Haem oxygenase-1: a novel player in cutaneous wound repair and psoriasis?

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Haem oxygenase (HO) is the rate-limiting enzyme in the degradation of haem. In addition to its obvious role in iron metabolism, a series of findings indicate an important role for HO in cellular protection against oxidative stress. This effect might be of particular importance during wound healing and also in inflammatory disease. Therefore we determined the expression of the two HO isoenzymes, HO-1 and HO-2, during the healing process of full-thickness excisional wounds in mice. We show a remarkable induction of HO-1 mRNA and protein expression within three days after skin injury. After completion of wound healing, HO-1 expression declined to basal levels. By contrast, expression of HO-2 was not significantly modulated by skin injury. *In situ* hybridization and immunohistochemistry revealed high HO-1 expression in inflammatory cells of the granulation tissue and in keratinocytes of the hyperproliferative epithelium. A strong overexpression of HO-1 was also observed in the skin of patients suffering from the inflammatory skin disease psoriasis. In addition, HO-2 mRNA levels were increased in the skin of psoriatic patients. Similar to wounded skin, inflammatory cells and keratinocytes of the hyperthickened epidermis were the major producers of HO-1 in psoriatic skin. *In vitro* studies with cultured keratinocytes revealed a potential role for reactive oxygen species (ROS), but not for growth factors and pro-inflammatory cytokines, as inducers of HO-1 expression in inflamed skin. Our findings suggest a novel role for HO in wound healing and inflammatory skin disease, where it might be involved in haem degradation and in the protection of cells from the toxic effects of ROS.

Key words: epidermis, inflammation, keratinocytes, skin.

INTRODUCTION

Injury to adult skin induces the release of various chemotactic factors that attract neutrophils and macrophages into the wound. The influx of these cells is beneficial, since they play an important role in the defense against contaminating bacteria by phagocytosis and by the production and release of various proteinases and reactive oxygen species (ROS). This increased production of ROS is described as the respiratory burst. The activated neutrophils and tissue macrophages use an NADPH cytochrome *b*-dependent oxidase for the reduction of molecular oxygen to superoxide anions [1]. In addition to these inflammatory cells, other cell types, such as fibroblasts, can also be stimulated to produce ROS in response to pro-inflammatory cytokines [2]. Although the oxidative burst is important for defence against microbial infection, prolonged production of high levels of ROS can cause severe tissue damage, as seen in chronic inflammatory disease. Most importantly, high levels of ROS cause DNA mutations that can lead to neoplastic transformation [3]. Therefore cells in injured tissues must be able to protect themselves against the toxic effects of ROS. For this purpose, two major strategies have been developed: (i) the use of small antioxidant molecules, such as ascorbate, polyunsaturated fatty acids or sugars, and (ii) the use of ROS-scavenging enzymes, including superoxide dismutases, catalase and several types of peroxidase [4,5].

Recent studies from our laboratory have provided evidence for an important role of ROS-detoxifying enzymes in cutaneous wound repair. We have demonstrated a striking increase in the expression of two types of superoxide dismutase, as well as of catalase and the selenoenzymes glutathione peroxidase I and phospholipid hydroperoxide glutathione peroxidase, after injury to adult mouse skin [6]. Furthermore, expression of a novel ROS-detoxifying enzyme (non-selenium glutathione peroxidase; also known as 1-Cys-peroxiredoxin and antioxidant protein 2) was shown to be expressed at high levels in wounded and psoriatic skin [7,8]. Although these enzymes are expressed by many cell types in the wound, a particularly high expression of most of these enzymes was detected in keratinocytes of the hyperproliferative epithelium [6,7]. The strong expression of ROS-detoxifying enzymes in keratinocytes of normal skin [9,10], and particularly in cutaneous wounds, might provide an explanation for the observed resistance of keratinocytes to fairly high doses of hydrogen peroxide [11]. This could be an important prerequisite for the ability of these cells to migrate and proliferate even in the presence of high levels of ROS, which are known to inhibit these processes [12].

Besides these 'classic' antioxidant enzymes, other oxidant-induced gene products might be involved in the protection of cells against oxidative stress. A series of recent studies has provided evidence for an important cytoprotective function of haem oxygenase (HO) [13]. HO is the rate-limiting enzyme in the degradation of haem into carbon monoxide, iron and biliverdin, which is subsequently reduced to bilirubin [14]. Three different isoforms of HO have been identified, including the inducible isoform HO-1, the constitutive isoform HO-2, and a recently identified isoform HO-3, with low enzymic activity [13]. Expression of HO-1, a protein also known as heat shock protein 32, is induced by its substrate haem, but also following exposure to a wide variety of stressful stimuli, including UV irradiation, hydrogen peroxide, nitric oxide, heavy metals, phorbol esters, lipopolysaccharide and organic chemicals [15,16]. Most

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; HO, haem oxygenase; KGF, keratinocyte growth factor; PECAM, platelet endothelial cell adhesion molecule; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α.

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interestingly, most of the known HO-1 inducers stimulate the production of ROS or lead to depletion of GSH levels, suggesting the involvement of HO-1 activity in cellular protection against oxidative stress. Given the high levels of ROS produced during the early phase of cutaneous wound repair and in inflammatory skin disease, as well as the role of HO described above in protection against ROS, we became interested in a possible role of HO in inflammatory processes of the skin. In the present study we provide evidence for a novel role of HO in wound repair, as well as in the inflammatory skin disease psoriasis, which is characterized by hyperproliferation and incomplete differentiation of epidermal keratinocytes.

