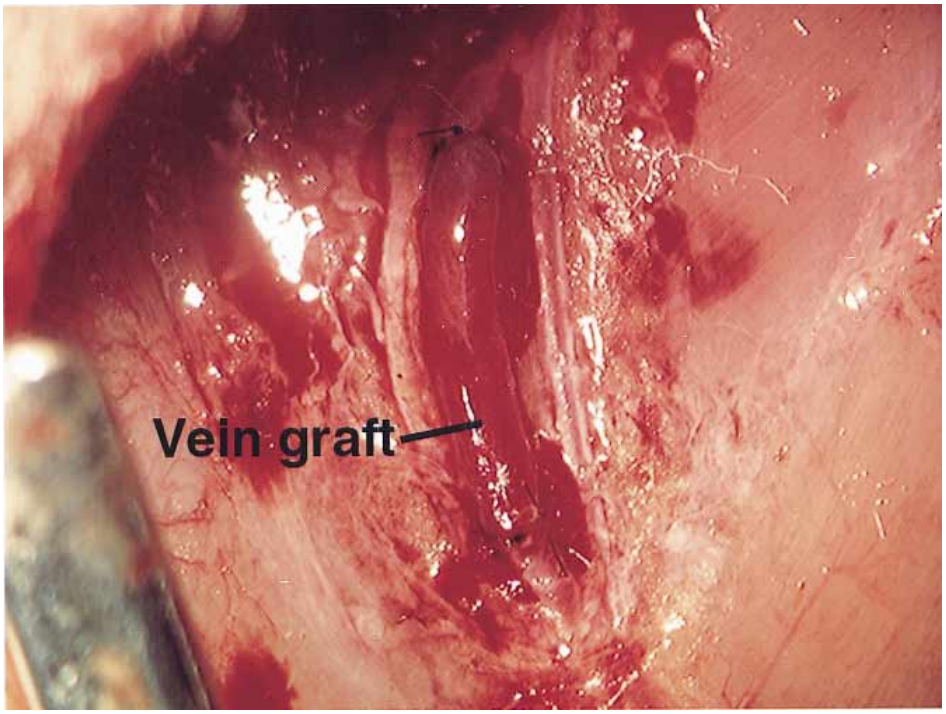

Fig 1. Photograph showing the epigastric interposition vein graft in the femoral artery of the Lewis rat vein graft model.

MATERIALS AND METHODS

The experimental rat vein graft model

Epigastric vein-to-common femoral artery interposition grafts were placed in 37 inbred male Lewis rats weighing 350 to 450 g (Fig 1) by the use of a method similar to that described by Hoch et al.[8] In brief, each animal was anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mL/kg). An 8-mm segment of ipsilateral epigastric vein was carefully harvested, gently irrigated with heparinized saline solution (100 U/mL), and placed as a reverse interposition graft into a segmental 3-mm defect of the femoral artery with 6 to 8 interrupted sutures of 10-0 nylon (Ethicon). The entire procedure was carried out with standard microsurgical techniques. The total ischemic time was kept to less than 1 hour. Patency was verified by means of an electromagnetic flow probe connected to a small animal flowmeter (Transonic Systems). Vein grafts were harvested at 6 hours (n = 3), 1 day (n = 5), 2 days (n = 5), 3 days (n = 5), 7 days (n = 4), 14 days (n = 4), 21 days (n = 4), 30 days (n = 4), and 70 days (n = 3). Thrombosed grafts (n = 2) were discarded. Contralateral epigastric veins were used as controls. Our protocol was approved by the Institutional Animal Care and Use Committee at the University of Arizona and was in compliance with federal guidelines (*Guide for the Care and Use of Laboratory Animals,* Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Washington: National Academy Press; 1966).

Histologic analysis

At harvest, specimens were perfusion fixed with either 4% paraformaldehyde or methyl carnoy solution, then paraffin-embedded. Serial 4-μm sections were stained with hematoxylin and eosin and Masson trichrome for gross morphologic examination. Individual microvessels were identified on light microscopy by means of systematic scanning of each sample with a 40× objective.

