Mice with a keratinocyte-specific deletion of Tak1 exhibit severe skin inflammation due to hypersensitivity to tumor necrosis factor (TNF) killing. Here we have examined the mechanisms underlying this hypersensitivity. We found that Tak1 deficiency up-regulates reactive oxygen species (ROS) resulting in cell death upon TNF or oxidative stress challenge. Because blockade of NF-κB did not increase ROS or did not sensitize cells to oxidative stress in keratinocytes Tak1 regulates ROS mainly through the mechanisms other than those mediated by NF-κB. We found that c-Jun was decreased in Tak1-deficient keratinocytes and that ectopic expression of c-Jun could partially inhibit TNF-induced increase of ROS and cell death. Finally, we show that, in an in vivo setting, the antioxidant treatment could reduce an inflammatory condition in keratinocyte-specific Tak1 deletion mice. Thus, Tak1 regulates ROS partially through c-Jun, which is important for preventing ROS-induced skin inflammation.

Tumor necrosis factor (TNF) plays a central role in inflammation, and also regulates cell death and survival (1–3). TNF initiates intracellular signaling by binding to its receptor, initiating the formation of the TNF receptor complex, which consists of several proteins including RIP1 kinase, Fas-associated death domain (FADD), and pro-caspase-8 (also called Flice). The TNF receptor complex in turn activates two opposing intracellular signaling pathways; one leads to up-regulation of the expression of anti-apoptotic genes such as cellular Flice-inhibitory protein (c-FLIP) (4) and caspase inhibitor IAPs (inhibitor of apoptosis proteins) (5) (anti-cell death pathway); another activates the caspase cascade to execute apoptotic cell death (pro-cell death pathway). TNF-induced activation of the transcription factor NF-κB is one of the major pathways of anti-apoptotic gene up-regulation and cell death inhibition. NF-κB is activated upon degradation of IκB (inhibitor of NF-κB), which is induced by its phosphorylation by IκB kinases (6).

The pro-cell death pathway is activated through FADD (1–3). FADD recruits the apoptosis-initiating protease caspase-8, which is in turn autoactivated by proteolysis. Caspase-8 cleaves and activates executor caspases such as caspase-3. c-FLIP is a specific inhibitor of caspase-8 (7). Additionally, the TNF receptor complex activates NADPH oxidase 1 and increases ROS production (8). ROS causes prolonged activation of JNK (9, 10). Prolonged JNK activation down-regulates the E3 ubiquitin ligase Itch, which degrades c-FLIP (11, 12). This ROS-JNK-mediated c-FLIP degradation facilitates the activation of caspase-8 (12). The ROS-facilitated caspase pathway is believed to be the major pathway of TNF-induced cell death.

TAK1 kinase is a member of the mitogen-activated protein kinase kinase family and is activated by innate immune stimuli including bacterial components and proinflammatory cytokines such as interleukin-1 and TNF (13, 14). TAK1 is an ubiquitin-dependent kinase and plays an essential role in innate immune signaling by activating both IκB kinases-NF-κB and mitogen-activated protein kinase (MAPK) pathways leading to activation of transcription factor AP-1 (15, 16). We have recently generated mice harboring a skin keratinocyte (epidermal)-specific deletion of Tak1 and found that Tak1 is essential for keratinocyte survival in vivo (17). We have found that Tak1 deficiency causes hypersensitivity to TNF-mediated killing in keratinocytes. TNF expressed in the skin kills Tak1-deficient keratinocytes and induces an inflammatory condition in the Tak1 mutant skin. However, the mechanism by which Tak1 deficiency increases the susceptibility to TNF killing has not yet determined. One plausible mechanism is that Tak1 deficiency impairs TNF-induced NF-κB resulting in activation of the pro-death caspase pathway. However, the degree of cell death is somewhat greater in Tak1-deficient mouse embryonic fibroblasts (MEFs) than NF-κB-deficient MEFs as described previously (18). Furthermore, we have recently found that Tak1 deletion in the intestinal epithelium causes epithelial cell death at a much higher degree compared with the intestinal epithelium-specific deletion of NEMO (19, 20). Therefore, we speculate that Tak1 may participate in cell survival pathways other than those mediated by NF-κB.
TAK1 Regulates ROS and Cell Death

