The Function of Nitric Oxide in Wound Repair: Inhibition of Inducible Nitric Oxide-Synthase Severely Impairs Wound Reepithelialization

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Recently, we demonstrated a large induction of inducible nitric oxide synthase (iNOS) during cutaneous wound repair. In this study, we established an *in vivo* model in mice to investigate the role of NO during the wound healing process. During excisional repair, mice were treated with L-N^6-(1-iminoethyl)-lysine (L-NIL), a selective inhibitor of iNOS enzymatic activity. Compared with control mice, L-NIL-treated animals were characterized by a severely impaired reepithelialization process, as the hyperproliferative epithelia at the wound edges appeared to be delayed and characterized by an atrophied morphology. Immunohistochemical labeling for detection of proliferating cells (BrdU-, Ki67-staining) revealed a strong reduction in proliferating keratinocyte cell numbers during the process of reepithelialization after inhibition of iNOS activity during repair. Western blot analysis of total wound lysates from PBS- and L-NIL-treated mice clearly demonstrated a reduction in proliferating cell nuclear antigen, representing a marker for cell proliferation, in lysates isolated from L-NIL-treated mice. The dependency between keratinocyte proliferation and NO availability observed during wound repair *in vivo* is further supported by the observation that proliferation of the keratinocyte cell line (HaCaT) is stimulated by low concentrations of NO-donors also *in vitro*. In summary, our data demonstrate that the presence of a functionally active iNOS is a crucial prerequisite for normal wound reepithelialization.

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To overcome tissue damage, the process of cutaneous wound repair represents a highly ordered process that is characterized by overlapping phases of important tissue movements like inflammation, reepithelialization, and granulation tissue formation. The latter processes involve macrophage accumulation, fibroblast ingrowth, matrix deposition, and angiogenesis. Central to wound closure is the reepithelialization that is closely associated to granulation tissue formation in a temporal and spatial manner (Clark, 1996; Martin, 1997). After a short lag period of several hours after injury, reepithelialization initiates with keratinocytes from the wound edge starting to migrate into the wound clot, which has formed as tissue damage is mostly associated with blood leakage from damaged peripheral blood vessels (Clark *et al*, 1982; Woodley, 1996). As reepithelialization moves on, keratinocytes at the wound margins proliferate to form a dense hyperproliferative epithelium, thus feeding the migrating epithelial tongue (Woodley, 1996). It is well established that, especially, two growth factors are central players to this process: epidermal growth factor (EGF) and keratinocyte growth factor (KGF), respectively, both factors representing potent mitogens to epithelial cells. EGF has been shown to accelerate epidermal regeneration in wound healing (Brown *et al*, 1986, 1989). More important, dermally derived KGF stimulates wound reepithelialization in a paracrine manner, as demonstrated by transgenic mice expressing the dominant-negative KGF-specific FGF-receptor IIIb in the skin (Werner *et al*, 1994). Besides these protein-type factors, an important role for the process of cutaneous wound repair has been demonstrated for nitric oxide (NO). NO is synthesized by three types of NO synthases (NOS). Two enzyme isoforms are constitutively expressed (endothelial and neuronal NOS), whereas one isoform is an inducible enzyme (iNOS) that is initially found in macrophages (Knowles and Moncada, 1994). All three isoforms are homodimeric proteins that depend on NADPH, reduced flavins, heme-bound iron, and (6R) 5,6,7,8-tetrahydrobiopterin as essential cofactors (Baek *et al*, 1993; Klatt *et al*, 1993). The enzymatic activity of the constitutively expressed NOS isoforms is strongly dependent on Ca^{2+} and calmodulin, whereas iNOS is regulated independent of changes in Ca^{2+} at the transcriptional level. The constitutively expressed NOS isoforms are expressed in keratinocytes, melanocytes, and endothelial cells, thus providing regulatory and homeostatic functions for NO in the skin like regulation of cutaneous blood flow (Goldsmith *et al*, 1996), or involvement in melanogenesis after ultraviolet irradiation (Romero-Graillet *et al*, 1996, 1997); however, a role for NO produced by enzymatic activities of the inducible NOS (iNOS) isoform has been implicated for pathologic states of the skin. Dysregulated expression of iNOS has been demonstrated for skin biopsies from psoriatic patients (Kolb-Bachofen *et al*, 1994; Bruch-Gerharz *et al*, 1996) or in the pathogenesis of lupus erythematosus (Kuhn *et al*, 1998). Additionally, expression of iNOS is largely

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Abbreviations: BrdU, 5-bromo-2′-deoxy-uridine; HE, hyperproliferative epithelium; iNOS, inducible nitric oxide synthase; KGF, keratinocyte growth factor; L-NIL, L-N^6-(1-iminoethyl)lysine; NO, nitric oxide; NOS, nitric oxide synthase; PCNA, proliferating cell nuclear antigen.

induced after skin injury during those stages of tissue regeneration, when inflammation, granulation tissue formation, and reepithelialization occur (Frank *et al*, 1998; Reichner *et al*, 1999), and, furthermore, iNOS-deficient mice are characterized by a severe delay in wound closure after skin injury (Yamasaki *et al*, 1998). Only very little is known, however, about the functional role of NO during the healing process.

A first line of evidence suggests an influence of NO on keratinocyte proliferation and differentiation, thus resembling effects mediated by protein-type factors EGF and KGF. But, in contrast to both epithelial mitogens, NO seems to mediate a biphasic effect on keratinocyte proliferation and differentiation, as only low doses of NO led to increased keratinocyte proliferation *in vitro* (Krischel *et al*, 1998). These observations demonstrated a possible involvement of NO in growth and differentiation of epidermal keratinocytes *in vitro*, which could be unique for these cells. In this study, we have identified NO as a potent mediator of keratinocyte proliferation during wound healing *in vivo*. Treatment of animals with the selective iNOS inhibitor L-N^6-(1-iminoethyl)-lysine (L-NIL) during wound repair resulted in a delayed and severely impaired reepithelialization of wounds, which is caused by a clearly reduced number of proliferating keratinocytes within the hyperproliferative epithelium during tissue regeneration.

MATERIALS AND METHODS

Inhibitor treatment of mice Mice were wounded as described below. During the wound healing period, female BALB/C mice (3 mo old) were injected intraperitoneally twice a day at 7 a.m and 7 p.m. with 2.5 mg L-N^6-(1-iminoethyl)lysine (L-NIL) in 0.5 ml phosphate-buffered saline (PBS) per injection for 13 d. L-NIL represents a highly selective inhibitor of iNOS enzymatic activity (Moore *et al*, 1994). At this concentration, L-NIL almost completely blocked the *in vivo* enzymatic activity of iNOS in lymph nodes and peripheral skin lesions of *Leishmania*-infected mice. This inhibition of iNOS has been shown to be potent and presumably irreversible *in vivo* and is not associated with weight loss and reduced water and food consumption of the treated animals (Stenger *et al*, 1995; Diefenbach *et al*, 1998). Control mice were injected with PBS. L-NIL was from Alexis (Grünberg, Germany).

