Reactive Oxygen Species and Their Detoxification in Healing Skin Wounds

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Injury to the skin initiates a cascade of events, which finally lead to at least partial reconstruction of the wounded tissue. The wound-healing process has been well described at the histological level, but the underlying molecular mechanisms are still poorly defined. To gain insight into these mechanisms we searched for genes, which are regulated by skin injury. Interestingly, some of the genes that we identified encode cytoprotective proteins, in particular enzymes, which detoxify reactive oxygen species (ROS). Since ROS are produced in high amounts at the wound site as a defense against invading bacteria, the expression of these genes is most likely important for the protection of cells against these toxic molecules. In this review, we summarize the results on the expression of cytoprotective genes in wounded skin, and we discuss their possible roles in the wound-healing process.

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INTRODUCTION

Cutaneous injury initiates a series of events, including inflammation, new tissue formation, and matrix remodeling, which finally lead to at least partial reconstruction of the wounded tissue (Martin, 1997). These processes are well described at the histological level, but the underlying molecular mechanisms are still poorly understood. Our previous studies suggested that genes, which are regulated by skin injury, are of functional importance for the repair process. Therefore, we performed differential display reverse transcriptase PCR, subtractive hybridization, and microarray analysis to identify genes, which are up- or downregulated within 24 hours after the generation of full-thickness excisional skin wounds in mice (Munz *et al.*, 1999; Thorey *et al.*, 2001; Kaesler *et al.*, 2004; Beer *et al.*, 2005). The identified genes encode, for example, various growth factors, cytokines, extracellular matrix molecules, cytoskeletal proteins, transcription factors, and enzymes. Interestingly, among the latter are several enzymes known to be involved in ROS detoxification. In addition, one of the injury-regulated genes encodes the transcription factor NF-E2-related factor 2 (Nrf2), a known regulator of ROS-detoxifying enzymes. In this review, we summarize the current knowledge on the expression of these genes in wounded skin and their potential function in the repair process.

ROS IN WOUND HEALING

During the inflammatory phase of wound-healing neutrophils and macrophages invade the wound. Neutrophils arrive first within a few minutes and are abundant at the wound site within 1–3 hours after wounding. Subsequently, lymphocytes and monocytes also invade the wound tissue and the latter differentiate into activated macrophages (Singer and Clark, 1999). Neutrophils and macrophages produce large amounts of superoxide radical anions, a phenomenon, which is often described as the ''respiratory burst''. Furthermore, other cells such as fibroblasts can be stimulated by pro-inflammatory cytokines to produce ROS (Meier *et al.*, 1989). The generation of these reactive molecules is part of the innate immune system and helps to rapidly clean the wound from invading bacteria (Clark, 1996). The driving enzyme for the conversion of molecular oxygen to superoxide radical anions is nicotinamide adenine dinucleotide phosphate (reduced form) oxidase, which resides within both phagosomal membranes and the plasma membrane of phagocytic cells (Wientjes and Segal, 1995). Superoxide radical anions can be dismutated by superoxide dismutases (SODs) to molecular oxygen and hydrogen peroxide. The latter is readily diffusible through biological membranes and may be converted to the highly reactive hydroxyl radical (Figure 1) (Fridovich, 1978).

Besides their beneficial role in microbial killing ROS can have a series of negative effects. For example, at low levels, hydrogen peroxide and other ROS inhibit migration and proliferation of various cell types, including keratinocytes (O'Toole *et al.*, 1996). At high levels ROS can lead to severe tissue damage and even neoplastic transformation (Cerutti and Trump, 1991). The proliferating and migrating cells in the

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Abbreviations: GPx, glutathione peroxidase; GSH, glutathione; HO, heme oxygenase; Nrf2, NF-E2-related factor 2; PhGPx, phospholipid hydroperoxide glutathione peroxidase; Prx, peroxiredoxin; ROS, reactive oxygen species; SeGPx, seleno-glutathione peroxidase; SOD, superoxide dismutase

