Reversal of Impaired Wound Repair in iNOS-deficient Mice by Topical Adenoviral-mediated iNOS Gene Transfer

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Abstract

Most evidence indicates that nitric oxide plays a role in normal wound repair; however, involvement of inducible nitric oxide synthase (iNOS) has not been established. Experiments were carried out to determine the requirement for iNOS in closing excisional wounds. Wound closure was delayed by 31% in iNOS knockout mice compared with wild-type animals. An identical delay in wound closure was observed in wild-type mice given a continuous infusion of the partially selective iNOS inhibitor N^6-(iminoethyl)-L-lysine. Delayed wound healing in iNOS-deficient mice was completely reversed by a single application of an adenoviral vector containing human iNOS cDNA (AdiNOS) at the time of wounding. Reverse transcription PCR identified iNOS mRNA expression in wild-type mice peaking 4–6 d after wounding, and confirmed expression of human iNOS in the adenoviral vector containing human iNOS cDNA–treated animals. These results establish the key role of iNOS in wound closure, and suggest a gene therapy strategy to improve wound healing in iNOS-deficient states such as diabetes, and during steroid treatment. (*J. Clin. Invest.* 1998. 101: 967–971.) Key words: nitric oxide • iNOS • iNOS knockout • wound repair • gene therapy

Introduction

Wound repair results from a series of highly orchestrated cellular and biochemical events, including increased synthesis of the bioregulatory molecule nitric oxide (NO).[1] Levels of nitrite and nitrate, the stable endproducts of NO synthesis, are elevated early and transiently in fluid obtained from sponges implanted in subcutaneous wounds (1), while urinary nitrate excretion increases in a sustained manner after excisional wounding (2) and burn injury (3, 4). Most evidence suggests that adequate rates of NO production are essential for normal wound healing. Arginine is the physiologic substrate for NO synthesis, and systemic arginine administration improves wound healing in normal individuals (5) as well as in individuals with impaired wound healing (6). NO promotes processes central to wound healing such as angiogenesis (7, 8) and endothelial (9) and epithelial (10, 11) cell proliferation and migration. Furthermore, urinary nitrate excretion is depressed in association with impaired wound healing after steroid treatment or diabetes induction in rats (12, 13). Nonspecific nitric oxide synthase (NOS) inhibitors applied to the surface of wounds (12) or given systemically (14) retard wound healing, while topical application of an NO donor has been shown to accelerate closure of excisional wounds in rats (15).

NO can be produced by one of three known NOS isoforms. The cytokine-inducible, or inflammatory, NOS (iNOS or NOS2) produces NO in a sustained manner independently of intracellular calcium elevations, and is expressed in many inflammatory conditions (16). Wound healing includes an inflammatory component, predicting a role for iNOS. Therefore, we studied the rate of excisional wound closure in mice with targeted disruption of the iNOS gene (iNOS knockout mice, KO; 17). These experiments were coupled with wounding studies using constant infusion of a partially selective iNOS inhibitor in wild-type (WT) mice to block induced NO production or gene transfer of human iNOS into wounds using an adenoviral vector to increase NO production. Our results establish a clear role for iNOS in closing excisional wounds, and introduce a gene transfer therapeutic strategy to promote wound healing in NO-deficient states.

Methods

Mice with targeted disruption of the iNOS gene were prepared as described (17). N^6-(iminoethyl)-L-lysine (NIL) was purchased from Torcis Cookson (Ballwin, MO). Alzet osmotic minipumps that deliver 0.5 μl/h for 7 d were obtained from Alzet Corp. (model 1007D; Palo Alto, CA).

Animal wound model. Animal protocols were approved by the Animal Use and Care Committee of the University of Pittsburgh. Mice of both genders were anesthetized with intraperitoneal pentobarbital (45 mg/kg), the dorsum was clipped free of hair, and the skin was prepped with providine-iodine and sterile H_2O. A full-thickness wound (1.5 × 1.5 cm) was created, and the surface area was measured after the skin edges had retracted. Adenovirus-mediated gene transfer was performed in some animals by placing 200 μl of viral solution directly on the wound for 30 min with the animal still anesthetized.

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Received for publication 23 October 1997 and accepted in revised form 7 January 1998.

1. *Abbreviations used in this paper:* AdiNOS, adenoviral vector containing human iNOS cDNA; BH_4, tetrahydrobiopterin; iNOS, inducible nitric oxide synthase; KO, iNOS knockout mice; NIL, N^6-(iminoethyl)-L-lysine; NO, nitric oxide; pfu, plaque-forming units; RT, reverse transcription.

