Venous Identity Is Lost but Arterial Identity Is Not Gained During Vein Graft Adaptation

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Objectives—Ephrin ligands and Eph receptors are signaling molecules that are differentially expressed on arteries and veins during development. We examined whether Eph-B4, a venous marker, and Ephrin-B2, an arterial marker, are regulated during vein graft adaptation in humans and aged rats.

Methods and Results—Eph-B4 transcripts and immunodetectable protein are downregulated in endothelial and smooth muscle cells of patent vein grafts in both humans and in aged rats, whereas Ephrin-B2 transcripts and protein are not strongly induced. Other markers of arterial identity, including dll4 and notch-4, are also not induced during vein graft adaptation in aged rats. Because VEGF-A is upstream of the Ephrin–Eph pathway, and expression of VEGF-A is induced only at early time points after exposure of the vein to the arterial environment, we inhibited VEGF-A in vein grafts using an siRNA-based approach. Vein grafts treated with siRNA directed against VEGF-A demonstrated a thicker intima-media containing α-actin, consistent with arterialization, but did not contain Eph-B4 or Ephrin-B2.

Conclusions—Venous identity is preserved in the veins of aged animals, but is lost during adaptation to the arterial circulation; arterial markers are not induced. Markers of vessel identity are plastic in adults and their selective regulation may mediate vein graft adaptation to the arterial environment in aged animals and humans. (Arterioscler Thromb Vasc Biol. 2007;27:1562-1571.)

Key Words: vein graft adaptation ■ venous identity ■ Eph-B4 ■ Ephrin-B2 ■ remodeling ■ smooth muscle cells

Veins are the most commonly used conduit for coronary and peripheral artery bypass; placement of a vein into the higher pressure and flow of the arterial circulation results in adaptation of the vein to the arterial environment, a process termed vein graft “arterialization” or “adaptation.”1,2 Successful vein graft adaptation is a complex process that includes cell proliferation and migration, programmed cell death, and changes in production and degradation of the extracellular matrix as well as coordinating growth factors, producing a thicker walled vessel.3–7 Some of the signal transduction pathways that are rapidly stimulated in the venous endothelium on exposure to the increased magnitude of pressure and shear stress in the arterial environment include activation of the mitogen-activated protein kinase,8 the phosphatidylinositol-3-kinase-Akt,9,10 and the vascular endothelial growth factor (VEGF) pathways.11,12 We have previously demonstrated that VEGF stimulates endothelial proliferation, migration, and actin reorganization by the Akt-nitric oxide pathway,13 and others have shown that VEGF inhibits vein graft intimal thickening in rabbits.14

The natural history of normal human vein graft adaptation is incompletely described, as excision of patent vein grafts that have adapted well to the arterial circulation is not ordinarily performed for ethical reasons; furthermore, reported specimens are often taken from abnormal or failing vein grafts.15 In addition, although in vitro and in vivo models of vein graft adaptation to the arterial circulation are well described, the regulation of this complex process by factors unique to aged hosts, which are relevant to elderly patients undergoing bypass surgery, is not well understood. Similarly, many of the in vitro models, noted above, have only examined arterial endothelial responses to shear stress, not venous endothelial responses.

Advances in developmental biology have demonstrated that there are genetic predeterminants of arterial and venous endothelium, and the molecular distinction between arteries and veins is becoming established.16 How genes are regulated during vein graft adaptation is beginning to be explored13,17,18; however, there are little data regarding regulation of genes associated with arterial or venous identity, in postnatal adults and aged animals. The Ephrin ligands and Eph receptors are an important class of signaling molecules that are differentially expressed on arteries and veins.19 Ephrin-B2 is a marker...
of arterial endothelium and smooth muscle and persists into adult vessels.\textsuperscript{20–22} Conversely, Eph-B4 is a marker of venous cells.\textsuperscript{22,23} Although it is not currently reported whether Ephrin-B2 or Eph-B4 expression in vessels is regulated during normal or pathological aging, nor during vein graft adaptation, it is likely that altered patterns of Ephrin or Eph expression may play a role in vein graft adaptation.

To establish the pattern of Ephrin-B2 and Eph-B4 gene expression during normal human vein graft adaptation, we report the analysis of several rare specimens of patent human vein grafts. We also describe an in vivo rat model of vein graft adaptation that considers host age and shows a similar pattern of Ephrin-B2 and Eph-B4 expression in remodeled rat and human vein grafts. Because VEGF-A may be an upstream regulator of Ephrin-B2 and Eph-B4,\textsuperscript{24,25} these results also suggest a mechanistic role for VEGF-A during vein graft adaptation in aged hosts.

