Focal Expression of Angiotensin II Type 1 Receptor and Smooth Muscle Cell Proliferation in the Neointima of Experimental Vein Grafts 
Relation to Eddy Blood Flow

S.Q. Liu

Abstract—Eddy flow has been shown to promote focal smooth muscle cell (SMC) proliferation and neointimal formation in experimental vein grafts. This study focuses on whether the angiotensin II type 1 (AT_1) receptor mediates these events. Experimental vein grafts with and without eddy flow were created in the rat. Losartan was used to assess the influence of the AT_1 receptor on SMC proliferation. In vein grafts with eddy flow, apparent focal expression of AT_1 mRNA and protein was found in the leading region of the proximal focal neointima, where eddy flow occurred, but not in the trailing region, where eddy flow diminished, at days 5, 10, 20, and 30. The rate of SMC proliferation in the leading region (10.9\pm 1.4\%, 19.5\pm 2.2\%, 12.2\pm 2.0\%, and 6.9\pm 1.3\% at these times, respectively) was significantly higher than that in the trailing region (9.5\pm 1.8\%, 15.3\pm 2.0\%, 8.2\pm 1.9\%, and 3.2\pm 0.7\% in these vein grafts. When eddy flow was prevented in engineered vein grafts, no apparent location difference was found in the distribution of AT_1 receptor mRNA and protein in the neointima, and the rate of SMC proliferation (5.3\pm 1.0\%, 5.8\pm 0.9\%, 3.4\pm 1.0\%, and 3.7\pm 0.9\% at days 5, 10, 20, and 30, respectively) was reduced significantly. In vein grafts with losartan, the rate of SMC proliferation in the leading region of the neointima (9.4\pm 1.8\%, 10.1\pm 1.3\%, 8.3\pm 0.9\%, and 4.2\pm 0.5\% at days 5, 10, 20, and 30, respectively) was significantly lower than that in vein grafts without losartan. These results suggested that eddy flow upregulated the AT_1 receptor, which in turn mediated focal SMC proliferation in the neointima of experimental vein grafts. (Arterioscler Thromb Vasc Biol. 1999;19:2630-2639.)

Key Words: fluid shear stress ■ tensile stress ■ intimal hyperplasia ■ vascular tissue engineering

Blood vessels develop and remodel under the influence of mechanical factors, including blood pressure and flow. It has long been hypothesized that these mechanical factors contribute to the development of vascular diseases such as atherosclerosis. Previous clinical and experimental investigations have provided convincing evidence for this hypothesis. These studies have demonstrated that atherosclerotic lesions in human and animal arteries develop mainly in curved and bifurcation regions where eddy or secondary blood flow occurs.\(^1\)\(^-\)\(^4\) The coincidence of eddy blood flow with atheroma suggests that eddy blood flow may play a role in the initiation and development of vascular atherosclerosis.

Vein grafts, which are commonly used to replace malfunctioned arteries, are subject to eddy blood flow. Experimental studies have demonstrated that the location and pattern of eddy blood flow vary in different vein graft models, depending on the vessel geometry for a given blood flow rate and blood viscosity.\(^5\)\(^-\)\(^8\) In an end-to-end anastomosed vein graft model, eddy blood flow occurs in the proximal region due to graft-host diameter mismatch, whereas in an end-to-side or side-to-side anastomosed vein graft, eddy blood flow occurs in the distal toe and heel regions due to geometric distortions and curvatures. It has been known that eddy blood flow in these regions is often associated with focal intimal hyperplasia, a pathological event causing vein graft restenosis.\(^5\)\(^-\)\(^8\) Thus, these vein graft models have been used to study the influence of blood flow on vascular remodeling. Recently, the author demonstrated, by using an end-to-end anastomosed vein graft model, that focal intimal hyperplasia and smooth muscle cell (SMC) proliferation were initiated in the proximal region where eddy flow was found. When eddy flow was eliminated by matching the graft-host diameters by using a biomechanical engineering approach, focal intimal hyperplasia and SMC proliferation were significantly prevented.\(^7\) These results further support the role of eddy blood flow in the regulation of intimal hyperplasia and SMC proliferation. However, the mechanisms that link fluid dynamics to focal SMC proliferation remain unclear.

