

laminin 5. In AM-LSE, both $\alpha 6$ and $\beta 4$ integrin subunits were restricted to the basal pole of the basal cells at days 7 and 21 (Fig. 3d,n,e,o). In conventional LSE, $\alpha 6$ and $\beta 4$ integrin proteins were synthesized and accumulated in the epidermis; they were distributed predominantly in the suprabasal layer and did not localize in a linear pattern (Fig. 3i,s,j,t).

The development of the BM zones of the LSEs was further elucidated by using transmission electron microscopy (TEM) (Fig. 1c,d,g,h). At day 7, a continuous lamina densa was formed along the plasma membrane of the keratinocytes, with many hemidesmosomes, in the AM-LSE. The hemidesmosomes were well developed structurally, with inner and outer plaques clearly being distinguished; keratin filaments were connected to the inner plaques (Fig. 1c), whereas in the conventional LSE, little lamina densa or BM structures were seen at day 7. Along the plasma membrane of the basal cells, some electron-dense hemidesmosome-like structures were seen; however, they did not possess the structural features of hemidesmosomes (Fig. 1g). At day 21, the lamina densa and hemidesmosomes in the AM-LSE were well developed, and the structural features of hemidesmosomes were clearly displayed (Fig. 1d), whereas in conventional LSE, an interrupted lamina densa was seen, with poorly developed hemidesmosomes (Fig. 1h).

De-epithelialized AM improved epidermal outgrowth

On the day of airlifting (day 0), the epidermis of both the AM-LSE and conventional LSE was 6 mm in diameter. The epidermis of the AM-LSE continued to outgrow faster than that of the conventional LSE (Fig. 4): at day 3, the epidermis measured 7.7 ± 0.4 mm (it had increased by 28.3%), and at day 10, it reached 18.3 ± 1.1 mm (over three times its original size). By contrast, the epidermis of the conventional LSE did not change in size by day 3, and only a 78.3% increase to 10.7 ± 0.2 mm was seen at day 10. These results indicated that the AM stimulated keratinocyte migration and proliferation.

AM-LSE grafts on nude mice exhibit better morphogenesis of the epidermis and BM

Two weeks after transplantation, the grafts were harvested and manipulated for observation by light microscopy and TEM. Both the AM-LSE and conventional LSE took well on the recipient nude mice. The fibroblast-populated gel in conventional LSE was prone to separate from the epidermis and was fragile during manipulation. The epidermis of the AM-LSE was tightly attached to the underlying tissue, and the graft was more resistant to rupture. The epidermis of both types of graft was well differentiated with a thick stratum corneum (Fig. 5a,b). The epidermis of the AM-LSE was better stratified than that of the conventional LSE, and the basal cells were columnar and aligned on the surface of the AM (Fig. 5a). By contrast, the epidermal

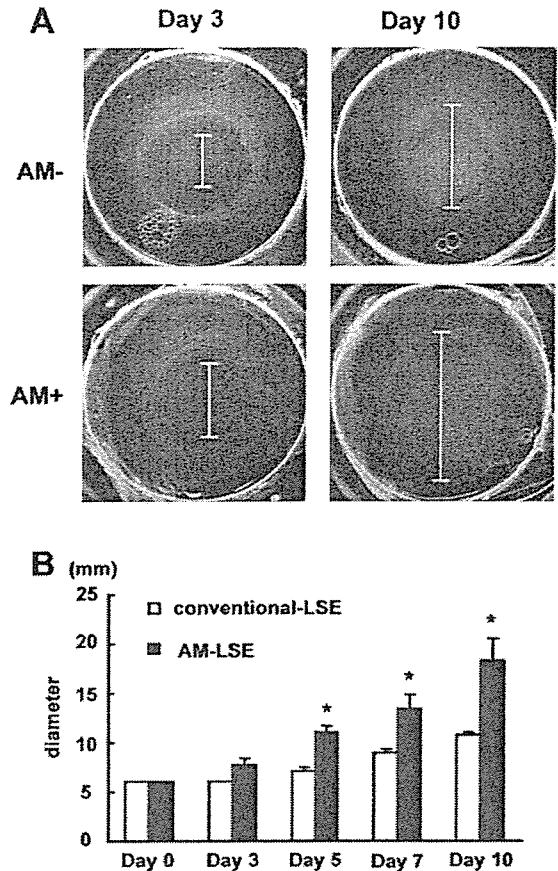


Fig. 4 Comparison of epidermal outgrowth of AM-LSE and conventional LSE. a Stainless steel rings with an inner diameter of 6 mm were placed on gels with (AM+) or without (AM-) AM, and 2×10^5 keratinocytes were seeded. When the keratinocytes reached confluence, the LSEs were lifted to the air-liquid surface, and the stainless steel rings were removed. Epidermal size (lines) was monitored after airlifting. b The epidermal diameter of AM-LSE and conventional LSE was measured at days 3, 5, 7, and 10, and the mean value for each day was calculated ($n=3$, $*P<0.05$)

stratification in the conventional LSE was disorganized, with few basal cells along the dermal-epidermal junction (Fig. 5b).

In the AM-LSE graft, an electron-lucent lamina lucida was clearly observed between the basal cell plasma membrane and the continuous electron-dense lamina densa. Hemidesmosomes were well developed structurally and dotted along the plasma membrane of the basal cells (Fig. 5c). By contrast, the BM in the conventional LSE graft was rudimentary and faint, and far fewer hemidesmosomes were present than in the AM-LSE graft, as seen in vitro (Fig. 5d).

The basal cell number per millimeter in three separate fields of view of both LSE grafts was calculated and showed a significantly higher cell number in AM-LSEs than in conventional LSEs (Fig. 5h). Since vascularization is essential for graft survival, we investigated the angiogenesis in AM-LSE and conventional LSE. We found the AM-LSE grafts were better vascularized, with even some capillaries infiltrating into the AM (Fig. 5e), whereas blood

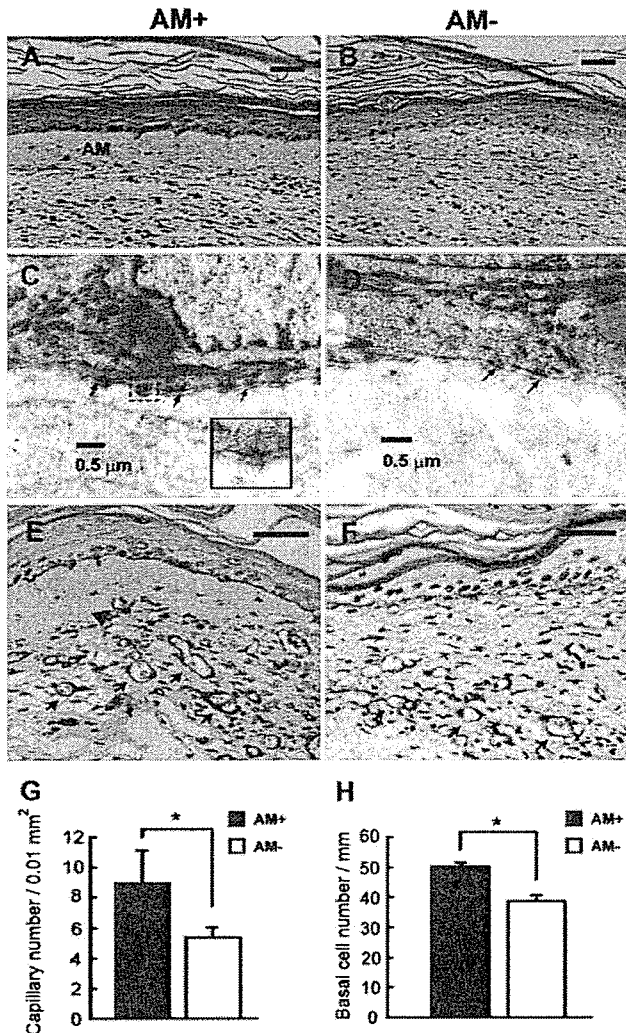


Fig. 5 Light-microscopic and ultrastructural views of AM-LSE (*AM+*) and conventional LSE (*AM-*) in vivo. AM-LSE or conventional LSE was grafted onto the back skin of nude mice. Two weeks after transplantation, samples were harvested and processed for HE staining (a, b), TEM (c, d), and immunohistochemical staining for type IV collagen to detect blood vessels (e, f). HE staining showed better organized epidermis (a, b) and more basal cells (b) in AM-LSE grafts than in conventional LSE grafts. TEM revealed a continuous lamina densa (arrows in c) with many structurally well-developed hemidesmosomes (inset in c) in the AM-LSE, and a faint interrupted lamina densa (arrows in d) with fewer, poorly developed hemidesmosomes in the conventional LSE. More blood vessels (arrows in e, f) were found in AM-LSE grafts than in conventional LSE grafts (e, f, g), with some vessels infiltrating into the AM (arrowhead in e). * $P < 0.05$ (g, h). Bars 50 μm (a, b, e, f), 0.5 μm (c, d)

vessels were fewer in conventional LSE grafts (Fig. 5f). The number of blood vessels/0.01 mm² in three separate fields of view was calculated and revealed significantly more blood vessels in AM-LSE grafts than in conventional LSE (Fig. 5g).

Discussion

Epithelial-mesenchymal interactions play important roles in controlling epidermal morphogenesis and homeostasis. The interaction between keratinocytes and the insoluble BM proteins contributes to the maintenance of tissue architecture and affects various biological processes, such as cell attachment, proliferation, differentiation, and migration (Blomme et al. 1998; Kim et al. 1994). In autologous split-thickness skin grafting for treating deep burns, a disturbance in BM reassembly is believed to be responsible for post-burn blisters (Bergman et al. 1997). In conventional LSEs constructed by seeding keratinocytes on a fibroblast-populated type I collagen gel, the BM is not well developed a few weeks after airlifting and is rarely seen after their transplantation onto nude mice (Amano et al. 2001; Medalie et al. 1997).

During the construction of a LSE, pre-existing BM components on the dermal matrix contribute to improving the morphology of the epidermis and are necessary for the formation of hemidesmosomes and the development of a lamina densa (Kim et al. 2001; Medalie et al. 1997; Ralston et al. 1999). The de-epithelialized AM is immunohistochemically positive for types VII and IV collagen, but negative for laminin 5 (data not shown), which is probably lost during the EDTA treatment and subsequent mechanical scraping used to remove epithelial cells from the AM. In this study, we have constructed LSEs with or without de-epithelialized AM to investigate the usefulness of AM. The same types of keratinocytes and fibroblasts and the same techniques have been used for both types of LSE in order to elucidate the roles of de-epithelialized AM in epidermal morphology, BM development, and epidermal migration. We have compared the two types of LSE morphologically, immunohistochemically, and ultrastructurally.

The results indicate that the presence of certain BM components on de-epithelialized AM improve the epidermogenesis of the overlying epidermis, which is more mature and better organized than epidermis without AM. Similar effects of BM proteins have also been documented in a composite skin-equivalent by using, as the substrate, acellular dermis, which retains several key BM components (Medalie et al. 1997; Ralston et al. 1999). The sustained growth of the keratinocytes in an LSE is maintained by a well-developed BM (Andriani et al. 2003), whose formation is facilitated by the pre-existing BM components.

Laminin 5 is also essential for epidermal-dermal adhesion. Laminin 5 bridges integrin $\alpha 6\beta 4$ with underlying connective tissue through an association with laminin 6 and 7 and direct binding to type VII collagen (Champlaud et al. 1996; Rousselle et al. 1997). The effect of pre-existing BM components in normalizing the deposition and polarization of laminin 5 has been documented in the construction of LSE by seeding keratinocytes on de-epithelialized dermis (Andriani et al. 2003, 2004). Moreover, the presence of laminin 5 initiates hemidesmosome formation and accelerates the formation of the lamina densa (Nishiyama et al. 2000; Tsunenaga et al. 1998). The de-epithelialized AM

also enhances the polarized basal distribution of $\alpha 6\beta 4$ integrin along the dermal-epidermal junction, as compared with the more suprabasal, albeit denser, distribution in conventional LSEs.

The well-developed basal lamina and hemidesmosomes and the polarized basal deposition of laminin 5 and $\alpha 6\beta 4$ integrin guarantee a stable association between the epidermis and the underlying tissues in AM-LSEs. This might explain why the epidermis is more resistant to separation from the underlying connective tissue during the manipulation of AM-LSEs, as compared with the loose connection in conventional LSEs.

