Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Accelerated Arteriosclerosis of Vein Grafts in Inducible NO Synthase–/– Mice Is Related to Decreased Endothelial Progenitor Cell Repair

Ursula Mayr, Yiping Zou, Zhongyi Zhang, Hermann Dietrich, Yanhua Hu and Qingbo Xu

Circ. Res. 2006;98;412-420; originally published online Dec 29, 2005;
DOI: 10.1161/01.RES.0000201957.09227.6d
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
Copyright © 2006 American Heart Association. All rights reserved. Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/cgi/content/full/98/3/412

Subscriptions: Information about subscribing to Circulation Research is online at
http://circres.ahajournals.org/subsriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, 351 West Camden Street, Baltimore, MD 21202-2436. Phone 410-5280-4050. Fax: 410-528-8550. Email:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/static/html/reprints.html

Downloaded from circres.ahajournals.org at King's College London on November 27, 2006

Accelerated Arteriosclerosis of Vein Grafts in Inducible NO Synthase$^{-/-}$ Mice Is Related to Decreased Endothelial Progenitor Cell Repair

Ursula Mayr, Yiping Zou, Zhongyi Zhang, Hermann Dietrich, Yanhua Hu, Qingbo Xu

***Abstract*—**Inducible NO synthase (iNOS) is expressed by macrophages and smooth muscle cells in atherosclerotic lesions. Previously, we have established a mouse model for vein graft arteriosclerosis by grafting autologous jugular veins or vena cava to carotid arteries. Using this model, we studied the role of iNOS in the development of vein graft arteriosclerosis in iNOS$^{-/-}$ mice. Four weeks after grafting, neointimal hyperplasia of vein grafts in iNOS$^{-/-}$ mice was increased 2-fold compared with that of wild-type controls. Neointimal lesions contained mainly MAC-1^{+} macrophages and α-actin^{+} smooth muscle cells (SMCs) in both vein grafts of iNOS$^{-/-}$ and iNOS$^{+/+}$ mice. Immunofluorescence analysis revealed that increased iNOS expression in neointimal macrophages and SMCs of wild-type, but not iNOS$^{-/-}$, mice coincided with increased vascular endothelial growth factor (VEGF) expression in vein grafts. When vein grafts were performed in iNOS$^{-/-}$/TIE2-LacZ transgenic mice expressing LacZ gene only in endothelial cells, the number of β-galactosidase^{+} cells in iNOS$^{-/-}$ vein grafts were significantly decreased. Furthermore, treatment with the NOS inhibitor N^{G}-nitro-L-arginine methyl ester resulted in delayed endothelial progenitor cell attachment, whereas L-arginine intake through drinking water enhanced endothelial repair. Interestingly, local application of VEGF to iNOS$^{-/-}$ vein grafts restored endothelial progenitor homing and reduced neointimal lesions, whereas the VEGF receptor inhibitor SU1498 increased the lesion formation. Additionally, iNOS-deficient SMCs showed a low level of VEGF production in response to interleukin 1β stimulation. Thus, iNOS deficiency accelerates neointima formation by abrogating VEGF production and endothelial progenitor cell attachment and differentiation. **(*Circ Res*. 2006;98:412-420.)**

Key Words: mouse models ■ iNOS ■ vein grafts ■ progenitor cells ■ neointimal hyperplasia

Autologous vein grafts are a common clinical procedure for vascular reconstruction, but their patency rate is limited by obliterative stenosis because of proliferative thickening of the intima. Neointimal hyperplasia develops rapidly once veins are subject to arterial blood pressure.[1] A hallmark of lesion initiation is endothelial cell damage, which occurs within the first week after surgery.[2] Previously, we established a mouse model of vein graft arteriosclerosis that resembles certain characteristics of human vein graft disease.[3] We demonstrated that 1 of the earliest events after grafting was vascular cell apoptosis followed by endothelial progenitor cell repair.[4,5] Venous endothelial cells became apoptosis in the arterial circulation and were replaced by circulating endothelial progenitors, which reached confluence within 4 weeks and differentiated into mature endothelial cells.[5] Thus, the balance between apoptosis and progenitor cell repair could be crucial in determining lesion development in vein grafts.

NO is thought to be a key regulator in the development of atherosclerosis.[6–9] Classical cardiovascular risk factors, such as hypertension, diabetes mellitus, hypercholesterolemia, and smoking all impair NO function. NO is formed from L-arginine by the enzyme NO synthase (NOS). Three distinct isoenzymes of NOS are known to exist: constitutive-type isoforms, such as neuronal NOS (nNOS) and endothelial cell NOS (eNOS), and the inducible type of the enzyme (iNOS). The latter is widely distributed in a variety of cell types, including SMCs and leukocytes, responds to a variety of stimuli, and can produce a very high output of NO.[10] NO is a pleiotropic signaling molecule involved in numerous processes,[11] including lipid oxidation, mononuclear cell infiltration, and SMC homeostasis.[12–16] Notably, eNOS is essential for the survival, migration, and angiogenic response of mature endothelial cells and has recently been implicated in endothelial progenitor cell mobilization.[17] However, it remains to be clarified whether iNOS might be important for endothelial progenitor cell recruitment to sites of injury.

To elucidate the role of iNOS in vascular graft-induced neointima formation, we performed vein bypass grafts in iNOS$^{-/-}$ and iNOS$^{+/+}$ mice and compared neointimal lesions

Original received June 20, 2005; revision received October 25, 2005; accepted December 15, 2005.

From the Department of Cardiac and Vascular Sciences (U.M., Z.Z., Y.H., Q.X.), St George's University of London, London, United Kingdom; and Institute for Pathophysiology (Y.Z., H.D.), Innsbruck Medical University, Austria.

Correspondence to Qingbo Xu, MD, PhD, Department of Cardiac and Vascular Sciences, St George's University of London, Cranmer Terrace, London SW17 0RE, United Kingdom. E-mail q.xu@sghms.ac.uk

© 2006 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/01.RES.0000201957.09227.6d