Studies using molecular and cellular approaches have demonstrated that growth-related factors regulate cell proliferation. In blood vessels, endothelial cells and SMCs are able to produce and release a number of growth-related factors,
including acidic and basic fibroblast growth factors, platelet-derived growth factor (PDGF), vascular endothelial cell growth factor, insulin-like growth factor, monocyte chemotactic protein-1, and endothelin. These growth factors have been shown to interact with their receptors in the cell membrane and to promote SMC proliferation and atherogenesis in human and animal arteries as well as in vein grafts. Further studies have demonstrated that growth factors may mediate mechanical stress--induced biological activities such as cell proliferation and hypertrophy. A typical example is the involvement of angiotensin II and the angiotensin II type 1 (AT₁) receptor in the regulation of cardiovascular hypertrophy. As shown in several studies, mechanical stretch elevated expression of the AT₁ receptor, which together with angiotensin II possibly mediated tensile stress--induced cardiac myocyte hypertrophy.

Several recent studies have demonstrated that the AT₁ receptor is upregulated in the neointima of experimental vein grafts, and an AT₁ receptor antagonist, L158809, significantly suppresses the rate of intimal hyperplasia. These studies suggest that the AT₁ receptor possibly plays a role in the regulation of intimal hyperplasia in vein grafts. On the basis of these investigations, it can be hypothesized that the AT₁ receptor may mediate eddy flow--related SMC proliferation in the neointima of experimental vein grafts. This study was designed to verify this hypothesis and to achieve these goals: (1) to demonstrate whether eddy flow influences the expression of AT₁ receptor mRNA and protein and (2) to investigate whether the AT₁ receptor influences the rate of SMC proliferation in the vein graft neointima that is subject to eddy flow.

Methods

Animals and Observation Schedules

Eighty male, 3-month-old Sprague-Dawley rats (Harlan, Indianapolis) were randomly divided into two experimental series, each consisting of 16 groups with 5 rats in each group. Four groups were used for each of the following experimental series: (1) nonengineered vein grafts with eddy flow and increased tensile stress; (2) engineered vein grafts without eddy flow and without increased tensile stress; (3) engineered vein grafts with eddy flow but without increased tensile stress; and (4) nonengineered vein grafts with administration of losartan. For each animal, the right jugular vein was used as a graft, and the left jugular vein was used as a normal vein control. Observations were carried out at 5, 10, 20, and 30 days after surgery. The experimental procedures were approved by the Animal Care and Use Committee of Northwestern University.

Experimental Models

Experimental Series 1: Nonengineered Vein Grafts With Eddy Flow and Increased Tensile Stress

Vein grafts were created by using conventional procedures. In brief, a rat was anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The right jugular vein was isolated, treated with heparinized host blood (100 U/mL) and papaverine/saline (1 mg/mL), and grafted into the abdominal aorta below the renal arteries by using an end-to-end anastomotic technique with 8 to 10 interrupted stitches (10-0 nylon sutures) at each end. After surgery, arterial blood flow was initiated, the external diameters of the vein graft and the host artery were measured, the abdominal cavity was closed, and the rat was allowed to recover.

In this model, the diameter of the vein graft was larger than that of the host aorta, with a graft-to-host diameter ratio of ~1.6. Eddy blood flow developed at the proximal region of the vein grafts due to graft-host diameter mismatch, and tensile stress in the vein graft wall increased owing to exposure to arterial blood pressure and enlargement of the vessel diameter.

Experiment Series 2: Engineered Vein Grafts Without Eddy Flow and Without Increased Tensile Stress

In this series, vein grafts were engineered to match the graft-host diameters and to increase the wall thickness by using an engineering approach as described previously. In brief, a flat piece of fixative-treated small intestine, with its stiffness similar to that of the aorta, was cut into a submucosa/SMC sheet with a thickness similar to that of the aorta by using a cryomicrotome. The width of the intestinal sheet was determined on the basis of the aortic diameter, and the length of the sheet was determined on the basis of the in vivo length of the jugular vein. After vein graft surgery, the intestinal sheet with desired dimensions was placed around the vein graft and sutured into a cylindrical sheath, with the 2 axial ends anchored to the adventitia of the host aorta by suture stitches. In all cases observed, the diameter of the vein graft was similar to that of the host aorta, and thus, no apparent eddy flow developed in this model. In addition, because the vein graft was restricted in a smaller and more rigid engineered sheath, tensile stress in the vessel wall was reduced.

