Impaired wound healing and angiogenesis in eNOS-deficient mice


A role for nitric oxide (NO) in wound healing has been proposed; however, the absolute requirement of NO for wound healing has not been determined. Experiments were carried out using eNOS gene knockout (KO) mice to determine the requirement for eNOS on wound closure and wound strength. Excisional wound closure was significantly delayed in the eNOS KO mice (29.4 ± 2.2 days) compared with wild-type (WT) controls (20.2 ± 0.4 days). At 10 days, incisional wound tensile strength demonstrated a 38% reduction in the eNOS KO mice. Because effective wound repair requires growth factor-stimulated angiogenesis, in vitro and in vivo angiogenesis assays were performed in the mice to assess the effects of eNOS deficiency on angiogenesis. Endothelial cell sprouting assays confirmed in vitro that eNOS is required for proper endothelial cell migration, proliferation, and differentiation. Aortic segments harvested from eNOS KO mice cultured with Matrigel demonstrated a significant reduction in endothelial cell sprouting and [3H]thymidine incorporation compared with WT mice at 5 days. Capillary ingrowth into subcutaneously implanted Matrigel plugs was significantly reduced in eNOS KO mice (2.67 ± 0.33 vessels/plug) compared with WT mice (10.17 ± 0.79 vessels/plug). These results clearly show that eNOS plays a significant role in facilitating wound repair and growth factor-stimulated angiogenesis.

Nitric oxide; nitric oxide synthase; endothelial nitric oxide synthase; wound repair; endothelial nitric oxide synthase knockout; endothelial cell

Attempts to elucidate the factors regulating wound healing have revealed a dependence on arginine metabolism and the nitric oxide (NO) synthase (NOS) pathway (1). End products of NO synthesis, nitrite, and nitrate are elevated early and transiently in fluid collected from sponges implanted in subcutaneous wounds (1). Adequate rates of NO production are necessary for intact wound healing. Systemic administration of arginine, a substrate for NO synthesis, enhances wound healing (3, 13). Topical application of an NO donor has been shown to accelerate closure of excisional wounds in rats (27), whereas NOS inhibitors given systemically (26) or applied to the surface of wounds (6) delay wound healing. Previous experiments in our laboratory (31) have demonstrated that wound closure was delayed by 31% in inducible NOS (iNOS) knockout (KO) mice compared with wild-type (WT) mice and that this delay could be reversed by transfection with an adenoviral vector containing the gene for human iNOS (AdiNOS). However, the requirement of endothelial NOS (eNOS) has not been determined in wound healing.

Effective wound repair has been shown to require growth factor-stimulated angiogenesis (2). Angiogenesis is the process of new blood vessel growth from preexisting vessels (10), with steps involving dissolution of basement membrane underlying endothelial cells, endothelial cell migration, adhesion, proliferation, and tube differentiation. Angiogenesis is an integral part of wound healing and must be present if injured tissues are to heal properly. The vasculature composes up to 60% of repair tissue in a wound (9), and the newly established blood supply is required to meet the metabolic demands of wound repair. Wound healing is accelerated on stimulation of endothelial cell migration and angiogenesis in vivo (17). An association between enhanced angiogenesis and wound collagen deposition was found from studying the beneficial effects of diphenhydantoin in healing wounds (7).

NO has been implicated recently as a crucial signaling molecule and regulator in angiogenesis. NO donors, such as nitroprusside, promote endothelial cell proliferation and migration; in contrast, inhibitors of NO suppress the response (33). Angiogenesis induced by human monocytes requires an L-arginine/NOS-dependent mechanism (16). Furthermore, Morbidelli et al. (19) and Ziche et al. (32) have established that NO lies downstream from and mediates the effects of vascular endothelial growth factor (VEGF), an important mitogen for vascular endothelial cells that is essential for angiogenesis. VEGF has also been shown to upregulate NO production, eNOS protein, and message (12, 24). Recently, Murohara and co-workers (20) have suggested that eNOS modulates angiogenesis in response to tissue ischemia and have demonstrated impaired angiogenesis in the ischemic hindlimb of mice lacking the eNOS gene.

