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Nitric oxide drives skin repair: Novel functions of an established mediator

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Nitric oxide drives skin repair: Novel functions of an established mediator. Wound healing of the skin represents a highly ordered process of important tissue movements that aims for a rapid closure of the wound site and a subsequent regeneration of the injured tissue. The factors ensuring the intercellular communication during repair are only known in part. However, although protein-type mediators are well-established players in this process, it has become evident that the diffusible, gaseous molecule nitric oxide (NO) participates in the orchestration of wound healing. The role of wound-derived NO that critically influences macrophage, fibroblast, and keratinocyte behaviour within the intercellular communication network during repair is subject of this review. Thus, cutaneous wound healing prototypically reflects processes that generally occur also in kidney injury and regeneration.

To overcome tissue damage, the process of cutaneous wound repair represents a highly ordered process that is characterized by temporally and spatially overlapping phases of tissue movements comprising hemorrhage, inflammation, re-epithelialization, granulation tissue formation, and the late remodeling phase of repair. As a result, the integrity of the body's protective layer is maintained, although repair fails to perfectly replace the original skin tissue. Loss of a functional healing process might lead to severe disabilities. Accordingly, chronic, non-healing wound conditions represent a situation of major clinical importance. A series of pathological changes accompanied with several diseases finally leads to severely disturbed wound healing conditions. Among those, the most prominent chronic wound situations are known as decubitus or pressure ulcers, venous ulcers, and diabetic ulcers [1]. Molecular and cellular biology and the use of different model systems, especially including genetically engineered animals, have greatly extended our knowledge on skin repair. Nevertheless, the most promising discoveries of growth factor actions for repair to date could not be successfully translated with respect to clinical outcome. To this end, our search for novel mediators has to proceed with the intent to develop novel concepts to improve healing of chronic skin wounds.

In most cases, skin injury results in the extravasation of blood constituents from damaged vessels. Blood coagulation then results from a cascade of events that are closely associated to platelet aggregation at the wound site. As a result, a clot is generated that covers the wounded area, plugs the injured vessels, represents a provisional matrix for infiltrating cells, and serves as a reservoir of chemotactic mediators, cytokines, and growth factors that initiate and promote early tissue movements [2–4].

Polymorphonuclear neutrophils (PMN) and monocytes represent the first cells that arrive at the wounded area. Both leukocytic cell types are attracted from the circulation by a variety of chemotactic factors. Among those, the CXC-chemokines and CC-chemokines represent potent attractors for both PMN and monocytes, respectively, within the process of skin repair in mice and humans [5]. Once they arrive at the site of injury, PMN process extracellular matrix (ECM) components, decontaminate bacteria, and produce cytokines. Arriving monocytes differentiate into mononuclear phagocytes at the wound site. Activated macrophages are central to wound repair, as they amplify the initial inflammatory response by release of a variety of signaling molecules, and, moreover, provide growth factors for fibroblast and endothelial cell proliferation and contribute to ECM degradation [2–4, 6].

After a short lag period of several hours after injury, the opening phase of re-epithelialization is marked by the first keratinocytes of the cut edges starting to migrate. Keratinocytes were guided into the wound by fibronectin and fibrin bundles in the provisional clot, which are sensed by the cells by expression of highly controlled integrin receptor subsets [2]. Migrating keratinocytes do not differentiate, and the leading edge keratinocytes express constituents of a very active fibrinolytic machinery such as plasminogen activators and matrix metalloproteinases (MMP) [3]. Keratinocytes at the wound margins

Key words: wound repair, cutaneous wound, immune response, injury, re-epithelialization.