Experiment Series 3: Engineered Vein Grafts With Eddy Flow but Without Increased Tensile Stress

In addition to eddy flow, increased tensile stress in the vessel wall influences vein graft remodeling. To distinguish the effect of eddy flow from that of tensile stress, vein grafts were engineered to reduce the graft diameter by ~25% with respect to the diameter of nonengineered vein grafts, leading to a graft-host diameter ratio of ~1.3. In such a case, eddy flow developed due to graft-host diameter mismatch, but tensile stress in the vein graft wall was reduced because the vein graft was restricted in a smaller and more rigid sheath, which carried the tensile load in the vessel wall. Thus, the influence of eddy flow could be examined independent of tensile stress. Methods described in the preceding section on experimental series 2 were used in this series of experiments as well, except for the difference in the graft-to-host diameter ratio.

Experiment Series 4: Nonengineered Vein Grafts With Losartan

Losartan, an AT₁ receptor antagonist, was used to examine the effect of the AT₁ receptor on SMC proliferation and intimal hyperplasia. Rats were administrated losartan (30 mg · kg⁻¹ · day⁻¹) in their drinking water for a period from 2 days before vein graft surgery to the end of observation. Nonengineered vein grafts were created by using the method described for experimental series 1. Observations were carried out at the same times as for the other experimental series described above.

Formation of Eddy Flow

The formation and characteristics of eddy flow in a vein graft were studied with the aid of a glass model. In a divergent blood vessel such as a vein graft, eddy flow develops under the influence of an adverse blood pressure gradient along the endothelial surface. The formation of an adverse pressure gradient in a vein graft depends on the wall slope at the proximal anastomosis or on the leading-edge slope of the proximal focal neointima. (Note that the definitions of these slopes are shown in Figure I, which can be found online at http://atvb.ahajournals.org/cgi/content/full/19/11/2630/DC1.) In this study, a relationship between wall slope and eddy flow formation was determined by using a glass model with actual vein graft geometry and size under a physiological Reynolds number, because it is difficult to characterize eddy flow formation in an actual rat vein graft.

To determine such a relationship, 23 glass models were constructed with various proximal anastomotic wall slopes ranging from 0 to 0.5. A Harvard rodent-blood pump (model 1407) was used to introduce pulsatile flow (200 beats/min) to the model, with flow and pressure magnitudes and waveform similar to those of the rat. The system was perfused with a mixture of glyceline, water, and 0.03 g/100 mL hollow glass beads of 30-μm diameter (Potter Industries, and a gift from Dr R.M. Lueptow of Northwestern University), with a fluid viscosity similar to that of rat blood (~0.06 poise). A slit-light
beam was applied to the graft model along the centerline in the axial direction. Images of glass bead streak lines were recorded in the direction perpendicular to the slit-light plane by using a Sony Betacam SP video recorder (UVW-1400 A) and a CCD camera (kindly provided by Dr M.R. Glucksberg, Northwestern University). The recorded images were analyzed, and a causal relationship between anastomotic wall slope and eddy flow was established.