Immunohistochemistry

Tissue sections were incubated with a number of cell type-specific antibodies against endothelial cells (anti-Factor VIII-related antigen, Dako), smooth muscle cells (α-smooth muscle actin, Sigma), and rat macrophages (ED1). Serial sections at different time points (2 to 3 sections for each specimen) were stained with a monoclonal antibody against the proliferating cell nuclear antigen (PCNA) as a means of assessing cell proliferation. Reactions were performed on an automated immunostainer (Ventana Medical Systems, Tucson, Ariz).[9] Detection of bound antibody was assessed by means of immunoperoxidase methodologies, with diaminobenzidine tetrahydrochloride serving as the color substrate. A PCNA index, in which the number of PCNA-positive cells was determined as a percentage of the total number of cells per high-power field times 100, was calculated.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

In situ detection of apoptosis in paraffin-embedded

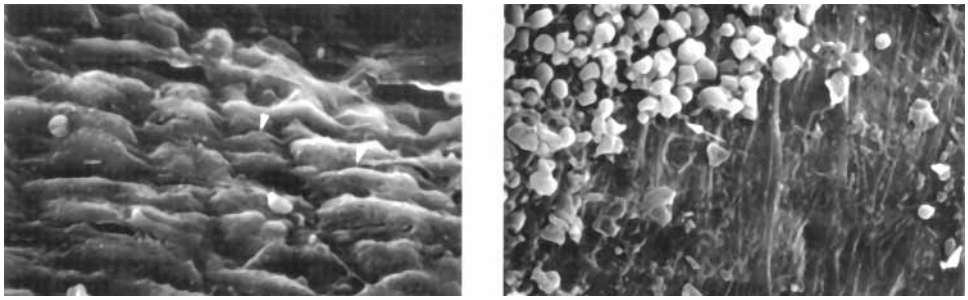

Fig 2. Scanning electron photomicrograph illustrating the surface characteristics of the rat epigastric vein **A,** before implantation and **B,** 1 day after implantation in the arterial circulation. Note the significant endothelial denudation and platelet deposition (*white arrow*; magnification, 4000×).

sections was performed by using the terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP-biotin nick end labeling (TUNEL) method. This technique relies on the occurrence of internucleosomal DNA fragmentation by an endonuclease into characteristic 180- to 200-bp segments. The different steps were performed with the automated immunohistochemical stainer with the rapid kinetic mode configuration (Ventana 320ES).[9] A TUNEL index was calculated; the number of TUNEL-positive cells was determined as a percentage of the total number of cells per high-power field times 100.

Electron microscopy

Transmission electron microscopy. A separate set of grafts was used for transmission electron microscopy (TEM), in which 1- to 2-mm segments were immersed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde buffered in 0.1 mol/L PO_4 (pH, 7.4) and postfixed in 1% osmium tetroxide. The specimens were subsequently dehydrated in graded ethanol solutions, then embedded in Epon-Araldite. Thin sections (60-90 nm) were obtained, placed on copper grids, and stained with uranyl acetate and lead citrate. They were examined with a JEOL-100CX II electron microscope (Japanese Electron Optical Laboratories), and representative photographs were taken. TEM studies were mainly used as a means of verifying the presence of apoptosis.

Scanning electron microscopy. For scanning electron microscopy (SEM), midportions of different specimens were fixed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer (pH, 7.4) and postfixed in 1% osmium tetroxide. They were dehydrated in graded ethanol solutions, critical point dried from CO_2, mounted on specimen stubs, and sputter-coated with gold-palladium by means of standard techniques. Specimens were examined in a ISI (Topcon) WB6 scanning electron microscope (International Scientific Instruments) to study the surface characteristics.

Total RNA isolation and reverse transcriptase polymerase chain reaction

Freshly isolated epigastric vein grafts were immediately frozen in liquid nitrogen. A minimum of 50 mg of tissue was homogenized in Trizol (Gibco/BRL), in the manner described by the manufacturer. After centrifugation, the RNA was precipitated with isopropan. Samples were dissolved in 15 μL of diethylpyrocarbonate-treated water, and the concentration was determined at 260 nm. The integrity of the RNA was determined by means of electrophoresis on a 1% agarose/formaldehyde gel and ethidium bromide staining.

