Interaction of Genetic Deficiency of Endothelial Nitric Oxide, Gender, and Pregnancy in Vascular Response to Injury in Mice

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Abstract

To begin to dissect atherogenesis as a complex genetic disorder affected by genetic makeup and environment, we have (*a*) generated a reproducible mouse model of neointimal growth; (*b*) evaluated the effect of disruption of a single gene, endothelial nitric oxide synthase, believed to be central to intimal growth, and (*c*) examined the modifying effects of gender and pregnancy upon the vascular response. Cuff placement around the femoral artery causes reproducible intimal growth. We assessed the response to injury by quantitative morphometry, measuring the intimal to medial (I/M) volume ratio. In wild-type mice, cuff placement causes pronounced intimal proliferation without affecting the media, resulting in I/M ratios of 31% (SV129 males) and 27% (C57BL/6 males). eNOS mutant male mice have a much greater degree of intimal growth (I/M ratio of 70%). Female mice show less intimal response than do males, although eNOS mutant female mice still have more response than do wild-type females. Most dramatic, however, is the effect of pregnancy, which essentially abolishes the intimal response to injury, even overriding the effect of eNOS mutation. We conclude that eNOS deficiency is a genetic predisposition to intimal proliferation that is enhanced by male gender, and that may be overridden by pregnancy. (*J. Clin. Invest.* 1998. 101:1225–1232.) Key words: vascular endothelium • animal disease models • transgenic mice • tunica intima • atherosclerosis

Introduction

Multiple genes interact with each other and with environmental factors to affect hereditary propensity to atherosclerosis. We are interested in studying the interaction of individual genes believed to function in vascular response to injury with modifiers, such as gender. Endothelial nitric oxide synthase (eNOS)[1] synthesizes nitric oxide (NO), which accounts for endothelium-derived relaxing factor activity. NO has physiologic effects that may normally serve to prevent atherosclerosis, including suppression of smooth muscle proliferation (1), inhibition of platelet aggregation and adhesion (2), and inhibition of leukocyte adhesion (3, 4). A reduction in endothelial NO levels would be expected to diminish these normally protective effects, and predispose to atherogenesis. Endothelial dysfunction, characterized by diminished vasodilatory responses to cholinergic stimulation and by diminished endothelial NO levels, is observed in atherosclerosis (5, 6), hypertension (7), diabetes mellitus (8), and hypercholesterolemia (9). It is unclear whether diminished endothelial NO levels are merely associated with endothelial dysfunction, or whether they cause increased propensity to atherosclerosis. This distinction is important, because if the latter is true, then approaches to restore endothelial NO levels may retard atherosclerosis.

In this report, we use eNOS mutant mice to test the hypothesis that reduction in endothelial NO production leads to increased propensity to atherosclerosis. To do this, we need a model that mimics features of human atherosclerosis with a quantitative and reproducible endpoint. In addition, the model should be one in which the endothelium itself is not directly injured so that we can study the effect of eNOS deficiency in the setting of an otherwise preserved endothelium. We adapted an external vascular cuff model previously used in rabbits (10, 11) to mice. We describe here the characterization of this model, and results using this model in eNOS mutant mice. We have found that eNOS deficiency markedly enhances intimal growth in response to injury, but this effect is completely obviated during pregnancy.

Methods

Animals. We have previously described the generation of eNOS mutant mice (12). These mice have a combined genetic background of both SV129 and C57BL/6 strains. Thus, both wild-type SV129 and C57BL/6 mice were used as controls to ensure that variation due to genetic background would not confound our results. Animals were divided into nine groups: SV129 males, SV129 females, SV129 pregnant females, C57BL/6 males, C57BL/6 females, C57BL/6 pregnant females, eNOS mutant males, eNOS mutant females, and eNOS mutant pregnant females. All animals were 7–8-wk old, and weighed 17–27 g. Pregnant mice were 6 or 7 d pregnant at the time of cuff placement (normal mouse gestation is 20–21 d).

Femoral artery cuff placement. Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The left femoral artery was isolated from surrounding tissues, loosely sheathed with a 2.0-mm polyethylene cuff made of PE-50 tubing (inner diameter, 0.56 mm; outer diameter, 0.965 mm; Becton Dickinson, Mountain

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; eNOS, endothelial nitric oxide synthase; I/M, intima to media ratio; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

View, CA) and tied in place with an 8-0 suture. The cuff is larger than the vessel, and does not obstruct blood flow. The right femoral artery was dissected from surrounding tissues (sham-operated), but a cuff was not placed. The femoral arteries were replaced, and the wounds were sutured. After recovery from anesthesia, the animals were given standard diet and water ad libitum.