EXPERIMENTAL

Cell culture experiments

HaCaT keratinocytes [17] were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) containing 1 % (w/v) penicillin/streptomycin and 10 % (v/v) fetal calf serum. They were rendered quiescent by serum starvation for 16–20 h and were subsequently treated with fresh DMEM containing 10 ng/ml recombinant human keratinocyte growth factor (KGF), 20 ng/ml epidermal growth factor (EGF), 1 ng/ml transforming growth factor-β1 (TGF-β1), 300 units/ml tumour necrosis factor-α (TNF-α), 50–500 μM hydrogen peroxide or 12.5–25 μM menadione. Cells were harvested at different time points after growth factor, cytokine, hydrogen peroxide or menadione stimulation and were used for RNA isolation. Each experiment was repeated at least twice. DMEM and serum were purchased fom Gibco BRL, growth factors were obtained from Roche Biochemicals (Mannheim, Germany), and hydrogen peroxide and menadione were purchased from Sigma.

RNA isolation and RNase protection assay

Isolation of total cellular RNA was performed as described previously [18]. RNase protection assays were carried out according to the method described in [19]. Autoradiograms were scanned and analysed with Scion Image software (Scion Corporation, Frederick, MD, U.S.A.).

cDNA templates

Murine and human cDNAs for HO-1 and HO-2 were amplified from the cDNA of HaCaT cells and mouse wounds respectively. The following fragments were amplified: a 301 bp fragment corresponding to nt 605–905 of the murine HO-1 cDNA (accession no. X13356); a 191 bp fragment corresponding to nt 431–621 of the human HO-1 cDNA (accession no. X06985); a 234 bp fragment corresponding to nt 380–613 of the murine HO-2 cDNA (accession no. AF029874); and a 234 bp fragment corresponding to nucleotides 223–456 of the human HO-2 cDNA (accession no. AF051306). The amplified cDNAs were cloned into the transcription vector pBluescript KSII(+) (Stratagene, La Jolla, CA, U.S.A.), and were used as templates for RNase protection assays and/or *in situ* hybridizations.

Animals

Balb/c mice were obtained from RCC (Füllinsdorf, Switzerland). They were housed and fed according to Federal guidelines, and all procedures were approved by the local authorities.

Wounding and preparation of wound tissue

Balb/c mice (8–12 weeks old) were anaesthetized with a single intraperitoneal injection of ketamine/xylazine. The hair on the back of the animals was cut and the skin was wiped with 70 % ethanol. Full-thickness excisional wounds (four; 5 mm in diameter, 3–4 mm apart) were generated on the back of each animal by excising skin and *panniculus carnosus*. The wounds were allowed to dry to form a scab. Animals were sacrificed at different time points after injury. For RNA isolation and for preparation of tissue lysates, complete wounds were isolated, including 2 mm of the wound margins, immediately frozen in liquid nitrogen and stored at -70 °C. For *in situ* hybridization, complete wounds were isolated, bisected and fixed overnight at 4 °C in 4 % (w/v) paraformaldehyde in PBS. They were subsequently incubated in 15 % (w/v) sucrose in PBS for 4 h at 4 °C, and were frozen in tissue-freezing medium (Jung, Nussloch, Germany). For immunohistochemistry, bisected wounds were directly frozen in tissue-freezing medium without prior fixation.

Human skin biopsies

Biopsies were obtained from stable psoriatic skin lesions located to the upper and lower extremities of psoriasis patients ($n = 7$; 33–65 years of age). Normal skin was obtained from skin transplants of patients attending the Dermatology Department for surgery ($n = 6$; 31–79 years of age). All samples included the dermis and the epidermis. The biopsies were immediately frozen in liquid nitrogen and stored at -80 °C until they were used for RNA isolation or embedding in tissue-freezing medium. None of the patients had received oral or topical treatment for the 8 weeks preceding biopsy. All patients signed informed consent for the Department of Dermatology, University of Cologne; approved by the Institutional Commission of Ethics (Az. 9645/96).

Preparation of HaCaT cell lysates, skin lysates and Western-blot analysis

HaCaT cells were treated with menadione as described above. For Western-blot analysis cells were lysed in 20 mM Tris/HCl (pH 8.0), 1 % (v/v) Triton X-100, 137 mM NaCl, 10 % (v/v) glycerol, 2 mM EDTA, 1 mM PMSF and 0.15 unit/ml aprotinin. Normal and wounded skin (see above) was frozen in liquid nitrogen. Preparation of tissue lysate was performed as described previously [19]. Protein (60 μg in total) from HaCaT cells or skin tissue was analysed by Western blotting, under reducing conditions, using polyclonal rabbit antibodies directed against HO-1 and HO-2 (StressGen Biotechnologies Corp., Victoria, BC, Canada; SPA-895 and OSA-200 respectively; 1:1000 dilution) or polyclonal goat antibodies directed against HO-1 (sc-1797 and sc-7695; Santa Cruz Biotechnology, La Jolla, CA, U.S.A.; 1:100 dilution). The anti-(HO-1) antibodies from Santa Cruz Biotechnology were used for the detection of the human protein, and the anti-(HO-1) antibody from StressGen Biotechnologies Corp. was used for the detection of the murine protein. The enhanced chemoluminescence (ECL®) detection system (Amersham International, Braunschweig, Germany) was used to visualize HO-1 and HO-2 proteins.