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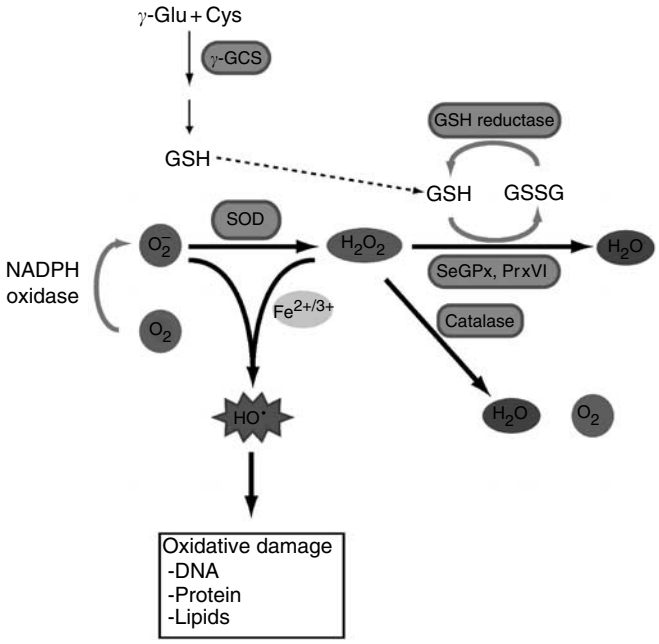

Figure 1. **Generation and detoxification of ROS.** Superoxide anions are generated at high levels by the inflammatory cell-derived nicotinamide adenine dinucleotide phosphate (reduced form) oxidase. These radicals are dismutated by SODs, resulting in the generation of hydrogen peroxide and molecular oxygen. Hydrogen peroxide is a substrate of catalase and of various peroxidases, including SeGPx and PrxVI. The latter use GSH as a substrate, which is concomitantly oxidized to GSH disulfide. *De novo* formation of the tripeptide GSH is achieved in two enzymatic reactions, with the formation of γ-glutamylcysteine as the rate-limiting step. If hydrogen peroxide is not rapidly detoxified by catalase and peroxidases, it can give rise to the highly toxic hydroxyl radical through Fenton reaction.

wound tissue are exposed to large amounts of ROS during the respiratory burst and thus have to develop strategies to protect themselves against these harmful insults.

ANTIOXIDANT DEFENSE SYSTEMS

To protect themselves from oxidative stress, cells have developed several systems to detoxify ROS. In general, there are two major strategies, which convey partial resistance against oxidative stress to most cell types: non-enzymatic and enzymatic. The first comprises small antioxidant molecules like vitamin E, vitamin C, β-carotene, glutathione (GSH), co-enzmye Q, and bilirubin, which function to quench ROS. The second consists of ROS-detoxifying enzymes, including among others SODs, the seleno-enzyme GSH peroxidase (SeGPx), and catalase (Fridovich, 1978; Michiels *et al.*, 1994; Shindo *et al.*, 1994). SODs catalyze the dismutation of superoxide anions to molecular oxygen and hydrogen peroxide. The latter can be further detoxified by catalase or by GSH peroxidases (GPx), which include, for example the SeGPx and members of the peroxiredoxin (Prx) family. The action of GPx involves oxidation of GSH to GSH disulfide (Figure 1). Normally the ratio between GSH and GSH disulfide is 10–100 in the cell. Disruption of this ratio, for example by enhanced formation of GSH disulfide, leads to an imbalanced redox state and oxidative stress. Therefore, the cell tries to maintain this ratio by enhanced activity of GSH reductase and/or removal of GSH disulfide (Schafer and Buettner, 2001). Furthermore, γ-glutamylcysteinyl synthase, the rate-limiting enzyme in GSH biosynthesis, is activated upon reduction of cellular GSH levels (Soltaninassab *et al.*, 2000).

Owing to the high levels of ROS present in wounded skin, the expression of ROS-detoxifying enzymes in healing skin wounds appears to be of particular importance, and we have therefore determined the expression and function of some of these genes in the wound-repair process.