Total RNA was pooled from two individual samples for analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as a means of analyzing expression of vascular endothelial growth factor (VEGF; Genbank Accession Number AF215725.1), glyceraldehyde 3-P dehydrogenase (GAPDH; Genbank Accession Number AF106860), and transforming growth factor beta-1 (TGFβ1; Genbank Accession Number X52498) with specifically designed oligonucleotide primers (gene Runner, Hastings Software). One microgram of total RNA was annealed with 1 μg/μL random hexamer. The product was reversed transcribed into complementary DNA (cDNA) with Maloney murine leukemia virus reverse transcriptase (Gibco BRL) at 42°C for 60 minutes. Then, 50 ng of the cDNA was amplified by means of polymerase chain reaction in PCR buffer (10 mmol/L Tris-HCL, pH = 7.4, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.1% gelatin), 20 pmol each of the 3′ and 5′ primers, dNTPs, and Taq DNA polymerase (Fisher Biotech). Samples were denatured at 94°C for 4 minutes, and amplification was carried out for 30 cycles on a Perkin Elmer-Cetus DNA thermal cycler. One cycle consisted of 1 minute at 94°C, 2 minutes at 50°C, and 4 minutes at 72°C. PCR products were analyzed by means of electrophoresis on 3% agarose gels, stained with ethidium bromide, and photographed under UV light.

Statistical analysis

PCNA and TUNEL results were expressed as the mean ± SD. The comparison of PCNA and TUNEL indices at each time point with that of their respective controls (nonarterialized epigastric veins) was performed by means of a 2-tailed Student *t* test with a Bonferroni adjustment. Values less than .006 were considered to be statistically significant.

RESULTS

General morphologic features. At 1 day and 2 days after grafting, endothelial denudation with focal platelet