Immunofluorescence

For the immunohistochemical detection of HO-1 and HO-2 we used the anti-(HO-1) and anti-(HO-2) antibodies described above, as well as an FITC-coupled antibody directed against platelet endothelial cell adhesion molecule (PECAM; Pharmingen, San Diego, CA, U.S.A.), an antibody directed against murine macrophages (anti-F4/80; Serotec, Kidlington, Oxford, U.K.), and an antibody against human macrophages (anti-CD68; BioGenex Laboratories, San Ramon, CA, U.S.A.). The

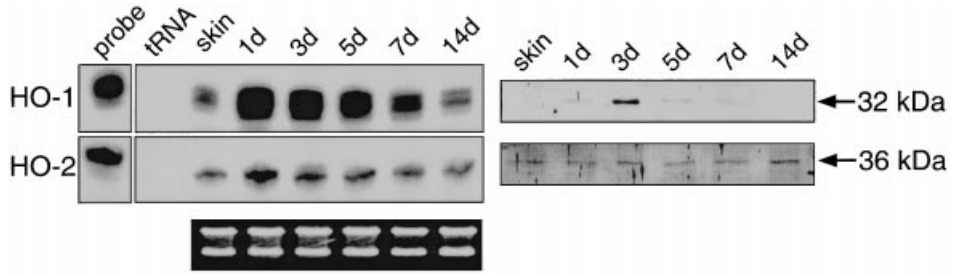

Figure 1 Increased expression of HO-1 mRNA and protein during cutaneous-wound healing in mice

Mice were wounded as described in the Experimental section and were sacrificed at different time points after injury. Left panel: total cellular RNA (20 μg) from normal and wounded skin was analysed by RNase protection assay for the expression of HO-1 and HO-2, as indicated. tRNA (20 μg) was used as a negative control. Hybridization probes (1000 c.p.m.) were loaded in the lanes labelled 'probe' and were used as size markers. The same set of RNAs were used for the HO-1 and HO-2 protection assays. Each RNA sample (1 μg) was loaded on to a 1% (w/v) agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays. Right panel: total protein (60 μg) from normal and wounded skin was analysed by Western blotting for the expression of HO-1 and HO-2. d, day-old.

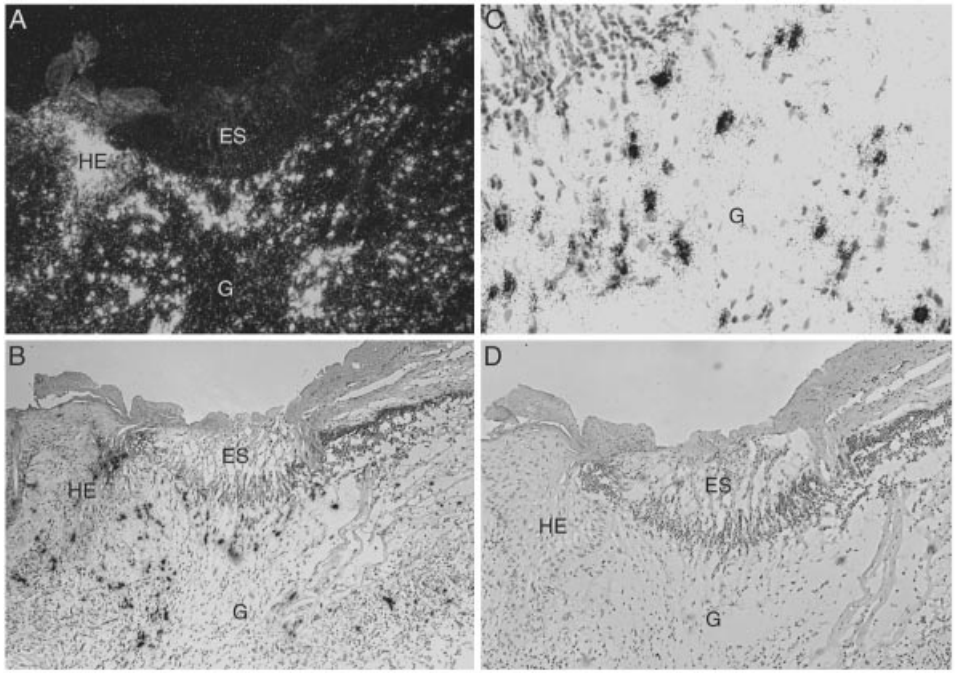

Figure 2 HO-1 mRNA is expressed in keratinocytes of the hyperproliferative epithelium and in the granulation tissue of full-thickness excisional mouse wounds

Frozen sections from the middle of 5-day-old full-thickness excisional wounds were hybridized with ^{35}S-labelled sense (**D**) and antisense (**A**–**C**) riboprobes. Overviews of the wound are shown in (**A**, **B**, and **D**) (65 × magnification). Details of the granulation tissue are shown in (**C**) (260 × magnification). Signals appear as black dots in the bright field surveys (**B**, **C** and **D**) and as white dots in the dark field survey (**A**). HE, hyperproliferative epithelium; G, granulation tissue; ES, eschar.

anti-(HO-1) antibodies from Santa Cruz Biotechnology were used for the detection of the human protein, and the anti-(HO-1) antibody from StressGen Biotechnologies Corp. was used for the detection of the murine protein. Double-immunofluorescence was performed with the following antibody combinations: (i) anti-(HO-1)/anti-(PECAM-FITC); (ii) anti-(HO-1)/anti-F4/80; and (iii) anti-(HO-1)/anti-CD68. Detection was performed with the following secondary antibodies: horse anti-rat IgG coupled to Cy3™, rabbit anti-goat IgG coupled to Cy3™ (both from Jackson Laboratories, West Grove, PA, U.S.A.), goat anti-rabbit IgG coupled to FITC (Roche Biochemicals), and rabbit anti-mouse IgG coupled to FITC (Sigma).