SUPEROXIDE DISMUTASES IN WOUND REPAIR

The superoxide radical anion is the major ROS generated during the respiratory burst of inflammatory cells. It can be detoxified by three types of SOD, the cytosolic Cu/Zn-dependent enzyme (SOD1), the mitochondrial Mn-dependent variant (SOD2), or an extracellular variant (SOD3). To gain insight into a potential role of SODs in cutaneous wound repair, we determined the expression of Cu/Zn-SOD and Mn-SOD in full-thickness skin wounds in mice. As shown by RNase protection assay (Figure 2), mRNAs encoding both enzymes were expressed at a low level in normal mouse back skin. After wounding, their expression increased with a similar kinetics, and highest levels of SOD mRNAs were found at the early stage of wound repair, when the oxidative burst occurs. The upregulation of Cu/Zn-SOD was more prolonged compared to that of Mn-SOD, and elevated mRNA levels were still found at day 7 after wounding (Steiling *et al.*, 1999). *In situ* hybridization of sections from 5-day wounds revealed the presence of particularly high levels of Cu/Zn-SOD mRNA in the hyperproliferating basal cells of the wound epidermis as well as in a few cells of the dermis and granulation tissue. mRNA encoding Mn-SOD was found in basal and suprabasal cells of the hyperproliferative wound epidermis. In early wounds, Mn-SOD mRNA was also seen in cells of the clot, which most likely represent neutrophils. Using immunohistochemistry, the sites of expression of the two types of SOD in the wound tissue were confirmed at the protein level (Steiling *et al.*, 1999). Thus, inflammatory cells and in particular keratinocytes of the wound epidermis appear to have a high capacity to detoxify superoxide radical anions.

CATALASE, SEGPx, AND PHOSPHOLIPID HYDROPEROXIDE GPx IN WOUND REPAIR

Although hydrogen peroxide, the product of the reaction catalyzed by SODs, is not a radical, it can give rise to the highly reactive hydroxyl radical in the presence of transition metals (Fenton reaction). Therefore, it is essential to rapidly detoxify hydrogen peroxide. This can be achieved by catalase as well as by different types of peroxidases. Of particular importance is the SeGPx I, which is also able to reduce a wide variety of organic peroxides (Wendel, 1981). As shown in Figure 2, expression of SeGPx increased after wounding with maximal mRNA levels being found between days 3 and 5 after injury. At 14 days after the insult, when the wound was completely healed, expression of this enzyme had almost

