Heparin-Binding EGF-Like Growth Factor Is Induced by Disruption of Lipid Rafts and Oxidative Stress in Keratinocytes and Participates in the Epidermal Response to Cutaneous Wounds

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Epidermal homeostasis and repair of the skin barrier require that epidermal keratinocytes respond to alterations of their environment. We report that cellular stress with methyl-β-cyclodextrin (MBCD), a molecule that extracts membrane cholesterol and thereby disrupts the structure of lipid rafts, strongly induces the synthesis of heparin-binding EGF-like growth factor (HB-EGF) in keratinocytes through the activation of p38 mitogen-activated protein kinase. Interesting parallels between lipid raft disruption and oxidative stress can be drawn as hydrogen peroxide induces p38 activation and HB-EGF synthesis in keratinocytes. Consistent with other studies, we show increased HB-EGF expression in keratinocytes located at the margin of wounded skin areas. Analyzing cultured keratinocytes exposed to rhHB-EGF, we report increased HB-EGF mRNA levels and alterations in the expression of differentiation markers. Interestingly, identical alterations in differentiation markers are shown to occur *in vivo* at the wound margin and in HB-EGF-treated cultures. In addition, *in vitro* sectioning of skin samples also induces the expression of HB-EGF at the border of the incisions. Altogether, our data suggest that expression of HB-EGF is a marker of the keratinocyte's response to a challenging environment and demonstrate that this growth factor alters the phenotype of keratinocytes in a manner similar to that observed during epidermal repair.

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INTRODUCTION

The skin fulfills essential barrier functions, as it protects internal tissues from environmental, physical, chemical, or biological stresses. The epidermis is formed by a keratinized squamous epithelium stratified into basal, spinous, granular, and cornified layers. The epidermis is mainly composed of keratinocytes that proliferate in the basal layer and undergo a complex differentiation program in the suprabasal layers leading to keratinization including cell death. Epidermal homeostasis is assured by an accurate lipid barrier regulation and a precise coordination between cell proliferation within the basal layer, differentiation in suprabasal layers, and desquamation of dead keratinocytes from the surface of the skin. On a cellular level, this coordination is due to a molecular interplay between cell adhesion, growth factors, and other particular differentiation signals.

Our previous study has shown that membrane cholesterol depletion perturbs keratinocyte differentiation in a p38 mitogen-activated protein kinase (MAPK)-dependent way (Jans *et al.*, 2004). On cellular level, cholesterol is an essential component of lipid rafts, plasma membrane microdomains. Methyl-β-cyclodextrin (MBCD), by its property to extract membrane cholesterol, disorganizes the structure of lipid rafts (Kabouridis *et al.*, 2000) and alters lipid raft-induced signaling pathways (Simons and Toomre, 2000). The crucial role of cholesterol in keratinocytes is particularly shown in animals unable to convert desmosterol into cholesterol. These animals exhibit a profoundly perturbed differentiation process in the epidermis (Mirza *et al.*, 2006), where desmosterol cannot replace cholesterol in lipid rafts (Vainio *et al.*, 2006). In epidermal wound healing, cholesterol probably also plays a physiological role, possibly by the intermediate of peroxisome proliferator-activated receptor-α (PPAR-α). In fact, impaired skin wound healing was shown in dominant-negative PPAR-α skin (Michalik *et al.*, 2005), suggesting a role for PPAR-α during epidermal repair. Recently, it has been shown that activated PPAR-α decreases cellular cholesterol content and concentration (Konig *et al.*, 2007).

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Abbreviations: HB-EGF, heparin-binding EGF-like growth factor HER-1, human epidermal growth factor receptor-1; rhHB-EGF, recominant human HB-EGF; HRP, horseradish peroxidase; K10, keratin 10; MAPK, mitogen-activated protein kinase; MBCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline

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Stresses applied to keratinocytes, for instance, during wounding, perturb epidermal homeostasis, and mechanisms are initiated to repair the epidermal tissue (Singer and Clark, 1999; Santoro and Gaudino, 2005). Cutaneous injury involves reactive oxygen species generation, and wound healing is subjected to redox control (Sen, 2003). Some years ago, it was discovered that hydrogen peroxide can act as a signaling molecule (Hancock *et al.*, 2001). Low concentrations of H_2O_2 seem to be beneficial for skin wound healing (Roy *et al.*, 2006), probably because H_2O_2 promotes the expression of wound healing-related growth factors such as vascular endothelial growth factor (Sen *et al.*, 2002). UVB-generated H_2O_2 is able to activate the EGFR (human epidermal growth factor receptor-1 (HER-1)) in keratinocytes (Peus *et al.*, 1998). For the re-epithelialization stage of cutaneous wound healing, HER-1 activation is crucial (Werner and Grose, 2003).

HB-EGF has been identified as a ligand and activator of HER-1 (Block *et al.*, 2004; Nishi and Klagsbrun, 2004), and HB-EGF acts as an autocrine growth factor in keratinocytes (Hashimoto *et al.*, 1994). Similar to all other members of the EGF family, HB-EGF is synthesized as a membrane-anchored precursor called proHB-EGF. ProHB-EGF has been suggested to act as a survival factor in epithelial cells (Higashiyama *et al.*, 1991; Takemura *et al.*, 1997). Scrape wounding, phorbol ester, and various other stress stimuli on cultured cells are able to induce HB-EGF mRNA levels and the shedding of proHB-EGF into soluble HB-EGF (Goishi *et al.*, 1995; Ellis *et al.*, 2001; Takenobu *et al.*, 2003; Xu *et al.*, 2004). Common stress-inducing stimuli (Kyriakis and Avruch, 2001), such as exposure to UV light, hypertonic osmotic pressure, or oxidative stress, induce the expression of HB-EGF (Takenobu *et al.*, 2003). Stress- and inflammatory cytokine-induced proHB-EGF shedding has been shown to be p38-dependent in Vero cells (Takenobu *et al.*, 2003). Moreover, wound-induced HB-EGF gene expression involves the action of MAPKs p38 and extracellular signal-regulated kinase 1/2 (Ellis *et al.*, 2001; Sharma *et al.*, 2003).

The importance of HB-EGF during cutaneous wound healing was first documented by Marikovsky *et al.* (1993), who detected HB-EGF in the wound fluid of pigs. HB-EGF is also present in the wound fluid derived from partial-thickness burns of humans (McCarthy *et al.*, 1996). Skin organ culture and wounding of epithelial cell cultures result in HER-1 activation through the ectodomain shedding of HB-EGF (Stoll *et al.*, 1997; Xu *et al.*, 2004). HB-EGF accelerates keratinocyte migration during the re-epithelization stage of cutaneous wound healing (Tokumaru *et al.*, 2000; Shirakata *et al.*, 2005), and *in vivo* HB-EGF treatment of mouse cutaneous wounds accelerates wound healing (Cribbs *et al.*, 1998). Moreover, HB-EGF neutralization experiments result in impaired wound healing (Block *et al.*, 2004).

