Peroxiredoxin 6 Is a Potent Cytoprotective Enzyme in the Epidermis

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Peroxiredoxin 6 is an enzyme that detoxifies hydrogen peroxide and various organic peroxides. In previous studies we found strongly increased expression of peroxiredoxin 6 in the hyperproliferative epidermis of wounded and psoriatic skin, suggesting a role of this enzyme in epidermal homeostasis. To address this question, we generated transgenic mice overexpressing peroxiredoxin 6 in the epidermis. Cultured keratinocytes from transgenic mice showed enhanced resistance to the toxicity of various agents that induce oxidative stress. However, overexpression of peroxiredoxin 6 did not affect skin morphogenesis or homeostasis. On skin injury, enhancement of wound closure was observed in aged animals. Most importantly, peroxiredoxin 6 overexpression strongly reduced the number of apoptotic cells after UVA or UVB irradiation. These findings demonstrate that peroxiredoxin 6 protects keratinocytes from cell death induced by reactive oxygen species *in vitro* and *in vivo*, suggesting that activation of this enzyme could be a novel strategy for skin protection under stress conditions. (Am J Pathol 2006; 169:1194–1205; DOI: 10.2353/ajpath.2006.060119)

Because of its exposure to the environment, the skin is permanently endangered by UV irradiation and noxious xenobiotics. Many of these insults induce the formation of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), oxyradicals, or organic hydroperoxides. Endogenous ROS are generated in the course of common metabolic processes, such as in the respiratory chain. Particularly large amounts of ROS are produced

by inflammatory cells in wounded and inflamed tissues as a defense against bacterial infection.^{1–3} Because excessive accumulation of ROS can lead to cell aging, severe cell damage, and even malignant transformation, the cells in the skin are equipped with a network of antioxidant enzymes to counteract oxidative stress and to maintain their redox balance. Of particular importance is the expression of ROS-detoxifying enzymes. The latter include, for example, different types of glutathione peroxidases, superoxide dismutases (SODs), as well as peroxiredoxins (Prdx). Prdx comprise a family of six enzymes that catalyze the reduction of a broad spectrum of peroxides. Whereas Prdx 1 to 5 contain two reactive cysteines and use thioredoxin and/or glutathione as a substrate,^{4–7} Prdx6 or 1-Cys-peroxiredoxin has a single redox-active cysteine and uses glutathione to catalyze the reduction of H₂O₂⁸ and various organic peroxides.⁹ In addition, Prdx6 has been reported to have phospholipase A₂ activity.^{10,11} Previous studies have shown that overexpression of Prdx6 in different cell types protects from ROS-induced cytotoxicity,^{12–14} whereas knockdown of this enzyme¹⁵ or ablation of the gene in mice resulted in enhanced sensitivity to oxidative injury.^{16–18} However, the role of Prdx6 in the skin is as yet unknown. Previous studies from our laboratory revealed a strong induction of *prdx6* expression in cultured keratinocytes on treatment with keratinocyte growth factor.¹⁹ *In vivo*, *prdx6* is overexpressed in the hyperproliferative epidermis of skin wounds²⁰ and of psoriatic patients.¹⁹ To determine the consequences of enhanced expression of this enzyme in keratinocytes, we generated transgenic mice overexpressing Prdx6 in the epidermis. Our results reveal a cytoprotective function of Prdx6 for keratinocytes *in vitro* and in wounded and UV-irradiated skin.

Supported by the Swiss National Science Foundation (grants 31-61358.00 and 3100A0-109340/1), the ETH Zurich Research Foundation, and the AETAS Foundation (to S.W.).

Accepted for publication July 12, 2006.

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Materials and Methods

Generation and Identification of Transgenic Mice

The full-length murine *prdx6* cDNA²⁰ was inserted into an expression cassette of the pBluescript KSII⁺ cloning vector (Stratagene, La Jolla, CA) that includes a 2-kb human keratin 14 promoter,²¹ followed by a 0.65-kb rabbit β -globin intron and a transcription termination/polyadenylation fragment [poly(A), 0.65 kb] of the human growth hormone gene.

Standard procedures were followed to generate transgenic mice. In brief, fertilized eggs were obtained after superovulation and mating of B6D2F1 or FVB/N females with males of the same genetic background. The 4.9-kb insert was separated from vector sequences, purified, and injected into the pronuclei of fertilized oocytes. Microinjected zygotes were transferred into the oviducts of pseudopregnant recipient females. Mouse tail DNA was analyzed for integration of the transgene by Southern blotting using the rabbit β -globin intron fragment as a probe. The progeny was genotyped by polymerase chain reaction using either 5'-GGATCCTGAGAACTTCAGGGT-GAG-3' as a 5' primer and 5'-CAGCACATAAACCAGC-ACGTTGCC-3' as a 3' primer or 5'-CGGGATCCCGGT-GACAGTGTTTTAC-3' as a 5' primer and 5'-CGGAATT-CAGCTTGTTCCCGAATAGAC-3' as a 3' primer. Both primer pairs were used at an annealing temperature of 53°C. The primers hybridize to the β -globin intron sequence or to the *prdx6* cDNA and the growth hormone poly(A) fragment and give products of 620 or 331 bp, respectively.