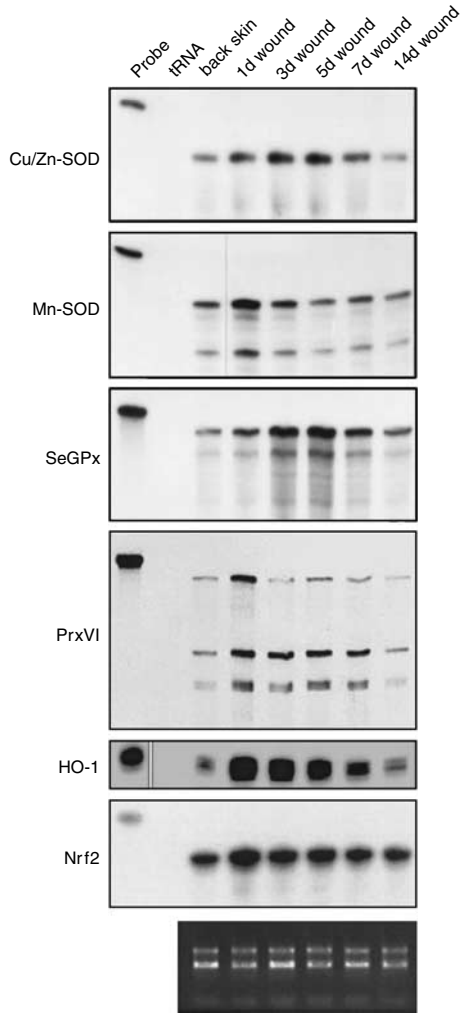

Figure 2. **Upregulation of ROS-detoxifying enzymes in healing skin wounds.** Full-thickness excisional skin wounds were generated on the back of Balb/c mice as described by Wankell *et al.* (2001). RNA was isolated from non-wounded skin (back skin) as well as from wound tissue obtained at different time points after injury as indicated on top of the figure. Twenty micrograms of total cellular RNA was analyzed by RNase protection assay for the presence of mRNAs encoding Cu/Zn-SOD, Mn-SOD, SeGPx, PrxVI, HO-1, and Nrf2. One microgram aliquots of the same batch of RNAs were analyzed on a 1% agarose gel and stained with ethidium bromide (shown below the RNase protection assays).

declined to the basal level. By contrast, expression of catalase as well as of phospholipid hydroperoxide GPx (PhGPx), which uses phospholipid hydroperoxides as a substrate, was not altered by skin injury (Steiling *et al.*, 1999). Similar to the expression pattern of SODs, the hyperproliferative wound epithelium and the hair follicles at the wound edge were also the predominant sites of expression of catalase, SeGPx, and phospholipid hydroperoxide glutathione peroxidase as shown by *in situ* hybridization of 5-day wounds. mRNAs encoding catalase and SeGPx were found in all layers of the wound epidermis, whereas phospholipid hydroperoxide glutathione peroxidase mRNA levels were particularly high in the basal and lower suprabasal layers. In addition, transcripts encoding all of these enzymes were found in the dermis and granulation tissue, although at lower abundance compared to the epidermis. The co-expression of SODs and peroxide-detoxifying enzymes strongly suggests that hydrogen peroxide produced by SODs is rapidly detoxified, thereby avoiding the generation of the aggressive hydroxyl radical.

PEROXIREDOXIN VI IN WOUND REPAIR

In addition to the classical peroxidases, recent studies revealed that members of the Prx family can also detoxify hydrogen peroxide as well as a wide variety of organic peroxides. Whereas, PrxI–V contain two reactive cysteines and utilize thioredoxin and/or GSH as a substrate (Hofmann *et al.*, 2002; Wood *et al.*, 2003), PrxVI or 1-Cys-Prx has a single redox-active cysteine and it uses GSH to catalyze the reduction of hydrogen peroxide and various organic peroxides. Additionally, PrxVI has been reported to have phospholipase A_2 activity (Manevich and Fisher, 2005). Previous studies have shown that overexpression of PrxVI prevents from ROS-induced cytotoxicity, whereas knockout of the gene in mice caused enhanced sensitivity to oxidative injury (Manevich *et al.*, 2002; Wang *et al.*, 2003, 2004). Thus, PrxVI is likely to have an important cytoprotective function.

A role of PrxVI in wound healing was first suggested by the identification of PrxVI as the product of a wound-regulated gene in our laboratory (Munz *et al.*, 1997). By RNase protection assay we found highest levels of the PrxVI mRNA at day 1 after injury. Elevated levels were still seen until day 7 after wounding, but expression declined to the basal levels seen in unwounded skin when the wound was fully healed (day 14 after wounding) (Figure 2). The peak of PrxVI protein expression was observed at day 5 after wounding. By contrast, expression of PrxI was unaltered after injury (data not shown). Using *in situ* hybridization we found highest levels of PrxVI mRNA in keratinocytes of the hyperproliferative wound epidermis (Munz *et al.*, 1997) (Figure 3). *In vitro* studies demonstrated that PrxVI expression is strongly enhanced in keratinocytes by keratinocyte growth factor (Frank *et al.*, 1997). Since the latter is also highly upregulated in wounded skin (Werner *et al.*, 1992), our findings suggest that this growth factor may also be responsible for the upregulation of PrxVI in wounded skin. Independent of the inducers, the expression pattern of PrxVI in healing skin wounds suggests that this enzyme protects keratinocytes against oxidative stress and acts together with SeGPx in the detoxification of the SOD product hydrogen peroxide. Furthermore, the potent activity of PrxVI towards phospholipid hydroperoxides further indicates that this enzyme also reduces peroxidation of membrane lipids. Functional studies to determine the role of PrxVI in the healing response are currently in progress.

