Flow-induced neointimal regression in baboon polytetrafluoroethylene grafts is associated with decreased cell proliferation and increased apoptosis

Scott A. Berceli, MD, PhD,[a] Mark G. Davies, MD, PhD, FRCSI,[b] Richard D. Kenagy, PhD,[c] and **Alexander W. Clowes, MD,[c]** *Seattle, Wash*

Objective: **We have previously shown that baboon grafts subjected to elevated shear stress exhibit an increase in luminal area through atrophy of the neointimal layer. This study was designed to investigate the smooth muscle cell (SMC) growth kinetics during early regression and evaluate the influence of nitric oxide (NO) in the regulation of this process.**
Methods: **Sixteen baboons underwent bilateral polytetrafluorethylene aortoiliac graft placement. After development of a neointima over an 8-week period, blood flow through one graft was increased with placement of a downstream arteriovenous fistula. Grafts were harvested at 4 (n = 6), 7 (n = 5), and 14 (n = 5) days and assessed for neointimal cross-sectional area, SMC proliferation and apoptosis, and macrophage infiltration. High-flow grafts were compared with contralateral normal-flow controls. Eleven baboons underwent an identical experimental preparation to evaluate the effect of NO inhibition. Eight weeks after graft implantation, the animals were treated with an initial bolus (100 mg/kg) followed by continuous infusion (60 mg/kg/d) of either N^G-nitro-L-arginine methyl ester (L-NAME; n = 6) or the inactive stereoisomer N^G-nitro-D-arginine methyl ester (n = 5). Grafts were harvested at 7 days and evaluated with the experimental endpoints detailed previously.**
Results: **Distal fistula placement resulted in a 3.8-fold increase in mean centerline velocity and wall shear stress. Grafts harvested during the initial 14 days after flow manipulation showed a progressive reduction in neointimal cross-sectional area. This change was associated with a decrease in subendothelial SMC proliferation and an increase in neointimal SMC apoptosis, the latter being in the region adjacent to the graft. Animals treated with L-NAME showed a 20% reduction in platelet cyclic guanosine monophosphate and a 17% reduction in serum nitrate/nitrite concentrations. Despite this inhibition of NO production, no effect on the flow-dependent changes in neointimal area, cell proliferation, or apoptosis was observed in the L-NAME–treated baboons.**
Conclusion: **The local hemodynamic environment within healing prosthetic grafts modulates neointimal SMC proliferation and apoptosis. An increase in graft flow leads to atrophy of the neointima. (J Vasc Surg 2002;36:1248-55.)**

In normal arteries, acute changes in blood flow induce a change in lumen diameter, a process that is dependent on the endothelium and may be an attempt to normalize shear stress.[1-3] In arteries where the geometry is constrained by calcification associated with progression of atherosclerosis or by an intraluminal stent, the vessels cannot contract or relax in response to changes in blood flow. Instead, luminal dimensions are altered only by changes in cell number and the amount of extracellular matrix in the wall.[4,5] The hemodynamic environment regulates the healing of prosthetic grafts placed in the arterial circulation. In response to an acute decrease in flow, polytetrafluorethylene grafts placed in the aortoiliac position of baboons show a reduction in luminal cross-sectional area because of neointimal thickening.[6,7] This increase in neointimal mass is the result of increased cell proliferation and synthesis of extracellular matrix. Conversely, grafts subjected to elevated shearing forces show an increase in lumen area through atrophy of the neointimal layer by undefined mechanisms.[8]

In the neonatal circulation, arterial development or atrophy is governed by the balance between cell proliferation and matrix protein synthesis on the one hand and cell death and matrix degradation on the other.[9-11] The local hemodynamic environment modulates these opposing processes by directly regulating the kinetics of tissue accumulation in these immature arteries. In this study, we examined whether flow-induced neointimal regression in prosthetic grafts occurs via a similar pertubation in this balance. We show that this atrophy is associated with a reduction in neointimal cell proliferation and an increase in cell apoptosis. On the basis of an established role of nitric oxide (NO) in the regulation of smooth muscle cell (SMC) growth kinetics[12-14] and our previous work showing increased expression of endothelial NO synthase in response to increased graft flows,[8] we tested the hypothesis that NO mediates atrophy in this situation of a constrained vascular

From the Division of Vascular Surgery, University of Florida[a]; the Center for Cardiovascular Research, Division of Vascular Surgery, University of Rochester[b]; and the Division of Vascular Surgery, University of Washington.[c]

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Reprint requests: Alexander Clowes, MD, University of Washington, Box 356410, 1959 NE Pacific St, Seattle, WA 98195 (e-mail: clowes@u.washington.edu).