Accordingly, we sought to determine the requirement of the constitutively expressed eNOS in wound healing and growth factor-stimulated angiogenesis. To eliminate the contribution of eNOS, our experimental approach focused on the use of eNOS KO mice. We demonstrated that closure of excisional wounds is significantly impaired in eNOS KO mice and that development of tensile strength in incisional wounds might also be somewhat impaired. We found that eNOS...
is required for growth factor-stimulated angiogenesis in vitro; aortic segments harvested from eNOS KO mice and cultured in Matrigel manifested a significant reduction in endothelial cell sprouting and proliferation compared with WT mice. We confirmed these findings in vivo with a Matrigel plug assay. Our results clearly show that eNOS plays a significant role in facilitating wound healing and that this may be due to alterations in angiogenesis.

METHODS

Animals. Mice homozygous (−/−) for disruption of the eNOS gene (parental background strains of 129 and C57BL/6) were a gift from Edward G. Seshely (Univ. of North Carolina at Chapel Hill, NC). Southern blot analysis and immunohistochemical tests for eNOS protein confirmed the homozygous mutant state (28). eNOS KO mice had been backcrossed onto the C57BL/6 background six times. WT mice (C57BL/6) were purchased from Charles River Laboratory (Wymington, MA). All mice used in the wounding experiments were 2–3 mo of age, weighing 20–26 g each, and were evenly distributed across both genders. Mice employed in the Matrigel plug assay were all male, with similar ages and weights. Animals were housed individually and provided food and water ad libitum. Anesthesia consisted of inhaled methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL). Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. All animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Research Council.

Excisional wound model. After induction of anesthesia, the dorsum of the mouse was dipped free of hair, and the skin was prepasted with povidone-iodine. A full-thickness wound (1.5 × 1.5 cm), including the panniculus carnosus, was created sharply with fine scissors. After the wound edges had retracted, the wound outline was traced onto an acetate sheet. To keep the area clean, the wounds were dressed with a clear, bioocclusive dressing (Op-Site; Owens Minor, Greensburg, PA). Wound closure was monitored by tracing the wound area every other day onto acetate sheets. The occlusive dressing was changed at the time of dressing. The tracings were digitized, and the areas were calculated in a blinded fashion using a computerized algorithm (Sigma Scan; Jandel Scientific, San Rafael, CA).

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Angiogenesis in vitro: endothelial cell sprouting assay. The in vitro angiogenesis assay was performed by the culture of murine aortic segments on Matrigel, modified from methods described by Brown et al. (4) and Nicosia and Ottinetti (22). Matrigel is a reconstituted basement membrane matrix that provides a substrate on which endothelial cells can migrate, proliferate, and form an array of capillaries. Matrigel matrix is derived from Engelbreth-Holm-Swarm tumor and contains collagen IV, laminin, entactin, and heparin sulfate proteoglycan (14). Matrigel is also known to contain an array of growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) I, platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β (30). Male WT C57BL/6 mice and eNOS KO mice were killed by CO2 asphyxia, and the entire thoracoabdominal aorta was harvested. Any residual clots were removed by rinsing the vessel segments in Hanks’ balanced salt solution. The aortas were cut into 1-mm sections, which were then embedded in 48-well Matrigel-coated plates (Becton Dickinson Labware, Bedford, MA). After incubation at 37°C for 30 min to promote jelling of Matrigel, medium (0.5 ml) containing 100 µg/ml endothelial cell growth supplement (Sigma, St. Louis, MO) and 20% fetal bovine serum (BioWhittaker, Walkersville, MD) was added to each well, and the plates were returned to the incubator. Sprouting of endothelial cells from the edge of the aortic segments was observed within 36 h of 37°C incubation. By 5 days, a capillary-like endothelial cell network was clearly visible at the margin of the vessel sections. Quantification of the endothelial cell sprouting area was performed at this time as a measurement of in vitro angiogenesis.