For determination of cellular proliferation, in additional animals a model 2002 Alzet pump containing a solution of bromodeoxyuridine (BrdU) was placed at the time of cuff placement for administering BrdU. The infusion rate was 0.5 μl/h, corresponding to a total BrdU dose of 350 mg/kg body wt over a 14-d period.

Tissue harvesting and histologic staining. 2 wk after cuff placement, animals were anesthetized and killed. Vessels were fixed in situ by constant pressure fixation at 100 mmHg with 10% formalin through a 22-gauge butterfly angiocatheter placed in the left ventricle of the heart. Both right and left femoral arteries were harvested. Each artery was embedded in paraffin, and cross-sections (10 μm) were continuously cut from one edge to the other edge of the cuffed portion, and in the corresponding segment of the contralateral control artery. Each section was mounted in order on five series of slides. Parallel sections were subjected to standard hematoxylin and eosin staining as well as to immunohistochemistry.

Morphometry. Morphometric analyses were performed on hematoxylin and eosin–stained tissue. For each animal, 10 cross-sections from the cuffed left femoral artery and the control right femoral artery were photographed, and the images were digitized using a Kodak RF 2035 Plus Film Scanner (Eastman Kodak Co., Rochester, NY). For each artery section, the thickness of the intima and media were measured. For area/vol calculations, four measurements were made using an image analysis computer program (NIH Image; National Institutes of Health; Bethesda, MD): luminal circumference, luminal area, area inside the inner elastic lamina, and area inside the outer elastic lamina. Mean vascular diameter was calculated as luminal circumference/π. The intima was defined as the area between the lumen and the internal elastic lamina. The media was defined as the area between the internal and external elastic laminas. The volumes of intima and media were calculated by integrating the areas over the length of the cuffed region. The observers of the sections were blinded to the gender or genotype of the mice. The intraassay variability, using independent observers or analyzing adjacent sections from the same vessel, was $< 3\%$.

Immunohistochemistry. Immunohistochemical stains were performed using the following antisera and dilutions: anti-alpha smooth muscle actin (1:500 dilution, monoclonal mouse; Sigma Chemical Co., St. Louis, MO), anti–von Willebrand factor (1:3,200 dilution, polyclonal rabbit; DAKO Corp., Carpinteria, CA), anti-inducible NOS (iNOS; 1:1,000 dilution, polyclonal rabbit; Transduction Laboratories, Lexington, KY), anti-BrdU (1:100 dilution, monoclonal rat; Harlan Sera-Lab, Loughborough, England). The secondary antibodies (biotinylated anti–mouse, anti–rat, or anti–rabbit) were applied followed by avidin-peroxidase complexes (Peroxidase Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA). The reaction was visualized with 3,3′-diaminobenzidine.

Statistical analysis. All values were expressed as mean±SEM. Luminal diameter, intimal and medial thickness, and the ratios of intimal to medial volumes of the 9 groups of mice studied 14 d after cuff placement were first tested by ANOVA. When the ANOVA demonstrated significant differences, Bonferroni/Dunn's analysis was used posthoc to compare groups. Luminal diameters and medial thickness of two groups (cuffed arteries and contralateral control arteries) were compared using a paired Student's *t* test. For all statistical analyses, $P < 0.05$ was considered significant.

Results

Characterization of cuff model. To characterize the cuff model, we studied both C57BL/6 and SV129 wild-type mice, since the eNOS mutant mice have a genetic background derived from both these strains. No baseline intima was present in the femoral arteries of either wild-type strain. There was reproducible neointimal formation 2 wk after placement of the cuff. We used morphometric analysis to make two types of measurements. First, we measured the thickness of the intima and media. Second, we determined the area of the intima and the media in 10 equally spaced sections along the cuffed region, and calculated the volume of intima and media. The degree of intimal formation was expressed as intimal/medial volume ratio.

Table I. Response to Vessel Injury in Wild-type Mice

	C57BL/6 strain		SV129 strain	
	Male	Female	Male	Female
n	7	7	15	9
Weight (g)	24.5±0.4	17.7±0.4	24.9±0.5	20.2±0.1
Control intimal thickness (μm)	0	0	0	0
Control medial thickness (μm)	24.6±1.5	30.0±1.5	28.5±1.6	30.2±1.3
Cuffed intimal thickness (μm)	7.3±1.8	5.3±0.5	9.2±0.9	7.0±2.3
Cuffed medial thickness (μm)	21.8±1.9	25.3±2.0	26.1±1.8	28.5±2.0
Cuffed I/M volume ratio (%)	27.7±5.2	18.2±1.5	31.1±3.0	17.2±4.7

Table I shows the results in wild-type mice. Male mice of both SV129 and C57BL/6 strains have more intima after cuff placement than do female mice. This result is apparent in terms of intimal thickness, as well as intimal to medial volume ratios. Male mice have intimal to medial (I/M) ratios of 27.7% (C57BL/6) and 31.1% (SV129), as compared with female mice with I/M ratios of 18.2% (C57BL/6) and 17.2% (SV129). There were no differences in luminal diameters or medial thickness among any of the groups. No intima developed on the sham-operated side in any of the mice.