J. Clin. Invest.

0021-9738/98/03/0967/05 $2.00
Volume 101, Number 5, March 1998, 967–971
http://www.jci.org

The solution was then removed, and the wounds were covered with a bioocclusive dressing to maintain sterility. Wound closure rates were measured every other day by tracing the wound area on acetate sheets. Dressings were changed at these times. Tracings were digitized, and areas were calculated in a blinded manner using a computerized algorithm (Sigma Scan™; Jandel Scientific, San Raphael, CA).

Adenoviral vectors. The human iNOS cDNA was previously cloned from cytokine-stimulated human hepatocytes (18). An E1- and E3-deleted vector carrying the human iNOS cDNA (AdiNOS) was designed and constructed. An adenovirus transfer plasmid with the cytomegalovirus promoter and an artificial splice sequence was designed to drive transcription of iNOS cDNA. To generate infectious virus, this plasmid was cotransfected into 293 cells (CRL. 1573; American Type Culture Collection, Rockville, MD) with the large Cla I fragment of AdlacZ DNA (19). Intracellular recombination of the plasmid with the Cla I fragment carrying adenoviral sequences generated a full-length recombinant adenoviral genome (20). Recombinant AdiNOS virus was double plaque-purified, and expression of the iNOS transgene was determined by screening for nitrite (NO_2^-) release from infected cells using the Griess reaction (21). Viral stocks were purified by triple banding on a cesium chloride gradient. Concentrations of AdiNOS and the control adenovirus AdlacZ that carries the bacterial β-galactosidase gene were determined by plaque assay. The titer of AdiNOS was $\sim 10^9$ plaque-forming units (pfu)/ml, and AdlacZ was $\sim 10^{10}$ pfu/ml. These stock solutions were diluted in OPTIMEM-I to a final concentration of 10^8 pfu/ml for the in vivo experiments.

Cell culture. National Institutes of Health (NIH) 3T3 cells were grown in Dulbecco's modified Eagle's medium with 10% (vol/vol) calf serum, 10 mM Hepes, penicillin (100 U/ml), and streptomycin (100 μg/ml). Subconfluent cultures were incubated with adenoviral vectors carrying the gene for either β-galactosidase or iNOS at an estimated multiplicity of infection of 100:1 for 60 min, at which time the media was replaced. After 24 h, the medium was collected for NO_2^- levels, and the RNA was isolated from the cells.

RNA isolation and reverse transcription(RT) PCR. Total RNA was isolated from NIH 3T3 cells, or frozen wound tissue using RNAzol B as described (21). cDNA synthesis was performed on 250 ng of RNA using 20 U/ml Moloney leukemic virus reverse transcriptase (Gibco Laboratories, Grand Island, NY). PCR was performed using 25 ng of cDNA with reactions carried out for 30 cycles of 92°C denaturation for 1 min, 57°C annealing for 2 min, and 72°C elongation for 3 min (22). PCR primers that recognize only human iNOS were used to detect adenovirally transferred iNOS mRNA in tissue (5′ primer sequence is 5′-AGGACATCCTGCGGCAGC-3′ and the 3′ primer sequence is 5′-GCTTTAACCCCTCCTGTA-3′, representing bp 3376–3691 of human hepatocyte iNOS sequence (18). Primers used to detect murine iNOS were 5′-ATGGACCAGTATAAGGCAAGC and 3′-GCTCTGGATGAGCCTATATTG. Primers designed specifically to recognize spliced human iNOS mRNA of adenoviral vector origin were used to detect transferred iNOS expression in some experiments. The 5′ primer (5′-CCGTGCCAAGAGTGACGTGTC) is complementary to the 5′adenoviral splice site, while the 3′ primer (3′-TGGCCTTATGGTGAAGTGTGT) is complementary for human iNOS sequences, and yields a PCR product 576 bp in size. To control for RNA quality and cDNA synthesis, β-actin mRNA was also amplified (652-bp product). The 5′ primer was labeled with γ-[^{32}P]dATP. The PCR products were separated by electrophoresis on a 5% polyacrylamide gel and visualized by autoradiography.

Statistical analysis. The times for wound closure were compared among groups using Student's *t* test. Analysis was performed using a computerized software package (Sigma Stat®; Jandel Scientific). $P \leq 0.05$ or less was statistically significant.