The outgrowth of corneal explants has been reported to be faster on de-epithelialized AM than on intact AM; this implies that the exposed extracellular matrix of de-epithelialized AM is more inductive to epithelial cell migration (Koizumi et al. 2000). Of the BM components, collagen IV had been shown to assist keratinocyte migration through an interaction with keratinocyte $\alpha 2\beta 1$ integrin (Kim et al. 1994). In AM-LSEs, the epidermis outgrows much faster than when on a fibroblast-populated type I collagen gel; AM-LSEs might thus better meet the clinical need to provide a skin-equivalent for a larger area in a shorter time.

AM-LSE therefore appears superior to conventional LSE in several ways (see above). We have transplanted AM-LSE and conventional LSE onto nude mice and found that both LSEs take well on full-thickness wounds of nude mice. A better-organized epidermis, continuous lamina densa and lamina lucida, better-formed hemidesmosomes, and more importantly, better vascularization are seen with AM-LSE. When manipulating the graft for transplantation, the AM-LSE is much more resistant to rupture, as compared with the fragile conventional LSE.

However, the lack of the BM zone in conventional LSE can be supplemented not only by the epidermal side of the de-epithelialized amnion, but also by the stromal side of the amnion, which can support the growth of fibroblasts (España et al. 2003; Kumar et al. 2003). This implies that amnion could provide a scaffold for LSE, greatly simplifying the procedures for making dermal matrix and avoiding the use of animal collagen, which is costly and ethically problematic. Moreover, the limited immunogenicity and immune-privilege of amnion (Kubo et al. 2001; Mahgoub et al. 2004), together with its anti-inflammatory effects (Park and Tseng 2000), make it a suitable material for transplantation and a biological immune barrier for xenotransplantation, which may facilitate allograft transplantation of AM-LSE. Amnion can be readily obtained from cesarean delivery under screening for viral diseases and can also be sterilized and preserved at low cost for long periods without obvious architectural changes (Ravishanker et al. 2003; Rejzek et al. 2001; von Versen-Hoynck et al. 2004), thereby ensuring the plentiful supply of amnion in clinical application. Usually, we can take five to six pieces of amnion of 40–50 mm in size in one delivery. Amnion of this size is easily manipulated, fits the commonly used transwell culture insert (diameter: 75 mm), and can be tailored and used in one piece to

cover fresh wounds or made into mesh grafts or stamp grafts to cover granulation tissue as split skin grafts.

In conclusion, the AM-LSE had good epidermal morphology and a well-developed BM zone and is easy to manipulate during transplantation. Since AM is readily obtainable, and since a large amount of AM can be supplied compared with other materials, an AM-LSE should be a good skin substitute for treating burns, ulcers, and other skin defects.

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Transforming Growth Factor- β -activated Kinase 1 Is Essential for Differentiation and the Prevention of Apoptosis in Epidermis*

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Transforming growth factor- β -activated kinase 1 (TAK1) is a member of the mitogen-activated protein (MAP) kinase family and is an upstream signaling molecule of nuclear factor- κ B (NF- κ B). Given that NF- κ B regulates keratinocyte differentiation and apoptosis, TAK1 may be essential for epidermal functions. To test this, we generated keratinocyte-specific TAK1-deficient mice from *Map3k7^{lox/lox}* mice and *K5-Cre* mice. The keratinocyte-specific TAK1-deficient mice were macroscopically indistinguishable from their littermates until postnatal day 2 or 3, when the skin started to roughen and wrinkle. This phenotype progressed, and the mice died by postnatal day 7. Histological analysis showed thickening of the epidermis with foci of keratinocyte apoptosis and intra-epidermal micro-abscesses. Immunohistochemical analysis showed that the suprabasal keratinocytes of the TAK1-deficient epidermis expressed keratin 5 and keratin 14, which are normally confined to the basal layer. The expression of keratin 1, keratin 10, and loricrin, which are markers for the suprabasal and late phase differentiation of the epidermis, was absent from the TAK1-deficient epidermis. Furthermore, the TAK1-deficient epidermis expressed keratin 16 and had an increased number of Ki67-positive cells. These data indicate that TAK1 deficiency in keratinocytes results in abnormal differentiation, increased proliferation, and apoptosis in the epidermis. However, the keratinocytes from the TAK1-deficient epidermis induced keratin 1 in suspension culture, indicating that the TAK1-deficient keratinocytes retain the ability to differentiate. Moreover, the removal of TAK1 from cultured keratinocytes of *Map3k7^{lox/lox}* mice resulted in apoptosis, indicating that TAK1 is essential for preventing apoptosis. In conclusion, TAK1 is essential in the regulation of keratinocyte growth, differentiation, and apoptosis.

The epidermis is a multilayered epithelial tissue, maintained by the precise regulation of keratinocyte proliferation, differentiation, and cell death. Cell growth is limited to the basal cell

layer, which attaches to the basement membrane. After leaving the basement membrane, keratinocytes differentiate and form a multilayered epidermis instead of undergoing apoptosis. This keratinocyte differentiation is regulated by intracellular signaling pathways involving nuclear factor- κ B (NF- κ B),² the MAP kinase family, phosphatidylinositol 3-kinase, and protein kinase C (1–3).

In the epidermis, NF- κ B is found in the cytoplasm of basal cells (1). The functional blockade of NF- κ B by expressing dominant negative NF- κ B in transgenic mouse epidermis produced a hyperplastic epithelium *in vivo* (1). With deficiency of the p65/RelA subunit of NF- κ B, the epidermis is hyperplastic (4). Conversely, the overexpression of active p50 (a subunit of NF- κ B) and p65 in the transgenic epithelium produced hypoplasia and growth inhibition, suggesting a role for NF- κ B in negative cellular growth control (1). Furthermore, the expression of active p50 and p65 in keratinocytes inhibits cell cycle progression to G₁ arrest *in vitro*, which was associated with an increased p21 level (5) and CDK4 down-regulation (6). This CDK4 regulation is dependent on both the TNFR1 and JNK pathways (7). In addition to regulating differentiation and cell growth, NF- κ B protects keratinocytes from apoptosis, and the blockade of NF- κ B function in the epidermis by the expression of the dominant negative mutant I κ B α provoked premature spontaneous cell death (8). Although the roles of the NF- κ B pathway have been studied in keratinocytes, the upstream signal of the NF- κ B pathway has not been clarified fully.

Transforming growth factor- β -activated kinase 1 (TAK1), a member of the MAP kinase kinase kinase family, is a signaling molecule upstream from NF- κ B and is encoded by the *Map3k7* gene. TAK1 was originally identified as a signaling molecule activated by transforming growth factor β (9). TAK1 is also involved in IL-1 signaling and TNF- α -induced activation of NF- κ B and MAP kinases (MAPKs). Activated TAK1 is recruited to TRAF6 and TRAF2, in response to the IL-1 and TNF receptors, respectively (10–12). TAK1 forms a complex

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² The abbreviations used are: NF- κ B, nuclear factor- κ B; TAK1, transforming growth factor- β -activated kinase 1; MAP, mitogen-activated protein; MAPK, MAP kinase; IKK, I κ B kinase; KO, knock-out; K, keratin; Ax, adenovirus vector; P, postnatal day; TAB, TAK1-binding protein; TEWL, trans-epidermal water loss; TUNEL, TdT-mediated dUTP nick end-labeling; RT, reverse transcriptase; JNK, c-Jun NH₂-terminal kinase; IL, interleukin; TNF, tumor necrosis factor; poly-HEMA, polyhydroxyethylmethacrylate; LDH, lactate dehydrogenase.

TAK1 Regulates Keratinocyte Differentiation and Apoptosis

with TAK1-binding protein (TAB) 1, TAB2, and TAB3 (10, 12–14). The activated TAK1 complex phosphorylates I κ B kinases (IKKs) and MAPK kinase 6, which activate NF- κ B and MAPKs, respectively. The transfection of TAK1 small interfering RNA markedly inhibited TNF- α - and IL-1-mediated activation of NF- κ B (11). Recently, TAK1 was shown to be essential for innate and adaptive immune responses by generating B cell-specific TAK1 deficiency (15).

Given that TAK1 is an upstream signaling molecule of the NF- κ B pathway, TAK1 might regulate keratinocyte growth, differentiation, and apoptosis. However, the function of TAK1 in epidermal keratinocytes has not been studied. To address this issue, we generated keratinocyte-specific TAK1-deficient mice by using Cre-recombinase transgenic mice under the control of the K5 promoter.

EXPERIMENTAL PROCEDURES

Generation of Keratinocyte-specific TAK1-deficient Mice Using Gene Targeting with the Cre Transgene—The targeting construct has been described previously (15). We generated keratinocyte-specific TAK1-deficient mice by breeding *Map3k7^{fllox/fllox}* mice with mice carrying the Cre transgene under the control of the keratin 5 promoter (*K5-Cre*) (16). The *Map3k7^{fllox/fllox}* mice were bred with *K5-Cre* mice to generate *K5-Cre/Map3k7^{fllox/+}* mice. Subsequently, the *K5-Cre/Map3k7^{fllox/+}* mice were bred to *Map3k7^{fllox/fllox}* mice to generate *K5-Cre/Map3k7^{fllox/fllox}* mice. The genotype of each mouse was confirmed using PCR and Western blot analysis. The TAK1 primer sequences were 5'-GGAACCCGTGGATAAGTGCACTTGAAT-3' and 5'-GGCTTTCATTGTGGAGGTAAGCTGAGA-3'. The amplified products were 320 bp for the floxed allele and 280 bp for the wild-type allele. The Cre-recombinase primers were 5'-TTACCGGTCGATGCAACGAGTGATG-3' and 5'-TTCCATGAGTGAACGAACCTGGTCG-3'. Isolated keratinocytes were cultured overnight, and the adherent keratinocytes were harvested for Western blot analysis (see Fig. 1D). This protocol was approved by the Institutional Review Board of Ehime University School of Medicine.

Trans-epidermal Water Loss (TEWL)—TEWL was measured using a Tewameter TM210 (Integral Co., Tokyo, Japan) according to the manufacturer's instructions. The data are expressed as the means \pm S.E. The statistical significance was determined using the paired Student's *t* test. The differences were considered statistically significant at *p* < 0.01.

Histological Analysis—Mouse skin was fixed in 3.6% formaldehyde, dehydrated, and embedded in paraffin. Four- μ m sections were stained with hematoxylin and eosin.

To analyze differentiation markers, keratin (K) 5, K14, K1, K10, and loricrin, the paraffin-embedded sections were deparaffinized, blocked with 10% goat serum, and reacted with the first antibodies overnight at 4 °C. After washing with phosphate-buffered saline, the first antibodies were detected using a peroxidase staining kit (ImmPRESS; Vector Laboratories, Burlingame, CA) and visualized with the chromogen 3-amino-9-ethyl-cabazole according to the manufacturer's instructions. For K16 and Ki67 staining, the deparaffinized sections were boiled in 10 mM citrate buffer, pH 6.0, for 40 min and cooled at

room temperature for 20 min for antigen retrieval. Subsequently, K16 and Ki67 were stained following the same procedure as for the differentiation markers.

Confocal Laser Scanning Microscopy—Frozen skin sections (4 μ m) were blocked with 10% goat serum and reacted overnight at 4 °C with rabbit anti p50 and p65. After washing with phosphate-buffered saline, the sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG for 30 min at room temperature. The stained specimens were observed under a LSM 510 microscope (Carl Zeiss, Jena, Germany). The images were captured using LSM 510 software.

TdT-mediated dUTP Nick End Labeling (TUNEL)—Keratinocyte apoptosis was detected by the TUNEL method using an *in situ* cell death detection kit (Roche Applied Sciences). After being deparaffinized, the sections were treated with 20 μ g/ml proteinase K in 10 mM Tris-HCl, pH 7.4, for 15 min at room temperature and labeled according to the manufacturer's instructions. The labeled cells were observed under a fluorescence microscope.

Antibodies—The following antibodies were used: K5, K14, K1, and K10 (rabbit; Covance, Berkeley, CA); K16 (mouse, clone number LL025; Chemicon International, Temecula, CA); loricrin (rabbit; Covance); Ki67 (mouse, clone number MMI, Novo Castra Laboratories, Newcastle, UK); β -actin (mouse, clone number AC-15, Abcam, Cambridge, UK); and p50, p65, and TAK1 (rabbit, Santa Cruz Biotechnology, Santa Cruz, CA).

Keratinocyte Culture—Primary mouse keratinocytes were isolated from newborn mouse skin. The skin samples were cut into 3 \times 5-mm pieces and incubated with 250 units/ml dispase (Godoohusei, Tokyo, Japan) in Dulbecco's modified Eagle's medium overnight at 4 °C. After the epidermis was separated from the dermis, the epidermal sheets were incubated in a 0.25% trypsin solution for 10 min at 37 °C and teased with forceps. The keratinocytes were collected by centrifugation and were cultured further in CnT-02 medium (CellnTec, Bern, Switzerland).