In this study, we provoked stresses on cultured human epidermal keratinocytes by MBCD or hydrogen peroxide. We observed in both cases rapid and transient synthesis of HB-EGF. Addition of HB-EGF to unstressed keratinocytes caused alterations of their phenotype that mimic the alterations detected in the re-epithelialization area during wound healing, where HB-EGF expression is precisely induced. These similarities suggest that HB-EGF could be considered as an actor in the stress response of keratinocytes and that this growth factor may act this way to promote epidermal healing.

RESULTS

Cholesterol depletion in differentiating keratinocytes induces the activation of the p38 MAPK and stimulates the expression of HB-EGF

Our previous study demonstrated that cholesterol depletion by MBCD in confluent differentiating keratinocytes causes the activation of p38 MAPK, which subsequently induces involucrin expression (Jans *et al.*, 2004). Here, we have further investigated whether cholesterol depletion induces the synthesis of the autocrine growth factor HB-EGF. Keratinocyte cultures around confluence were depleted of their membrane cholesterol for 18 hours and proteins and mRNA were analyzed. Figure 1a shows that cholesterol depletion strongly activates p38 phosphorylation, but only in confluent and postconfluent cultures. p38 phosphorylation is accompanied by increased amounts of proHB-EGF and involucrin. Thus, the activation of p38 could be responsible for both involucrin and proHB-EGF enhancements. Accordingly, augmented *involucrin* and *HB-EGF* mRNA expression levels are observed when confluent and postconfluent cultures are cholesterol-depleted (Figure 1b).

Potential off-target effects of cholesterol depletion are controlled for by checking the phosphorylation state of p38 in cholesterol-repleted keratinocyte cultures (Figure 1c). In fact, these control experiments were performed with MBCD–cholesterol complexes after a protocol edited by Klein *et al.* (1995). As the complexes include cholesterol in the hydrophobic cage of the MBCD molecule, the MBCD–cholesterol complexes are not able to extract plasma membrane cholesterol any more. Figure 1c shows that the MBCD–cholesterol complexes do not induce p38 phosphorylation and thereby we show that the cholesterol-sequestrating agent MBCD does not induce unspecific signaling events.

Lipid raft disruption and oxidative stress induce a transient p38 activation followed by HB-EGF expression during the first hours of the recovery period

Figure 1a shows that p38 MAPK is still phosphorylated 18 hours after cholesterol depletion when the synthesis of cholesterol is inhibited by lovastatin, indicating that this treatment constitutes a stress factor for keratinocytes. Thus, we investigated the time courses of p38 activation and proHB-EGF expression when cells were cholesterol-depleted for a short-term and then allowed to recover (Figure 2a–c). Simultaneously, we wondered whether an oxidative stress by H_2O_2 could produce similar effects as disruption of lipid rafts by MBCD (Figure 2d–f). H_2O_2 is a known activator of EGFR and p38 MAPK in keratinocytes (Peus *et al.*, 1998, 1999). For this purpose, we performed time-course experiments after short-term cellular stresses (1 hour MBCD or 20 minutes H_2O_2) followed by increasing recovery periods in normal autocrine culture medium (Figure 2). Figure 2a illustrates a fast p38 phosphorylation immediately after the disruption of

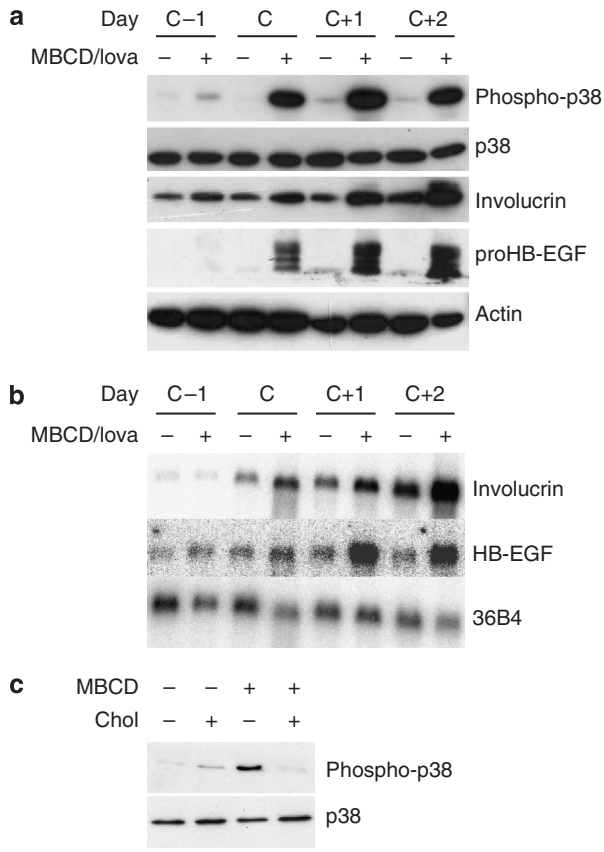

Figure 1. p38 MAPK activation and HB-EGF synthesis induced by long-term cholesterol depletion in confluent and postconfluent cultures. (**a** and **b**) Keratinocyte cultures were either left untreated or cholesterol-depleted for 18 hours by a combined treatment (1 hour 7.5 mM MBCD/17 hours 10 μM lovastatin (lova)) at confluence minus 1 day (C−1), confluence (C), or confluence plus one respectively two days (C + 1 resp. C + 2). (**a**) Protein extracts were analyzed by Western blotting using specific antibodies for phospho-p38, p38, involucrin, proHB-EGF, and actin (loading control). The results shown are representative data obtained in three independent experiments. (**b**) After mRNA extraction, samples were analyzed by Northern blotting using serial hybridizations of the membrane with cDNA probes specific for *involucrin* and *HB-EGF*. Gel loading was controlled by hybridization of cDNA specific for the reference gene *36B4*. (**c**) Confluent keratinocyte cultures were treated for 1 hour with 7.5 mM MBCD, 7.5 mM cholesterol (chol), or 7.5 mM MBCD–cholesterol complexes. The data are representative of three independent experiments as analyzed by Western blotting using specific antibodies for phospho-p38 and p38.

membrane lipid rafts, which is maintained for 2 hours during the recovery phase. In comparison to p38 phosphorylation, proHB-EGF expression is delayed with a maximal expression between 2 and 8 hours of recovery times (Figure 2a and b). HB-EGF mRNA expression correlates with protein expression, as it is strongly increased during the first hour of the recovery phase but then decreases rapidly during the next hours (Figure 2c). The rather quick decrease of the HB-EGF mRNA after the stress compared with the later decrease of the HB-EGF protein suggests that either the encoded protein is stable for approximately 8 hours or proHB-EGF is processed and thus becomes undetectable by that time.