Wounding and Preparation of Wound Tissues

At 10 to 12 weeks or 1 year of age, mice were anesthetized with a single intraperitoneal injection of ketamine/xylazine. Two full-thickness excisional wounds, 5 mm in diameter, were made on either side of the dorsal midline by excising skin and panniculus carnosus as described previously.²² Wounds were left uncovered and harvested at different time points after injury. At least two independent wounding experiments with different transgenic mouse lines were performed. For expression studies, the complete wounds including 2 mm of the epithelial margins were excised and immediately frozen in liquid nitrogen and stored at -80°C until used for RNA isolation or preparation of protein lysates. Nonwounded back skin served as a control. For histological analysis, the complete wounds were excised and either fixed overnight in 95% ethanol/1% acetic acid or in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) followed by paraffin-embedding. Sections (6 μ m) from the middle of the wound were stained with hematoxylin and eosin (H&E) or by the Masson trichrome procedure or used for immunofluorescence analysis. Only littermates of the same sex were used for direct histological comparison. All experiments with animals were performed with permission from the local veterinary authorities.

Determination of Wound-Bursting Strength

Mice were anesthetized by an intraperitoneal injection of ketamine/xylazine. For the generation of incisional wounds, the hair on the back was removed by depilation cream (Pilca Perfect; Stafford-Miller Continental, Oevel, Belgium). Two full-thickness incisional wounds (1-cm length, 3 to 4 mm apart) were made at each site of the dorsal midline. Wound margins were fixed together with a Fixomull stretch plaster (Beiersdorf AG, Hamburg, Germany). After 5 days, animals were sacrificed, and wound-bursting strength was measured with a biomechanical tissue characterization device (BTC 2000; Surgical Research Laboratory Incorporation, Nashville, TN).

Preparation of Dermis and Epidermis from Tail Skin

Tail skin was separated from bone and incubated for 30 minutes at 30°C in 2 mol/L NaBr. After this incubation, the epidermis was mechanically separated from the dermis, and both samples were snap-frozen in liquid nitrogen and used for the preparation of protein lysates.

UVA and UVB Irradiation of Mice

Mice (10 to 12 weeks old) were anesthetized by intraperitoneal injection of ketamine/xylazine and subsequently shaved. Thereafter, the mice were irradiated with 75 J/cm² UVA using a UVA1 lamp (GP-12H; Cosmedico Licht GmbH, Stuttgart, Germany) emitting wavelengths in

Figure 1. Generation of transgenic mice overexpressing Prdx6 in the epidermis. **A:** Scheme of the transgene construct. Functional elements include the human keratin 14 promoter, the rabbit β -globin intron, the murine *prdx6* cDNA, and the human growth hormone poly(A). **B:** Total RNA (10 μ g) from back skin of transgenic mice from different lines and from wild-type animals was analyzed for the presence of Prdx6 mRNA by RPA. tRNA (20 μ g) was used as a negative control. The hybridization probe (1000 cpm) was loaded in the lane labeled "probe" and used as a size marker. One μ g of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide. The band corresponding to the transgene-derived mRNA is indicated with an arrow. **C and D:** Western blot analysis of back skin lysate (20 μ g of protein) from wild-type and transgenic mice of different founder lines (**C**) or of lysates from isolated tail skin epidermis (10 μ g of protein) (**D**) using an antibody against Prdx6. As a loading control the membranes were reprobed with an antibody against β -actin.

Figure 2. Normal skin morphogenesis and keratinocyte differentiation in K14-Prdx6 transgenic mice. Ethanol/acetic acid-fixed paraffin sections from tail skin of K14-Prdx6 transgenic mice and control littermates were stained using the Masson trichrome procedure (**A**) or analyzed by immunofluorescence using antibodies against K14, K10, and K6 (**B**). To visualize the epidermis and the hair follicles, the K6 staining was combined with Hoechst staining of the nuclei. D, dermis; E, epidermis; HF, hair follicle. The basal cell layer of the epidermis is indicated with **arrows**. Scale bars = 100 μ m.

the 340 to 400 nm range. Alternatively, they were irradiated with 100 mJ/cm² UVB using a Medisun FH-54 lamp (Schulze & Böhm, Huerth, Germany), equipped with six UVB-TL/12 bulbs (9 W each; Philips, Amsterdam, The Netherlands), which emit UVB light in the range of 280 to 315 nm with a peak emission at 312 to 315 nm. Twenty-four hours later, the irradiated mice were sacrificed and the tissue was either fixed in 4% PFA or 95% ethanol/1% acetic acid, or immediately frozen in liquid nitrogen as described above. To identify apoptotic keratinocytes PFA-fixed paraffin sections were stained with H&E or analyzed using the *In Situ* Cell Death Detection kit, fluorescein (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions. The number of apoptotic cells per μ m of basement membrane was determined by counting the cells in 10 to 15 independent microscopic fields per mouse.