Frozen sections were fixed for 10 min in ice-cold acetone (−20 °C) and washed three times for 10 min with PBS. The primary antibodies were diluted in PBS containing 1% BSA and 0.2% Tween 20 [anti-(HO-1) (StressGen Biotechnologies Corp.), 1:100 dilution; anti-(HO-1) (Santa Cruz Biotechnology), 1:50 dilution; anti-(HO-2), 1:100 dilution; anti-F4/80, 1:100 dilution; anti-(PECAM-FITC), 1:200 dilution; and anti-CD68, 1:200 dilution] and the slides were incubated overnight at 4 °C with these antibodies. After three 10 min washes with PBS/0.2% Tween 20 the sections were incubated for 45 min with the secondary antibodies (diluted 1:100 in PBS/12% BSA/0.2% Tween 20). The slides were rinsed three times with PBS/

0.2% Tween 20 and overlaid with coverslips using mounting medium.

In situ hybridization

Sense and antisense murine and human HO-1 and HO-2 riboprobes were generated using T3 or T7 RNA polymerases and [α-^{35}S]UTP. Frozen sections (6 μm) from the middle of 5-day-old wounds were hybridized as described previously [20]. After hybridization, sections were coated with NTB2 nuclear emulsion (Kodak) and exposed in the dark for 4 weeks. After development they were counterstained with hematoxylin/eosin and mounted.

RESULTS

To gain insight into a possible role for HO in cutaneous wound repair, we first determined the expression of HO-1 and HO-2 in normal and wounded mouse skin. RNase protection assays with RNAs from non-wounded skin and from full-thickness excisional wounds at different stages of the healing process revealed a similar level of expression of HO-1 and HO-2 mRNAs in non-wounded skin. After skin injury, a slight (2–3-fold) upregulation of HO-2 expression was observed within 1 day after wounding, but the mRNA levels rapidly declined to basal levels (Figure 1, left-hand panel). By contrast, a striking induction (5–10-fold) of HO-1 expression was seen at day 1 after wounding. HO-1 mRNA levels were still elevated at day 7 after injury. At day 14 after wounding, when the wound was completely re-epithelialized, HO-1 mRNA levels had declined to basal levels (Figure 1, left-hand panel). These results were reproduced in three independent RNase protection assays using RNAs from different wound-healing experiments.

We subsequently determined whether the increase in HO-1 mRNA levels correlated with the induction of the corresponding protein. For this purpose Western-blot experiments were performed with lysates from non-wounded skin and from skin wounds at different stages after injury. As shown in Figure 1 (right-hand panel), increased amounts of the 32 kDa HO-1 protein were detected in lysates from 1-, 3-, 5- and 7-day-old wounds. Maximal levels were seen at day three after wounding. This result was reproduced with another antibody from a different company (results not shown). In contrast to HO-1, no significant increase in the level of the 36 kDa HO-2 protein was detected (Figure 1, right-hand panel). Taken together, these protein data correlate well with the mRNA results obtained by RNase protection assay.

To localize HO-1 and HO-2 mRNAs we analysed the expression of these enzymes by *in situ* hybridization of 3-day-old and 5-day-old mouse wounds. As shown in Figures 2(A)–2(C) for 5-day-old wounds, HO-1 mRNA was expressed in clusters of cells in the clot and the granulation tissue. In addition, strong signals were seen throughout the hyperproliferative epithelium at the wound edge, with particularly high mRNA levels being present in the upper-most layers of living cells (Figures 2A and 2B). A gradient effect for HO-1 expression in keratinocytes was observed at the wound edge, and HO-1 transcripts could not be detected in the non-wounded epidermis (results not shown). No signal was obtained with the sense probe (Figure 2D). A similar distribution of HO-1 expression was seen in 3-day-old wounds (results not shown). In contrast with HO-1, HO-2 transcripts were present at low levels in almost every cell within normal and wounded skin (results not shown).

To localize HO-1 and HO-2 proteins within the skin, we stained wounded back skin at day 5 after injury with antibodies directed against HO-1 and HO-2. As shown in Figure 3(A), strong HO-1 signals were seen in a certain population of cells

