Graft-Extrinsic Cells Predominate in Vein Graft Arterialization

Lisheng Zhang, Neil J. Freedman, Leigh Brian, Karsten Peppel

Objective—Vein graft disease involves neointimal smooth muscle cells, the origins of which are unclear. This study sought to characterize and quantitate vein graft infiltration by cells extrinsic to the graft in a mouse model of vein graft disease.

Methods and Results—Inferior vena cava-to-carotid artery interposition grafting between C57BL/6 and congenic β-galactosidase–expressing ROSA26 mice was performed. Vein grafts were harvested 6 weeks postoperatively and stained with X-gal. More than 60% of neointimal cells derived from the recipient, and 50% of these cells expressed smooth muscle α-actin. The distribution of donor and recipient-derived cells within this vein graft wall layer was distinctly focal, consistent with focal infiltration and expansion of progenitor cells. When bone marrow transplantation with congenic green fluorescent protein (GFP)-expressing cells was used in vein graft recipients 1 month before surgery, abundant GFP-expressing cells appeared in the media, but not the neointima, of mature grafts. Endothelial cells in mature grafts derived from graft-intrinsic and graft-extrinsic sources and were, in part, of bone marrow origin.

Conclusions—Cells extrinsic to the graft, including bone marrow-derived cells, predominate during vein graft remodeling.

Key Words: smooth muscle cells ■ vein graft neointimal hyperplasia ■ mouse models ■ bone marrow transplantation ■ endothelium

A utologous vein grafting ranks among the most common of surgical procedures, but its long-term success remains compromised by vein graft disease.1 Up to 30% of grafts become stenotic and require intervention within 2 years because of hemodynamically significant neointimal hyperplasia.2 Ten years after surgery, only approximately 50% of venous grafts are still patent, and only half of these are free of significant stenosis.1,3–5 Vein graft failure in humans has been intimately linked to atherosclerosis-predisposing neointimal hyperplasia, involving smooth muscle cells (SMCs) and extracellular matrix.1,3

The origin of neointimal SMCs remains incompletely characterized, despite its therapeutic implications. SMCs proliferating in the vein graft media have been shown to migrate into the neointima.6,7 In addition, arterial myofibroblasts contribute to neointimal hyperplasia in a pig endoluminal coronary artery injury model,8 and bone marrow (BM)-derived cells contribute neointimal SMCs in models of atherosclerosis9 or transplant arteriopathy.10,11 To date, however, there has been no quantitation of vein graft neointimal cell precursors.

To address this issue, we recently developed a unique murine model of vein grafting that uses sutured arteriovenous anastomoses, and neointimal hyperplasia mimics early human vein graft disease by reaching steady-state thickness without engendering significant luminal stenosis.12 In this model, we used β-galactosidase–expressing ROSA26 mice as vein graft donors and/or recipients to quantitate the extent to which the mature vein graft wall and neointima comprise cells derived from precursors residing either within or outside of the vein graft at the time of its implantation. To further differentiate the origins of graft-extrinsic cells, we replaced the bone marrow of vein graft recipient mice with that of green fluorescent protein (GFP)-expressing animals.

Methods

Animals

Adult male C57BL/6 (wild-type [WT]) mice, and congenic mice that either express Lac Z (βgal, ROSA26 strain) or GFP, were purchased from Jackson Labs. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals.

Vein Graft Surgery

Interposition vein graft surgery was performed as described previously.12 Inferior vena cavae (IVC) from C57BL/6 or ROSA26 donor mice were anastomosed to the right common carotid artery of recipient mice, yielding four different donor/graft recipient groups, as follows: (1) WT/WT; (2) WT/βgal; (3) βgal/WT; and (4) βgal/βgal. All mice were between 12 and 20 weeks old.

