The Transcription Factor E26 Transformation–Specific Sequence-1 Mediates Neointima Formation in Arteriovenous Fistula

Wenguang Feng,* Phillip Chumley,* Michael Allon,* James George,*† David W. Scott,‡ Rakesh P. Patel,‡ Silvio Litovsky,‡ and Edgar A. Jaimes*§

*Division of Nephrology, †Department of Surgery, ‡Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama; and §Research Service, Birmingham Veterans Affairs Medical Center, Birmingham, Alabama

ABSTRACT

Hemodialysis vascular access dysfunction contributes to increased morbidity and mortality in hemodialysis patients. Arteriovenous fistula (AVF) is the preferred type of vascular access for hemodialysis but has high rates of dysfunction, in part because of excessive neointima formation. The transcription factor E26 transformation–specific sequence-1 (ETS-1) is a mediator of proinflammatory responses in hypertension and endovascular injury. We examined the role of ETS-1 in the formation of neointima in AVF. Right carotid artery to internal jugular vein fistulas were created in C57BL/6 mice and assigned to treatment with an ETS-1–dominant negative peptide (ETS-DN), an inactive mutant peptide (ETS-MU), or vehicle (n=6 per group). After 7 and 21 days, AVFs or contralateral internal jugular veins were processed for PCR, immunofluorescence, immunohistochemistry, and morphometry. In AVFs, ETS-1 mRNA increased 2.5-fold at 7 days and 4-fold at 21 days. By immunofluorescence, we confirmed increased expression of ETS-1 predominantly in the neointima and overlying endothelium. Similarly, ETS-1 expression increased in human AVFs compared with normal veins. In mice, ETS-DN, but not ETS-MU, reduced neointima formation at days 7 and 21 and reduced the expression of nitric oxide synthase 2, NADPH oxidase (NOX) 2, NOX4, E-selectin, and monocyte chemotactic protein-1. Shear stress increased ETS-1 phosphorylation in human umbilical vein cells in a NOX-dependent manner, demonstrating a role for reactive oxygen species in ETS-1 activation. These results unveil the role of ETS-1 as a mediator of neointima formation in AVF and may result in the development of novel strategies for the treatment of AVF dysfunction.


Vascular access dysfunction is an important contributor to morbidity and mortality in hemodialysis patients,1–7 accounting for 20% of hospitalizations in this population.8 Although arteriovenous fistulas (AVFs) are the preferred type of vascular access for hemodialysis, they are associated with high rates of nonmaturation, leading to prolonged catheter dependence. Up to 50% of AVFs do not mature to the point that they can provide effective hemodialysis (primary nonfunction), and in those that mature, their primary patency is decreased to 75% after 2 years.1 One of the most important pathologic lesions contributing to the pathogenesis of primary or secondary failure of an AVF is excessive neointima formation, which thickens the venous wall, narrows the luminal area, and predisposes to intravascular thrombosis.1,9–11 The mechanisms involved in the pathogenesis of neointima formation in AVF are, however, not well understood.

E26 transformation–specific sequence-1 (ETS-1) is the founding member of a family of transcription factors that share a highly conserved DNA-binding domain, called the ETS domain, that recognizes a
ETS-1 is well recognized as an oncogene, with more recent studies showing its upregulation in the vasculature by the systemic administration of angiotensin II. We previously demonstrated that ETS-1 mediates the formation of neointima after endovascular injury of the carotid arteries by regulating the activation of proinflammatory cytokines and adhesion molecules, including IL-6, monocyte chemotactic protein (MCP)-1, P-selectin, and E-selectin.

The present studies evaluated the hypothesis that ETS-1 plays an important role as a mediator of neointimal formation after AVF creation by modulating the expression of different mediators involved in these processes. This hypothesis was tested in both an experimental mouse AVF model and in patients with CKD receiving a new AVF.

RESULTS

ETS-1 Expression Is Increased in AVF

To determine whether ETS-1 expression is increased in mouse AVFs, ETS-1 mRNA expression was measured 1 and 3 weeks after creation of the fistulas. As shown in Figure 1A, mRNA expression for ETS-1 in AVFs increased significantly at both time points. By immunofluorescence we determined that these changes were accompanied by concomitant changes in the expression of the total and phosphorylated forms of ETS-1 protein (Figure 1B).