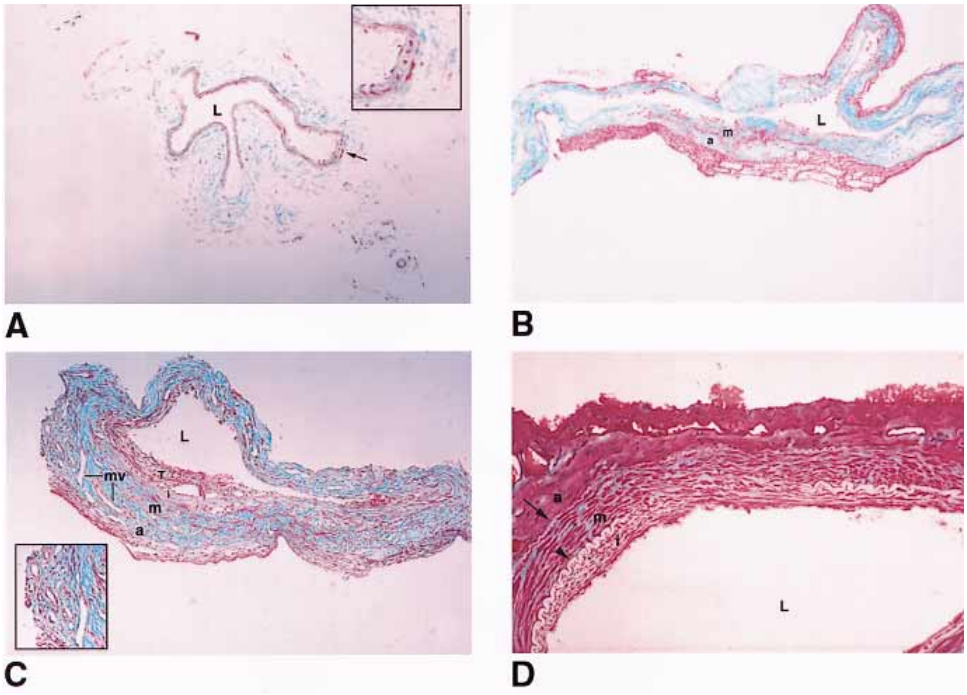

Fig 3. Morphologic assessment of arterialized vein graft in the Lewis rat model by means of Masson trichrome stain. **A,** Normal epigastric vein before implantation (original magnification, 31×). Insert shows a segment of the vein wall at higher magnification (80×). **B,** Arterialized vein graft at 2 days postimplantation. Note significant smooth muscle loss in tunica media (original magnification, 31×). **C,** Arterialized vein graft at 7 days after implantation. Note presence of layered nonocclusive recanalized thrombus. There is abundant microvasculature throughout the vein graft wall (original magnification, 31×). Insert shows microvessels in a segment of the graft wall (magnification, 40×). **D,** Arterialized vein graft at 70 days. There is a developed neointima, and the tunica media has well regenerated. The internal elastic lamina is well delineated (*arrowhead*). The *arrow* indicates external elastic lamina (original magnification, 80×). *L*, Lumen; *T,* thrombus; *i,* intima; *m,* media; *a,* adventitia; *mv,* microvessels.

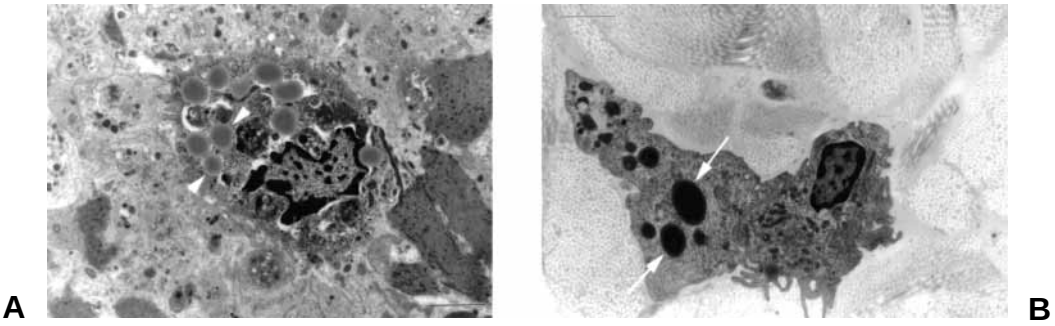

Fig 4. Transmission electron micrograph illustrating typical features of apoptosis in a rat vein graft specimen harvested 2 days after implantation. **A,** At an earlier stage, there is chromatin condensation, cell shrinkage, and vacuolization of the cytoplasm. **B,** At a later stage, apoptotic bodies have been engulfed by a neighboring cell (magnification, 5400×).

aggregates present on the surface was shown by means of SEM (Fig 2, *B*). Loss of SMCs was noted in the media, as first evidenced by means of Masson trichrome staining (Fig 3, *B*). By means of TEM, many smooth muscle-like cells were found to display key morphologic features of apoptosis in the media, including pyknosis and karyorrhexis, preservation of structure of cytoplasmic organelles, cell shrinkage, and presence of apoptotic bodies (Fig 4, *A* and *B*). Apoptotic cells were also present in the adventitia and in the vessel lumen near the surface. Typical necrotic cells were also present. Infiltration of mononuclear cells was noted through the vein graft wall at 1 day. At 7 days,