RNA Isolation and RNase Protection Assay

RNA isolation and RNase protection assays were performed as previously described.^{23,24} All protection assays were performed at least in duplicate with different sets of RNAs from independent experiments. The following templates were generated by reverse transcriptase-polymerase chain reaction and cloned: a murine *prdx6* cDNA fragment (allows distinction between the endogenous and the transgene-derived *prdx6* mRNAs) and a murine *prdx1* cDNA fragment [nucleotides 369 to 563 of the cDNA (accession number D16142)]. Other templates were described previously: murine interleukin (IL)-1 β ,²⁵ murine fibronectin, and collagen 1 α (I),²⁶ murine vascular endothelial growth factor,²⁷ murine heme oxygenase 1 (HO-1),²⁸ murine glutathione peroxidase I, murine Cu/Zn superoxide dismutase (Cu/Zn-SOD), and murine Mn-SOD.²⁹ As a loading control, the RNA was hybridized with a probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*).³⁰ Multi-probe RNase protection assays were performed with mCK-5b and mCD-1 template sets (BD Bioscience, Basel, Switzerland) according to the manufacturer's recommendations.

Culture of Primary Mouse Keratinocytes, Menadione Treatment, and UVA Irradiation

Murine epidermal keratinocytes were isolated from pools of K14-Prdx6 transgenic mice from lines 11 and 55 and control littermates as described previously,^{30,31} with the exception that 2- to 4-day-old mice were used instead of embryos and that cells were seeded at a density of 5×10^4 cells per cm² on collagen IV (2.5 μ g per cm²)-coated dishes. The freshly isolated cells were incubated for 30 minutes at 37°C. Thereafter the medium was replaced, and cells were grown to 90% confluence in defined keratinocyte serum-free medium (Invitrogen, Basel, Switzerland) supplemented with 10 ng/ml epidermal growth factor and 10^{-10} mol/L cholera toxin and rendered quiescent by overnight incubation in defined keratinocyte serum-free basal medium without additives. Subse-

quently, menadione was added to the medium, and 6 hours later the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed or the cells were fixed with 4% PFA for further analysis.

For UVA irradiation 90% confluent cells were starved overnight, 20 mmol/L HEPES (Sigma, Munich, Germany) were added to the medium, and cells were subsequently irradiated at a distance of 5 cm using a Medisun HF-54 UVA lamp from Schulze & Böhm. The device is equipped with six UVA-TL/08 bulbs (9 W each; Philips) that emit UVA light in the range of 315 to 400 nm with a peak emission at 350 to 352 nm. Irradiation was monitored with a UVA dosimeter. After, irradiation cells were incubated at 37°C/5% CO₂ for different time periods and subsequently analyzed.

Determination of Apoptotic Cells, Cell Viability Assay, and Oxyblot Analysis

To determine the number of apoptotic keratinocytes, the cells were fixed in 4% PFA in PBS for 30 minutes, per-

Figure 3. Peroxiredoxin 6 overexpression protects keratinocytes from menadione- and UVA-induced cell damage. **A:** Primary keratinocytes from K14-Prdx6 transgenic mice (lines 11 and 55) and from wild-type littermate controls were seeded into 96-well plates at a density of 5×10^4 cells per well, cultured to 90% confluence, rendered quiescent overnight, and subsequently challenged with different concentrations of menadione as indicated or solvent control, and subjected to MTT assay. All measurements were performed in quadruplicates ($n = 4$). The values obtained with nontreated wild-type and K14-Prdx6 cells were arbitrarily set as 100. Statistical analysis was performed using the GraphPad Prism4 software and applying the Student's *t*-test. *** $P < 0.0001$ for both concentrations of menadione, * $P = 0.0137$ for 37.5 μ mol/L menadione, and ** $P = 0.0089$ for 62.5 μ mol/L menadione. **B:** Primary keratinocytes from K14-Prdx6 transgenic mice (line 11) and from wild-type littermate controls were treated with 25 μ mol/L menadione for 6 hours and subsequently analyzed by immunofluorescence for the presence of cleaved caspase-3-positive cells. The percentage of cleaved caspase-3-positive cells was counted in 10 independent microscopic fields. Statistical analysis was performed using the Mann-Whitney *U*-test. *** $P = 0.0001$. Representative stainings are shown below. **C:** Primary keratinocytes from K14-Prdx6 transgenic mice (line 11) and from wild-type littermate controls were irradiated with UVA (20 J/cm²) for different time periods as indicated. Lysates from these cells (8.3 μ g of total protein) were analyzed for the presence of oxidized proteins by Oxyblot analysis. Reprobing of the membrane with an antibody to β -actin was performed as a loading control. Scale bars = 100 μ m.