***In vivo* 5-bromo-2′-deoxy-uridine (BrdU)-labeling of proliferating cells** Mice were wounded as described below. During the wound healing period, female BALB/C mice (3 mo old) were injected intraperitoneally twice a day at 7 a.m and 7 p.m. 2.5 mg with L-N^6-(1-iminoethyl)lysine (L-NIL) in 0.5 ml PBS per injection for 3 d. At day 3, mice received 1.6 mg BrdU simultaneously with the last intraperitoneal injection of L-NIL/PBS 2 h before animals were sacrificed.

Wounding and preparation of wound tissues To examine gene expression during the wound healing process, six full-thickness wounds were created on each animal, and skin biopsy specimens from four animals were obtained 1, 3, 5, 7, and 13 d after injury. To investigate the effect of L-NIL on gene expression, wounds from four L-NIL-treated mice, and four PBS-treated mice, respectively, were obtained 1, 3, 5, 7, and 13 d after injury. Mice were anesthetized with a single intraperitoneal injection of Ketamin (80 mg per kg body weight)/Xylazin (10 mg per kg body weight). The hair on the back of these mice were cut, and the back was subsequently wiped with 70% ethanol. Six full-thickness wounds (5 mm diameter, 3–4 mm apart) were made on the backs of these mice by excising the skin and the underlying *panniculus carnosus*. The wounds were allowed to dry to form a scab. An area of 7–8 mm in diameter, which included the scab and the complete epithelial margins, was excised at each time point. As a control, a similar amount of skin was taken from the backs of four nonwounded mice. For every experimental time point, four wounds each from four animals (n = 16 wounds) and the nonwounded back skin from four animals, respectively, were combined, frozen immediately in liquid nitrogen, and stored at −80°C until used for RNA isolation. All animal experiments were carried out according to the guidelines and with the permission from the local government of Hessen.

Nitrite determination in wound lysates Nitrite, a stable NO oxidation product, was determined in wound lysates using the Griess reaction (Green *et al*, 1982). Normal and wounded back skin was frozen in liquid nitrogen. Every experimental time point represents eight wounds (n = 8) from four different animals that have been pooled for nitrite determination. Skin samples were homogenized in 2 × lysis buffer (1 × lysis buffer: 1% Triton X-100, 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 15 μg leupeptin per ml). The tissue extract was cleared by centrifugation and the supernatant diluted 1:1 with water. One milliliter of lysate from unwounded skin and from wounds harvested 3, 5, 7, and 13 d after injury were cleared by a centrifugation step at 100 000 × *g* for 30 min. Cleared lysates (200 μl) were adjusted to 4°C, mixed with 20 μl sulfanilamide (dissolved in 1.2 M HCl), and 20 μl *N*-naphtylethylendiamine dihydrochloride. After 5 min at room temperature, the absorbance was measured at 540 nm with a reference wavelength at 690 nm. Phenylmethylsulfonyl fluoride, leupeptin, sulfanilamide, and *N*-naphtylethylendiamine dihydrochloride were from Sigma Biochemicals (Deisenhofen, Germany).

Preparation of wound lysates Biopsies of normal back skin and wounds isolated from L-NIL-treated or PBS-treated animals (n = 4 wounds) were taken and frozen immediately in liquid nitrogen. Total skin samples were homogenized in 2 × homogenizing buffer (1 × homogenizing buffer: 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 15 μg leupeptin per ml). The tissue extract was cleared by centrifugation and the supernatant diluted 1:1 with water. The amount of protein in the cytoplasmic fractions of the lysates was determined using the Bio-Rad protein assay (Bradford method).

Western blot analysis Twenty-five micrograms of protein from unwounded back skin and 1 d, 3 d, 5 d, and 7 d wounds of L-NIL- or PBS-treated mice was separated using SDS-gel-electrophoresis. After transfer to a PVDF membrane, proliferating cell nuclear antigen (PCNA)-specific protein was detected using a monoclonal antiserum directed against human recombinant PCNA (Santa Cruz, Biotechnology, Heidelberg, Germany). A secondary antibody coupled to horseradish peroxidase and the ECL detection system were used to visualize PCNA protein. Phenylmethylsulfonyl fluoride, and leupeptin were supplied from Sigma Biochemicals, the antibody coupled to horseradish peroxidase was from Biomol (Hamburg, Germany), and the ECL detection system was obtained from Amersham (Braunschweig, Germany).

RNA isolation and RNase protection analysis RNA isolation was performed as described (Chomczynski and Sacchi, 1987). Thirty micrograms of total RNA from wounded or nonwounded skin were used for RNase protection assays. Every experimental time point represents 16 wounds (n = 16) from four different animals that have been pooled for RNA isolation. RNase protection assays were carried out as described previously (Werner *et al*, 1992). Briefly, DNA probes were cloned into the transcription vector pBluescript II KS (+) (Stratagene, Heidelberg, Germany) and linearized. An antisense transcript was synthesized *in vitro* using T3 or T7 RNA polymerase and [α-^{32}P]UTP (800 Ci per mmol). RNA samples were hybridized at 42°C overnight with 100 000 cpm of the labeled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30°C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% acrylamide/8 M urea gels and analyzed using a PhosphoImager (Fuji). All protection assays were carried out with at least three different sets of RNA from independent wound healing or cell culture experiments. A 316 bp fragment corresponding to nucleotides 139–455 of the murine $VEGF_{120}$ cDNA (Breier *et al*, 1992) was used as a template. RNases A and T1 were from Boehringer Biochemicals (Mannheim, Germany).

Histology Mice were wounded as described above. Animals were sacrificed at days 3 and 5 after injury. Complete wounds were isolated from the middle of the back, bisected, and frozen in tissue freezing medium. Six-micrometer sections from the middle of the wound were stained with hematoxylin and eosin.