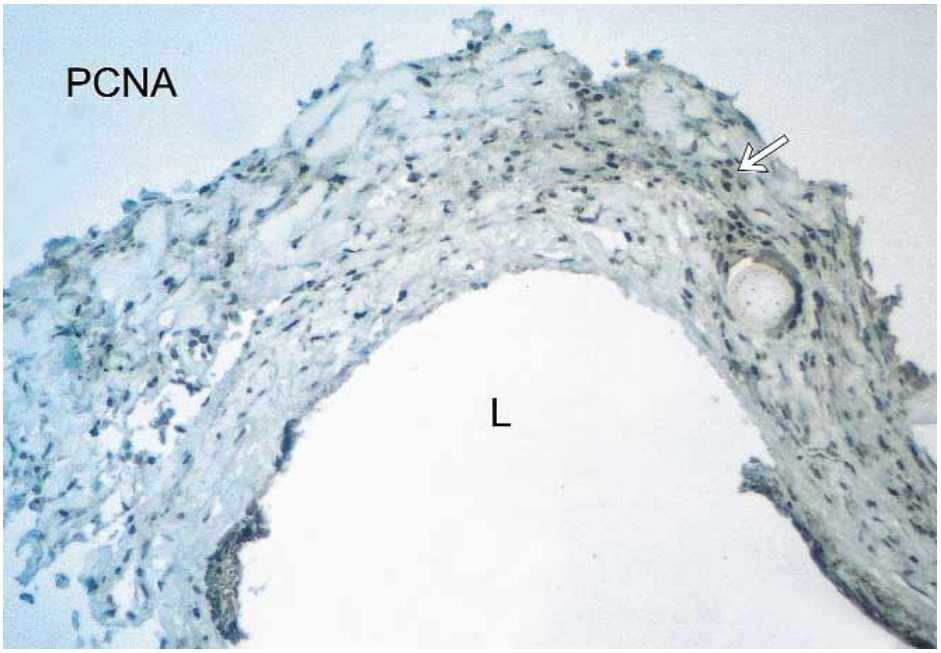

Fig 5. Immunolabeling for the proliferating cell nuclear antigen. *Arrows* indicate replicating nuclei appearing as dark black (original magnification, 80×). *L*, Lumen; *PCNA*, proliferating cell nuclear antigen.

microvessels had become abundant in the adventitia and the media (Fig 3, *C*). A developing neointima, consisting predominantly of alpha actin-positive SMCs, was present at 14 days. Intimal thickening continued to develop, with no apparent luminal compromise at 70 days (Fig 3, *D*). Reendothelialization was complete at 30 days, as evidenced by means of SEM.

Profiles of proliferating cell nuclear antigen and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling immunostaining. There was minimal or no detectable cell replication in control epigastric veins. PCNA-positive cells first appeared in the adventitia of arterialized vein grafts, then in the media. Both mitogenic and apoptotic activity were most intense during the first week after implantation (Figs 5 and 6), then decreased to lower levels (Fig 7). TUNEL-positive cells were rare in control veins (< 1%). At 1 day, apoptosis exceeded proliferation; TUNEL- and PCNA-positive cells became concordant at 7 days and later maintained comparably low levels as long as 30 days after implantation (Fig 7).

At 7 days, similar topographic profiles were also noted; both PCNA- and TUNEL-positive cells were mainly dispersed throughout the media and the adventitia. But at later points, cellular proliferation, although of lesser degree, continued to be present in the media and the adventitia, whereas TUNEL-positive cells were found mostly in the developing neointima.

Selected growth factors associated with vascular remodeling and angiogenesis. Early in the course of the study, our findings of microvascular endothelial cell proliferation prompted us to study the expression of VEGF, a potent angiogenic factor,[10] by the use of RT-PCR. TGFβ was also selected because of its described ability to stimulate the expansion of extracellular matrix components during repair of arterial injury.[11] Total RNA was isolated from the arterialized graft at various time points and analyzed with oligonucleotide primers designed to amplify the respective cDNA. Grafts amplified with TGFβ1 oligonucleotide primers produced a 378-bp product[12]; the gene appeared to be upregulated approximately 24 hours after graft implantation and remained elevated until 72 hours (Fig 8). The same samples amplified with the VEGF oligonucleotide primers produced a 446-bp product.[13] In contrast to the slow increasing concentration of TGFβ1 mRNA, the VEGF gene was dramatically upregulated at 24 hours and did not diminish significantly until 7 days after surgery (Fig 8). For comparison, the samples were amplified with GAPDH, a constitutively active gene, to ensure the similar levels of the cDNA; Fig 8 shows the product of 534 bp. These results suggest that the effects of these growth factors may begin early after the initiation of the vein arterialization process.