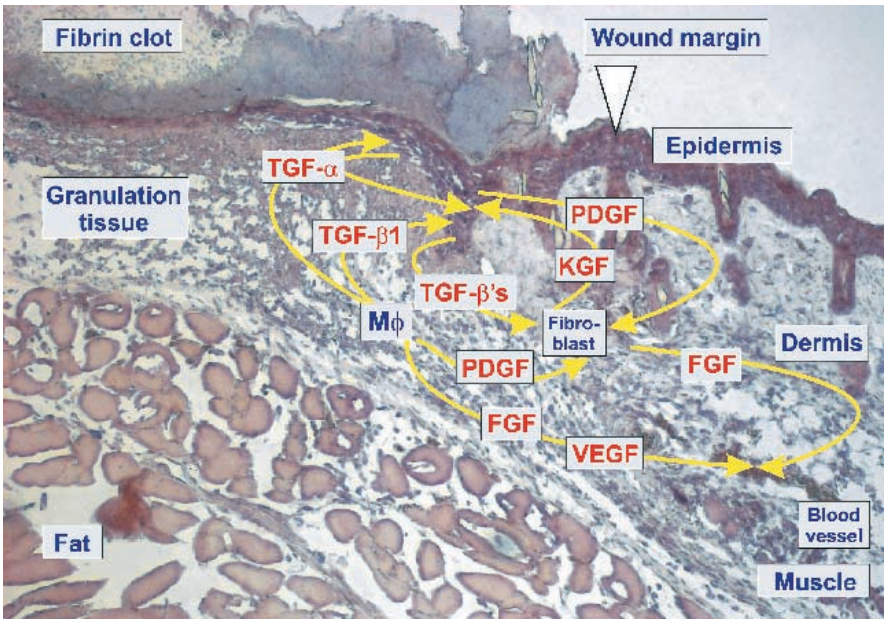

Fig. 1. Intercellular communication network in a skin wound three days after injury. Protein-type growth factors are well established to orchestrate cell movements at the wound site. Abbreviations are: FGF, fibroblast growth factor; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

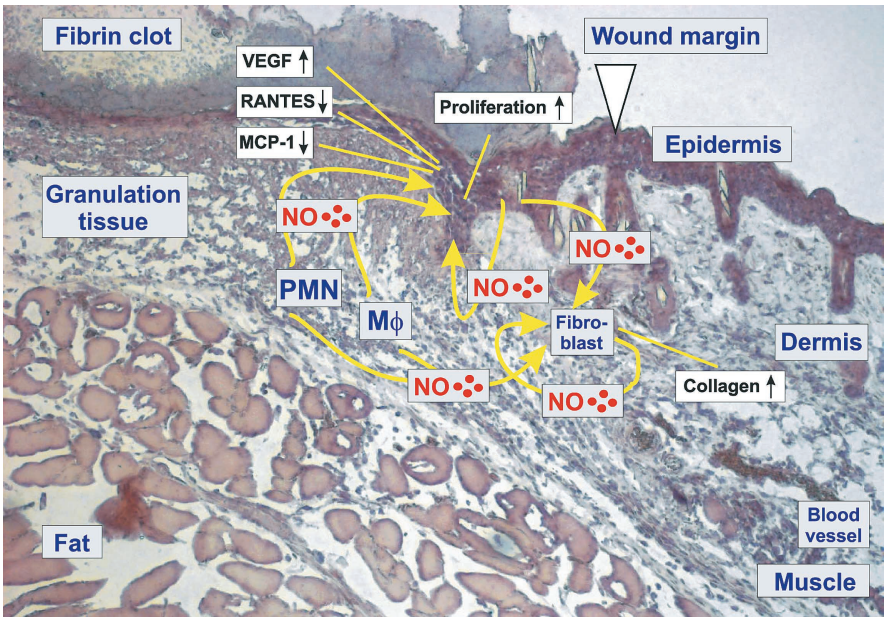

Fig. 2. Nitric oxide (NO) as a signaling molecule at the wound site. The gaseous molecule NO serves as a mediator that regulates gene expression and proliferation in keratinocytes, and collagen synthesis in fibroblasts. Abbreviations are: NO, nitric oxide; MCP-1, macrophage chemoattractant protein-1; RANTES, regulated upon activation, normal T-cell expressed and secreted; VEGF, vascular endothelial growth factor.

start to proliferate one or two days after injury. Re-epithelialization is closely controlled by growth factors, predominantly by members of the epidermal growth factor (EGF)- [3], and fibroblast growth factor (FGF)- [7] family of growth factors. However, unexpected mediators such as the cytokine leptin [8] or the small gaseous molecule nitric oxide (NO) [9], as reviewed below, crucially contribute to cutaneous re-epithelialization also.