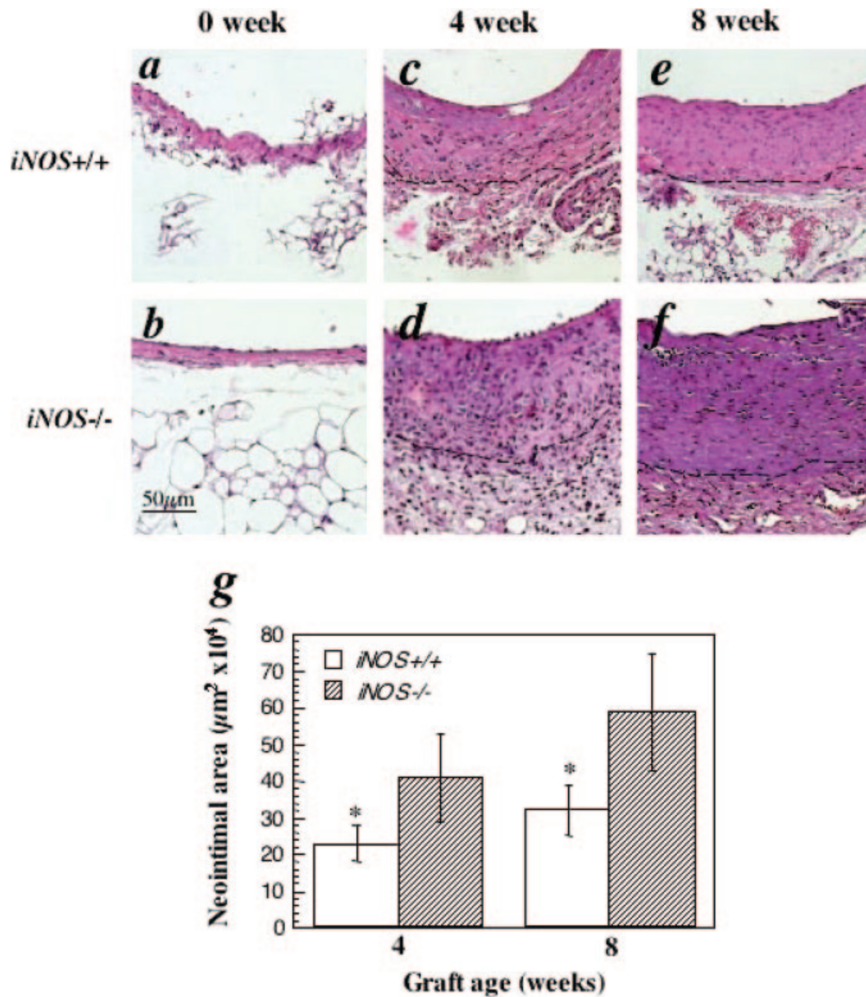

Figure 1. Neointima formation in iNOS$^{+/+}$ and iNOS$^{-/-}$ vein grafts. Mouse vena cava was isografted into carotid arteries and harvested 0, 4, or 8 weeks after surgery. Vein grafts of iNOS$^{+/+}$ (a, c, e) and iNOS$^{-/-}$ (b, d, f) were sectioned and stained with hematoxylin/eosin. The lesion area was measured microscopically. Data in g represent mean values ($\pm$SD) of the neointimal area obtained from 8 animals per group, respectively. *Significant difference between iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, $P<0.05$.

4 and 8 weeks after surgery. We investigated whether altered progenitor cell repair in iNOS-null mice contributed to neointima formation in vivo and studied the molecular mechanism by comparing VEGF expression in vivo and in vitro.

Materials and Methods

Mice

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. iNOS$^{+/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and used as breeders. The mice were maintained on a light/dark (12/12 hour) cycle at 22°C receiving food and water ad libitum.

Three genotypes of iNOS$^{-/-}$, iNOS$^{+/-}$, and iNOS$^{+/+}$ mice were identified using the PCR protocol of The Jackson Laboratory, with a slight modification. Briefly, genomic DNA was isolated from tail tissues by enzyme digestion and precipitation. DNA (50 ng) in reaction buffer was thermocycled with 0.4 μmol/L of each primer (0IMR1216: 5′-ACA TGC AGA ATG AGT ACC GG-3′; 0IMR1217: 5′-TCA ACA TCT CCT GGT GGA AC-3′; and OIMR1218: 5′-AAT ATG CGA AGT GGA CCT CG-3′) at optimal temperature and separated by electrophoresis on agarose gels producing a single 108 and 270 bp band in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, respectively, whereas both bands were visible in iNOS$^{+/-}$ mice.

TIE2-LacZ mice[18] expressing β-galactosidase (gal) under the control of the endothelial-specific protein TIE2 promoter were purchased from The Jackson Laboratory. β-Gal activity of cells from mice is mainly localized in the nucleus. Three genotypes of LacZ$^{-/-}$, LacZ$^{+/-}$, and LacZ$^{+/+}$ mice were identified using the PCR protocol of The Jackson Laboratory (primers: 5′-ATC CTC TGC ATG GTC AGG TC-3′ and 5′-CGT GGC CTG ATT CAT TCC-3′). iNOS$^{-/-}$ mice were crossed with TIE2-LacZ mice in our laboratory, and heterozygous offspring were mated to produce iNOS-deficient mice expressing β-gal in endothelial cells (TIE2-LacZ/iNOS$^{-/-}$).

Vein Graft Procedure and Treatment

For vein grafts, animals of the same genotype as donors and recipients were used. The procedure was similar to that described previously.[3] Briefly, the vena cava from 3-month-old mice was harvested. The right common carotid artery was mobilized free from the bifurcation at the distal end toward the proximal, cut in the middle, and a cuff placed at the end. The cuff was made of an autoclavable nylon tube 0.63 mm in diameter outside and 0.5 mm inside (Portex Ltd, Hythe-Kent, UK). The vein segment was grafted between the 2 ends of the carotid artery by sleeving the ends of the vein over the artery cuff and ligating them together with the 8-0 suture. Immediately after vessel grafting, SU1498 (10 μmol/L; Calbiochem-EMD Biosciences) or vascular endothelial growth factor (VEGF)-165 (100 ng/mL; Sigma) was applied to the adventitia dissolved in a 20% pluronic-127 gel (pH 7.2) as described previously.[19,20] On contact with the tissues, the solutions gelled immediately, generating a translucent layer that enveloped the grafted vessel segment. In addition, 2 groups of mice received either N^G-nitro-L-arginine methyl ester (L-NAME) (1 g/L) or L-arginine (25 g/L; Sigma) in their drinking water, a dose previously shown to reduce lesion formation in animal models.[21] Supplementation was started 3

days before the vein graft procedure and continued until mice were euthanized.

Histology and Lesion Quantification

For histological analysis, in vivo perfusion with 4% phosphate-buffered formaldehyde was performed, as described previously.[22] Vein grafts were harvested by cutting the implanted segments from the native vessels at the cuff end. The grafts were dehydrated in graded ethanol baths, cleared in xylol, embedded in paraffin, and sectioned. Neointimal lesions were defined as the region between the lumen and media, which contains 2 to 3 layers of condensed cells without microvessels. For lesional area measurement, sections were reviewed using a BX60 microscope (Zeiss) equipped with a camera and television monitor. Images were first scanned, saved and then overlaid by different linings to trace the lumen and media. The lesion area was determined by subtracting the area of the lumen from the area enclosed by the line inside of the media.

Reverse-Transcription Polymerase Chain Reaction

The procedure used for RT-PCR was similar to that described elsewhere.[23] Total RNA was prepared with absolutely RNA RT-PCR Miniprep Kit (STRATAGENE). The following primers were used: iNOS (5′-CGA GGA GGC TGC CTG CAG ACT TGG-3′ and 5′-CTG GGA GGA GCT GAT GGA GTA GTA-3′) and GAPDH (5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ and 5′-AGC CTT CTC CAT GGT GGT GAA GAC).

