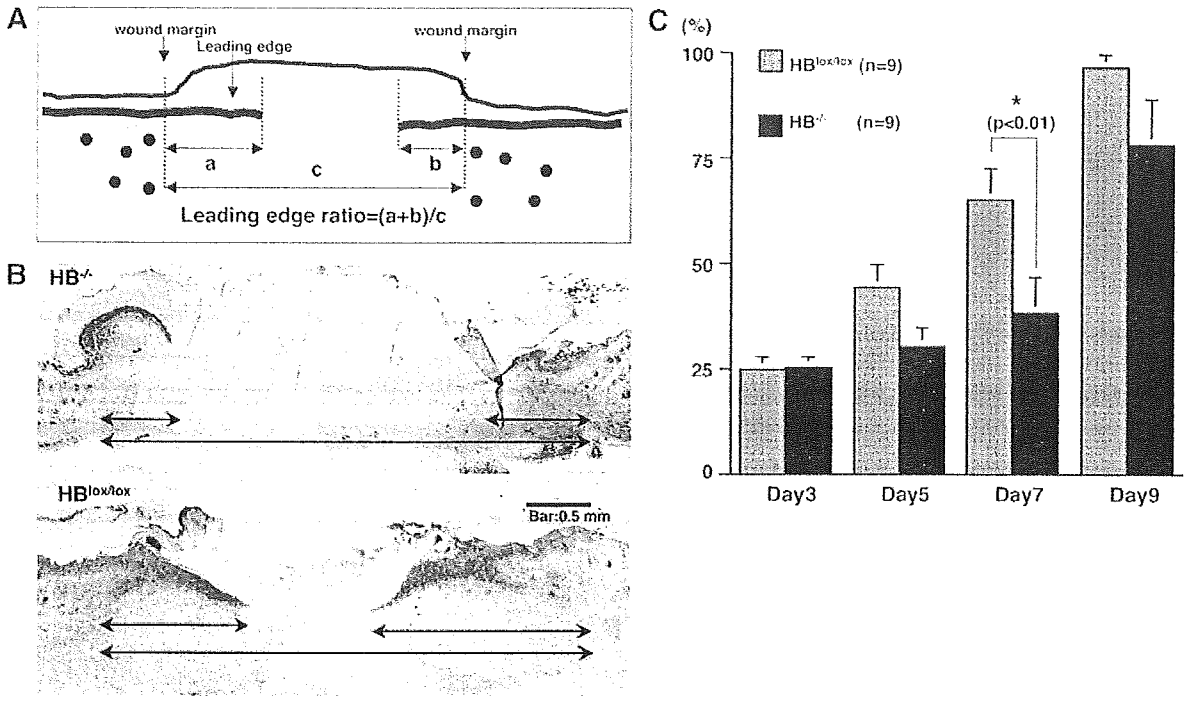

Fig. 5. Impaired keratinocyte migration in $HB^{-/-}$ mice. (A) Serial sections were prepared, and the epidermis was stained with anti-keratin antibody. Computer-assisted morphometric analysis was performed and the ratio of the leading edge to initial wound length was calculated. (B) Immunohistochemical staining of wound healing assay at day 7. Scale bar: 500 μm. (C) Measurements of leading edge ratio in $HB^{lox/lox}$ and $HB^{-/-}$ mice. The leading edge ratio was significantly decreased in $HB^{-/-}$ mice (n=9) at day 7. *P<0.01.

and +0.2 mm. From day 5 to day 7, BrdU+ cells were located at the leading edge, although they always appeared just behind the β-gal+ cells (Fig. 6B,C).

We also examined β-gal+ and BrdU+ cells in the wound-healing assay using $HB^{-/-}$ mice. As in $HB^{+/-}$ mice, in $HB^{-/-}$ mice, on day 2 post-wounding, β-gal+ cells were localized mostly 0 to +0.2 mm into the leading edge, whereas BrdU+ cells were detected mostly between –0.2 and –0.4 mm into the peripheral skin. On day 3, the peak of the β-gal+ cells were localized at 0 to +0.2 mm, whereas the peak of the BrdU+ cells were between 0 and –0.2 mm. On days 5 and 7, the peak aggregation of BrdU+ cells in $HB^{-/-}$ mice was at almost the same location as the β-gal+ cells, at the leading edge (Fig. 6D,E). The overlapping distribution patterns of BrdU+ and β-gal+ cells in these mice may be due to the impaired cell migration (Fig. 5B,C). However, the total counts of BrdU+ cells were similar in $HB^{lox/lox}$ and $HB^{-/-}$ mice (Fig. 4B,C).

Discussion

Wound healing is a complex process involving a number of coordinated events including inflammation, cell migration, cell proliferation, matrix production and angiogenesis (Singer and Clark, 1999). A complex array of cells, growth factors, cytokines and matrix components are involved in wound healing, and a number of transgenic and knockout mouse models have revealed the contribution of several molecules to wound healing (Scheid et al., 2000). Impaired wound healing was observed in mice transgenic for several molecules, such as BMP-6, follistatin, truncated FGF receptor and thorombospondin-1, as well as in mice with activin, β1 integrin, and TGF-β1 knockouts, among others (Grose and Werner, 2003; Scheid et al., 2000; Werner and Grose, 2003). EGF family members such as EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, epiregulin and their receptor EGFR mainly regulate migration, proliferation and differentiation of many cell types involved in wound healing. EGFR knockout mice showed striking abnormalities, such as wavy hair and thin skin (Miettinen et al., 1995; Sibilia and Wagner, 1995). In contrast to this striking phenotype in EGFR knockout mice, EGF-disrupted mice showed no phenotypic abnormalities (Luetteke et al., 1999). No differences in wound healing were found in TGF-α knockout mice with either excisional dorsal wounding or ear-punch wounding (Luetteke et al., 1993; Mann et al., 1993). These unexpectedly minor differences in phenotypes in wound healing in EGFR-ligand knockout mice are probably due to the known functional redundancy among the EGF family members, including HB-EGF.

HB-EGF is produced and secreted by human keratinocytes and acts as an autocrine growth factor (Hashimoto et al., 1994). HB-EGF mRNA was rapidly and dramatically induced after scrape-wounding, although slight increases in TGF-α, amphiregulin and epiregulin mRNAs were observed. Furthermore, blocking HB-EGF by addition of neutralizing antibody to the medium inhibited keratinocyte migration (Tokumaru et al., 2000). In contrast, the addition of recombinant HB-EGF to the medium accelerates keratinocyte migration (Tokumaru et al., 2000). These results indicate that

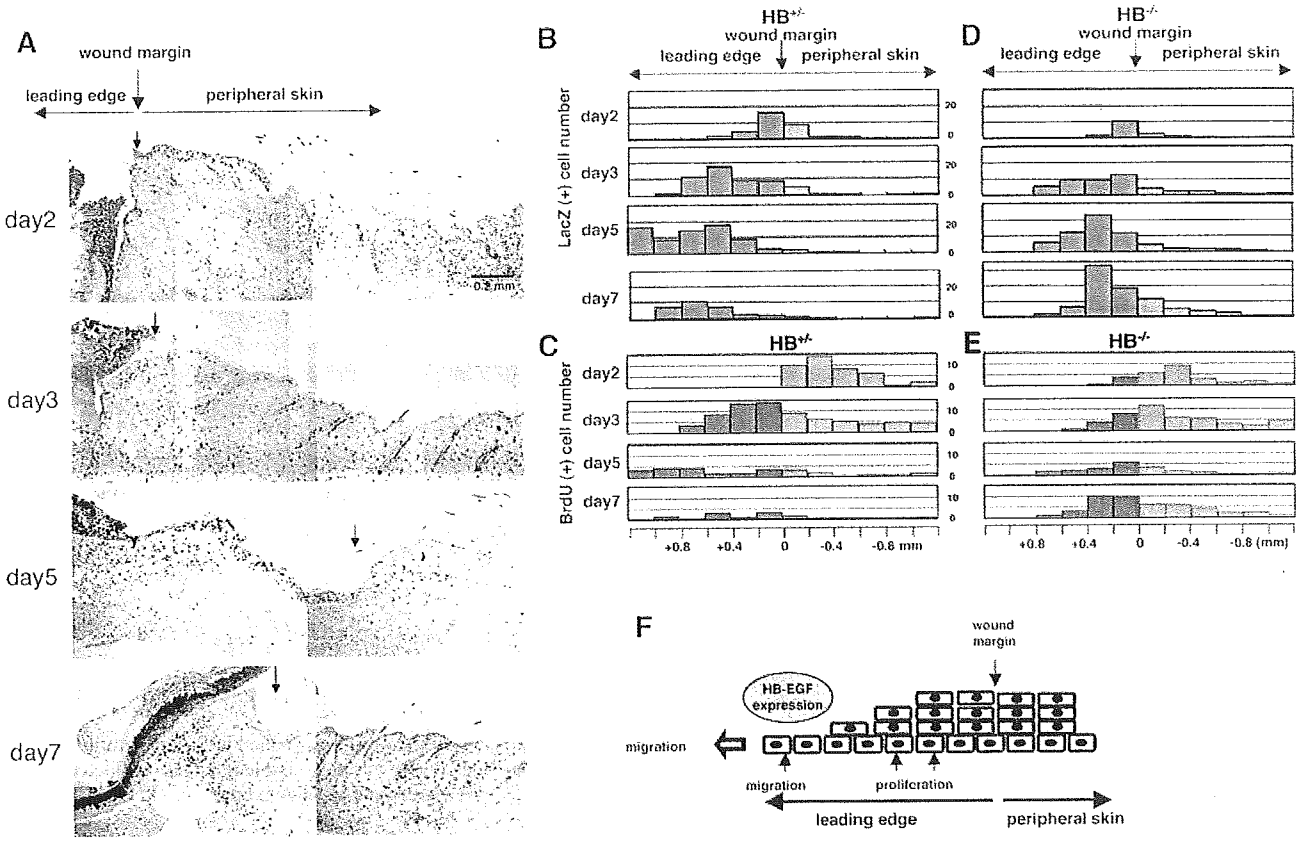

Fig. 6. HB-EGF expression in skin wound healing. The targeting vector contained the *lacZ* gene as a reporter for the expression of HB-EGF. When HB-EGF cDNA is deleted by Cre-recombinase, HB-EGF expression can be identified by X-gal staining in $HB^{+/-}$ mice. (A) Double staining for X-gal and BrdU at the wound healing stage. Scale bar: 0.2 mm. Arrow, wound margin. (B-E) Distribution of β-gal-positive [lacZ(+)] cells (B) and BrdU-positive cells (C) from day 2 to 7 in $HB^{+/-}$ mice. HB-EGF is expressed predominantly at the tip of the leading edge, whereas BrdU-positive cells were distributed mainly at wound margin. Distribution of β-gal-positive cells (D) and BrdU-positive cells (E) in $HB^{-/-}$ mice. There was no significant difference in the number of BrdU-positive cells between $HB^{+/-}$ and $HB^{-/-}$ mice at any stage of wound healing. (F) A proposed schematic illustration of skin wound healing. After injury, keratinocytes at the wound margin begin to migrate and express HB-EGF without proliferation. Next, focal release of HB-EGF may signal further migration and up-regulate HB-EGF expression in an autocrine manner. Values in B-E are number of cells per indicated area.