Formation of the granulation tissue starts about three to four days after injury (Figs. 1 and 2) [2–4]. The main cell types driving the generation of new stroma are macrophages, fibroblasts, and endothelial cells. Interaction of these cell types leads to deposition of newly synthesized connective tissue and ingrowth of new blood vessels. Wound fibroblasts own three main capacities: proliferation, migration and production of ECM. Additionally, fibroblasts contribute to the "growth factor cocktail" by secreting important mediators such as keratinocyte growth factor (KGF), connective tissue growth factor (CTGF), or activin. To make their way into the wound site, migratory fibroblasts express a series of proteolytic enzymes (MMP-1, -2, -3). As repair progresses, the mi-

gratory phenotype is subsequently changed into a profibrotic one, and fibroblasts start to deposit collagen, a process that is potently regulated by the action of transforming growth factor-β1 (TGF-β1). A proportion of wound fibroblasts specializes into myofibroblasts, which finally decrease the overall wound size by contracting the granulation tissue. Neovascularization has to accompany granulation tissue formation, as the newly forming cellular complex must be supplied with oxygen and nutrients. Outgrowth of endothelial cells from pre-existing vessels at the wound margins is triggered by a series of angiogenic factors that activate endothelial cell proteases that are crucial for the cells to transmigrate the basement membrane and infiltrate the wound. Especially vascular endothelial growth factor (VEGF), which is expressed in large amounts by epidermal cells during repair, regulates endothelial cell action at the wound site. Finally, the late phase of granulation arises when wound fibroblasts undergo apoptosis, and, thus, mark the transition from a cell-rich granulation tissue to a more or less acellular scar.

NITRIC OXIDE AND SKIN

In mammals, the synthesis of nitric oxide (NO) is catalyzed by NO synthase (NOS) that exists in three distinct isoforms [10]. NOS catalyzes the oxidation of the amino acid L-arginine to finally release citrulline and NO. Molecular cloning has revealed the existence of three different NOS isoforms: the constitutively expressed neuronal (nNOS) and endothelial (eNOS) NOS isoforms, and the inducible NOS (iNOS) isoenzyme, respectively [10, 11]. All three NOS isoenzymes are homodimeric proteins that depend on nicotinamide adenine dinucleotide phosphate (NADPH), reduced flavins, heme-bound iron, and 6(R) 5,6,7,8-tetrahydrobiopterin as essential cofactors. The constitutively expressed NOS isoforms are activated by increasing Ca^{2+} levels via calmodulin. It is well established that all three NOS isoforms are expressed in skin tissue. Expression of nNOS has been observed in keratinocytes and melanocytes [12, 13], expression of eNOS could be detected in keratinocytes of the basal epidermal layer, dermal fibroblasts, endothelial capillaries and eccrine glands [14, 15], whereas iNOS can be induced in keratinocytes [16, 17], fibroblasts [15], Langerhans [18] and endothelial cells [19]. It is now reasonable to assume that NO participates in the regulation of skin homeostatic functions such as circulation, UVB-mediated melanogenesis, sunburn erythema, and the maintenance of the protective barrier against microorgansims [20]. In line, NO restricts pathogen growth after cutaneous infection [21]. In general, the presence of iNOS is associated with pathological conditions, a situation that also is true of skin tissue. Accordingly, the expression of iNOS has been described for inflammatory diseases of the skin, including psoriasis, lupus erythematosus, inflammatory dermatoses, and hypersensitivity reactions [20].

L-ARGININE AND WOUND HEALING

Wound healing of the skin is a highly coordinated process, finally leading to an at least partial reconstruction of the injured tissue. The factors mediating the intercellular communication during wound repair are known in part, but their number is still increasing. Proinflammatory cytokines and various peptide growth factors are known to be key players in this process [3]. However, these protein-type factors are not unique in regulating cellular behavior in wound repair, and evidence is emerging for an important role of small diffusible molecules in wound repair. One of them is NO, a free radical gas, which has become one of the most studied molecules in biomedical sciences during the past years.