For suspension culture, keratinocytes were plated onto 6-cm polyhydroxyethylmethacrylate (poly-HEMA)-coated plates (3). The poly-HEMA-coated plates were made by adding 4 ml of a solution containing 10 mg/ml poly-HEMA (Sigma-Aldrich) in ethanol to the dish, drying, and repeating once, followed by extensive phosphate-buffered saline washes. After 24 h, the cells were harvested by pipetting.

Western Blotting—Keratinocytes were harvested on ice with lysis buffer containing 1.0% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% protease inhibitor (Sigma-Aldrich). The proteins (20 μ g) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.05% Tween 20 and was incubated with the appropriate first antibody. After washing, the membrane was incubated with fluorescein-labeled goat anti-mouse IgG (1:2,500 dilution) for 1 h. The signal was amplified with an anti-fluorescein antibody conjugated with alkaline phosphatase, followed by the fluorescent substrate AttoPhos (Molecular Dynamics, Sunnyvale, CA). The membrane was scanned using FluoroImager (Molecular Dynamics),

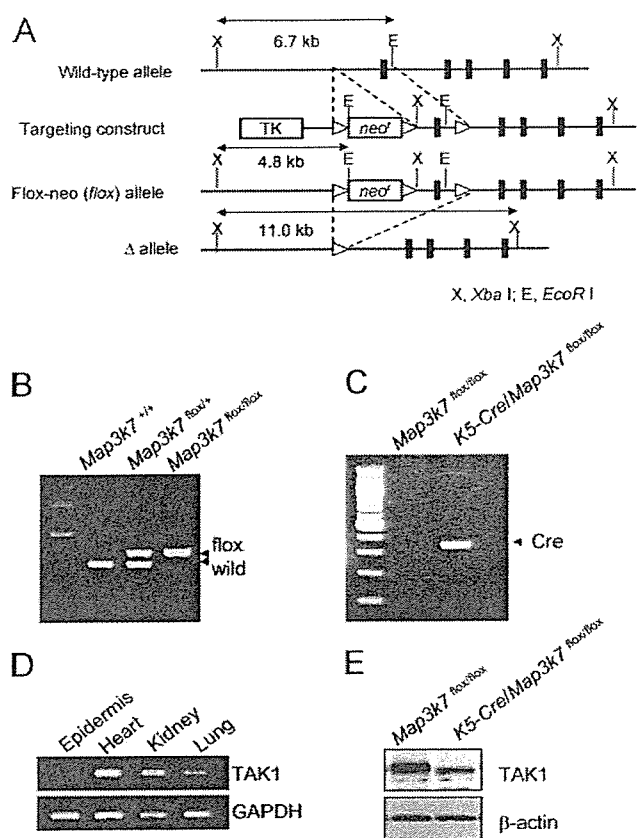


FIGURE 1. Establishment of mice with keratinocyte-specific TAK1 deficiency. Keratinocyte-specific TAK1-deficient mice were generated by breeding *Map3k7^{fllox/fllox}* mice with mice carrying the *Cre* transgene under the control of the keratin 5 promoter (*K5-Cre*) (16). *A*, schematic representation of the targeting strategy. A targeting construct was designed to flank exon 2 of the *Map3k7* gene and the *neo'* gene with *loxP* sites (triangles). Cre-mediated removal was performed to generate the deleted (Δ) allele of *Map3k7*. The genotype of each mouse was confirmed by PCR (*B* and *C*). *B*, a primer pair was designed to include the *loxP* site (triangle), so that the PCR products represent the floxed (320 bp) and wild-type (280 bp) alleles of *Map3k7*. *C*, the PCR product represents Cre-recombinase. *D*, RT-PCR analysis showed the Cre-mediated keratinocyte-specific deletion of TAK1 in *K5-Cre/Map3k7^{fllox/fllox}* mice. mRNA isolated from the epidermis, heart, kidney, and lung of *K5-Cre/Map3k7^{fllox/fllox}* mice at birth was subjected to RT-PCR to determine TAK1 expression. The faint band from the epidermis detected at the expected size is likely the result of the presence of nonkeratinocyte cells in the epidermis, such as Langerhans cells or melanocytes. Glyceraldehyde-3-phosphate dehydrogenase is an internal standard. *E*, Western blot analysis showed the Cre-mediated deletion of TAK1 from the keratinocytes of *K5-Cre/Map3k7^{fllox/fllox}* mice. Keratinocytes isolated from *Map3k7^{fllox/fllox}* and *K5-Cre/Map3k7^{fllox/fllox}* mice at birth were subjected to Western blot analysis to detect TAK1. There was a faint band in the keratinocytes from *K5-Cre/Map3k7^{fllox/fllox}* mice, which was slightly lower than that for *Map3k7^{fllox/fllox}* mice. This may represent TAK1 Δ (*A*), which lacks the ATP-binding site required for kinase activity (15). β -Actin is an internal standard. In this manuscript, *K5-Cre/Map3k7^{fllox/fllox}* mice and *Map3k7^{fllox/fllox}* mice are referred as TAK1-KO and control mice, respectively.

and the intensity of each band was quantified with ImageQuant (Molecular Dynamics), referring to the control as one unit.

Reverse Transcriptase (RT)-PCR—Total RNA samples were isolated using Isogen (Nippon Gene, Tokyo, Japan), and the TAK1 mRNA expression was analyzed by RT-PCR using RT-PCR High Plus (Toyobo, Osaka, Japan). The PCR product (337 bp) was sequenced to confirm the accuracy of amplification. The primer sequences for TAK1 were 5'-AGTGCTGACATG-TCTGAAAT-3' and 5'-TTCGAACACTGCCATGGATT-3',

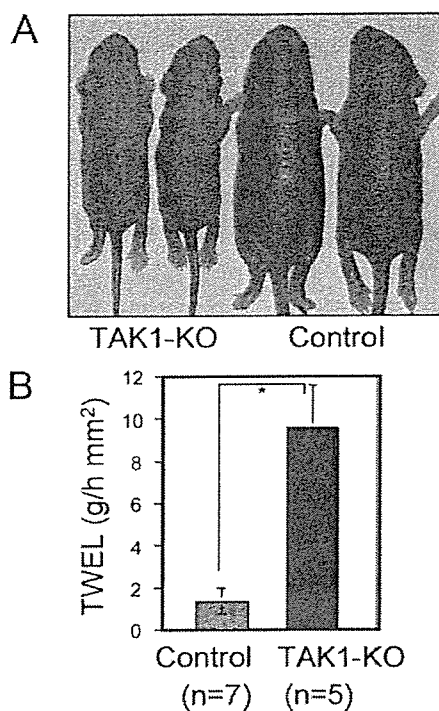


FIGURE 2. The macroscopic phenotype of TAK1-KO mice. *A*, appearance of TAK1-KO mice at P6. The skin is rough and wrinkled, with mild scaling. The TAK1-KO mice weighed approximately half the control weight. *B*, TEWL in TAK1-KO mice at P6. The TEWL value, a measure of epidermal barrier function, in TAK1-KO mice ($n = 5$) was 6.9-fold that in the control mouse ($n = 7$). The data are expressed as the means \pm S.E. The statistical significance was determined using the paired Student's *t* test. * The difference was considered statistically significant at $p < 0.01$.

and those for the internal standard glyceraldehyde-3-phosphate dehydrogenase were 5'-ACCACAGTCCATGCCAT-CAC-3' and 5'-TCCACCACCCTGTGCTGTA-3'. The intensity of each band was quantified using NIH Image. The data are presented as fold induction relative to the control signal, set at 1 unit.

Adenovirus Vector—Adenovirus vector (Ax) encoding Cre-recombinase was generated using the COS-TPC method (17). Virus stocks were prepared by standard procedures. Concentrated, purified virus stocks were prepared using a CsCl gradient, and the virus titer was checked using a plaque formation assay. We infected keratinocytes with Ax at a multiplicity of infection of 100. Empty Ax-1W vector was used as a control.

LDH Assay—Cell death was analyzed quantitatively by measuring LDH release using an LDH assay kit (Kyokutokogyo, Tokyo, Japan). Keratinocytes were cultured on 6-cm dishes, and Ax was transfected. At the indicated time, 100 μ l of supernatant were harvested, centrifuged, and stored at -70°C until the LDH assay was performed according to the manufacturer's instructions. The LDH of living cells was obtained by cell lysis with 0.1% Tween 20[®]. LDH release was expressed as a percentage of the total LDH, which was obtained by summing the LDH released and the LDH of living cells. The data are expressed as the means \pm S.E. The statistical significance was determined using the paired Student's *t* test ($n = 5$). The differences were considered statistically significant at $p < 0.01$.

TAK1 Regulates Keratinocyte Differentiation and Apoptosis

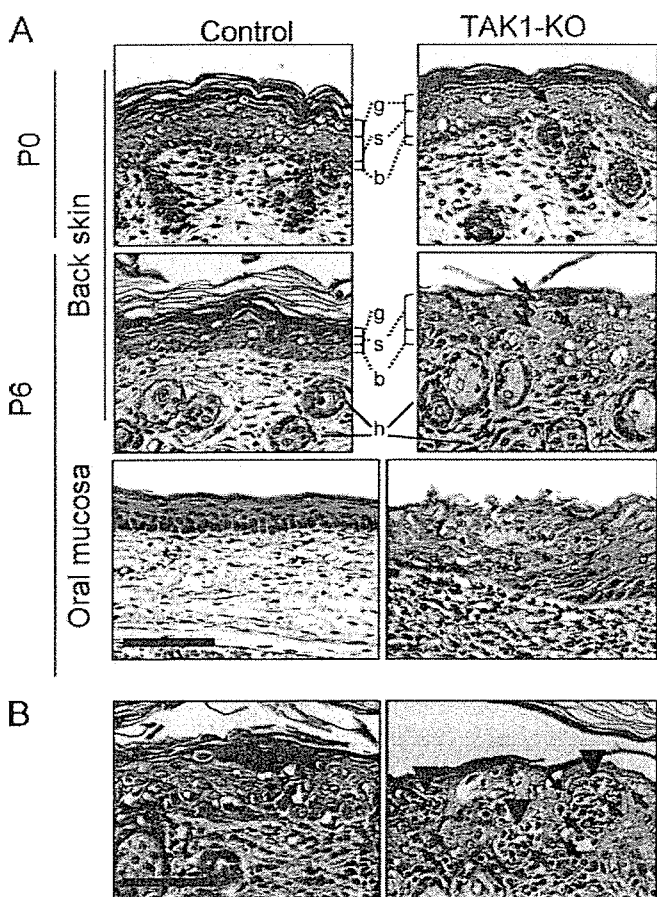


FIGURE 3. Hyperplasia, apoptosis, and micro-abscesses in the epidermis of TAK1-KO mice. A, histological analysis of TAK1-KO mouse skin at P0 and P6, using hematoxylin and eosin staining. Normal epidermis consists of granular (g), spinous (s), and basal cell (b) layers. h indicates a hair follicle in the dermis. TAK1-KO mice at P6 showed marked thickening of the epidermis with no granular layer, and apoptosis (arrow). The oral mucosa showed a phenotype similar to that of the epidermis. B, the thickened epidermis of TAK1-KO mice contains foci of keratinocyte apoptosis (left panel, arrow) and intra-epidermal micro-abscesses (right panel, arrowhead), which were associated with apoptotic cells (arrow). Scale bar, 20 μ m.

RESULTS

Generating Mice with Keratinocyte-specific TAK1 Deficiency—The germ line targeting of the *Map3k7* gene encoding TAK1 results in embryonic lethality (15); therefore, we generated keratinocyte-specific TAK1-deficient mice (Fig. 1) by breeding *Map3k7^{fllox/fllox}* mice with mice carrying the *Cre* transgene under the control of the keratin 5 promoter (*K5-Cre*) (16). Western blot analysis and RT-PCR showed the *Cre*-mediated deletion of TAK1 in keratinocytes from *K5-Cre/Map3k7^{fllox/fllox}* mice at birth (Fig. 1, D and E). Here, the *K5-Cre/Map3k7^{fllox/fllox}* mice and *Map3k7^{fllox/fllox}* mice are referred to as TAK1-KO and control, respectively.