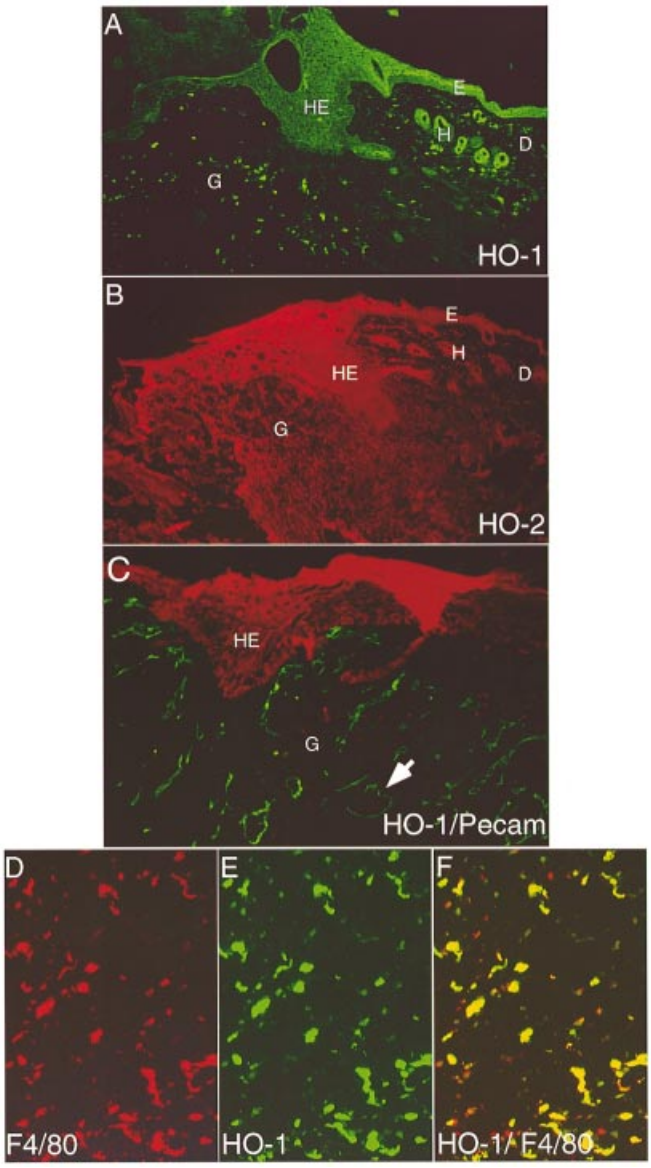

Figure 3 Expression of HO-1 and HO-2 proteins in 5-day-old wounds

Frozen sections were taken from the middle of the wound and incubated with antibodies against HO-1 (**A**, **C**, **E** and **F**), HO-2 (**B**), F4/80 (**D** and **F**) and PECAM (**C**). Double-immunofluorescence with antibodies against HO-1 (green) and the macrophage marker F4/80 (red) is shown in (**F**). Double-immunofluorescence with antibodies against HO-1 (red) and the endothelial cell marker PECAM (green) is shown in (**C**). E, epidermis; H, hair follicle; D, dermis; HE, hyperproliferative epithelium; G, granulation tissue. Magnification 59 × (**A**–**C**) and 236 × (**D**–**F**).

within the granulation tissue. The majority of these cells appear to be macrophages, as demonstrated by double-immunofluorescence with antibodies against HO-1 (green) and the macrophage marker F4/80 (red) (Figures 3D–3F), but other cells in the granulation tissue also expressed HO-1 (Figure 3F, green colour). These cells are not endothelial cells, as demonstrated by double-staining with the HO-1 antibody (red) and an antibody against the endothelial cell marker PECAM (green) (Figure 3C). In addition to the granulation tissue, HO-1 was strongly expressed in hair follicle keratinocytes at the wound edge and throughout the hyperproliferative wound epidermis (Figures 3A and 3C).

In contrast with the restricted expression pattern of HO-1, HO-2 protein was found to be ubiquitously expressed in normal and wounded skin (Figure 3B).

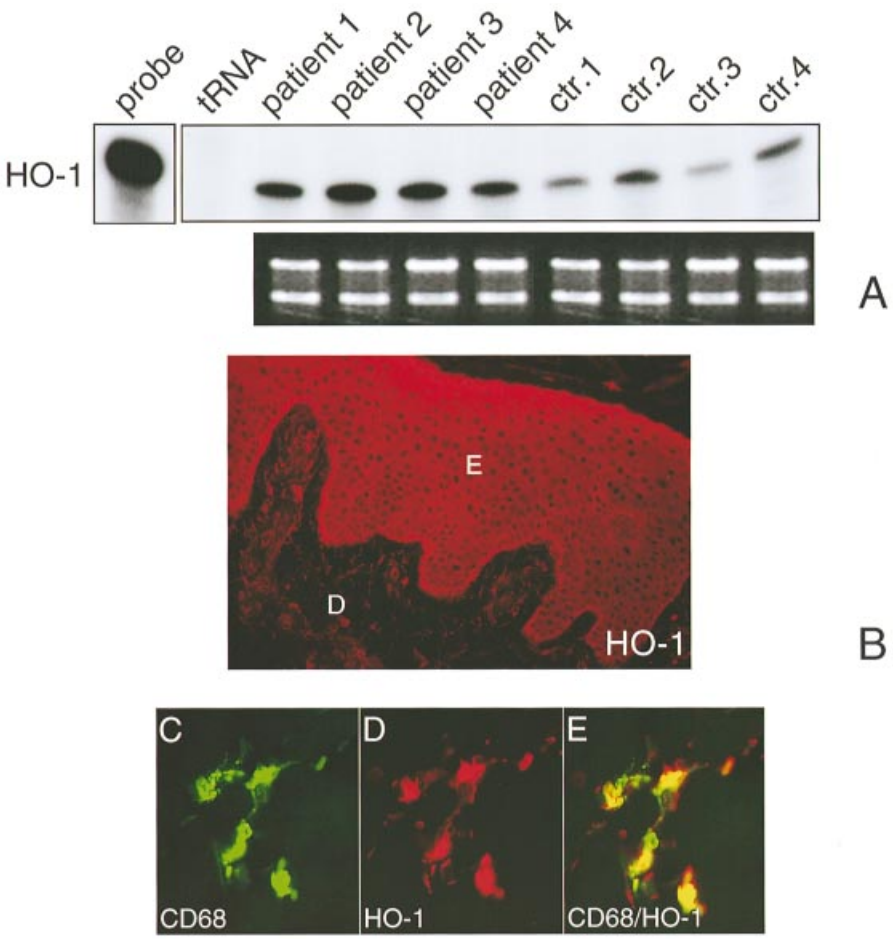

Figure 4 Increased expression of HO-1 mRNA and protein in psoriatic skin

(**A**) RNA (10 μg) isolated from skin biopsies of normal (ctr.) and psoriatic skin (patient) was analysed by RNase protection assay for the presence of HO-1 mRNA. tRNA (20 μg) was used as a negative control. Hybridization probe (1000 c.p.m.) was loaded into the lane labelled 'probe' and was used as a size marker. Each RNA sample (1 μg) was loaded on to a 1% (w/v) agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays. Frozen sections (6 μm) from psoriatic skin were analysed by indirect immunofluorescence with a HO-1-specific antiserum. HO-1 expression in the hyperproliferative epithelium is shown in (**B**). Staining of the dermis with antibodies against HO-1 (red) and the macrophage-specific antigen CD68 (green) are shown in (**C** and **D**). HO-1-expressing macrophages appear yellow in the overlay (**E**). Magnification 140 $\times$ (**B**) and 280 $\times$ (**C**–**E**). ctr., control.