Effect of eNOS gene deficiency. Neointimal formation after cuff injury was greater in eNOS mutant male mice compared with male mice of either wild-type strain ($P < 0.05$, Table II). Male eNOS mutant mice had I/M volume ratios of 70.0%, as compared with wild-type values of 27.7% (C57BL/6) and 31.1% (SV129). Female eNOS mutant mice had less intimal proliferation (I/M volume ratio of 42.7%) than did male eNOS mutant mice, but had more than did wild-type female mice (I/M volume ratios of 18.2% for C57BL/6 and 17.2% for SV129). There were no significant differences in luminal diameters or medial thickness among the groups. As for wild-type mice, eNOS mutant mice did not have any visible intima at

Table II. Effect of eNOS Gene Disruption on Response to Vessel Injury

	eNOS mutant	
	Male	Female
n	9	9
Weight (g)	23.9±0.9	24.6±0.4
Control intimal thickness (μm)	0	0
Control medial thickness (μm)	30.2±1.2	23.8±3.0
Cuffed intimal thickness (μm)	20.3±1.5	11.1±1.8
Cuffed medial thickness (μm)	23.4±0.8	20.7±2.8
Cuffed I/M volume ratio (%)	70.0±6.4	42.7±1.8

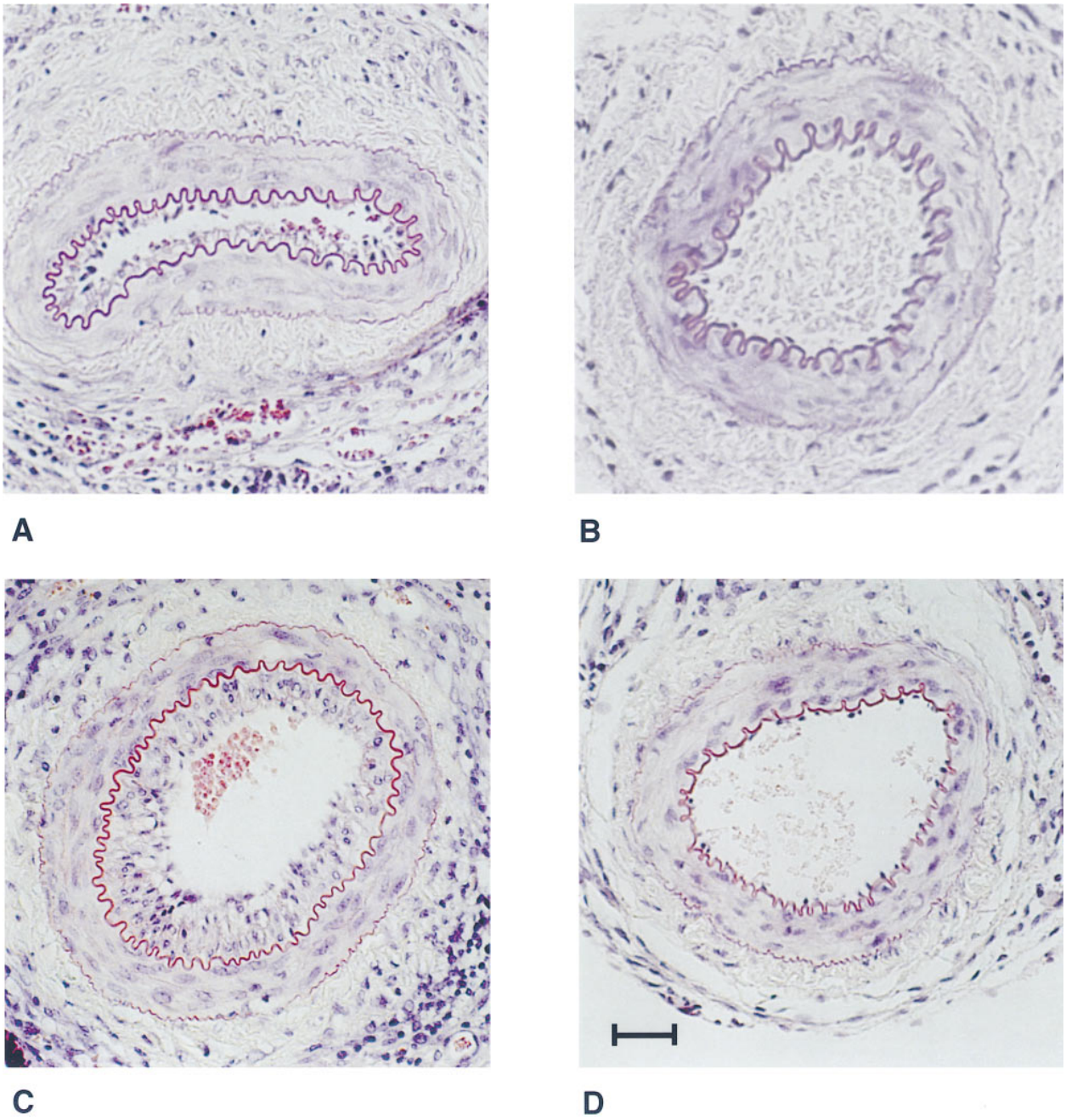

Figure 1. Response of wild-type and eNOS mutant mice to cuff injury. Hematoxylin and eosin–stained sections from male wild-type injured (*A*), sham-operated (*B*), and eNOS mutant injured (*C*) and sham-operated (*D*) vessels. The intima is seen within the internal elastic lamina. eNOS mutant mice show substantially more neointimal formation in response to cuff injury than wild-type mice. *Bar*, 30 μm.

baseline, nor did sham-operated vessels show intimal proliferation. Fig. 1 shows representative sections of sham-operated and cuff-injured vessels.

Histologic characterization of intimal response. Fig. 2 shows sections of cuff-injured and sham-operated control arteries from an eNOS mutant male mouse. Smooth muscle cells in the media normally stain for alpha actin (Fig. 2 *F*). In the corresponding cuff-injured artery (Fig. 2 *E*), not only do cells in the media (inside the internal elastic lamina) stain, but so do the majority of cells in the neointima (outside the internal elastic lamina). On the other hand, staining for von Willebrand factor occurs only in the endothelial layer in both cuff-injured and sham-operated vessels. In the cuff-injured vessel, the vWF staining is separated from the internal elastic lamina by the neointima (Fig. 2 *G*), whereas on the sham-operated side, the vWF-stained cells directly appose the internal elastic lamina because there is no intima (Fig. 2 *H*). The endothelial layer is intact in both the sham-operated and the cuff-injured vessels.

Fig. 3 shows a high-power magnification of a section from a wild-type cuff-injured vessel 7 d after cuff placement. The in-