Expression of AT1 Receptor mRNA
At scheduled times, vein graft specimens were fixed in vivo under in vivo arterial blood pressure by using 4% formaldehyde in PBS. The proximal portion (10 mm) of each vein graft was selected, cut into several axial strips, and embedded in 5% gelatin in PBS at 37°C to prevent specimen distortion during preparation. After gelatin solidification, the gelatin-embedded specimens were fixed for 15 minutes and cut into histological sections along the vessel axis, with each section containing the graft-host junction, by using a cryomicrotome. Several sections with the thickest intimal hyperplasia were selected from each graft for in situ hybridization of AT1 receptor mRNA. All solutions used for in situ hybridization were treated with diethyl pyrocarbonate (0.02%), and all tools and glassware were autoclaved. A rat vascular AT1 receptor cDNA (Ca18b) sample, kindly provided by Dr T.J. Murphy of Emory University, Atlanta, Ga,20 was used to produce digoxigenin-conjugated sense and antisense cDNA probes by using a polymerase chain reaction (PCR) method.21 In brief, ~0.1 ng of the template AT1 receptor cDNA was mixed on ice with AT1 receptor oligonucleotide primers 1 and 2 (5'-GTCATGATCCTCTACCCCTCTACAGC-3' and 5'-CCGTAGAACAGGGTTACGAGCAG-3', respectively; 1 μmol/L each), 1× PCR buffer with 2.5 mmol/L MgCl2, dNTPs with digoxigenin-11-dUTP (200 μmol/L of each dNTP), and 2.5 U Taq DNA polymerase (Boehringer Mannheim). The mixture was placed in a PCR tube and subjected to 30 cycles of denaturation (94°C, 1 minute), annealing (60°C, 2 minutes), and primer extension (72°C, 3 minutes) in a Perkin-Elmer 2400 thermocycler. The PCR product was verified by restriction enzyme digestion and agarose gel electrophoresis in accordance with the restriction map of the AT1 receptor gene.20 The AT1 receptor cDNA probe was heat denatured at 95°C for 2 minutes and mixed with a hybridization solution (50% deionized formamide, 0.3 mol/L NaCl, 10 mmol/L Tris-Cl, 1 mmol/L EDTA, 1× Denhardt's solution, 500 μg/mL sonicated salmon sperm DNA, 50 mmol/L DTT, and 10% polyethylene glycol of molecular weight 6000) for specimen labeling.

Selected specimens were mounted onto microscopic slides; acetated in a mixture of 0.1 mol/L triethanolamine hydrochloride, 0.25% acetic anhydride, and 0.9% NaCl; washed in 2× SSC (2 changes) and 1× SSC (2 changes) at 37°C for 2 hours and in 0.1× SSC (2 changes) at 37°C for 10 minutes, and incubated with a blocking solution (2% sheep serum and 0.3% Triton X-100 in 100 mmol/L Tris-Cl and 150 mmol/L NaCl) for 30 minutes, and washed in PBS. The specimens were then incubated with a hybridization mix containing the AT1 receptor cDNA probe for 16 hours at 37°C. Washed in 2× SSC (2 changes) and 1× SSC (2 changes) at 37°C for 2 hours and in 1× SSC (2 changes) at 37°C for 10 minutes, and incubated with a blocking solution (2% sheep serum and 0.3% Triton X-100 in 100 mmol/L Tris-Cl and 150 mmol/L NaCl) for 30 minutes, and washed in PBS. The specimens were then incubated with a mixture of 5 μg/mL anti-α-actin antibody (Boehringer Mannheim),13,24 1% BSA, and PBS at 37°C for 1 hour; washed with PBS; incubated with 1:20 fluorescein-conjugated anti-IgG antibody (Boehringer Mannheim) at 37°C for 1 hour; washed in PBS; and examined with an Olympus BX40 fluorescence microscope. Specimens incubated with only the secondary antibody were used as controls.

SMC Identification
Specimens used for AT1, mRNA and protein labeling were also used to identify SMCs by using an antibody against the SMC isoform of α-actin. In brief, selected specimens were incubated with a mixture of 5 μg/mL anti-α-actin antibody (Boehringer Mannheim),13,24 1% BSA, and PBS at 37°C for 1 hour; washed with PBS; incubated with 1:20 fluorescein-conjugated anti-IgG antibody (Boehringer Mannheim) at 37°C for 1 hour; washed in PBS; and examined with an Olympus BX40 fluorescence microscope. Specimens incubated with only the secondary antibody were used as controls.