Immunofluorescent Staining

For frozen section preparation, vein grafts were harvested without perfusion and immediately frozen in liquid nitrogen. The procedure used for immunofluorescent staining was similar to that described previously.[24] Briefly, serial 5-μm thick frozen sections were labeled with rat monoclonal antibodies against mouse MAC-1^+ (CD11b/18) leukocytes (BD Biosciences Pharmingen), CD31 (Abcam) or a mouse monoclonal antibody against α-actin conjugated with Cy3 (Sigma), and a rabbit antibody against iNOS (BD Transduction Laboratories) or VEGF (Santa Cruz Biotechnology). The sections were visualized with swine anti-rat Ig-Cy3 and anti-rabbit Ig conjugated with FITC (DakoCytomation).

Immunohistochemistry

The procedure used in the present study was similar to that described previously.[3] Briefly, serial 5-μm thick frozen sections were cut from cryopreserved tissue blocks. The sections were overlayered with rat monoclonal antibodies against mouse MAC-1^+ leukocytes (CD11b/18) or endothelial marker CD31. After washing with PBS, sections were incubated with rabbit anti-rat Ig (DakoCytomation) for 1 hour and developed with APARP. For SMC staining, a mouse monoclonal antibody against α-actin (Sigma) labeled with phosphatase was used. Semiquantitive evaluation was performed at 10×25 magnification. Positive stained cells in the intima were counted on 2 regions of each section and expressed as the percentage of total nuclei per 100 μm of the vessel wall. For CD31$^+$ cells, staining intensity was measured using a computer software AxioVision.

En Face Preparation and X-Gal Staining

Mice were anesthetized and perfused with 0.9% NaCl solution and subsequently perfusion fixed with 2% formaldehyde and 0.2% glutaraldehyde (pH 7.2) for 2 and 10 minutes, respectively. The procedure for en face preparation is similar to that described elsewhere.[25,26] In short, vein segments were harvested and the samples were fixed with 2% formaldehyde and 0.2% glutaraldehyde at 4°C for 24 hours. Each vessel segment (≈5×5 mm^2) was prepared free from the adventitia and cut open. Vessel segments were mounted with the endothelium up on a glass slide (2.6×7.5 cm). The procedure for X-gal staining was similar to that described previously.[27] Briefly, vein segments were incubated at 37°C for 18 hours in PBS supplemented with 1 mg/mL X-gal (Sigma). Vessel segments were rinsed with 3% DMSO in PBS and mounted with the endothelium up on a glass slide (2.6×7.5 cm). Positive stained (blue) cells were enumerated under the microscope.

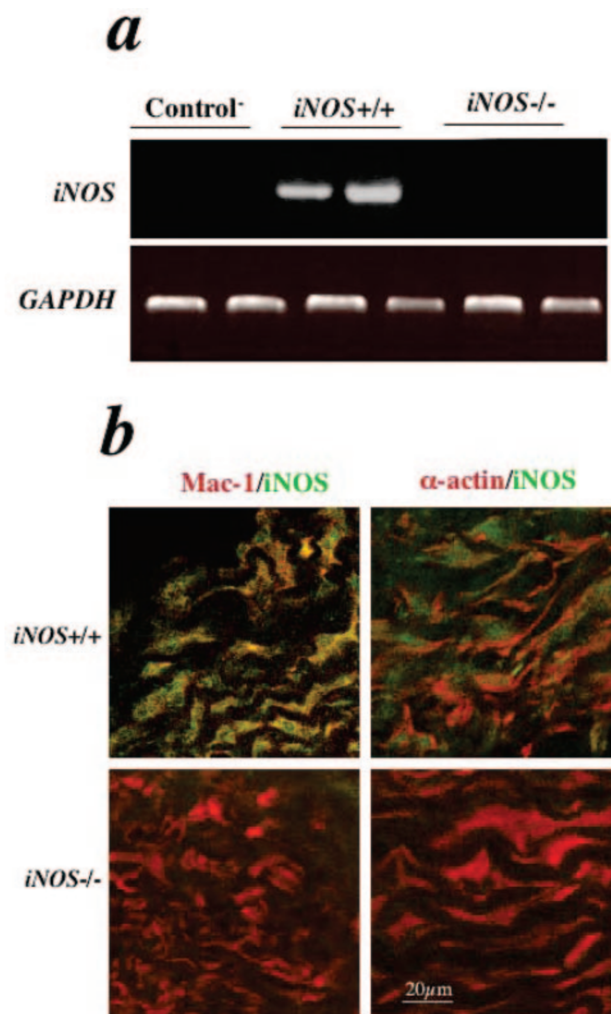

Figure 2. RT-PCR and immunofluorescence staining for iNOS in vein grafts. a, Total RNA was extracted from 4-week vein graft tissues. Levels of iNOS mRNA were detected by RT-PCR. b, Double immunofluorescence staining. Cryostat sections from 4-week vein grafts of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice were labeled with a rat monoclonal antibody MAC-1 or mouse monoclonal antibody against α-actin conjugated with Cy3 (red fluorescence) and a polyclonal rabbit anti-iNOS antibody visualized by a swine anti-rabbit Ig-FITC conjugate (green fluorescence).

Western Blot Analysis

Preparation of extracts from whole vessel wall for Western blotting was performed as described previously.[28] Briefly, 20 μg of protein were separated on a 10% polyacrylamide gel, blotted, and incubated with a rabbit polyclonal antibody to VEGF (1:100; Santa Cruz Biotechnology) followed by a rabbit IgG secondary antibody. To confirm equal loading, membranes were stained with antibody against actin (Santa Cruz Biotechnology).

Cell Culture and ELISA

Vascular SMCs were isolated by collagenase digestion of the mouse thoracic aorta and cultured in DMEM with 15% FCS at 37°C in an atmosphere of 5% CO_2 as described previously.[19] Cells were used for experiments between passage 10 and 20. On subconfluence, SMCs were placed in DMEM medium with 0.5% FCS for 24 hours (the presence of a low concentration of FCS is necessary to stabilize VEGF released into the medium) and subsequently exposed to