### Methods

#### Human Vein Grafts

Patent saphenous vein grafts were explanted from human patients undergoing cardiac transplantation, or from patients with peripheral bypass undergoing amputation for distal ischemia without infection or a thrombosed vein graft. Vein grafts were fixed in 10\% formalin. Approval of the institutional Human Investigation Committee was obtained.

#### Animal Vein Graft Model

Young adult (6-month) and aged (24-month) male Fischer 344 strain rats (380 to 430 gm) were obtained from the National Institute of Aging Rodent Colony (Bethesda, Md).\textsuperscript{26} Rats were raised in pathogen-free conditions at the NIA and housed locally at least 1 week before surgery. All experiments were approved by the Institutional Animal Care and Use Committee.

Aged and young adult rats underwent autologous jugular vein to carotid artery reverse interposition grafting using intraperitoneal ketamine/xylazine/acepromazine anesthesia. Briefly, the distal right carotid artery reverse interposition grafting using intraperitoneal ketamine/xylazine/acepromazine anesthesia. Briefly, the distal right carotid artery was dissected and side branches were ligated via a midline neck incision. An 8-mm segment of vein was harvested and sutured into the divided ipsilateral common carotid artery using interrupted 10–0 nylon suture. After measurement of diameter and flow, the wound was closed. No heparin was used during the procedure.

Vein grafts were harvested at 6 to 72 hours, or 7 or 21 days after surgery using accepted euthanasia technique. After exsanguination under anesthesia, vein grafts were flushed with heparinized saline and fixed in 10\% formalin. No heparin was used during the procedure.

### Hemodynamic Measurement

Because anesthesia using xylazine can produce significant reduction in cardiac output,\textsuperscript{27} xylazine and acepromazine were avoided for these measurements, and the combination of ketamine (100 mg/kg) and diazepam (10 mg/kg) was used. Mean arterial pressure (MAP) was measured by catheterization of the right femoral artery and recorded continuously; the external zero reference was placed at the level of the heart. Cardiac output (CO) was measured by the thermodilution technique as previously reported.\textsuperscript{28}

### Functional Study

Jugular veins, vein grafts, and carotid arteries were carefully exposed and quickly excised. Vessels were mounted in a myograph (Danish Myo Technology) by cannulating both ends and placed in modified Krebs-Ringer solution (pH 7.4, 37°C, 95\%O\textsubscript{2}/5\%CO\textsubscript{2}). Vessel diameters were determined by measuring the external diameters; the pressure at both ends of the vessel was monitored using 2 pressure transducers, placed equidistantly at the vessel ends, and the mean of these pressures is representative of the lumen pressure. Vessels were equilibrated for 30 minutes, and the pressure was increased in step-wise fashion from 0 to 200 mm Hg, both in regular and calcium-free buffer containing EGTA (2 mmol/L). Cross-sectional compliance was calculated as \(((\pi\times D^2/4)-(\pi\times D^2/4))\times(P_1-P_0)\) for each step in pressure.

### Immunohistochemistry

Five-micron cross sections were taken from the center of the graft, and staining was performed for Hematoxylin & Eosin, Masson trichrome, and van Gieson elastin, as well as immunohistochemistry for von Willebrand factor (vWF), alpha smooth muscle actin, calponin, sm22 alpha, PCNA, monocyte/macrophage (ED1), and Nogo-B. Antigen retrieval was performed using 10 mmol/L citrate buffer at pH 6.0. Sections were counterstained with Mayer’s Hematoxylin. Negative control specimens were included for all groups.

### Immunofluorescence

Samples were fixed with 4\% paraformaldehyde, embedded in OCT, and sections cut at 5 \micron thickness. Primary antibodies included Ephrin-B2, Eph-B4, and alpha actin. Alexa Fluor 488 and 568 were used for fluorescence. All samples were treated with autofluorescent eliminator reagent (Chemicon) and counterstained with DAPI. Images were captured with an Axioimager A1 (Carl Zeiss) under identical conditions.

### Morphometry

Vessel cross sections were measured for intima, media, and total vessel thickness in at least 8 sections, using NIH ImageJ software. Areas of positive immunostaining were measured using image analysis (MetaMorph software) in blinded fashion. Relative density was graphed in arbitrary units.

### RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was isolated from each vessel with TRIzol Reagent (Invitrogen) and was cleaned with the RNeasy Mini kit (Qiagen). For quantification of total RNA, each sample was measured using the Ribogreen RNA assay kit (Molecular Probes), and RNA quality was confirmed by 1.2% formaldehyde-agarose gel electrophoresis. Reverse transcription was performed using the SuperScript III First-Strand Synthesis Supermix kit (Invitrogen). Primers were used as previously described.\textsuperscript{29,30} Real-time quantitative polymerase chain reaction (PCR) was performed by using SYBR Green Supermix (BioRad) and amplified with the iQ5 Real-Time PCR Detection System (BioRad). All samples were confirmed by sequence analysis and 2\% agarose gel electrophoresis for correct target amplification and exclusion of non-specific amplification. All samples were normalized to GAPDH amplification and graphed in arbitrary units, setting the amount of GAPDH amplification to 1.0.

### VEGF siRNA Transfection In Vitro and In Vivo

RNA oligonucleotides were synthesized by Dharmacon using the siRNA sequence for rat VEGF-A AUGUGAUGCGACGACAAA-GAA as previously described.\textsuperscript{31} The control RNA primer was siCONTROL Nontargeting siRNA #1 (Dharmacon). For in vitro testing, rat aortic smooth muscle cells (SMCs) were isolated from male adult rats using the collagenase technique, seeded at a density of 1×10\textsuperscript{5} cells/mL in complete growth medium on 24-well plates, and were exposed, at \~60\% confluence, to VEGF siRNA/polyethyleneimine (PEI; Polys plus transfection) (100 mmol/L), or nontargeting siRNA/PEI, in the presence of Opti-MEM I reduced serum medium. After overnight transfection, cells were exposed to hypoxia (1.2\% O\textsubscript{2}/94\% N\textsubscript{2}/5\% CO\textsubscript{2}), lipopolysaccharide (LPS; 100 ng/mL), or deferoxamine (50 \mu mol/L) for 48 hours. The supernatant was taken from control and treated cells and analyzed for VEGF secretion by ELISA.
For in vivo experiments a mixture of 70 μg/H9262 L VEGF siRNA/PEI at N/P ratio of 10 (50 μg/H9262 g of siRNA/11001 10/192 μL of PEI) was dissolved in 130 μL of 30% pluronic F-127 gel (Invitrogen) in 5% glucose solution. The complex (200 μL) was applied to the surface of the vessel after completion of the jugular vein anastomoses and restoration of flow, or directly on control vessels. The control vein graft groups were treated with either saline, 200 μL pluronic gel, or 200 μL of nontargeting siRNA/PEI/pluronic gel. Control vessels that were transfected in vivo were harvested after 48 hours; vein grafts were harvested after 21 days.

Statistical Analyses

Results are reported as mean±SEM. Groups were compared by analysis of variance. All tests were 2-tailed and probability values ≤0.05 were considered statistically significant (Statview 5.0, SAS Institute).

Results

Human Vein Graft Adaptation Is Associated With Decreased Eph-B4

To describe the natural history of normal human vein graft adaptation, we examined several patent human vein grafts, derived from patent coronary or peripheral vein grafts that were explanted for medical reasons unrelated to the graft (n=4). Representative sections of these patent vein grafts, as well as control saphenous veins, are shown in Figure 1A. Saphenous veins typically used for vein graft bypass often demonstrate, before surgical implantation as a bypass graft, a basally thickened intimal layer (I), above the internal elastic lamina (IEL, yellow arrowheads; first column of supplemental Figure IA, available online at http://atvb.ahajournals.org). Vein graft adaptation in patent human vein grafts results in significantly more thickening of all vessel layers compared with preimplantation saphenous veins, although most noticeably in the thickened intima (TI; second column of Figure 1A). The intima of preimplantation saphenous vein does not demonstrate strong immunostaining for smooth muscle α-actin, SM22α, calponin, or Nogo-B (first column of Figure 1B and supplemental Figure IB). However, patent human vein grafts demonstrate significantly more immunostaining for intimal SMCs, staining strongly positive for α-actin, SM22α, and calponin, compared with the saphenous veins (second column of Figure 1B and supplemental Figure IB). In addition, we examined Nogo-B, a protein that is downregulated in injury-associated neointima,32 in the patent human vein graft; intimal SMCs demonstrated increased immunoreactive Nogo-B (supplemental Figure IB). The increased density of intimal α-actin and Nogo-B is quantified in supplemental Figure IC. CD45 leukocytes were only sparsely found in the adventitia of the saphenous vein and vein graft, without significant accumulation in the patent vein grafts (data not shown), consistent with lack of ongoing inflammation in patent vein grafts.