The presence of inflammatory cells and epithelial hyperthickening is also a characteristic feature of psoriatic skin. Therefore we determined the expression of HO-1 and HO-2 in the skin of patients suffering from this inflammatory skin disease. RNA was isolated from 4 mm biopsies of healthy control skin and from lesioned skin and was analysed by RNase protection assay for the presence of HO-1 mRNA. As shown in Figure 4(A), expression of HO-1 was low in control skin ($n = 6$; 4 samples shown). However, 2–5-fold elevated HO-1 mRNA levels were found in psoriatic skin ($n = 7$; 4 samples shown). The two control samples (ctr. 2 and 4), which were characterized by a slightly higher HO-1 mRNA expression, had been taken from highly sun-exposed skin. The distribution of HO-1 protein in psoriatic skin was similar to wounded skin with highest expression being found in the hyperproliferative epidermis (Figure 4B). Furthermore, dermal cells of psoriatic lesions expressed HO-1 (Figure 4C). Double-immunofluorescence with antibodies against HO-1 and the macrophage-specific antigen, CD68, revealed that most of these cells are macrophages (Figures 4C–4E), although other dermal cells also expressed HO-1. In addition, increased levels of HO-2 mRNA were also seen in psoriatic skin, although the extent of overexpression was low compared with HO-1 (Figure 5A). Similarly to wounded skin, HO-2 expression was seen in almost every cell in normal human skin (results not shown) and in psoriatic lesions (Figure 5B).

The experiments described above demonstrate a strongly increased expression of HO-1 in keratinocytes of the wound epithelium and in the epidermis of psoriatic patients. These tissues are characterized by the presence of increased levels of various growth factors and cytokines and by multiple inflammatory cells which produce ROS (for review see [21–23]). To determine whether any of these molecules might be responsible for the strong expression of HO-1 in keratinocytes, we analysed the effects of growth factors, cytokines and ROS on the expression of HO-1 in the human immortalized, but non-transformed, HaCaT keratinocyte cell line. Consistent with previous results obtained with human primary keratinocytes [24], expression of HO-1 mRNA was hardly detectable in quiescent HaCaT cells. This low basal expression was not increased by the addition of the epithelial mitogens KGF and EGF. Furthermore, the pro-inflammatory cytokine TNF-α had no effect on HO-1 expression. The only response was seen with TGF-β1. This factor caused a slight and transient increase in the expression of this enzyme (results not shown). These data suggest that growth factors and

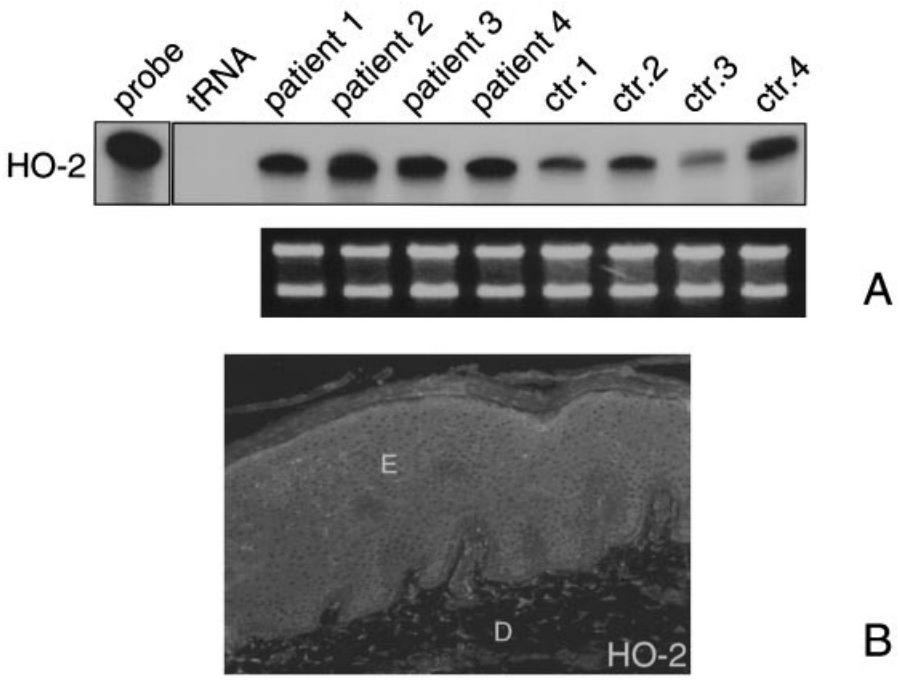

Figure 5 Increased expression of HO-2 mRNA in psoriatic skin

(**A**) RNA (10 μg) isolated from skin biopsies of normal (ctr.) and psoriatic skin (patient) was analysed by RNase protection assay for the presence of HO-2 mRNA. tRNA (20 μg) was used as a negative control. Hybridization probe (1000 c.p.m.) was loaded into the lane labelled 'probe' and was used as a size marker. Each RNA sample (1 μg) was loaded on to a 1% (w/v) agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays. (**B**) Frozen sections (6 μm) from psoriatic skin were analysed by indirect immunofluorescence using a HO-2-specific antiserum. Magnification 140 $\times$. ctr., control.

cytokines are not responsible for the increase in HO-1 expression after wounding and for the strong expression of this enzyme in psoriatic skin. In contrast with HO-1, HO-2 mRNA was expressed at significantly higher levels in HaCaT cells. This fairly high basal expression level was further increased by addition of KGF (3.5-fold) and particularly by EGF (7-fold) (Figure 6). TGF-β1 and TNF-α caused only very weak HO-2 induction (results not shown).