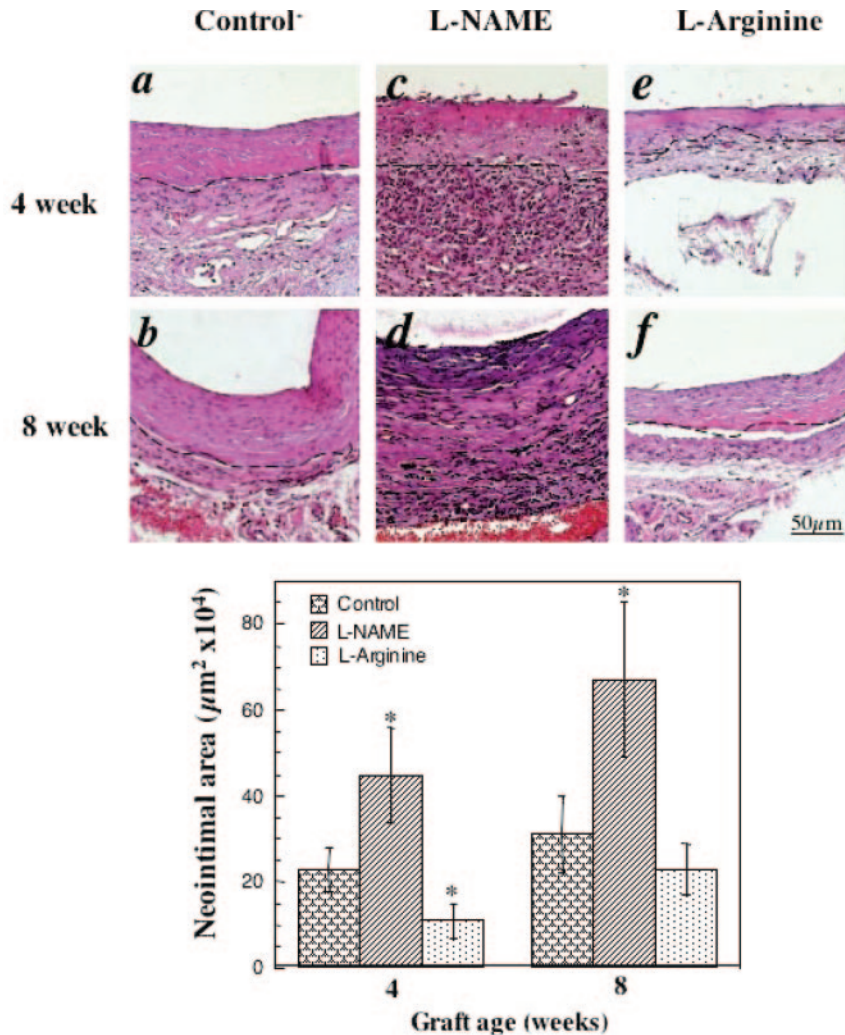

Figure 3. Effects of L-NAME and L-arginine on neointimal lesions. Wild-type mice received either L-NAME (1 g/L) or L-arginine (25 g/L) in their drinking water 3 days before the operation until animals were euthanized to harvest the vein grafts after 4 or 8 weeks. a through f, Representative pictures of hematoxylin/eosin sections, and the graph presents means±SD of the neointimal area obtained from 8 animals per group, respectively. *Significant difference from untreated controls, $P<0.05$.

interleukin (IL)-1β (20 ng/mL; R&D Systems). In some experiments, exposure to IL-1β occurred in the presence of the NOS inhibitor L-NAME (5 mmol/L) or L-arginine (10 mmol/L), respectively. VEGF was measured in the conditioned medium using a commercial ELISA kit (R&D Systems).

Statistical Analysis

Statistical analyses were performed using the Student's *t* test, the Mann–Whitney *U* test, or ANOVA, respectively. A probability value of <0.05 was considered significant.

Results

Accelerated Lesions in iNOS-Deficient Mice

Before grafting, the composition of the venous wall was similar in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (Figure 1a and 1b). Four weeks after grafting, neointimal hyperplasia, ie, thickening of the venous wall to more than 10 cell layers, was observed in wild-type mice (Figure 1c). Notably, neointima formation and cell density were markedly increased in vein grafts of iNOS$^{-/-}$ mice (Figure 1d). At 8 weeks, a loss of cell density was observed in both wild-type and knockout mice (Figure 1e and 1f). Figure 1g summarizes quantitative data on neointima thickness as measured microscopically. The lesion area of vein grafts derived from iNOS$^{-/-}$ mice was approximately doubled compared with wild-type controls at 4 and 8 weeks, and total cell numbers in 4-week grafts of iNOS$^{-/-}$ mice were markedly higher than those of wild-type animals (253±23 versus 108±16, counted over 100 μm in length).

Cell Composition and iNOS Expression in Vein Grafts

Intensive immunostaining for MAC-1^{+} macrophages was observed especially in the surface area of vein grafts from iNOS$^{+/+}$ mice, whereas α-actin^{+} SMCs were found in the deeper layers of the neointima. MAC-1^{+} cells were the predominant cell type in neointimal lesions at 4 weeks (52% versus 69%), whereas α-actin positive SMCs constituted approximately 10% of cells in lesions (11% versus 9%). Moreover, SMCs became dominant cells in neointimal lesions of 8-week vein grafts in both knockout and wild-type mice (Table I in the online data supplement available at http://circres.ahajournals.org). No significant difference in ratios of MAC-1^{+} macrophages and α-actin^{+} cells were observed between iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, indicating that iNOS deficiency does alter neointimal size but not cell composition.

iNOS is known to be highly expressed in atherosclerotic lesions,[7] but its presence in neointimal lesions of vein grafts remains to be determined. RT-PCR data presented in Figure

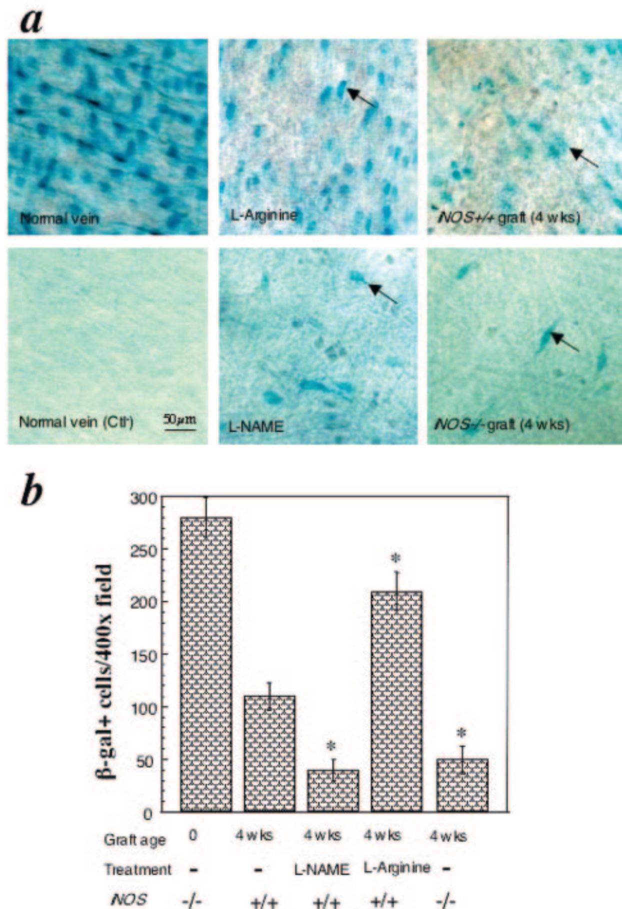

Figure 4. NO-influenced endothelial cell repair in vein grafts. Freshly harvested vena cava segment from iNOS$^{-/-}$/TIE2-LacZ, iNOS$^{+/+}$/TIE2-LacZ, or TIE2-LacZ mice were isografted into carotid arteries with or without L-NAME or L-arginine treatment. The grafts were harvested at 4 weeks and incubated with substrate X-gal as described in Materials and Methods. The blue color on en face photographs indicates β-gal^{+} cells (a). Arrows highlight examples of positive cells. The number of endothelial cells present per $\times$400 field was quantified and data are given as means$\pm$SD (n=5) (b). *Significant difference from untreated group, $P<0.01$.