The cellular stress induced by H_2O_2 produces results similar to MBCD. Indeed, Figure 2d illustrates p38 phosphorylation immediately after oxidative stress with sustained phosphorylation during the first 2 hours of the recovery phase. In comparison to p38, proHB-EGF expression is delayed with maximal HB-EGF enhancements between 4 and 8 hours after the stress (Figure 2d and e). HB-EGF mRNA expression corroborates the proHB-EGF expression profile, as mRNA levels increase transiently during the second hour of the recovery phase (Figure 2f).

Thus, oxidative stress and disruption of membrane lipid rafts provoke fast and strong HB-EGF expression at mRNA and protein levels. These results suggest that *HB-EGF* is an early response gene induced by cellular stresses like perturbations of the plasma membrane or oxidative stress, and indicate that in keratinocytes, *HB-EGF* mRNA is translated into membrane-bound proHB-EGF protein with a delay between 1 and 2 hours.

HB-EGF synthesis induced by disruption of lipid rafts is dependent on p38 MAPK activity

Since p38 phosphorylation and HB-EGF expression are sequentially stimulated by short-term MBCD treatment (Figure 2a), we wondered whether HB-EGF expression could be a consequence of p38 signaling. p38 MAPK inhibition by the specific inhibitor PD169316 strongly impedes the increase in proHB-EGF protein induced by lipid raft disruption (Figure 3a and b). Efficiency of the p38 inhibitor has been controlled by checking phosphorylation of HSP27, a known phosphorylation target of p38 in keratinocytes (Garmyn *et al.*, 2001). PD169316 inhibits only the kinase activity of p38 and does not hinder upstream kinases to phosphorylate p38, so it is not surprising to detect p38 phosphorylation in the presence of PD169316. On the mRNA level, the expression of *involucrin* and *HB-EGF*, previously shown to be increased after long-term cholesterol depletion, was clearly decreased in the presence of PD169316 (Figure 3c). These results indicate that cholesterol depletion-induced HB-EGF and involucrin synthesis are dependent on p38 activity in keratinocytes.

The expression of HB-EGF is induced in the epidermal margin of wounded human skin and associated with alterations in the expression of differentiation markers

Combining data showing that HB-EGF is induced in keratinocytes during cutaneous wound healing (McCarthy *et al.*, 1996) and our data showing that HB-EGF is induced in keratinocytes after extraction of membrane cholesterol, we wondered whether there could be other similarities between keratinocytes at the margin of a wound and cholesterol-depleted keratinocytes. Epithelial margins of burned skin areas were immunolabeled for HB-EGF, keratin 10 (K10), and involucrin expression (Figure 4). The control sample (Figure 4a and b) was labeled with unspecific serum and a secondary antibody. We observed a non-specific staining in the wound area of the control sample (Figure 4a and b), which apparently corresponds to the scab, but the dermis and epidermis were devoid of labeling. As a supplementary