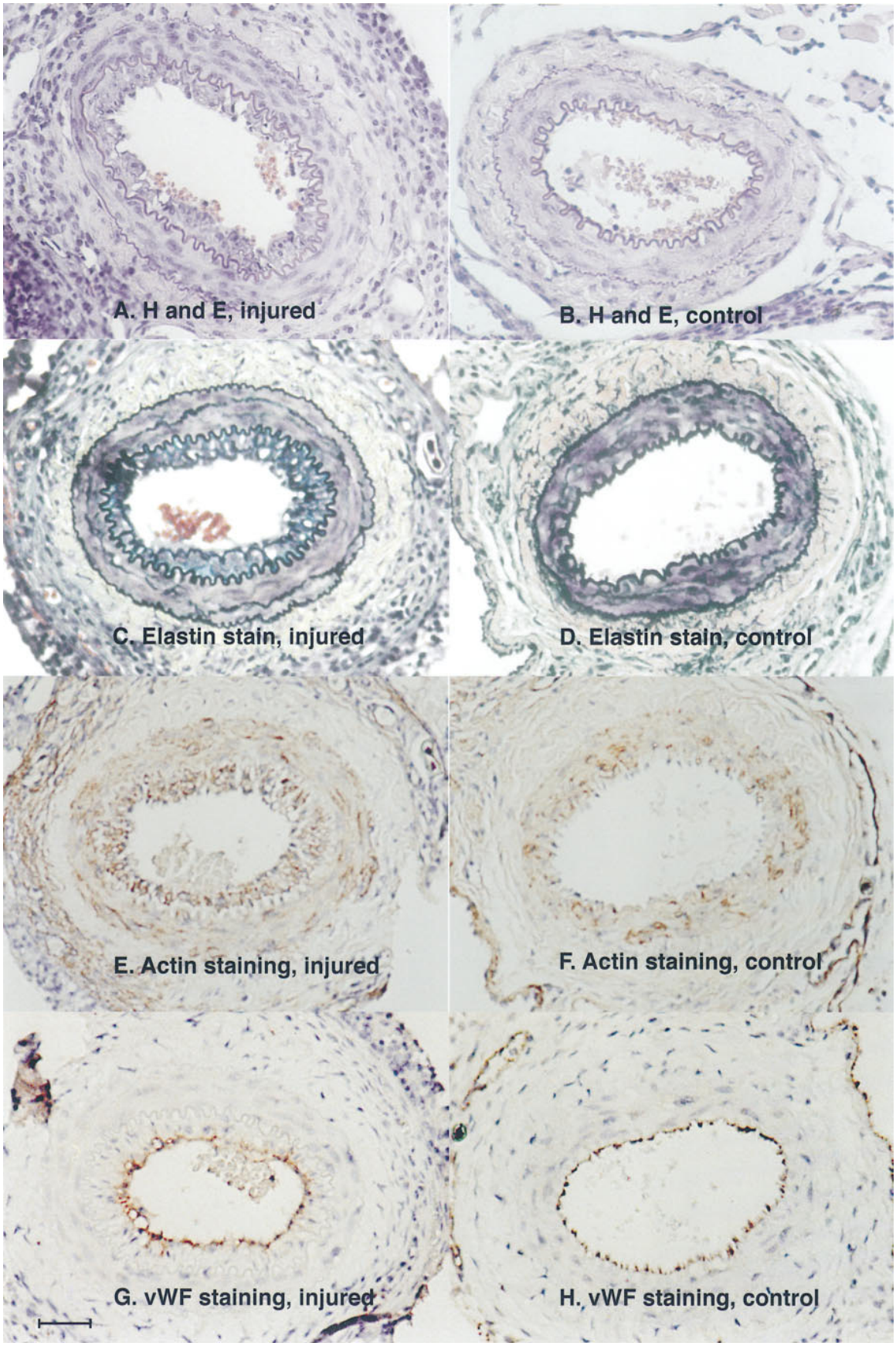

Figure 2. Histology and immunochemical staining of injured and control vessels. One injured (*left side*) and one sham-operated (*right side*) femoral artery was dissected from an eNOS mutant male mouse. The sections were adjacent sections processed for hematoxylin and eosin staining (*A* and *B*), elastin staining (*C* and *D*), immunohistochemical staining for alpha actin (*E* and *F*), and immunohistochemical staining for von Willebrand factor (*G* and *H*). *Bar*, 30 μm.

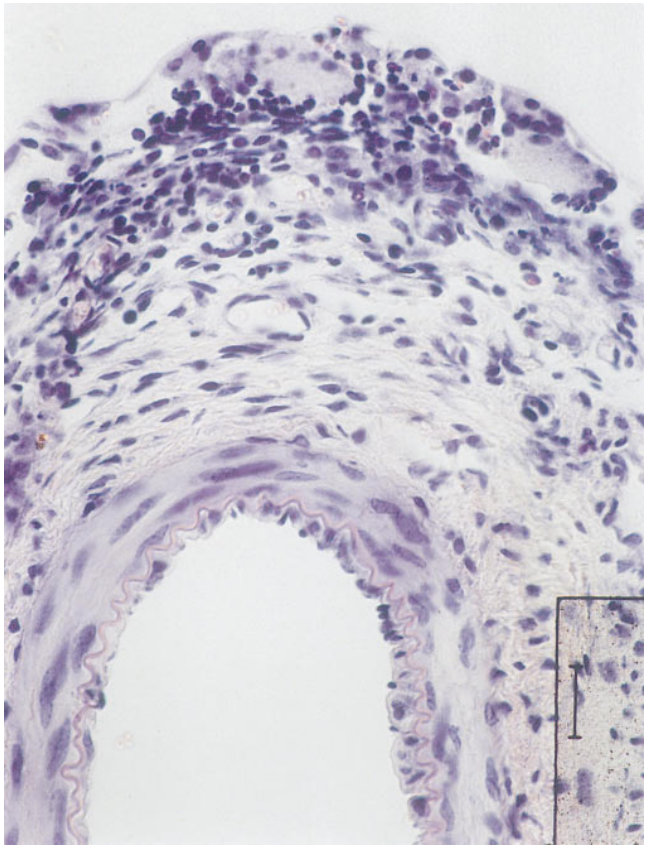

Figure 3. High-power magnification of cuffed femoral artery from wild-type mouse 7 d after cuff placement. This section was stained with hematoxylin and eosin. Inflammatory cells are seen in the adventitia. At 7 d, there is less intima present than at the 14-d endpoint. *Bar*, 30 μm.