HEME OXYGENASES IN WOUND REPAIR

Besides the ROS-detoxifying enzymes described above, additional antioxidant proteins are likely to be key players in wound repair. One of them is heme oxygenase (HO), the rate-limiting enzyme in the degradation of heme into carbon

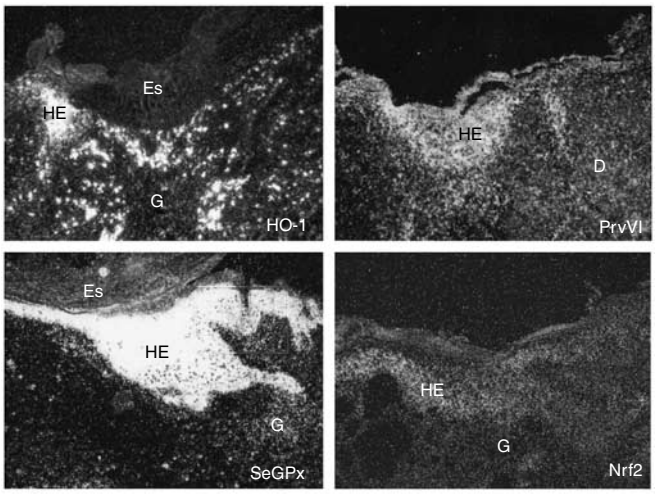

Figure 3. Genes encoding cytoprotective proteins are predominantly expressed in the hyperproliferative wound epidermis. Paraformaldehyde-fixed frozen sections from 5-day full-thickness excisional skin wounds were analyzed by *in situ* hybridization for the presence and localization of mRNAs encoding HO-1, PrxVI, SeGPx, and Nrf2. Note the strong expression of all of these genes in the hyperproliferative wound epidermis. D: dermis; Es: eschar; G: granulation tissue; HE, hyperproliferative wound epidermis. Original magnification $\times 100$.

monoxide, iron, and biliverdin that is subsequently reduced to bilirubin, a potent antioxidant (Tenhunen *et al.*, 1968; Baranano *et al.*, 2002). Three different HO isoforms have been described, including the inducible isoform HO-1, the mostly constitutive isoform HO-2, and HO-3, with low enzymatic activity (Elbirt and Bonkovsky, 1999). Expression of HO-1 is induced by its substrate heme, but also following exposure to various stressful stimuli, including UV irradiation, hydrogen peroxide, nitric oxide, phorbol esters, heavy metals, and organic chemicals (Applegate *et al.*, 1991). Most interestingly, many of the known HO-1 inducers stimulate the production of ROS or lead to depletion of GSH, suggesting an involvement of HO-1 activity in the cellular protection against oxidative stress. Therefore, we determined the expression of HO-1 and HO-2 during the healing process of full-thickness excisional wounds in mice (Hanselmann *et al.*, 2001). We found a remarkable induction of HO-1 mRNA and protein expression one day after skin injury (Figure 2 and data not shown). After completion of wound healing, HO-1 expression declined to basal levels (Figure 2). By contrast, expression of HO-2 was not significantly modulated by skin injury (data not shown). *In situ* hybridization and immunohistochemistry revealed high HO-1 expression in inflammatory cells of the granulation tissue and in keratinocytes of the hyperproliferative epithelium (Figure 3 and Hanselmann *et al.*, 2001). Using *in vitro* studies with cultured keratinocytes we identified hydrogen peroxide and the ROS-producer menadione as strong inducers of HO-1 expression in keratinocytes, suggesting that ROS are also responsible for the upregulation *in vivo*. By contrast, proinflammatory cytokines and growth factors had no effect (Hanselmann *et al.*, 2001). Taken together, our findings suggest a novel role of HO-1 in wound-healing where it might be involved in the protection of cells from the toxic effects of ROS through formation of the antioxidant bilirubin. In addition, a recent study suggests an additional role of HO in wound-healing through its function in heme degradation (Wagener *et al.*, 2003). These investigators found a strong accumulation of heme at the edges of skin wounds, resulting in heme-induced influx of leukocytes. Therefore, the enhanced expression of HO at the wound site is likely to be required to attenuate the inflammatory response.