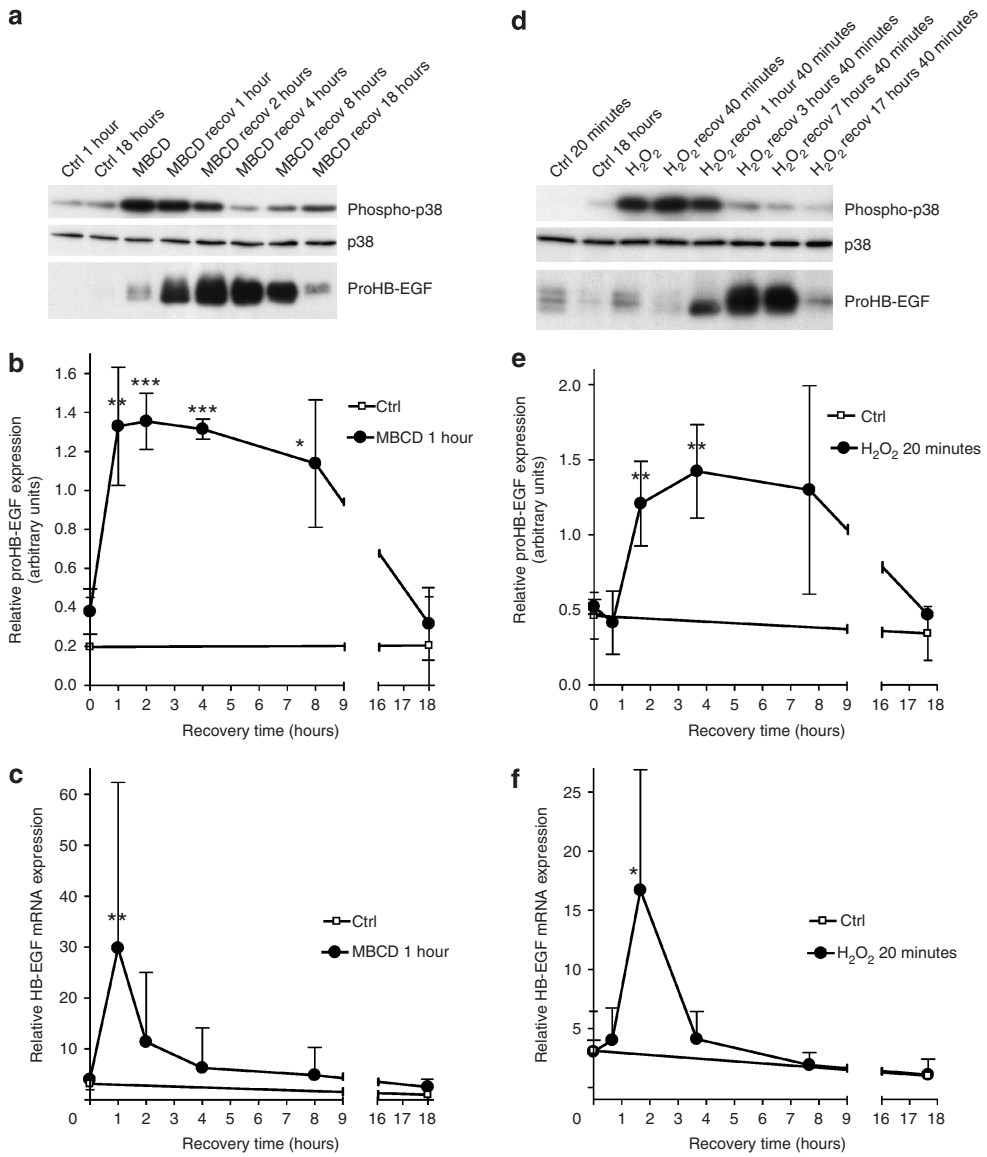

Figure 2. Lipid raft disruption and oxidative stress induce early p38 activation and subsequent HB-EGF expression during recovery periods. (**a**–**c**) Confluent keratinocyte cultures were either left untreated or treated with 7.5 mM MBCD for 1 hour. After the MBCD treatment, the cells were incubated in autocrine culture medium during various periods allowing increasing recovery (recov) times. (**d**–**f**) Confluent keratinocyte cultures were either left untreated or subjected to oxidative stress for 20 minutes with 1 mM H_2O_2. After the applied stress, cells were incubated in autocrine culture medium during various recovery periods. (**a** and **d**) Protein extracts were analyzed by Western blotting using specific antibodies for phospho-p38, p38, and proHB-EGF, p38 serving at the same time as a loading control. The results shown are representative data obtained in three independent experiments. (**b** and **e**) Illustration of mean relative proHB-EGF expression (normalized to p38) ± SD from three independent experiments of which one is shown in (**a**) and (**d**), respectively. Statistical analysis was performed by Student's *t*-test comparing the values to the expression level of Ctrl for 18 hours ($n = 3$, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**c** and **f**) Total RNA extracts prepared from three independent cultures were transcribed into cDNA and analyzed as triplicates by real-time PCR to determine relative *HB-EGF* mRNA expression (normalized to *36B4*). Data show the mean relative *HB-EGF* mRNA expression ± SD from three independent experiments. Student's *t*-test was performed comparing mRNA levels to mRNA levels of Ctrl for 18 hours ($n = 3$, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

control, we labeled one sample with an isotype-matched primary antibody; the result obtained was identical to that shown in Figure 4a and b (data not shown). The immunolabeling of HB-EGF in Figure 4c reveals that this growth factor is present in the re-epithelializing area, with weak labeling detected in areas distant from the wound and strong labeling at the extremity of the leading edge within epidermal cells. At a higher magnification of the wound margin, HB-EGF is found to be present mainly in the cell periphery, very likely in the plasma membrane of keratinocytes migrating above the granulation tissue (Figure 4d, arrows). These results confirm the production of HB-EGF in keratinocytes involved in the re-epithelialization of a skin wound and underline a potentially important function of this growth factor in epidermal healing.

The immunolabeling of K10 (Figure 4e) reveals that in areas located at some distance from the wound, this keratin is expressed typically in all suprabasal layers of the epidermis.

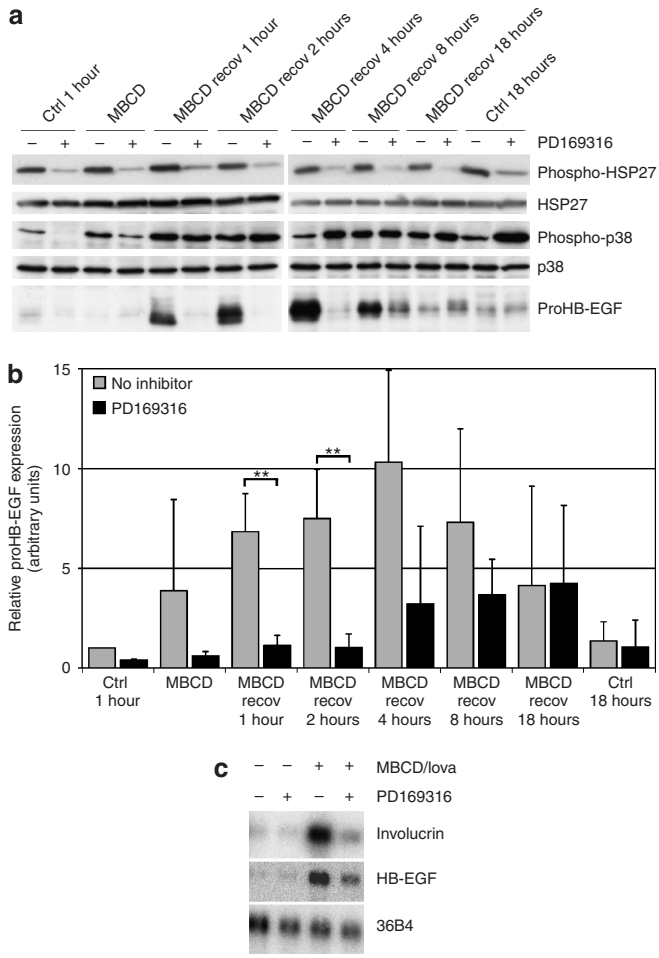

Figure 3. HB-EGF synthesis in lipid raft-disrupted keratinocytes is dependent on p38 MAPK. (**a**) Confluent keratinocyte cultures were pretreated for 30 minutes with 15 μM PD169316, a selective p38 inhibitor, and then cultures were treated for 1 hour with 7.5 mM MBCD the in presence of PD169316, which stayed also present in the recovery medium. Protein extracts were analyzed by Western blotting using specific antibodies for phospho-HSP27, HSP27, phospho-p38, p38, and proHB-EGF. p38 served at the same time as a loading control. The results shown are representative data obtained in three independent experiments. (**b**) Illustration of mean relative proHB-EGF expression (normalized to p38) $\pm$SD from three independent experiments of which one is shown in (**a**) ($n=3$, $^{**}P<0.01$). (**c**) Confluent keratinocyte cultures were pretreated with 15 μM PD169316 and then cultures were subjected to long-term cholesterol-depletion (1 hour 7.5 mM MBCD/17 hours 10 μM lova) in the presence of PD169316. Subsequently, RNA messengers were extracted and analyzed by Northern blotting using selective serial hybridization probes for *involucrin*, *HB-EGF*, and housekeeping gene *36B4*.