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geometry with the NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME).[15]

MATERIALS AND METHODS

Experimental model. Sixteen male baboons (Papio cynocephalus) weighing approximately 10 kg were used. Bilateral aortoiliac polytetrafluorethylene grafts (5 cm length, 4 mm diameter, 60 μm internodal distance; W. L. Gore, Newark, Del) were placed as previously described.[6] Animals were anesthetized with ketamine hydrochloride (10 mg/kg, intramuscularly) and maintained on isoflurane inhalation anesthetic during all the operative procedures. After 8 weeks, a superficial femoral artery to vein fistula (10 mm length) was created on one side to increase blood flow. The contralateral graft was maintained as a normal flow control.

Specimens were collected 4, 7, and 14 days after fistula placement. At 17, 9, and 1 hour before necropsy, animals were administered 5-bromo-2′deoxyuridine (BrdU; 30 mg/kg/dose, intramuscularly). Animals were killed with sodium pentobarbital (160 mg/kg, intravenously). Proximal aorta, graft, and external iliac artery specimens were harvested en bloc, and representative samples were either frozen or immersion fixed in 10% buffered formalin. Animal care and handling complied with the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council.

Hemodynamic monitoring. Duplex ultrasonography was used to measure center-stream velocities and diameter in the mid portion of each graft. Measurements were performed 24 hours before placement of the distal fistula and graft harvest. A time-averaged velocity for each graft scan was determined through graphic integration of the velocity waveforms over five cardiac cycles. Shear stress was calculated with the Hagen-Poiseuille equation, $\tau = 2 \times \mu \times v_{tav}/r$; where τ is the mean wall shear stress, μ is the viscosity of blood (0.035 poise), v_{tav} is the time-averaged centerline velocity, and r is the graft radius.

Histology. Formalin-fixed tissue segments were embedded in paraffin, and histologic cross sections were stained with hematoxylin/eosin. Digitized images were collected and analyzed to determine luminal and neointimal cross-sectional areas for each specimen. Mean areas were obtained through averaging the results from two mid-graft segments.

Immunohistochemical procedures were performed with the avidin-biotin-peroxidase method (Vector Laboratories, Burlingame, Calif). Proliferating cells in the neointima were evaluated with an antibody to the thymidine analogue BrdU (Boehringer-Mannheim, Basil, Switzerland).[16] Detection of apoptotic cell death was evaluated with in situ terminal deoxy nucleotidyl transferase-mediated deoxy-uridine-triphosphate nick end labeling of fragmented DNA (TUNEL, Boehringer-Mannheim).[17] Sections were counterstained with hematoxylin to assist in the quantification of total nuclear density. Cells undergoing apoptosis were also identified in frozen tissue sections with an anti-active caspase-3 polyclonal antibody (PharMingen, San Diego, Calif).[18] Macrophage content within the neointima was evaluated with HAM56 (Enzo, Farmingdale, NY).

BrdU-stained, TUNEL-stained, and total nuclei were manually counted in four regions per graft. Regions for analysis were chosen from two separate mid-graft specimens, located 1.5 cm from either the proximal or distal anastomosis, respectively. Two regions of each graft specimen oriented at 0 degrees and 180 degrees around the circumference of the wall were analyzed. To evaluate the radial variation in BrdU and TUNEL staining within the neointima, each region (200 μm in width) was analyzed throughout the thickness of the neointima. Neointimal thicknesses ranged from 190 μm to 980 μm. On average, 3353 nuclei (with a mean of 117 BrdU positive and 265 TUNEL positive cells) over an area of 1.35 mm^2 were examined for each graft. These data served as input to determine an average BrdU or TUNEL labeling index, expressed as the ratio of stained to total nuclei, for each graft.