DISCUSSION

Material available on the use of human vein grafts depends on the occurrence of preocclusive stenotic lesions leading to surgical graft revision.[3] This consideration precludes the analysis of early pathologic changes associated with myointimal hyperplasia. The use of an experimental vein graft model allows analysis of the early injury response

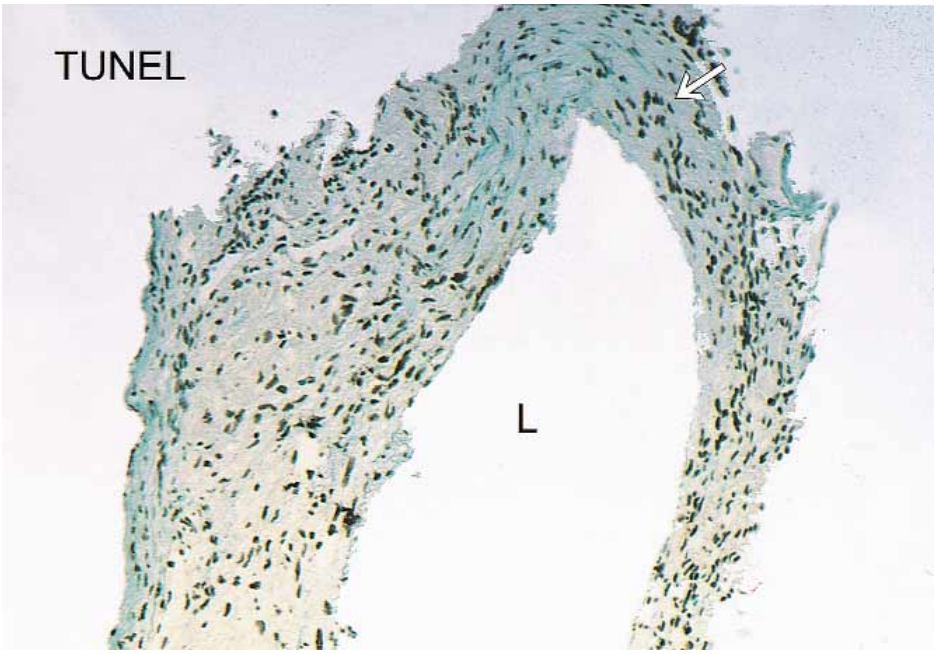

Fig 6. Detection of apoptosis. Arterialized vein graft at 7 days after implantation showing apoptosis by means of the TUNEL method. *Arrows* indicate TUNEL-positive nuclei appearing as dark brown (original magnification, 80×). *L*, Lumen; *TUNEL*, transferase-mediated dUTP-biotin nick end labeling.

after arterialization. Other investigators, such as Hoch et al,[8] Hirsch and Karnovsky,[14] and Faries et al,[15] have also used a rat epigastric vein interposition graft model as a means of examining various aspects of the development of intimal hyperplasia. Factors such as mechanical stretch caused by increased tensile stress after exposure to arterial blood pressure, vessel wall ischemia, and free radical oxidative injury associated with reperfusion contribute to endothelial and smooth muscle cell injury.[16-21] Interestingly, the amount of SMC loss in the media contrasts with the limited medial damage observed in the balloon angioplasty model, even when the angioplasty is performed experimentally in a vein.[22] The early injury response and subsequent cell loss have generally been considered to consist of necrosis, but our findings confirm that it may also include apoptosis. This genetically encoded cell death program has been described in endothelial cells exposed to hypoxia-reperfusion conditions[23] and in animal ischemia-reperfusion models of various organs.[24-26] It has been shown to be an important mechanism of cellular regulation in a rat balloon-injury model[5] and in a variety of hyperplastic conditions and atherosclerosis.[27-30] Clowes et al earlier demonstrated in the balloon-injured rat carotid artery that replicative activity of vascular SMCs persisted at relatively high levels (3.8%) for as long as 3 months after injury. However, the total arterial SMC content at 2 weeks remained unchanged at 12 months, which suggested that cell death was occurring.[29] Hoch and Stark,[30] in a preliminary report, described their findings of apoptosis in rat vein grafts harvested at 4 days and at later points. A novelty of our study was the study of the cellular proliferation in a freshly arterialized vein graft and the comparison of the respective profiles of cell replication and apoptosis. To our knowledge, similar comparisons had only been performed in a balloon angioplasty model,[31] in which the injury response appears to differ from the vein adaptation described in these studies. A quantitative difference in levels of apoptosis and proliferation was seen at 1 day after implantation, with apoptosis greater than proliferation by a ratio of 3 to 1. However, subsequently at 3 days, proliferation indices exceeded TUNEL indices by a ratio of 2 to 1. At 7 days, 14 days, 21 days, and 30 days, levels of proliferation and apoptosis were grossly concordant. This apparent mismatch between proliferation and apoptosis at 3 days may partly explain a net cellular accumulation early in the development of intimal hyperplasia. However, fundamental differences may also account for these results; the respective duration of these two biological events (proliferation requires 14-18 hours, whereas apoptosis requires 2 hours[32]), the longer half-life of PCNA positivity,[33-34] and apoptotic cells being quickly engulfed by neighboring macrophages could certainly lead to an underestimation of the degree of apoptosis present.