SMC Proliferation
A 5-bromo-2'-deoxyuridine (BrdU) labeling method was used to determine the rate of SMC proliferation in the neointima of vein grafts. At scheduled times, a rat was injected intramuscularly with 30 mg/kg BrdU (Boehringer Mannheim) 3 times at 17, 9, and 1 hour before the rat was killed.25 Vein graft specimens were prepared by using methods described above. Selected vein graft sections were digested in 0.5% pepsin in 0.1N HCl for 30 minutes at 37°C, incubated in 1.5N HCl for 30 minutes at 37°C, washed in 0.1 mol/L borax buffer (pH 8.5) and then in Tris-buffered saline (pH 7.6), and blocked with 10% goat serum in PBS. The sections were incubated with an anti-BrdU antibody (Boehringer Mannheim) at a dilution of 1:20 in 1% BSA–PBS at 37°C for 30 minutes, washed in PBS, incubated with a secondary anti-IgG antibody conjugated with fluorescein (Boehringer Mannheim) at a dilution of 1:20 at 37°C for 30 minutes, and washed in PBS again. The specimens were examined with an Olympus BX40 fluorescence microscope. Two types of specimen were used as BrdU labeling controls: (1) specimens without BrdU injection, which were processed by the method described above, and (2) specimens with BrdU injection and incubated with only the secondary antibody. SMCs were identified by positive anti-α-actin antibody labeling in the same specimens.

Hoechst 33258 was used to label the cell nuclei.27 The numbers of BrdU-labeled and Hoechst 33258–labeled SMCs, which were identified by positive anti-α-actin antibody labeling, were measured in the leading and trailing regions of the area with proximal focal neointima. The proximal focal neointima was defined as that found in the proximal region of the vein graft. The leading and trailing regions were separated by a line perpendicular to the vein graft wall and passing through the maximal convex curvature of the focal neointima. (Note that the definitions of these regions are shown in Figure I, which can be found online at http://atvb.ahajournals.org/cgi/content/full/19/11/2630/DC1.) In engineered vein grafts, no apparent proximal focal neointima was found. In such a case, the proximal region, with the axial length equivalent to the average axial length of the proximal focal neointima in the nonengineered vein grafts at the same observation time, was measured and divided into 2 subregions of equal length. The 1 region adjacent to the anastomosis was defined as the trailing region, and the distal 1 was defined as the leading region. The percentage of BrdU-labeled cells in each region was calculated on the basis of the number of BrdU-labeled cells and the number of Hoechst 33258–labeled cell nuclei.

Average Thickness of the Proximal Focal Neointima
Axial histological sections of engineered and nonengineered vein grafts were used to measure the average thickness of the proximal

Expression of AT1 Receptor Protein
Axial cryosections of vein grafts were prepared by using a method described above, incubated with a blocking solution (10% goat serum in PBS) for 30 minutes, reacted with a rabbit anti-rat AT1 receptor antibody (AB1525, Chemicon) in 1% BSA–PBS (1:100) at 37°C for 1 hour, washed in PBS, incubated with a rhodamine-conjugated goat anti-rabbit IgG antibody (Chemicon) at 37°C for 1 hour, washed in PBS, and examined with an Olympus BX40 fluorescence microscope. Specimens incubated with only the secondary antibody were used as controls.
Results from actual vein grafts showed that the anastomotic wall slope and the leading-edge slope of the proximal focal neointima of nonengineered vein grafts were larger than the critical value of 0.2 for eddy flow formation at days 5, 10, 20, and 30 (see Figure 1). Flow visualization in actual vein grafts showed that eddy blood flow appeared at the proximal region or in front of the leading edge of proximal focal intimal hyperplasia of nonengineered vein grafts, but not in engineered vein grafts, in all observed cases. This result was similar to that reported in a previous study.2

Influence of Eddy Flow on AT_1 Receptor Expression

Figure 2 shows the distribution of AT_1 receptor mRNA in the proximal focal neointima of nonengineered vein grafts with eddy flow. At days 10, 20, and 30 after surgery, focal expression of AT_1 receptor mRNA was found in the leading region of the proximal focal neointima, whereas the level of AT_1 mRNA in the trailing region was apparently lower than that in the leading region. Similar results were found in engineered vein grafts with eddy flow. Note that AT_1 receptor mRNA was also highly expressed in the host aorta. In specimens labeled with a sense AT_1 receptor RNA probe, little positive labeling of AT_1 receptor mRNA was found in the leading region of proximal focal intimal hyperplasia of nonengineered vein grafts (see Figure III, which can be found online at http://atvb.ahajournals.org/cgi/content/full/19/11/2630/DC3). Simultaneous examination of AT_1 receptor mRNA and SMC α-actin localization showed that the major cell type that expressed AT_1 receptor mRNA was the SMC in the neointima of vein grafts (photomicrographs available on request). In engineered vein grafts without eddy blood flow, no apparent difference in the distribution of AT_1 receptor mRNA was found in the neointima at all observation times (see Figure 3).