To evaluate and analyze the radial variations in staining, BrdU, TUNEL, and total nuclear concentration profiles of varying thickness were mathematically transformed to a fixed coordinate system. Within this scheme, the luminal surface was assigned a depth of 0, the neointimal-graft interface was assigned a depth of 1.0, and nuclear counts were converted to 10 data points equally spaced along the depth of the neointima. Normalized profiles were then pooled to obtain group-averaged profiles for each experimental condition.

All data are expressed as the mean ± the standard error of the mean. Statistical differences in mean graft velocity and wall shear stress were calculated with a paired Student *t* test. Differences in neointimal thickness and global BrdU and TUNEL staining were evaluated with two-way repeated measures analysis of variance with a Tukey post hoc analysis (Sigma Stat, San Rafael, Calif). Differences in standardized BrdU and TUNEL profiles were assessed individually at each time point with a two-way analysis of variance (Sigma Stat). *P* values of less than .05 were considered significant.

NOS inhibition experiments. Bilateral aortoiliac polytetrafluorethylene bypass grafts were placed in 11 male baboons. After 8 weeks, a unilateral increase in flow was induced with placement of a femoral arteriovenous fistula. One hour before fistula placement, the NOS inhibitor, L-NAME (Sigma, St Louis, Mo), was infused intravenously at a dose of 100 mg/kg over 10 minutes. L-NAME (60 mg/kg/d) was administered continuously for the remainder of the experiment (7 days) with the intraperitoneal placement of osmotic pumps (Alzet Model 2ML1, Cupertino, Calif). The continuous L-NAME infusion rate was selected on the basis of initial experiments showing that a dose of 60 mg/kg/d reduced platelet cyclic guanosine monophosphate (cGMP) levels (>50% reduction from baseline). Similar doses of L-NAME have produced pharmacologic effects in other animal species (rat 10 to 50

Table I. Hemodynamic changes in response to unilateral fistula placement

	Before fistula		*After fistula*	
	Control-side graft	*Fistula-side graft*	*Control-side graft*	*Fistula-side graft*
Mean centerline velocity (cm/s)	43 ± 5	43 ± 5	29 ± 3	112 ± 9
Shear stress (dynes/cm^2)	15 ± 2	15 ± 2	10 ± 1	39 ± 3

Control-side graft: before versus after fistula, $P < .01$.
Fistula-side graft: before versus after fistula, $P < .0001$.
Values are mean ± standard error of mean.

Table II. Neointimal areas 4, 7, and 14 days after unilateral fistula placement

	Neointimal area (mm^2)		*Ratio high to normal flow*	P *value*
	Normal flow	*High flow*		
Day 4 (n = 6)	4.3 ± 0.3	3.8 ± 0.1	0.87 ± 0.06	NS
Day 7 (n = 5)	4.8 ± 0.1	3.9 ± 0.1	0.80 ± 0.05	.02
Day 14 (n = 5)	4.8 ± 0.1	3.2 ± 0.1	0.67 ± 0.05	<.001

Two-way analysis of variance: effect of flow ($P < .001$).
Values are mean ± standard error of mean.
NS, Not significant.

mg/kg/d,[19-21] rabbit 2 to 12 mg/kg/d,[22,23] pig 40 mg/kg/d[24]). The initial 100 mg/kg loading dose was determined with a single-compartment pharmacokinetic model of L-NAME metabolism, assuming a serum half life of 20.7 hours, volume of distribution of 1.76 L/kg, and a clearance rate of 1.57 L/kg/d.[25-27] Equal doses of the inactive stereoisomer N^G-nitro-D-arginine methyl ester (D-NAME; Sigma) were administered in control animals.

Blood pressure and heart rate were noninvasively monitored during the initial administration of L-NAME and every other day for the remainder of the experiment. Blood samples were collected before drug infusion and at 3 and 7 days after fistula placement. These specimens served to monitor the biologic effect of the administered L-NAME with an enzyme immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ) to measure cGMP levels in platelets[28] and a Griess reagent-based colorimetric assay (Cayman Chemical, Ann Arbor, Mich) to measure nitrate/nitrite levels in serum.[29]

Seven days after fistula placement, animals were killed and the grafts retrieved. BrdU (30 mg/kg/dose) was administered at 17, 9, and 1 hour before graft harvest. Assessment of neointimal cross-sectional areas, BrdU, and TUNEL staining in graft specimens was performed as described previously.