HB-EGF plays an important role in skin wound healing, and led us to investigate the in vivo function of HB-EGF. Since germline targeting of the HB-EGF gene resulted in embryonic lethality (Iwamoto et al., 2003), we generated keratinocyte-specific HB-EGF-deficient mice ($HB^{-/-}$) using Cre/loxP technology in combination with the keratin 5 promoter (Takeda et al., 2000). There was no difference in wound closure between $HB^{-/-}$ and $HB^{lox/lox}$ mice on day 3. However, wound closure was markedly retarded in $HB^{-/-}$ mice compared to $HB^{lox/lox}$ mice. We clearly demonstrated for the first time that endogenous HB-EGF is the most important growth factor in the epithelialization of skin wound healing in vivo, using keratinocyte-specific HB-EGF-deficient mice.

EGF family members are well known to promote keratinocyte growth in vitro (Hashimoto, 2000). It has been reported that TGF-α, amphiregulin, HB-EGF and epiregulin are autocrine growth factors in normal human keratinocytes (Coffey et al., 1987; Cook et al., 1991; Hashimoto et al., 1994; Shirakata et al., 2000). In vitro observation suggests that these EGF family members play important roles in development, epidermal morphogenesis, skin homeostasis and wound healing. In this study, we investigated HB-EGF function in cell migration and proliferation. HB-EGF stimulates keratinocyte migration in vitro and in vivo. However, there was little difference in proliferation between $HB^{lox/lox}$ and $HB^{-/-}$ mice. HB-EGF promoter activity was up-regulated at the migrating epidermal edge, whereas the distribution of proliferating cells (BrdU-positive) was not identical to that of HB-EGF mRNA-positive cells. Interestingly, the wound margin of normal epidermis expressed HB-EGF mRNA and was positive for BrdU, although HB-EGF promoter activity could not be detected in normal skin far from the wound margin. Therefore, normal skin does not require much HB-EGF, but after injury HB-EGF is induced and plays a crucial role in wound healing by up-regulating keratinocyte migration but not proliferation.

Combined, the evidence suggests that the synthesis of HB-EGF at the leading epithelial edge stimulates cells, via an autocrine loop, to migrate towards the center of the wound rather than to proliferate. Interestingly, there were few β-gal-positive cells and little HB-EGF expression in normal skin far

from the wound margin. Therefore, HB-EGF expression induced by wounding might itself stimulate further expression of HB-EGF at the leading edge via an autocrine loop. Fig. 6F shows a schematic illustration of our proposed skin wound healing mechanism. After injury, keratinocytes at the wound margin begin to express HB-EGF and migrate toward the wound site without proliferating. Next, the focal release of HB-EGF may trigger the migration of additional cells, rather than cell proliferation at the leading edge. Therefore, we conclude that HB-EGF is rapidly induced after injury and plays an important role in wound healing by up-regulating keratinocyte migration.

Nuclear transcription factors play important roles in almost all biological events resulting from growth factor signaling, and several nuclear transcription factors are thought to be involved in skin wound healing. Several mouse models with gene-targeted disruption of nuclear transcriptional factors have been analyzed for skin wound healing. Sano et al. (Sano et al., 1999) reported severe retardation of wound healing in keratinocyte-specific STAT3 knockout mice. D'Souza et al. (D'Souza et al., 2002) reported impaired skin wound healing in E2F-1 knockout mice. Recently, the development of keratinocyte-specific c-jun knockout mice was reported (Li et al., 2003; Zenz et al., 2003). These mice showed retarded wound healing, and the activation of EGFR was greatly decreased (Li et al., 2003). Since EGF itself is not produced by keratinocytes, the autocrine loop consisting of HB-EGF, EGFR and c-jun might be one of the major regulatory signal transduction mechanisms in skin wound healing.

In conclusion, HB-EGF is an important growth factor in epithelialization during skin wound healing in vivo, and acts mainly by stimulating migration, rather than proliferation, of keratinocytes.

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New mechanisms of skin innate immunity: ASK1-mediated keratinocyte differentiation regulates the expression of β-defensins, LL37, and TLR2

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Epidermal keratinocytes differentiate and form a multilayered epidermis, which is the primary barrier between the body and the outer environment. As the epidermis is constantly exposed to a variety of microbial pathogens, its function of resisting microbial pathogens is vital. This characteristic feature is formed during differentiation. Immunohistochemical analysis revealed that the upper epidermis of normal human skin expresses β-defensins 1–3 and LL37. We hypothesized that epidermal keratinocytes develop an innate immune barrier based on human β-defensins (hBD) and LL37 during differentiation. To prove this, we introduced an active form of the apoptosis signal-regulating kinase-1 (ASK1), an intracellular regulator of keratinocyte differentiation, into cultured normal human keratinocytes. Transfection of this active form, ASK1-ΔN, significantly enhanced the expression of hBD1–3 and LL37. In addition, a p38 inhibitor abolished this induction, indicating that the ASK1-p38 cascade regulates the expression of hBD1–3 and LL37. Furthermore, the ASK1-p38 pathway also regulated the expression of Toll-like receptor (TLR)2 in keratinocytes. Contact between *S. aureus* and keratinocytes resulted in the phosphorylation of p38 and induced the expression of hBD2 and hBD3. Moreover, the p38 inhibitor reduced this induction. In conclusion, the ASK1-p38 cascade regulates the innate immunity of the skin by forming an immune barrier consisting of hBD, LL37, and TLR2 during epidermal differentiation.

Key words: ASK1 · Keratinocyte · β-defensins · LL37 · TLR2

Introduction

The epidermis is the primary barrier between the body and the outer environment. As the epidermis is constantly exposed to a variety of microbial pathogens, its function of resisting microbial pathogens is vital. Epidermal keratinocytes differentiate and form a physical barrier consisting of a multilayered epidermis. This multilayered structure is characteristic of keratinocytes and is totally different from the simple epithelia that cover most of the gastrointestinal tract, the respiratory tract, and the urinary system. In addition to this physical barrier, the epidermis functions as an innate immune barrier to resist microbial pathogens. This characteristic feature also arises during differentiation. Dysregulation of innate immunity in the epidermis may increase susceptibility to skin infection, such as in atopic dermatitis (AD) [1]. Few recent studies have

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Abbreviations: **AD:** Atopic dermatitis · **ASK1:** Apoptosis signal-regulating kinase-1 · **BHE:** Bovine hypothalamic extract · **GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase · **hBD:** Human β-defensins · **JNK:** Jun N-terminal kinase · **LTA:** Lipoteichoic acid

considered the regulation of the immune function of epidermal keratinocytes by differentiation and the alterations with abnormal differentiation in the pathogenesis of disease.

Epidermal keratinocytes produce antimicrobial peptides, such as β-defensins and LL37. Antimicrobial peptides are the effector molecules of the innate immune system. The major human antimicrobial peptides are defensin and cathelicidin [2–5]. Defensins are cysteine-rich cationic peptides and are further classified into α- and β-defensins by their structure. α-Defensins (HNP1–4) are produced by neutrophils [2, 6], and α-defensins 5 and 6 are found in Paneth cells of the gastrointestinal tract [7, 8]. Human β-defensins (hBD)1–3 are also produced in the epithelium [9–11]. LL37 is the only human antimicrobial peptide identified in the cathelicidin family; it is produced in the epithelium and in neutrophils [5]. LL37 is secreted in human eccrine sweat to deliver innate effector molecules in the absence of inflammation [12]. Although these peptides were originally identified as effector molecules against microorganisms, it was recently found that they also mediate inflammatory responses, similar to the action of cytokines or chemokines. hBD are chemotactic for dendritic cells; LL37 is chemotactic for neutrophils, monocytes, and T cells, but not for dendritic cells [4, 13]. In addition to its effects on leukocytes, LL37 activates endothelial cells directly, resulting in increased proliferation and formation of vessel-like structures in cultivated endothelial cells and neovascularization [14]. Furthermore, LL37 binds and neutralizes LPS and lipoteichoic acids (LTA) [5], which are mediators of inflammation.