Rough and Wrinkled Skin with Disrupted Barrier Function, Growth Retardation, and Early Mortality in TAK1-KO Mice—At birth, the TAK1-KO mice were macroscopically indistinguishable from their littermates until postnatal day 2 or 3 (P2–P3), when the skin started to roughen and wrinkle. This phenotype progressed with mild scaling, and the TAK1-KO mice subsequently died by P7 (Fig. 2A). At this stage, the mice weighed

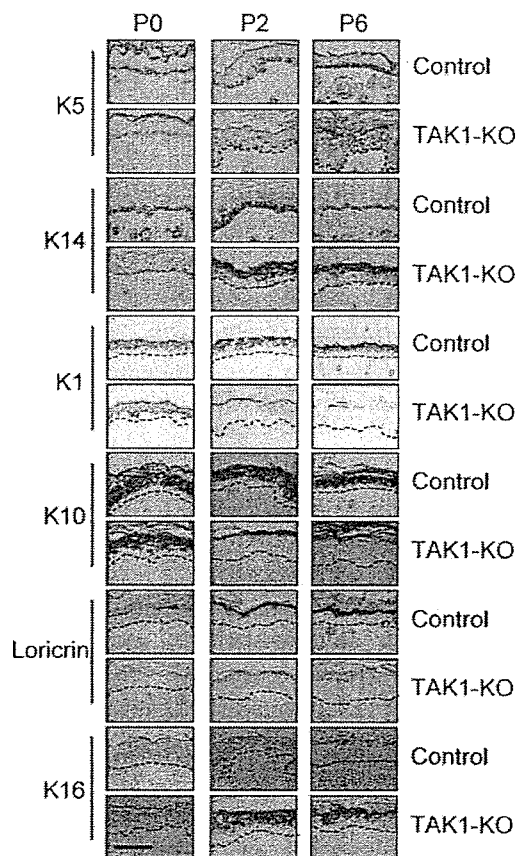


FIGURE 4. Abnormal expression of differentiation markers in the epidermis of TAK1-KO mice. Paraffin-embedded skin sections from TAK1-KO mice at P0, P2, and P7 were stained immunohistochemically with antibodies against K5, K14, K1, K10, loricrin, and K16. The expression of K5 and K14 is normally confined to the basal cell layer. The expression of K1, K10, and loricrin marks the suprabasal and late-phase differentiation. K16 is a marker for inflammatory and hyperproliferative epidermal keratinocytes. The dotted lines indicate the basement membrane. Scale bar, 30 μ m.

approximately half the control weight. To evaluate epidermal barrier function, we measured TEWL at P6 (Fig. 2B). The TEWL value in TAK1-KO mice was 6.9-fold that in the control, indicating that the barrier function had been disrupted. Given that the epidermal barrier function arises during keratinocyte differentiation, the regulation of keratinocyte differentiation might be disturbed in TAK1-KO mice. The *Map3k7^{fllox/+}* and *Map3k7^{+/+}* mice with or without *K5-Cre* showed no pathological phenotypes.

Hyperplasia, Apoptosis, and Micro-abscess Formation in the Epidermis of TAK1-KO Mice—The epidermis of TAK1-KO mice at P0 was histologically indistinguishable from that of control mice, except for a few apoptotic cells (arrow). However, the histological analysis of the skin of TAK1-KO mice at P6 (Fig. 3A) showed many apoptotic cells (arrow) and marked thickening of the epidermis without a granular layer (g), indicating abnormal epidermal differentiation. The thickened epidermis contained foci of keratinocyte apoptosis (Fig. 3B, left panel, arrow) and intra-epidermal micro-abscesses (Fig. 3B, right panel, arrowhead), which were associated with apoptotic cells (arrow). The oral mucosa showed a similar phenotype to that of the epidermis (Fig. 3A). Clinically, there were no apparent erosions or ulcers on the oral mucosa (data not shown).

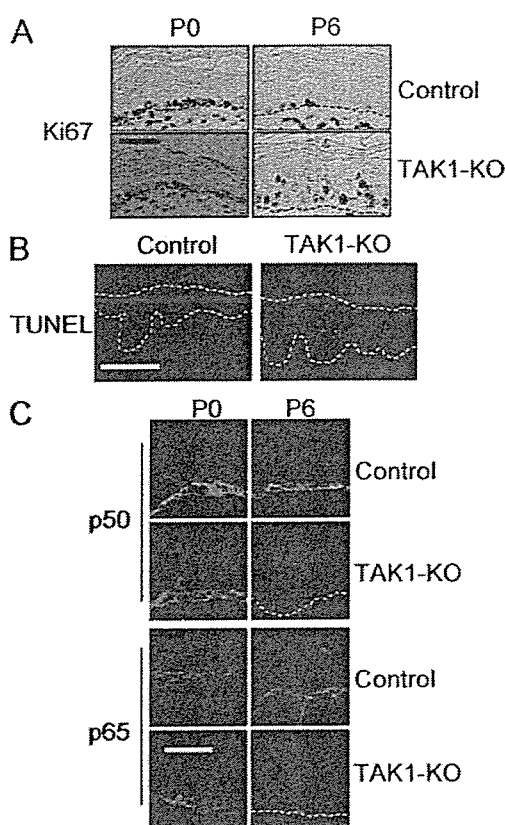


FIGURE 5. Immunohistochemical analysis of the epidermis of TAK1-KO mice. *A*, increased proliferation of keratinocytes in the epidermis of TAK1-KO mice. Paraffin-embedded skin sections from TAK1-KO mice at P0 and P6 were stained immunohistochemically with anti-Ki67 antibody. The dotted lines indicate the basement membrane. Scale bar, 10 μ m. *B*, increased apoptosis in the epidermis of TAK1-KO mice. Keratinocyte apoptosis was detected in paraffin-embedded skin sections of TAK1-KO mice at P6 by using the TUNEL method. The signal was observed under a fluorescence microscope. The dotted lines indicate the basement membrane and the surface of the epidermis. Scale bar, 20 μ m. *C*, decreased expression of p50 and p65 in the epidermis of TAK1-KO mice. Frozen skin sections from TAK1-KO mice at P0 and P6 were stained with anti-p50 and anti-p65 antibodies. The primary antibody was detected with Alexa Fluor 488-labeled second antibody. Fluorescence was observed under a confocal laser scanning microscope. Scale bar, 10 μ m.

No microbial pathogens were seen in the micro-abscesses on staining the specimens with methylene blue (data not shown), indicating that the micro-abscesses were not caused by infection. Because the micro-abscesses were associated with keratinocyte apoptosis, one possible mechanism for micro-abscess formation is the release of cytokines or chemokines from dead keratinocytes.

Abnormal Differentiation and Increased Proliferation of Keratinocytes in TAK1-KO Mice—To analyze the differentiation status of epidermal keratinocytes, we performed immunohistochemical analysis of the skin using antibodies against differentiation markers (Fig. 4). The expression of differentiation markers of TAK1-KO epidermis at P0 was similar to that of controls. The expression pattern started to change at P2, and the changes became more marked at P6. The expression of K5 and K14 is normally confined to the basal cell layer, as seen in the control. However, the suprabasal keratinocytes of TAK1-KO mice at P6 expressed K5 and K14. K1, K10, and loricrin are markers for the suprabasal and late phase differentia-

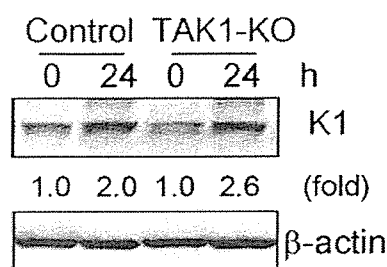


FIGURE 6. Induction of K1 in TAK1-KO mouse keratinocytes in suspension culture. Freshly isolated keratinocytes were seeded on culture plates and incubated overnight. To induce differentiation, the adherent cells were subjected to suspension culture using poly-HEMA-coated plates. Western blot analysis was performed to analyze the expression of K1. β -Actin is an internal standard. The intensity of each band was quantified relative to the control, set at one unit.

tion of keratinocytes and are normally expressed on the suprabasal keratinocytes and in the upper epidermis, respectively. In addition to the ectopic expression of K5 and K14, the expression of K1, K10, and loricrin was absent on the viable epidermal keratinocytes of TAK1-KO mice at P6. Furthermore, the epidermis of TAK1-KO mice at P6 expressed K16, a marker for inflammatory and hyperproliferative epidermal keratinocytes; K16 expression was absent from control mice. These data indicate that keratinocyte differentiation was disturbed by removing TAK1 from keratinocytes.

We further analyzed growth and apoptosis in the epidermis using Ki67 staining and the TUNEL method (Fig. 5). The staining of Ki67 revealed increased proliferation of keratinocytes in the epidermis of TAK1-KO mice at P6 (Fig. 5A), in contrast to the increased apoptosis shown with the TUNEL method (Fig. 5B). Furthermore, Ki67-positive keratinocytes were present in the suprabasal layer of the epidermis of TAK1-KO mice. The expression of p50 and p65 in the basal layer of the epidermis of TAK1-KO mice was decreased at P6 (Fig. 5C).

Induction of K1 in the Keratinocytes of TAK1-KO Mice in Suspension Culture—Based on the immunohistochemical results, we postulated that the keratinocytes of TAK1-KO mice do not express K1 by differentiation. To test this, we isolated and cultured keratinocytes from the epidermis of newborn mice. Although the number of Ki67-positive keratinocytes was increased in the epidermis of TAK1-KO mice, the *in vitro* growth of keratinocytes obtained from TAK1-KO mice was significantly reduced. Therefore, to study the ability of keratinocytes to differentiate, freshly isolated keratinocytes were seeded on culture plates and incubated overnight; adherent cells were subjected to suspension culture (3) to induce differentiation. Unexpectedly, the keratinocytes from TAK1-KO mice induced K1 in the suspension culture, as did those from control mice (Fig. 6).

Apoptosis of Keratinocytes on Depletion of TAK1 in Culture—Because of the difficulty in culturing TAK1-KO keratinocytes, we depleted TAK1 in cultured keratinocytes obtained from the control mice (*Map3k7^{fllox/fllox}*). An adenovirus vector carrying Cre-recombinase (Ax-Cre) was transfected to the cultured keratinocytes. Empty Ax-1W vector was used as a control. As shown in Fig. 7, the expression of TAK1 was reduced to 0.4-fold that in control mice at 48 h after the transfection of Ax-Cre. The reduction of TAK1 in cultured keratinocytes resulted in apo-

TAK1 Regulates Keratinocyte Differentiation and Apoptosis

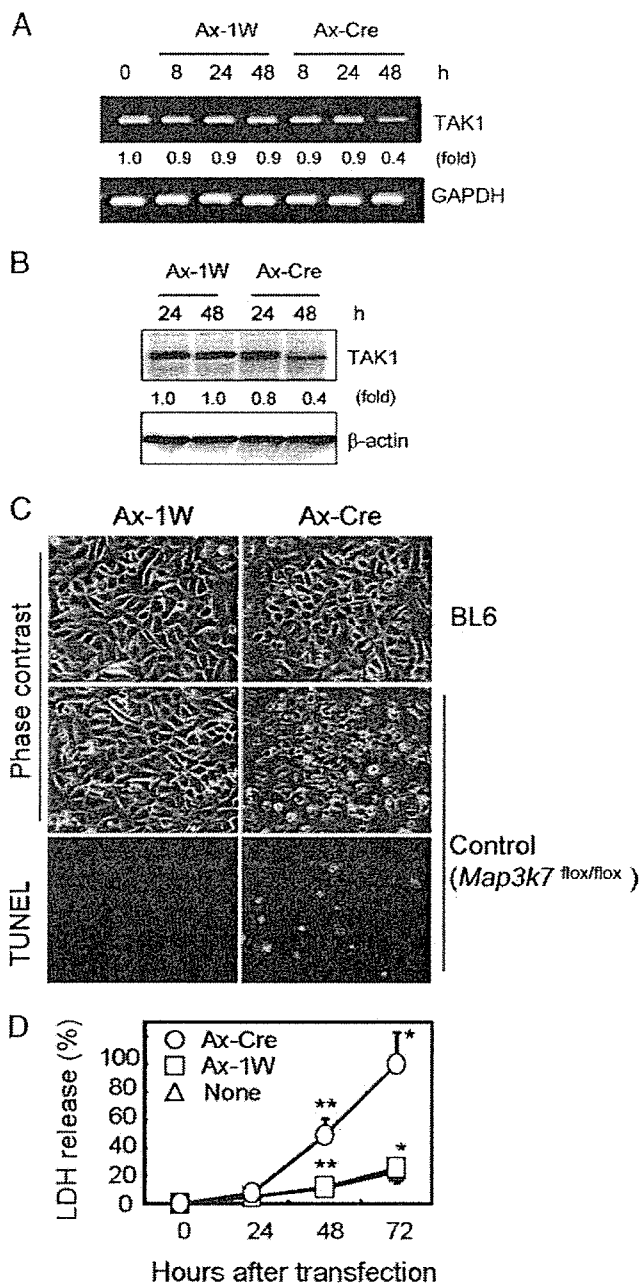


FIGURE 7. Apoptosis on depletion of TAK1 from cultured keratinocytes. TAK1 expression was depleted in cultured keratinocytes of the control mice (*Map3k7^{flox/flox}*). An adenovirus vector carrying Cre-recombinase (Ax-Cre) was transfected into the cultured keratinocytes at a multiplicity of infection of 100. The empty vector Ax-1W was used for the control. The expression of TAK1 mRNA and protein were analyzed using RT-PCR (A) and Western blotting (B). The intensity of each band was quantified relative to the control, set at one unit. Glycerinaldehyde-3-phosphate dehydrogenase and β -actin are internal standards. There was a weak band in the cells treated with Ax-Cre that was slightly lower than that of Ax-1W at 48 h (B). This might represent TAK1 Δ (15), as in Fig. 1E. At 72 h after transfection, the cell morphology was observed under a phase contrast microscope, and apoptotic cells were detected using the TUNEL method (C). Keratinocytes of C57/BL6 (BL6) mice were also transfected with Ax vector as a control. Cell death was quantified by measuring LDH release (D). Ax was transfected to the cultured keratinocytes, and the culture supernatant was harvested for LDH assay at the indicated time. LDH release was expressed as the percentage of total LDH (mean \pm S.E.), which was obtained by summing the LDH release and the LDH of living cells. The statistical significance was determined using the paired Student's *t* test ($n = 5$). * and **, differences were considered statistically significant for $p < 0.01$.