RESULTS

Hemodynamic parameters. Creation of the femoral arteriovenous fistula resulted in an approximate four-fold increase in mean centerline velocity within the graft, with a corresponding increase in fluid shear stress at the neointimal surface (Table I). Accompanying these changes was a modest but statistically significant ($P < .01$) reduction in blood velocity within the contralateral graft.

Morphometry. A progressive reduction in graft neointimal areas was observed over the 14-day period in response to elevated flow rates (Table II). This result confirmed our previous observation, which showed significant neointimal atrophy after an 8-week exposure to increased flows.[8]

BrdU and TUNEL staining. High-flow grafts showed a decrease in BrdU labeling at day 4, and the reductions observed at days 7 and 14 did not reach statistical significance (Fig 1, *A*). This was accompanied by a significant increase in TUNEL labeling, which persisted above baseline levels at all time points examined (Fig 1 , *B*). Average nuclear density was relatively independent of the imposed hemodynamics throughout the regression period (Fig 1 , *C*).

Both BrdU and TUNEL staining were localized to specific regions within the graft neointima (Fig 2). Proliferating neointimal cells were located near the lumen of the graft, and apoptotic cells were present in the juxtagraft region, deep within the neointima (Fig 3). Nuclear density was elevated in the subendothelial region, reaching a minimum in the juxtagraft portion of the neointima. Immunostaining for activated caspase 3 confirmed apoptosis to be the mechanism of cell death within this juxtagraft region (Fig 2 , *C*).

Because macrophage factors might contribute to SMC apoptosis, we counted the number of monocyte/macrophages in the neointima with HAM56 as a marker. The number of macrophages was small and was not altered by increasing flow (0.03 ± 0.02% and 0.05 ± 0.02% for day 4 normal and high flow, respectively; 0.04 ± 0.0.2% and 0.05 ± 0.03% for day 7 normal and high flow, respectively; and 0.10 ± 0.06% and 0.05 ± 0.02% for day 14 normal and high flow, respectively; mean ± the standard error of the mean as a percentage of the number of SMC).

Examination of external iliac arteries, distant to the distal graft anastomosis and exposed to normal flow conditions, showed only rare BrdU positive nuclei (<0.5%) and an absence of TUNEL positive nuclei (data not shown). Increases in iliac artery flow rates, induced by the distal fistula, did not alter these staining patterns.

NOS inhibition experiments. Six animals underwent treatment with L-NAME and five control animals underwent treatment with D-NAME before placement of the femoral fistula. After the initial infusion of L-NAME, a significant increase in mean arterial pressure was observed (before infusion, 72 ± 3 mm Hg; after infusion, 100 ± 9 mm Hg; $P = .016$). No change in pressure was noted after D-NAME administration (before infusion, 69 ± 6 mm Hg; after infusion, 72 ± 3 mm Hg; $P =$ not significant). This increase in blood pressure was only transient. The mean arterial pressures returned to baseline by day 2 and remained at baseline through the completion of the 7-day