Previously, we showed that apoptosis signal-regulating kinase-1 (ASK1), a member of the MAP kinase superfamily, regulates the late-phase differentiation of epidermal keratinocytes [15]. ASK1 was identified as a MAP kinase kinase kinase involved in the stress-induced apoptosis signaling cascade that activates the SEK1-Jun N-terminal kinase (JNK) and MKK-p38 MAP kinase cascade [16]. ASK1 is activated by death receptor ligands, such as TNF-α and Fas ligand, and by various cytotoxic stresses, such as hydrogen peroxide, anticancer drugs, and growth factor deprivation [17]. Furthermore, endoplasmic reticulum stress and G protein-coupled receptor signaling were recently shown to activate ASK1 [17]. In normal human keratinocytes, ceramide increases the expression and activity of ASK1, and in turn, ASK1 induces keratinocyte differentiation. As ASK1 is expressed in the upper epidermis, it has been suggested that ASK1 regulates late-phase differentiation [15]. Recently, a tie between ASK1 and innate immunity has been reported. *C. elegans* has an ASK1 homologue, NSY-1, and a p38 homologue, PMK-1. Mutations of these genes resulted in enhanced susceptibility to microbial pathogens in *C. elegans* [18]. Therefore, we hypothesized that the ASK1-p38 cascade regulates the innate immune response in epidermal keratinocytes via differentiation. To prove this, we transfected ASK1 into cultured normal human keratinocytes and asked whether ASK1 regulates the skin innate immunity.

Results

Expression of hBD1–3, LL37, and ASK1 in normal human epidermis

Polyclonal anti-hBD1–3 and anti-LL37 antibodies were raised against synthetic peptides. The specificity of the antibodies was evaluated by Western blot (Fig. 1). Neither of the antibodies reacted to the other peptide.

We first analyzed the localization of antimicrobial peptides in normal human epidermis. hBD1, hBD2, hBD3, and LL37 were expressed in the upper epidermis (Fig. 2), suggesting that the expression is induced by differentiation. ASK1 is also expressed in the upper epidermis, as previously described [15].

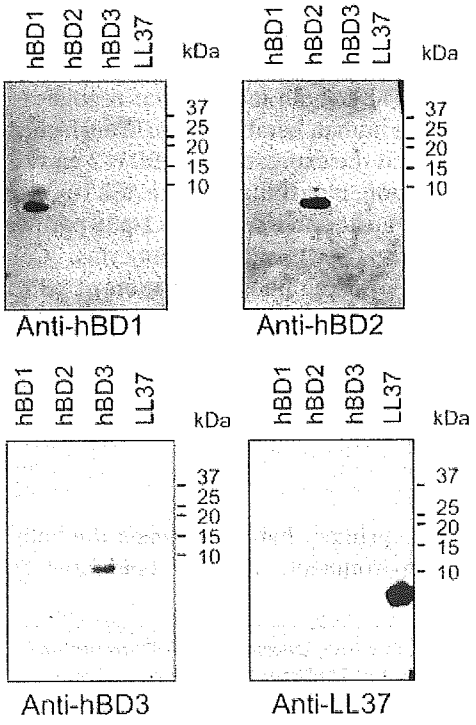

Fig. 1. Specificity of anti-hBD1–3 and LL37 antibodies. hBD1–3 and LL37 (100 ng/well) were applied to 15% SDS-PAGE with Tris/tricine buffer and transferred to PVDF membranes. After blocking, the membranes were reacted with anti-hBD1–3 or anti-LL37 antibodies overnight at 4°C. The signals were detected with an ECL detection kit.

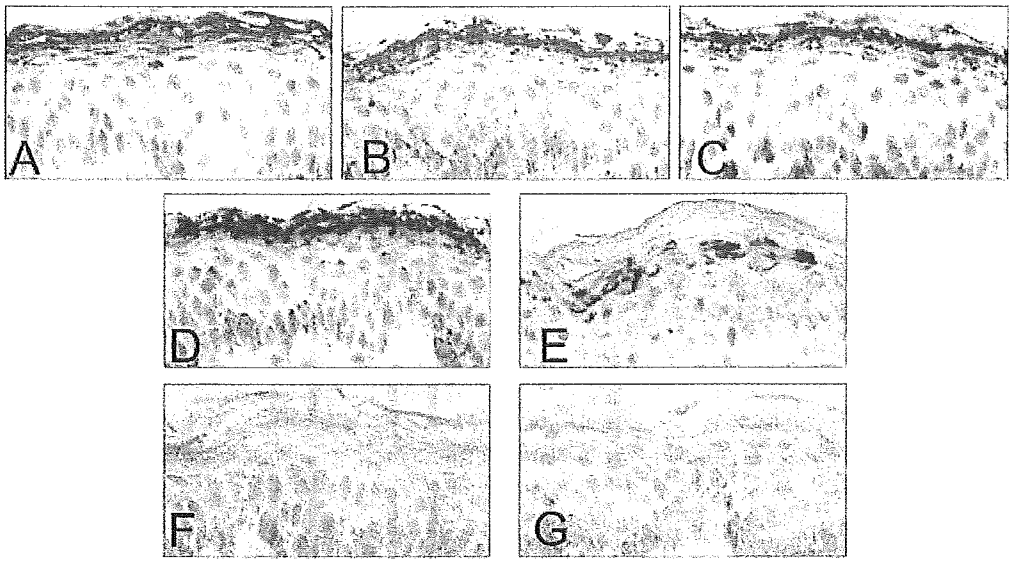

Fig. 2. Expression of hBD1–3, LL37, and ASK1 in normal human epidermis. Frozen sections of normal human skin were reacted overnight with anti-hBD1–3, anti-LL37, and anti-ASK1 antibodies. The first antibodies were detected with the corresponding second antibody using streptavidin-biotin-peroxidase and visualized with AEC. The nucleus was counterstained with hematoxylin. (A) hBD1, (B) hBD2, (C) hBD3, (D) LL37, (E) ASK1, (F) mouse pre-immune serum, and (G) rabbit pre-immune serum (600×).

Induction of hBD1–3 and LL37 by the ASK1-p38 pathway

Therefore, we hypothesized that ASK1 regulates the expression of hBD and LL37. To prove this, we introduced an active form of ASK1 (ASK1-ΔN) into cultured normal human keratinocytes using an adenovirus vector (Ax-ASK1-ΔN). Ax-β-gal carrying β-galactosidase was used as a control vector. After transfecting ASK1-ΔN into normal human keratinocytes, the mRNA expression of antimicrobial peptides was analyzed using an RNase protection assay. Transfection of ASK1-ΔN significantly enhanced the expression of hBD1, hBD2, hBD3, and LL37 mRNA (Fig. 3). The control vector had no effect on the mRNA expression. To further confirm the induction of enhanced production of antimicrobial peptides by ASK1, culture supernatants were concentrated and applied to membranes for dot blot analysis (Fig. 4). The hBD1-3 signals in supernatants of keratinocytes transfected with Ax-ASK1-ΔN were significantly higher than the signals of the control (Ax-β-gal). However, the LL37 signal of cells transfected with Ax-ASK1-ΔN was almost the same as that of the control. The discrepancy between the data for mRNA expression (Fig. 3) and the dot blot analysis for LL37 may be attributable to the mechanism of production of LL37. As LL37 is produced by extracellular cleavage of full-length cathelicidin hCAP18 by proteolytic activity after secretion [5], enhanced mRNA expression and translation of the premature form alone may not be sufficient to increase the level of LL37 in the culture supernatant *in vitro*. The supernatant of the control (Ax-β-gal) was positive for both anti-hBD1–3 and anti-LL37 antibodies when tested at higher concentrations, suggesting that

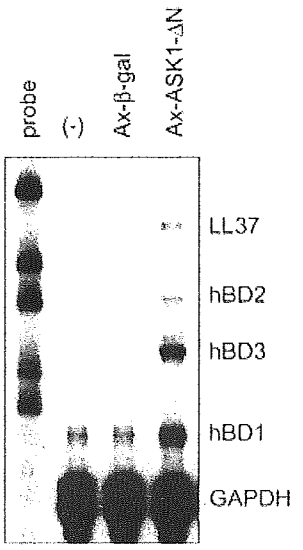

Fig. 3. Induction of hBD1-3 and LL37 by ASK1. Cultured normal human keratinocytes were infected with Ax-ASK1-ΔN or Ax-β-gal at an MOI of 5. Ax-β-gal is a control vector. After infection with the adenovirus vectors for 24 h, mRNA expression of hBD1–3 and LL37 was analyzed using an RNase protection assay. GAPDH is an internal standard.

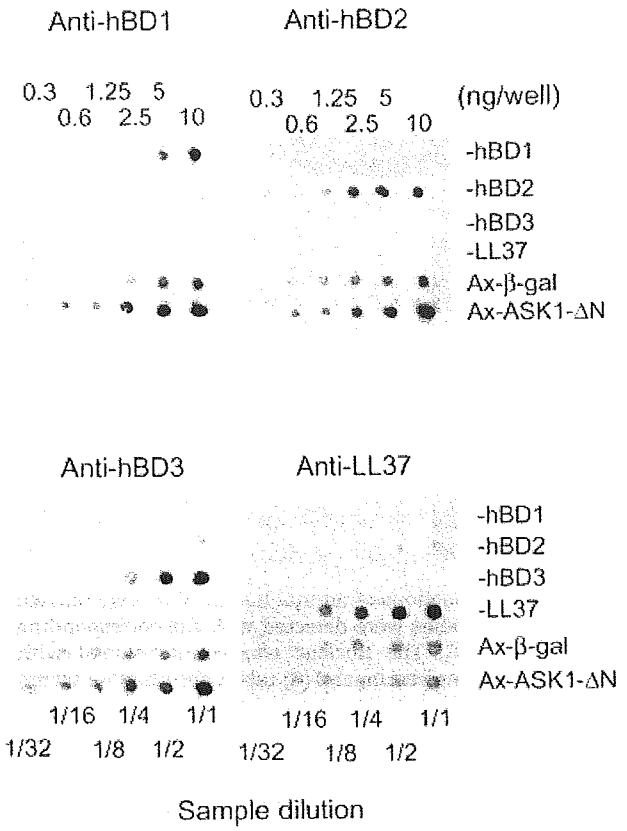

Fig. 4. Dot blot analysis of hBD1–3 and LL37. After transfection of the adenovirus vectors into normal human keratinocytes in 10-cm dishes at an MOI of 5, the medium was replaced with 5 ml medium without BHE, and the cells were cultured for an additional 2 days. After adjusting the pH of the supernatants to 7.2, 1.0 ml of pre-treated MacroPrep beads was added to 100 ml of the culture supernatants, gently shaken overnight at 4°C, washed with 25 mM ammonium acetate buffer at pH 7.4 and then eluted with 1.0 ml 5% acetic acid. The eluates were freeze-dried and redissolved in 1.0 ml distilled water. Samples (1 μl) of serially diluted, reconstituted supernatants or control solutions containing synthetic peptides (hBD1–3 and LL37) were applied to nitrocellulose membranes and air-dried. After blocking, the membranes were reacted overnight at 4°C with anti-hBD1–3 or anti-LL37 antibodies diluted 1:1,000. After extensive washing, the signals were detected using an ECL kit.