Results

Rate of wound closure in iNOS-deficient mice. To examine the consequence of iNOS deficiency in the rate of wound closure, a standard 1.5 × 1.5 cm–full thickness wound was created on the dorsum of either WT or KO mice (17). Immediately after skin excision, all wounds measured between 2.33 and 2.43 cm^2 in size, with no significant difference among groups. The rate of wound closure was monitored by measuring the reduction in wound surface area by planemetry. Wound areas were traced serially on acetate sheets. Tracings were digitized, and areas were calculated in a blinded manner using a computerized algorithm (Sigma Scan™, Jandel Scientific). As shown in Table I, the average time of wound closure was significantly delayed in the KO animals. The disparity in closure rates between WT and KO mice became greater with time, and KO mice required an average of 5.3 d longer for complete wound healing. Southern blot analysis was used to confirm the disruption of the iNOS gene in the KO group (not shown).

Table I. Rate of Excisional Wound Closure in Wild-type and iNOS Knockout Mice

% Closure	Wild-type	iNOS Knockout
30	3.5±0.2*‡	4.4±0.3
50	6.1±0.2	7.0±0.4
80	9.9±0.3	12.0±0.6§
100	17.0±0.4	22.3±0.8§

*Data represent time in days that it took for the wound to close by the indicated percentage. ‡Data are the mean±SEM for nine mice in each group. §$P < 0.01$ vs. wild-type.

iNOS inhibition mimics iNOS knockout mice. To assure that the differences in wound healing rates were not due to random variability in the C57/B6 × 129 genetic background between the WT or KO groups, or to the influences of sequences linked to the target gene in the KO mice that could affect the phenotype, wounding experiments were carried out using constant infusion of an iNOS inhibitor in WT mice. Alzet osmotic pumps containing either saline or NIL were placed intraperitoneally at the time of excisional wounding. NIL, which has been shown to be 33 to 50 times more selective for iNOS than constitutive endothelial NOS (23), was infused at a rate of 20 μmol/kg/h. Because the pumps infused for only 7 d, the first pump was removed after 7 d and was replaced with a full pump, permitting constant infusion of the inhibitor for the first 14 d, a time period that included maximal iNOS expression (see below). Pump placement was always carried out using the smallest incision that allowed pump insertion. Infusion of NIL resulted in a 1.8-d delay in wound closure to 50%, and a 4.5-d delay in complete closure compared with WT mice with saline-containing pumps (Fig. 1). Thus, NIL infusion resulted in a delay in wound closure in the WT mice almost identical to the delay observed in the KO animals.

Gene transfer using human iNOS reverses the iNOS-deficient state. We used in vivo gene transfer to determine if delayed wound healing in the KO mice could be reversed by locally expressing the iNOS gene. An AdiNOS was constructed and tested for expression in murine NIH 3T3 cells. As shown in Fig. 2, the iNOS-transduced cells released large amounts of NO_2^-, whereas the control cells transduced with an adenoviral vector carrying the β-galactosidase (AdlacZ) gene did not. The AdiNOS-transduced NIH 3T3 cells also expressed human iNOS mRNA as shown by RT-PCR using primers specific for

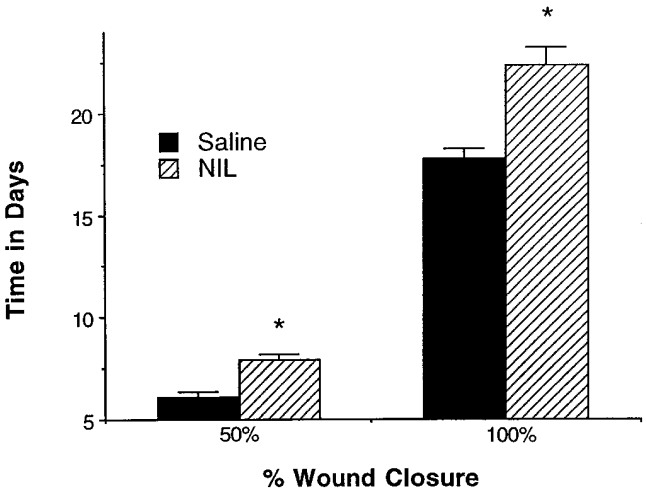

Figure 1. Wound closure rates in WT mice receiving a constant infusion of NIL. WT mice underwent cutaneous wounding as described in Methods. At the same time, an Alzet osmotic pump containing either saline or NIL (20 μmol/kg/h) was placed in the peritoneal cavity. After 7 d, the pump was replaced with a new full pump, permitting 14 d of continuous infusion. Wound closure rates were monitored by measuring the wound surface every other day. Results show the mean± SEM to 50 or 100% closure (n = 8/group; *$P < 0.001$ vs. saline group).

the adenoviral iNOS transcript (Fig. 2). Consistent with our previous work with iNOS gene transfer into NIH 3T3 cells (24), production of NO in the transduced cells required addition of the NOS cofactor tetrahydrobiopterin (BH_4). This requirement was not unexpected because this cell line does not express GTP cyclohydrolase I, the rate-limiting enzyme for BH_4 biosynthesis (24).