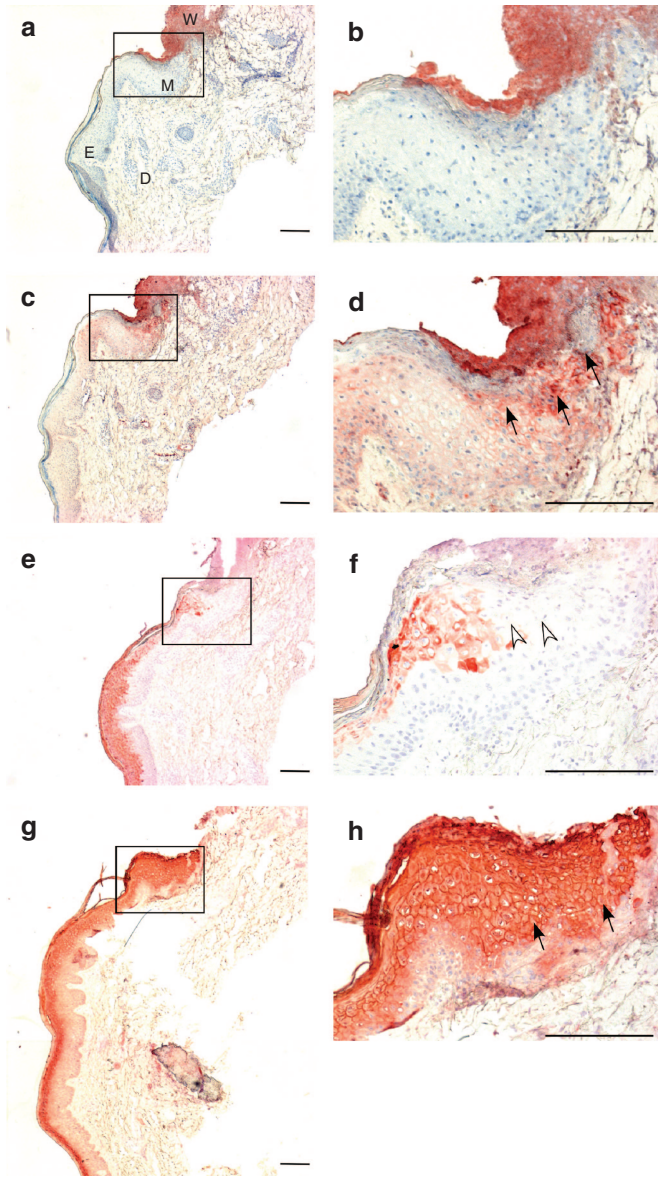

Figure 4. Wounded skin shows an increased HB-EGF and involucrin expression and a decreased K10 expression in the wound margin. Serial sections of human burned skin at day 5 of wound healing are depicted (**a**, **c**, **e**, and **g**); the rectangles indicate higher magnifications of the area of interest illustrated in (**b**, **d**, **f**, and **h,** respectively). Arrows (**d** and **h**) indicate particularly strongly labeled areas. Arrowheads (**f**) indicate a decreased labeling. (**a** and **b**) Illustration of the wound margin immunostained with BSA and secondary antibody as a control (E = epidermis, D = dermis, W = wound, and M = margin of the wound). (**c** and **d**) Same area immunostained for HB-EGF. (**e** and **f**) Same area immunostained for K10. (**g** and **h**) Same area immunostained for involucrin. Bar = 200 μm. Similar results were obtained in wound healing skin from a second severely burned patient.

Analysis of the epidermal leading edge, particularly at high magnification (Figure 4f), illustrates that the expression of K10 is delayed in the re-epithelializing area and even absent from cells forming the extremity of the leading edge (Figure 4f, arrowheads).

The immunostaining of involucrin also reveals normal localization in the epidermis at some distance from the wound, where this late marker of differentiation is expressed in the upper spinous and granular layers of the tissue (Figure 4g). However, in the re-epithelializing margin area, the expression of involucrin is intense and distributed in all the suprabasal layers (Figure 4h, arrows).

Thus, the immunolabeling of wound margins indicates that the phenotype of re-epithelializing keratinocytes is profoundly altered. Whether these alterations of keratinocyte

differentiation markers could be the result of the stimulated expression of HB-EGF in this area is an interesting question that should be investigated. Meanwhile, however, we investigated whether the expression of HB-EGF can be stimulated by the presence of some free border or epidermal margin after wounding. With this aim in mind, we mimicked epidermal injury by sectioning with scissors samples of human epidermis (Figure 5a). After sectioning the tissue into smaller pieces, these were incubated in autocrine culture medium for various periods of time before protein extraction and analysis of proHB-EGF expression. Before injury, proHB-EGF is only detected in minor quantities in the epidermal tissue, but its synthesis is strongly upregulated during the first 2 days after injury as illustrated in Figure 5b and c. The sectioning causes increases in HB-EGF synthesis with a significant increase 24 hours after the sectioning (Figure 5c). Immunohistochemical HB-EGF labeling on sectioned skin samples has been performed immediately after the sectioning or 24, 48, and 72 hours after the sectioning (Figure 5d–g). The control sample (Figure 5h) is labeled with an isotype-matched primary antibody and exhibits no staining. HB-EGF labeling is detected 24 hours after the wound is performed and is particularly strong after 48 hours. Note that HB-EGF staining is strongest at the border of the incision and disappears progressively when studying keratinocytes away from the wound as illustrated in Figure 5f.

Involucrin expression and *HB-EGF* expression are both upregulated by HB-EGF or EGF, but the expression of *K10* is downregulated in the same conditions

The addition of HB-EGF to keratinocyte cultures modifies their cell phenotype and increases motility in a way similar to recombinant EGF (Shirakata *et al.*, 2005 and our unpublished data). The data presented in Figure 6 show that HB-EGF or EGF treatments significantly increase *HB-EGF* mRNA levels, thereby confirming the existence of a positive feedback mechanism for *HB-EGF* in keratinocytes (Hashimoto *et al.*, 1994). Moreover, analysis of mRNA levels of differentiation markers shows that both growth factors inhibit significantly the expression of *K10*, whereas they conversely stimulate *involucrin* expression (Figure 6). These data concur with the observation of K10 and involucrin expression at the wound margin where HB-EGF expression is induced (Figure 4).

DISCUSSION

To gain insight into the response of keratinocytes facing epidermal homeostasis perturbation, we studied skin burn injury and *in vitro* stresses, that is, membrane cholesterol depletion, oxidative stress, and epidermal injury. Our aim was to investigate whether HB-EGF could be involved in restoring epidermal homeostasis and thus be an actor in the keratinocyte's response to environmental stress.

Indeed, our data illustrate that HB-EGF synthesis is induced at RNA and protein levels after oxidative stress or lipid raft disruption by MBCD. We show that the effects engendered by lipid raft disruption are dependent on p38 phosphorylation (Figure 3) and on cell density-determined differentiation state of the cultures (Figure 1; Jans *et al.*, 2004). Analysis of HB-EGF expression kinetics after MBCD or H_2O_2 treatments reveals an elevated but quickly vanishing HB-EGF expression (Figure 2), which confirms that *HB-EGF* is an early response gene (Hashimoto *et al.*, 1994; Wang *et al.*, 2006). Moreover, experiments with conditioned media are currently under investigation to provide evidence that HB-EGF can be secreted into the culture medium subsequent to disruption of lipid rafts by MBCD.

In the second part of our study, we analyzed the expression of HB-EGF in cutaneous wounds and studied the effects engendered by HB-EGF treatments. In *in vivo* cutaneous wounds, we detect a strong HB-EGF expression in keratinocytes of the wound margin (Figure 4), and *in vitro* wounding by sectioning of the epidermis also generates increased HB-EGF expression (Figure 5). So, we can deduce that epidermal wounding generates HB-EGF expression in keratinocytes. Recent data have underlined the crucial role of HB-EGF as an autocrine growth factor for keratinocytes in wound healing (Tokumaru *et al.*, 2000; Shirakata *et al.*, 2005). Thus, the function of HB-EGF in keratinocytes is very likely in relation with the restoration of epidermal tissue homeostasis after stressing situations.