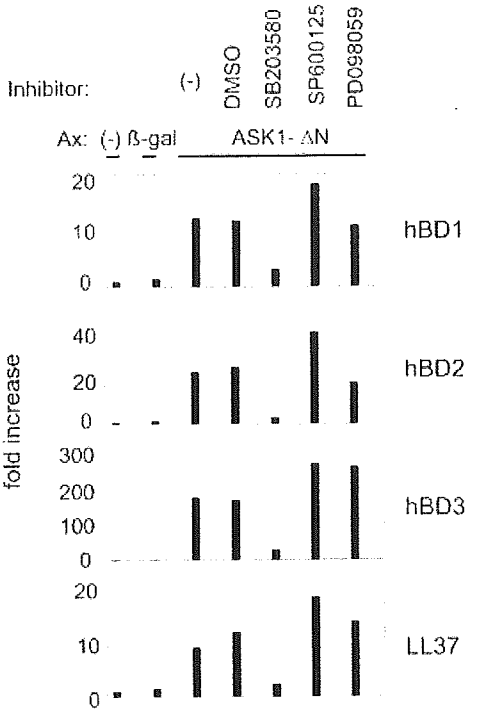

Fig. 5. Inhibition of ASK1-induced hBD and LL37 by MAP kinase inhibitors. The involvement of p38, JNK, and MEK was analyzed using specific inhibitors: SB203580 (200 nM), SP600125 (20 μM), and PD098059 (30 μM), respectively. DMSO was the vehicle. Normal human keratinocytes were pretreated with the inhibitors for 1 h. Then, the cells were transfected with the adenovirus vectors. After 24 h, hBD and LL37 mRNA expression was analyzed using quantitative RT-PCR. The data are adjusted to the internal standard GAPDH and represent the fold increase compared with the control. There were three samples in each group. Three independent experiments were carried out, and similar results were obtained in each.

keratinocytes produce small amounts of these peptides even at non-differentiated conditions.

As p38 is located downstream of ASK1 and is activated by ASK1 in keratinocytes [15], we next analyzed whether p38 is involved in the induction of antimicrobial peptides by ASK1. Normal human keratinocytes were pretreated with a p38 inhibitor (SB203580), a JNK inhibitor (SP600125), and a MAPK/ERK kinase (MEK) inhibitor (PD098059), and then ASK1-ΔN was transfected. mRNA expression was analyzed using quantitative RT-PCR. The p38 inhibitor almost completely abolished the induction of hBD1, hBD2, hBD3, and LL37 (Fig. 5). Conversely, the JNK and MEK inhibitors did not reduce the induction of hBD or LL37. Therefore, the ASK1-p38 pathway regulates the expression of hBD and LL37.

Regulation of TLR2 expression by the ASK1-p38 pathway

Next, we tested whether the ASK1-p38 pathway regulates the expression of TLR2, a receptor for the cell wall components of gram-positive bacteria [19]. Although peptidoglycans were previously shown to be a ligand for TLR2, it has recently been suggested that the sensing of peptidoglycans by TLR2 was attributable to the binding of contaminating lipoproteins or LTA from bacterial cell walls that were present in the peptidoglycan preparations [20]. ASK1 transfection enhanced TLR2 mRNA expression (Fig. 6A). The p38 inhibitor, SB203580, abolished this enhancement (Fig. 6B). Therefore, in addition to hBD and LL37, the ASK1-p38 cascade regulates TLR2 expression in human keratino-

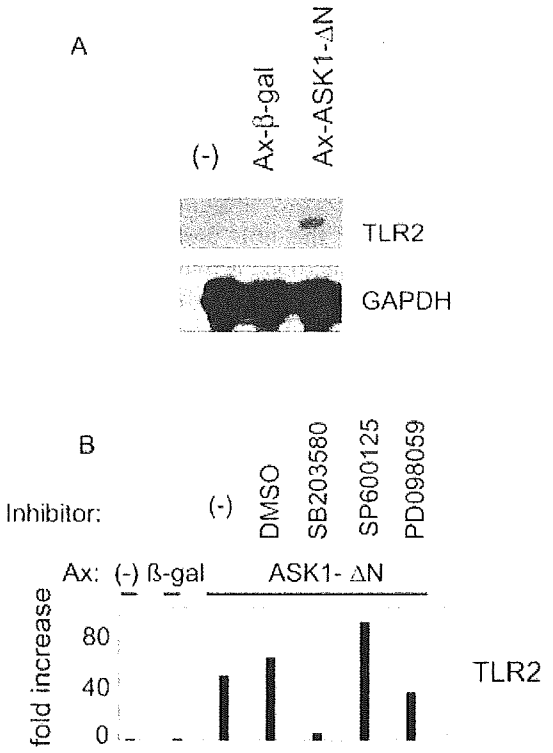

Fig. 6. Regulation of TLR2 expression by the ASK1-p38 pathway. (A) Induction of TLR2 expression by ASK1. Induction of TLR2 expression by ASK1 was analyzed by transfecting cultured normal human keratinocytes with Ax-ASK1-ΔN at an MOI of 5. Ax-β-gal was the control vector. After 24 h, TLR2 mRNA expression was analyzed using an RNase protection assay. GAPDH was the internal standard. (B) Inhibition of ASK1-induced TLR2 expression by MAP kinase inhibitors. The involvement of p38, JNK, and MEK was analyzed using specific inhibitors: SB203580 (200 nM), SP600125 (20 μM), and PD098059 (30 μM), respectively. DMSO was the vehicle. Normal human keratinocytes were pretreated with the inhibitors for 1 h. Then, the cells were transfected with the adenovirus vectors. After 24 h, TLR2 mRNA expression was analyzed using quantitative RT-PCR. The data are adjusted to the internal standard GAPDH and represent the fold increase compared with the control. There were three samples in each group. Three independent experiments were carried out, and similar results were obtained in each.

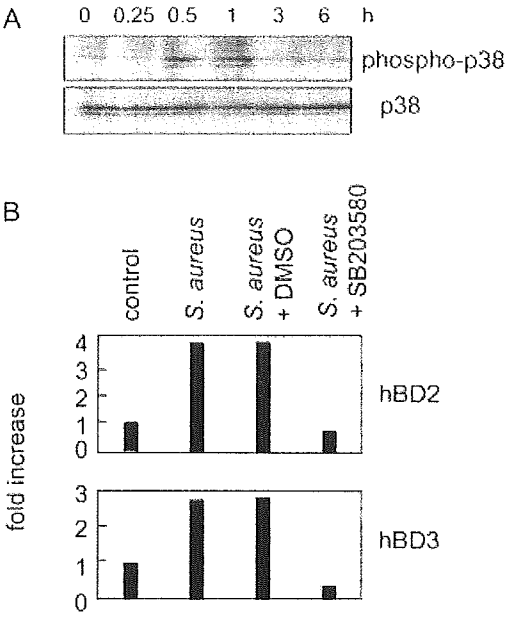

Fig. 7. Induction of hBD2 and hBD3 by *S. aureus*. Overnight cultures of *S. aureus* (209P) grown in TSB at 37°C were washed with PBS and then incubated at 68°C for 30 min to inactivate the bacteria. The heat-inactivated bacteria (final concentration, 10^8 cells/ml) were added to subconfluent keratinocytes in MCDB153 medium without supplements, and the keratinocytes were cultured and harvested for analysis of mRNA expression and p38 phosphorylation. (A) Phosphorylation of p38 was analyzed using Western blotting with anti-phospho-p38 and anti-p38 antibodies at the indicated times for up to 6 h. (B) Normal human keratinocytes were pretreated with the inhibitor SB203580 (200 nM) for 1 h; heat-killed *S. aureus* was then added to the cultures. The induction of hBD2 and hBD3 was analyzed at 12 h using quantitative RT-PCR. Three independent experiments were carried out, and similar results were obtained in each.

cytes. However, ASK1 transfection did not enhance the expression of TLR3, 4, 5, 7, and 9 (data not shown).

Inhibition of *S. aureus*-induced hBD2 and hBD3 by SB203580

To study the role of p38 further, we used the model of induction of antimicrobial peptides by *S. aureus*. Direct contact of *S. aureus* with keratinocytes induces antimicrobial peptides [21]. We studied whether p38 is involved in this induction. After contact with *S. aureus*, the phosphorylation of p38 started to increase at 0.5 h and lasted until 1 h (Fig. 7A). Furthermore, SB203580 inhibited *S. aureus*-induced hBD2 and hBD3 expression (Fig. 7B). These results indicate that p38 is involved in *S. aureus*-induced hBD2 and hBD3 production.

Discussion

The epithelium of the skin (epidermis), the respiratory tract, the gastrointestinal tract, and the urinary system serves as the frontline for immune system resistance to microbial pathogens. The epithelium produces antimicrobial peptides, such as hBD and LL37. The mechanisms most commonly regulating the production of antimicrobial peptides are contact with the pathogenic bacteria and cytokines. Colon epithelial cells produce hBD2 upon stimulation by bacteria [22]. In the small intestine, Paneth cells secrete α-defensins in response to bacteria or bacterial antigens [23]. Tracheo-

bronchial epithelial cells produce hBD2 after contact with bacterial lipopeptide [24]. In epidermis, bacterial contact [9, 21, 25], cytokines including TNF-α and IL-1 [9, 25, 26], and wounding [27] are reported to stimulate keratinocytes to produce antimicrobial peptides. Our study clarified a new mechanism: ASK1-p38 cascade-mediated differentiation regulates the production of antimicrobial peptides in epidermal keratinocytes.