Quantification of endothelial cell sprouting area. Digital images of the endothelial cell sprouting were obtained using the Nikon TMS inverted microscope with the ×20 objective coupled to a high-sensitivity, integrating three-chip Sony color video camera (Sony, Park Ridge, NJ; 700 × 600 pixels). Angiogenesis was measured by area analysis of the endothelial cell sprouting with the use of BioScan Optimas 4.02 image analysis software (Optimas, Seattle, WA). Endothelial cell sprouting was defined by branching structures containing cell nuclei. Measurements were taken from 10 random sites within each well and recorded as the mean endothelial cell sprouting area expressed in arbitrary area units. Each experiment was repeated three times with the use of vessels from separate animals for each group.

[3H]Thymidine incorporation assay. A modified [3H]thymidine incorporation assay was used to measure cell proliferation in cultured aortic segments. [3H]thymidine (5 µCi/ml; NEN, Boston, MA) was added to the medium before incubation. After 5 days, the medium was removed, and the aortic segments along with the surrounding sprouts were carefully separated from the Matrigel and digested with TS-2 tissue solubilizer (Research Products International, Mount Prospect, IL). Radioactivity was then measured by the Beckman LS-3801 scintillation counter and expressed as counts per minute (cpm) per milligram of tissue. Each experiment was repeated three times.

Nitrite measurement. After release, NO reacts with O2 to form the stable metabolite nitrite. Nitrite concentrations were measured by the Griess reaction to evaluate the total amounts of NO in the media released from murine aortic segment cultures. Nitrite content of the medium by itself was also measured to determine the background nitrite level. After 5 days, 100 µl of the medium in duplicate were removed and mixed with 100 µl of Griess reagent (1% sulfanilamide-0.1% naphthylethylendiamine-5% phosphoric acid; Sigma) and incubated for 10 min at room temperature, and absorbance was measured at 550 nm by using Spectra MAX 340 (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were determined by comparison with sodium nitrite standards. Each experiment was repeated three times.
Angiogenesis in vivo: Matrigel plug assay. After induction of anesthesia, the abdomen of the mouse was clipped free of hair, and the skin was prepped with alcohol. Matrigel (0.5 ml) was injected subcutaneously near abdominal midline of WT and eNOS KO mice (n = 6/group), as previously described (25). A subcutaneous plug formed within seconds after injection as the Matrigel solidified at body temperature. After 10 days, the mice were killed by CO₂ asphyxia. The Matrigel plugs were recovered by dissection, fixed with 2% paraformaldehyde for 4 h, and cryoprotected with 2.3 M sucrose overnight. The plugs were then rapidly frozen with liquid nitrogen in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) for later histological and immunohistochemical analysis.

Histology. Six separate sections (10 µm) were cut from different layers through the Matrigel plug with Microm cryotome. Sections were then stained with hematoxylin and eosin (H/E) and examined with light microscopy.

Immunohistochemistry. Immunohistochemistry was performed on cell sprouts from cultured murine aortic segments and 10-µm Matrigel plug cryostat sections with the use of an immunofluorescence technique. Aortic segments along with the surrounding sprouts were separated from the Matrigel and mounted on glass slides. All samples were washed three times with PBS containing 0.15% glycine and 0.5% BSA, pH 7.4 (buffer A). To minimize nonspecific binding of the primary and secondary antibodies, purified goat IgG (50 mg/ml) was used as the blocking solution, with 40-min incubation at 25°C followed by three washes with buffer A. The samples were then incubated for 60 min with the primary antibody to von Willebrand factor (vWF; 1:800 dilution, Sigma). This was followed by three washes in buffer A and a 60-min incubation with the secondary antibody that was fluorescently tagged (Alexa 488 conjugate, 1:500 dilution; Molecular Probes, Eugene, OR, or Fluorophore Cy3, 1:3,000 dilution; Sigma). Hoechst bisbenzimide 33258 dye (Sigma; 1–2 drops) was then added to samples of cell sprouts. The samples were then washed with buffer A three times, mounted in Gelvatol (Monsanto, St. Louis, MO), coverslipped, and examined under a Nikon Microphot-FXL immunofluorescence microscope.