Immunohistochemistry Mice were wounded as described above. Animals were sacrificed at days 3 and 5 after injury. Complete wounds were isolated from the middle of the back, bisected, and frozen in tissue freezing medium. Six-micrometer frozen sections were fixed with acetone and treated for 10 min at room temperature with 1% H_2O_2 in PBS to inactivate endogenous peroxidases. They were subsequently incubated for 60 min at room temperature with a polyclonal antiserum raised against murine Ki67 (Gerdes *et al*, 1984) from Dianova (Hamburg, Germany) (1:50 diluted in PBS, 0.1% bovine serum albumin). The slides were subsequently stained with the avidin-biotin-peroxidase complex system from Santa Cruz (Heidelberg, Germany) using 3-amino-9-ethylcarbazole as a chromogenic

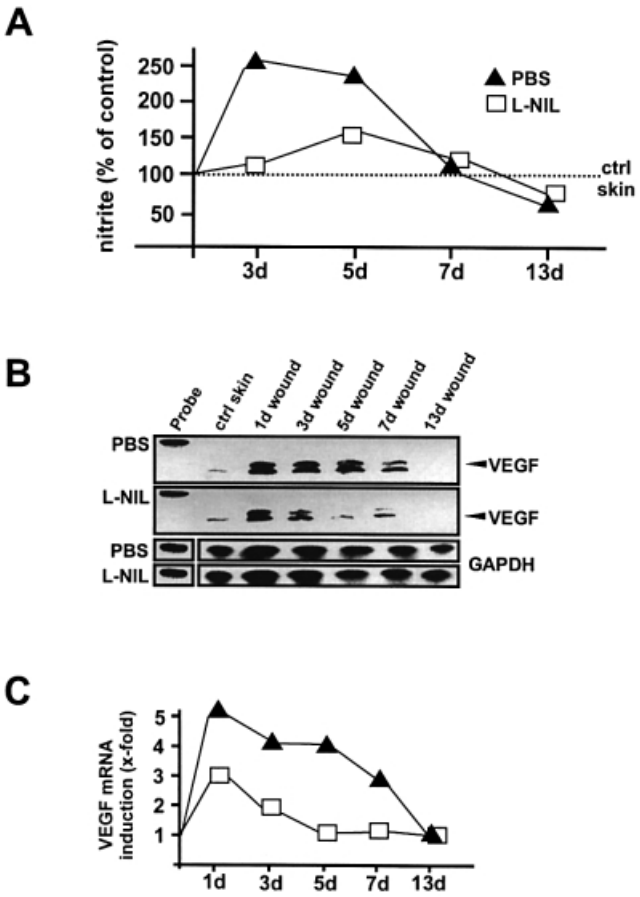

Figure 1. L-NIL is functionally active. (*A*) BALB/C mice were treated with L-NIL as described under *Materials and Methods*. Mice injected with PBS were used as a control. Wound lysates from wounded skin of control mice and L-NIL-treated mice (n = 8 wounds were pooled for each experimental time point) were used for nitrite determination as indicated. Normal back skin was used as control. Nitrite accumulation in wound lysates (200 μl) during the wound healing process was measured as a readout for iNOS enzymatic activity. (*B*) Total cellular RNA (30 μg) from nonwounded and wounded back skin of normal and L-NIL-treated mice was analyzed by RNase protection assay with an RNA hybridization probe complementary to the 3′-end of murine $VEGF_{120}$ mRNA. For one representative experiment, the regulation of VEGF mRNA expression in normal and L-NIL-treated mice is shown in (*B*). For every experimental time point, four wounds each from four animals (total of n = 16 wounds) were pooled for analysis. The time after injury is indicated on top of each lane. Control skin refers to nonwounded skin of normal mice. One thousand counts per min of the hybridization probe were added to the lane labeled *probe*. The gels were exposed simultaneously on the same imaging plate for 16 h. The degree of VEGF mRNA induction as assessed by PhosphoImager (Fuji) analysis of the radiolabeled gels is shown in (*C*).

substrate. After development, slides were rinsed with water, counterstained with hematoxylin, and mounted. For immunohistochemical detection of BrdU-labeled cells, we used the 5-bromo-2′-deoxy-uridine labeling and detection kit II (Boehringer Mannheim Biochemicals, Germany) according to the instructions of the manufacturer.

Proliferation assay Keratinocytes (HaCaT cell line) (Boukamp *et al*, 1988) were seeded into 96 well plates (Greiner, Frickenhausen, Germany). Each well contained 2×10^3 cells in a total volume of 100 μl Dulbecco's modified Eagle's medium/10% fetal calf serum. Under these conditions, cells remained proliferative and undifferentiated. Subsequently, cells were grown for 24 h. The proliferation rate of incubated cells was determined after stimulation of cells with either S-nitroso-glutathione (GSNO, 100 μM, 500 μM) (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)-amino] diazen-1-ium-1,2-diolate (DETA-NO, 100 μM, 500 μM), or sodium nitroprusside (SNP, 100 μM, 500 μM) for 24 h using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Mannheim, Germany) according to the instructions of the manufacturer. Briefly, proliferation was quantified by measuring the amount of soluble formazan produced by cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS).

GSNO synthesis S-nitroso-glutathione was synthesized as described previously (Hart, 1985). Briefly, glutathione was dissolved in 0.625 N HCl at 0°C to a final concentration of 625 mM. $NaNO_2$ was added equimolar and the mixture was stirred at 0°C for 40 min. After the addition of 2.5 volumes of acetone stirring went on another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally three times with diethylether and was dried under vacuum. GSNO was characterized by UV-spectroscopy.

Statistical analysis Data are shown as means ± SD. The data are presented as percent induction compared with the unstimulated control (100%). Data were analyzed by unpaired Student's t test on raw data using Sigma Plot (Jandel Scientific, Erkrath, Germany).

RESULTS

Effects of L-NIL on wound nitrite production and gene expression To determine a possible function for NO during wound healing and tissue regeneration in the skin, we established an *in vivo* model in mice. During excisional repair, animals were treated with L-NIL, a selective inhibitor of iNOS enzymatic activity. Although it has been well described that L-NIL, used at concentrations of 2.5 mg in 0.5 ml PBS per injection for every 12 h, almost completely blocked the *in vivo* activity of iNOS in lymph nodes and skin in mice (Stenger *et al*, 1995), we had to demonstrate the inhibitory activity of the selective iNOS inhibitor in our experimental set-up. We decided to prove the inhibitory potency of L-NIL during wound repair at two different levels. First, we measured nitrite, a stable end product of nitric oxide generation, spectrophotometrically in wound fluids that have been cleared by ultracentrifugation ($100\,000 \times g$) using the Griess reaction (Green *et al*, 1982). Note that for every experimental time point eight wounds (n = 8) from four different animals have been pooled for nitrite determination. As shown in **Fig 1(*A*)**, we observed a strong reduction in wound nitrite concentrations at day 3 and day 5 after injury, clearly indicating an inhibitory effect of L-NIL on iNOS enzymatic activity at time points when iNOS levels are highest during repair (Frank *et al*, 1998; Reichner *et al*, 1999). As a second step, we decided to investigate the potential inhibitory activity of L-NIL during repair at the transcriptional level. For these reasons, we have chosen to investigate the expression of vascular endothelial growth factor (VEGF) during wound repair in L-NIL-treated mice, as we could demonstrate recently that VEGF is up-regulated during repair and represents an NO-inducible gene in keratinocytes (Frank *et al*, 1995, 1998). For this purpose, we isolated RNA from excisional full-thickness wounds at different intervals after wounding from L-NIL-treated mice and PBS-injected control mice and performed RNase protection assays. Sixteen wounds (n = 16) from the backs of four mice were excised for each time point and used for RNA isolation. Non-wounded back skin from the same area was used as a control. As shown in **Fig 1(*B*)**, two different protected fragments were obtained with RNA from normal and wounded skin, corresponding to different forms of VEGF mRNA. The longer protected fragment is generated by RNA encoding the murine $VEGF_{120}$, whereas the shorter protected fragment is generated by mRNA encoding murine $VEGF_{164}$ and $VEGF_{188}$. In PBS-injected control mice, a strong induction of VEGF mRNA expression was observed within 24 h after injury and highest VEGF mRNA levels were found between day 1 and 7 after injury. These data are consistent with previously published data (Frank *et al*, 1995); however, VEGF mRNA expression is severely altered in wounds isolated from L-NIL-treated mice. As shown in **Fig 1(*C*)**, the initial increase and sustained high level expression of VEGF mRNA found in PBS-injected