Although several reports have shown the expression of hBD2 [28, 29] and LL37 [30] in normal human epidermis, the expression patterns of these peptides remain controversial, because it has also been reported that the expression levels of hBD2 and LL37 are low in the epidermis in organotypic cultures [26] or absent in normal epidermis [27, 31, 32]. This discrepancy may be at least partially attributable to the low levels of expression of hBD2 and LL37 in normal epidermis and to the immunostaining methods used by the different groups. Some authors used frozen sections [30, 31] and others used samples processed without freezing, such as paraffin-embedded tissue sections [26–29, 32]. Furthermore, different antibodies were used in these studies. Apparently, hBD2, hBD3, and LL37 are inducible by inflammation or infection [26, 28, 30–32], as we have also shown in Fig. 7 and in a previous report [21]. Therefore, the relatively low expression of these peptides in normal tissues, as compared with the expression levels in the epidermis with inflammation or infection, may not be detectable using some techniques. Taken together, the findings indicate that the expression levels of hBD2, hBD3, and LL37 in the normal epidermis, shown in Fig. 2, should be considered the basal level, which may be further increased by stimulation by inflammation or infection.

A low level of hBD1 mRNA expression was seen in keratinocytes before ASK1 transfection (Fig. 3). This suggests that regulatory mechanisms other than ASK1 affect hBD1 expression. As hBD1 expression in keratinocytes is reported to be regulated by calcium [33] and cell confluency [34], it is most probable that ASK1 is not the sole factor regulating hBD1 expression. In the present study, ASK1 was shown to participate only in the induction phase of hBD1 expression and may not be involved in the regulation of the basal expression of hBD1.

Keratinocytes produce antimicrobial peptides in response to two different stimuli: differentiation and bacterial contact. Although ASK1-mediated differentiation induces hBD1–3, bacterial contact induces only hBD2 and 3 [21]. This indicates that the mechanisms to induce antimicrobial peptides by differentiation differ from that by bacterial contact. Therefore, keratinocytes have at least two different mechanisms to induce antimicrobial peptides. However, both pathways involve p38, suggesting that signals downstream of p38 are the same in both pathways.

In contrast to the idea that differentiation regulates the expression of the antimicrobial peptides, there is an interesting report suggesting that hBD1 drives the differentiation of keratinocytes [34]. As hBD1 is localized to the upper epidermis, it is possible that hBD1 is involved in ASK1-mediated differentiation mechanisms in keratinocytes. Recently it became evident that the roles of the hBD and LL37 are not limited only to their antimicrobial activities but include certain roles in adaptive and innate immunity [4, 5, 13, 14]. The concept that hBD1 drives keratinocyte differentiation suggests a new aspect of the functions of antimicrobial peptides.

Bacterial contact activates p38, as shown in Fig. 7. Although p38 regulates the expression of involucrin and differentiation in keratinocytes [15, 35], bacterial contact does not induce involucrin expression or differentiation (data not shown). Similar inconsistencies are also observed with cellular stresses, such as UV irradiation. UV irradiation activates p38, but does not induce involucrin or differentiation (data not shown). However, ASK1-induced p38 activation differentiates keratinocytes [15]. Although p38 phosphorylation occurs with UV irradiation, bacterial contact, and differentiation, the strength or duration of the signal may be important in the differential reaction to each stimulus.

Although ASK1 enhanced TLR2 mRNA expression in keratinocytes, we failed to detect TLR2 on keratinocytes by cell sorter analysis (data not shown) using the monoclonal antibody TL2.1, the most commonly used antibody against TLR2, as previously described [36]. However, several groups have shown TLR2 expression on primary cultured keratinocytes [37] and normal human epidermis [38] using polyclonal goat anti-TLR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). In light of the observations that keratinocytes react with gram-positive bacteria [9, 21, 25] and peptidoglycans [37] (or contaminating lipoproteins or LTA in peptidoglycan preparations [20]), the presence of TLR2 on keratinocytes can be assumed. This issue should be studied further, assisted by the development of new antibodies against or ligands for TLR2.

Since skin innate immunity is regulated by keratinocyte differentiation as shown in the present study, abnormal keratinocyte differentiation may disturb skin innate immunity. Among the diseases with abnormal differentiation and innate immunity, AD is the most common skin disease. AD is a highly pruritic chronic inflammatory skin disease. With its increased prevalence, AD has become a major public health problem. AD is complicated by recurrent skin infections, including bacterial, fungal, and viral infections [1]. As a skin infection triggers the exacerbation of AD, there is considerable interest in the mechanisms underlying the

increased colonization of bacteria on the AD skin lesion. The mechanism that eliminates *S. aureus* from the epidermis is impaired in AD. This immune abnormality is the result, at least in part, of the reduced expression of antimicrobial peptides in the epidermis [31, 39]. The mechanism of reduced expression of antimicrobial peptides is thought to consist in increased levels of IL-4 and IL-13 [31]. These Th2-type cytokines inhibit the expression of antimicrobial peptides in human keratinocytes [31]. In addition, epidermal differentiation is impaired in AD. A hallmark of epidermal differentiation is the cornified envelope in the stratum corneum, an insoluble structure that replaces the plasma membrane of differentiated keratinocytes [40]. The cornified envelope in the involved area of AD is immature [41]. This physical barrier is formed during keratinocyte differentiation; thus, the barrier disruption in AD [42] is due to impaired differentiation, making AD a disease involving abnormal keratinocyte differentiation. As shown here, an innate immune barrier is formed during differentiation; consequently, the dysregulation of epidermal differentiation in AD would increase the susceptibility to skin infection. This hypothesis should be further studied as a new pathogenesis of AD.

In conclusion, the ASK1-p38 cascade forms an innate epidermal immune barrier consisting of hBD, LL37, and TLR2 during epidermal differentiation.

Materials and methods

Synthesis of antimicrobial peptides

hBD1, 2, and 3 and LL37 were synthesized using a peptide synthesizer (Shimazu, Tokyo, Japan), as described [21]. The peptides were purified using reversed-phase high-performance liquid chromatography with an octadecyl-4PW column (Tosoh, Tokyo, Japan) and a linear gradient from aqueous 0.05% trifluoroacetic acid (TFA) to 100% acetonitrile containing 0.05% TFA. The peptides were then lyophilized to completely remove the organic solvent. To confirm the purity and quality of the peptides, mass spectrometry using the MALDI/TOF-MS method was performed with Voyager (PerSeptive Biosystems, MA).

Antibodies

Antiserum to ASK1, called PEL, was raised in rabbits against the peptide sequence PELRPHFSLASESDTAD (amino acids 1172–1188 of human ASK1), as described [16]. Rabbit anti-hBD2 and anti-LL37 antibodies were raised against synthetic hBD2 and LL37, respectively. Mouse polyclonal anti-hBD1 and anti-hBD3 antibodies were also raised against synthetic hBD1 and hBD3, respectively.

Immunohistochemical staining

Frozen skin sections were fixed with methanol containing 3% H_2O_2 at 4°C for 5 min, washed with PBS and blocked with 10% goat serum. The sections were reacted overnight at 4°C with rabbit or mouse first antibody diluted 1:1,000 in PBS. After washing with PBS, the first antibodies were detected using a streptavidin-biotin-peroxidase staining kit (Nichirei Co. Inc., Tokyo, Japan) and visualized with 3-amino-9-ethyl-carbazole (AEC) according to the manufacturer's instructions. Control staining with pre-immune serum showed no positive signal. The nucleus was counterstained with hematoxylin.

Adenovirus vectors

An adenovirus vector (Ax) encoding a constitutively active form of ASK1 (Ax-ASK1-ΔN) was prepared as described [15]. The adenovirus vector expressing a bacterial β-galactosidase gene (Ax-β-gal) was used as a control to exclude the effect of the vector itself. Virus stocks were prepared using a standard procedure. Concentrated, purified virus stocks were prepared using the CsCl gradient method, and the virus titer was checked with the plaque formation assay. Normal human keratinocytes were infected with adenovirus vector at a concentration of multiplicity of infection (MOI) equaling 5. Protein expression was confirmed by Western blot analysis.

Keratinocyte culture

Human skin samples were obtained after plastic surgery under a protocol approved by the Institutional Review Board of Ehime University School of Medicine. Primary normal human keratinocytes were isolated from neonatal surgical-discard skin. Normal human keratinocytes were cultured with MCDB153 medium supplemented with insulin (1 μg/ml), hydrocortisone (0.5 μg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (BHE) (50 μg/ml), and Ca^{2+} (0.1 mM), as described [43].

RNase protection assay

Total RNA was prepared using Isogen (Nippon Gene Co., Tokyo, Japan). The analysis was performed using the Multi-Probe RNase Protection Assay System (PharMingen Co.) according to the manufacturer's guidelines. Oligonucleotide probes were prepared by inserting PCR-amplified human cDNA corresponding to oligonucleotides 151–265 of hBD1 (GenBank accession no. U73945), 44–200 of hBD2 (NM004942), 65–204 of hBD3 (AF295370), 321–517 of CAP18/LL37 (NM004345), and 2204–2513 of TLR2 (AF051152) into the Eco RI and Hind III sites of the pPMG vector. Total RNA (5 μg) was hybridized with a ^{32}P-labeled riboprobe and digested with RNase. The hybridization products were separated on 5% polyacrylamide-8 M urea gels and exposed to film. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal standard.