ptosis 72 h after transfection (Fig. 7C), indicating that TAK1 prevents keratinocyte apoptosis. The quantitative analysis of cell death based on measurements of LDH release revealed that the apoptosis started at 48 h and was completed at 72 h (D).

DISCUSSION

Although TAK1 is evolutionarily conserved and is thought to be an important intracellular signaling molecule, little is known about its physiological roles. In *Xenopus* embryonic development, TAK1 is involved in mesoderm induction mediated by bone morphogenetic protein, a member of the transforming growth factor β family (18). TAK1-deficient *Drosophila* are viable and fertile, but they do not produce antibacterial peptides and are highly susceptible to Gram-negative bacteria infection (19). In HeLa cells, it is thought that TAK1 is involved in the TNF receptor and IL-1 receptor/Toll-like receptor-mediated signaling pathways upstream from NF- κ B and MAPKs, based on small interfering RNA inhibition of TAK1 expression (11). Because TAK1-deficient mice are embryonically lethal (15), by utilizing embryonic fibroblasts, a loss of response to IL-1 β and TNF in TAK1-deficient cells was demonstrated (15). To study the role of TAK1 in B cell function, mice with B cell-specific TAK1 deficiency were generated (15). The B cell-specific TAK1-deficient mouse showed that TAK1 was indispensable for B cell activation in response to Toll-like receptor ligands (CpG DNA, poly(I:C), and LPS), CD40, and B cell receptor cross-linking (15). Furthermore, TAK1-deficient B cells failed to activate NF- κ B and JNK in response to IL-1 β , TNE, and Toll-like receptor ligands. Therefore, TAK1 has essential functions in the Toll-like receptor-, IL-1 receptor-, TNF receptor-, and B cell receptor-mediated cellular responses. In addition, we showed that TAK1 is essential for regulating keratinocyte differentiation and preventing keratinocyte apoptosis in the epidermis.

TAK1-KO mice die by P7. Because the barrier function of the epidermis was severely disturbed, as shown by TEWL, the impaired homeostasis of body fluids resulting from water loss through the skin may have caused the early mortality. Another possible cause of the early mortality is impaired feeding. The body weight at death was approximately half that of the control mice. The lips of the TAK1-KO mice showed signs of scaling, and the histological study revealed massive keratinocyte apoptosis (data not shown), as in the oral mucosa. These findings suggested that the diseased lip and oral mucosa affected nursing, resulting in severe growth retardation, which might cause the early mortality.

TAB1 and TAB2 were identified as adaptor proteins of TAK1 using yeast two-hybrid screening (10, 13). TAB1 binds to TAK1 constitutively and induces kinase activity on stimulation with IL-1. TAB2 is an adaptor molecule linking TRAF6 and TAK1. With IL-1 stimulation, TAB2 translocates from the cell membrane to the cytosol and binds to TRAF6 and TAK1. TAB3 is a TAB2 homologue that interacts with TRAF6 and TRAF2 on stimulation with IL-1 and TNF, respectively (12). Co-transfection with small interfering RNA s directed against both TAB2 and TAB3 inhibited both the IL-1- and TNF-induced activation of TAK1 and NF- κ B (12). These results suggest that TAB2 and TAB3 play redundant roles as mediators of TAK1 activation in IL-1 and TNF signal transduction. Although TABs have been

identified as TAK1 adaptor proteins, the genetic study of these proteins suggested that they do not always function together as a TAK1 complex. The neural tube development was abnormal in the TAK1-deficient embryo (20). This phenotype is substantially different from those of TAB1-deficient (abnormal cardiovascular and pulmonary morphogenesis) and TAB2-deficient (liver degeneration and apoptosis) mice (21, 22). These data suggested not only that TAK1 and the TAB complex function as a single unit, but that each component has a distinct role during development (20). Furthermore, the roles of TABs in keratinocytes have not yet been studied. Therefore, molecular interaction between TABs and TAK1 and their roles in keratinocytes should be clarified.

Genetic studies have shown that mutations in the human *NEMO/IKK- γ* gene are the cause of incontinentia pigmenti or Bloch-Sulzberger syndrome (23). Disruption of the *NEMO/IKK- γ* gene causes female mice to develop patchy skin lesions with massive granulocyte infiltration and hyperproliferation and increased apoptosis of keratinocytes (24, 25). Diseased animals show severe growth retardation and early mortality (24, 25). Although the skin lesion of TAK1-KO mice was not patchy, the clinical appearance, histological findings, and early mortality were similar to those in IKK- γ deficiency. The formation of patchy lesions in IKK- γ deficiency is thought to be the result of a chimerism in the IKK- γ -deficient mouse. Furthermore, the epidermis-specific deletion of IKK- β /IKK2 increased apoptosis and the abnormal expression of differentiation markers, including K6, K14, K10, and loricrin (26), resembling the characteristics of TAK1-KO mice. The development of similar skin phenotypes among mice with TAK1-, IKK- β -, or IKK- γ -deficient epidermal keratinocytes indicates the disruption of a common pathway in these mice. Given that TAK1 activates the IKK complex, the signal from TAK1 to NF- κ B might be disrupted in the IKK- β - or IKK- γ -deficient epidermis.

Although apoptosis was almost 100% at 72 h in Fig. 7D, TAK1 expression was still 0.4-fold 48 h after Ax-Cre transfection (Fig. 7, A and B). There are three possibilities for this discrepancy: 1) The band in the Western blot of cells transfected with Ax-Cre was slightly lower than that of Ax-1W at 48 h (B); this may represent TAK1 Δ , which lacks the ATP-binding site required for kinase activity (15), as in Fig. 1E. Therefore, the active TAK1 has already disappeared, and a small amount of inactive TAK1 (TAK1 Δ) appeared instead. 2) Cells started to die 48 h after Ax-Cre transfection. Because the dead cells detached spontaneously or on washing the culture dishes, and only living cells were subjected to analysis, it is most likely that cells expressing 0.4-fold TAK1 were still alive, whereas the complete loss of TAK1 had resulted in the apoptosis of cells that were not included in the analysis. At 72 h, TAK1 knockdown may be complete, and all the cells underwent apoptosis. 3) The transfection efficiency of Ax-Cre might not be equal for all cells. In less transfected cells, TAK1 expression might not be reduced. This might cause the 0.4-fold TAK1 expression at 48 h. Moreover, the high expression of Cre caused the complete loss of TAK1 and apoptosis at 48 h, but these cells were not included in the analysis, as discussed in the second possible explanation. One reason or a combination of these reasons might cause this discrepancy.

A possible role for NF- κ B in negative cellular growth control

via cell cycle regulation has been suggested (1, 5, 6). Growth inhibitory genes are induced by p65 in keratinocytes but not in other cell types (27). In the p65-deficient mouse, the number of Ki67-positive keratinocytes increased in the epidermis, and keratinocyte proliferation increased in culture (4). Based on the increased number of Ki67-positive cells in TAK1-KO mice seen in this study, we postulate that cell growth increased. However, the proliferation of TAK1-KO keratinocytes is reduced *in vitro*. IKK- β -deficient keratinocytes are also hypoproliferative *in vitro*, despite an increased number of Ki67-positive cells in the epidermis (26). One possible mechanism to account for an increased number of Ki67-positive cells and decreased proliferation *in vitro* is the induction of apoptosis that overtakes proliferation. However, this cannot explain the discrepancy between TAK1 and IKK- β deficiencies and p65 deficiency. Similar phenotypic discrepancy has also been found between IKK α /IKK1-deficient mice and others; the phenotypes of IKK α -deficient mice are somewhat different from those of mice deficient in p65, I κ B kinase 2, or IKK β . Mice lacking IKK α died perinatally and showed severely impaired limb outgrowth and abnormal epidermal differentiation (28, 29). These results suggest that the phenotypic differences among these deficient mice result from the loss of individual upstream signaling molecules of NF- κ B that do not completely impair NF- κ B function.

The loss of adhesion to the extracellular matrix in suspension culture strongly induces keratinocyte differentiation (3). Although the epidermis of TAK1-KO mice showed abnormal differentiation, the keratinocytes of TAK1-KO mice differentiated to express K1 in suspension culture. Similarly, IKK- β -deleted keratinocytes retained the ability to differentiate in suspension culture despite the failure of epidermal differentiation (26). These data indicate that the regulation of keratinocyte differentiation by TAK1 and IKK- β is independent of the initiation of differentiation by the loss of adhesion to the extracellular matrix.

Keratinocytes in the basal cell layer of control mice expressed p50 and p65, whereas the expression of p50 and p65 in the basal cell layer of TAK1-KO mice was decreased. One possible cause of the decreased expression is abnormal differentiation. High expression levels of p50 and p65 are normally found in the keratinocytes of the basal layer. However, the keratinocytes in the TAK1-KO basal layer did not possess a characteristic feature of the basal layer, the expression of K5 and K14. This abnormal differentiation might cause decreased expression of p50 and p65. As a result of decreased p50 and p65 expression, the NF- κ B signal is scarcely transduced to the downstream signaling pathway, in addition to the lack of TAK1. In conclusion, TAK1 is essential in regulating keratinocyte growth, differentiation, and apoptosis.

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SOCS1-Negative Feedback of STAT1 Activation Is a Key Pathway in the dsRNA-Induced Innate Immune Response of Human Keratinocytes

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Toll-like receptor (TLR)3 is a receptor for virus-associated double-stranded RNA, and triggers antiviral immune responses during viral infection. Epidermal keratinocytes express TLR3 and provide an innate immune defense against viral infection. Since the intracellular regulatory mechanism is unknown, we hypothesized that the signal transducers and activators of transcription (STAT)-suppressors of cytokine signaling (SOCS) system regulates the innate immune response of keratinocytes. Treatment with polyinosinic-polycytidylic acid (poly(I:C)) resulted in the rapid translocation of IFN regulatory factor (IRF)-3 into the nucleus, followed by phosphorylation of STAT1 and STAT3. The activation of STATs by poly(I:C) probably occurs in an indirect fashion, through poly(I:C)-induced IFN. We infected cells with the dominant-negative forms of STAT1 (STAT1F), STAT3 (STAT3F), and SOCS1 using adenovirus vectors. Infection with STAT1F suppressed the induction of macrophage inflammatory protein (MIP)-1 α by poly(I:C), whereas STAT3F had a minimal effect, which indicates that STAT1 mediates MIP-1 α induction. SOCS1, which is a negative feedback regulator of STAT1 signaling, was induced by treatment with poly(I:C). SOCS1 infection inhibited the phosphorylation of STAT1 and significantly reduced poly(I:C)-induced MIP-1 α production. Furthermore, STAT1-SOCS1 regulated poly(I:C)-induced TLR3 and IRF-7 expression. However, SOCS1 did not affect NF- κ B signaling. Thus, the STAT1-SOCS1 pathway regulates the innate immune response via TLR3 signaling in epidermal keratinocytes.