In this study, we investigated the mechanisms by which TAK1 regulates sensitivity to TNF-induced cell death. Particularly, we are interested in whether TAK1 deficiency induces cell death through the mechanisms mediated by other than lack of NF-κB activation in TAK1-deficient keratinocytes. To this end, we generated NF-κB-deficient keratinocytes by expressing non-degradable IκB (IκBΔN) that completely blocks activation of NF-κB, and compared them with TAK1-deficient keratinocytes. We show here that TNF-induced ROS is mainly regulated by a non-NF-κB mechanism in TAK1-deficient keratinocytes, and that this TAK1-dependent ROS regulation is important for preventing the inflammatory conditions in mice having an epidermal specific Tak1 deletion.

EXPERIMENTAL PROCEDURES

Mice—Tak1+/+ and Tak1Δ/Δ keratinocytes were isolated from Tak1+/+ and K5-Cre Tak1Δ/Δ mice described previously (17). Spontaneously immortalized keratinocytes derived from the skin of postnatal day 0–2 mice were cultured in Ca2+-free minimal essential medium (Invitrogen) supplemented with 4% Chelex-treated bovine growth serum (Hyclone), 10 ng/ml human epidermal growth factor (Invitrogen), 0.05 mM calcium chloride, and penicillin-streptomycin at 37 °C in 5% CO2.

Reagents—Reagents used were TNF-α (human recombinant; Roche), N-acetyl-L-cysteine (Calbiochem), BHA (Sigma), tert-butyl hydroperoxide (tBHP; Sigma), and 4-hydroxytamoxifen (Sigma). Polyclonal antibodies were Tak1 described previously (13), JNK1 (FL), p65 (F-6; Santa Cruz), caspase-3 (Cell Signaling), c-Jun (BD Bioscience), phospho-c-Jun (Ser73; Cell Signaling). Monoclonal antibodies were c-FLIP (Abcam), phospho-JNK (Thr183/Tyr185; Cell Signaling), c-Jun (BD Bioscience), β-actin (Sigma), and nigem (Chemicon).

Annexin V Binding Assay—To determine apoptotic cells, Annexin V-Alexa Fluor 488 binding and propidium iodide staining were performed according to the manufacturer’s protocol (Invitrogen). Images were taken using an inverted fluorescent microscope (TE2000-S; Nikon). 3–5 randomly selected areas were photographed with the same exposure time. The images were processed using the fixed threshold in all samples in each experiment, and more than 1000 cells were counted for each sample.

Crystal Violet Assay—The viable adherent cells were fixed with 10% formalin and stained with 0.1% crystal violet. The stain was solubilized by adding 25% ethanol containing 50 mM sodium citrate, and the absorbance of each plate was determined at 595 nm.

Real-time PCR Analysis—Total RNA was prepared from keratinocytes using the RNeasy protect mini kit (Qiagen). cDNA was synthesized using the reverse transcription reagents (Applied Biosystems). Real-time PCR analysis was performed using the ABI PRISM 7000 sequence detection system and the Assays-on-Demand gene expression kit (Applied Biosystems). All samples were normalized to the signal generated from glyceraldehyde-3-phosphate dehydrogenase.

Electrophoretic Mobility Shift Assay—The binding reactions contained radiolabeled 32P-NF-κB oligonucleotide probe (Promega), cell extracts, 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 500 ng of poly(dI-dC) (GE Healthcare), and 10 μg of bovine serum albumin to a final volume of 15 μl. The reaction mixtures were incubated at 25 °C for 30 min, separated by 5% (w/v) polyacrylamide gel, and visualized by autoradiography.

ROS Measurement—Keratinocytes were stimulated with TNF and incubated with 10 μM CM-H2DCFDA (Invitrogen) for 30 min at 37 °C, harvested, and analyzed by flow cytometry.

Immunoblotting—Cells were washed once with ice-cold phosphate-buffered saline and whole cell extracts were prepared using a lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EGTA, 10 mM sodium fluoride, 2 mM dithiothreitol, 1 mM Na3VO4, 1 mM phenylmethylsulfonfluryl fluoride, 20 μM aprotinin, 0.5% Triton X-100). Cell extracts were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Analysis of Cytochrome c Release—Cytosolic fraction was obtained using cytochrome c release apoptosis assay kit (Calbiochem). Briefly, keratinocytes were collected by trypsinization and sonicated in cytosol extraction buffer. Homogenates were fractionated into cytosolic and mitochondrial fractions. Immunoblot was performed using monoclonal antibody against cytochrome c (BD Bioscience).