There were also differences noted within each layer of the vein graft. The apoptotic cells were initially identified in all three layers of the vein graft, then predominantly in the neointima at later points. The mitogenic activity began in the outer layers (adventitia then tunica media) as early as 1 day after implantation, involved all layers at 7 days, and then decreased to lower levels. Levels of proliferation and apoptosis after 21 days were remarkably low (< 1%).

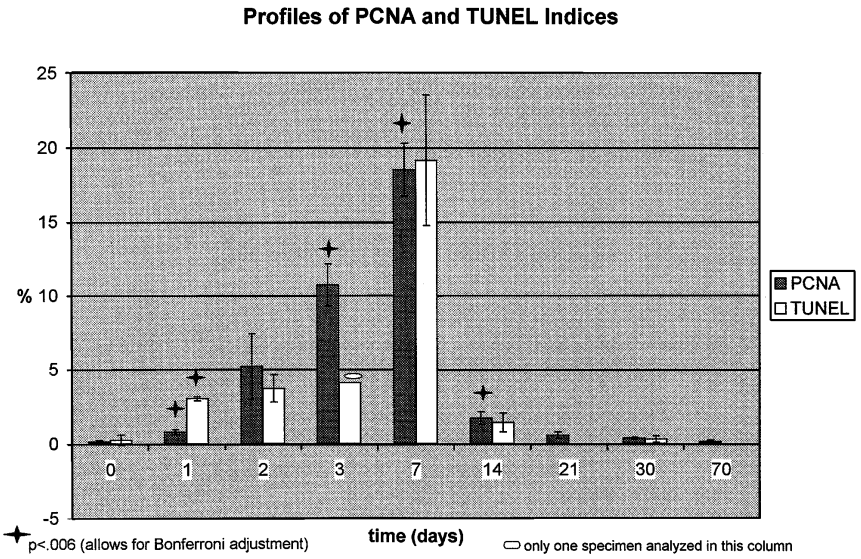

Fig 7. Profiles of PCNA and TUNEL indices represented graphically in a 30-day period. Results are expressed as mean ± SD. PCNA and TUNEL labeling indices (%) are represented on the y axis, and time is indicated on the x axis. *Statistical differences ($P < .006$) compared with controls. *PCNA,* Proliferating cell nuclear antigen; *TUNEL,* transferase-medicated dUTP-biotin nick end labeling.

These findings also contrast with those described after balloon angioplasty in arteries. In the arterial balloon-injury model, the development of the neointima has been shown to result from proliferation and migration of vascular SMCs from the media to the intima, with half of the SMCs continuing to proliferate after reaching the intima.[35] Moreover, the replicative activity continued at relatively high levels for as long as 3 months after injury.[5] Later in the development of the neointima, much of the hyperplastic response is contributed to by a marked increase in extracellular matrix from the secretory activity of SMCs and fibroblasts.[36] We observed no apparent diminution of the luminal area as long as 70 days after implantation, consistent with adequate vascular remodeling without development of stenosis. This may represent a limitation of our animal model to study vein graft stenosis, because no pathologic remodeling was apparent at 70 days. However, these observations still bring important implications. In contrast with a low PCNA index (< 1%) found at 21 days and at later points in our rat experimental vein graft, an average PCNA index of 1.34% has been found in human vein graft stenoses harvested between 6 and 18 months after implantation, with a peak recorded at 5.2%.[3] Combining these two observations, one can speculate that persistent or renewed cellular replication in arterialized vein grafts may be among the best biologic predictive markers of the development of vein graft stenosis.