Because Ephrin-B2 is a marker of arteries and Eph-B4 is a marker of veins that have been described during embryonic and young animal development,19–23 we examined whether these markers were present on saphenous veins and patent vein grafts in adult humans. Eph-B4, a venous marker, was expressed in the endothelium of saphenous veins and strongly expressed in the media, and was significantly diminished in vein grafts placed in the arterial circulation; Ephrin-B2, an arterial marker, was weakly detectable in saphenous vein...
endothelium and media and not induced in patent vein grafts (higher magnifications in Figure 1C); Ephrin-B2 was detectable in human arteries (data not shown). Both Ephrin-B2 and Eph-B4 in these specimens, where detected in the media, colocalized with α-actin+ SMC (yellow color in Figure 1C). These immunostaining changes are quantified in Figure 1D (left panel). To determine whether reduced Eph-B4 immunostaining reflected reduced gene expression, we used quantitative PCR to examine transcripts for Eph-B4 and Ephrin-B2; similar to the changes in immunodetectable protein, there was reduced transcript expression of Eph-B4 and no induction of Ephrin-B2 (Figure 1D, right panel). These results are consistent with strongly diminished expression of the venous phenotype marker Eph-B4 and no induction of the arterial phenotype marker Ephrin-B2 during normal human vein graft adaptation.

**Vein Graft Adaptation in Aged Rats Mimics Human Vein Graft Adaptation**

To establish our in vivo model, the distal jugular vein of aged (22 to 24 months) Fischer 344 rats was placed as an autologous interposition graft into the ipsilateral carotid artery, and explanted and examined after 7 or 21 days (Figure 2). Figure 2A demonstrates early cellular infiltration at 7 days, depicted by H&E staining, and intima-media thickening at both 7 and 21 days after implantation of the vein into the arterial circulation. The intima and media are measured together as the rat jugular vein has no well-defined IEL, only a single “external” elastic lamina (EL) between the media and adventitia, as noted with the yellow arrowheads (supplemental Figure II); the intima-media in this model is analogous to the human thickened intima and media layers in human patent vein grafts (Figure 1A). Figure 2B demonstrates quantitative analysis of remodeling in this model, with increased thickness of the vein graft intima-media at 7 and 21 days. Young adult (6 months) rats were similarly used to place interposition vein grafts in the carotid artery; however, there was less cellular infiltration at 7 days and a thinner intima-media layer at both 7 and 21 days compared with that present in aged rats (supplemental Figure II, bottom panels).

The thickened intima-media of aged rats, compared with young adult rats, occurred under identical hemodynamic conditions in the different age animals, with no differences in graft diameter, flow, or shear stress either immediately after implantation or after 21 days of adaptation (Table). In addition, aged and young adult rats had similar heart rates (377±24 bpm versus 376±6 bpm; P=0.96), blood pressure (116±10 mm Hg versus 113±1 mm Hg; P=0.83), and cardiac output (129±11 mL/min versus 110±13 mL/min; P=0.31). Compliance of the vein grafts after 21 days was similar in aged and young adult rats, under both venous (low pressure) and arterial (high pressure) conditions (Table). There was no difference in mean serum values of white blood cell count, hematocrit, or platelets, nor of creatinine, total protein, or albumin, between aged and young adult rats (data not shown).

Supplemental Figure III demonstrates the preservation of the endothelial integrity, with immunostaining for vWF, at 7
and 21 days in this model; preservation of the endothelial monolayer demonstrates that intima-media thickening in this model reflects thickening of the media. Also seen are the presence of underlying SMCs at both 7 and 21 days using several SMC markers of the contractile phenotype, including α-actin, SM-22α, and calponin, as well as Nogo-B. At 21 days, the thickened intima-media of the vein graft has several layers of SMCs, consistent with mature SMCs and the process of vein graft adaptation to the arterial environment. In addition, there are rare α-actin–containing cells in the vein graft adventitia after 21 days; vein grafts in young adult rats demonstrated the presence of SMC markers only in the adventitia of the vein grafts (data not shown). Supplemental Figure III (fifth row) also shows that Nogo-B is detectable in the adventitia of the vein grafts (data not shown).