meabilized with 0.1% Triton X-100 for 30 minutes, blocked with PBS containing 3% bovine serum albumin, and incubated with the primary antibody directed against cleaved caspase 3 (Cell Signaling Technology, Beverly, MA) diluted in blocking solution overnight at 4°C. Detection was performed by immunofluorescence as described below. The number of caspase 3-positive cells in relation to the total cell number was determined by counting 10 independent microscopic fields.

Cell viability was quantified using the MTT assay. For this purpose, 20 μ L of MTT (5 mg/ml in PBS; Sigma) were added directly to the culture medium in each 96-well plate followed by a 1.5-hour incubation at 37°C/5% CO₂. The supernatant was removed, and the cells were lysed with 0.04 mol/L hydrochloric acid/2-propanol. After 10 minutes of incubation at room temperature, water was added, and the absorption was measured at 595 nm in an enzyme-linked immunosorbent assay reader.

Oxidized proteins were detected using the Oxyblot assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The method is based on the detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine to convert carbonyl groups to dinitrophenylhydrazone derivatives. Derivatized protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Western blot analysis using an antibody against dinitrophenylhydrazone.

Preparation of Protein Lysates and Western Blot Analysis

Cells were lysed and tissues were homogenized in 20 mmol/L Tris/HCl (pH 8.0), 1% Triton X-100, 137 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 10% glycerol, 0.5 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.15 U/ml aprotinin, 0.15 U of 1% leupeptin, and 1% pepstatin. For Oxyblot analysis, 50 mmol/L dithiothreitol were added to the lysis/homogenization buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Antibody incubations were performed in 5% nonfat dry milk in TBST (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween 20). A mouse monoclonal antibody against β -actin (Sigma) and a rabbit polyclonal antibody against Prdx6 were used. The Prdx6 antibody was raised in rabbits against the C-terminal peptide NH₂-CELPSPGKKYLRYPQ-COOH coupled to bovine serum albumin. The final bleed was directly used for Western blot analysis at a dilution of 1:2000 to 1:5000.

Immunofluorescence and Immunohistochemistry

Dewaxed ethanol/acetic acid-fixed paraffin sections from tail skin were incubated overnight at 4°C with the primary antibodies diluted in PBS containing 3% bovine serum albumin and 0.025% Nonidet P-40. After three 10-minute washes with PBS/0.1% Tween 20, sections were incubated for 1 hour with the Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), washed again, and mounted with Mowiol (Hoechst, Frankfurt, Germany). Mouse monoclonal antibodies against keratin 10 (K10) (DAKO, Hamburg, Germany), and rabbit polyclonal antibodies against keratin 6 (K6) and keratin 14 (K14) (both from BAbCO, Richmond, CA) were used. For p53 immunohistochemistry PFA-fixed paraffin sections in combination with a polyclonal antibody against p53 [p53 (CM5); Novocastra, Newcastle, UK] and a biotinylated secondary antibody (Jackson ImmunoResearch) were used, followed by counterstaining with hematoxylin.

5'-Bromo-2'-Deoxyuridine (BrdU) Incorporation Assay

Mice were injected intraperitoneally with BrdU (250 mg/kg in 0.9% NaCl; Sigma) and sacrificed 2 hours after injection. Bisected wounds were fixed in ethanol/acetic acid as described above. Sections were incubated with a peroxidase-conjugated monoclonal antibody directed against BrdU (diluted 1:4; Roche Diagnostics, Rotkreuz, Switzerland) and stained using the 3,3-diaminobenzidine peroxidase substrate (Sigma). Counterstaining was performed with hematoxylin.

Results

Generation of Transgenic Mice Overexpressing Prdx6 in the Epidermis

In previous studies, we showed a strong up-regulation of *prdx6* expression after cutaneous injury, in particular in keratinocytes of the hyperproliferative wound epithelium.²⁰ Highest expression levels were seen during the inflammatory phase of repair, suggesting a role of Prdx6 in the protection of keratinocytes against ROS. To determine whether Prdx6 is indeed able to protect cells from the toxic effects of ROS *in vivo*, we generated transgenic mice overexpressing this enzyme in the epidermis. For this purpose we used the well-characterized keratin 14 (K14) promoter^{21,32,33} (Figure 1A), which targets the ex-