Quantitative RT-PCR analysis

To quantify the mRNA expression *in vivo*, we performed quantitative RT-PCR using the ABI PRISM 7700 sequencer detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). RT-PCR mixtures were prepared according to the manufacturer's instructions for the TaqMan One-Step RT-PCR Master Mix Reagent kit (Perkin-Elmer Applied Biosystems). Briefly, 50 ng total RNA were added to each 50-μl reaction mixture containing Master Mix, MultiScribe, and RNase Inhibitor Mix, 200 nM of each primer, and 100 nM hybridization probe specific for the target cDNA. The probe was labeled with a reporter fluorescent dye [6-carboxyfluorescein (FAM)] at the 5′ end. For GAPDH detection, Pre-Developed TaqMan Assay Reagent (Perkin-Elmer Applied Biosystems) was added. The thermal conditions were 48°C for 30 min for reverse transcription and 95°C for 10 min, followed by 45 amplification cycles of 95°C for 15 s for denaturing and 55°C for 1.5 min for annealing and extension. The PCR products were sequenced to confirm proper amplification. To compare mRNA expression, the results were determined as relative values using GAPDH as an internal reference. There were $n=3$ samples in each group. The sense and antisense primers and probes for hBD1–3 and CAP18/LL37 were as follows: HBD1–67F: TCG CCA TGA GAA CTT CCT ACC T, HBD1–195R: CTC CAC TGC TGA CGC AAT TGT A, HBD1–136T: CCA CCT GAG GCC ATC TCA GAC AAA AGT AAG, HBD2–16F: TGA AGC TCC CAG CCA TCA G, HBD2–144R: GGC TCC ACT CTT CCA AAG GA, HBD2–107T: CAC CAA AAA CAC CTG GAA GAG GCA TCA, HBD3–346F: TCA GCT GCC TTC CAA AGG A, HBD3–414R: TTC TTC GGC AGC ATT TTC G, HBD3–367T: AAC AGA TCG GCA AGT GCT CGA CGC, CAP18–284F: CAC AGC AGT CAC CAG AGG ATT G, CAP18–366R: GGC CTG GTT GAG GGT CAC T, and CAP18–341T: ATA CAC CGC TTC ACC AGC CCG TCC. The primers and probe for TLR2 were purchased from Applied Biosystems (Assays-on-Demand).

Western blotting

The analysis of p38 was performed using a Vistra ECF kit (Amersham Life Science, Inc., Arlington Heights, IL) according to the manufacturer's instructions. Proteins (20 μg) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-HCl pH 7.4, 0.15 M NaCl, and 0.05% Tween-20, and incubated with rabbit anti-p38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-phospho-p38 (Cell Signaling Technology, Inc., Beverly, MA) antibodies. After washing, the membrane was incubated with fluorescein-labeled goat anti-mouse IgG (diluted 1:2,500) for 1 h. The signal was amplified using an anti-fluorescein antibody conjugated with alkaline phosphatase, followed by the fluorescent substrate AttoPhos (Amersham Life Science). Then, the membrane was scanned using a FluoroImager (Molecular Dynamics, Inc., Sunnyvale, CA).

For Western blotting of hBD1–3 and LL37, the synthetic peptides (100 ng) were applied to 15% SDS-PAGE with Tris/tricine buffer and transferred to PVDF membranes. After blocking with 5% non-fat dry milk, the membranes were reacted overnight at 4°C with anti-hBD or anti-LL37 antibodies diluted 1:1,000. After extensive washing, the signals were detected using an ECL Advance Western blotting detection kit (Amersham Biosciences, Piscataway, NJ).

Dot blot analysis

Antimicrobial peptides were prepared for dot blot analysis by partial purification from the culture supernatants. MacroPrep CM Support beads (Bio-Rad Laboratories, Richmond, CA) were washed with distilled water and then pretreated with 25 mM ammonium acetate buffer, pH 7.4. After transfection of the adenovirus vectors into normal human keratinocytes in 10-cm dishes at an MOI of 5, the medium was replaced with 5 ml medium without BHE, and the cells were cultured for an additional 2 days. The culture supernatants were centrifuged and filtered through 0.22-μm filters to remove the cellular debris. After adjusting the pH of the supernatants to 7.2, 1.0 ml of pretreated MacroPrep beads was added to 100 ml of the culture supernatants, gently shaken overnight at 4°C, washed with 25 mM ammonium acetate buffer at pH 7.4 and then eluted with 1.0 ml 5% acetic acid. The eluates were freeze-dried and redissolved in 1.0 ml distilled water.

Samples (1 μl) of serially diluted, partially purified supernatants or control solutions containing synthetic peptides (hBD1–3 and LL37) were applied to nitrocellulose membranes and air-dried. After blocking with 5% non-fat dry milk, the membranes were reacted overnight at 4°C with anti-hBD or anti-LL37 antibodies diluted 1:1,000. After extensive washing, the signals were detected using an ECL Advance Western blotting detection kit (Amersham Biosciences).

Induction of antimicrobial peptides by *S. aureus*

Induction of antimicrobial peptides by *S. aureus* was analyzed as described [21]. We used FDA 209P (ATCC6538), a laboratory strain, for *S. aureus*. Overnight cultures of *S. aureus* (209P) grown in tryptic soy broth (TSB) at 37°C were harvested and washed twice with PBS. Then, the *S. aureus* cells were suspended in PBS and incubated at 68°C for 30 min to kill the bacteria. The *S. aureus* suspension was subjected to mild sonication, and the dissociation of clumps was confirmed by microscopic observation. Before bacterial contact (12 h), the confluent keratinocyte medium was replaced with MCDB153 medium without supplements, and then the heat-killed bacteria were added (final concentration, 10^8 cells/ml) to the medium. The culture was incubated for an appropriate time period, and mRNA expression and p38 phosphorylation were analyzed. This experiment was carried out three times independently.

Inhibitors

SB203580, PD098059, and SP600125 were purchased from Calbiochem-Novabiochem International Co. (San Diego, CA), dissolved in dimethyl sulfoxide (DMSO) at 2, 30, and 20 mM, respectively, as stock solutions, and used at final concentrations of 200 nM, 30 μM, and 20 μM, respectively.

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Microbubble-enhanced ultrasound for gene transfer into living skin equivalents

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Summary

Background: Gene transfer to skin is an attractive therapeutic approach because of the accessibility of the skin and the high rate of cure for many cutaneous diseases. However, safety concerns over viral vectors and the low efficiency of most non-viral gene transfer techniques have encumbered their clinical application for gene transfer. By contrast, efficient gene transfers into various cell types using microbubble-enhanced ultrasound has been reported.
Objectives: The purpose of this study was to investigate whether ultrasound with microbubble enhancement allowed effective transfer of foreign genes into living skin equivalents (LSEs).
Methods: Microbubbles and plasmid DNA encoding green fluorescent protein (GFP) were added to the dermal–epidermal junctions of LSEs, which were then exposed to ultrasound. The LSEs were harvested at different time points to investigate transgene expression using confocal laser microscopy. Transfected LSEs were also transplanted onto nude mice, and the in vivo transgene expression was observed.
Results: From days 2 to 7 after transfection, most GFP-positive cells continued to migrate upward from the basal layer, while other GFP-positive cells lagged behind or remained in the basal layer on days 5 and 7. Transfection resulted in 20–30% GFP-

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positive cells. Multiple transfections further increased the percentage of transfected cells and resulted in multi-layer transgene expression. Grafts from the transfected LSEs survived on nude mice and continued to express GFP up to 2 weeks post-transplantation.

Conclusion: Gene transfer into LSE using ultrasound with microbubble enhancement is an effective alternative to viral and non-viral methods.

1. Introduction

A human living skin equivalent (LSE) consists of keratinocytes seeded upon a fibroblast-populated type I collagen gel. LSEs are now readily cultured and applied in the treatment of burn wounds, ulcers, and other skin defects [1–3]. It has been suggested that this three-dimensional skin substitute could be genetically modified and transplanted in order to deliver therapeutic proteins locally and systemically for the treatment of cutaneous diseases and systemic disorders characterized by low or absent levels of circulating polypeptides [4,5]. LSE is an attractive ex vivo approach to gene delivery, as it allows confirmation of the efficiency of gene transfer and avoids the need to administer the vectors directly to patients, with the concomitant risk of systemic dissemination [6]. Virally transduced keratinocytes have been deposited onto dermal matrices to construct genetically engineered LSEs that expressed the transgene in vitro and on nude mice after transplantation [7,8] and that corrected the abnormal morphogenesis and malfunction of LSEs comprising cells expressing certain genodermatoses [9,10]. However, safety concerns regarding viral vectors, such as immunogenicity, insertional mutagenesis, and cytopathic effects, as well as the low efficiency of most non-viral gene transfers, have encumbered their use in clinical application [11–13].

Several non-viral approaches for gene transfer to skin have been introduced [14,15], among which, in vivo intradermal injection has successfully transduced naked DNA into keratinocytes [16] and the cytokine transgene expression has provided biological effects in the treated skin [17,18]. However, non-viral gene transfer into living skin equivalent has not been established. Recently, microbubble-enhanced ultrasound, as a new non-viral gene delivery approach, has efficiently transferred foreign genes into cardiac cells, muscle cells, and prostate cancer cells [19–22]. In the present study, we demonstrate that this approach is also effective in delivering genes into LSE.

2. Materials and methods

2.1. Monolayer cell culture

Normal human epidermal keratinocytes were cultured in MCDB 153 type II medium as previously described [23,24]. Keratinocytes in their fourth passage were used for LSE cultures.

Normal human dermal fibroblasts were isolated from normal human skin and cultured in DMEM (Gibco) supplemented with 10% FCS. Fourth- or fifth-passage cells were used in constructing the LSEs.