To characterize the cells that express ETS-1 in AVF, we performed colocalization studies using specific markers for vascular endothelium (platelet endothelial cell adhesion molecule-1, CD31), macrophages (F4/80), vascular smooth muscle cells (α-smooth muscle actin [SMA]), and neutrophils (myeloperoxidase [MPO]) 3 weeks after AVF creation. As shown in Figure 2, A–C, the normal jugular veins expresses low levels of ETS-1 that colocalize exclusively with CD31, indicating endothelial-specific expression. In AVFs, a greater intensity of ETS-1 staining was evident, which colocalized with CD31 (Figure 2, D–F) and SMA (Figure 2, G–I). We also observed occasional positive neutrophils (Figure 3, A–C) and macrophages that were also weakly positive for ETS-1 (Figure 3, D–F). These findings indicate that most of the expression of ETS-1 in AVF occurs in the neointima and overlying endothelium and to a lesser degree in inflammatory cells.

Human AVFs Express ETS-1

For these studies, we took advantage of the availability of venous samples obtained at the time of initial surgery for AVF from patients with CKD and 2–6 months later from AVFs of the same patients at the time of surgical revision. As shown in Figure 4, we observed low expression of ETS-1 in human veins used to create an AVF. In contrast, we observed substantial ETS-1 expression, mainly in areas of neointima, in all six venous samples obtained from nonmaturing AVFs at the time of surgical revision. In these samples, ETS-1 colocalized with CD31 (Figure 5, A–C) and SMA (Figure 5, D–F). We also observed occasional neutrophils (Figure 6, A–C) and macrophages in some but not all samples available for analyses that were weakly positive for ETS-1 (Figure 6, D–F). In the aggregate these findings demonstrate that ETS-1 expression is also increased in human AVFs.

ETS-1 Blockade Reduces Neointima Formation after AVF

We previously demonstrated that blockade of ETS-1 using a specific dominant negative peptide reduces the formation of neointima after balloon injury of the carotid artery. To determine whether ETS-1 blockade reduces the formation of neointima after AVF, mice were infused with ETS-1–dominant negative peptide (ETS-DN) for 1 or 3 weeks or with an ETS-1–inactive mutant peptide (ETS-MU) as described in Concise Methods. As shown in Figure 7, the administration of ETS-DN, but not of the inactive ETS MU peptide, resulted in significant reductions in neointima and increases of the lumen to cross-sectional ratio at both time points tested.

ETS-1 Mediates the Expression of Proinflammatory Mediators in AVFs

To assess the role of ETS-1 on the expression of several mediators involved in the formation of neointima in AVF, we mea-
sured the expression of the inducible nitric oxide synthase (NOS) isoform NOS2, the NADPH oxidase (NOX) isoforms (NOX2 and NOX4), the NOX subunits p47Phox and p67Phox, the adhesion molecules E-selectin and vascular cell adhesion molecule-1 (VCAM), and MCP-1 by immunohistochemistry in control veins as well as in AVF from the different groups: AV F, AVF + ET S– DN , and AVF +ETS–MU. We chose these mediators on the basis of previous studies showing the role of the aforementioned molecules or their products in formation of neointima in AVF.17–22 As shown in Figure 8, the expression of NOS2 increased significantly in AVFs compared with control veins. ETS-1 blockade resulted in a significant reduction in NOS2 expression, suggesting that ETS-1 is linked to increased expression of the inducible NOS isoform NOS2. Mouse AVFs also had significant increases in the expression of MCP-1 and E-selectin that were reduced by treatment with ETS-DN but not by the inactive control peptide ETS-MU (Figure 9). As shown in Figure 10, AVF also had increased expression of NOX2 and NOX4, which were also reduced by the administration of ETS-DN but not of ETS-MU. The expression of p47phox, p67phox, and VCAM increased significantly (Figure 11), but ETS-1 blockade did not modify their expression, indicating that they were not regulated by ETS-1. Together these findings demonstrate that ETS-1, directly or indirectly, regulates the expression of several mediators involved in the pathogenesis of neointima formation in AVF.