Quantification of angiogenesis in vivo. Matrigel plug sections stained with H/E and vWF were examined. Capillaries were defined as tubular structures containing red blood cells or staining positive with vWF. Angiogenesis was quantified by the number of capillaries penetrating the Matrigel plug, as averaged by the six different sections.

Statistical analysis. The results are presented as means ± SE. Comparisons between two means were performed using the unpaired Student's t-test. Analysis was performed using a computerized software package (Sigma Stat, Jandel Scientific). P ≤ 0.05 was considered to be statistically significant.

RESULTS

eNOS is required for normal wound repair. We examined the requirement of eNOS for normal wound repair. First, the rate of closure of excisional wounds was examined in WT (n = 11) and eNOS KO (n = 10) mice. No significant weight loss or evidence of infection occurred throughout the study period. Analysis of the digitized wound areas revealed a 9.2-day delay in wound closure in the eNOS KO mice (29.4 ± 2.2 days) compared with the WT mice (20.2 ± 0.4 days; Fig. 1A). As a separate measure of wound repair, the tensile strength of healing incisions was tested at postwounding days 5 and 10 (n = 9 for each of 4 groups). No animals developed evidence of wound infection throughout the study period. One animal from each of the day 10 groups could not be analyzed because of complete wound separation. Day 5 wounds demonstrated no significant differences between WT and eNOS KO mice (fresh 7.7 ± 0.9 vs. 9.7 ± 2.2 g/mm² and Formalin-fixed strips 16.2 ± 1.6 vs. 17.1 ± 2.2 g/mm², respectively; Fig. 1B). Fresh wound strips from day 10 animals did not reveal a significant difference; however, Formalin-fixed wound strips, which assessed efficiency of collagen cross-linking, demonstrated a 38% reduction in tensile strength in eNOS KO mice compared with WT mice (34.5 ± 7.6 vs. 55.9 ± 6.6 g/mm², respectively; Fig. 1B).
eNOS is required for angiogenesis in vitro. We demonstrated that eNOS is required for effective wound repair, a physiological process known to rely on intact angiogenesis stimulated by growth factors. With the essential role of growth factor-stimulated angiogenesis in wound healing, we sought to define more precisely with an in vitro angiogenesis assay the role of eNOS in endothelial cell migration, proliferation, and differentiation. We chose the endothelial cell sprouting assay, since it allows us to assess the integrity of each of these steps. Sprouts of cells from the cut edge of murine aortic segments were observed within 36 h of culture.
with Matrigel. Nicosia and Ottinetti (22) have described in the rat aorta model the mixed nature of these outgrowths, which contain not only endothelial cells but also pericytes, smooth muscle cells, and fibroblasts. However, ~90% of the sprouting cells from our murine aorta model were identified as endothelial in origin, staining positive for vWF by immunohistochemistry (Fig. 2). Over time, the sprouts branched and anastomosed, giving rise to a complex endothelial cord network resembling capillaries. As shown in Fig. 3, an obvious endothelial cord network formed at 5 days in WT aortic segments but failed to form in eNOS KO aortic segments. The endothelial cell sprouting area was quantified as a measurement of angiogenesis in vitro (Fig. 4A). In eNOS KO mice, there was a significant reduction compared with WT mice: 378 ± 67 vs. 674 ± 84 area units (n = 6 vessels/group, P < 0.05). This disparity in endothelial cell sprouting patterns between eNOS KO and WT mice was further investigated by a modified [³H]thymidine incorporation assay to determine cell proliferation. As shown in Fig. 4B, there was a significant reduction in [³H]thymidine incorporation among the aortic segments taken from eNOS KO mice compared with WT controls: 8,288 ± 1,098 vs. 26,123 ± 3,595 cpm/mg (n = 4 vessels/group, P < 0.01). Because ~90% of the sprouting cells were endothelial in origin by vWF staining, the reduction in [³H]thymidine incorporation seen in eNOS KO mice reflected mostly a decrease in endothelial cell proliferation. To assess the effect of eNOS unavailability in NO production, we measured the stable NO metabolite nitrite in the media of aortic segment cultures (Fig. 5). After 5 days, the nitrite level was significantly reduced in the eNOS KO mice compared with WT mice: 7.36 ± 0.07 vs. 9.54 ± 0.38 µM (n = 4 and 6 vessels, respectively; P = 0.002). In this assay, the nitrite level measured in the eNOS KO mice reflected the basal nitrite content of the medium: 7.43 ± 0.08 µM (n = 4). Overall, these in vitro results show that eNOS is required for proper endothelial cell migration, proliferation, and differentiation, steps critical in angiogenesis.