IVC Graft Harvest and Analysis

Grafts were harvested 2 or 6 weeks postoperatively and prepared for histochemical and immunofluorescent analysis as described previ-
souly. For analysis of β-galactosidase expression, the grafts were excised without fixation and incubated at 37°C (16 hours) in x-gal staining solution (10 mmol/L K₄Fe(CN)₆, 10 mmol/L K₃Fe(CN)₆, 2 mmol/L MgCl₂, and 500 µg/mL x-gal [5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside] in phosphate-buffered saline (PBS). Grafts were subsequently fixed in 10% formalin for 24 hours and photographed en bloc using RT SPOT 2 CCD camera (Diagnostic Instruments). Grafts were then embedded in optimal cutting temperature (OCT) compound to facilitate cryosectioning for light and immunofluorescence microscopy.

**Bone Marrow Transplantation**

C57Bl/6 mice received 950 rads of gamma irradiation at age 14 weeks and were infused with 10⁷ BM cells procured from femurs of GFP mice. Successful BM engraftment was confirmed 3 weeks later by examining blood smears. Mice thus made chimeric for BM GFP expression received WT IVC grafts 4 weeks after bone marrow transplantation (BMT). Grafts were harvested 6 weeks after operation, perfused briefly with saline, and frozen in OCT compound.

**Vein Graft Microscopic Analysis**

Immunofluorescence and immunoperoxidase staining and microscopy were performed as previously described. T cells and macrophages were identified with rabbit anti-CD3-ε antibody (DAKO) or BM8 (Research Diagnostic), respectively. Endothelial cells were identified with rabbit anti-factor VIII (DAKO). Grafts from GFP BM chimeric mice were OCT-embedded, sectioned at 5 to 10 µm, and imaged for GFP fluorescence. Immediately thereafter, sections were processed for immunofluorescence. Positive controls for CD3 and macrophage staining were murine spleen and raw 264.7 macrophages, respectively. Negative controls were incubated with cognate non-immune IgG in lieu of primary antibody. The nuclear DNA dye Hoechst 33342 (10 µg/mL) was added to secondary antibody incubations. Sequential images of single microscopic fields were taken as described. In overlays of fluorescent and bright-field images, the black background of fluorescence images was removed to facilitate identification of non-SMCs in the vein grafts.

**Quantitation of Graft Donor-Derived and Recipient-Derived Cells**

Vein graft sections from 5 graft regions were each imaged at 4 to 8 different clock hours, at ×440 magnification. Five different locations taken over the length of the graft were analyzed for each specimen. Both WT IVC grafts placed into ROSA26 recipients (WT/βgal) and ROSA26 IVC grafts placed into WT recipients (βgal/WT) were analyzed. Consequently, percent recipient-derived cells was calculated in either of 2 ways: (1) 100 × (number of x-gal-stained cells)/(number of total [ie, Hoechst-stained] cells) for ROSA26/WT specimens; and (2) 100 × [1 − (number of x-gal-stained cells)/(number of total cells)] for WT/ROSA26 specimens. More than 100 cells were counted per ×440 field. Cells were counted only within the vein graft neointima.