**Shear Stress Increases ETS-1**

Shear stress rapidly changes once the AVF is established.23 To determine the role of shear stress on ETS-1 activation, human umbilical vein endothelial cells (HUVECs) were exposed to...
static or shear stress (6 dyn/cm²) for 15 or 30 minutes, and protein extracted from the HUVECs was collected for Western blot analysis of total and phospho-ETS-1. As shown in Figure 12A, introduction of shear stress resulted in significant increases in the expression of the phosphorylated form of ETS-1 and had no significant effect on total ETS-1 levels. In separate experiments, HUVECs were exposed to shear stress for 15 minutes, fixed and stained with anti-phospho-ETS-1 primary antibody and an Alexa Fluor secondary antibody. DAPI (4’,6-diamidino-2-phenylindole) stain was used to assess nuclear stain. As shown in Figure 12B, introduction of shear stress increased the nuclear expression of phospho-ETS-1. To determine whether reactive oxygen species (ROS) contributes to the activation of ETS-1 by shear stress, HUVECs were pretreated with diphenyleneiodonium (DPI, a general flavoprotein inhibitor) and then exposed to shear stress. Figure 11, C and D, shows that DPI significantly decreased the expression of phospho-ETS-1, consistent with the concept that NOX-derived

---

**Figure 3.** ETS-1 is weakly expressed in inflammatory cells in AVF. Colocalization studies were performed to determine ETS-1 expression in neutrophils as identified by MPO and macrophages as identified by F40/80. (A–C) Positive stains for ETS-1 (A, red) and occasional MPO-positive cells (B, green, arrows) that are also slightly positive for ETS-1 (C, arrows). F4/80-positive were scarce and present only in some of the samples examined. (D–F) Positive immunofluorescence for ETS-1 (D, red) and F4/80 (E, green, arrows) that colocalize with ETS-1 (F, arrows). Original magnification, ×40.

---

**Figure 4.** ETS-1 expression in normal human veins and venous limb of AVFs. Hematoxylin and eosin (H&E) stain of normal vein and AVF sample showing increased wall thickness and neointima. Original magnification, ×20. Consecutive sections of the same samples were used for immunofluorescence. Low baseline expression of ETS-1 (red) in normal human veins is observed in scattered cells throughout the vessel wall. Strong ETS-1 expression (red) is observed in the venous limb of AVFs. Original magnification, ×40.
Figure 5. Vascular endothelium and neointima express ETS-1 in human AVFs. Human AVFs have positive immunofluorescence for ETS-1 (A, red) that colocalizes with DAPI stain, indicating nuclear expression. (B) Positive CD31-positive cells (green) that are also positive for ETS-1 (C, arrows). Colocalization immunofluorescence for SMA shows that positive areas for ETS-1 (D, red) and SMA (E, green) colocalize in human AVFs (F), indicating ETS-1 expression in subendothelial smooth muscle cells. Original magnification, ×40.

Figure 6. Inflammatory cells express ETS-1 in human AVFs. Colocalization studies were performed to determine ETS-1 expression in neutrophils, as identified by MPO, and macrophages, as identified by F40/80, in human AVF samples. (A–C) Positive stain for ETS-1 (A, red) and occasional MPO-positive cells (B, green, arrows) that are also positive for ETS-1 (C, arrows). F4/80-positive were scarce and present only in some of the samples examined. (D–F) Positive immunofluorescence for ETS-1 (D, red) and F4/80 (E, green, arrows) that colocalize with ETS-1 (F, arrows). Original magnification, ×40.
ROS play an important role in ETS-1 activation as result of increased shear stress.

**DISCUSSION**

To our knowledge, these studies demonstrate for the first time that the transcription factor ETS-1 plays a major role as mediator of proinflammatory responses and neointima formation in a mouse model of AVF. We show that AVF is associated with a significant increase in ETS-1 mRNA and protein expression and that blockade of ETS-1 with a specific ETS-DN reduces the expression of the chemokine MCP-1, the adhesion molecule E-selectin, the NOS isoform NOS2, and the NOX isoforms NOX2 and NOX4. In addition we show that ETS-1 blockade reduces the formation of neointima in AVF. Moreover, our studies demonstrate that human AVFs also have increased ETS-1 expression compared with normal veins before AVF creation and that shear stress increases ETS-1 phosphorylation in HUVECs.