To confirm the decrease in Eph-B4 gene expression and lack of stimulation of Ephrin-B2 gene expression during vein graft adaptation in aged rats, we used immunofluorescence to confirm the localization and changes of these markers. Ephrin-B2, an arterial marker, was weakly diminished, but not completely eliminated, in the intima-media of vein grafts placed in the arterial circulation (Figure 3C); Eph-B4 was strongly diminished, but not completely eliminated, in the intima-media of vein grafts placed in the arterial circulation (Figure 3B); Ephrin-B2 was detectable in both the endothelium and the media of the aged rat carotid artery (Figure 3B). These results, strongly diminished detection of Eph-B4 and little as expected; the vein graft had less detectable Eph-B4 than the jugular vein and similar amounts as expressed in the carotid artery (Figure 3A), suggesting downregulated expression of transcripts for the venous marker Eph-B4 during vein graft adaptation in aged rats. The arterial marker Ephrin-B2 was detectable in higher amounts in the carotid artery compared with the jugular vein; however, no increase in Ephrin-B2 mRNA was detectable in the vein graft compared with jugular vein (Figure 3A). As a control experiment, because expression of Ephrin-B2 is not induced during vein graft adaptation, we confirmed that Ephrin-B2 expression is detectable in cultured rat arterial SMCs. RT-PCR was performed using mRNA isolated from rat arterial SMC; Ephrin-B2, an arterial marker, but not Eph-B4, a venous marker, was detectable in cultured cells (data not shown).

To confirm the decrease in Eph-B4 gene expression and lack of stimulation of Ephrin-B2 gene expression during vein graft adaptation in aged rats, we used immunofluorescence to confirm the localization and changes of these markers. Eph-B4, a venous marker, was expressed in aged rat jugular veins (Figure 3B), both in the endothelium and the α-actin–positive medial SMCs (Figure 3C); Eph-B4 was strongly diminished, but not completely eliminated, in the intima-media of vein grafts placed in the arterial circulation (Figure 3B and 3C). Ephrin-B2, an arterial marker, was weakly detectable in the endothelium and medial SMCs of aged rat jugular veins and not strongly induced in vein grafts (Figure 3B); Ephrin-B2 was detectable in both the endothelium and the media of the aged rat carotid artery (Figure 3B). These results, strongly diminished detection of Eph-B4 and little
induction of Ephrin-B2 in rat vein grafts, confirm the similar results seen with gene expression (Figure 3A) and are consistent with strongly diminished expression of venous phenotype markers and no induction of arterial phenotype markers during normal human vein graft adaptation (Figure 1C and 1D). During vein graft adaptation in young adult rats, changes in Eph-B4 immunofluorescence were more limited, with less inhibition of Eph-B4 expression and limited to adventitial SMC in vein grafts (data not shown).

Because the expression of Ephrin-B2, a marker of arterial identity, is not induced by 21 days during vein graft adaptation, we determined whether there were changes in expression of other markers of arterial identity, including dll-4, jagged-1 and -2, and notch-1, -3, and -4. There were no significant changes in expression of any of these other markers of arterial identity, compared with expression levels in the preimplantation vein (Figure 3D). These results confirm that expression of markers of arterial identity is not stimulated during vein graft adaptation in aged rats.

VEGF-A Is Required for Diminished Eph-B4 Expression in Vein Grafts

Because VEGF-A can stimulate expression of the Ephrin pathway members, we determined whether VEGF-A expression was detectable during vein graft adaptation. Although VEGF-A expression was induced during vein graft adaptation by 6 hours of implantation, there was strongest expression of VEGF-A at 24 hours after implantation that was afterward downregulated by 72 hours (Figure 4A); diminished VEGF-A expression at 72 hours was coincident with diminished Eph-B4 expression (Figure 4B). VEGF-A expression remained decreased at 21 days (Figure 4C), coincident with continued diminished Eph-B4 expression at 21 days (Figure 3A); expression of VEGFR2 transcripts was unchanged at 21 days (Figure 4C). These results suggest that VEGF-A gene expression is induced early in vein graft adaptation, but that its expression is downregulated during later phases of vein graft adaptation in aged rats, and that VEGF-A downregulation during later phases may be associated with intima-media thickening.