By means of immunohistochemistry, proliferation of microvessels was already seen at 2 days. These observations prompted us to analyze VEGF gene expression, and upregulation of VEGF mRNA was noted at 24 hours (Fig 8). These data suggest an important role of VEGF as a direct promoter of angiogenesis in arterialized vein grafts. VEGF has been identified as a potent modulator of the formation of endothelial cell-lined channels.[37] Previous studies have indicated that it is among the factors secreted by the vessel wall in response to injury[38] and also in hypoxic conditions, providing a physiologic feedback mechanism to accommodate insufficient tissue oxygenation by promoting blood vessel formation.[39] VEGF has also been implicated in a variety of inflammatory and neoplastic conditions.[10,38,40,41]

TGFβ was also selected for analysis because of its ability to stimulate extracellular matrix expansion, because an early accumulation of extracellular matrix was noted. TGFβ1 mRNA was expressed as early as 6 hours after vein graft implantation and was upregulated at 24 hours (Fig 8). Another reason for its selection is its known interaction with VEGF. TGFβ1 may contribute to an angiogenic response by stimulating VEGF production in adventitial fibroblasts.[42] However, its role in angiogenesis remains unclear, because exogenous TGFβ1 inhibits rat aortic angiogenesis[43] and has been shown to downregulate expression of the VEGF receptor flk-1 in endothelial cells.[44] A more detailed analysis of VEGF and TGFβ is in progress, and it will characterize their relationships and their exact role in the development of intimal hyperplasia.

In summary, we have shown that early during the process of vein graft arterialization in a rat model there is endothelial and smooth muscle cell death by both necrosis and apoptosis and infiltration of mononuclear cells in the vein graft wall. Cellular proliferation begins early in the adventitia and the tunica media, resulting in increased microvessel density and SMC regeneration. Neovascularization is an early part of the injury response and con-

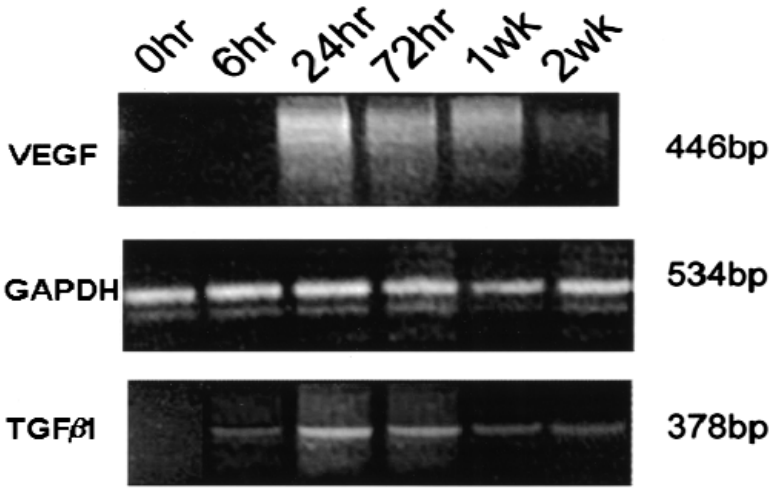

Fig 8. RT-PCR analysis of VEGF and TGFβ1 transcripts in rat vein graft specimens at different time points after arterialization. There is upregulation of both VEGF and TGFβ1 mRNA at 1 day. No expression is detected in the control vein. GAPDH primers are used for reference. *VEGF,* Vascular endothelial growth factor; *TGFβ1;* transforming growth factor beta-1.

tributes to vascular remodeling, rather than being a consequence of neointimal formation that appears later during the process. Apoptosis participates in regulation of cellularity early during the first week, when the mitogenic response is the most intense. Then, myointimal enlargement takes place, while cellular proliferation decreases to low levels (< 1% after 21 days), which implies a relative increase in the amount of extracellular matrix. The ultimate means of controlling neointimal thickness remains unknown, but models such as this may provide a useful experimental system for further investigation.

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