The ETS factors are a family of transcription factors that share the E26 ETS sequence DNA-binding domain. Although this family is composed of several members, ETS-1 in particular has emerged as a critical transcription factor involved in the regulation of multiple biologic and pathologic processes and in the pathogenesis of different types of cancer. The transcriptional activity of ETS factors can be modulated through posttranslational modifications. Phosphorylation
of threonine-38 by the mitogen-activated kinases extracellular signal regulated kinases 1 and 2 increases the transcriptional activity of ETS-1, while calmodulin-dependent kinase II serine phosphorylation inhibits its activity. In addition, nuclear localization sequences regulate ETS-1 activity via facilitating its movement from the cytoplasm into the nucleus.

In previous studies we demonstrated that ETS-1 is an important mediator of neointima formation in a rat model of balloon injury of the carotid artery. This model is characterized by adventitial accumulation of neutrophils and monocytes and subsequent neointima formation. We also demonstrated that balloon injury of the carotid artery induces ETS-1 expression and that ETS-1 blockade reduces the formation of neointima and the mRNA and protein expression of MCP-1, E-selectin, and P-selectin. These previous studies clearly demonstrated the important role of ETS-1 as a mediator of the inflammatory responses to mechanical arterial endovascular injury. Moreover, studies by others have demonstrated that the systemic administration of angiotensin II to ETS-1–deficient mice is associated with marked reductions in medial hypertrophy and inflammatory responses compared with wild-type mice. In addition, we recently demonstrated that angiotensin II increases the glomerular expression of ETS-1 in vivo and that ETS-1 blockade reduces angiotensin II–induced proinflammatory responses, including macrophage infiltration, oxidative stress, and cell proliferation and reduces the expression of profibrotic cytokines, such as TGF-β and connective tissue growth factor. Moreover, in vitro, we demonstrated that ETS-1 blockade reduces the production of fibronectin in response to angiotensin II in rat mesangial cells, and that ETS-1 directly binds to and activates the fibronectin gene promoter. Together, these studies demonstrate the role of ETS-1 as an important mediator of inflammation and fibrosis in response to diverse types of injury.

Although AVF is the preferred vascular access for hemodialysis, it has high rates of primary nonfunction, dysfunction, and eventual failure. A fundamental pathologic lesion that contributes to the pathogenesis of primary or secondary failure of an AVF is neointimal hyperplasia, which thickens the venous wall, narrows the luminal area, and predisposes to intravascular thrombosis. The formation of neointima results from endothelial activation, inflammation, increased cell proliferation and migration, and matrix accumulation. The formation of excessive neointima plays a major role in the maturational failure of AVFs, in which the AVFs may never fully acquire the capacity to accommodate and sustain the heightened rate of blood flow required for effective dialysis; it also accounts for late AVF failure after variable periods of adequate function.

Figure 8. ETS-1 blockade reduces NOS2 expression in AVF. Three weeks after the AVFs were created, mice were euthanized and perfusion-fixed, and samples (normal internal jugular vein and AVF) were collected. The upper panel shows the representative photomicrographs of immunohistochemistry sections for NOS2 (brown stain). The lower panel shows the quantitative analysis of NOS2 immunohistochemistry of normal mouse vein and 3-week AVF with and without administration of ETS-DN or ETS-MU peptide. NOS2 expressions were increased in AVF; ETS-1 blockade significantly reduced NOS2 expression (data is shown as mean±SEM; n=6 per group; *P<0.05 versus control; #P<0.05 versus AVF, n=6 per group). Original magnification, ×40.
In the current studies we have expanded on our previous observations and demonstrated that ETS-1 expression is increased in a mouse model of AVF as well as in human AVFs. We first demonstrated strong expression of ETS-1 in mouse AVF that colocalized with endothelial (CD31) and SMA markers, consistent with expression in the neointima and overlying endothelium. We also observed occasional inflammatory cells, including neutrophils and macrophages, that were also positive for ETS-1. Similar cellular distribution of ETS-1 was observed in samples obtained from human AVFs.