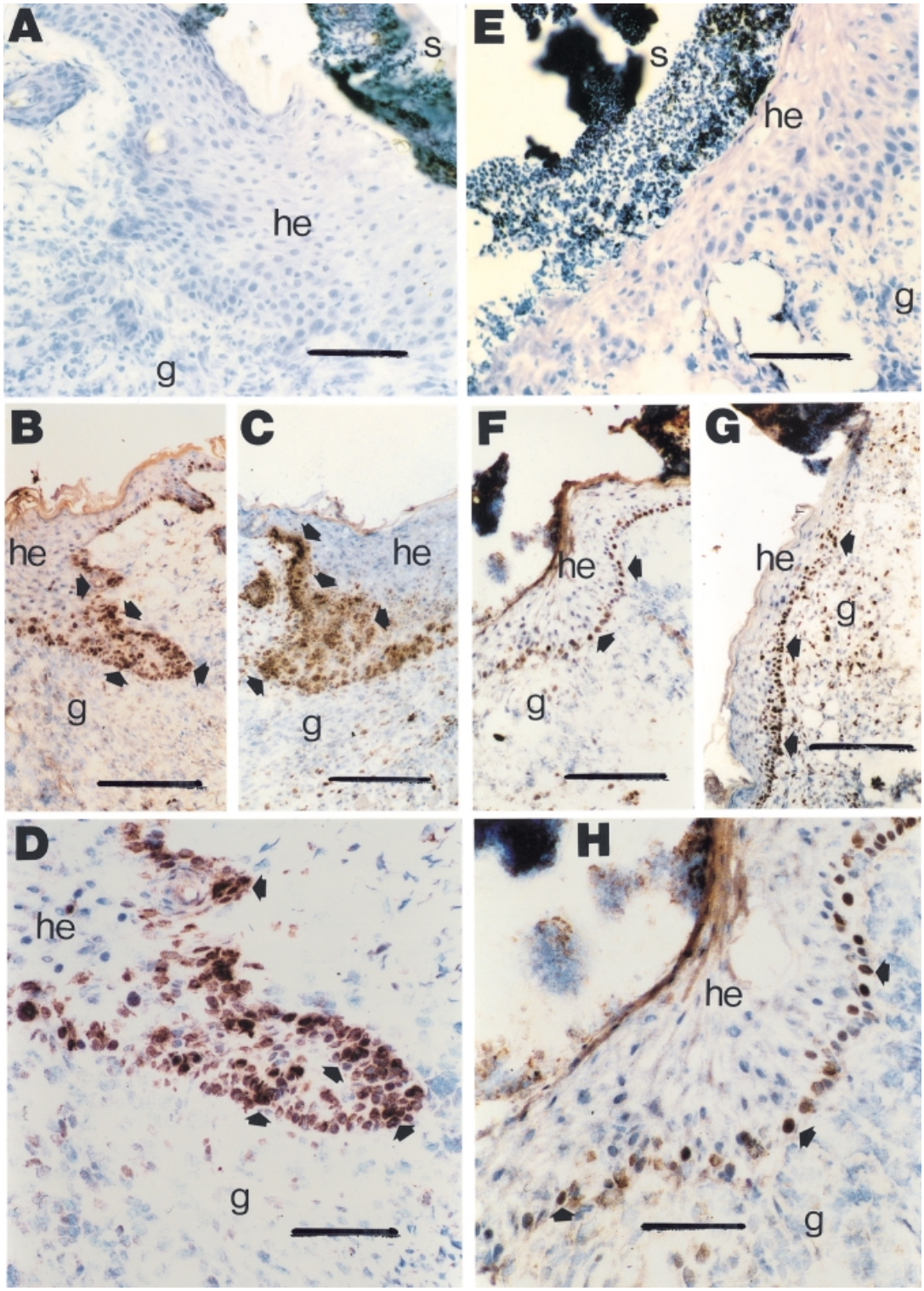

Figure 2. Epithelial proliferation in 5 d mouse wounds. (*A–D*; *left panels*) Frozen serial sections from 5 d wounds isolated from PBS-treated control mice; (*E–H*; *right panels*) frozen serial sections from 5 d wounds isolated from L-NIL-treated mice. (*A*, *E*) Frozen serial section from a 5 d wound hyperproliferative epithelium stained with eosin and hematoxylin. (*B*, *C*, *D*, *F*, *G*, *H*) Frozen serial sections were incubated with a monospecific, polyclonal antibody directed against murine Ki67 and stained with the avidin-biotin-peroxidase complex system using 3-amino-9-ethylcarbazole as a chromogenic substrate. Nuclei were counterstained with hematoxylin. *Scale bars*: (*B*, *C*, *F*, *G*) 200 μm; (*A*, *E*) 100 μm; (*D*, *H*) 50 μm. Strongly immunopositive signals within the sections are indicated with *arrows*. g, granulation tissue; he, hyperproliferative epithelium; s, scab.

control mice were markedly reduced (50%) in L-NIL-injected animals. Thus, L-NIL could be demonstrated to be effective in our experimental set-up, as we observed a, at least partial, reduction of iNOS activity as demonstrated by reduced nitrite levels and subsequent alteration of gene expression within the wound tissue.