Figure 4. Overexpression of Prdx6 in the epidermis does not affect the wound-healing process in young mice but in aged mice. Full-thickness excisional wounds were generated on the backs of female K14-Prdx6 transgenic mice and wild-type littermates. Sections from the middle of 5-day (A) and 13-day (C) wounds were stained with H&E. B: Mice were injected intraperitoneally with BrdU 2 hours before sacrifice. Sections from the middle of 5-day wounds were analyzed by immunohistochemistry for the presence of BrdU-positive cells. H&E-stained sections from the middle of representative 5-day wounds of aged mice are shown in D. Arrowheads indicate the wound margins. D, dermis; E, epidermis; Es, eschar; G, granulation tissue; HE, hyperproliferative wound epidermis; HF, hair follicle. Scale bars = 100 μ m.

pression of transgenes to stratified epithelia, in particular to the basal epidermal keratinocytes and the outer root sheath keratinocytes of the hair follicles.³⁴ Several transgenic mouse lines were generated with FVB/N inbred and B6D2F2 hybrid background. Expression of the transgene was determined by RNase protection assay (Figure 1B) and Western blot analysis using total skin lysates (Figure 1C) or isolated epidermis from the tail skin (Figure 1D). One transgenic line from each genetic background expressed high levels of the transgene (L11 in FVB/N and L55 in B6D2 background), whereas the other lines expressed only low levels (L21 in FVB/N and L50 in B6D2 background). The transgene had integrated at different sites of the genome in the different founder mice, as determined by Southern blotting (data not shown). All founders gave rise to transgenic offspring. As expected from the activity of the promoter, expression of the transgene was only seen in the skin as well as in the tongue (data not shown).

Lack of Skin Abnormalities in K14-Prdx6 Transgenic Mice

K14-Prdx6 transgenic mice showed no visible abnormalities and had the same average life span as their wild-type littermates. The fur appeared normal, and a histological analysis of tail and back skin revealed no differences in skin architecture as shown by Masson trichrome staining (shown for tail skin in Figure 2A). These findings were confirmed by immunofluorescence analysis of the differentiation-specific keratins 14 and 10, which were expressed in the basal or suprabasal layers of the epidermis, respectively, in mice of both genotypes (Figure 2B, top and middle panels). Keratin 6 (K6) is restricted to hair follicles in normal skin but is transiently expressed in the hyperproliferative epithelium of wounds and permanently up-regulated in the suprabasal epidermal layers of hyperplastic, neoplastic, and psoriatic skin.³⁵ No interfollicular expression of this keratin was found in the transgenic mice (Figure 2B, third panel). Furthermore, no difference in the rate of keratinocyte proliferation was observed as determined by BrdU incorporation studies (data not shown). In summary, overexpression of Prdx6 in the epidermis had no obvious effect on keratinocyte proliferation and differentiation.

Primary Keratinocytes Derived from Transgenic Animals Are More Resistant to Oxidative Stress

We next determined whether overexpression of Prdx6 in keratinocytes enhances the resistance of these cells to oxidative stress. This was first tested *in vitro* using isolated primary keratinocytes from newborn transgenic and wild-type mice. Under normal culture conditions, the proliferation rate was identical in Prdx6-overexpressing cells and control keratinocytes as determined by BrdU incorporation assay (data not shown). However, on treatment with menadione, which generates oxidative stress,³⁶ primary cells from wild-type animals showed a significant

Figure 5. Quantitative analysis of the wound-healing process. **A:** Morphometric analysis of the area of the hyperproliferative wound epidermis. Six wound halves from three wild-type mice and eight wound halves from four transgenic mice derived from line 11 were used, as well as 20 wound halves from 10 wild-type and 13 wound halves from nine transgenic mice from line 55. Differences between the four groups are not significant. **B:** Analysis of wound-bursting strength. Incisional wounds were created on the backs of five K14-Prdx6 transgenic mice and five control littermates before postmortem measurement of bursting strength using a suction device. Differences between the two groups are not significant. **C:** Analysis of wound closure rates in young mice. For the analysis of wound closure, eight wound halves from four wild-type mice and seven wound halves from five transgenic mice of line 11 were analyzed. For line 55, six wound halves from four wild-type mice and seven wound halves from four transgenic mice were used. Differences are not statistically significant. **D:** Analysis of wound closure rates in aged mice. Analysis of wound closure in aged mice was performed with 10 wound halves from five wild-type animals and 10 wound halves from five transgenic mice from line 55. *P* value according to unpaired *t*-test: **P* = 0.0416. Measurements in **A**, **C**, and **D** were performed using the Openlab software. Shown are median values, 25th to 75th percentile (boxes) and range (vertical bars). Statistical analysis was performed with GraphPad Prism4 software. **E:** Twenty µg of protein from lysates of total skin of aged and young wild-type and transgenic mice were analyzed by Western blotting for the presence of Prdx6.