As previously shown by others, we demonstrated increased expression of several mediators that are linked to neointima formation in AVF. For these studies, and as we have done in previous studies, we took advantage of the availability of an ETS-1–dominant negative peptide to block the effects of ETS-1 and of a mutant inactive peptide that was used as control. Our studies demonstrate that the administration of ETS-DN reduces the expression of NOS2, NOX2, NOX4, MCP-1, and E-selectin 3 weeks after the creation of AVF. These findings indicate that ETS-1 regulates the expression of several mediators involved in the formation of neointima in AVF, including chemoattractant molecules, adhesion molecules, and vascular sources of ROS and NO. Although these molecules decreased significantly, it is not clear whether ETS-1 regulates the expression of these molecules by directly activating their transcription or indirectly by regulating the expression of other genes; this will be the subject of future studies in our laboratory. ETS-1 blockade did not significantly modify the expression of the NOX subunits p47phox and p67phox or the adhesion molecule VCAM, suggesting that ETS-1 regulates the expression of these molecules by directly activating their transcription or indirectly by regulating the expression of other genes; this will be the subject of future studies in our laboratory. ETS-1 blockade did not significantly modify the expression of the NOX subunits p47phox and p67phox or the adhesion molecule VCAM, suggesting that ETS-1 regulates the expression of these molecules by directly activating their transcription or indirectly by regulating the expression of other genes; this will be the subject of future studies in our laboratory. ETS-1 blockade did not significantly modify the expression of the NOX subunits p47phox and p67phox or the adhesion molecule VCAM, suggesting that ETS-1 regulates the expression of these molecules by directly activating their transcription or indirectly by regulating the expression of other genes; this will be the subject of future studies in our laboratory.
with angiotensin II. In our studies we could not demonstrate that ETS-1 regulates p47phox, which suggests the presence of tissue differences in the regulation of this NOX subunit by ETS-1. Of note, in our studies we analyzed the expression of these molecules in the venous limb of AVF and in a model that is not accompanied by hypertension. We postulate that ETS-1 has different effects in the setting of hypertension and increased angiotensin II, which is associated with the activation of myriad pathways that could explain some of the observed differences with our current studies.

Excessive neointima hyperplasia contributes to functional vessel stenosis and AVF dysfunction.9,34,37 The administration of ETS-DN resulted in significant reductions in the formation of neointima at both 1 and 3 weeks after the creation of AVF, demonstrating that ETS-1 plays an important role in the adaptive vascular changes that occur after the creation of AVF and lead to the formation of neointima. These results would suggest that ETS-1 could be a target for the treatment and prevention of excessive neointima formation in AVF and improvement of AVF maturation. Because neointima formation is part of the expected vascular response after AVF, we postulate that ETS-1 participates in this normal process but that at the same time excessive or dysregulated expression of ETS-1 could play a role in the excessive formation of neointima stenosis. All the human samples we had available for study were from patients who required surgical revision because of insufficient fistula maturation. The samples collected were from stenotic areas, and therefore it is unclear at this time whether these patients had increased ETS-1 expression compared with patients with appropriate fistula maturation.

Our studies were performed in mice with normal renal function; however, as shown by studies by others, CKD is associated with a more robust neointima formation, likely the result of more aggressive smooth muscle migration.37,38 Whether the vascular expression of ETS-1 is further upregulated under uremic conditions is not known. We speculate,

![Figure 10](image-url)
however, that ETS-1 probably plays a role in the accelerated formation of neointima under uremic conditions. Increased shear stress and turbulent flow as a result of the shunt of arterial blood flow into the venous circulation is the most important initiating factor in the process that leads to neointima formation in AVF. To determine the effects of increased shear stress on ETS-1 activation in HUVECs, we exposed the HUVECs in elevated shear stress. Our findings clearly demonstrate that shear stress increases ETS-1 phosphorylation and nuclear translocation. These effects are prevented by pretreatment with the NOX inhibitor DPI, demonstrating a role for NOX-derived ROS on these effects. Although these results indicate that ROS participate in ETS-1 activation, it is unclear at this time which specific isozyme (NOX2 or NOX4) is responsible for these effects. These results also suggest the presence of positive feedback in which ROS participate in ETS-1 expression and activation while at the same time ETS-1 activates enzymes involved in the production of ROS, resulting in further ETS-1 activation and an amplified biologic response.