eNOS is required for angiogenesis in vivo. To determine the requirement of eNOS in growth factor-stimulated angiogenesis in vivo, we used a Matrigel plug assay in eNOS KO mice. Matrigel (0.5 ml) was injected subcutaneously into the abdomen of WT and eNOS KO mice. The Matrigel plug that subsequently formed provided a substrate on which endothelial cells could migrate, proliferate, and differentiate into an array of capillaries. As shown in Fig. 6A (top), after gross sectioning of the Matrigel plugs at 10 days, a marked reduction was seen in the number of capillaries infiltrating the plugs taken from eNOS KO mice in contrast to WT mice. Representative H/E-stained images are shown in Fig. 6A (bottom). There was a marked paucity of endothelial cells and neovascularization within the Matrigel plugs taken from eNOS KO mice. The number of capillaries penetrating the Matrigel plug was quantified as a measure of angiogenesis in vivo. In addition to H/E-stained sections, the presence of capillaries within Matrigel plugs was detected and confirmed by immunohistochemistry with the use of the anti-vWF antibody (Fig. 7). As shown in Fig. 6B, a significant reduction in the number of vessels was observed within the plugs taken from eNOS KO mice (2.67 ± 0.33 vessels/plug; n = 6 mice) compared with WT mice (10.17 ± 0.79 vessels/plug; n = 6 mice, P < 0.001). These results demonstrate that loss of the eNOS isoform impairs angiogenesis.
DISCUSSION

In this study, we demonstrated that eNOS is required for effective wound repair, a physiological process known to rely on intact angiogenesis stimulated by growth factors. In vivo, this was supported by our findings that both excisional wound closure and incisional wound strength were impaired in eNOS KO mice. We then demonstrated with in vitro and in vivo angiogenesis assays that eNOS indeed plays a significant role in facilitating growth factor-stimulated angiogenesis. The endothelial cell sprouting assay confirmed in vitro that eNOS is required for proper endothelial cell migration, proliferation, and differentiation. This finding is confirmed by growth factor-stimulated angiogenesis in vivo; capillary ingrowth into subcutaneously implanted Matrigel plugs was significantly reduced in eNOS KO mice compared with WT mice.

Closure of excisional wounds reflects the processes of both wound contraction and wound reepithelialization, which occur in response to the production of multiple growth factors, including bFGF, PDGF, IGF-I, VEGF, TGF-β, and EGF among others. Within hours of wounding, keratinocytes at wound margins begin migrating through the provisional fibrin matrix formed by extravasated fibrinogen (18). In situ hybridization performed on these keratinocytes reveals significant increases in the levels of VEGF mRNA, and biopsies of healing wounds reveal increased vascular permeability in the proximity of advancing keratinocytes (5, 11). This suggests a dynamic state in which leaky vessels allow formation of the matrix across which reepithelialization may proceed and suggests that keratinocytes, in turn, augment both proliferation and permeability of wound capillaries. Deficiency in eNOS could thus impair wound closure by reducing the efficacy of VEGF, and perhaps other growth factors, thus delaying reepithelialization. Furthermore, reduction in angiogenesis could result in persistent wound hypoxia and retard progression into or through the proliferative phase.

Incisional wounds from eNOS-deficient animals exhibited only a decrease in fixed tensile strength, not fresh tensile strength, indicating that collagen cross-linking, and hence, collagen organization, was impaired. How this may relate to angiogenesis is uncertain; however, study of the beneficial effects of diphenylhydantoin in healing wounds revealed an association between increased wound collagen deposition and enhanced angiogenesis (7). It is possible that early and sustained angiogenesis in healing wounds promotes collagen formation through the indirect mechanism of improved substrate delivery. Together, these observations suggest that eNOS plays a significant role in wound angiogenesis.