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INTRODUCTION

Toll-like receptors (TLRs) play critical roles in innate and adaptive immunity by detecting microbial pathogens. These signals are classified into MyD88-dependent and MyD88-independent pathways, which lead to the transcription of appropriate host-defense genes (Takeda *et al.*, 2003). TLR3 is a receptor for virus-associated double-stranded RNA (dsRNA) and activates NF- κ B, mitogen-activated protein kinase, and IFN regulatory factor (IRF)-3 in a MyD88-independent manner to control viral infection (Matsumoto *et al.*, 2004).

Keratinocytes are the major constituent of the epidermis and the target of viruses, such as herpes simplex virus (Mikloska *et al.*, 1998), human papillomavirus (Cho *et al.*,

2001), and varicella-zoster virus (Nahass *et al.*, 1996). Human keratinocytes express TLR3 both *in vitro* and *in vivo* (Mempel *et al.*, 2003; Tohyama *et al.*, 2005), and cultured keratinocytes produce IFN- α/β followed by macrophage inflammatory protein (MIP)-1 α (CCL3) in response to the ligand of TLR3, dsRNA (Tohyama *et al.*, 2005). MIP-1 α is chemotactic for monocytes, T lymphocytes, and natural killer cells and is critical for antiviral defense (Menten *et al.*, 2002). Previously, we showed that the lesional epidermis of virus-infected skin expresses MIP-1 α , and that dsRNA-treated keratinocytes produce MIP-1 α (Tohyama *et al.*, 2005), which indicates that keratinocytes play crucial roles in viral infection. Since chemokines recruit immune cells to attack virus-infected tissues, regulatory mechanisms must exist to avoid aberrant reactions to viral infection, otherwise the innate immune reaction would cause serious tissue damage. However, the negative regulatory mechanism of TLR3 signaling in keratinocytes has not been clarified.

The signal transducers and activators of transcription (STAT) family and its negative regulators, the suppressors of cytokine signaling (SOCS)/cytokine-inducible SH2-containing protein family of proteins, play central roles in regulating cytokine production in various cell types. STATs are crucial molecules for the IFN/cytokine-signaling pathways (Takaoka and Taniguchi, 2003). The SOCS family is induced by STAT activation, and SOCS proteins bind directly to cytokine

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Abbreviations: dsRNA, double-stranded RNA; IRF, IFN regulatory factor; MIP, macrophage inflammatory protein; poly(I:C), polyinosinic-polycytidylic acid; PKR, dsRNA-dependent protein kinase; RIG-I, retinoic acid-inducible gene; RT, reverse transcriptase; SOCS, suppressors of cytokine signaling; STAT, signal transducers and activators of transcription; TLR, Toll-like receptor

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receptors or to the catalytic domain of the Janus kinase (JAK) protein, thereby inhibiting the phosphorylation of tyrosine kinase and blocking cytokine signaling (Gadina *et al.*, 2001).

There is some evidence for a link between SOCS and TLR signaling. It has been reported that macrophages from SOCS1-deficient mice exhibit increased LPS sensitivity (Kinjyo *et al.*, 2002), and that the overexpression of SOCS1 impairs TLR triggering (Kinjyo *et al.*, 2002; Baetz *et al.*, 2004). Despite these reports, the inhibitory profile of the SOCS family differs according to cell type and growth conditions. It is not yet known whether or how the SOCS family proteins negatively regulate TLR3 signaling in keratinocytes.

Several IRF molecules are involved in the immune response to viral infection (Sato *et al.*, 2000), among which IRF-3 and IRF-7 regulate the transcription of IFN- α/β genes (Taniguchi *et al.*, 2001). IRF-3 is primarily responsible for the induction of IFN- α/β in the early induction phase of viral infection (Juang *et al.*, 1998). IRF-7 is a short-lived protein, and its expression is dependent on the activation of the transcription factor ISGF3 by type I IFN (Lu *et al.*, 2000). During viral infection, *de novo*-produced IRF-7 is phosphorylated, similarly to IRF-3, which further activates the IFN- α promoter and induces IFN-inducible genes (Marie *et al.*, 1998). Therefore, massive production of IFN- α/β can be achieved through this positive-feedback loop.

Given the importance of IFNs in viral infection and the ability of polyinosinic-polycytidylic acid (poly(I:C)) to induce IFN- α/β production in human keratinocytes, we hypothesized that the STAT family mediates TLR3 signaling and that SOCS family negatively regulates TLR3 signaling in keratinocytes. To support this hypothesis, we utilized adenovirus vectors (Axs) carrying the dominant-negative forms of STAT1, STAT3, and wild-type SOCS1. In this study, we demonstrate for the first time that dsRNA activates the JAK-STAT pathway, which is negatively regulated by SOCS1, in human keratinocytes.

RESULTS

Poly(I:C) activation of IRF-3 in cultured normal human keratinocytes

We studied whether IRF-3 was activated by poly(I:C) in normal human keratinocytes, since IRF-3 is a key transcription factor for virus-induced IFN expression (Sato *et al.*, 1998, 2000) and poly(I:C)-treated keratinocytes produce IFN- α and IFN- β (Tohyama *et al.*, 2005). We used confocal laser scanning microscopy to investigate the localization of IRF-3 after treatment with poly(I:C). As shown in Figure 1, IRF-3 was located in the cytoplasm of unstimulated keratinocytes. IRF-3 translocated into the nucleus as early as 30 minutes after treatment with poly(I:C), and translocation lasted for 6 hours, which confirms the previous findings in HeLa cells (Iwamura *et al.*, 2001). This result suggests that the activation of IRF-3 mediates poly(I:C)-induced IFN- α/β production in human keratinocytes.

Poly(I:C) activation of the JAK-STAT pathway is dependent upon IFN

We examined whether STATs were phosphorylated in keratinocytes in response to poly(I:C) treatment, as

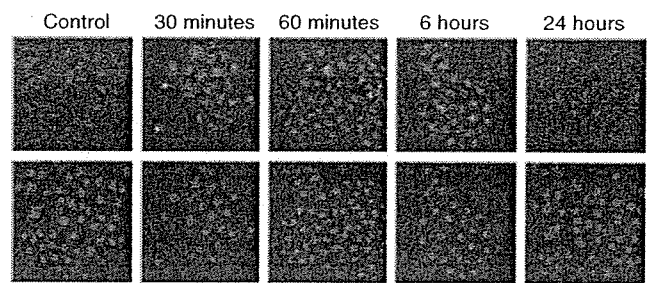


Figure 1. Poly(I:C) induces the nuclear translocation of IRF-3. Poly(I:C)-treated keratinocytes were fixed, and immunofluorescence was performed to detect the nuclear translocation of IRF-3. Green: IRF3 staining; red: 4,6-diamidino-2-phenylindole staining.

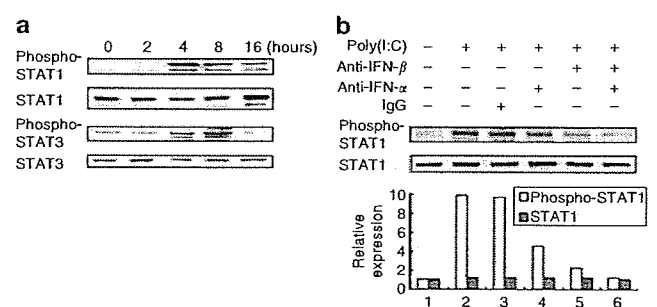


Figure 2. Poly(I:C)-provoked STAT activation is IFN-dependent. (a) Keratinocytes were stimulated with poly(I:C), and cell lysates were collected. Immunoblotting was performed with antibodies against STAT1, phospho-STAT1, STAT3, and phospho-STAT3. (b) The cultures were neutralized with normal IgG, anti-IFN- α antibody (5 μ g/ml), and/or anti-IFN- β antibody (5 μ g/ml) for 2 hours before the addition of poly(I:C). The keratinocytes were incubated for another 4 hours, and the cells were collected for evaluation of the STAT1 and phospho-STAT1 levels. The intensity of each band was quantified using the NIH Image software. The relative levels of STAT1 and phospho-STAT1 were normalized against the control, which was designated as one unit.

IFN receptors transduce signals via STATs (Takaoka and Taniguchi, 2003). As expected (Figure 2a), poly(I:C) treatment resulted in the phosphorylation of not only STAT1 but also STAT3. We also found that poly(I:C)-induced phosphorylation of STAT1 is dependent upon the expression of IFNs, as pretreatment with neutralizing antibodies against IFN- α or IFN- β significantly inhibited the phosphorylation of STAT1. Furthermore, almost complete blockage of STAT1 phosphorylation was detected when the anti-IFN- α and anti-IFN- β antibodies were added in combination into the culture, whereas the total STAT level was not affected (Figure 2b). Our data also suggest that IFN- β is more important than IFN- α for the activation of STAT1. Blocking the IFN signal also suppressed poly(I:C)-provoked STAT3 phosphorylation (data not shown). Therefore, the poly(I:C)-activated JAK-STAT pathway is type I IFN dependent.

Inhibition of poly(I:C)-induced MIP-1 α production by STAT1F but not by STAT3F

Next, we investigated whether the activation of STATs was involved in poly(I:C)-induced MIP-1 α production. For this

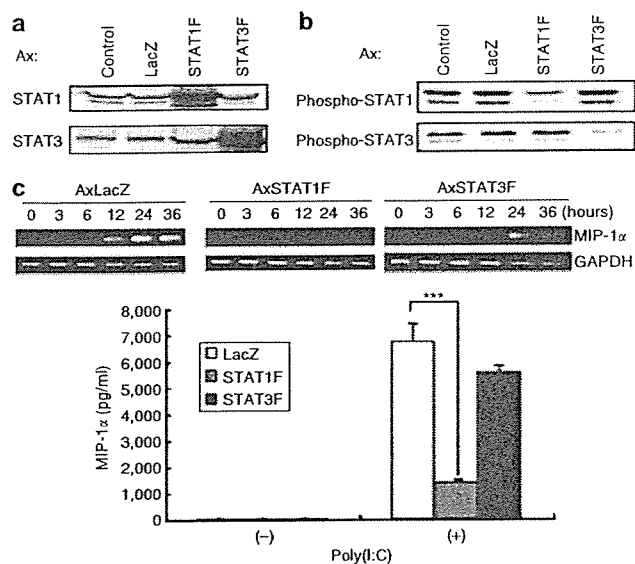


Figure 3. Infection with STAT1F blocks poly(I:C)-induced MIP-1 α production. (a) Keratinocytes were infected with AxLacZ, AxSTAT1F, and AxSTAT3F for 24 hours. Cell lysates were analyzed for the levels of STAT1 and STAT3 proteins. (b) Keratinocytes were infected with AxLacZ, AxSTAT1F, and AxSTAT3F for 24 hours, and the cultures were then stimulated with poly(I:C) for 4 hours. The phosphorylation levels of STAT1 and STAT3 were determined by Western blotting. (c) Keratinocytes were infected with AxLacZ, AxSTAT1F, and AxSTAT3F, respectively, and then stimulated with poly(I:C). Total RNA samples were extracted at the indicated times, and the supernatants were collected at 36 hours post-treatment. The production of MIP-1 α was examined using real-time RT-PCR and ELISA.

purpose, we constructed AxSTAT1F and AxSTAT3F. Infection with AxSTAT1F and AxSTAT3F resulted in significant expression of the STAT1 and STAT3 proteins, respectively (Figure 3a), and abolished the poly(I:C)-provoked phosphorylation of STAT1 and STAT3 (Figure 3b), respectively. Therefore, STAT1F and STAT3F specifically inhibit the STAT1 and STAT3 pathways. Poly(I:C) strongly stimulated the production of MIP-1 α , which was completely abolished by STAT1F; however, STAT3F had little effect on MIP-1 α production (Figure 3c). These data suggest that the STAT1-signaling pathway is essential for poly(I:C)-induced MIP-1 α production.