It has long been known that the amino acid L-arginine, representing the only substrate for NOS enzymatic activity, is implicated in wound healing. An initial study from nearly 25 years ago demonstrated the influence of L-arginine supplementation on healing of wounds in a rat model of incisional repair [22]. Arginine-deficient animals showed an impaired healing, as assessed by markedly decreased wound breaking strength and collagen deposition, which was thought to be associated with an L-arginine–induced growth hormone release [22]. A few years later, Albina and colleagues determined enzymatic activities of the oxidative L-arginine deiminase (OAD) and arginase in wound fluids isolated from implanted sponges in the rat [23]. OAD catalyzes the reaction of L-arginine into citrulline and nitrogen intermediates and, thus, clearly resembles the more recently identified NOS. In this model of repair, wound fluids were characterized by low arginine levels, which directly implicate a consumption of L-arginine at the wound site. Moreover, citrulline and nitrite, products of the OAD or NOS activity, respectively, peaked within the early, inflammatory phase of healing [23, 24]. L-Arginine levels remained low, as the amino acid is subject to the catalytic activity of arginase later in repair, releasing measurable amounts of ornithine into the wound fluid [23]. However, the potency of L-arginine to improve wound healing must be tightly linked to both the NOS and arginase enzyme systems, as the loss of a functional iNOS gene in iNOS-deficient mice abrogates the beneficial effects of L-arginine in wound healing [25]. Consistently, the beneficial effects of L-arginine observed in animal models also were confirmed in humans. Clinical studies demonstrated an enhanced wound healing in L-arginine supplemented patients that was characterized by a markedly increased wound collagen deposition [26, 27].

SPECIFIC ROLES OF iNOS AND eNOS ISOENZYMES IN WOUND HEALING

Although an important role for NO in skin repair has been well defined within the last decade, the specific roles of distinct NOS isoenzymes for wound-derived NO production could only be addressed when NOS-deficient knockout mice or NOS isoenzyme-specific inhibitors became available. Nevertheless, utilization of wound healing models in either normal or transgenic animals, or of specific iNOS inhibitors, respectively, predominantly revealed the first insights in the regulation and possible roles of the iNOS isoform during repair.

Data obtained from iNOS-deficient mice clearly indicated an important role for iNOS-derived NO in wound healing. The average time of wound closure was significantly delayed in the knockout animals in a model of full-thickness excisional wounding. The disparity between normal and knockout mice upon injury became greater with time finally resulting in an average delay of 31% in iNOS-deficient animals [28]. Inhibition of iNOS enzymatic activity during repair in wild-type mice using N^6-(iminoethyl)-L-lysine (L-NIL) resulted in a similar delay in complete wound closure as compared to iNOS kockout mice. More important, functional evidence for iNOS-derived NO in skin repair was evidenced by an adenoviral-mediated expression of a human iNOS cDNA at wound sites in iNOS-deficient mice, which led to an accelerated wound closure that was comparable to healing in control animals [28]. These data were consistent with the induction of iNOS expression upon cutaneous injury in a murine model of normal healing [29]. iNOS levels remained elevated during the inflammatory phase of repair with infiltrating PMN and macrophages as one major source of iNOS expression [29, 30]. Interestingly, iNOS expression was not restricted to immune cells, as proliferating keratinocytes of the wound margins, and to a smaller extent also wound fibroblasts expressed iNOS [29]. In line with data obtained from iNOS knockout mice [28], a glucocorticoid-mediated delay in wound closure in wild-type animals was associated with a markedly reduced presence of iNOS at the wound site [29]. For the first time, application of an iNOS inhibitory substance, L-NIL, during wound healing revealed an important role of iNOS in regulation of epithelial tissue movements [9]. L-NIL is now established as a partially selective inhibitor of iNOS, with a 50-fold selectivity for iNOS versus eNOS, or 20-fold selectivity versus nNOS, respectively [10]. Compared with control mice, L-NIL-treated animals were characterized by a severely impaired re-epithelialization process, as the hyperproliferative epithelia at the wound edges appeared to be delayed and characterized by an atrophied morphology. Moreover, proliferating keratinocyte cell numbers were strongly reduced during re-epithelialization after inhibition of iNOS during repair [9].