Further examination with the use of an immunohistological method demonstrated that the distribution pattern of AT_1 receptor protein was similar to that of AT_1 receptor mRNA in nonengineered and engineered vein grafts, although the location of expression between AT_1 receptor protein and AT_1 receptor mRNA did not match exactly at each observation time. Photomicrographs of AT_1 receptor protein distribution in the neointima of nonengineered vein grafts are presented in Figure IV, which can be found online at http://atvb.ahajournals.org/cgi/content/full/19/11/2630/DC4.

Influence of Eddy Flow on SMC Proliferation

Figure 8 shows the distribution of BrdU-labeled cells in a normal jugular vein and in nonengineered vein grafts with eddy flow at days 5, 10, 20, and 30 after surgery. In normal jugular veins, BrdU-labeled SMCs were rarely seen. In nonengineered vein grafts with eddy flow, the density of BrdU-labeled SMCs in the proximal focal neointima increased significantly from days 0 to 10 and then decreased toward the normal level (P<0.001, ANOVA). Furthermore, SMC density in the leading region was significantly higher than that in the trailing region of the proximal focal neointima of the same specimens of nonengineered vein grafts at all observation times except on day 5 (see Figures 4 and 5). In specimens with control labeling, no BrdU-labeled cells were found (photomicrographs available on request).

In engineered vein grafts without eddy flow, as shown in Figure 5, the density of BrdU-labeled SMCs was significantly higher than that in normal veins but significantly lower than that in the nonengineered vein grafts in the leading and
trailing regions of the proximal focal neointima. No difference was found in the density of BrdU-labeled SMCs between the leading and trailing regions of engineered vein grafts at all observation times (see Figure 5). An immunohistological examination with the use of an anti–α-actin antibody showed that the major type of proliferating cell was the SMC in the neointima in both nonengineered and engineered vein grafts (photomicrographs available on request).

Influence of Losartan on SMC Proliferation and Intimal Hyperplasia

As shown in Figure 5, administration of losartan significantly lowered the density of BrdU-labeled SMCs in the proximal focal neointima of nonengineered vein grafts with eddy flow at all observation times except for day 5, despite the locally increased expression level of AT\(_1\) receptor mRNA and protein in the leading region. The distribution pattern of AT\(_1\) mRNA and protein in these specimens was similar to that in the neointima of nonengineered vein grafts without losartan. In specimens with losartan, the density of BrdU-labeled SMCs in the leading region of the proximal focal neointima was significantly higher than that in the trailing region of the same specimens at days 10, 20, and 30 after surgery.

Figure 6 shows the influence of losartan on the average thickness of the proximal focal neointima in nonengineered vein grafts with eddy flow. The average thickness was...
significantly reduced in nonengineered vein grafts with losartan compared with vein grafts without losartan at all observation times except day 5.

Discussion

Eddy Flow and Focal Intimal Hyperplasia

The formation of eddy blood flow in a blood vessel depends on several factors, including vessel geometry, flow velocity, and blood viscosity. For a given flow velocity and blood viscosity, eddy blood flow occurs because of adverse pressure gradients in blood vessels with divergent geometry, such as those distal to bifurcations and bypass grafts with a graft-host diameter mismatch. Previous studies showed that eddy blood flow often coincided with focal intimal hyperplasia in experimental vein grafts.5–8 To understand the role of eddy blood flow in the regulation of this pathological event, a number of investigators have studied the fluid dynamic features of eddy flow and demonstrated that it is associated with local oscillatory, low, fluid shear stress and large shear gradients in experimental vein grafts.28–30 Thus, it has been hypothesized that oscillatory, low, fluid shear stress and/or large shear gradients28–31 may play a role in the regulation of vein graft intimal hyperplasia. Although no direct evidence is available for this hypothesis, supporting data have been obtained from related experimental and clinical observations. For instance, lowered fluid shear stress, with reference to physiological blood shear stress, promoted SMC proliferation and intimal hyperplasia, whereas increased fluid shear stress prevented these events in experimental polymer vascular grafts in nonhuman primates.26,32,33 Investigations on human and animal arterial atherosclerosis have supported these observations.1–4 However, how oscillatory, low, fluid shear stress and increased shear stress gradients differentially regulate vein graft remodeling remains to be investigated.