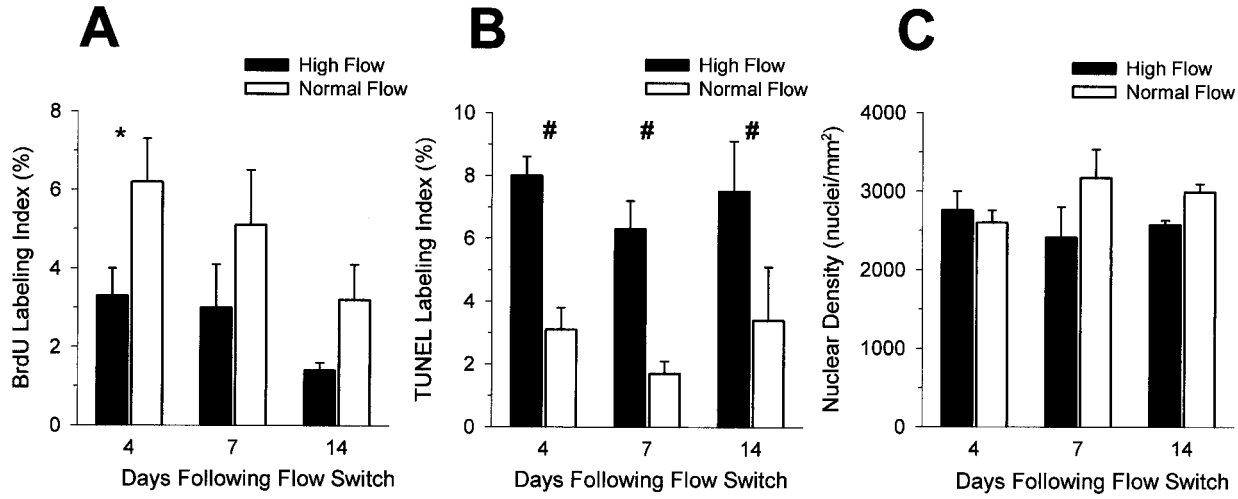

Fig 1. Neointimal SMC proliferation (BrdU labeling index; **A**), apoptosis (TUNEL labeling index; **B**), and nuclear density **(C)** in normal-flow and high-flow grafts after fistula placement. Values are mean ± standard error of mean.
*$P < .05$, normal-flow versus high-flow graft.
#$P < .001$, normal-flow versus high-flow graft.

experiment. Blood velocity and shear stress changes after fistula placement were not significantly different in the L-NAME and D-NAME groups (Table III).

In agreement with our earlier observations, increased graft flow resulted in a significant reduction in neointimal cross-sectional area (Table IV). Administration of L-NAME, however, did not prevent the flow-induced change in neointimal area. An effect of the elevated graft flow rate was also observed in the BrdU and TUNEL labeling indices. High-flow grafts showed a significant reduction in cell proliferation and a trend towards an increase in cell death. Neither the BrdU nor TUNEL index was influenced by the infusion of L-NAME.

The animals administered L-NAME showed a significant reduction in platelet cGMP concentrations (before infusion, $4.1 \pm 0.2 \times 10^{-9}$ pmole/platelet; after infusion, $3.3 \pm 0.7 \times 10^{-9}$ pmole/platelet; $P < .005$), and the animals receiving D-NAME showed no difference (before infusion, $2.6 \pm 0.4 \times 10^{-9}$ pmole/platelet; after infusion, $2.3 \pm 0.5 \times 10^{-9}$ pmole/platelet; P = not significant). In animals receiving L-NAME, a significant reduction in the concentration of inactive NO end products, NO_2 and NO_3, within the serum was observed (before infusion, 25.5 ± 1.5 mmol/L; after infusion, 21.2 ± 1.2 mmol/L; $P = .05$). Serum NO_x concentrations were unaffected by D-NAME administration (before infusion, 25.4 ± 2.8 mmol/L; after infusion, 24.7 ± 1.4 mmol/L; P = not significant).

DISCUSSION

We have shown that prosthetic grafts heal and that the neointima grows in a normal flow environment largely because of subendothelial SMC replication. Our findings support the hypothesis that the rate at which neointimal hyperplasia develops is dependent on the relative balance between subendothelial cell replication and cell death in the region adjacent to the graft. Through modulation of the hemodynamic environment, and more specifically through changes in shearing forces, the balance between these processes appears to be altered and result in a change in neointimal thickness within the graft. Regression within our model occurs via simultaneous reduction in cell number and loss of surrounding matrix, as supported by the stable cell density throughout the 2-week period. This agrees with our recent findings that reveal an increase in matrix metalloproteinases (MMP; MMP-2 and MMP-9) under conditions of elevated flow.[30]

Our findings are similar to observations made in developing arteries, where cell proliferation and death are closely regulated by fluid shear.[10] This study is the first documentation of the importance of this balance in vascular graft healing. A similar inverse relationship between neointimal thickness and shear stress has been observed in healing stented human coronary arteries.[5]