To date, information dealing with an in vivo function of eNOS-derived NO in skin repair is clearly restricted. However, eNOS also contributes to wound-related NO production, as eNOS knockout mice were characterized by an even more pronounced delay in wound closure compared to iNOS-deficient animals as assessed by the same model of full-thickness skin wounding [31]. In parallel to the delayed wound closure, the tensile strength of the regenerating tissue was markedly reduced in eNOS knockout mice [31], indicating an important role for eNOS-derived NO for ECM production (see the section, **Nitric oxide and wound fibroblasts**). The study suggests defects in angiogenic processes during repair that are associated with the absence of eNOS. Thus, in vitro and in vivo experiments implicated that eNOS contributes to granulation tissue formation by triggering endothelial migration, proliferation and differentiation and, thus, it participates in capillary ingrowth into the wound site during repair [31].

NITRIC OXIDE AND WOUND MACROPHAGES

Interestingly, the observed wound-related OAD/NOS activity was exclusively connected to viable macrophages at the wound site in early studies [23]. More recent data confirmed the wound macrophage as a prominent source of iNOS expression in the early inflammatory phase of repair [30]. This observation might relate to findings that define sites of inflammation with prominent macrophage infiltration as uniquely deficient in L-arginine. Consistently, the L-arginine metabolism via NOS and arginase appears to be closely counter-regulated in macrophages. Factors increasing macrophage iNOS activity, such as interferon-γ (IFN-γ) or bacterial lipopolysaccharide (LPS), decrease arginase activity in the cells, whereas transforming growth factor-β1 (TGF-β1)–mediated iNOS inhibition resulted in increasing ornithine levels [32]. This hypothesis is supported by yet unpublished observations from our laboratory, where chronic, non-healing wound situations in diabetic *db/db* mice were characterized by tremendous numbers of macrophages at the wound site that were associated with a lack of iNOS expression in the presence of an overshooting expression of arginase (S. Frank, H. Kämpfer, unpublished observations).

NITRIC OXIDE AND WOUND FIBROBLASTS

In vitro studies have convincingly demonstrated that human skin-derived fibroblasts spontaneously possess the capacity to produce NO that is either linked to the Ca^{2+}-dependent eNOS isoenzyme and—after stimulation of the cells with IFN-γ and LPS—to the iNOS isoform [15]. Independent from the cellular source it derives from during repair, NO predominantly regulates collagen synthesis in wound fibroblasts, the cell type that

represents a central player in the formation of new stroma [2–4]. Most of the data dealing with the role of NO for the dermal compartment derive from a well established wound healing model that utilizes implanted polyvinyl alcohol sponges that are implanted into subcutaneous pockets after dorsal incision of the skin [24]. NOS enzymatic activities could be clearly demonstrated using this model system, as cells infiltrating the sponge structures were responsible for the formation of nitrite and nitrate in the wound fluids [33]. Besides a large number of wound macrophages [24], wound fibroblasts also invade the implanted spongy structures [34]. Consistently in all studies, the inhibition of wound NO synthesis was paralleled by a lowered wound breaking strength and a markedly decreased collagen deposition [33–35]. These data suggest that NO synthesis is critical to wound collagen accumulation and acquisition of wound mechanical strength. Accordingly, sponge-isolated wound fibroblasts express iNOS and produce NO in vitro, and inhibition of iNOS significantly decreased collagen synthesis [34]. Moreover, transfection of these sponges with an iNOS cDNA resulted in a marked increase in collagen deposition in vivo [36], and NO-donating agents enhanced fibroblast collagen synthesis in vitro [37]. These studies again demonstrate that increased iNOS expression precedes an increase in fibroblast collagen synthesis.

In general, fibroblasts are highly resistant to NO-induced necrosis and apoptosis [38]. Nevertheless, NO is most likely to mediate an antiproliferative effect on skin fibroblasts. The increased mitogenic potency of hypertrophic scar fibroblasts was clearly associated with reduced eNOS expression [39], and fibroblast cytostasis can be mediated in vitro using NO-donating agents [37, 38]. Interestingly, NO-mediated cytostasis and collagen synthesis might be coupled, as NO-mediated cytostasis is associated with a net increase of collagen synthesis by the cells [37].