Impaired reepithelialization in wounds of L-NIL-treated mice As a next step, we were interested in the morphology of wounds isolated from L-NIL-treated animals. For this purpose, we isolated wounds from L-NIL-treated mice and PBS-treated control animals 3 d and 5 d after injury. Remarkably, the first and most striking observation could be made already in hematoxylin and

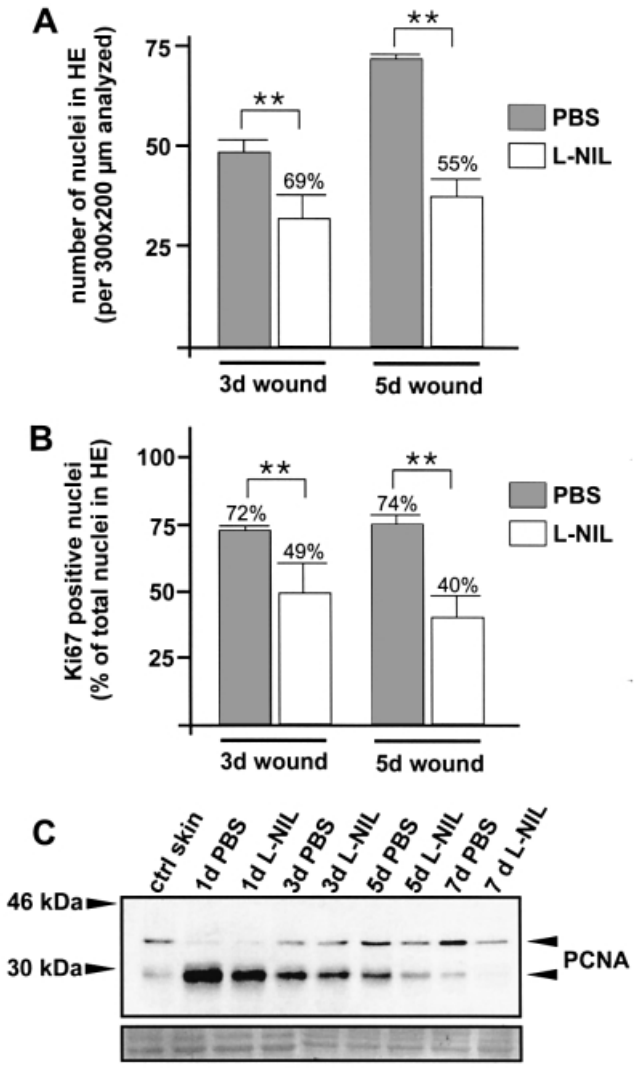

Figure 3. Keratinocyte proliferation in 3 d and 5 d wounds. (*A*) Low magnification photographs (100×) of frozen serial sections were analyzed for total keratinocyte cell numbers within the hyperproliferative epithelia (HE) from PBS-treated control mice and L-NIL-injected mice (n = 3) as indicated. This was done by counting hematoxylin-stained keratinocyte nuclei in a defined area (300 × 200 µm). Data are expressed as total number of keratinocyte nuclei ±SD (n = 3). **$p < 0.01$ as indicated by the brackets, per cent means compared with PBS-treated animals. (*B*) Low magnification photographs (100×) of frozen serial sections were analyzed for total proliferating keratinocyte cell numbers within the hyperproliferative epithelia (HE) from PBS-treated control mice and L-NIL-injected mice (n = 3) as indicated. This was done by counting Ki67-immunostained keratinocyte nuclei in a defined area (300 × 200 µm). Data are expressed as total number of Ki67-positive keratinocyte nuclei ±SD (n = 3). **$p < 0.01$ as indicated by the brackets. (*C*) PCNA protein expression in wound tissues from PBS-treated and L-NIL-treated mice. Twenty-five micrograms of total protein from lysates of nonwounded and wounded back skin from PBS- and L-NIL-treated mice, respectively (days 1, 3, 5, and 7 after injury) were analyzed by immunoblotting for the presence of PCNA protein. The time after injury and treatment of mice is indicated at the top of each lane. Reduced amounts of PCNA protein were detected in wound lysates from L-NIL-treated mice. PCNA specific bands are indicated by *arrows*.

eosin stained sections from wounds of L-NIL-treated mice. We observed that the formation of the hyperproliferative epithelium at the wound edges was disturbed in wounds from inhibitor-treated animals. This observation is pointed out in **Fig 2**(***A***, ***E***), showing a representative, hematoxylin and eosin-stained 5 d wound section isolated from a PBS-treated control animal (**Fig 2*A***) and a L-NIL-treated animal (**Fig 2*E***), respectively. The hyperproliferative epithelia from wounds of PBS-treated control mice were characterized by high numbers of keratinocytes, which formed a dense and highly organized mass of cells that spread out deeply into the wounded area (**Fig 2*A***, see also immunohistologic panels **2*B***, ***C***). In contrast to this physiologic situation of wound repair, we detected atrophied ''hyperproliferative epithelia'' in wounds from L-NIL-treated mice that were markedly reduced in size compared with the situation found in control animals (**Fig 2*E***). This was, in part, due to reduced keratinocyte cell numbers (**Fig 3*A***). Furthermore, keratinocytes did not form a compact cell mass, as the cells appeared to display a disorganized morphologic pattern within the atrophied hyperproliferative epithelium. Note that the reduced epithelium failed to spread out deeply into the wound bed, because the amorphic mass of keratinocytes was reduced to a flat cell tongue that covers the wound (see **Fig 2*E***, see also **2*F***, ***G***).

Thus, we speculated that inhibition of iNOS activity during wound repair might cause a dysregulation of keratinocyte proliferation, indicating an important role for NO for the process of wound reepithelialization. Therefore, we decided to investigate the proliferative behavior of keratinocytes during normal wound healing and wound healing in L-NIL-treated mice. A first line of evidence for a possible correlation between the presence of NO within the wound and keratinocyte proliferation was supported by immunohistochemistry of wounds isolated from BrdU-labeled animals. PBS- and L-NIL-injected mice were labeled *in vivo* using the nucleotide analog BrdU. BrdU is incorporated into DNA in place of thymidine during DNA synthesis, thus specifically labeling proliferating cells. Subsequently, monoclonal antibodies directed against BrdU were used in immunohistochemistry to specifically detect proliferating cells. As shown in **Fig 4**(***A***, ***D***), *in vivo*-labeling of 3 d wounds revealed a clear reduction in proliferating (that means BrdU-labeled nuclei) keratinocytes within the hyperproliferative epithelium of L-NIL-injected mice (**Fig 4*D***). Moreover, we used the Ki67 protein as a second cellular marker to investigate keratinocyte proliferation, as Ki67 is known to be specifically expressed in nuclei of proliferating cells during cell-cycle (Gerdes *et al*, 1984). We could observe comparable expression patterns for Ki67 positive cells in both 3 d and 5 d wounds, although the inhibitory effects of L-NIL on keratinocyte proliferation appeared to be enhanced within time during repair. As shown in **Fig 2** and **Fig 4**(***B***, ***C***, ***E***, ***F***) (*left panels*, wounds from PBS-injected mice; *right panels*, wounds from L-NIL-treated mice), we could observe a markedly reduced number of proliferating keratinocytes within the ''hyperproliferative epithelia'' after inhibition of iNOS activity in the wounds (**Fig 2*F*–*H***, **Fig 4*E***, ***F***). Note that the dense and massive epithelia, which are characterized by the intense spreading into the wound bed at the wound margins, are nearly completely built up from highly proliferative keratinocytes in the normal wound healing situation. In contrast, inhibition of iNOS enzymatic activity in the wound was clearly associated to reduced numbers of Ki67 positive, proliferating keratinocytes, and, hence, to a suppression of the well-known outgrowth of keratinocytes towards the granulation tissue.