To determine whether VEGF-A plays a mechanistic role during vein graft adaptation in aged rats, we used an siRNA-based approach to reduce VEGF-A expression, and test whether early reduction in VEGF-A levels would change later Eph-B4 expression and the extent of intima-media thickening in vein grafts. The specificity of an siRNA targeting VEGF-A was determined by the ability to reduce
VEGF-A secretion in rat aortic SMC under 3 different stimuli that normally induce VEGF-A secretion.\textsuperscript{35} Rat aortic SMCs were transfected with this siRNA using polyethylenimine, or control noncoding, scrambled siRNA; transfection efficiency was directly counted and found to be approximately 98% (supplemental Figure IVA). VEGF-A secretion was reduced compared with control siRNA for each of these 3 stimuli (supplemental Figure IVB), confirming the specificity and function of the siRNA in vitro. In addition, we confirmed the ability of this siRNA to inhibit VEGF-A expression in vivo; siRNA dissolved in pluronic gel was placed on the adventitia of carotid arteries and found to diminish endogenous expression of VEGF-A by approximately 40% at 48 hours (supplemental Figure IVC).

siRNA directed against VEGF-A, or noncoding control siRNA, was dissolved in pluronic gel and placed on the adventitia of newly surgically placed vein grafts; grafts were harvested 21 days later. Consistent with the hypothesis that Eph-B4 is downstream of VEGF-A, siRNA directed against VEGF-A almost completely prevented detection of what little immunoreactive Eph-B4 was still present after 21 days (higher magnification and exposure, Figure 5A, first row). However, grafts treated with siRNA that inhibits VEGF-A also had greatly increased intima-media thickness compared with grafts treated with pluronic gel alone or control siRNA (Figure 5A, second row); this intima-media stained positively for α-actin (Figure 5A, third row). Quantitative morphometric data are shown in Figure 5B and confirms that VEGF-A knockdown results in increased intima-media thickening. These results suggest that early expression of VEGF-A is a mechanism by which vein grafts downregulate Eph-B4 expression during long-term adaptation to the arterial environment.

Discussion

We demonstrate that intima-media thickening during vein graft adaptation to the arterial environment is associated with loss of the venous phenotype marker Eph-B4, but without induction of expression of the arterial phenotype marker Ephrin-B2, in both human and aged rats. Patent human vein grafts normally develop intimal, medial, and adventitial thickening in response to arterial pressure and shear stress, and the increased number of intimal-medial SMC stain positively for α-actin, SM-22α, calponin, and Nogo-B. Aged rats and humans express similar changes of Ephrin-B2 and Eph-B4 expression in remodeled rat and human vein grafts, suggesting that loss of venous identity is a crucial mechanism in vein graft adaptation; early expression of VEGF-A may mediate these long-term changes during vein graft adaptation. These findings also suggest that vascular adaptation in elderly humans and animal models may not exactly recapitulate changes in gene expression that occur during embryogenesis, but that selective expression of subsets of vascular determinant genes may be adequate to mediate plasticity in the adult vascular system.

Using patent human vein grafts, we determine that human vein graft adaptation is associated with intimal thickening, largely comprised of mature SMCs that spatially localize with decreased Eph-B4 expression, and show little induction of Ephrin-B2 expression (Figure 1C and 1D). These results suggest that vein graft adaptation is largely associated with loss of venous identity, rather than stimulation of arterial identity. Because a shift from venous to arterial identity has been associated with pathological transformation, such as the induction of Ephrin-B2 expression during development of Kaposi sarcoma and hepatocarcinoma, increased expression of arterial determinants may not be consistent with or necessary for normal vein graft adaptation.\textsuperscript{36,37} Our finding that Ephrin-B2 mRNA is reduced during human vein graft adaptation, whereas the protein is unchanged, may reflect biological variation in our small number of samples or potential differences in time points between specimen collection; however, both rat and human data show that Ephrin-B2 expression is not induced. The consistency of Eph-B4 mRNA and protein downregulation in both human and rat vein graft adaptation suggests the utility of the rat as a model for the human process. As additional markers of arterial and venous identity are described, and these markers have specificity for the rat, they could be used to more completely characterize
vein graft adaptation. For example, neuropilin-1 has a similar distribution to arteries and Ephrin-B2 in the chick embryo, whereas neuropilin-2 has a similar distribution to veins. However, in our rat model, both neuropilin-1 and neuropilin-2 are strongly induced during vein graft adaptation, in both young adult and aged rats (data not shown).

Nonetheless, our finding that Eph-B4 is downregulated during vein graft adaptation in humans and aged animals suggests that determinants of vessel identity expressed during embryogenesis may be plastic, even in aged adults.