Role of Signaling Molecules and Growth Factors in Mechanical Stress–Induced Vascular Remodeling

For the last decade, numbers of studies have demonstrated that fluid shear stress and strain rate influence the structure and regulate a variety of activities of vascular endothelial cells and SMCs.34–42 For instance, altered fluid shear stress influences the activities of mitogen-activated protein kinases43,44 and G proteins,45 the production rate of prostacyclin,46 the activities of growth factors,26,33,35,47,48 and the morphology49,50 and cytoskeletal structure51 of endothelial cells. These changes have been implicated in the mediation of mechanical stress–related vascular atherogenesis. Several studies have provided experimental evidence for this mechanism. In these studies, a cis-acting, shear stress–response element has been identified in the promoter region of the PDGF B-chain gene by a gene-deletion method.48 Fluid-shear stress was shown to activate nuclear factor-κB, a transcription regulatory factor activated through signal transduction pathways. Activated nuclear factor-κB further interacted with the shear stress–responsive element of the PDGF B-chain gene, leading to transcription of this gene.52 The PDGF B chain is a well-known growth factor that promotes cell proliferation and vascular hypertrophy. Thus, it becomes clear that a regulatory cascade, which involves fluid shear stress, signaling transduction pathways, transcription regulatory factors, and growth factors, may transduce fluid shear signals from the cell membrane to the cell nucleus and induce DNA synthesis and cell proliferation. It is expected that further studies may reveal the roles of other signaling molecules and growth factors in the mediation of mechanical stress–related vascular SMC proliferation.

Influence of Eddy Blood Flow on AT1 Receptor Expression

The AT1 receptor is a potent promoter of vascular SMC proliferation53,54 and has been implicated in the mediation of tensile stress–induced biological events in cardiac myocytes.14–16 However, the role of this factor in the mediation of blood flow–related SMC proliferation has not been well studied. Thus, this study was designed to investigate this issue. By using experimental vein graft models with and without eddy flow, the present study demonstrated that eddy

Figure 3. Fluorescence photomicrographs showing the distribution of AT1 receptor mRNA in the neointima of engineered vein grafts without eddy flow at days 5, 10, 20, and 30 (A through D, respectively). The endothelium is facing up in all photomicrographs. Blood flow direction is from left to right. a indicates host aorta; v, vein graft; and IS, intestinal sheath. Scale=100 μm for all panels.
Flow was associated with the focal upregulation of AT_1 receptor mRNA and protein in the proximal focal neointima. When eddy flow was prevented by restricting the vein graft into an engineered sheath whose diameter was identical to that of the host artery, no difference in the distribution of AT_1 receptor mRNA and protein was found. These results suggest that eddy flow possibly promotes AT_1 receptor expression in the neointima of vein grafts. The influence of blood flow on the expression of growth factors has been studied by using different experimental models. These studies showed that reduced blood flow upregulated the PDGF gene, whereas increased blood flow exerted an opposite effect in nonhuman primate polymer vascular grafts. These results clearly demonstrated that blood flow played a role in the regulation of mitogen expression in blood vessels.

The present study showed that AT_1 receptor mRNA and protein were highly expressed in SMCs that were not directly exposed to altered fluid shear stress. This result suggests that certain local mediators may be involved in the transduction of the shear stress signal from endothelial cells to SMCs. It is possible that signaling molecules and mitogenic factors may be involved in the transduction of a shear stress signal from endothelial cells to SMCs in vein grafts. However, the exact mechanisms remain to be determined.