Impaired reepithelialization after L-NIL treatment of mice is due to reduced keratinocyte proliferation As a next step, statistical analysis was performed on the immunohistochemical data obtained from the wound healing experiments. For this purpose, wound sections from three independent animals (n = 3 for PBS- and L-NIL-treated animals) were photographed at the same magnification (×100) and an equal square (300 × 200 µm) within the hyperproliferative epithelia was subsequently analyzed by counting total keratinocyte cell numbers, or Ki67 positive keratinocyte nuclei. Cell numbers were determined by counting hematoxylin-, or Ki67-stained keratinocyte nuclei after immunohistochemistry. As shown in **Fig 3**(***A***), we observed a 45% reduction in total keratinocyte cell numbers in the hyperproliferative epithelia isolated from L-NIL-treated mice. Moreover, inhibition of iNOS enzymatic activity during wound repair led to a markedly reduced number of proliferating keratinocytes during repair (**Fig 3*B***). Whereas the number of Ki67-stained keratinocytes was reduced by 23% in 3 d wounds

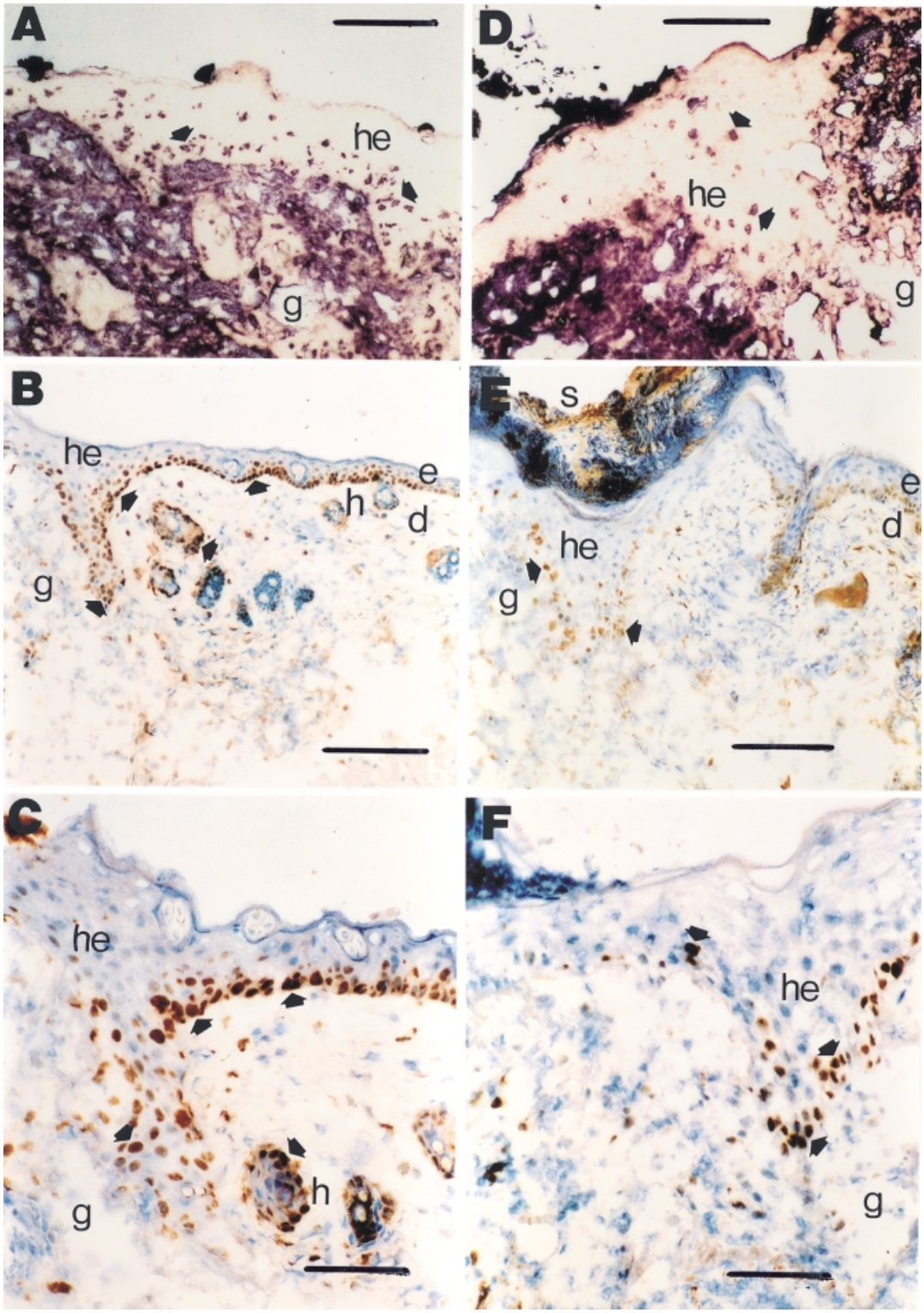

Figure 4. Epithelial proliferation in 3 d mouse wounds. (*A–C*; *left panels*) Frozen serial sections from 3 d wounds isolated from PBS-treated control mice, (*D–E*; *right panels*) frozen serial sections from 3 d wounds isolated from L-NIL-treated mice. (*A*, *D*) Frozen serial sections from BrdU-labeled animals were incubated with a mouse monoclonal antibody directed against BrdU and stained with an alkaline phosphatase system using NBT/X-phosphate as a chromogenic substrate. (*B*, *C*, *E*, *F*) Frozen serial sections were incubated with a monospecific, polyclonal antibody directed against murine Ki67 and stained with the avidin-biotin-peroxidase complex system using 3-amino-9-ethylcarbazole as a chromogenic substrate. Nuclei were counterstained with hematoxylin. *Scale bars*: (*B*, *E*) 200 μm; (*A*, *D*) 100 μm; (*C*, *F*) 50 μm. Strongly immunopositive signals within the sections are indicated with *arrows*. d, dermis; e, epidermis; g, granulation tissue; h, hair follicle; he, hyperproliferative epithelium; s, scab.