We demonstrate a difference in vein graft adaptation between young adult and aged rats, with increased intima-media thickening in aged rats compared with young adult rats (Figure 2). In addition, aged rats do not show the adventitial α-actin–positive cells (“adventitial myofibroblasts”) that are seen in young adult rats. Because we do not examine late time points, because of the mortality of aged rats, it is possible that young adult rats might develop similar intima-media thickening at later time points. However, our finding that intima-media thickening in aged rats is associated with incomplete induction of several members of the Notch pathway, such as dll-4 and notch-4, and Ephrin-B2 expression (Figure 3A and 3D), compared with strong induction in young adult rats (data not shown), suggests that 21 days is an adequate end point to assess changes in gene expression during vein graft adaptation. Because changes in morphology and gene expression during vein graft adaptation in aged rats appears similar to those seen in human vein graft adaptation, we believe that
using aged rats as a model for humans is reasonable, although limited because of rat specificity of many reagents as well as the inability to perform complex genetic manipulations in the rat.

Our finding that VEGF-A expression is induced at 24 hours and downregulated at 21 days is consistent with the data from Westerband et al.6 Strong induction of VEGF-A expression at 24 hours is consistent with the interpretation that early expression of VEGF-A mediates normal vein graft adaptation, and provides rationale to inhibit expression at early time points using an siRNA-based approach. Using this approach we demonstrate that early inhibition of VEGF-A in vivo leads to even further decreased detection of Ephrin-B2 (data not shown) and Eph-B4 (Figure 5A) during long-term vein graft adaptation, which is not surprising as Ephrin-B2 and Eph-B4 are downstream of VEGF-A in other reports. However, we find that intima-media thickening at 21 days is strongly induced by early inhibition of VEGF-A and the concomitant later decrease in Eph-B4. This finding leads us to speculate that long-term changes in Eph-B4 expression may inhibit intima-media thickening, or that Ephrin-B2 may stimulate intima-media thickening; because Ephrin-B2 is not detectable (data not shown), we favor the former explanation. The finding that diminished VEGF-A gene expression during long-term vein graft adaptation (Figure 4C) is associated with intima-media thickening is consistent with previous reports demonstrating that exogenous VEGF-A inhibits intima-media thickening in vein grafts.14

It is possible that the Notch pathway may mediate the changes of VEGF-A on Eph-B4 expression. This is consistent with in vitro data showing that VEGF-A downregulates Eph-B4 expression in HUVECs by the Notch pathway.37 We also find that the Notch pathway is induced in young adult rats, consistent with its downstream stimulation by VEGF-A, but that expression of the Notch ligand dll-4 and the Notch receptor notch-4 are not induced in aged rats (Figure 3D). Age-dependent differences in activation of Notch has been previously described, and our data are consistent with this finding.40,41 Because Ephrin expression can be induced by members of the Notch pathway,37,42 it is possible that the effects of VEGF-A during vein graft adaptation are at least partially mediated by the Notch pathway. In addition, the diminished induction of the Notch pathway in aged rats, compared with the induction in young adult rats, may enhance diminution of Eph-B4 and promote vein adaptation to the arterial environment.34

Acute exposure to increased pressure and shear stress stimulates endothelial cell signal transduction in multiple in vitro and in vivo models.8–11,13 This work has led to the recognition of the importance of hemodynamic factors in determining endothelial phenotype.44 Kwei et al have reported endothelial expression of E-selectin and vascular cell adhesion molecule (VCAM)-1 as early characteristics of vein graft adaptation to arterial flow.7 Although human data remains elusive, early dog models of vein graft adaptation demonstrate that preservation of a functional endothelial monolayer during vein graft adaptation is a critical determinant of vein graft function.1,2 The importance of the endothelial monolayer to graft patency has been demonstrated by the ability of this monolayer to provide superior patency for prosthetic grafts.45 Because chronic exposure to arterial magnitudes of shear stress increases the ability of endothelial cells lining vascular grafts to adhere to their underlying substratum,46,47 our finding of a preserved endothelial monolayer in our model (supplemental Figure III) suggests that endothelial cells may be the source of early VEGF-A expression in vein grafts (Figure 4). This is also consistent with our finding of a confluent monolayer in patent human vein grafts (supplemental Figure 1A), as well as downregulated expression of the VEGF receptor (Figure 4C). The endothelial monolayer may also directly stimulate Eph-B4 downregulation, potentially by a shear stress–induced mechanism directly sensed by endothelial cells; however, it is also possible that smooth muscle cells directly downregulate Eph-B4 by a pressure- or stretch-induced mechanism. Little is known regarding regulation of Eph-B4 expression in adult cells, whereas Ephrin-B2 is expressed during adult angiogenesis, both in endothelial cells as well as in smooth muscle cells.20 Although expression of Ephrin-B2 on aortic endothelial cells is modified by contact with smooth muscle cells,25 we demonstrate that expression of Ephrin-B2 is not induced during vein graft adaptation in spite of accumulation of SMC below the endothelium (Figures 1 and 3). The relative importance of Eph-B4 in venous adaptation to the arterial environment suggests that selective expression of vascular determinant genes mediates plasticity of the adult vascular system even though these changes do not entirely recapitulate those that occur during embryogenesis. However, definitive genetic determination of whether the endothelium is the source of VEGF-A, and downstream Ephrin signaling, is currently beyond the capability of the rat model.