Inhibition of poly(I:C)-induced MIP-1 α production by SOCS1

The expression of SOCS1-3 in poly(I:C)-treated keratinocytes was examined by real-time reverse transcriptase (RT)-PCR. The expression of SOCS1 mRNA was enhanced 150-fold at 24 hours and more than 400-fold at 36 hours, compared with the control level at 0 hour; the level of SOCS2 mRNA was increased slightly after 36 hours, and SOCS3 expression was increased several-fold after 12 hours (Figure 4a). Western blot analysis confirmed the induction of SOCS1 (Figure 4b), which is certainly dependent upon the activation of STAT1 (Figure 4c). It has been reported that SOCS1 and SOCS3 inhibit IFN-mediated antiviral activities and that the inhibitory activity of SOCS3 toward STAT1 activation is weaker

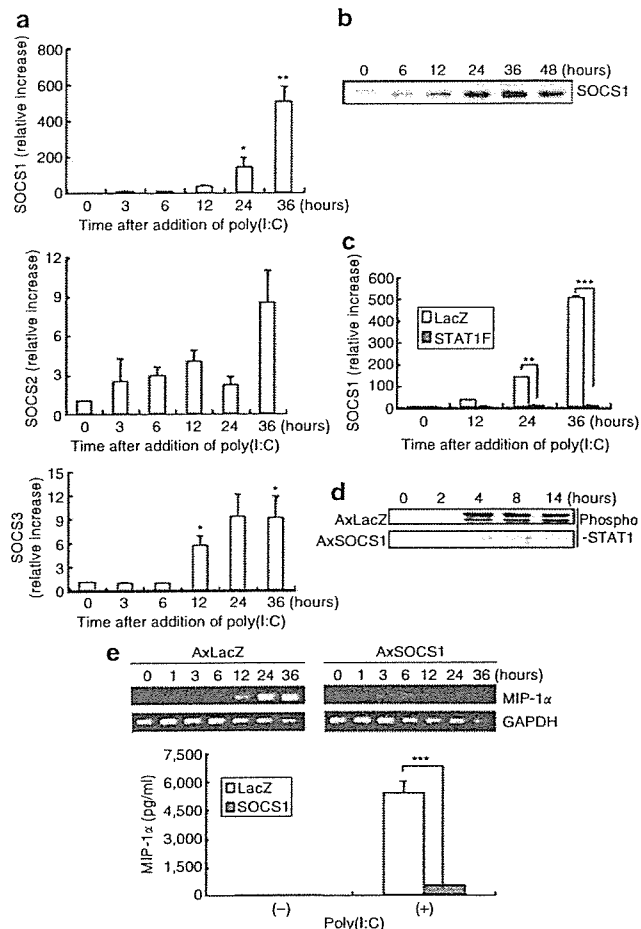


Figure 4. Poly(I:C)-induces SOCS1 expression, and SOCS1 infection inhibits poly(I:C)-induced MIP-1 α production. (a) Keratinocytes were treated with poly(I:C) for the indicated time periods. The mRNA expression levels of SOCS1, SOCS2, and SOCS3 were examined using real-time RT-PCR. (b) Keratinocytes were treated with poly(I:C), and the SOCS1 protein levels were assayed by Western blotting. (c) Keratinocytes were infected with AxLacZ or AxSTAT1F for 24 hours before the addition of poly(I:C). Real-time RT-PCR was performed to evaluate the levels of SOCS1 mRNA. (d) Keratinocytes were infected with AxLacZ and AxSOCS1 before incubation with poly(I:C). Cell lysates were examined using the phospho-STAT1 antibody. (e) Keratinocytes were infected with adenovirus for 24 hours before the addition of poly(I:C). Total RNA samples were extracted, and real-time RT-PCR was performed for MIP-1 α mRNA. Supernatants were collected at 36 hours post-treatment, and the MIP-1 α protein levels were determined by ELISA.

than that exhibited by SOCS1 (Song and Shuai, 1998). Therefore, we investigated the effect of SOCS1 on poly(I:C)-induced MIP-1 α production.

As shown in Figure 4d, the expression of SOCS1 virtually abolished poly(I:C)-induced STAT1 phosphorylation. Furthermore, SOCS1 almost completely blocked poly(I:C)-induced MIP-1 α production (Figure 4e). Taken together, these results suggest that the expression of SOCS1 is dependent upon the activation of STAT1, and that SOCS1 negatively regulates poly(I:C)-provoked MIP-1 α production by inhibiting STAT1 phosphorylation.

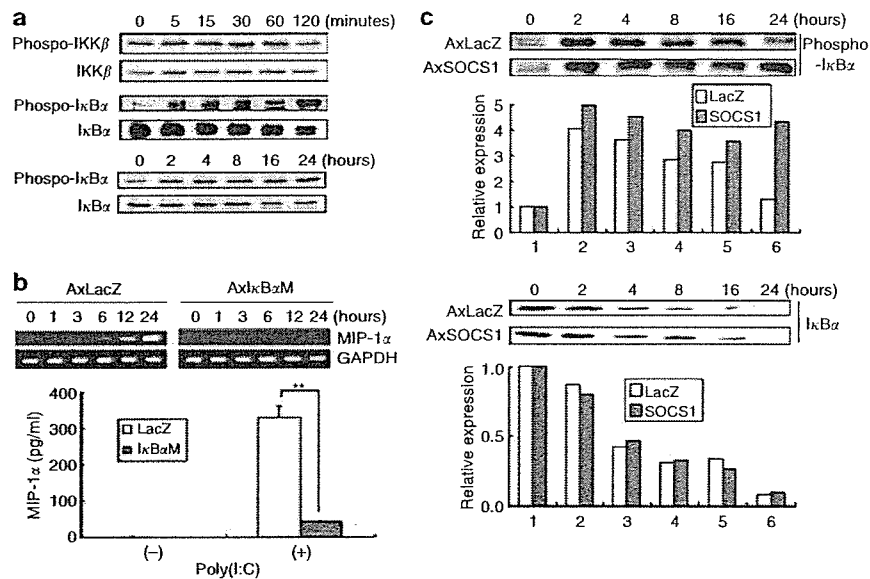


Figure 5. The activation of NF- κ B regulates MIP-1 α production, but SOCS1 does not affect the NF- κ B signal. (a) Poly(I:C) activates the NF- κ B signal. Keratinocytes were treated with poly(I:C), and cell lysates were prepared. Immunoblotting was performed with antibodies directed against IKK β , phospho-IKK β , I κ B α , and phospho-I κ B α . (b) Infection with I κ B α M suppresses poly(I:C)-induced MIP-1 α production. Keratinocytes were infected with AxLacZ or AxI κ B α M for 24 hours, and the cultures were stimulated with poly(I:C). RNA samples were collected at the indicated times, and supernatants were collected at 36 hours post-treatment. The mRNA and protein levels of MIP-1 α were measured. (c) SOCS1 does not inhibit the phosphorylation of I κ B α . Keratinocytes were infected with AxLacZ and AxSOCS1 for 24 hours before the addition of poly(I:C). Cell lysates were prepared, and Western blotting was performed with antibodies directed against phospho-I κ B α and I κ B α . The graph is constructed as described in Figure 2b.

Involvement of NF- κ B in poly(I:C)-induced MIP-1 α production

TLR signaling phosphorylates I κ B α and activates the NF- κ B signal (Alexopoulou *et al.*, 2001). The activation of the NF- κ B signal was detected in poly(I:C)-treated keratinocytes (Figure 5a). The promoter for the gene that encodes MIP-1 α contains several NF- κ B-binding sites, and NF- κ B has been demonstrated to activate MIP-1 α transcription in response to cytokines (Guo *et al.*, 2003). Therefore, we studied whether NF- κ B was involved in poly(I:C)-provoked MIP-1 α production. We introduced the Axs that carries a dominant-negative form of I κ B α (I κ B α M) to block NF- κ B signaling (Dai *et al.*, 2004a). As shown in Figure 5b, the production of MIP-1 α was suppressed by I κ B α M.

NF- κ B is not involved in the inhibition of poly(I:C)-induced MIP-1 α production by SOCS1

As SOCS1 has been shown to function as a negative-feedback regulator of TLR4 by suppressing LPS-provoked I κ B α phosphorylation and STAT activation in macrophages (Kinjyo *et al.*, 2002; Kimura *et al.*, 2004), we investigated whether the inhibitory effect of SOCS1 on poly(I:C)-induced MIP-1 α production was attributable to the inhibition of I κ B α phosphorylation. Unexpectedly, infection with AxSOCS1 showed no effect on the level of total I κ B α but slightly increased the phosphorylation of I κ B α (Figure 5c). This outcome may be explained by the possibility that the inhibition of SOCS1, as a negative regulator for the indirect or secondary effect of TLR3 signaling, benefits the activation of direct signals provoked by dsRNA (Sen and Sarkar, 2005).

Neither infection with STAT1F nor blocking of the IFN signal affected the phosphorylation of I κ B α (data not shown). Therefore, although NF- κ B is involved in MIP-1 α production, the inhibition of MIP-1 α production by SOCS1 does not occur via the NF- κ B signal.

Regulation of poly(I:C)-induced TLR3 expression by the STAT1-SOCS1 pathway

As described previously (Tanabe *et al.*, 2003; Tohyama *et al.*, 2005), type I IFNs are required for poly(I:C)-induced TLR3 upregulation (Figure 6a). Thus, we investigated whether the SOCS1-STAT1 pathway regulates TLR3 expression in poly(I:C)-treated keratinocytes. STAT1F completely abolished the induction of TLR3 mRNA, while STAT3F also inhibited TLR3 expression, albeit less efficiently than STAT1F (Figure 6b). Moreover, SOCS1 expression significantly reduced poly(I:C)-induced TLR3 mRNA expression (Figure 6c). These data indicate that the induction of TLR3 expression by poly(I:C) is also regulated by the STAT1-SOCS1 pathway in keratinocytes.

Inhibition of poly(I:C)-induced IRF-7 expression by SOCS1

Although the IRF-7 protein is undetectable in normal cells, treatment with type I IFNs or viral infection induces the accumulation of IRF-7 (Taniguchi and Takaoka, 2002). The IRF-7 mRNA level in the keratinocytes increased beginning 6 hours post-treatment with poly(I:C); this induction process was dependent upon the type I IFN signal (Ousman *et al.*, 2005), as evidenced by the suppression of IRF-7 expression

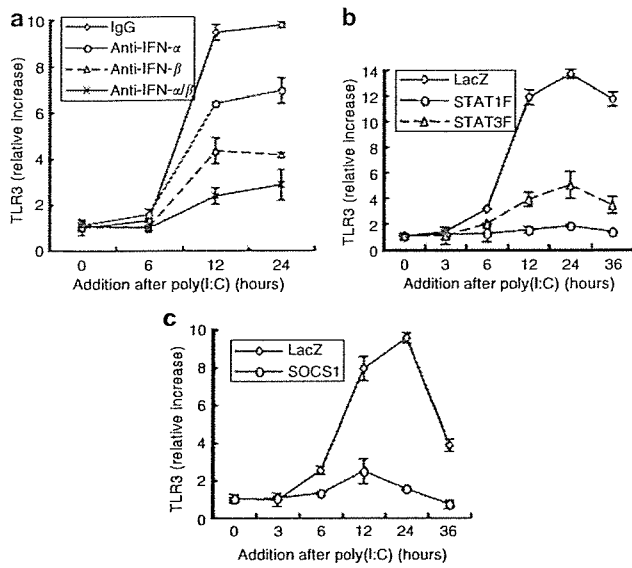


Figure 6. STAT1F and SOCS1 inhibit poly(I:C)-induced TLR3. (a) Keratinocytes were pretreated with normal IgG or neutralizing antibody against type I IFN for 2 hours before the addition of poly(I:C). (b) The protocols for keratinocyte treatment and total cellular RNA preparation were as described in Figure 3c. (c) The protocols for keratinocyte treatment and total cellular RNA preparation were as described in Figure 4e. The expression of TLR3 mRNA was determined by real-time RT-PCR.

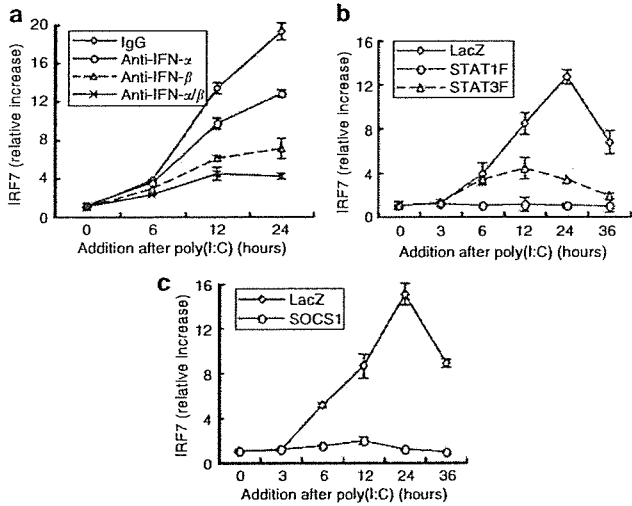


Figure 7. STAT1F and SOCS1 inhibit poly(I:C)-induced IRF-7. The samples described in Figure 6 were used for the detection of IRF-7 mRNA by real-time RT-PCR. (a) The samples were same as Figure 6a. (b) The samples described in Figure 6b were used. (c) RNA samples were prepared as described in Figure 4e. The expressions of IRF-7 mRNA were detected in real time RT-PCR.

by neutralizing antibody against type I IFNs (Figure 7a). STAT1F completely blocked poly(I:C)-induced IRF-7 mRNA expression, whereas STAT3F partially suppressed its expression (Figure 7b). SOCS1 also significantly inhibited IRF-7 expression (Figure 7c). Therefore, activation of the STAT1-SOCS1 pathway is an essential step in this process.