We also show that recombinant HB-EGF alters the expression of differentiation markers in keratinocytes, in fact *K10* expression is decreased, whereas expression of *involucrin* is increased (Figure 6). Of highest interest, similar alterations of these two differentiation markers are clearly observed *in vivo* in the re-epithelialization area of burn wounds (Figure 4). This kind of alterations in differentiation markers is not unique, as the addition of retinoic acid to cultured keratinocytes also inhibits *K10* expression while increasing the expression of *involucrin* (Poumay *et al.*, 1999), and it is intriguing that retinoic acid also stimulates the expression of *HB-EGF* in keratinocytes (Stoll and Elder, 1998). Consequently, with regard to our data, we speculate that *HB-EGF*, owing to its early induction in keratinocytes after stress situations, is involved in the observed alterations of the epidermal differentiation markers.

Interestingly, *HB-EGF* is overexpressed in lesional psoriatic skin (Stoll and Elder, 1998). Psoriatic skin also exhibits diminished *K10* (van Erp *et al.*, 1989) and increased *involucrin* (Watanabe *et al.*, 1991) expression. Intriguingly, psoriasis also reveals links between cholesterol and oxidative stress. On the one hand, psoriatic patients present a perturbed cholesterol metabolism, lipid-lowering drugs aggravate psoriatic lesions (Fisher *et al.*, 1988; Jacobi and Highet, 2003), and elevated levels of plasma cholesterol are detected. On the other hand, high levels of reactive oxygen species and lipid peroxidation products are detected in the psoriatic epidermis (Vanizor Kural *et al.*, 2003).

As our results show that oxidative stress, lipid raft disruption by cholesterol depletion, and injury increase HB-EGF expression in keratinocytes, we wondered whether a change in cholesterol metabolism and/or in the oxidative state of cells is relevant during cutaneous injury. Indeed, cutaneous burn injury induces oxidative stress (Pintaudi *et al.*, 2000) and interestingly burn injury also causes HB-EGF expression (Figure 4) and p38 MAPK activation in leading

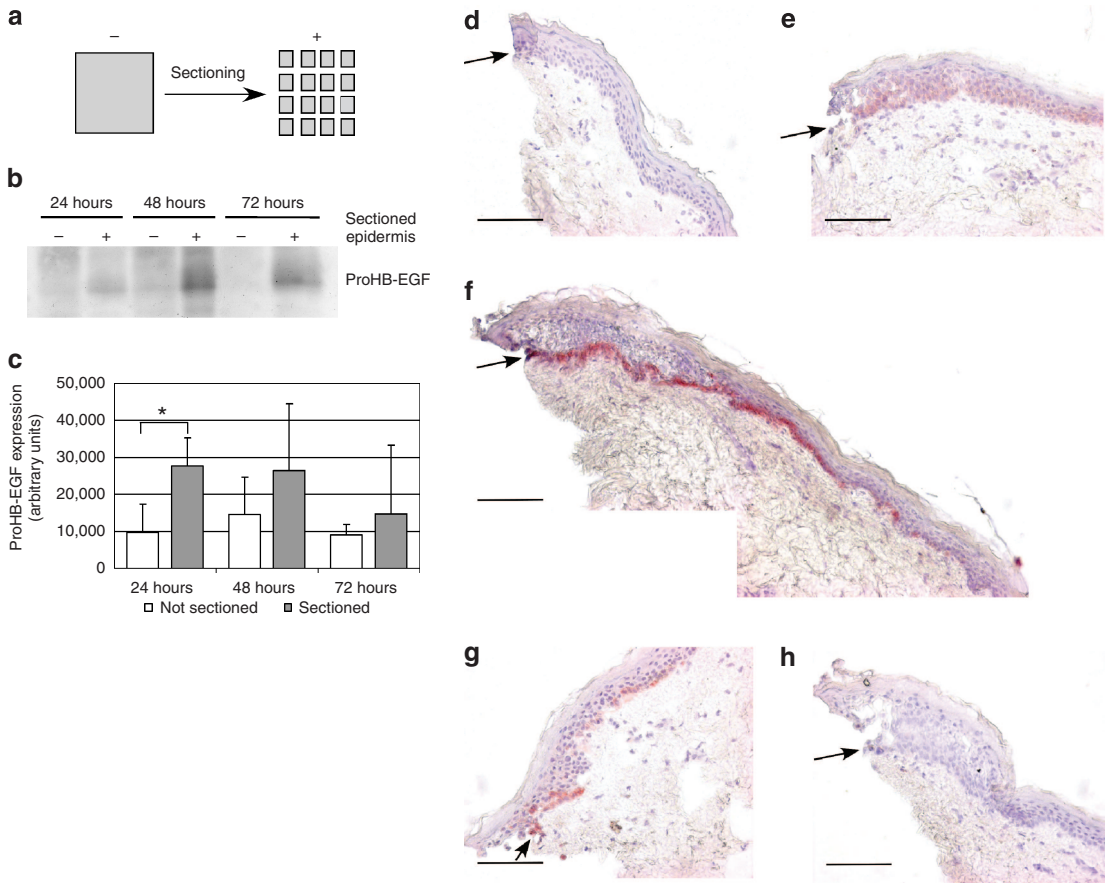

Figure 5. Presence of HB-EGF in *in vitro*-wounded epidermis. (**a**) Normal human epidermis was cut into six parts of identical size: three pieces remained as such (−), and the three other pieces were wound-induced by being sectioned each into 16 smaller squares (+). Subsequent to this sectioning, the epidermal pieces were cultured in autocrine culture medium for 24, 48, or 72 hours before homogenization and protein extraction. (**b**) Analysis of proHB-EGF expression by Western blotting. The results shown are representative data obtained in three independent experiments. (**c**) Illustration of mean densitometric values of proHB-EGF expression ± SD from three independent experiments, of which one is shown in (**b**) (*$P<0.05$). (**d–h**) The border of sectioned skin samples of humans cultured for (**d**) 0 hours, (**e**) 24 hours, (**f** and **h**) 48 hours, and (**g**) 72 hours after the sectioning in autocrine culture medium is depicted. Panels (**d–g**) labeled for HB-EGF and panel (**h**) 48-hour sample stained with isotype-matched primary antibody. The arrows point at the epidermal–dermal junction exposed after sectioning. The images are representative of three independent experiments. Bar = 100 μm.