2a suggest increased iNOS mRNA transcription in wild-type, but not knockout, vein grafts. Similarly, immunostaining for iNOS was enhanced in neointimal macrophages and SMCs 4 weeks after grafting (Figure 2b) compared with freshly harvested veins. Vein segments stained with normal rat serum served as a negative control (data not shown). As expected, no staining for iNOS was detectable in vein grafts of iNOS$^{-/-}$ mice (Figure 2b).

Role of NO in Vein Graft Arteriosclerosis

It is well established that the activity of all NOS isoforms can be enhanced by L-arginine supplementation in the drinking water and inhibited by administration of the nonselective NOS inhibitor L-NAME. Whereas treatment with L-NAME accelerated neointima formation in wild-type mice and resulted in increased cell density, mainly mononuclear cells and SMCs, compared with untreated controls (Figure 3a-d), L-arginine supplementation reduced neointimal lesion size and inhibited mononuclear cell infiltration (Figure 3e and 3f). The graph in Figure 3 summarizes the data derived from 8 mice per group.

NO Influences Endothelial Progenitor Cell Homing

We demonstrated previously that endothelial cells in vein grafts are replaced by circulating progenitor cells. Because NO was recently implicated in endothelial progenitor cell mobilization,[17] we wanted to determine whether iNOS deficiency would interfere with progenitor cell recruitment to vein grafts. Endothelial integrity was monitored using TIE2-LacZ/iNOS$^{-/-}$ mice, which express the LacZ gene in only endothelial cells. As expected, extensive endothelial loss occurred in wild-type vein grafts (data not shown) and endothelial cells were regenerated in vein grafts 4 weeks after surgery (Figure 4a). Whereas L-arginine supplementation accelerated restoration of the endothelial monolayer after grafting, the number of newly formed endothelial cells was markedly reduced in iNOS$^{-/-}$ mice and in wild-type mice treated with L-NAME (Figure 4a and 4b). These findings suggest that iNOS influences endothelial progenitor cell homing or attachment.

iNOS Influences VEGF Production

Because VEGF is a key chemokine and growth factor for EPCs, VEGF expression in vein grafts was measured by Western blot analysis and immunostaining. VEGF expression peaked in vein grafts 3 days after surgery and was significant lower in vein grafts of iNOS$^{-/-}$ mice compared with wild-type controls (Figure 5a). Interestingly, microvessels were abundant in the adventitia of vein grafts in iNOS$^{+/+}$ mice but rare in iNOS$^{-/-}$ vein grafts (Figure 5b, top). Cells within these microvessels were identified to be CD31^{+} (red) expressing VEGF (green) (Figure 5b, middle). Neointimal SMCs also expressed high levels of VEGF at 4 weeks (Figure 5b, bottom). This local VEGF expression is likely to contribute to the recruitment of circulating endothelial progenitors to vein graft surface. To further analyze the effects of iNOS on vasa (microvessel) formation in vein grafts, sections of control vessels and vein grafts were labeled with anti-endothelial marker CD31 and quantified for both iNOS$^{-/-}$ and iNOS$^{+/+}$ mice. Data shown in Figure 6a through 6d are representative images indicating that microvessels are mainly localized in the adventitia, and much less in neointimal lesions. Figure 6e summarized data of 8 animals per group indicate the significant higher in microvessel density in vein grafts derived from wild-type mice compared with knockouts.

NO-Induced VEGF Expression Plays a Role in Lesion Formation

Because SMCs in neointimal lesions expressed high levels of VEGF (Figure 5b), we cultivated aortic SMCs from both iNOS$^{-/-}$ and iNOS$^{+/+}$ mice and measured VEGF production in response to IL-1β stimulation. Figure 7a demonstrates that levels of VEGF in the conditioned medium of iNOS$^{+/+}$ SMCs reached a maximum after 24 and 48 hours but were significantly lower for iNOS$^{-/-}$ SMCs. Furthermore, IL-1β–induced VEGF production in iNOS$^{+/+}$ SMCs was inhibited by preincubation with NOS inhibitor L-NAME, whereas L-arginine significantly enhanced VEGF induction in

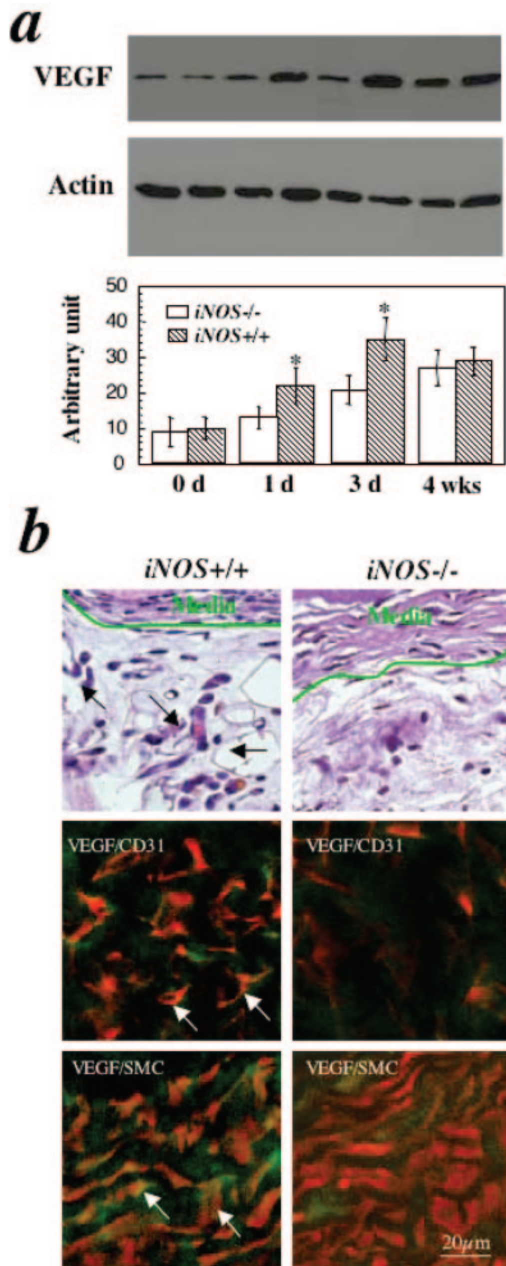

Figure 5. Western blot analysis and immunofluorescence staining for VEGF in vein grafts. a, Protein extracts from vein grafts were probed for VEGF in Western blot analysis. Bands were quantified by scanning and normalizing for actin. b, Double immunofluorescence staining. Cryostat sections from 4-week vein grafts of $iNOS^{+/+}$ and $iNOS^{-/-}$ mice were labeled with a rat monoclonal antibody CD31 or mouse monoclonal antibody against α-actin conjugated with Cy3 (red fluorescence) and a polyclonal rabbit anti-VEGF antibody visualized by a swine anti-rabbit Ig-FITC conjugate (green fluorescence).