2.2. Preparation of LSEs

1. Collagen gel was prepared as follows: six volumes of ice-cold porcine collagen type I solution (Nitta Gelatin Co., Osaka, Japan) were mixed with one volume of 8× DMEM (Gibco), 10 volumes of 1× DMEM supplemented with 20% FCS, and one volume of 0.1N NaOH. One millilitre of the resulting solution was added to each culture insert (Transwel, collagen membrane bottom, diameter: 24 mm, membrane pore size: 3 μm, Costar) in a six-well culture plate (Costar). After polymerization of the solution on the insert at 37 °C, 3.5 ml of fibroblast-containing collagen solution was applied onto each insert, followed again by polymerization at 37 °C. DMEM supplemented with 10% FCS and ascorbic acid (final concentration 50 ng/ml) was then added to the fibroblast-rich gel. After 5 days of culture, the fibroblasts had contracted the gel.
2. Five days after the dermal component was prepared, 6.0×10^5 keratinocytes in 60 μl MCDB 153 type II were seeded onto the concave surface of the contracted gel. The resulting LSE was maintained submerged in culture medium for 2 days. When the keratinocytes reached confluence, the LSE was raised to the air–liquid interface, and cornification medium (CM), composed of a 1:1 mixture of Ham's F-12 and DMEM supplemented with 2% FCS, 0.1 mM ethanola-

mine, 0.1 mM *o*-phosphoethanolamine, 0.4 μg hydrocortisone/ml, 5 μg insulin/ml, 12.2 μg adenine/ml, 5 μg transferrin/ml, 6.83 μg selenious acid/ml, 13.46 pg triiodothyronine/ml, 89.34 μg choline chloride/ml, 105.1 μg serine/ml, 20 μg linoleic acid/BSA/ml, and 50 μg ascorbic acid/ml was added. The medium was changed every other day.

2.3. Transfection of GFP plasmid DNA

A commercial reporter plasmid, pEGFP-C2 vector 6083-1 (Clontech, Japan), encoding green fluorescent protein (GFP), was used for transfection. Plasmid DNA was obtained from *Escherichia coli* JM 109 cultures and prepared with a kit (Qiagen Midi; Qiagen, Chatsworth) according to the manufacturer's instructions. Agarose gel electrophoresis was performed before and after restriction endonucleases digestion to verify the identity and purity of the plasmid DNA.

GFP plasmid DNA was transfected into the LSE on day 7 after air-lift. As stated in the protocol accompanying the MB-3 albumin-coated octafluoropropane-gas-filled microbubbles (purchased from Neppa Gene Co., Japan), GFP plasmid was mixed with the microbubbles (final DNA concentration, 200 μg/ml). The plasmid DNA and microbubbles (10 μl) were added to the dermal–epidermal junction of the LSEs, which were then immediately transferred to a six-well plate (Costar) and exposed to ultrasound using an ultrasound generator (Ultax Model No. UX-301, Celcom Inc., Japan), with the probe applied to the bottom of the plate. The optimal conditions for gene transfection were determined by testing various combinations of exposure parameters: frequency (from 1 to continuous), intensity (from 1 to 10), and exposure duration (from 1 to 60 s). Following the exposure to ultrasound, the transfected LSEs were kept in culture for 2 more days, after which the epidermis was peeled off and checked for GFP expression using confocal laser microscopy (LSM 510, Zeiss, Germany). The remaining layers of the LSEs were cultured continuously in air-lifted status and harvested on days 2, 5, and 7 after transfection.

Plasmid DNA re-transfection was also conducted. LSEs were divided into three groups. The first group was transfected on day 7 after air-lift; the second group was re-transfected on days 7 and 9, and the third group on days 7, 9, and 11. All the LSEs were harvested on day 13 after air-lift.

In the transfection and re-transfection trials, the epidermis was peeled off of the dermal matrix of the LSEs and cut into two pieces. One piece was mounted directly for observation of horizontal sections of the LSE; the other was snap frozen and sectioned vertically at 7 μm intervals. The sections were evaluated by confocal laser microscopy. Three independent experiments were performed.

2.4. Transplantation of GFP-transfected LSEs onto nude mice

On day 7 after air-lift, GFP-containing plasmid was transfected into the LSEs by microbubble-enhanced ultrasound as described above. On the 2nd day of transfection, the epidermal layers of the LSEs were peeled off and grafted onto full-thickness wounds on the backs of 8-week-old, female BALB/cAJc1-nu nude mice. The grafting protocol was approved by the Ethics Committee of Ehime University School of Medicine. Each mouse was anesthetized by an intraperitoneal injection of 0.3 ml avertin (1.25% tribromoethanol, 2.5% 2-methyl-2-butanol). An 8 mm skin biopsy punch was used to create a full-thickness wound deep to the fascia of the dorsal musculature,

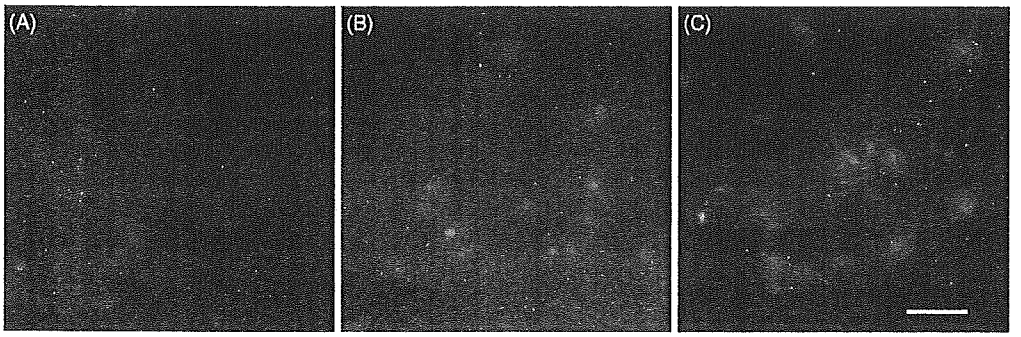

Fig. 1 The influence of the ultrasound exposure duration on gene transfer efficiency in LSEs. Microbubble-enhanced ultrasound gene transfer to living skin equivalents (LSEs) was performed at 2 Hz and an intensity of 8 for 10 s (A), 30 s (B), and 60 s (C). Green fluorescent protein (GFP) expression in the peeled epidermis is observed 2 days later using fluorescence microscopy. Scale bar, 50 μm.

and an LSE graft of corresponding size, removed by the same punch, was applied to the wound and covered with transparent film. On days 3, 5, 7, and 14 after transplantation, the nude mice were killed, the transplanted grafts were excised, and each graft was cut into two pieces.

One piece was fixed in 20% formalin, embedded in wax, and sectioned at 5 μm intervals for H&E staining. The other piece was frozen in OCT compound and sectioned at 7 μm intervals for observation using confocal laser microscopy. Three independent experiments were performed.

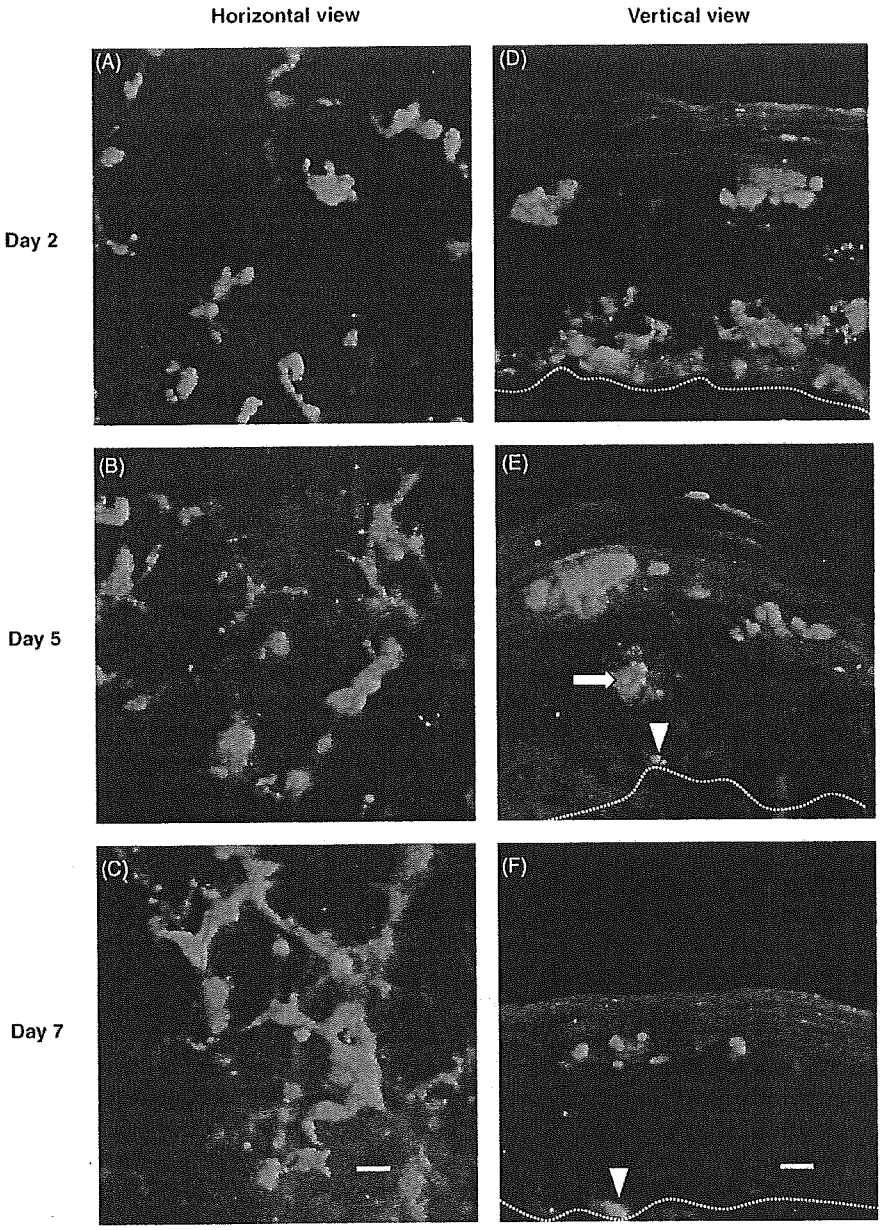

Fig. 2 The dynamics of GFP expression in LSEs after transfection. Samples were collected on day 2 (A, D), day 5 (B, E), and day 7 (C, F) after transfection. For each sample, horizontal (A–C) and vertical (D–F) microscopy sections were examined. The horizontal sections show that fluorescence-positive cells had spread and merged with each other from day 2 (A) to day 5 (B) to day 7 (C). The vertical sections show that most fluorescence-positive cells are located in the basal or suprabasal layer on day 2 (D) and move to the stratum corneum on day 5 (E) and day 7 (F), while some positive cells either lag behind in their upward migration (arrow in Fig. 2E) or remain in the basal layer on day 5 (arrow head in E) and day 7 (arrow head in F). The white-dotted line indicates the dermal–epidermal junction. Scale bar, 50 μm.