NRF2 IN WOUND REPAIR

The expression of various ROS-detoxifying enzymes and other cytoprotective proteins in healing skin wounds raises the question about their regulation. Interestingly, many of these genes are under the control of the Nrf2 transcription factor. The latter is a member of the ''cap'n' collar'' family of transcription factors, which, among others, also includes the closely related proteins Nrf1 and Nrf3 (Motohashi and Yamamoto, 2004). Together with their heteromeric interaction partners, the small Maf proteins, they bind to *cis*-acting elements in the promoters of target genes, called antioxidant response elements. At least the binding of Nrf1 and Nrf2 activates the expression of these genes, which encode, for example GSH *S*-transferase Ya subunit, γ-glutamylcysteinyl synthase, HO-1 and PrxI (Nguyen *et al.*, 2003). In addition, a recent study suggests that PrxVI is also under the control of Nrf2 (Lee *et al.*, 2003).

In the absence of specific activators, Nrf2 is localized in the cytosol via binding to the actin-binding protein Keap1, which also mediates its degradation via the ubiquitin-proteasome pathway. Upon addition of electrophilic substances, which couple to Keap1 through Michael addition, and possibly through oxidation of Keap1 via ROS, Nrf2 becomes liberated and shuttles to the nucleus, where it activates its target genes. In addition, phosphorylation of Nrf2 by different kinases can also result in liberation from Keap1 (Itoh *et al.*, 2004; Nguyen *et al.*, 2004). In a recent study, we demonstrated a strong increase in the expression of Nrf2 as well as of its target genes HO-1 and GSH *S*-transferase Ya in healing skin wounds (Figure 2 and Braun *et al.* (2002)). Highest expression levels of Nrf2 were seen in cells of the granulation tissue, which most likely represent macrophages, as well as in the hyperproliferative wound epithelium (Figure 3) (Braun *et al.*, 2002). The expression of Nrf2 in wound keratinocytes correlates with the expression of many of its target genes (see above), suggesting that the enhanced levels of Nrf2 seen at the site of injury result in upregulation of ROS-detoxifying enzymes and other cytoprotective proteins. To determine the importance of Nrf2 for the wound-healing process, we performed wound-healing studies in Nrf2 knockout mice. The expression of several key players involved in wound repair was strongly reduced in early wounds of Nrf2-deficient mice, and prolonged inflammation was seen in the late phase of repair. Surprisingly, however, these differences in gene expression and inflammation were not reflected by wound-healing abnormalities. This may be explained by the observed upregulation of Nrf3, which was

co-expressed with Nrf2 in the hyperproliferative wound epidermis (Braun *et al.*, 2002). Therefore, this transcription factor may compensate for the lack of Nrf2 during wound healing. Studies using genetically modified animals, which lack both Nrf2 and Nrf3 and possibly also Nrf1 in the wound will help to identify the role of Nrf transcription factors in the healing response.

CONCLUSION

The results described above suggest that a thorough regulation of the levels of ROS in wounded skin is required for efficient repair. On the one hand, production of ROS by inflammatory cells and other cell types in the wound is required for the defense against invading bacteria (Figure 4), and at physiological levels ROS are also important regulators of various intracellular signaling pathways (Forman *et al.*, 2002). On the other hand, enhanced levels of ROS are detrimental and can lead to severe cell damage. This effect may be further enhanced by ROS-mediated inhibition of the activity of various ROS-scavenging enzymes (Pigeolet *et al.*, 1990; Vessey and Lee, 1993). Therefore, the observed upregulation of various genes encoding antioxidative proteins in keratinocytes and other cell types of the healing skin wound might be a strategy for efficient ROS detoxification in the harsh wound environment (Figure 4). The increased mRNA expression of several ROS-scavenging enzymes that we observed after skin injury seems to be in contrast to findings of other investigators (Shukla *et al.*, 1997), who found reduced SOD and GPx activities in wound tissue between days 2 and 7 after cutaneous injury in rats. The discrepancy between these results and our mRNA data may be explained by inhibition of the enzymatic activities of these enzymes at the wound site by the high levels of ROS (Pigeolet *et al.*, 1990; Vessey and Lee, 1993).