Either AdiNOS or AdlacZ (2×10^7 pfu in 0.2 ml of solution) was placed on fresh wounds for 30 min. The solution containing the vector was then removed, and the rate of wound closure was monitored as before. Transfer of AdlacZ into wounds in WT mice had no effect on the rate of wound healing (WT vs. WT/AdlacZ < 17.0±0.4 vs. 17.3±0.6 d to 100% closure), but it slightly accelerated the closure rate in the KO mice (KO vs. KO/AdlacZ, 22.3±0.8 vs. 20.2±0.6 d to 100% closure), although this value was not statistically significant. Whereas transfer of iNOS into wounds of WT mice increased wound healing rates minimally, transfer of iNOS into wounds of KO mice accelerated the complete closure rate by an average of 4.6 d compared with the KO/AdlacZ group (Fig. 3), and 6.7 d compared with the KO mice not subjected to gene transfer.

Expression of iNOS in wounds. To determine the time course for iNOS mRNA expression in the various groups, total RNA was isolated from excised wounds of mice killed on every other day from days 2 –18 after wounding (except day 14). The quantity of RNA obtained precluded quantitative analysis in individual mice by Northern blot analysis. Therefore, ^{32}P-labeled RT-PCR was performed on the cDNA synthesized from the RNA. Oligonucleotide primers specific for β-actin, murine iNOS, or human iNOS (see Methods) were used. Wounds from two mice were analyzed for each time point, and Fig. 4 demonstrates the expression pattern for a representative animal for each day. A strong β-actin band indicated that adequate cDNA was obtained from all wounds. Murine iNOS mRNA was present in the WT wounds between days 2 and 10, peaking on days 4–6 independently of *AdlacZ* or *AdiNOS* gene transfer, but was absent in the wounds of KO animals. Human iNOS mRNA was detectable only in the WT or KO mice treated with AdiNOS, with expression detected from days 2–10.

Discussion

Our results indicate that iNOS is expressed in the healing wound, with peak expression on days 4–6 after wounding. Furthermore, we show that inducible NO production accelerates