reduction in mitochondrial metabolic activity as determined by the MTT assay (Figure 3A). At intermediate levels of menadione, primary cells from transgenic mice were more protected against the toxicity of this substance. This result was obtained with two independent transgenic mouse lines. The concentration of menadione required for efficient cell death was between 12 and 37.5 µmol/L, depending on the age of the mice. Furthermore, keratinocytes of mice with B6D2 background (line 55) were generally more resistant to menadione toxicity compared with mice with FVB/N background (line 11). Nevertheless, we reproducibly observed that cells from transgenic mice are more resistant to menadione-induced toxicity compared to cells from their wild-type littermates (Figure 3A and data not shown). Consistent with this finding, we also found a reduced number of apoptotic cells in menadione-treated keratinocytes from transgenic

mice compared to wild-type mice as shown by staining for cleaved caspase 3 (Figure 3B and data not shown). Because proteins are among the major targets of ROS, we wondered whether Prdx6 overexpression is able to reduce protein oxidation in response to oxidative stress. For this purpose primary cells were irradiated with UVA, which is known to induce oxidative stress,^{37–39} and the cells were subsequently analyzed for the presence of oxidized proteins using the Oxyblot assay. As shown in Figure 3C the amount of oxidized proteins in UVA-treated Prdx6-overexpressing cells was much lower compared with control cells. A similar difference was seen in response to menadione (data not shown).

Wound Closure Is Enhanced in Aged K14-Prdx6 Transgenic Mice

Because Prdx6-overexpressing keratinocytes are more resistant to oxidative stress *in vitro*, we determined if this is also the case *in vivo*. Therefore, we subjected the animals at 8 weeks of age to full-thickness excisional wounding because high levels of ROS are produced by inflammatory cells in wounded skin.⁴⁰ Wounds were generated on the back and analyzed at different time points after injury. At day 1 after injury, which is characterized by a massive inflammatory response, we did not detect obvious histological differences (not shown). Day 5 after wounding was characterized by ongoing granulation tissue formation and re-epithelialization. No macroscopic abnormalities in wound appearance were observed at this time point (Figure 4A). Furthermore, keratinocyte proliferation in the wound epidermis was not affected as revealed by BrdU staining and subsequent counting of the BrdU-positive cells (Figure 4B and data not shown). Finally, 13 days after injury, excisional wounds in control and transgenic mice were closed and showed a similar area of granulation tissue (Figure 4C). Subsequently, we determined the consequences of enhanced Prdx6 expression on wound healing in aged mice (1 year of age). Day 5 was chosen for this purpose because re-epithelialization is maximal at this stage in young mice. As shown in Figure 4D, re-epithelialization appeared to be enhanced in aged transgenic mice compared with aged wild-type mice.

To quantify the histological results, we performed a detailed morphometric analysis of the healing skin wounds at day 5 after injury. We found a slight increase in the area of hyperproliferative epidermis as well as in the percentage of wound closure in young transgenic animals at this time point, although these parameters were not statistically significant (Figure 5, A and C). In addition, we measured the wound-bursting strength of 5-day full-thickness incisional wounds to determine the stability of the new matrix. Consistent with the normal granulation tissue formation, no significant difference in wound-bursting strength was seen (Figure 5B).

In wild-type mice, the rate of wound closure was lower in aged animals compared to young animals (Figure 5, compare C and D). This age-dependent reduction was less pronounced in the transgenic mice. Thus, aged transgenic animals showed a significantly higher rate of wound closure compared to their wild-type littermates (Figure 5D), which confirms the impression from the histological analysis (Figure 4D). Enhanced wound healing was also seen in aged mice of another transgenic mouse line, which expresses only very low levels of the transgene, although the difference was not statistically significant in this case ($P = 0.123$, data not shown). The age-dependent effect of the Prdx6 transgene on wound healing was not attributable to reduced levels of endogenous Prdx6 in aged compared to young animals as determined by Western blot analysis of total skin lysates from young and aged wild-type and transgenic mice (Figure 5E). Rather, it seems to be related to protection from accumulated oxidative damage by the overexpressed Prdx6. These findings suggest that free radical protection is not rate limiting in young mice, but obviously in aged mice, and that overexpression of Prdx6 enhances wound healing in aged mice in a dose-dependent manner.