isolated from L-NIL-treated animals, we detected a further increase in reduction of proliferating cells (35%) after 5 d of repair. It is important to note that these data represent the percentage relation between total number of keratinocyte nuclei and number of Ki67-immunolabeled nuclei within the analyzed epithelial area. That means that, additionally, the absolute number of Ki67-stained, proliferating keratinocytes located within the analyzed epithelial area of L-NIL-injected mice was more clearly reduced, because

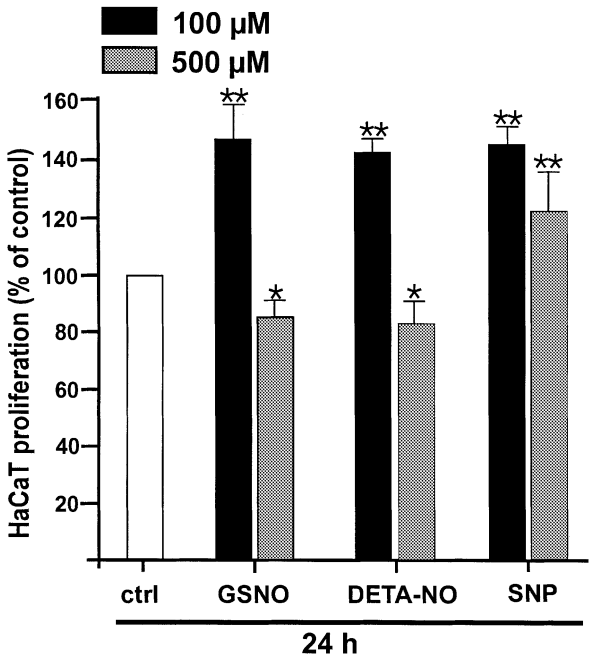

Figure 5. Biphasic effect of NO on keratinocyte (HaCaT) proliferation. Keratinocytes were seeded into 96 well plates using 2×10^3 cells in a total volume of 100 µl Dulbecco's modified Eagle's medium/10% fetal calf serum per well. After 24 h, fresh medium supplemented with different concentrations of NO-donating agents (GSNO, DETA-NO, SNP as indicated) was added. After an additional 24 h, 20 µl of MTS reagent per well was added. Generation of soluble formazan was used as direct readout for cell proliferation. Data are expressed as per cent of unstimulated control (100%). Mean per cent change in proliferation ±SD are shown (n = 6). *$p < 0.05$; **$p < 0.01$ compared with control.

total keratinocyte cell numbers were decreased by approximately 50% in wounds from L-NIL-injected mice (**Fig 3*A***).

Reduction of PCNA-specific proteins in total wound lysates of L-NIL-treated mice Finally, in order to further strengthen the observation that the presence of NO during wound healing might be directly correlated to the proliferation rate of keratinocytes during reepithelialization, we analyzed total wound lysates (day 1, 3, 5, and 7 after injury) from PBS- and L-NIL injected mice for the presence of PCNA-specific protein, also known as cyclin. This protein is well known to be expressed in early G1 and S phases of the cell cycle (Woods *et al*, 1991). As shown in **Fig 3(*C*)**, the antibody specifically detected two specific bands in the western blot analysis of total wound lysates from PBS- and L-NIL-injected animals, with molecular weights of approximately 30 and 36 kDa. The appearance of two PCNA-specific bands might be due to the presence of two basic forms of PCNA protein, a soluble form sensitive to organic fixation and not involved in replication, and a second form that is insoluble and associated with ongoing protein synthesis (Bravo and MacDonald-Bravo, 1987; Woods *et al*, 1991). The immunoblot against PCNA-specific protein strongly supported the observation of reduced keratinocyte proliferation during reepithelialization after inhibitor treatment of mice, as the total amount of PCNA-specific protein was clearly reduced in wound lysates from L-NIL-treated mice compared with PBS-injected control animals. As we had observed the majority of cellular proliferation to be present in the epithelium, and not in the granulation tissue (see Ki67-staining of 3 d and 5 d wound in **Fig 4** and **Fig 2**), the reduced amounts of PCNA-specific protein in wound lsyates of L-NIL-treated mice are most likely to be due to the reduced cellular proliferation seen for keratinocytes in these mice.

Biphasic effect of NO on keratinocyte (HaCaT) proliferation To further support our *in vivo* observation of a possible correlation between NO and keratinocyte proliferation, we investigated the effects of low (100 µM) and high (500 µM) concentrations of NO-donating agents on the proliferation of the stable keratinocyte cell line HaCaT (Boukamp *et al*, 1988) *in vitro*. As shown in **Fig 5**, we observed a markedly increased proliferation rate of cells (about 40%) incubated at low concentrations (100 µM) of GSNO, DETA-NO, or SNP, which is independent of the NO-donor used. In contrast, high concentrations of NO reduced keratinocyte proliferation. For SNP, a cytostatic effect could be observed for concentrations higher than 500 µM (data not shown), therefore indicating that the behavior of cells depends, at least partially, on the NO donor used. Taken together, the data obtained from the *in vitro* experiments clearly support the *in vivo* relevance of NO for keratinocyte proliferation observed during wound repair *in vivo*.

DISCUSSION

Over the last years, an increasing number of studies have pointed out a role for nitric oxide synthase (NOS) pathways for the regulation of skin homeostasis and function. An improved understanding of NO regulatory roles has been a direct consequence from the identification of various cell types in the skin to be involved in the production of NO. The homeostatic activities of NO in human skin include regulation of vasodilatation, melanogenesis, and protection against invading pathogens (Goldsmith *et al*, 1996; Romero-Graillet *et al*, 1996, 1997; Weller *et al*, 1996). Accordingly, many studies have implicated a participation of NO for the pathogenesis of several skin diseases via dysregulation of NOS expression. Besides inflammatory dermatoses (Rowe *et al*, 1997), iNOS has been shown to be overexpressed in psoriatic skin lesions (Kolb-Bachofen *et al*, 1994; Bruch-Gerharz *et al*, 1996; Ormerod *et al*, 1998), indicating a function for NO in the progression of psoriasis, a chronic inflammatory skin disease that is characterized by hyperproliferation and incomplete differentiation of epidermal keratinocytes in the involved lesions. Moreover, very recent studies even demonstrated a crucial role for the iNOS in the process of tissue regeneration during cutaneous wound healing. Tissue movements of inflammation, granulation tissue formation, and reepithelialization are accompanied by a large induction of iNOS during repair (Frank *et al*, 1998), and, in line with these observations, iNOS-deficient mice are characterized by a delayed cutaneous wound closure after injury (Yamasaki *et al*, 1998). The possibility that iNOS-derived NO is indeed a key player during tissue regeneration is convincingly demonstrated by reversal of the wound healing defects observed in iNOS-deficient mice by a single application of an adenoviral vector system mediating a human iNOS cDNA into the wounded area (Yamasaki *et al*, 1998). Although a crucial role for NO during tissue repair becomes evident, the molecular and physiologic basis of NO action, however, remains nearly completely unknown.