tima is visible at 7 d of injury, although it is not as thick as it was 14 days after cuff placement. Inflammatory cells are visible in the adventitia of the vessel. To determine whether iNOS is expressed after cuff injury, and how this relates temporally to cellular proliferation, we performed a separate series of time-course experiments. Vessels were stained for iNOS immunohistochemistry and BrdU uptake at 3, 7, 10, and 14 d after injury. The results were the same in wild-type and eNOS mutant mice. There was no significant BrdU staining or iNOS staining in the sham-operated vessels. As seen in Fig. 4, iNOS expression is absent at 3 d, but is detectable in adventitial cells at 7 d and in neointimal and adventitial cells at 10 and 14 d. Staining for BrdU, on the other hand, is clearly present at 3 d. It occurs in the adventitia, media, and intimal layers, and increases with time. Thus, cellular proliferation occurs before iNOS expression.

Table III. Effect of Vessel Injury in Pregnant Female Mice

	C57BL/6	SV129	eNOS mutant
n	8	7	7
Weight (g)	19.6±0.5	22.3±0.5	23.2±1.0
Control intima (μm)	0	0	0
Control media (μm)	28.1±1.2	24.7±3.7	30.5±2.0
Cuffed intima (μm)	0.3±0.1	0.7±0.2	2.0±0.3
Cuffed media (μm)	26.3±0.7	26.6±2.2	26.9±0.7
Cuffed I/M vol. ratio (%)	0.9±0.4	2.1±0.5	6.5±1.2

Effect of pregnancy. Pregnant mice showed a striking lack of intimal proliferation after cuff placement. As seen in Table III, pregnant female wild-type mice had I/M ratios of 0.9% (C57BL/6) and 2.1% (SV129), as compared with pregnant eNOS mutant mice that had I/M ratios of 6.5%. These values are markedly lower than the I/M ratios of nonpregnant female mice of the same age (Tables I and II).

Discussion

In this report, we describe and characterize a new mouse model of vessel injury that involves placing a nonocclusive cuff around the femoral artery. This model has been previously described in rabbits (10, 11). In our hands, it results in predictable formation of neointima in mice over a 14-d period. It differs from other rodent models such as the filament model (13) and the balloon model (14, 15) in several important ways. In the present cuff model, the endothelial cells are not directly manipulated or removed, allowing study of the effect of individual endothelial factors, including endothelium-derived NO. In contrast, the filament and balloon models involve removing the endothelial layer. In the cuff model, the primary endpoint is neointimal formation. In the filament model, intimal formation occurs to varying degrees, but the primary endpoint is an increase in medial thickness (13, 16).

We believe that the cuff model is complementary to the filament model, and that both may be useful animal models with which to examine vessel responses to injury that are relevant to atherosclerosis. The cuff model is reproducible, easily quantitated, and lends itself well to analysis of individual gene products that can be manipulated by transgenic approaches and targeted gene disruption. In our study, the C57BL/6 and the SV129 mouse strains, often used to generate transgenic and null mutant mice, showed similar responses (Table I). Thus, these genetic backgrounds should not affect the quantitative analysis of vascular intimal formation in mice generated using SV129 embryonic stem cells and bred using C57BL/6 mice.

The mechanism of intimal formation after cuff injury is not known. Booth et al. propose that neointimal formation in the rabbit model of cuff injury may be mediated by obstruction of the adventitial vasa vasorum with the creation of a localized ischemic region (10). In addition, local hemodynamic changes may be involved (17). Kockx et al. report that the rabbit cuff model is characterized by smooth muscle cell replication in the media before neointimal formation (11). The temporal sequence suggests that medial proliferation is followed by migration of cells through the internal elastic lamina into the intima. The adventitia of the cuff-injured, but not sham-operated, vessels shows infiltration of inflammatory cells, in agreement with our observations in mice.

Our results indicate that absence of eNOS increases the neointimal proliferative response to vessel injury from cuff placement, consistent with protective roles for eNOS that normally serve to prevent vascular intimal formation in vivo. These results support the notion that the reduction in endothelial NO levels observed in hypertension, diabetes, and hypercholesterolemia is not only associated with atherosclerosis, but