Expression of Major Genes Involved in Wound Healing Is Not Altered in Prdx6 Overexpressing Mice

We next determined whether the enhanced levels of Prdx6 in the epidermis affect the expression of other

Table 1. Expression of Major Genes Involved in Wound Repair Is Unaltered in Prdx6 Overexpressing Mice Compared to Wild-Type Littermates

Gene	Skin		1 Day after wounding		5 Days after wounding		13 Days after wounding	
	wt	tg	wt	tg	wt	tg	wt	tg
IL-1 β	–	–	++++	++++	++	++	–	–
VEGF	+	+	++	++	++	++	+	+
Fibronectin	++	++	++	++	++	++	++	++
Collagen α 1(I)	+	+	+	+	++	++	+	+
Prdx1	+++	+++	+++	+++	+++	+++	+++	+++
HO-1	+	+	++	++	++	++	+	+
SeGpx	+	+	++	++	++	++	+	+
Mn-SOD	++	++	+++	+++	++	++	++	++
Cu/Zn-SOD	+	+	+	+	+	+	+	+

Expression levels were determined by RNase protection assays and are defined as –, not detectable; +, weakly expressed; ++, expressed at moderate levels; +++, strongly expressed; +++++, very strongly expressed.

ROS-detoxifying enzymes in normal or wounded skin. However, expression of Cu/Zn-SOD, Mn-SOD, HO-1, glutathione peroxidase I, and Prdx1 was not affected as shown by RNase protection assays. Because alterations in oxidative stress are often reflected by a different inflammatory response, we also determined the expression of the proinflammatory cytokine IL-1 β and of various chemokines but could not observe a difference. Furthermore, the expression of marker genes for angiogenesis [vascular endothelial growth factor, fibroplasia, collagen 1 α (I), and fibronectin] and keratinocyte differentiation (K6, K10, K14) was unaltered (Table 1).

Prdx6 Overexpression Protects from UVA and UVB Cytotoxicity

Finally, we determined if overexpression of Prdx6 is beneficial under conditions in which the epidermis is challenged with very high levels of ROS. Therefore, we irradiated the mice with UVA (Figure 6, A–C) or UVB (Figure 6, D–F) and determined the number of apoptotic sunburn cells 24 hours after irradiation. Indeed, we found a significantly reduced number of sunburn cells in the epidermis of transgenic mice when compared to wild-type littermates on treatment with either UVA (Figure 6, A and B) or UVB (Figure 6D). This result was consistent with the number of terminal dUTP nick-end labeling (TUNEL)-positive cells in the epidermis (Figure 6, C and E; and data not shown). Moreover, significantly reduced numbers of keratinocytes with nuclear p53 staining were seen in the transgenic mice after UVB irradiation in comparison to wild-type mice (Figure 6F). The result obtained by quantification of the number of keratinocytes with nuclear p53 was similar to the results obtained after counting sunburn cells or TUNEL-positive cells. No nuclear p53 staining was seen in nonirradiated skin (data not shown). The most likely explanation for this protective effect of Prdx6 is direct detoxification of UV-induced ROS by the overexpressed enzyme. In conclusion, our results demonstrate that enhanced levels of Prdx6 protect keratinocytes from oxidative stress *in vitro* and *in vivo*.

Discussion

Cell damage resulting from oxidative stress is thought to underlie the pathogenesis of various types of human disease, including neurodegenerative disorders and cancer.^{41,42} Therefore, cells need to develop efficient strategies to cope with enhanced levels of ROS. One mechanism to achieve this goal is the up-regulation of ROS-detoxifying enzymes, and recent studies suggest that enhanced expression of Prdx6 may be of particular importance. Thus, up-regulation of this enzyme is a physiological response to hyperoxia in the lung and kidney of

newborn rats^{43,44} and to skin and corneal injury in rodents.²⁰ In addition, strong expression of Prdx6 was observed in affected tissues of patients suffering from various diseases, including psoriasis,¹⁹ sporadic Jakob-Creutzfeld disease,⁴⁵ Parkinson's disease,⁴⁶ Pick disease,⁴⁷ and cancer.⁴⁸ All these conditions are associated with enhanced levels of ROS.^{49,50} Thus, the strong expression of Prdx6 in these diseases may be a mechanism to limit the severity of the condition. These findings suggested that forced expression of Prdx6 in endangered tissues and organs could protect cells from ROS-mediated cell damage, a hypothesis that is supported by the observation that adenoviral overexpression of Prdx6 in the lung protected mice from hyperoxic injury.¹³

ROS detoxification is of particular importance in the epidermis, which is frequently exposed to ROS-generating xenobiotics and UV irradiation, resulting in accumulation of oxidative damage. A role of Prdx6 in the antioxidant defense of keratinocytes was suggested by our previous studies, which demonstrated a strong overexpression of Prdx6 in keratinocytes of wounded and psoriatic skin.^{19,20} To determine whether these enhanced levels of Prdx6 in keratinocytes are beneficial, we generated and characterized transgenic mice, which overexpress Prdx6 in the epidermis. The transgenic mice did not reveal any obvious abnormalities in the skin, demonstrating that the enhanced levels of this enzyme do not affect normal skin homeostasis. This is an important finding, because recent studies showed that Prdx1 and Prdx2 are negative regulators of growth factor/cytokine-induced intracellular signal transduction, attributable to the reduction of the signaling molecule H₂O₂.⁵¹ Thus, it seems likely that Prdx6 does not affect signaling by growth factors, which are important for skin morphogenesis, and this hypothesis is also supported by the lack of obvious skin abnormalities in transgenic mice overexpressing Prdx6 in all cell types as well as in Prdx6 knock-out mice (our own unpublished data).^{16,18,52}