To determine a potential role for ROS in the induction of HO-1 expression in keratinocytes of wounded and psoriatic skin, we treated HaCaT cells with different concentrations of hydrogen peroxide. As shown in Figure 7 (upper panel), a slight and transient increase in HO-1 mRNA expression was observed within 1.5 h after the addition of 50 μM hydrogen peroxide to these cells. A 10-fold higher hydrogen peroxide concentration caused a much stronger increase, but the time course of induction was identical (Figure 7, upper panel). These concentrations of hydrogen peroxide did not lead to increased cell death within the treatment period as determined by Trypan Blue staining (results not shown).

Since keratinocytes are resistant to fairly high doses of hydrogen peroxide [11], and since the half-life of hydrogen peroxide in the medium is very short, we subsequently treated the cells with 12.5 and 25 μM of menadione. The latter is a potent xenobiotic which leads to continuous production of superoxide anions [25]. This treatment resulted in a minor induction of HO-2 expression (results not shown), but in a striking (85-fold) and long-lasting (8 h) increase in HO-1 mRNA expression (Figure 7, middle panel). This result was confirmed by Western blotting with an antibody directed against HO-1 (Figure 7, lower panel). HO-1 protein was not detectable in non-treated cells, but was strongly upregulated within 5–8 h after addition of the xenobiotic. These findings suggest that the continuous presence of high levels of ROS in wounds and in psoriatic skin might be, at least partially, responsible for the upregulation of HO-1 expression in keratinocytes *in vivo*.

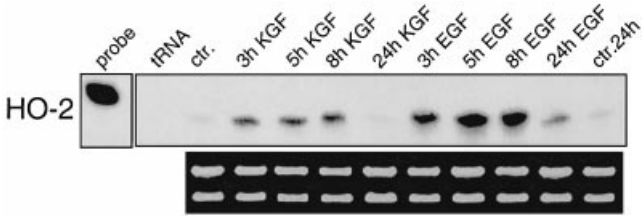

Figure 6 KGF and EGF induce HO-2 mRNA expression in keratinocytes

HaCaT keratinocytes were rendered quiescent by serum starvation. They were subsequently treated with KGF and EGF as described in the Experimental section. Total RNA was harvested after 3, 5, 8 and 24 h. Total cellular RNA (20 μg) was analysed by RNase protection assay for the expression of HO-2 mRNA. Each RNA sample (1 μg) was loaded on to a 1% (w/v) agarose gel and stained with ethidium bromide (shown below the protection assay). tRNA (20 μg) were used as a negative control. Hybridization probe (1000 c.p.m.) was loaded into the lane labelled 'probe' and was used as a size marker. ctr., control.

DISCUSSION

Injury to the skin is accompanied by disruption of blood vessels and subsequent release of haemoglobin and myoglobin from erythrocytes. The free haem can promote free radical formation and lipid peroxidation, resulting in severe tissue damage. Therefore rapid degradation of the free haem is required, a function which can exclusively be fulfilled by the action of HO [26]. Thus

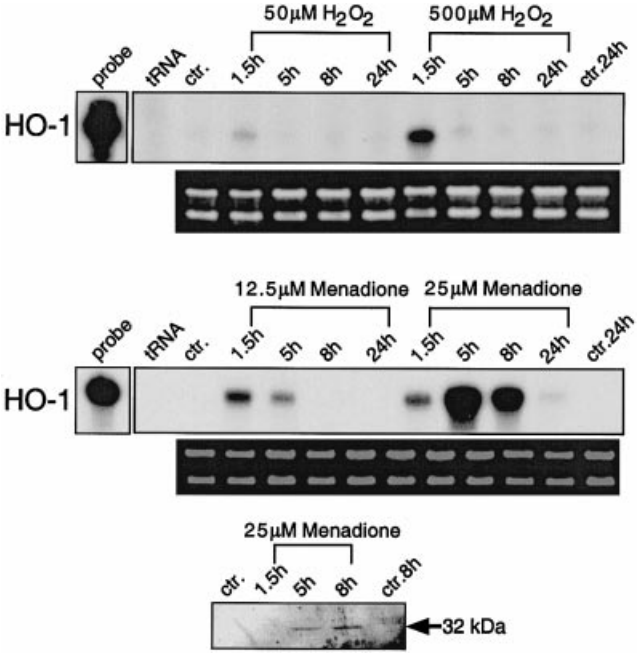

Figure 7 HO-1 expression is upregulated by hydrogen peroxide and menadione in cultured keratinocytes

HaCaT keratinocytes were rendered quiescent by serum starvation. They were subsequently treated with hydrogen peroxide or menadione as described in the Experimental section. Upper and middle panels: total RNA was isolated 1.5, 5, 8, and 24 h after the addition of hydrogen peroxide or menadione, as indicated. Total cellular RNA (20 μg) was analysed by RNase protection assay for the expression of HO-1 and HO-2 mRNAs. Each RNA sample (1 μg) was loaded on to a 1% (w/v) agarose gel and stained with ethidium bromide (shown below the protection assays). tRNA (20 μg) were used as a negative control. Hybridization probes (1000 c.p.m.) were loaded into the lanes labelled 'probe' and were used as size markers. Lower panel: total protein (60 μg) from non-treated (ctr.) and menadione-treated HaCaT cells was analysed by Western blotting for the expression of HO-1.