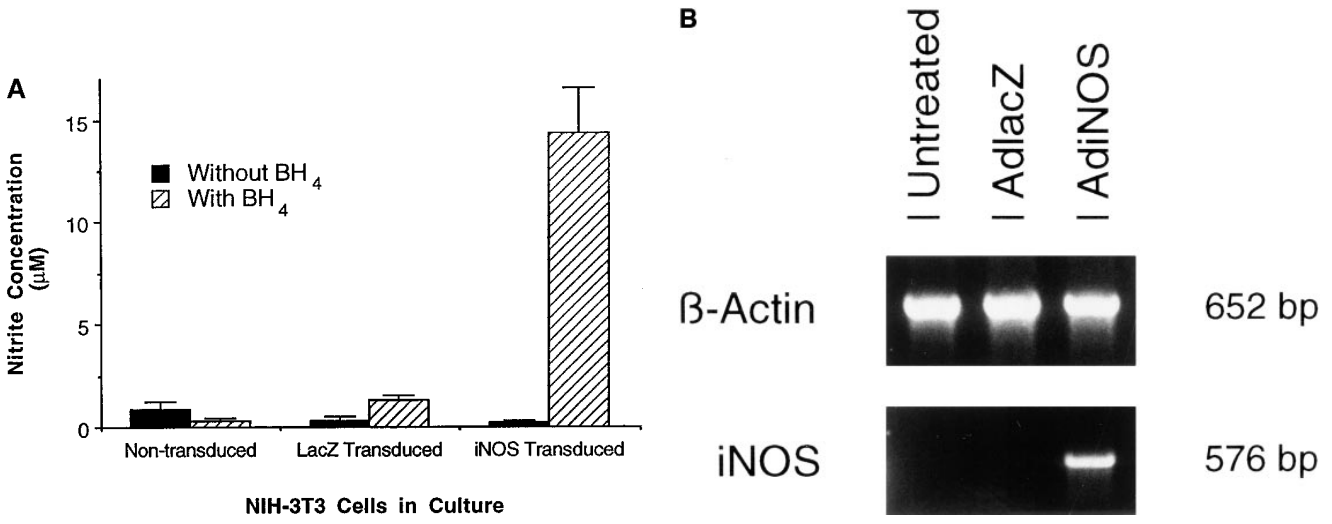

Figure 2. Adenovirus-iNOS transduces NIH 3T3 cells to express functional iNOS. Subconfluent cultures of NIH 3T3 cells were transduced with adenovirus- lacZ or adenovirus iNOS at a multiplicity of infection of 100:1 in the presence or absence of 10 μM tetrahydrobiopterin (BH_4). After 24 h, the supernatant was removed, NO_2^- concentrations were measured (*A*), and RNA was isolated from the cells for detection of human iNOS mRNA (*B*). Results are representative of three separate experiments with duplicate cultures.

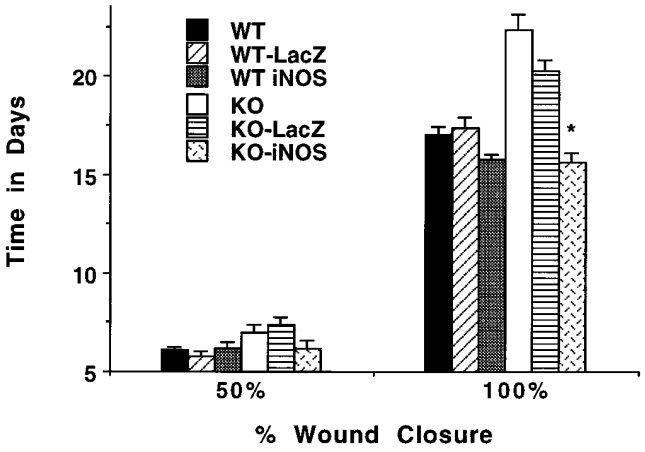

Figure 3. Adenovirus-mediated iNOS gene transfer reverses the iNOS-deficient state. At the time of cutaneous wounding, the wounds of both WT and iNOS KO mice were exposed to either adenovirus β-galactosidase (lacZ) or adenovirus iNOS (iNOS), 2×10^7 pfu in 200 μl media. After 30 min, the media was removed and wound closure rates were monitored as described in the Methods. *Bars* representing WT and KO groups from Table I are included to demonstrate the effect of transfection ($n = 9$/group; mean±SEM; $^*P < 0.001$ vs. KO AdlacZ).

the closure rate of excisional wounds. This conclusion is supported by our observation that wound closure is delayed by about 30% in iNOS KO mice, and in WT mice treated with a continuous infusion of a NOS inhibitor with partial selectivity for iNOS. Wound repair is an extremely efficient process in rodents, and this 4–5-d prolongation is highly significant ($P < 0.001$) and greater than the delay in excisional wound healing described in diabetic rats (15). In addition, delayed healing in the iNOS-deficient animals could be completely reversed by transient expression of iNOS in the wounds using gene transfer.

Increased NO production resulting from upregulation of iNOS expression could promote wound healing by several mechanisms. Angiogenesis is a key component of normal wound healing, and NO has been shown to be necessary for expression of macrophage angiogenic activity (7). Furthermore, NO donors increase angiogenesis induced by PGE_2 or agonist of the tachykinin NK_1 receptor (8). The angiogenic effect of NO may be due to the capacity of NO to stimulate endothelial cell proliferation and migration (9–11). Injured keratinocytes express iNOS (25), and NO appears to function as a signal to switch stationary epithelial cells to a locomoting phenotype (10, 11). NOS inhibitors suppress nuclear expression of proliferating cell nuclear antigen in keratinocytes after ultraviolet photodamage to skin (10), suggesting a role for NO in keratinocyte proliferation as well. Both epithelial migration and proliferation are required for reepithelialization of cutaneous wounds. Inhibition of NO synthesis results in a decrease in collagen content in sponges implanted in subcutaneous wounds (14) and a decrease in collagen synthesis by wound fibroblasts in vitro (26), suggesting that NO stimulates collagen synthesis by wound fibroblasts. Even though the greatest difference in wound closure rates in the KO and NIL animals was detected beyond 12 d, iNOS expression was most prominent in the first 10 d, raising the possibility that the effects of NO on earlier events in wound healing such as angiogenesis and collagen synthesis may be more important. Experiments are currently underway to establish the mechanisms by which induced NO promotes wound healing.