Smooth muscle cell apoptosis has been identified as a prominent feature in both primary atherosclerotic[31] and restenotic arterial lesions.[32,33] Examination of these lesions show the frequent colocalization of macrophages with apoptotic SMCs, and recent studies suggest that upregulation of proinflammatory chemokines during programmed cell death may be the underlying mechanism for this association.[34] Although baseline apoptosis in healing prosthetic grafts proceeded in the relative absence of surrounding macrophages and enhancement of cell death after increasing fluid shear occurred without a change in macrophage content, it is interesting that apoptosis was confined to the neointima adjacent to the macrophage-rich graft matrix. Further studies are needed to determine whether the localization of apoptotic SMCs is the result of a gradient of

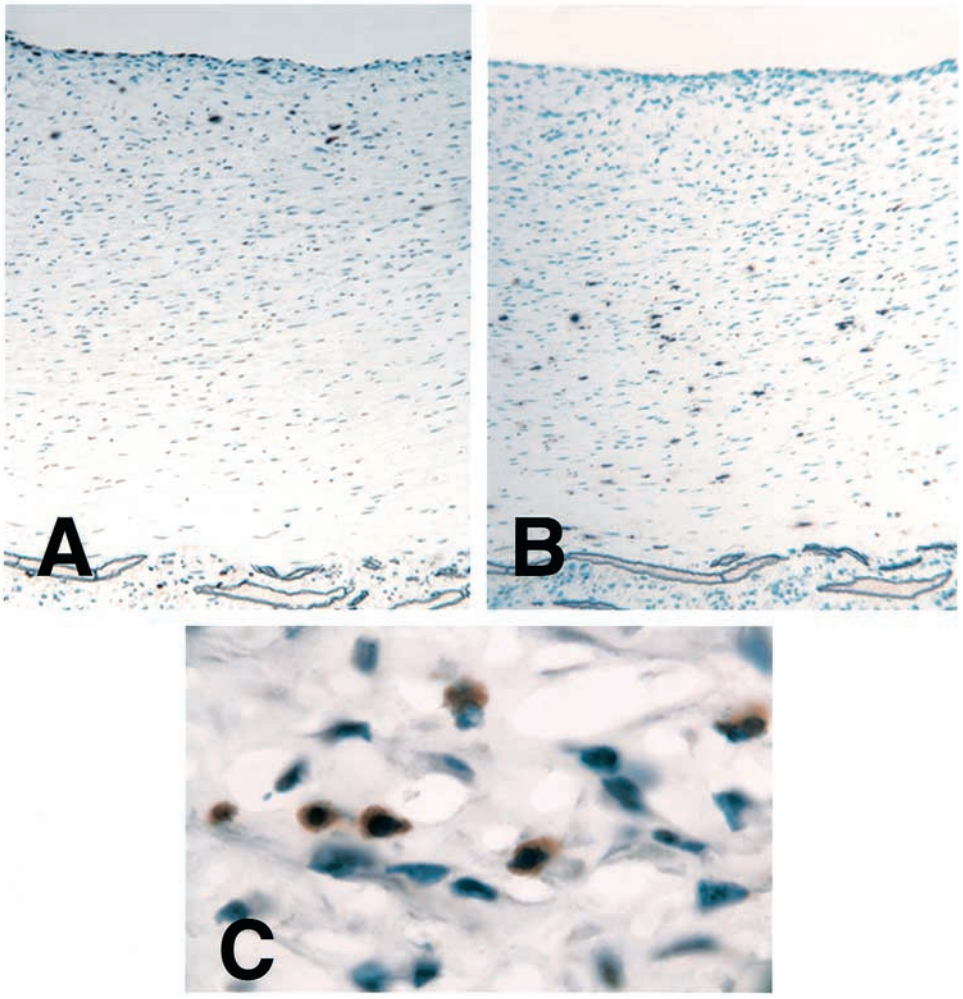

Fig 2. BrdU **(A)** and TUNEL **(B)** staining within neointima of consecutive histologic sections. Note cell proliferation located primarily in subendothelial region and cell death in neointima in deep juxtagraft region. Activated caspase-3 antibody staining **(C)** over neointimal cells adjacent to polytetrafluorethylene graft.

proapoptotic cytokines secreted by macrophages in the graft matrix.