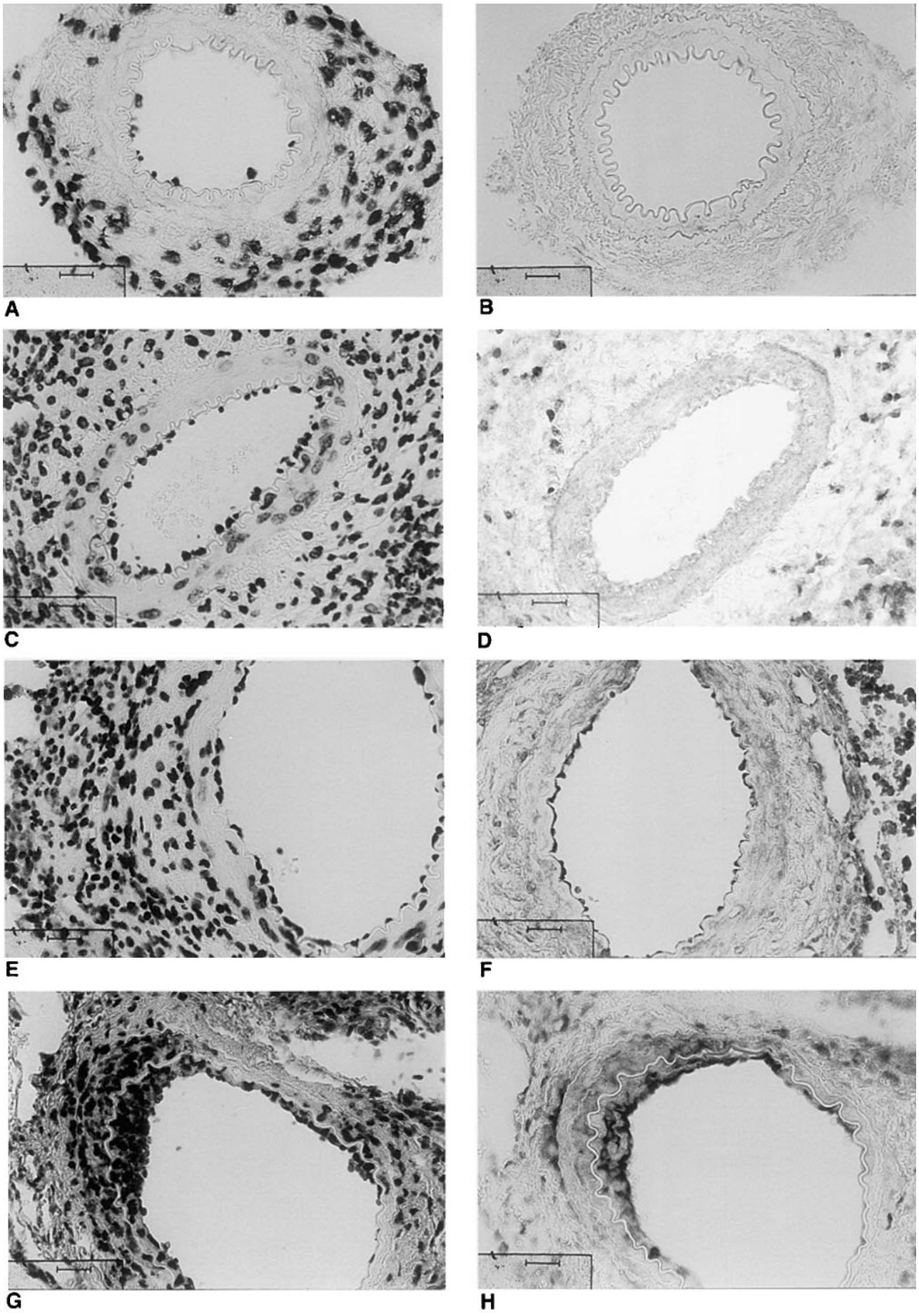

Figure 4. Time course of cellular proliferation and iNOS expression after cuff placement. Vessels were harvested at 3 (*A* and *B*), 7 (*C* and *D*), 10 (*E* and *F*), and 14 d (*G* and *H*) after cuff placement from eNOS mutant mice. Sections from the same cuffed vessel at each time point were stained for BrdU (*A*, *C*, *E*, and *G*) and for iNOS immunohistochemistry (*B*, *D*, *F*, and *H*). BrdU-positive cells are seen at 3 d, and increase with time. iNOS expression is not detected at 3 d, but is visible in the adventitia at 7 d and in the intima at 10 and 14 d. *Bar*, 30 μm.

may indeed be involved in its pathogenesis. There are several possible mechanisms for this involvement. First, NO suppresses smooth muscle cell proliferation. The effect of NO donors on smooth muscle proliferation and on neointimal formation has been demonstrated in a rat model of intimal injury (18). Second, NO inhibits platelet adhesion and aggregation (2). Third, NO suppresses leukocyte–endothelial interactions (3, 4). NO markedly attenuates leukocyte rolling along the endothelium and leukocyte adhesion in part by inhibiting expression of P-selectin on the vascular endothelium (19–21), and in part by inhibiting ICAM-1 and VCAM-1 (22). Each of these mechanisms would have the effect of suppressing responses to vessel injury. Reductions in endothelial NO levels would therefore cause increased responses to injury.