NITRIC OXIDE AND WOUND KERATINOCYTES

Skin keratinocytes are capable of expressing all three NOS isoforms [12–14, 16, 17]. Upon cutaneous injury, proliferating keratinocytes of the wound margins have been shown to strongly express iNOS [29]. Thus, it was reasonable to hypothesize that NO might be implicated in the control of keratinocyte proliferation. About a decade ago, a first line of evidence that argues for NO as a regulator of keratinocyte mitogenicity came from a study demonstrating that inhibition of keratinocyte proliferation by inflammatory cytokines could be overcome by NOS inhibitors or EGF, both of which blunt NO production in the cells [40]. Moreover, in vitro studies using NO-donating agents have convincingly shown that keratinocytes respond to NO in a biphasic manner with increased proliferation at low and cytostasis associated with differentiation at high NO concentrations [38], a regulatory phenomenon that might be directly implicated in keratinocyte behavior during wound healing. Remarkably the mitogenic capacity of NO on keratinocytes appeared to result from its interference with intracellular superoxide levels [41, 42]. Most important, the mitogenic potency of NO for the keratinocyte as a target cell could be proven in different models of wound repair, as inhibition of NOS enzymatic activity resulted in decreased epithelial proliferation during repair of photodamaged skin [43], of skin excisions [9], and also of burn wounds [44].

Obviously, there is evidence for a close correlation between NO-mediated keratinocyte proliferation and keratinocyte gene expression during skin repair in vivo. Exogenously added NO is capable of inducing the expression of important mediators of healing such as the angiogenic VEGF [45] and the PMN-attracting CXC-chemokine IL-8 [46] in HaCaT keratinocytes. Basal expression levels of the CC-chemokine macrophage chemoattractant protein-1 (MCP-1) were simultaneously reduced [46]. Remarkably, when keratinocytes were exposed to exogenously added or endogenously produced NO in the presence of cytokines, the cells completely change their expression patterns, as cytokine-induced expression of VEGF and IL-8 was enhanced [45, 46], whereas cytokine-triggered monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated upon activation, normal T cell expressed and secreted) expression levels were markedly reduced [46, 47]. It is reasonable to hypothesize that wound keratinocytes might respond to NO at the wound site in a similar manner, as keratinoycte-derived VEGF, MCP-1 and also RANTES expression have been shown to be under the regulatory control of iNOS-released NO during wound healing in vivo [45–47].

PERSPECTIVES

Although a central role for protein-type growth factors and mitogens during wound repair has been well known for years, the application of these factors in treatment of wound healing disorders did not provide a breakthrough in clinical studies. One possible explanation for the failure of protein-type mitogens to markedly accelerate closure of chronic wounds may be due tremendously elevated protease activities in the wound fluids of chronic wounds [48]. This finding implicates that the molecular environment of chronic wounds may impair the ability of endogenous and exogenously applied growth factors to stimulate healing. In this context, NO might represent a novel target molecule that circumvents the difficulties that arise due to proteolytic cleavage of therapeutically applied growth factors in chronic wound situations. The

small and short-lived radical molecule NO represents a potent mitogen for keratinocytes (comparable to the mitogenic activities of EGF, or KGF) during wound healing in vivo [9], a property that might be applied beneficially to improve the re-epithelialization of chronic wounds. Moreover, data from our laboratory implicate a capacity for NO that is most likely unique to this small molecule, and that makes it distinguishable from protein-type growth factors: NO clearly modulates keratinocyte responses to extracellular stimuli. Thus, mitogen-, or cytokine-triggered gene expression in the cells can be dramatically altered by NO, indicating that a nitric oxide-mediated "pre-adjustment" of the keratinocyte is crucial for its cellular response [45–47]. A closely controlled and therefore homeostatic concentration of NO in the wound fluid during repair is most likely to adjust the wound keratinocytes in the sense of "fine-tuning" the cells to the available external stimuli. Accordingly, one would expect that disturbed wound healing conditions could be improved by application of exogenously added NO-donating agents, as impaired repair is associated with a reduced availability of NO [49–51]. Promising results have been obtained from preliminary studies using non-soluble, polymeric NONOates as NO-donating agents during cutaneous healing in rats [52]. In addition to the well-investigated protein-type factors and mitogens, NO may provide a novel therapeutical target to improve severely disturbed wound healing conditions.

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