the presence of high levels of HO in the skin [24], and the rapid induction of HO-1 expression after skin injury (the present study) is likely to be of major importance for the prevention of haem toxicity. However, this is unlikely to be the only function of HO in wound repair, due to the multiple functions of the products generated by HO. Thus one product of HO action, biliverdin, is rapidly reduced to the potent anti-oxidant bilirubin [27]. This antioxidant function could be of major importance during cutaneous wound repair and in inflammatory skin disease, since large amounts of ROS are produced by granulocytes and macrophages under these conditions. Due to the high toxicity of these aggressive molecules, the ROS-producing cells, as well as neighbouring cells, need to develop strategies to protect themselves against ROS. Indeed, previous studies by ourselves and others have demonstrated a strong overexpression of various ROS-detoxifying enzymes in wounded and psoriatic skin [6–8,28]. In addition to these classical protective enzymes, a series of recent data have provided evidence for a cytoprotective role of HO-1 *in vitro* [29–32] and also *in vivo*. Thus mice lacking a functional HO-1 gene can survive to adulthood, but they reveal significant defects in iron re-utilization [33], and they are vulnerable to mortality and hepatic necrosis on treatment with endotoxin [34]. Furthermore, cardiomyocytes from HO-1 null mice have a maladaptive response to hypoxia and subsequent pulmonary hypertension [35]. Finally, the important role of HO-1 in the defence against oxidative stress was strongly supported by the hypersensitivity of HO-1-deficient fibroblasts to cytotoxicity caused by haemin, hydrogen peroxide, serum deprivation, staurosporine or etoposide [34,36]. In addition to HO-1, HO-2 knockout animals were also sensitized to hyperoxia-induced oxidative injury and mortality [37]. Thus it seems likely that HO can indeed protect cells under stress situations.

To determine a possible cytoprotective role for HO in inflamed skin, we analysed the expression of HO-1 and HO-2 during the healing process of mouse skin wounds. Highest HO-1 mRNA and protein levels were observed during the early inflammatory phase of wound repair and in psoriatic skin where particularly high levels of ROS are produced. *In situ* hybridization and immunohistochemistry revealed strong HO-1 signals in macrophages. Since these cells produce high levels of ROS during the oxidative burst, they need to be particularly protected against these molecules. Recent *in vitro* studies suggested that induction of HO-1 expression by oxidative stress is part of this defence mechanism [38], and our *in vivo* findings support this hypothesis.

High HO-1 mRNA and protein levels were also detected in the hyperproliferative epithelium. This is remarkable, since cultured primary keratinocytes [24], and HaCaT cells (the present study) express extremely low levels of this enzyme. Thus it seems likely that the strong HO-1 expression in the wounded epidermis and in keratinocytes of psoriatic skin is due to stimulation of HO-1 expression by factors present in the inflamed skin. Our *in vitro* results suggest that growth factors and pro-inflammatory cytokines that are present at high levels in the wound and in psoriatic skin are not responsible for this increase. By contrast, ROS might be important inducers. Although hydrogen peroxide treatment resulted only in a transient and rather weak induction of HO-1 expression in primary keratinocytes [24], and HaCaT cells (the present study), addition of the xenobiotic menadione, which leads to continuous production of superoxide anions, caused a strong and long-lasting expression of HO-1 mRNA and protein. Since large amounts of superoxide anions are continuously generated by inflammatory cells [1] and other cell types [39] in wounded and psoriatic skin, these molecules are likely to be involved in the strong HO-1 expression seen in these tissues. Furthermore, nitric oxide, a potent stimulator of HO-1 expression in keratinocytes and other cell types [40,41], might play a role in HO-1 induction in inflamed skin, since this radical has been shown to be produced in wounded and psoriatic skin [42]. Finally, haem derived from the haemoglobin that is released from erythrocytes in the bleeding skin, as well as the hypoxic conditions present in the wounded tissue, could further increase HO-1 expression [43,44].

In contrast with HO-1, the increased levels of HO-2 mRNA seen in psoriatic skin are unlikely to be the result of the elevated levels of ROS in inflamed skin, since neither hydrogen peroxide nor menadione induced HO-2 expression in cultured keratinocytes. By contrast, growth factors, such as KGF and EGF receptor ligands, which have been shown to be overexpressed in psoriatic skin [45,46], might be responsible for the increase, since both KGF and EGF stimulated HO-2 expression in HaCaT keratinocytes.

In summary, the striking upregulation of HO-1 expression in inflamed skin, together with the fairly high levels of HO-2 in normal, wounded and diseased skin, suggest an important role for HO activity in skin homoeostasis and particularly in skin repair and disease. Given the emerging evidence for a cytoprotective role of HO under conditions of oxidative stress, it seems likely that the high levels of HO present in inflamed skin play an important role in the protection of various cell types from the toxic effects of ROS. In addition, HO might be involved in the hyperproliferation of keratinocytes during wound healing and in psoriasis, since recent data have suggested a role for the

HO pathway in keratinocyte proliferation mediated by nitric oxide [40]. Finally, carbon monoxide, one of the three main by-products of the catabolism of haem by HO, has recently been shown to mediate potent anti-inflammatory effects [47]. Thus the production of this molecule as a result of increased HO activity could be a novel defence mechanism of cells in inflamed tissues which might lead to a limitation of the inflammatory process.

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