In conclusion, we demonstrate for the first time that vein graft adaptation results in loss of venous identity, but not in gain of molecular markers of arterial identity. Our work supports the plasticity of vascular markers and demonstrates the modulatory influence of hemodynamic changes on developmentally-determined vascular identity.

Sources of Funding

This work was supported by the Dennis W. Jahniyen Career Development Scholarship Program, which is administered by the American Geriatrics Society through an initiative funded by The John A. Hartford Foundation of New York City and The Atlantic Philanthropies, the Wylie Scholar in Academic Vascular Surgery Award, the Pacific Vascular Research Foundation, the National Institutes of Health, and the American Vascular Association William J. von Liebig Award, as well as with resources and the use of facilities at the VA Connecticut Healthcare System, West Haven, Conn.

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2007;27:1562-1571; originally published online April 26, 2007;
doi: 10.1161/ATVBAHA.107.143032
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Venous identity is lost but arterial identity is not gained during vein graft adaptation

**Figure 1.** Human vein graft adaptation.  **A)** Photomicrographs of saphenous veins (left column) and patent vein grafts (right column). First row, Masson’s trichrome stain; second row, Elastin stain; third row, immunohistochemistry for Von Willebrand factor. Yellow arrowheads denote the internal elastic lamina. Scale bar, 200 microns. n=4; representative samples are shown.  **B)** Photomicrographs of saphenous veins (left column) and patent vein grafts (right column). Samples are processed for immunohistochemistry staining to detect: First row, SM-22 α; second row, calponin; third row, Nogo-B.  **C)** Bar graphs depicting density of α-actin (left panel) and Nogo-B (right panel) staining in both saphenous veins and patent vein grafts.
**Supplementary Figure 2.**

<table>
<thead>
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<th>Jugular Vein</th>
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<th>Vein Graft (Day 21)</th>
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**Figure 2.** Vein graft adaptation in aged and young adult rats. Photomicrographs of jugular vein (first column), vein graft explanted at 7 days (second column), vein graft explanted at 21 days (third column), and carotid artery (fourth column) in aged (top panel) and young adult (bottom panel) rats. First row, H&E stain; second row, Masson’s trichrome stain; third row, Elastin stain. I+M, intima-media; A, adventitia; LC, loose connective tissue. Yellow arrowheads denote the elastic lamina. Scale bar, 50 microns. n=7; representative samples are shown.
Supplementary Figure 3.

Figure 3. Analysis of vein graft adaptation in aged rats. Photomicrographs of jugular vein (first column), vein graft explanted at 7 days (second column), vein graft explanted at 21 days (third column), and carotid artery (fourth column) in aged rats. Samples are processed for immunohistochemistry staining to detect: First row, Von Willebrand factor; second row, $\alpha$-actin; third row, SM-22 $\alpha$; fourth row, calponin; fifth row, Nogo-B. Scale bar, 50 microns. n=7; representative samples are shown.

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Supplementary Figure 4.

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**Figure 4.** VEGF-A is required for Eph-B4 expression in vein grafts. **A)** Photomicrographs of rat aortic SMC transfected with siRNA, fluorescently labeled. Top panel, low magnification (200x); bottom panel, high magnification (630x). **B)** Bar graph depicting relative VEGF-A secretion into the medium by rat aortic SMC pretreated with control siRNA or siRNA directed against VEGF-A (100nM), and with additional treatment (48 hours) with hypoxia (1.2% O₂), deferoxamine (100ng/ml), or lipopolysaccharide (50µM) (n=6). **C)** Bar graph depicting relative VEGF-A mRNA transcript levels in carotid arteries, either untreated or after 48 hours of treatment with control siRNA or siRNA directed against VEGF-A (n=3 samples per group, repeated twice; p=.01).