Given the obvious importance of ROS detoxification in healing skin wounds, it seems likely that reduced levels of ROS-detoxifying enzymes result in healing impairments, a hypothesis, which is supported by the observation of reduced activities of SOD, catalase, and GPx in wounds of aged rats compared to young rats (Anamika and Shukla, 2000); by the beneficial effect of antioxidants on wound healing in ischemic rat skin (Senel *et al.*, 1997) and by the restoration of the delayed wound healing seen in diabetic mice by adenoviral delivery of Mn-SOD to the wound site (Luo *et al.*, 2004). Furthermore, a large number of observations suggest a role of enhanced ROS levels in the pathogenesis and pathophysiology of chronic leg ulcers (Wlaschek and Scharffetter-Kochanek, 2005). Consistent with this hypothesis, preliminary studies from our laboratory indeed suggest that the lack of PrxVI in mice is associated with wound-healing abnormalities (A.K. and S.W., unpublished data). Wound-healing studies in other genetically modified animals with altered expression of ROS-detoxifying enzymes will allow to determine the function of individual components of the ROS defense system for the wound-healing process. An even more elegant approach will be the use of inducible systems, which allow the induction of a transgene or the deletion of an endogenous gene in a time- and tissue-specific manner (Lewandoski, 2001). This strategy will circumvent the problem of systemic defects and will also prevent abnormal organ development. The latter is important, since a wound-repair phenotype might be secondary to a defect already present in non-wounded skin or to systemic abnormalities, which influence the repair process. Thus, by induction of a transgene or deletion of an endogenous gene prior to injury, the role of this particular gene in the healing response can be analyzed in the absence of secondary abnormalities. This type of study will most likely resolve a number of the issues that we brought up in this review as well as unravel exciting novel functions of ROS-detoxifying enzymes in tissue repair.

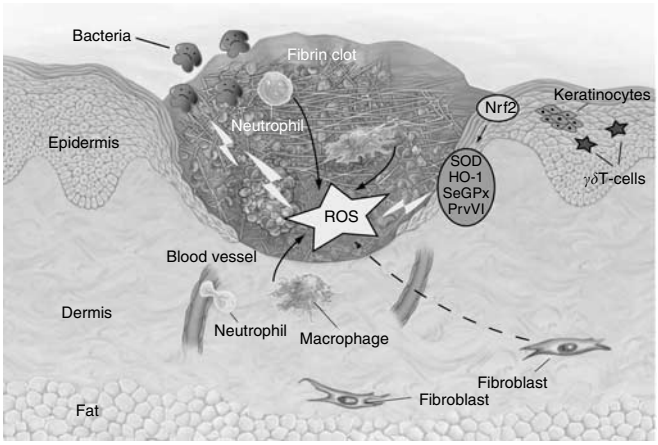

Figure 4. ROS defense in the healing skin wound. As a defense against invading bacteria, inflammatory cells produce ROS via nicotinamide adenine dinucleotide phosphate (reduced form) oxidase. To protect themselves against these harmful molecules, cells at the wound site, in particular keratinocytes, express various ROS-detoxifying enzymes and other antioxidant proteins. The expression of some of these genes is regulated by the Nrf2 transcription factor, which is also upregulated after skin injury. The wound background of this figure was taken from Figure 1 of Singer and Clark (1999), and modified to include our results and additional knowledge on the role of ROS in wound repair.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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