Our model permits us to evaluate the divergent adaptive responses in prosthetic grafts and normal iliac arteries that occur in response to changes in flow. In agreement with previous observations, SMC proliferation and death in iliac arteries exposed to normal flow were barely detectable.[9] After an acute increase in flow, wall shear within these arteries is normalized by vasodilation. This is in notable contrast to the adaptive response seen in rigid prosthetic grafts. We think that an increase in wall shear induces atrophy of the neointima, mediated through reduced cell replication and increased cell death, because lumen diameter could not be increased by vasodilation. Although the mechanisms of atrophy have not been well characterized, we have hypothesized that those mediators that regulate vascular tone in intact arteries might also regulate flow-induced remodeling of the graft neointima.[35] We have previously found an association between neointimal atrophy and increased expression of endothelial NO synthase,[8] and this observation served as a basis for this investigation.

NO and flow-induced neointimal atrophy. In this study, we showed that pharmacologic blockade of NO production to the modest level achieved via systemic administration of L-NAME did not affect the flow-mediated reduction in neointimal cross-sectional area, SMC proliferation, or apoptosis. This observation seems contrary to the perceived role of NO in vascular remodeling. Most research has focused on the role of NO in the development of neointimal hyperplasia and provided support for the conclusion that increasing NO production inhibits this process.[12,36,37] However, the role of NO in the vascular remodeling associated with increased flow, and more

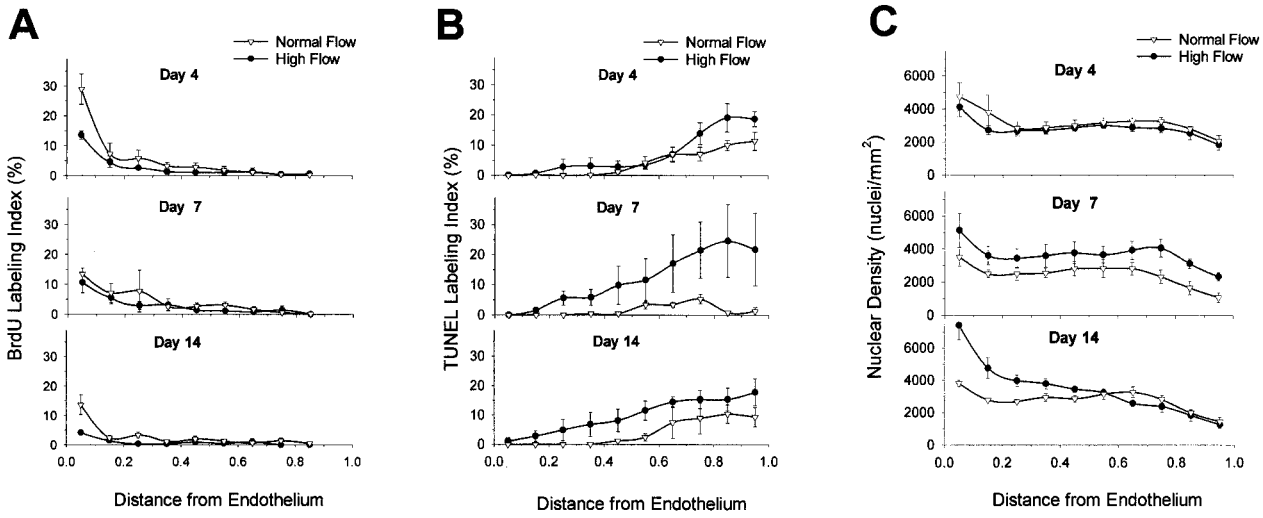

Fig 3. SMC proliferation (BrdU labeling index; **A**), apoptosis (TUNEL labeling index; **B**), and nuclear density **(C)** in neointima. Depth is measured from luminal surface and normalized to neointimal thickness. Values are mean ± standard error of mean.
BrdU staining: $P = .005$ (day 4) and $P < .001$ (day 14) for high versus normal flow.
TUNEL staining: $P = .001$ (day 4), $P < .001$ (day 7), and $P < .001$ (day 14) for high versus normal flow.
Nuclear density: $P = .003$ (day 7) and $P < .001$ (day 14) for high versus normal flow.