Notably, we observed a particularly strong expression of iNOS in the basal keratinocytes of the epidermis adjacent to the wound and of the hyperproliferative epithelium at the wound edge during repair, whereas iNOS was less abundant in the suprabasal keratinocytes (Frank *et al*, 1998). That means, interestingly, that iNOS expression is closely located and targeted to those sites, where keratinocyte proliferation has to be regulated during reepithelialization. To this end, one has to consider another situation, where iNOS is described to be involved in keratinocyte proliferation, although this phenomenon was observed for pathologic conditions associated to the inflammatory skin disease psoriasis. *In situ* hybridization of psoriatic lesions against iNOS revealed keratinocytes of the highly proliferative basal epidermal layer at the tips of the rete ridges to express highest amounts of iNOS mRNA (Bruch-Gerharz *et al*, 1996). Obviously, these data strongly implicate a close association, or, at least, participation of the small radical-type mediator NO to keratinocyte proliferating and differentiating processes.

In vitro studies using primary keratinocytes clearly strengthen a functional correlation between the availability of NO and a stimulation of keratinocyte proliferation (Clark *et al*, 1997; Krischel *et al*, 1998; Vallette *et al*, 1998). Moreover, because NO has been shown to act as a negative regulator of cellular growth by either inhibition of cellular growth or induction of terminal differentiation for a variety of different cells, like liver cells, endothelial cells, or neurons (Feder and Laskin, 1994; Peunova and Enikopolov, 1995; Kawada *et al*, 1998), a pro-proliferative function might be unique to epidermal keratinocytes. Exogenously applied NO mediated a biphasic effect on proliferation and differentiation to primary skin-derived keratinocytes, but not fibroblasts *in vitro*. Primary keratinocytes exposed to low concentrations of NO donors (<0.25 mM) in the cell culture medium increased proliferation, whereas high concentrations of NO donors (<0.5 mM) mediated a cytostatic effect on the cells. Accordingly, expression of the keratinocyte-specific differentiation marker keratin 6 decreased at lower, but increased at higher concentrations of the applied NO donors, clearly indicating an induction of keratinocyte differentiation at higher NO concentrations (Krischel *et al*, 1998). Thus, one might speculate that the potency of NO action on keratinocytes is somewhat janus-like, as the same player dose-dependently mediates opposite effects within the process of keratinocyte proliferation and differentiation. Furthermore, NO is most likely to also mediate cell migration, as migratory activities of epithelial and endothelial cells as well as neutrophils could be stimulated by NO *in vitro* (Beauvais *et al*, 1995; Noiri *et al*, 1996, 1997).

But what about the situation of wound repair? The process of keratinocyte proliferation and differentiation represents one central process during tissue regeneration after cutaneous injury. Reepithelialization of the wounded area is driven by highly proliferative keratinocytes, which start to proliferate from the cut edges of the injured tissue and subsequently migrate into the wound bed. This highly dynamic process finally leads to the formation of a massive bulk of proliferating keratinocytes, the hyperproliferative epithelium, which serves as a cellular source to drive the epithelial tongue to cover the wounded area (Clark, 1996; Martin, 1997). Obviously, reepithelialization, essentially occurring in a close spatial and functional connection to the underlying granulation tissue, is central to wound closure. Therefore, formation of new epithelial structures has to be tightly controlled. Key players driving these processes are protein-type cytokines, growth factors, and mitogens (Moulin, 1995; Martin, 1997). Among these factors, one is well known to be unique for the epithelial repair process: keratinocyte growth factor (KGF), which represents a potent mitogen for epithelial cells (Rubin *et al*, 1989; Werner, 1998). KGF is largely induced in dermal fibroblasts early during wound repair and shows highly elevated levels during the process of reepithelialization. Accordingly, the KGF receptor is found to be expressed in proliferating wound keratinocytes, thus suggesting a paracrine mode of action (Werner *et al*, 1992). But how is the wound situation altered when function of this well-characterized key player is inhibited? Reepithelialization is severely impaired. Targeting a dominant-negative KGF receptor into stratified squamous epithelia using a keratin 14 promoter, finally led to reduction of keratinocyte proliferation rates, and subsequently to a delayed reepithelialization in these animals (Werner *et al*, 1994). For these reasons, it was exciting for us to observe comparable and severe defects in wound reepithelialization after inhibition of iNOS enzymatic activity. Comparable with the functions mediated by KGF *in vivo*, wound depletion for NO led to a disturbed migration of keratinocytes into the wound, a markedly reduced proliferation of keratinocytes, and, subsequently, a nearly completely suppressed formation of hyperproliferative epithelia, which are characterized by an atrophied morphology. Thus, NO might represent a potent mediator during tissue regeneration, which develops its actions independent from the presence of protein-type mitogens. This is supported by the observation that treatment of animals with L-NIL did not result in a reduced expression of KGF during repair (S. Frank, unpublished observation). Moreover, it is remarkable that NO, during repair, is nearly as potent in regulation of keratinocyte growth during reepithelialization as the well-investigated keratinocyte-specific mitogen KGF. An obvious correlation between NO and keratinocyte proliferation *in vivo* is further supported by the observation that wound repair in UV-irradiated, photodamaged skin revealed a reduced immunostaining for proliferating cell nuclear antigen (PCNA) in keratinocytes after local application of the NOS inhibitor N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Benrath *et al*, 1995). Noteably, a vectorial NO release is discussed to be essential for the spatial and temporal coordination of locomoting epithelial cells. It is conceivable that NO might represent a cellular switch that changes the stationary to the locomoting epithelial phenotype (Noiri *et al*, 1996). Accordingly, a reduction of keratinocyte migration in L-NIL-treated mice might further contribute to the impaired reepithelialization process that occurs after inhibition of iNOS in these animals. Thus, the observed defects in epithelial regeneration processes after inhibition of iNOS enzymatic activity during repair are most likely to be involved in the delayed wound closure described for iNOS-deficient mice (Yamasaki *et al*, 1998). Moreover, our data might provide an explanation for the beneficial effects of exogenously added NO-donating agents into wounds (Shabani *et al*, 1996). In this study, we have identified NO as a potent mediator of keratinocyte proliferation during wound healing *in vivo*. Therefore, this study extends the list of mediators that are known to be central to the process of tissue regeneration during wound repair for an additional factor: the gaseous radical NO. Because depletion of NO from the wound is accompanied by an impaired repithelialization, one has to consider NO, besides the well-investigated protein-type factors and mitogens, as a novel therapeutical target to improve severely disturbed wound healing conditions.

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