Tensile stress is another mechanical factor that is increased in the wall of nonengineered vein grafts and that promotes leukocyte activation and intimal hyperplasia. Previous studies showed that relief from increased tensile stress in the vein graft wall by use of a rigid, external support could partially prevent intimal hyperplasia and medial thickening, indicating a role for tensile stress in the regulation of vein graft remodeling. Several recent studies further showed that increased tensile stress induced SMC actin filament degradation and SMC death, influenced the orientation of newly generated SMCs, and might contribute to medial and adventitial hypertrophy in experimental vein grafts. Thus, tensile stress is another potential factor that regulates expression of the AT_1 receptor.

To distinguish the influence of eddy flow from that of increased tensile stress, an experimental model with eddy flow but without increased tensile stress was also created by using an engineering technique in the present study. In this model, the vein graft was restricted in a cylindrical engineered sheath that was larger than the host artery in diameter, leading to the formation of eddy flow in the proximal region of the vein graft. However, tensile stress in the vessel wall was reduced because the vein graft was restricted in a smaller and more rigid sheath that carried the tensile load due to arterial blood pressure. In such a model, focal expression of the AT_1 receptor was found in the leading edge of the area of proximal focal intimal hyperplasia, where eddy flow occurred. This result provided further evidence for the role of eddy flow in the regulation of focal AT_1 receptor expression in the neointima of experimental vein grafts. Tensile stress, which is relatively uniformly distributed in the vessel wall, may not be directly related to the focal AT_1 receptor expression in the neointima of experimental vein grafts. Whether and how tensile stress

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**Figure 4.** Fluorescence photomicrographs showing the distribution of BrdU-labeled cells in a normal jugular vein (A1) and in proximal focal intimal hyperplasia of nonengineered vein grafts with eddy flow at days 5, 10, 20, and 30 (panels B1 through E1, respectively). Each white dot is a BrdU-labeled cell. Hoechst 33258-labeled cell nuclei of the same specimens are shown in A2 through E2. Cells in the area of focal intimal hyperplasia were identified as SMCs by using an anti-α-actin antibody. Blood flow direction is from left to right. The endothelium is facing up in all micrographs. v indicates vein graft; IH, proximal focal intimal hyperplasia; arrow, leading edge of the area of proximal focal intimal hyperplasia. Scale=100 μm for all panels.
influences the activities of the AT₁ receptor in experimental vein grafts remain to be investigated.

It should be noted that fluid shear stress in regions with and without eddy blood flow was not measured and analyzed in this study because of the lack of available techniques for small blood vessels with complex geometry. Several experimental methods, including particle tracing and photochromic tracing methods, have been used to assess fluid shear stress in large blood vessels. However, for a rat vein graft of 2 to 3 mm in diameter, the accuracy of fluid shear stress measurements is always an issue to be considered. Thus, further studies are necessary for the development of experimental techniques that will allow fluid shear stress measurements in small blood vessels with complex geometry. Although fluid shear stress was not analyzed, this study provides information into the role of eddy blood flow in the regulation of vascular SMC proliferation and atherogenesis.

Influence of the AT₁ Receptor on SMC Proliferation and Intimal Hyperplasia

The present study demonstrated that focal expression of the AT₁ receptor was associated with an increase in the rate of SMC proliferation in the leading region of the proximal neointima, whereas the activity of the AT₁ receptor and the rate of SMC proliferation were both reduced in the trailing region of the same neointima in vein grafts. These results suggest that the AT₁ receptor possibly regulates SMC proliferation. As observed in a previous study as well as in the present study, the proximal focal neointima elongated gradually toward the distal anastomosis in experimental vein grafts after surgery. Dynamic expression of the AT₁ receptor and SMC proliferation in the leading region likely facilitate the elongation process of the focal neointima.

The role of the AT₁ receptor in the regulation of SMC proliferation in vein grafts was further verified by using losartan, an AT₁ receptor antagonist. In nonengineered vein grafts with losartan, the rate of SMC proliferation and the degree of intimal hyperplasia were significantly reduced compared with those in nonengineered vein grafts without losartan. However, losartan did not completely prevent SMC proliferation and intimal hyperplasia in nonengineered vein grafts. This observation suggested that, in addition to angiotensin II and its AT₁ receptor, other local mediators are possibly involved in the regulation of blood flow–related focal SMC proliferation and intimal hyperplasia in vein grafts.

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S. Q. Liu

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