Repair of excisional wounds is impaired under circumstances associated with reduced iNOS expression and reduced NO availability, such as steroid administration and diabetes (12, 13). Reducing NO availability by topical application of a nonspecific NOS inhibitor in rats also delays wound closure (12). Our results significantly extend these previous observations by showing that complete absence of iNOS or continuous iNOS inhibition leads to a similar delay in closure rates in mice, indicating the importance of the iNOS isoform as an NO source in wounds. Transient iNOS expression achieved with adenoviral-mediated iNOS gene transfer using a single, brief exposure to low-titer virus was adequate to reverse the delayed wound closure in the iNOS-deficient animals. This result suggests that a minimal level of sustained NO production is required for normal wound healing. The failure of gene transfer to promote wound healing in WT mice while NO donors were effective in normal rats (15) may be due to species differences in wound healing or failure to achieve supranormal levels of

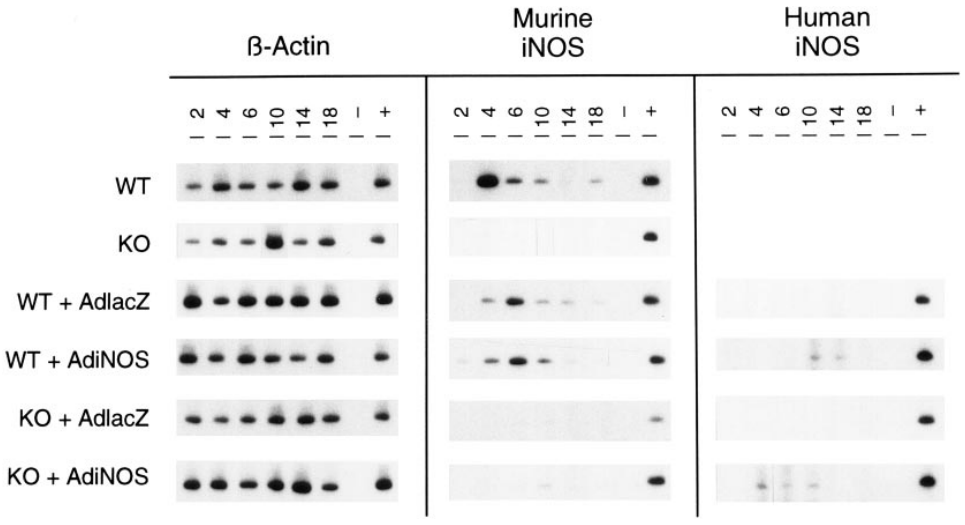

Figure 4. RT-PCR for detecting murine or human iNOS expression. On the days shown, the wounds were excised, total RNA was isolated, and RT-PCR was performed to detect either murine or human iNOS mRNA. Two animals were analyzed for each time point, and the figure demonstrates the results from a representative animal.

NO by gene transfer in these experiments. Higher iNOS expression might be achieved by longer exposure to higher viral titers. It is even possible that BH_4 availability limits NO synthesis, although cofactor levels were clearly adequate to support some iNOS activity in the KO mice receiving adenovirus–iNOS gene transfer.

Gene transfer using iNOS offers some attractive advantages over other therapeutic approaches to promote wound repair based on increasing wound NO levels in NO-deficient states. Topical application of the iNOS gene in an efficient vector such as an adenovirus should increase local NO production without systemic effects. Dietary supplementation or local application of NO donors requires continuous therapy, and may not effectively increase wound NO levels in a sustained manner. We found that a single application of iNOS in an adenoviral vector resulted in iNOS mRNA expression for up to 10 d. The benefits of a single topical application of a therapy may be even more apparent in circumstances where the wound is not continuously accessible, such as an internal wound created during a surgical procedure. iNOS may also have distinct advantages over the two other NOS isoforms for wound-healing gene therapy applications. Unlike the constitutive NOS isoforms, iNOS exhibits activity independently of intracellular calcium elevations, and exhibits high-level sustained NO synthesis in the presence of adequate cofactors and substrate levels (16). Thus, even low-efficiency transfer could provide a constant source of adequate local NO to influence wound healing rates.

Acknowledgments

The authors gratefully acknowledge the helpful comments from Dr. Carl Nathan and Dr. John Mudgett.

This work was supported by National Institutes of Health grants R01-GM-44100 and R01-GM-37753.

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