To determine whether Prdx6 is cytoprotective under stress conditions, primary keratinocytes isolated from transgenic mice were challenged with different types of oxidative stress. Indeed, Prdx6-overexpressing keratinocytes were more resistant to the cytotoxicity of various agents, which generate ROS, including menadione and UVA. This result is in accordance with previous studies, in which Prdx6 overexpression in cultured fibroblasts and in a lung-derived cell line protected from H₂O₂ and UVB toxicity.^{12,14,53} This protective effect required the peroxidase activity but not the phospholipase A2 activity of the enzyme.⁵³

Surprisingly, however, we could not find any alterations in the wound-healing process of our transgenic mice at the age of 8 to 10 weeks, although ROS are abundant in healing wounds, in particular during the early stage of repair.^{40,54} Even wound-healing studies with transgenic mice, which overexpress Prdx6 in all tissues,⁵² did not

Figure 6. Overexpression of Prdx6 in keratinocytes protects from UVA- and UVB-induced cell death *in vivo*. K14-Prdx6 transgenic mice from line 11 and sex-matched control littermates were irradiated with 75 J/cm² UVA (**A–C**) or 100 mJ/cm² UVB (**D–F**). PFA-fixed paraffin sections from the back skin of the irradiated mice were stained with H&E (**A**) or analyzed by TUNEL staining (**C**, **E**) or by immunohistochemistry with an antibody against p53 (**F**). D, dermis; E, epidermis; HF, hair follicle. **B** and **D**: The number of apoptotic sunburn cells in nine wild-type and nine transgenic animals were counted. Statistical analysis was performed using Mann-Whitney *U*-test for non-Gaussian distribution. **B**: ***P* = 0.004; **D**: ***P* = 0.0019. Scale bars = 20 μ m (**A**, **E**, **F**); 50 μ m (**C**).

reveal significant alterations of the wound-healing process (data not shown). These findings suggest that the high levels of endogenous Prdx6 in the epidermis are sufficient to protect keratinocytes against ROS in wounds of young animals under normal laboratory conditions. Although the levels of endogenous Prdx6 in the skin did not decline on aging (Figure 5E), wound closure was reduced in aged wild-type mice compared to young wild-type mice. This age-dependent reduction in the rate of wound closure was much less pronounced in the transgenic mice. Therefore, it seems likely that the continuous overexpression of an antioxidant enzyme reduces the accumulation of oxidative damage in the epidermis, thereby allowing a normal wound healing rate in the aged animal. It may be possible that Prdx6 levels are even more limiting in wounds with higher levels of ROS, eg, in infected wounds or in chronic human ulcers.⁵⁴

Finally, we determined if Prdx6 overexpression protects keratinocytes from more severe ROS-mediated insult, as seen for example after UV irradiation. Absorption of UVA by endogenous chromophores leads to the formation of ROS, in particular singlet oxygen and H₂O₂. By contrast, UVB directly damages DNA, but it can also induce oxidative stress, especially in the form of H₂O₂.⁴¹ Keratinocytes from Prdx6-overexpressing mice were efficiently protected against UVA- and UVB-induced cell death. This protective function is most likely due to Prdx6-mediated detoxification of ROS, which are generated in response to UV irradiation, as suggested by the reduced levels of oxidized proteins in irradiated keratinocytes (Figure 3C). Whether such an antiapoptotic effect is beneficial or deleterious for the tissue is under debate. On the one hand it prevents severe tissue damage, but on the other hand it may also enhance the risk of skin cancer development, because cells with damaged DNA are more prone to malignant transformation.⁵⁵ Thus, the effect of Prdx6 overexpression on skin tumorigenesis remains to be determined. In particular, it will be interesting to determine the consequences of long-term UV irradiation in wild-type and transgenic mice. Independent of a potential effect of Prdx6 overexpression on tumor formation, our results identified Prdx6 as a potent cytoprotective enzyme for keratinocytes. Thus, short-term overexpression or activation of this enzyme may be a novel strategy for the improvement of wound healing and/or for skin protection from chemical or physical insults.

Acknowledgments

We thank Christiane Born-Berclaz for excellent technical assistance, Dr. Philippe Bugnon for performing the wound-bursting strength experiments, Dr. Reinhard Dummer for providing the UVA light source, and Drs. Thomas and Agatha Schwarz for helpful suggestions with the UV irradiation experiments.

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