Table III. Hemodynamic changes in response to unilateral fistula placement: L-NAME and D-NAME treated animals

		Before fistula		*After fistula*	
		Control-side graft	*Fistula-side graft*	*Control-side graft*	*Fistula-side graft*
L-NAME	Mean centerline velocity (cm/s)	37 ± 9	38 ± 9	20 ± 3	123 ± 16
L-NAME	Shear stress (dynes/cm^2)	26 ± 6	27 ± 6	14 ± 2	86 ± 11
D-NAME	Mean centerline velocity (cm/s)	34 ± 10	39 ± 12	23 ± 4	83 ± 13
D-NAME	Shear stress (dynes/cm^2)	24 ± 7	27 ± 9	16 ± 3	58 ± 9

L-NAME: control-side graft, before versus after fistula, P = not significant. Fistula-side graft, before versus after fistula, $P < .01$.
D-NAME: Control-side graft, before versus after fistula, P = not significant. Fistula-side graft, before versus after fistula, $P < .01$.
Values are mean ± standard error of mean.

Table IV. Effect of NOS inhibition on graft neointimal area and percentage of BrdU and TUNEL positive nuclei

	L-NAME (n = 6)		*D-NAME (n = 5)*		*ANOVA* P *values*	
	Normal flow	*High flow*	*Normal flow*	*High flow*	*Effect of L-NAME*	*Effect of flow*
Neointimal area (mm^2)	6.6 ± 0.6	4.4 ± 0.8	5.7 ± 0.3	5.0 ± 0.1	NS	<.001
BrdU labeling index (%)	3.7 ± 1.5	2.9 ± 1.0	4.6 ± 1.3	2.7 ± 0.6	NS	.05
TUNEL labeling index (%)	4.1 ± 1.5	4.5 ± 1.4	3.3 ± 1.0	5.7 ± 2.1	NS	NS

Values are mean ± standard error of mean.
ANOVA, Analysis of variance; *NS*, not significant.

specifically in flow-mediated neointimal regression, has not been well defined. Several studies have confirmed the importance of NO in mediating the early changes in lumen diameter that occur in response to acute elevations in flow.[38,39] Yet to be clearly delineated is the importance of NO in the remodeling process associated with chronic increases in flow. Although some studies have suggested that the structural remodeling in flow-loaded arteries is in part NO dependent,[19,40,41] other investigators have failed to confirm this finding.[42]

Few investigators have performed both a physiologic and pharmacologic evaluation after the administration of NOS inhibitors; however, two recent manuscripts may provide some insight into our findings. In a study by Nishiyama et al,[43] the effect of L-NAME, given as a 30-mg/kg bolus and 50-mg/kg/h infusion, on renal hemodynamics was evaluated. Administration of L-NAME resulted in a 35% decrease in renal blood flow and a 24% increase in renal artery pressure. These changes were associated with a modest 16% reduction in cGMP levels within the renal parenchyma. In an ex vivo experimental system, Akiyama et al[44] evaluated the impact of L-NAME on endothelin-induced vasoconstriction and correlated their finding to arterial cGMP levels. They found that treatment of arterial segments in a bath of 0.3 mmol/L L-NAME resulted in a significant inhibition in the contractile response (averaging an approximate one-log inhibitory shift in the dose response curve). This inhibition was associated with a 30% reduction in cGMP levels.

Although our data suggest that flow-mediated graft neointimal regression is not NO dependent, several additional explanations must be explored. The early time point of this experiment was chosen to examine both the early impact on cell kinetics and the later morphologic changes in neointimal thickness. The negative findings in this study may have related to the short duration of the treatment period. Although significant reductions in platelet cGMP and serum nitrate/nitrite levels were seen, we have not documented pharmacologic blockade of NOS within the graft neointima. Despite use of an L-NAME dose in this experiment at the upper end of the range used by other investigators,[19-24] this dose may have been insufficient to completely inhibit NO production. This potential concern is also important in light of the relative sensitivity of the various NOS isoforms to L-NAME. L-NAME is a more selective inhibitor for the constitutive over the inducible form of NOS,[45] and insufficient blockade of an upregulated iNOS may have yielded only partial blockade of NO production. Other investigators have observed similar findings.[46]

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