In addition to local tissue effects, eNOS deficiency has systemic hemodynamic effects. The eNOS mutant mice are hypertensive (12) compared with wild-type animals. In the current study, we have not demonstrated that systemic hypertension does not affect intimal formation in the cuff model. However, because hypertension itself is associated with endothelial dysfunction and reduced endothelial NO levels, it would be difficult to separate the hemodynamic effects of hypertension from its endothelial effects. The effect of hypertension in this model needs to be further evaluated by experiments involving local gene transfer of eNOS into vessels of eNOS mutant animals. This transfer would separate the local effects of eNOS expression on suppressing smooth muscle cell proliferation from systemic hypertension and its neurohumoral effects. In the current study, the animals studied were young adults (7–8 wk), and we did not note any histologic evidence of vascular abnormality or increased wall thickness secondary to hypertension.

To determine the time course of neointimal proliferation and the possible role of the iNOS isoform, we performed a separate series of experiments in mice in which Alzet pumps were placed for administration of BrdU. We examined vessels at 3, 7, 10, and 14 d after cuff placement for BrdU staining for cellular proliferation and iNOS staining by immunohistochemistry. Neither significant BrdU staining nor iNOS expression was noted in any of the sham-operated control vessels. BrdU-positive cells reflecting cellular proliferation were present in the cuffed vessels as early as day 3, and occurred in the intima, media, and adventitia. In contrast, iNOS staining was not detected at 3 d, but was present at later time points in both the intima and the adventitia. Thus, the proliferative response begins to occur in the cuffed vessels before iNOS expression.

These results are in agreement with a recent report on the cuff model, in which iNOS expression was found in cuffed arteries only after cellular proliferation and neointimal formation. In the rabbit model, as we find in the mouse model, iNOS is induced not only in the adventitia, but also in the intima. One unresolved question is why iNOS expression is not sufficient to overcome the absence of eNOS expression in the eNOS mutant mice. The first explanation is that iNOS expression clearly occurs after cellular proliferation has started, so it may not be expressed early enough to suppress the initial events in neointimal formation. A second possibility is that the manner in which eNOS generates NO in response to physiologic signals such as flow, shear stress, stretch, and circulating signals is important. Despite its ability to generate larger quantities of NO, iNOS may not generate it in a manner or location that triggers the same results as eNOS in suppressing smooth muscle proliferation. Finally, while we have documented the presence of iNOS protein by immunohistochemistry, we have not shown whether it is active. It is possible that iNOS protein induced may remain as monomers or otherwise inactive forms, or that by virtue of its high turnover, the amount of L-arginine substrate may become limiting.

Our results show a clear gender difference in the response of wild-type mice to cuff injury, with I/M ratios of 28 and 31% in males and 17 and 18% in females. Gender differences have also been noted in the filament injury model, the rat balloon injury model (16), and the cuff model (24), paralleling the atheroprotective effects of estrogens observed in humans. Recent work suggests that these effects of estrogen persist in estrogen receptor alpha mutant mice, suggesting that they do not require the estrogen alpha receptor (25). The eNOS mutant mice still show a significant difference in neointimal formation between male and female mice, indicating that the effects of estrogen do not require eNOS expression.

Inhibition of vascular intimal formation by pregnancy was impressive. To our knowledge, there has been no report about vascular intimal formation during pregnancy. Coronary artery disease is rare among pregnant women, in part due to the younger age of pregnant women. In our initial studies, we noted a remarkable reduction in intimal formation in a mouse that was pregnant, so we systematically examined this reduction in all three mouse strains studied. We placed the cuff around the femoral artery on day 7 of pregnancy. Normal mouse gestation lasts 20 d, and the endpoint for the model is 14 d, so the mouse delivered pups within 1 d of vessel harvesting. Further studies will be necessary to define why pregnancy suppresses intimal proliferation in response to vessel injury, and whether this effect is due to circulating estrogens, chorionic gonadotropin, or other changes.

In summary, we have characterized a cuff model of vessel injury in mice that results in reproducible formation of neointima in 14 d. Male mice show more intimal formation than female mice, and the response of C57BL/6 and SV129 mouse strains are similar. Mice in which the eNOS gene is disrupted show substantially increased vascular neointimal formation in response to cuff injury, with more intima in male mice than female mice. Pregnancy suppresses the response to vessel injury in both wild-type and eNOS mutant mice.

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