$iNOS^{+/+}$, but not $iNOS^{-/-}$, SMCs (Figure 7b). Taken together, our results indicate that NO generated by iNOS contributes to VEGF production in SMCs.

To further clarify the functional relevance of NO-induced VEGF production in vivo, vein grafts were locally treated with VEGF receptor inhibitor SU1489. As shown in Figure 8a, SU1489 treatment markedly reduced the number of endothelial cells at 4 weeks after grafting in wild-type mice, whereas local VEGF transfer improved endothelial repair in $iNOS^{-/-}$ mice. Thus, VEGF treatment restored endothelial progenitor homing to the surface of $iNOS^{-/-}$ vein grafts. Concomitantly, neointima formation was enhanced in the SU1489-treated group compared with untreated controls, whereas local VEGF treatment reduced lesion formation in $iNOS^{-/-}$ mice (Figure 8b). In addition, VEGF treatment slightly increased the density of microvessels in the adventitia (data not shown). These findings support the notion that loss of VEGF expression may, at least partially, be responsible for impaired endothelial progenitor cell recruitment and increased neointima formation in $iNOS^{-/-}$ mice.

Discussion

We reported previously that 1 of the earliest cellular events in neointima formation of vein bypass grafts is cell death,[4] triggering an inflammatory response[26] followed by endothelial progenitor cell repair.[5] In this process, circulating progenitor cells are largely responsible for replacing the endothelium in vein grafts.[5,29] In the present study, we demonstrate in our murine vein graft model that iNOS deficiency accelerates neointima lesion formation by abrogating endothelial progenitor cell repair and facilitating mononuclear cell infiltration and SMC accumulation. Thus, iNOS is a crucial enzyme that attenuates lesion development in vein grafts by stimulating endothelial progenitor cell homing and differentiation.

Accumulating evidence indicates that endothelial progenitor cells in the blood have the capacity to proliferate, migrate, and differentiate into mature endothelial cells.[30–33] Previously, our group demonstrated that the number of β-gal$^+$ cells was reduced at 3 days and disappeared completely by 4 weeks after grafting, when a vein fragment from a TIE2-LacZ transgenic mouse expressing the marker gene LacZ only in endothelial cells was isografted into the carotid artery of wild-type mice.[5] Conversely, β-gal$^+$ cells were observed on the surface of vein segments donated by wild-type mice isografted into TIE2-LacZ mice at 1 week and reached confluence by 4 weeks. These data show that circulating progenitor cells cover the surface of neointimal and arteriosclerotic lesions of vein grafts.[34] Furthermore, other groups have demonstrated that eNOS is essential for progenitor cell mobilization[17] and that iNOS contributes to endothelial progenitor cell recruitment into ischemic tissues.[35] In the present study, we found that endothelial cell numbers on the surface of vein grafts are significantly reduced in $iNOS^{-/-}$ mice and in L-NAME–treated wild-type mice, suggesting that NO is needed for endothelial progenitor cell attachment and differentiation.

Another important observation is the finding that locally produced VEGF in vein grafts is significantly reduced in $iNOS^{-/-}$ mice, which is related to decreased endothelial attachment. It has been shown that VEGF production in SMCs could be attenuated by inhibition of NOS activity,[36] indicating a relationship between these 2 factors. NO was also shown to be involved early in angiogenesis, where inhibition of NOS activity abolished the increase in capillary proliferation.[37] NO is released from endothelium by increased shear stress[38,39] and in vivo in response to increased blood flow.[40]

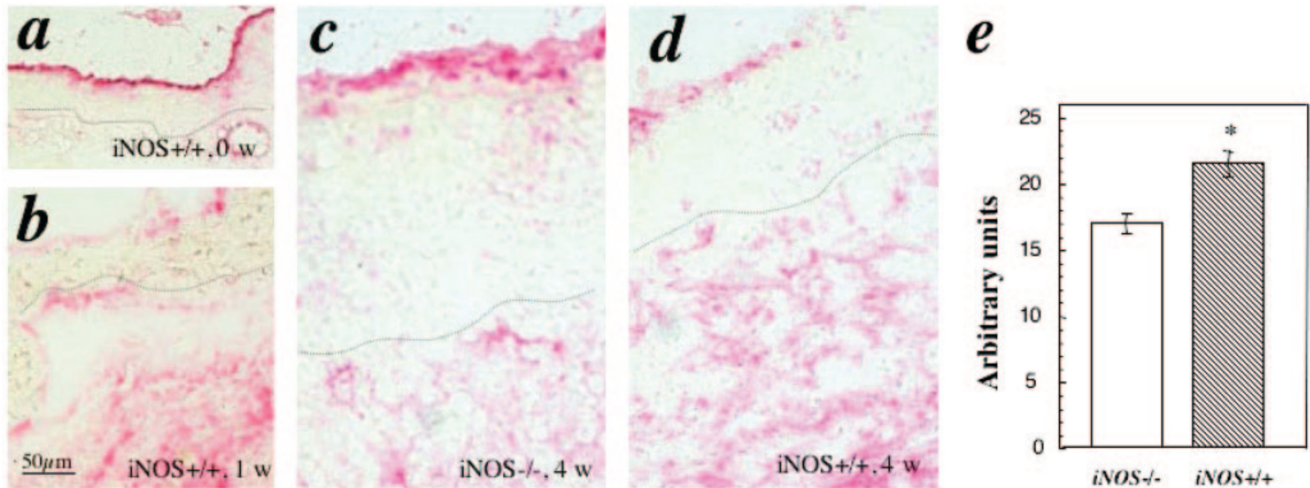

Figure 6. Immunohistochemical staining for CD31. Sections from freshly harvested vein (a) and 1-week (b) and 4-week (c and d) vein grafts of iNOS$^{+/+}$ (a, b, and d) and iNOS$^{-/-}$ (c) mice were labeled with a rat monoclonal antibody CD31 and visualized by alkaline phosphatase–anti-alkaline phosphatase techniques. Dotted lines indicate the border between media and adventitia. Note abundant vasa vasorum in the adventitia. e, Statistical data of means±SEM (n=8). *Significant difference between 2 groups, $P<0.05$.