In conclusion, the results of our studies support a critical role for ETS-1 as a transcriptional mediator of endoluminal vascular injured induced inflammation and neointima formation. We have identified several downstream effectors of the model, including MCP-1, NOS2, NOX2, NOX4, and E-selectin, that are regulated by ETS-1. Future studies will be directed at identifying protein-protein interactions of ETS-1 with other transcription factors or cofactors and to additional mechanisms by which ETS-1 could play a role in neointima formation. Of note, our studies unveil a potential therapeutic
microsurgical sutures, end-to-side anastomosis was completed. Male C57BL/6 mice (Jackson Labs) weighing 20–25 g were divided into the following groups (n=6 per group for histology analysis; n=6 per group for mRNA analysis): group 1: AVF mice infused with vehicle; group 2: AVF mice treated with ETS-DN, 10 mg/kg per day, via osmotic mini-pump; and group 3: AVF mice treated with ETS-MU, 10 mg/kg per day, via osmotic mini-pump. Mice belonging to the different groups were euthanized 1 or 3 weeks after surgery. The ETS-DN and inactive ETS-MU peptides were synthesized (CPC Scientific, Inc., San Jose, CA) following the sequences described by Ni et al.

**Human Samples**

For these studies we took advantage of the availability of vein samples obtained from six patients with CKD at the time of AVF creation and venous samples obtained from the same six patients at the time of surgical AVF revision 2–6 months later due to AVF nonmaturation. The AVF samples were obtained from sites slightly proximal to the prior anastomosis for pathologic examination. The Institutional Review Board for Human Use at the University of Alabama at Birmingham approved the protocol for collection and study of these samples.

**Neointima Measurement**

Fistula samples were collected at 1 or 3 weeks after perfusion and fixation in situ. The area of vascular anastomosis (approximately 1 mm in length) was sectioned at 5-μm intervals (approximately 200 sections per sample). The section with the greatest neointima area as identified by bright field microscopy and four additional sections (two proximal and two distal) situated at 100-μm intervals were stained with hematoxylin and eosin. In several of the slides we observed the presence of fibrin near the area of the AVF. The neointima was distinguished from the media because the latter has circularly arranged smooth muscle cells while the intima has some smooth muscle cells admixed in an extracellular matrix. The wall thickness was measured at 12 evenly spaced radial zones at the five predetermined levels, and the results were averaged. The wall thickness for each sample consisted of the average of the values obtained in the five different levels. To calculate the luminal area to cross-sectional area ratio, the circumferential profiles of the lumen and the lamina externa of the venous wall were delineated (Supplemental Figure 1A), the areas encompassed by these boundaries were determined, and the ratios of the luminal area to the venous wall cross-sectional area at the five predetermined levels were calculated by Image software (Media Cybernetics and National Institutes of Health, Bethesda, MD) and averaged for each sample.

**CONCISE METHODS**

**Experimental Protocol**

All animal experiments were approved by the Institutional Committee for Use and Care of Laboratory Animals at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. AVFs were constructed by an end-to-side anastomosis between the right carotid artery and jugular vein as previously described by others. Briefly, mice were anesthetized with isoflurane and through a midline incision of the neck; the right common carotid artery and external jugular vein were dissected and exposed. Using target that could be used to modulate excessive neointima formation in AVF, a common cause of stenosis and subsequent AVF failure.