3. Results

3.1. Determination of optimal ultrasound exposure parameters

After testing various combinations of ultrasound frequency, intensity, and duration, an exposure of 2 Hz at an intensity of 8 for 60 s was found to most efficiently transfer the GFP plasmid into LSEs. Fig. 1 shows the influence of the ultrasound exposure duration on the GFP gene transfer efficiency. At a frequency of 2 Hz and an intensity of 8, more fluorescence-positive cells were found in the epidermis of the LSE exposed to ultrasound for 60 s (Fig. 1C) than for 10 s (Fig. 1A) or 30 s (Fig. 1B).

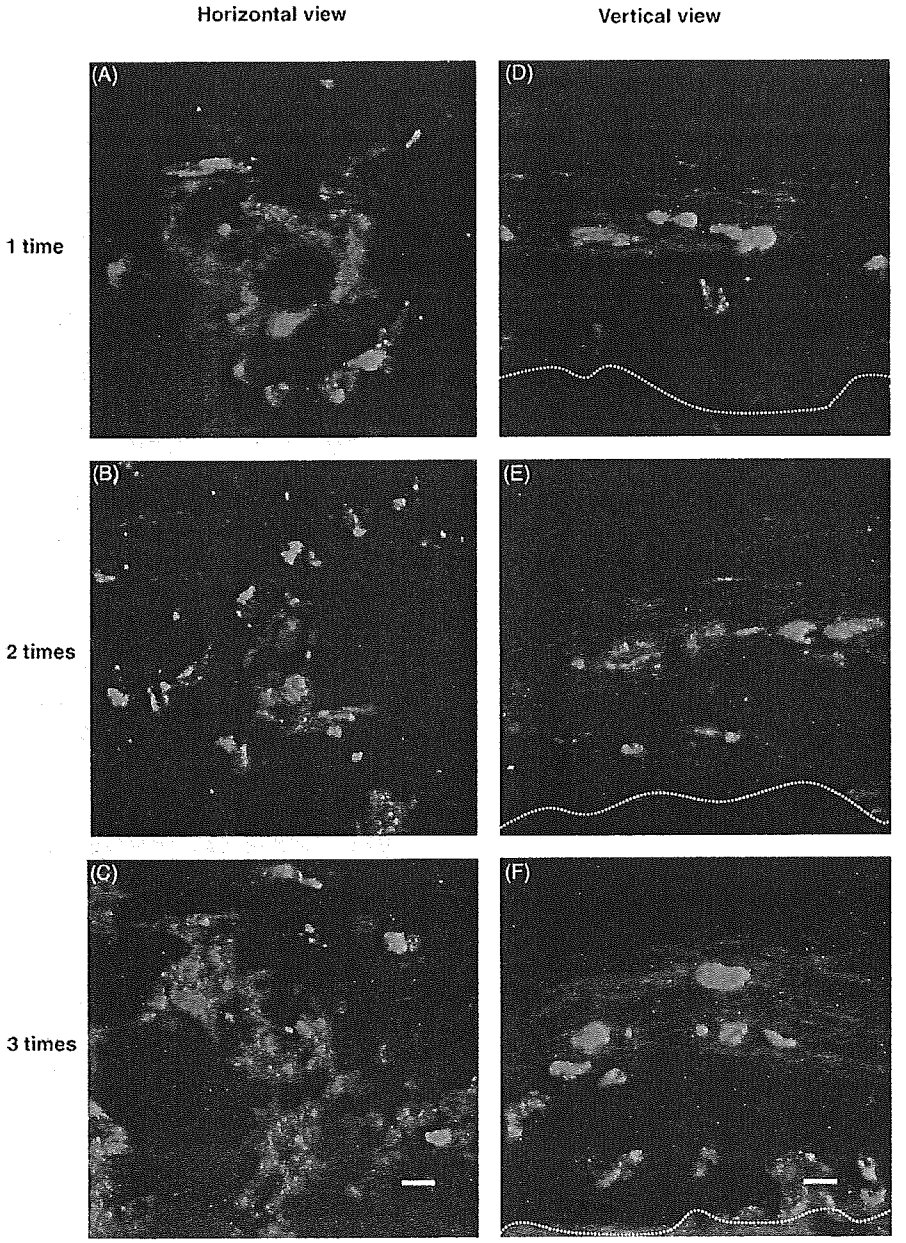

Fig. 3 GFP expression after multiple transfections. LSEs were transfected once (A, D), twice (B, E), or three times (C, F). The horizontal microscopy sections show that cells transfected three times (C) expressed more GFP than did those transfected once (A) or twice (B). The vertical sections show that the fluorescence-positive cells are located mainly in the stratum corneum in cells transfected once (D), in both the stratum corneum and the suprabasal layer in cells transfected twice (E), and in the stratum corneum, suprabasal, and basal layers in cells transfected three times (F). The white-dotted line indicates the dermal–epidermal junction. Scale bar, 50 μm.

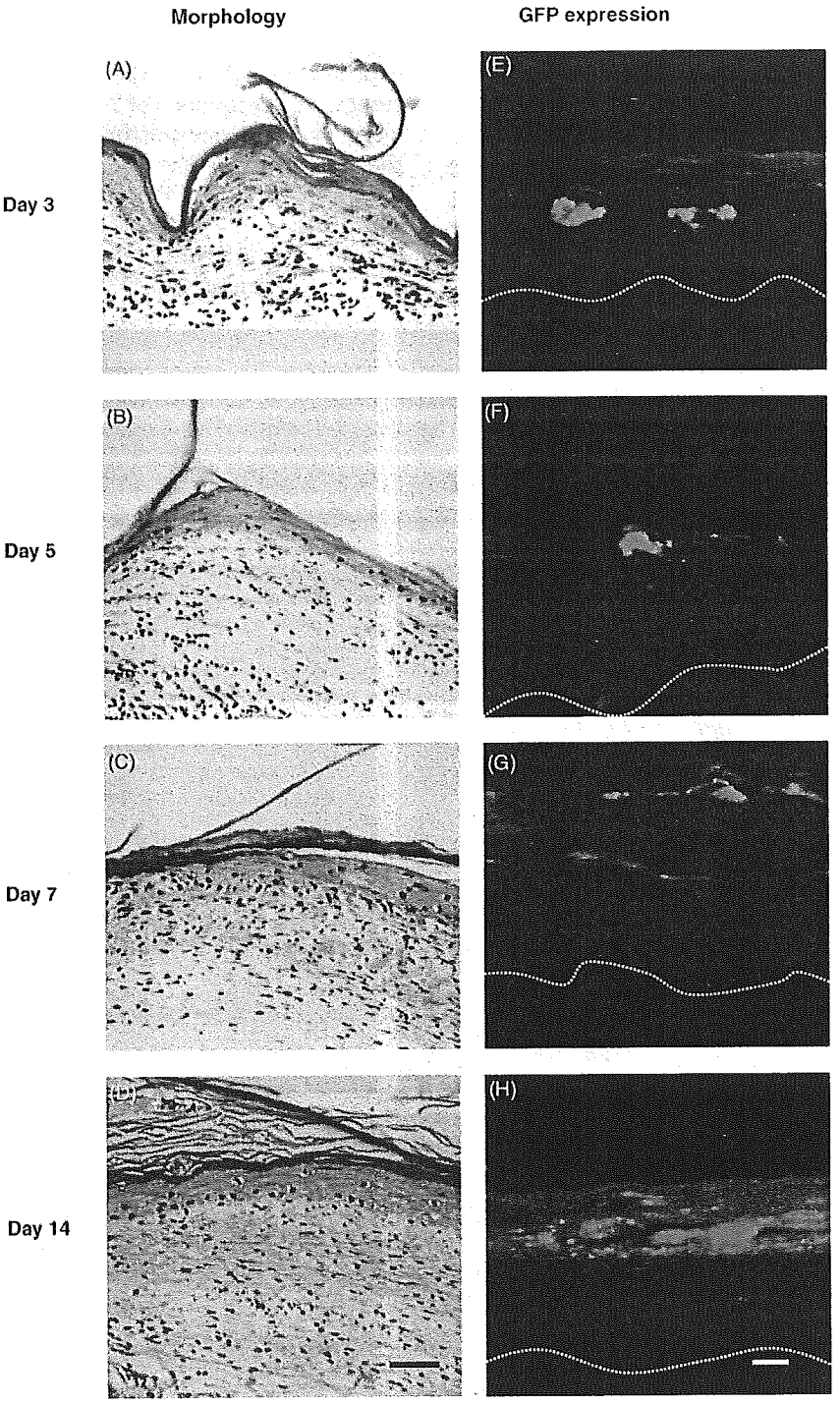

Fig. 4 Morphology of the epidermis and GFP expression in transfected LSE grafts in vivo. Transfected LSE grafts were collected on day 3 (A, E), day 5 (B, F), day 7 (C, G), and day 14 (D, H) after transplantation. H&E staining (A–D) shows that the transfected LSE grafts survive on nude mice and that the epidermis is well stratified and differentiated. Frozen sections (E–H) viewed by confocal laser microscopy show that the fluorescence-positive cells stayed in the suprabasal layer on day 3 (E) and moved to the stratum corneum on day 5 (F) and day 7 (G). By day 14, all of the cells were restricted to the stratum corneum (H). The white-dotted line indicates the dermal–epidermal junction. Scale bar, 50 μm.