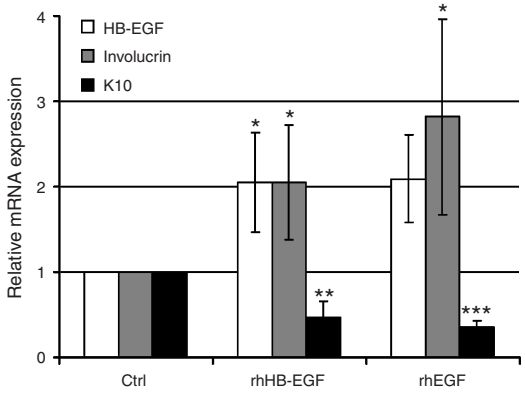

Figure 6. Recombinant HB-EGF upregulates *involucrin* and *HB-EGF* mRNA expression, whereas *K10* mRNA expression is downregulated. Three independent confluent cultures of keratinocytes untreated (Ctrl) or treated for 18 hours with 10 ng/ml recombinant HB-EGF or 10 ng/ml recombinant EGF. Total RNA was extracted, reverse transcription was performed, and cDNA was analyzed in triplicate by real-time PCR. Student's *t*-test was performed comparing the relative mRNA expression of rhHB-EGF- or rhEGF-treated cells to the untreated control cells (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

keratinocytes of the wound edge (Harper *et al.*, 2005). Skin injury generates reactive oxygen species, and wound healing is subjected to redox control (Sen, 2003). Moreover, HB-EGF (Figure 2e) and vascular endothelial growth factor (Sen *et al.*, 2002), both important growth factors in cutaneous wound healing, are induced in keratinocytes by H_2O_2 treatments.

Membrane cholesterol, as an essential component of lipid rafts and of the plasma membrane in general, is likely to play an important role in response to extracellular stresses, as cholesterol regulates the plasma membrane fluidity and permeability, and initiates numerous signaling pathways via lipid rafts. Actually, low membrane cholesterol content increases the cell's sensitivity to oxidative stress (Yang *et al.*, 2006; Hinzpeter *et al.*, 2007), which could be mediated by perturbed lipid raft signaling and/or changes in plasma membrane permeability toward H_2O_2 (Branco *et al.*, 2004). Cells can probably control cholesterol synthesis in response to extracellular stimuli to assure membrane integrity and fluidity of the bilayer (Robichon and Dugail, 2007).

Our previous study has shown that cholesterol depletion activates EGFR in keratinocytes (Jans *et al.*, 2004) and

EGFR–ligand interaction generates extracellular H_2O_2 (Bae *et al.*, 1997; DeYulia and Carcamo, 2005), which is required for EGFR activation and cell survival in keratinocytes (Peus *et al.*, 1998, 1999). As H_2O_2 possesses second messenger properties (Hancock *et al.*, 2001), cholesterol depletion may initiate H_2O_2-mediated signaling pathways. Further support for this hypothesis comes from a study in keratinocytes that shows that cholesterol depletion enhances UVA-induced ROS synthesis (Gniadecki *et al.*, 2002).

Besides EGFR activation, PPARs play crucial roles in epidermal wound healing. PPARs belong to the nuclear steroid hormone receptor superfamily and are involved in epidermal wound healing, redox control, and cholesterol metabolism. On the one hand, PPAR-α expression in keratinocytes at wound edges is essential for epidermal healing (Michalik *et al.*, 2005). On the other hand, activation of PPAR-α decreases cellular cholesterol concentration and synthesis (Konig *et al.*, 2007), so it is possible that epidermal injury changes the cellular cholesterol content through PPAR-α activation. Interestingly, PPAR-α activators reduce inflammatory responses and increase antioxidant enzymes in the skin (Sheu *et al.*, 2002), which again suggest a link with inflammatory skin diseases such as psoriasis.

Lipid raft disruption by cholesterol extraction, oxidative stress, and cutaneous injury all induce HB-EGF expression in keratinocytes (Figure 7). Moreover, HB-EGF is also induced by 12-*O*-tetradecanoylphorbol-13-acetate (Goishi *et al.*, 1995), retinoic acid (Stoll and Elder, 1998; Rittie *et al.*, 2006), and chemotherapy (Wang *et al.*, 2006). It is thus likely that stress situations induce HB-EGF to restore cell and epidermal tissue homeostasis, a function for HB-EGF that remains to be confirmed.

MATERIALS AND METHODS

Chemicals and antibodies

Paraformaldehyde, acetone, and dimethyl sulfoxide were purchased from Merck (Overijse, Belgium). MBCD, cholesterol, BSA, lovastatin, hydrogen peroxide, and mouse anti-human β-actin antibody were obtained from Sigma-Aldrich (Munich, Germany). PD169316 no. 513030 was purchased from Calbiochem (Bierges, Belgium), whereas rhEGF no. 236-EG, recombinant human HB-EGF no. 259-HE, goat anti-human HB-EGF antibody no. AF-259-NA and goat anti-mouse IgG antibody no. AF007 were obtained from R&D Systems (Abingdon, UK). Rabbit anti-human MAPK p38 antibody no. 9212, rabbit anti-human MAPK phospho-p38 Thr180/Tyr182 antibody no. 9211S, and rabbit anti-human HER-1 antibody no. 2232 were obtained from Cell Signaling (Leiden, The Netherlands). Rabbit anti-human phospho-HER-1 Tyr1173 antibody no. 44-794G was purchased from Biosource (Nivelles, Belgium). Rabbit anti-human phospho-HSP27 Ser82 antibody no. 07-489 was obtained from Upstate (Brussels, Belgium). Goat anti-human HSP27 antibody no. sc-1048 was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-human involucrin antibody no. 5001 was purchased from Harbor Bio-Products (Boechout, Belgium). AEC, glycergel, mouse anti-human K10 antibody no. M7002, and horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-goat antibodies were purchased from Dako (Heverlee, Belgium). Keratinocyte growth medium was from Lonza (Verviers, Belgium). Keratinocyte complete culture medium (Epilife and HKGS) and keratinocyte autocrine culture medium (Epilife without HKGS) were from Cascade Biologics (Mansfield, UK).

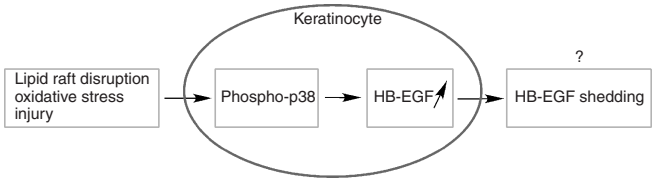

Figure 7. Epidermal stresses induce HB-EGF expression through p38 MAPK activation in normal human keratinocytes. Stresses like disruption of membrane lipid rafts, oxidative stress, or injury induce HB-EGF expression in a p38-dependent manner in normal human keratinocytes, possibly leading to shedding of HB-EGF into the extracellular environment.

Culture of human keratinocytes

After written informed consent of patients (Dr Bienfait, Clinique St Luc, Namur-Bouge, Belgium), normal human abdominal skin samples were obtained from plastic surgery. The Medical Ethical Committee of Clinique St Luc, Namur-Bouge, approved all described studies, and all experiments were carried out according to the Declaration of Helsinki Principles.

Skin samples were cut with a dermatome and keratinocytes were isolated by the trypsin float technique (Wille *et al.*, 1984). For experiments, second passage normal human keratinocytes were cultivated in complete culture medium until 50% confluence and then cultures were switched to autocrine culture medium, which does not contain any peptide factors (autocrine culture conditions; Cook *et al.*, 1991). These keratinocyte culture conditions are characterized by cell growth arrest and commitment to differentiation when keratinocytes reach cell confluence (Poumay and Pittelkow, 1995). All described experiments were performed at confluence in autocrine culture conditions.