**Figure 12.** Shear stress increases ETS-1 phosphorylation in HUVECs. (A) Cells were exposed to static or shear stress (SS) conditions for 15 or 30 minutes. Cell lysates were used for Western Blot for total and phosphorylated ETS-1. Shear stress increased phospho-ETS-1 expression at both 15 and 30 minutes without modifying total ETS-1. (B) In other experiments, after exposure to shear stress for 15 minutes, HUVECs were alcohol fixed and immunofluorescence was performed using an anti-phospho-ETS-1 antibody; nuclei were stained with DAPI. As shown in photomicrograph under static conditions, there is low expression of phospho-ETS-1 that is increased after shear stress. Original magnification, ×64. (C) Representative Western blot of HUVECs pretreated with or without the NOX inhibitor DPI (10^{-6} M) for 1 hour before static or shear stress for 15 minutes. NOX inhibition with DPI prevented the effects of shear stress on ETS-1 phosphorylation. (D) Densitometry analysis of Western blots for phospho-ETS-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as loading control in HUVECs exposed to shear stress with and without NOX inhibition. DPI significantly reduced phospho-ETS-1 expression in HUVECs exposed to shear stress for 15 minutes (data is expressed as mean±SEM; *P<0.05 versus static; #P<0.01 versus shear stress only; n=3 in duplicate).
Immunofluorescence
Five-micrometer sections of normal veins (contralateral mouse internal jugular vein or human basilica or cephalic veins) and AVF (mouse and human) were prepared from paraffin-embedded tissues. Sections were incubated with a primary antibody against ETS-1 (mouse monoclonal, sc-55581; Santa Cruz Biotechnology; 1:50), in combination with one of the primary antibodies—CD31 (rabbit polyclonal; Abcam; 32457, 1:100), SMA (Sigma-Aldrich; A2547), MPO (rabbit polyclonal; Abcam 9535, 1:100), or F4/80 (rat monoclonal antimouse; Abcam; 6640, or rat monoclonal antihuman; Abcam;16911, 1:100)—in blocking buffer at 4°C overnight. Sections were incubated with the appropriate secondary antibodies and mounted with Vectashield DAPI mounting medium (Vector Laboratories). Two controls, in which we omitted the primary or secondary antibody, were included in each experiment. Images were acquired using a Leica DM6000 epifluorescence microscope (Leica Microsystems, Bannockburn, IL) with a Hamamatsu ORCA ER cooled CCD camera and SimplePCI software (Comipsx, Inc., Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence. Relative fluorescent intensities were measured using Simple PCI software.

Immunohistochemistry
The avidin-biotin-peroxidase immunohistochemical technique (ABC kit; Vector Laboratories) was used to detect MCP-1, E-selectin, NOX2, NOX4, NOS2, p47phox, p67phox, and VCAM. In brief, after deparaffinization and heat-mediated antigen retrieval, positive areas were immunolocalized by incubation with the respective primary antibody: Anti–MCP-1 (sc-28879; Santa Cruz Biotechnology), anti–E-selectin (sc14011; Santa Cruz Biotechnology), anti–NOX2 (sc-651; Santa Cruz Biotechnology), anti–NOX4 (BN110–58851; Novus Biologicals), anti–p47phox (sc14015; Santa Cruz Biotechnology), anti–p67phox (sc15342; Santa Cruz Biotechnology), anti–VCAM (sc1054R), and anti–NOS2 (sc-651; Santa Cruz Biotechnology). For quantification of the immunohistochemistry stain, the same levels (50–150 μm downstream of the anastomosis) as an area that included the neointima and adventitia were delineated for each sample. Background was subtracted and the stain quantified (ImageJ) (Supplemental Figure 1, B and C) under identical settings for all samples examined and adjusted for the total area delineated.15

Real-Time PCR
Total RNA was extracted from normal veins and AVFs with TRIzol (Invitrogen, Carlsbad, CA), treated with DNase I and then purified with an RNA purification kit (Invitrogen). The protein- and DNA-free RNA was reverse-transcribed to cDNA (Invitrogen) and amplified by PCR with specific primers and quantified using SYBR Green and a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) as previously described.40 Levels of specific mRNAs were normalized using glyceraldehyde 3-phosphate dehydrogenase as an internal control.

In Vitro Studies
For the in vitro experiments, HUVECs (Lonza Corporation, Rockville, MD) were seeded onto a 0.2% gelatin-coated disc and then exposed to static or shear stress (6 dyn/cm²) for 15 or 30 minutes by using a Glycotech flow chamber as previously described.41 Cells were saved for Western blot or immunofluorescence. In separate experiments, HUVECs were fixed and stained with anti–phospho-ETS-1 primary antibodies and an Alexa Fluor secondary antibody. Nuclear accumulation of p-ETS-1 was assessed using confocal fluorescence microscopy. In other experiments, HUVECs were pretreated with the NOX inhibitor DPI (10⁻⁶ M) for 1 hour before exposure to static control condition or shear stress for 15 minutes, and phospho-ETS expression was assessed by Western blot.

ACKNOWLEDGMENTS
These studies were funded by a Merit Review Award (I01BX001073-03) and a Program Project Award from the Veterans Affairs Administration (IP1BX001595-01A1) to E.A.J., a National Institutes of Health Research Grant (DK085027-03) to M.A., and the UAB-UCSD O’Brien Core Center for Kidney Research Grant (P30DK079337-05).

DISCLOSURES
None.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013040424/-/DCSupplemental.