**Results**

To address whether vein graft SMCs derive from progenitors extrinsic to the vein graft, we placed IVC segments from ROSA26 (βgal) or C57Bl/6 (WT) donor mice as interposition grafts into the right common carotid artery of βgal or WT recipient mice (Figure 1). The ROSA26 strain is congenic to C57Bl/6, and thus transplantation of vascular grafts between these two strains does not engender graft rejection and confounding transplant arteriopathy. Therefore, this model system is well suited to assess infiltration of vein grafts by graft-extrinsic, recipient-derived cells. Grafts were harvested 6 weeks postoperatively, at a time when vein graft wall thickness and neointimal hyperplasia have reached steady-state in this model. To determine the relative contribution of βgal-expressing cells to the vein graft wall, intact vein grafts were stained en bloc for βgal expression (upper photographs, Figure 1A through 1D). WT IVCs grafted into WT recipient mice did not display any blue staining, either in whole explants (Figure 1A, bottom) or in cross-sections (Figure 1A, top), demonstrating the absence of activated macrophages in these specimens. All vein grafts involving βgal mice as either donors or recipients showed some degree of blue staining, which traversed the entire length of the graft (Figure 1B through 1D, arrowheads), and varied by specimen group (Figure 1B through 1D, lower photos). Blue cellular staining evident in the WT IVC-to-βgal recipient specimens (Figure 1C) clearly demonstrated that vein graft wall cells may derive from sources that are extrinsic to the vein graft at the time of implantation. Similarly, the prevalence of blue cellular stain-
Prevalence of SMCs among cells derived from vein grafts. A, Vein graft paraffin section was stained with a modified connective tissue stain (see Methods) and imaged at 110× original magnification. B, C, The area boxed in (A) was imaged at 440× original magnification, and a serial section was stained with Hoechst 33342 and Cy3-conjugated anti-SM α-actin. The fluorescence and light images are aligned, with their luminal surfaces upward. Images are representative of 7 different vein graft samples. D–G, Six-week-old WT IVC-to-βgal and βgal IVC-to-WT specimens were processed as in Figure 1, with the omission of eosin counterstain. Each specimen was then imaged sequentially under fluorescence (D, F) and bright-field conditions (E) to identify nuclei (D), SM α-actin–positive cells (F) and βgal-positive cells (E) within the same microscopic field. G, Images in (E) and (F) were digitally merged, as described in the Methods section, to allow identification of SMCs (red staining cells) that are graft extrinsic (blue) or graft intrinsic (non-blue). Arrow indicates graft intrinsic SMCs; arrowhead, graft extrinsic SMCs; open arrow, graft extrinsic non-SMCs.

In patients, the developing vein graft neointima predominately comprises cells expressing smooth muscle α-actin and represents an atherosclerosis prone area that forms the foundation for the later development of graft atheroma. Similarly, smooth muscle α-actin–expressing cells predominate in the neointima of murine grafts (Figure 2). Indeed, smooth muscle α-actin–expressing cells reside almost exclusively in the collagen-poor, cytoplasm-rich neointima of mouse vein isografts (Figure 2C) and rabbit jugular vein autografts, which have long been used to model human vein graft disease (data not shown). To quantify the percentage of graft-intrinsic and graft-extrinsic cells in the neointima of these murine vein grafts, we superimposed x-gal stained bright-field and red fluorescence SM α-actin–stained images of identical WT-to-βgal vein graft cross-sections (Figure 2G). Total cellularity of the sections was assessed by quantitating nuclei after incubation with Hoechst 33342 (Figure 2D). Analysis of βgal-to-WT and WT-to-βgal grafts revealed that approximately 60% of cells in the vein graft neointima develop from graft-extrinsic precursors (Table). Moreover, smooth muscle α-actin expression was evident in 66% of graft donor-recipient and 56% of graft recipient-derived neointimal cells (Table). Thus, graft-intrinsic and graft-extrinsic precursors give rise predominantly to SMCs of the mature vein graft neointima. There was only scant evidence of inflammatory cell infiltration at 6 weeks (Table). βgal IVC-to-WT grafts were analyzed in parallel and yielded congruent results.

Multiple possibilities exist for the origin of these prevalent graft-extrinsic precursor cells. It is conceivable that some of the graft-infiltrating cells derived by inward migration from the media of the adjacent carotid artery. To examine this possibility, we quantitated the percentage of graft-intrinsic and graft-extrinsic cells over the entire length of the vein graft (Figure 2H). If vascular cell migration into the graft from the anastomoses were a predominant source of graft-extrinsic precursor cells, then we would expect to see a higher prevalence of recipient cells in graft sections near the anastomoses, as compared with sections obtained from the center of the grafts (because graft-extrinsic cells constituted only 60% of total graft wall cells). The center of the vein grafts (segment 3) showed only 20% to 25% fewer recipient-derived cells than the peri-anastomotic portions of the graft (segments 1 and 5). Thus, although not excluding a contributing role of lateral-migrating arterial SMCs to the formation of the βgal IVC-to-WT recipient specimens (Figure 1B) was substantially less than that obtained in βgal IVC-to-βgal recipient specimens (Figure 1D). Thus, the contributions of graft recipient (ie, graft-extrinsic) cells to the vein graft wall were observed in WT IVC-to-βgal and βgal IVC-to-WT specimens.