Lipid raft disruption by cholesterol depletion and oxidative stress

Keratinocyte cultures were short-term cholesterol-depleted by 7.5 mM (1% wt/vol) MBCD for 1 hour followed by incubation in autocrine culture medium for different periods (recovery times). Repletion experiments were done with MBCD–cholesterol complexes following a protocol edited by Klein *et al.* (1995). Long-term cholesterol depletion was done by incubation of keratinocytes in 7.5 mM MBCD for 1 hour followed by incubation in 10 μM lovastatin for 17 hours (Jans *et al.*, 2004). Oxidative stress was performed by incubating cultures with 1 mM H_2O_2 for 20 minutes followed by various recovery periods.

Organ culture of human epidermis

The epidermis of an abdominal skin sample was cut into six squared pieces of identical size. Three pieces remained as such (ctrl), and the other three pieces were each sectioned by scissors into 16 smaller squares increasing thereby four-fold the length of the epithelial margin (cf Figure 5a). All samples were incubated at 37°C/5% CO_2 in autocrine culture medium for 24, 48, or 72 hours. For Western blotting, the tissues were homogenized in Laemmli sample buffer using an Ultra-Turrax machine and centrifuged to discard insoluble

material, and the supernatants were analyzed for proHB-EGF by Western blotting. For immunohistochemical analysis, abdominal skin was sectioned in squares and then incubated at 37°C/5% CO_2 in autocrine culture medium for 0, 24, 48, or 72 hours. After the respective incubation time, the tissues were immediately embedded in optimal cutting temperature (OCT) compound for future HB-EGF labeling as described in ''Immunohistochemical labeling of human wounded skin sections.''

Protein extraction and Western blotting

Immediately after the indicated treatments, cells were washed with phosphate-buffered saline (PBS) and harvested in twice concentrated Laemmli sample buffer (62.5 mM Tris–HCl, 2% SDS, 8.7% glycerol, 0.05% bromophenol blue, 0.2% dithiothreitol). The proteins of the cell lysates were analyzed by SDS-PAGE and by blotting onto polyvinylidene difluoride membranes (GE Healthcare Bio-Sciences, Uppsala, Sweden). Blocking of the membrane in PBS/1% Tween 20/5% skimmed milk (blocking buffer) was followed by incubation of the membrane with primary antibody diluted in blocking buffer. After three washing steps, the membrane was incubated with an HRP-conjugated secondary antibody in blocking buffer. The results were visualized using the POD Chemoluminescence Blotting Substrate (Roche Diagnostics, Mannheim, Germany) on Hyperfilm ECL (GE Healthcare Bio-Sciences, Uppsala, Sweden). Equivalent gel loading was evaluated by detection of actin or p38 total protein levels. Densitometric values of Western blot data were measured by ImageJ software and represented with GraphPad Prism 3 software.

mRNA isolation and Northern blotting

RNA messengers were extracted from keratinocyte cultures and analyzed by Northern blotting using serial hybridizations of the membrane with randomly labeled radioactive probes as described by Poumay and Pittelkow (1995). Hybridization signals were visualized by a Cyclone apparatus (Packard BioScience Company, Meriden, CT). Gene expression levels were compared to the expression of housekeeping gene *36B4*.

Total RNA isolation and relative quantitative real-time PCR

Total RNA was extracted by the RNeasy kit (Qiagen, Hilden, Germany). RNA concentrations were measured spectrophotometrically, and total RNA was reverse transcribed into cDNA using the Super Script II RNase H-Reverse transcriptase kit (Invitrogen, Merelbeke, Belgium). Sample cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Lennik, Belgium) and primer sense and antisense sequences (300 nM; Sigma-Aldrich, Bornem, Belgium) in a 7300 real-time PCR machine (Applied Biosystems, Lennik, Belgium). The following sequences for real-time PCR primers were used: HB-EGF sense, 5′-TGGCCCTCCACTCCTC ATC-3′, HB-EGF antisense 5′-GGGTCACAGAACCATCCTAG CT-3′, involucrin sense 5′-TGAAACAGCCAACTCCAC-3′, involucrin antisense 5′-TTCCTCTTGCTTTGATGGG-3′; K10 sense 5′-AATCA GATTCTCAACCTAACAAC-3′, K10 antisense 5′-CTCATCCAGCA CCCTACG-3′, 36B4 sense 5′-ATCAACGGGTACAAACGAGTC-3′, 36B4 antisense 5′-CAGATGGATCAGCCAAGAAGG-3′. mRNA levels were normalized to *36B4* (housekeeping gene) mRNA levels, using the real-time PCR software 7300 v1.3.1 (Applied Biosystems, Lennik, Belgium) and data were represented with GraphPad Prism 3 software.

Immunohistochemical labeling of human wounded skin sections

In severely burned patients, the healing margins of the wound must be excised with a dermatome before autologous skin grafting. Healing skin samples were kindly provided by Dr Fauville from IMTR (Loverval, Belgium). The collected samples were embedded in OCT compound for future preparation with a cryostat of frozen 6 μm sections transversal to the margin. Serial sections were immunolabeled for HB-EGF, K10, and involucrin. For control, HB-EGF, and K10 labelings, sections were fixed in 4% paraformaldehyde (15 minutes) followed by washes in 0.1 M glycine. For involucrin labeling, fixation was done in acetone at −20°C (5 minutes). After the fixation step, all slides were incubated in 3% H_2O_2 to inhibit endogenous peroxidases. For control, HB-EGF, and K10 immunolabelings, the skin sections were blocked and permeabilized in PBS/BSA (0.1%)/Triton X-100 (0.02%), whereas slides for involucrin immunostaining were not permeabilized (incubation in PBS/BSA (0.1%)). After this blocking step, every slide was incubated for 1 hour with its respective primary antibody diluted in PBS/BSA/Triton X-100 (negative control, HB-EGF, K10) or PBS/BSA (involucrin). The samples for the supplementary control for HB-EGF labeling shown in Figure 4 and the control illustrated in Figure 5h were incubated in isotype-matched primary antibody at a concentration identical to the HB-EGF antibody. The control illustrated in Figure 4 was incubated in BSA instead of a primary antibody. Then, HB-EGF controls and HB-EGF immunolabelings were incubated in HRP-secondary antibody using a biotin–streptavidin amplification system (Dako LSAB® + System, HRP, Dako, Heverlee, Belgium). K10 and involucrin immunolabelings were incubated, respectively, in anti-mouse and anti-rabbit HRP-conjugated secondary antibodies in a biotin–streptavidin amplification system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Detection of HRP was done using AEC resulting in a red-brown staining. Counterstaining with hemalun was performed for 5 minutes and slides were mounted in glycergel for observation in an Olympus AX70 microscope. Micrographs were taken by a digital camera.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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