NO and VEGF are known to be synergistic and/or complementary in other angiogenic situation.[17,41] Our results demonstrated that iNOS-mediated NO production is a key event for VEGF production in SMCs stimulated by IL-1β. Thus, NO together with NO-induced VEGF may synergistically serve as chemokines for endothelial progenitor homing and growth factors for cell proliferation and differentiation in vein grafts.

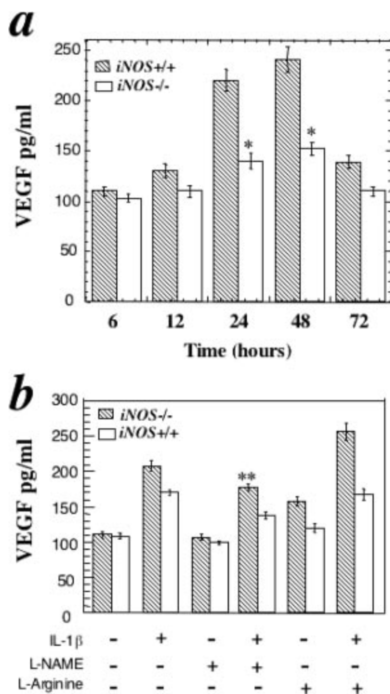

Figure 7. VEGF induction in aortic SMCs from iNOS$^{-/-}$ and iNOS$^{+/+}$ mice. SMCs were treated with IL-1β (20 ng/mL). VEGF was measured in the conditioned medium by ELISA at various time points (a). *Significant difference from iNOS$^{+/+}$ SMCs, $P<0.001$. b, Inhibition of NOS activity by L-NAME (5 mmol/L) reduces VEGF synthesis. Supplementation of L-arginine (10 mmol/L) increases VEGF production. Values are means±SD of 3 independent experiments, each performed in triplicate. **Significant difference from L-arginine–treated group, $P<0.01$.

Because local concentration of VEGF could influence both endothelial repair on the surface of vein grafts and angiogenesis or vasa formation in the vessel wall, we have therefore compared the density of microvessels in grafts, and demon-

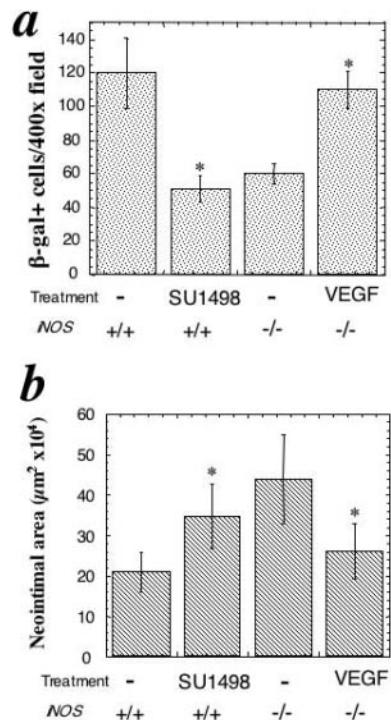

Figure 8. VEGF-influenced endothelial repair and neointimal lesions in vein grafts. Vein segment from iNOS$^{-/-}$/TIE2-LacZ, iNOS$^{+/+}$/TIE2-LacZ, or TIE2-LacZ mice were isografted into carotid arteries with or without local SU1498 or VEGF treatment. The grafts were harvested at 4 weeks and incubated with substrate X-gal as described in Materials and Methods. The number of endothelial cells present per ×400 field was quantified (a). In addition, vein grafts with or without treatment were processed for hematoxylin/eosin sections. Neointimal lesions were quantified as described in Materials and Methods (b). Data are given as means±SD (n=5). *Significant difference from untreated group, $P<0.05$.

strated a lower level of microvessels in iNOS$^{-/-}$ mice (Figure 6). These results indicate iNOS could also be an important enzyme for endothelial progenitor cell functions in terms of vasa formation in the adventitia. Concomitantly, Ii et al[35] demonstrated that iNOS produced from EPCs appears to play a crucial role in cardioprotection, possibly via the involvement in angiogenesis. Is there any relationship among endothelial repair, neointimal formation, and vasa function in vein grafts? As mentioned above, decreased microvessel density in iNOS$^{-/-}$ vein grafts may directly relate to lower level of local VEGF production that influences endothelial repair on the surface of vein grafts. On the other hand, microvessels within the vessel wall might be involved in transporting mononuclear cells and smooth muscle progenitors from the adventitial side based on observations that there are some microvessels in the media and neointima of 4-week vein grafts. Previously, we demonstrated that mononuclear cell recruitment into neointima was significantly reduced in ICAM-1$^{-/-}$ mice,[26] suggesting these cells are mainly derived from endothelial side of the vein grafts. Thus, we hypothesize that neointimal cells may be mainly recruited from endothelial side of the vessel, although vasa vasorum could be involved as well.

The role of iNOS in vascular pathology is variable, and cholesterol levels appear to be among the factors determining whether iNOS is pro- or antiatherosclerotic. Although iNOS deficiency has no effect on atherosclerosis development in apoE/iNOS-dKO mice on normal chow,[42] it substantially reduced atherosclerosis in apoE-null mice on Western diet.[14,15] In contrast to spontaneous atherosclerosis, iNOS plays a protective role in transplant atherosclerosis and chronic cardiac rejection is accelerated in iNOS$^{-/-}$ mice.[13] Similarly, our findings demonstrate that neointima formation is enhanced in vein grafts of iNOS-deficient mice. These observations suggest that iNOS plays different role in hyperlipidemia-induced atherosclerosis and vascular graft arteriosclerosis. In hyperlipidemia-induced atherosclerosis, reduction in lesion formation was associated with a decrease in plasma lipoperoxide concentrations.[21] Hyperlipidemia is known to cause endothelial dysfunction, but, unlike the vein graft model, no acute endothelial damage occurs. The extensive loss of endothelial cells after grafting veins to arteries[4] is followed by rapid progenitor cell repair.[5] Therefore, different strategies for modulating iNOS activity would be needed to treat native atherosclerosis and vein graft stenosis.

In summary, we demonstrated that iNOS deficiency results in exacerbated arteriosclerosis in vein grafts in a murine model. The mechanisms of iNOS-enhanced lesion formation involve delayed endothelial repair, ie, progenitor homing and differentiation, in which NO-mediated VEGF production by vascular cells is crucial. Our findings provide basic information for developing a new strategy for treatment of vein graft disease.

Acknowledgments

This work was supported by grants from the British Heart Foundation (RG/04/008) and Oak Foundation.