Shown is a single microscopic field from one WT IVC-to-βgal specimen, representative of 4 samples analyzed from n = 5 mice in each group. Original magnification ×440; scale bar in (G) 100 μm. H, The percent graft-extrinsic cells was calculated as in Methods for WT IVC-to-βgal and βgal IVC-to-WT specimens (n = 5 for each group) at 1-mm intervals across the length of the grafts, as indicated. Plotted at each location are the mean±SE. Scale bar is 200 μm (A) and 100 μm (B, G).
of vein graft lesions, this analysis does not support the notion of a predominance of anastomotic vascular cells in the population of cells originating outside of the graft.

To address the origin of graft-extrinsic cells further, we examined serial sections of 2-week-old vein grafts, in which the media and neointima are still enlarging. We reasoned that if returned again to an analysis of mature, 6-week-old vein cellular expansions observed in these immature grafts, we would be able to examine the origin of the focal mitosis. Therefore, to examine the origin of the focal cellular expansions observed in 2-week-old specimens, isolated foci of cells are evident in many sections—even in sections remote from the anastomoses (Figure 3A). These foci show numerous actively proliferating cells, as assessed by staining for proliferating cell nuclear antigen (PCNA) (Figure 3C, red arrow). This cell proliferation distinguishes vein graft proliferative foci from adjacent cells that constitute part of postsurgical adhesions (Figure 3, yellow arrow). Staining with smooth muscle α-actin reveals that these foci are composed of SMCs and non-SMCs. These focal, cellular expansions of the graft wall could, of course, originate from either graft-extrinsic or graft-intrinsic cells. If these foci represent expansions of recipient-derived cells, however (please see later), then this finding suggests that graft-extrinsic precursor cells do infiltrate the graft and originate from sources distinct from the peri-anastomotic carotid artery—either the circulating blood or adventitial adhesions. Unfortunately, x-gal staining of these foci was inadequate to determine whether their progenitor cells originated in the graft donor or recipient—perhaps because insufficient βgal is present in these cells undergoing relatively rapid mitosis. Therefore, to examine the origin of the focal cellular expansions observed in these immature grafts, we returned again to an analysis of mature, 6-week-old vein grafts in which remodeling has ceased. We reasoned that if focal cellular expansions in the arterialized vein graft enlarge to constitute the mature graft wall, then these mature vein grafts should evince focal staining for donor-derived and recipient-derived cells. This expectation was fulfilled in our 6-week-old vein grafts stained with x-gal (Figure 3D). Quantitatively, graft-extrinsic neointimal foci demonstrated a 60% relative increase in graft-extrinsic cells, compared with the neointima taken as a whole (Figure 3E). Thus, focal cellular vein graft wall expansions observed in 2-week-old specimens almost certainly did develop from both graft-intrinsic and graft-extrinsic precursor. The focal cellular nodules (remote from anastomoses) in 2-week-old vein grafts and focal expansions of graft-intrinsic and graft-extrinsic cell popula-
tions in mature vein grafts together support a model in which graft-extrinsic cells originate from precursors entering the graft via a transendothelial and/or transadventitial route in addition to cells entering the vein graft via migration from the anastomoses. What is the derivation of the graft extrinsic cells that do not originate from the adjacent carotid artery? Recent interest and controversy has focused on the role of BM-derived cells in vascular repair. To examine the potential contribution of BM-derived circulating precursor cells to vein graft remodeling, we performed vein graft surgeries in C57Bl/6 WT recipient mice that had their BM replaced with that of congenic GFP-expressing mice. Six-week-old mature grafts harvested from these chimeric mice showed prominent, albeit inhomogeneous, infiltration of BM-derived cells into the media and adventitia, but notably not the neointima, of the grafts (Figure 4C). These BM-derived cells comprised primarily cells of the macrophage lineage and did not express SMC/actin (Figure 4D).