NO donors promote endothelial cell proliferation and migration (33), which are important steps for angiogenesis. Evidence put forth by Noiri et al. (23) has suggested that NO induces in the endothelial cells a switch from a stationary to a locomotive phenotype, resulting in directional migration. Ziche et al. (32) have also demonstrated that NO is downstream from and medi-
Fig. 6. In vivo angiogenesis within Matrigel plugs (MP). Matrigel (0.5 ml) was injected subcutaneously into abdomen of mice. After 10 days, plugs were recovered by dissection. A: top shows representative images of MP obtained from WT and eNOS KO mice taken under Olympus dissection scope. There is a marked reduction in number of newly formed capillaries (arrows) within MP recovered from eNOS KO mice. Figure is representative of 6 separate mice/group. Bottom shows representative hematoxylin and eosin (H&E) sections of MP obtained from WT and eNOS KO mice (original magnification, ×100). Arrows, capillaries containing red blood cells. There was a paucity of infiltrating endothelial cells and capillaries within MP recovered from eNOS KO mice. B: angiogenesis was quantified by number (#) of vessels penetrating Matrigel plugs recovered from WT and eNOS KO mice, as averaged by 6 different sections/plug (n = 6/group, * P < 0.001).
ates the mitogenic effects of VEGF on endothelial cells. Furthermore, VEGF has been shown to upregulate the constitutively expressed eNOS and endothelial NO production (12, 24). Consistent with these observations, angiogenesis in the ischemic hindlimb model of eNOS-deficient mice was impaired compared with WT mice (20). The Matrigel used in our assays is known to contain an array of growth factors, including bFGF, EGF, IGF-I, PDGF, and TGF-β. This permitted a direct analysis of the requirement of eNOS in growth factor-stimulated angiogenesis. Our in vivo and in vitro findings provide definitive proof that eNOS plays an important role in growth factor-stimulated angiogenesis. Both cell proliferation, determined by [3H]thymidine incorporation, and the angiogenic process of endothelial cell migration and differentiation, demonstrated by sprouting assay, were impaired in vessels obtained from eNOS-deficient mice. One possible mechanism explaining these findings could be that endothelium-derived NO maintains the surface expression of certain adhesion molecules in endothelial cells that are necessary for cell migration. With the use of flow cytometry, the surface expression of integrin αvβ3 in cultured human umbilical vein endothelial cells (HUVEC) was shown to be downregulated by NOS inhibitor Nω-nitro-L-arginine methyl ester (21). NO also acts as a survival factor for endothelial cells and may be necessary to prevent apoptosis (8, 29) during the initial phases of angiogenesis.

In the endothelial cell sprouting assay, we have demonstrated that NO production by murine aortic segments in culture is dependent on eNOS availability. Compared with WT aortic segments, nitrite in the media of cultured eNOS KO aortic segments was significantly reduced to a level nearly identical to the basal nitrite content of the media. If iNOS had contributed to NO generation in this assay, the nitrite levels in even the eNOS KO specimens should have been higher than media alone, suggesting that iNOS did not contribute substantially to the nitrite measurements. Together, these observations suggest that NO produced by eNOS in our in vitro sprouting assay plays an important role in promoting endothelial cell migration, proliferation, and differentiation, which are necessary steps in angiogenesis.

In summary, we have demonstrated that eNOS KO mice manifest impaired wound healing and angiogenesis. Diseases in which a deficit in VEGF expression has been identified, such as diabetes (11), may benefit from a therapeutic increase in eNOS activity or even delivery of NO to promote wound healing. Recent work by Kullo et al. (15) has demonstrated successful transfection of vascular tissues with adenoviral vectors encoding eNOS (AdeNOS). AdeNOS gene transfer in the setting of impaired wound healing could potentially increase eNOS activity in wounds and thereby allow normal wound repair to occur.

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