References

1. Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation*. 1998;97:916–931.
2. Davies MG, Hagen PO. Pathobiology of intimal hyperplasia. *Br J Surg*. 1994;81:1254–1269.
3. Zou Y, Dietrich H, Hu Y, Metzler B, Wick G, Xu Q. Mouse model of venous bypass graft arteriosclerosis. *Am J Pathol*. 1998;153:1301–1310.
4. Mayr M, Li C, Zou Y, Huemer U, Hu Y, Xu Q. Biomechanical stress-induced apoptosis in vein grafts involves p38 mitogen-activated protein kinases. *FASEB J*. 2000;14:261–270.
5. Xu Q, Zhang Z, Davison F, Hu Y. Circulating progenitor cells regenerate endothelium of vein graft atherosclerosis, which is diminished in apoE-deficient mice. *Circ Res*. 2003;93:e76–e86.
6. Wever RM, Luscher TF, Cosentino F, Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation*. 1998;97:108–112.
7. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*. 1997;17:2479–2488.
8. Yan Z, Hansson GK. Overexpression of inducible nitric oxide synthase by neointimal smooth muscle cells. *Circ Res*. 1998;82:21–29.
9. West NE, Qian H, Guzik TJ, Black E, Cai S, George SE, Channon KM. Nitric oxide synthase (nNOS) gene transfer modifies venous bypass graft remodeling: effects on vascular smooth muscle cell differentiation and superoxide production. *Circulation*. 2001;104:1526–1532.
10. Nathan C. Inducible nitric oxide synthase: what difference does it make? *J Clin Invest*. 1997;100:2417–2423.
11. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem*. 1994;63:175–195.
12. Shears LL, Kawaharada N, Tzeng E, Billiar TR, Watkins SC, Kovesdi I, Lizonova A, Pham SM. Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. *J Clin Invest*. 1997;100:2035–2042.
13. Koglin J, Glysing-Jensen T, Mudgett JS, Russell ME. Exacerbated transplant arteriosclerosis in inducible nitric oxide-deficient mice. *Circulation*. 1998;97:2059–2065.
14. Detmers PA, Hernandez M, Mudgett J, Hassing H, Burton C, Mundt S, Chun S, Fletcher D, Card DJ, Lisnock J, Weikel R, Bergstrom JD, Shevell DE, Hermanowski-Vosatka A, Sparrow CP, Chao YS, Rader DJ, Wright SD, Pure E. Deficiency in inducible nitric oxide synthase results in reduced atherosclerosis in apolipoprotein E-deficient mice. *J Immunol*. 2000;165:3430–3435.
15. Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL. Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E-knockout mice. *Circulation*. 2001;103:3099–3104.
16. Niu XL, Yang X, Hoshiai K, Tanaka K, Sawamura S, Koga Y, Nakazawa H. Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions. *Circulation*. 2001;103:1115–1120.
17. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003;9:1370–1376.
18. Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN. Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci U S A*. 1997;94:3058–3063.
19. Hu Y, Zou Y, Dietrich H, Wick G, Xu Q. Inhibition of neointima hyperplasia of mouse vein grafts by locally applied suramin. *Circulation*. 1999;100:861–868.
20. Torsney E, Mayr U, Zou Y, Thompson WD, Hu Y, Xu Q. Thrombosis and neointima formation in vein grafts are inhibited by locally applied aspirin through endothelial protection. *Circ Res*. 2004;94:1466–1473.
21. Chen J, Kuhlencordt P, Urano F, Ichinose H, Astern J, Huang PL. Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knockout mice. *Arterioscler Thromb Vasc Biol*. 2003;23:97–103.
22. Dietrich H, Hu Y, Zou Y, Huemer U, Metzler B, Li C, Mayr M, Xu Q. Rapid development of vein graft atheroma in ApoE-deficient mice. *Am J Pathol*. 2000;157:659–669.
23. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature*. 2000;408:92–96.
24. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia contribute to atherosclerosis

of vein grafts in ApoE-deficient mice. *J Clin Invest*. 2004;113: 1258–1265.
25. Seitz CS, Kleindienst R, Xu Q, Wick G. Coexpression of heat-shock protein 60 and intercellular-adhesion molecule-1 is related to increased adhesion of monocytes and T cells to aortic endothelium of rats in response to endotoxin. *Lab Invest*. 1996;74:241–252.
26. Zou Y, Hu Y, Mayr M, Dietrich H, Wick G, Xu Q. Reduced neointima hyperplasia of vein bypass grafts in intercellular adhesion molecule-1-deficient mice. *Circ Res*. 2000;86:434–440.
27. Hu Y, Baker AH, Zou Y, Newby AC, Xu Q. Local gene transfer of tissue inhibitor of metalloproteinase-2 influences vein graft remodeling in a mouse model. *Arterioscler Thromb Vasc Biol*. 2001;21:1275–1280.
28. Li C, Hu Y, Mayr M, Xu Q. Cyclic strain stress-induced mitogen-activated protein kinase (MAPK) phosphatase 1 expression in vascular smooth muscle cells is regulated by Ras/Rac-MAPK pathways. *J Biol Chem*. 1999;274:25273–25280.
29. Hu Y, Davison F, Zhang Z, Xu Q. Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. *Circulation*. 2003;108:3122–3127.
30. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.
31. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003;348:593–600.
32. Szmitko PE, Fedak PW, Weisel RD, Stewart DJ, Kutryk MJ, Verma S. Endothelial progenitor cells: new hope for a broken heart. *Circulation*. 2003;107:3093–3100.
33. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol*. 2003;23: 1185–1189.
34. Zhang L, Freedman NJ, Brian L, Peppel K. Graft-extrinsic cells predominate in vein graft arterialization. *Arterioscler Thromb Vasc Biol*. 2004;24:470–476.
35. Ii M, Nishimura H, Iwakura A, Wecker A, Eaton E, Asahara T, Losordo DW. Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via “imported” nitric oxide synthase activity. *Circulation*. 2005;111:1114–1120.
36. Dulak J, Jozkowicz A, Dembinska-Kiec A, Guevara I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wojtowicz A, Szuba A, Cooke JP. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2000;20:659–666.
37. Jozkowicz A, Cooke JP, Guevara I, Huk I, Funovics P, Pachinger O, Weidinger F, Dulak J. Genetic augmentation of nitric oxide synthase increases the vascular generation of VEGF. *Cardiovasc Res*. 2001;51: 773–783.
38. Chiu JJ, Wung BS, Hsieh HJ, Lo LW, Wang DL. Nitric oxide regulates shear stress-induced early growth response-1. Expression via the extracellular signal-regulated kinase pathway in endothelial cells. *Circ Res*. 1999;85:238–246.
39. Davis ME, Cai H, Drummond GR, Harrison DG. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *Circ Res*. 2001;89:1073–1080.
40. Johnson DL, McAllister TN, Frangos JA. Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. *Am J Physiol*. 1996;271: E205–E208.
41. Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem*. 1998;273: 4220–4226.
42. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest*. 2000;105: 451–458.