Vein graft remodeling is initiated, in part, by hemodynamic and ischemic injury to the endothelium of the graft. Re-endothelialization is thought to play an important part in limiting vascular remodeling. We therefore sought to determine the origin of the endothelial cells that participate in vein graft remodeling. In WT IVC grafts that were placed into gal recipients and stained with x-gal, we found evidence of graft-intrinsic (non-stained) and graft-extrinsic (blue-stained) endothelial cells (Figure 5A and 5B). In addition, when we examined WT grafts that were placed into GFP BM chimeric recipients (Figure 5C through 5F), we found a small prevalence (≈10%) of GFP-positive cells among the vein graft endothelial cells. This indicates that re-endothelialization in this model derives from multiple sources, including cells of the graft donor and endothelial progenitor cells that originate from BM intrinsic and extrinsic compartments of the graft recipient. These findings thus contrast with those of Xu et al, who recently used Tie2-driven gal expression to show that, in their vein grafts, endothelium is essentially replaced by recipient cells. Hemodynamic differences between vein...
The finding that vein graft neointima formation proceeds without bone marrow contribution to a large part from graft extrinsic cells distinguishes vein graft remodeling from other forms of arterial remodeling. In arterial remodeling associated with transplant arteriopathy and mechanical injury, BM-derived cells contribute to neointimal hyperplasia. Vascular progenitor cells have been isolated from other organs as well. Majka et al demonstrated the potential for skeletal muscle-derived progenitor cells to differentiate into endothelial cells as well as SMCs. This study also demonstrated the slow replacement of skeletal progenitor cells by cells derived from the bone marrow. Thus, our data support the notion that vascular injury elicits divergent repair responses, dependent on the type of injury inflicted.

Approximately 30% of our vein graft neointimal cells did not express SM α-actin and may comprise fibroblasts or fibrocytes. Adventitial fibroblasts have been reported to migrate into the neointima of balloon-injured rat and pig arteries, and ultimately transdifferentiate into myofibroblasts that express SM α-actin. Because adventitial fibroblasts in our model could originate from the vein graft adventitia itself or the peri-graft (postsurgical) adhesions, these fibroblasts could derive from either graft-intrinsic or graft-extrinsic cells. Moreover, graft-extrinsic fibroblasts originating from surgical adhesions need not originate from the BM—consistent with the absence of neointimal GFP fluorescence in grafts from BM-chimeric mice (Figure 4).

The novel finding of focal proliferation in arterializing vein grafts (Figure 3) recalls data regarding the clonal origins of fibrous plaques in atherosclerosis. Proliferative cellular foci in vein grafts were demonstrable by congruent data from 2- and 6-week-old vein grafts (Figure 3), which evince foci originating from either graft-intrinsic or graft-extrinsic precursor cells. Whether these distinct foci of cells represent clonal, or rather colonial, cell growth currently transcends the scope of this investigation.

Because most vein graft neointimal cells derive from precursors extrinsic to the vein graft, we can make novel mechanistic inferences about successful therapies for vein graft neointimal hyperplasia, like the E2F decoy. Successful therapies must, of course, target proliferation, migration, and extracellular matrix secretion by cells intrinsic to the implanted vein graft, because these cells ultimately constitute 40% of neointimal cells. In addition, however, these therapies must target recruitment to the graft of cells initially extrinsic to the graft, perhaps by reducing the inflammatory reaction associated with vein graft endothelial damage and/or the chemokine/growth factor secretion by vein graft cells.

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References


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