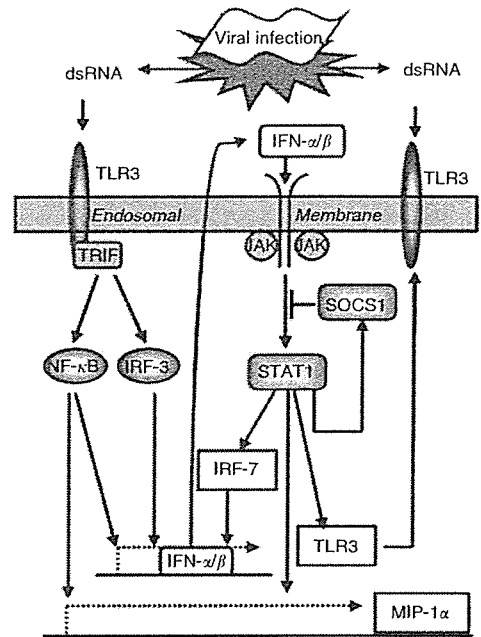


Figure 8. Regulation of dsRNA-provoked innate immune reactions by the STAT1-SOCS1 pathway in normal human keratinocytes. After infection with virus or treatment with dsRNA, the IRF-3, and NF-κB signals are activated, which results in IFN-α/β production. The *de novo*-expressed IFNs stimulate MIP-1α expression by activating the JAK-STAT pathway, which also increases the transcription of the SOCS family members, TLR3, and IRF-7. TLR3 and IRF-7 promote signal amplification, while SOCS1 inhibits STAT1-dependent TLR3, IRF-7, and MIP-1α production by blocking the JAK-STAT pathway. Thus, SOCS1 negatively regulates the immune responses of keratinocytes to dsRNA during viral skin infection.

DISCUSSION

Keratinocytes, which are the primary target of viral infections of the skin, are likely to play a pivotal role in virus-induced skin inflammation. Figure 8 summarizes the intracellular signaling mechanisms of the dsRNA-provoked innate immune responses of normal human keratinocytes. Initially, dsRNA enhances IFN-α/β production via TLR3-activated IRF-3 and NF-κB. IFNs activate the JAK-STAT pathway, which results in the expression of genes related to antiviral function. The activation of STAT1 by IFNs not only induces chemokine production, but also results in the expression of IRF-7 and TLR3, thus amplifying the dsRNA-provoked reaction in a positive-feedback manner during viral infection. However, SOCS1, which is another inducible gene, not only blocks STAT1 activation but also inhibits STAT1-dependent TLR3, IRF-7, and MIP-1α. The SOCS1 negative-feedback mechanism for the STAT activation pathway probably impairs autocrine secondary signaling via type I IFN, thereby avoiding exaggerated reactions and regulating the antiviral immune response to viral infection of the skin. This is the first report to indicate that the SOCS-STAT pathway regulates MIP-1α production in dsRNA-stimulated keratinocytes.

Since the induction of SOCS was initially attributed to the JAK/STAT pathways, SOCS has generally been thought to act on JAK/STAT cascades. However, this idea has recently been

challenged by the finding that SOCS^{-/-} mice show enhanced responses to LPS, with strongly enhanced phosphorylation of I κ B α , p38, and JNK (Kinjyo *et al.*, 2002). Moreover, overexpression of SOCS1 suppresses LPS-induced NF- κ B activation, a process through which SOCS1 inhibits TLR4-specific cytokine induction (Kinjyo *et al.*, 2002). However, two other groups have presented contrasting data (Baetz *et al.*, 2004; Gingras *et al.*, 2004), which are consistent with our observations that SOCS1 has no negative effects on the poly(I:C)-stimulated rapid activation of NF- κ B signaling. In this study, we show the almost complete inhibition by SOCS1 of STAT1 activation in TLR3 signaling, and demonstrate that SOCS1 inhibits MIP-1 α expression by disabling the STAT1 signal and not the NF- κ B signal, although the NF- κ B signal is also required for MIP-1 α production.

The continuous expression of IRF-7 through type I IFN results in the amplification of the IFN signal, which plays an important role in ensuring a robust immune response during viral infection. In this study, we found that poly(I:C) induces IRF-7 expression, which is dependent upon type I IFN-stimulated STAT1 activation. It has been reported that virus-induced regulated upon activation, normal T-cell expressed and secreted (RANTES) gene expression is blocked in airway epithelial cells through the inhibition of IRF-7 expression (Casola *et al.*, 2001). We propose that SOCS1 partially regulates MIP-1 α expression by inhibiting IRF-7. SOCS1 may inhibit the JAK/STAT-dependent autocrine and paracrine amplification loops, for example, additional activation via type I IFNs. Based on these findings, we suggest that the STAT-SOCS pathway plays a role in avoiding the unnecessary amplification of the immune response and in keeping host defenses balanced by regulating the activities of the IRF family and TLR3.

The dsRNA-dependent protein kinase (PKR) has been implicated in dsRNA-activated signaling (Chu *et al.*, 1999). PKR exists in most cell types and *trans*-autophosphorylates in response to poly(I:C). PKR has been shown to regulate poly(I:C)-induced tumor necrosis factor- α (Meusel *et al.*, 2002). However, PKR is not the kinase responsible for phosphorylating and activating IRF-3 (Chu *et al.*, 1999). Furthermore, we did not detect any notable level of phosphorylated PKR in the poly(I:C)-treated keratinocytes (data not shown). All of these events support the notion that the poly(I:C)-stimulated signal in keratinocytes is independent of PKR.

The existence of a viral sensor other than TLR3 has been proposed (Levy and Marie, 2004). The RNA helicase retinoic acid-inducible gene (RIG-I) probably serves as this type of sensor, as RIG-I has been demonstrated to activate IRF-3 and induce IFN- β production (Levy and Marie, 2004; Yoneyama *et al.*, 2004). It has been reported that the responses to viral infection or to intracellular dsRNA are reduced in the absence of RIG-I, whereas the extracellular dsRNA-stimulated responses mediated by TLR3 remain intact (Yoneyama *et al.*, 2004). Both TLR3 and RIG-I sense the *in vivo* immune responses to viruses. It is possible that MIP-1 α production and JAK-STAT activation in virus-infected keratinocytes are mediated partially via RIG-I. Therefore, the involvement of

RIG-I in the virus-induced innate immune responses of keratinocytes should be clarified.

Our investigations suggest that SOCS1 mainly suppresses the indirect effects of the dsRNA signal and negatively regulates the viral immune response to avoid exaggerated reactions. Therefore, the STAT-SOCS1 pathway plays a critical role in the maintenance of homeostasis in the innate immune system of the skin.

MATERIALS AND METHODS

Reagents

Normal IgG, as well as the anti-IFN- α and anti-IFN- β antibodies were purchased from R&D Systems (Minneapolis, MN), and the anti-IRF-3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The remaining antibodies used in this study were obtained from Cell Signaling Technology (Beverly, MA).

Cell culturing and treatment

Normal human keratinocytes were cultured in MCDB153 medium that was supplemented with insulin (5 μ g/ml), hydrocortisone (5 $\times 10^{-7}$ M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μ g/ml), and Ca²⁺ (0.03 mM), as described previously (Shirakata *et al.*, 2004). Cells that had been passaged four times were used in the experiments, and subconfluent keratinocyte cultures were treated with 100 ng/ml of poly(I:C) (Amersham Biosciences Corp., Piscataway, NJ) for a predetermined period of time. The study was conducted according to the Declaration of Helsinki Principles. All of the procedures that involved human subjects received prior approval from the Ethical Committee of Ehime University School of Medicine, and all the subjects provided written informed consent.

Adenovirus construction and infection

The cosmid cassette pAxCAW and the parental virus Ad5-dlX were kind gifts from Dr Izumu Saito (Tokyo University, Japan) (Miyake *et al.*, 1996). The full-length coding regions of STAT1F, STAT3F, and SOCS1 cDNA were subcloned into pAxCAW. The STAT1F and STAT3F genes encode dominant-negative mutant proteins, in which the tyrosine phosphorylation sites have been changed to phenylalanine (Yamasaki *et al.*, 2003). Axs that contained the CA promoter and STAT1F, STAT3F, or SOCS1/JAB were generated using the cosmid cassettes and Ad DNA-TPC method (Miyake *et al.*, 1996). The cosmid DNA was mixed with the EcoT221-digested DNA-terminal Ad5-dlX protein complex, which was used to transfect 293 cells. The recombinant viruses were generated through homologous recombination in 293 cells, and the virus stocks were prepared using a standard procedure (Miyake *et al.*, 1996). Concentrated, purified virus stocks were prepared by CsCl gradient centrifugation, and the virus titers were estimated in a plaque formation assay.

Cultured normal human keratinocytes were infected with AxSTAT1F, AxSTAT3F, or AxSOCS1 at a multiplicity of infection of 10, as described previously (Yamasaki *et al.*, 2003), and AxLacZ was used as the control vector. The AxI κ B α M vector was constructed and infected into keratinocytes as described previously (Dai *et al.*, 2004a).

Immunofluorescence microscopy

The keratinocytes were seeded on chamber slides. The treated cells were fixed for 5 minutes in methanol:acetone (1:1), and treated with

the anti-IRF-3 antibody overnight at 4°C. After washing with phosphate-buffered saline, the cells were incubated with fluorescein-labeled goat anti-rabbit IgG for 30 minutes at 37°C. 4,6-Diamidino-2-phenylindole staining of the nucleus was also performed. The stained cells were visualized at an original magnification of $\times 40$ under an LSM 510 microscope (Carl Zeiss, Jena, Germany). Images were captured using the LSM 510 software.

RT-PCR

Total RNA samples were isolated using Isogen (Nippon Gene, Tokyo, Japan), and RT-PCR was performed using RT-PCR High Plus (Toyobo, Osaka, Japan) (Dai et al., 2004b). The expression levels of *MIP-1 α* and glyceraldehyde-3-phosphate dehydrogenase mRNA were detected using specific primers (primer list: Table S1). The PCR products were sequenced to confirm the accuracy of amplification.

Real-time RT-PCR

Real-time RT-PCR was performed in an ABI PRISM 7700 sequence detector (PE Applied Biosystems, Foster City, CA). The primers and probes for glyceraldehyde-3-phosphate dehydrogenase, *TLR3*, and *IRF-7* were obtained from Applied Biosystems (Norwalk, CT). The primers and probes for members of the human *SOCS* family are shown in Table S2. The RNA analysis was carried out using the TaqMan RT-PCR Master Mix reagents kit (Applied Biosystems, Norwalk, CT) and the quantification of gene expression was performed using the comparative computed tomography method, as described previously (Dai et al., 2004a). The level of target gene expression in the test samples was normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase level and is reported as the fold difference. In this study, each assay was performed in triplicate, and the factorial change of each sample was normalized against that of the vehicle as one unit.

ELISA

Culture supernatants were collected at the indicated times after treatment and were stored at -70°C until subjected to ELISA. The ELISA kit for *MIP-1 α* was purchased from Endogen (Auburn, MA). ELISA was performed according to the manufacturer's instructions.

Protein preparation and Western blot analysis

The cells were harvested by transfer into extraction buffer that contained 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The analysis was performed using the Vistra ECF kit (Amersham Biosciences, Tokyo, Japan) and a Fluorolmager (Molecular Dynamics, Sunnyvale, CA).

Statistical analyses

In this study, at least three independent experiments were performed, with similar results, and one representative experiment is shown in each of the figures. The quantitative ELISA data and the relative mRNA expression levels detected by real-time RT-PCR are expressed as the mean \pm SD ($N=3$). Statistical significance was determined by the paired Student's *t*-test. Differences were

considered to be statistically significant for $P<0.05$. The levels of statistical significance are indicated as follows in the figures: * $P<0.05$; ** $P<0.01$; and *** $P<0.001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Primer pairs for RT-PCR.

Table S2. Primer and probe list for real time RT-PCR.

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