Retroviral Infection—Retroviral vectors for c-Jun (pMX-puro-c-Jun) and IκBΔN (pQCIXP-IκBΔN) was generated by inserting c-Jun cDNA into the retroviral vector pMX-puro (22) or IκBΔN into the retroviral vector pQCIXP (Clontech). IκBΔN cDNA was a gift from Dr. Ballard, Vanderbilt University (23). EcoPack293 cells (BD Biosciences) were transiently transfected with pMX-puro-c-Jun or pQCIXP-IκBΔN. After 48 h culture, growth medium containing retrovirus was collected and filtered with 0.45-μm cellulose acetate membrane to remove packaging cells. Keratinocytes were incubated with the collected virus-containing medium with 8 μg/ml Polybren for 24 h. Uninfected cells were removed by puromycin selection.
Histology and TUNEL Staining—Paraffin sections were stained with hematoxylin and eosin for histological analysis. dUTP nick-end labeling (TUNEL) assay was performed on frozen sections using an apoptotic cell death detection kit (Promega) according to the manufacturer’s instructions. Immunohistochemical analysis was performed on paraffin sections using polyclonal antibodies against K5, K6, Loricrin (Convance), and cleaved caspase-3 (Cell Signaling). Sections were counterstained with hematoxylin.

RESULTS

TNF Induces Pro-cell Death Events in Tak1Δ/Δ Keratinocytes—We first determined which cell signaling events were induced in Tak1Δ/Δ keratinocytes following TNF stimulation. Tak1 wild-type and Δ/Δ keratinocytes were isolated from Tak1flox/flox or K5-Cre Tak1flox/flox mice, respectively. In this floxed Tak1 system, Cre recombinate catalyzes the deletion of the Tak1 ATP binding site, amino acids 41–77, resulting in the generation of a truncated form of TAK1 (TAK1Δ) (Fig. 1A, bottom panel). As reported previously (17), TNF activated NF-κB in Tak1 wild-type keratinocytes, but this activation was largely abolished in Tak1Δ keratinocytes (Fig. 1A, top panel).

We next examined pro-cell death events, including activation of JNK, degradation of c-FLIP, and activation of caspase in Tak1 wild-type and Δ/Δ keratinocytes. TNF activated JNK in a transient manner, peaking at 10 min post-stimulation in Tak1 wild-type keratinocytes, whereas no transient JNK activation was detected in Tak1Δ keratinocytes (Fig. 1A, third panel). Thus, Tak1 is essential for TNF-induced transient JNK activation, consistent with earlier studies using TAK1-deficient MEFs (14, 18). However, JNK was greatly activated at 3–6 h after TNF stimulation in Tak1Δ but not in wild-type keratinocytes. It has been reported that ROS accumulation activates JNK in a prolonged manner following TNF stimulation (9, 10). Prolonged activation of JNK has been linked to c-FLIP degradation and subsequent caspase activation (10). We examined c-FLIP degradation and caspase-3 activation (Fig. 1A, fifth and sixth panels). As anticipated, c-FLIP was degraded and caspase-3 was activated in Tak1Δ but not in wild-type keratinocytes. We measured ROS production using CM-H$_2$DCFDA, a substrate that exhibits increased fluorescence when oxidized by intracellular ROS (Fig. 1B). The levels of ROS were greatly increased in Tak1Δ but not in wild-type keratinocytes following TNF stimulation. We also examined whether cell death is through the apoptotic or necrotic pathways. Apoptosis and necrosis were assessed by Annexin V binding and propidium iodide staining (Fig. 1C). The numbers of both apoptotic and necrotic cells were greatly increased in Tak1Δ keratinocytes at 2–6 h post-TNF stimulation. Cytochrome c was increased in the cytoplasm in TNF-stimulated Tak1Δ but not in wild-type keratinocytes (Fig. 1D), suggesting that TNF activates the mitochondrial apoptosis pathway in Tak1Δ keratinocytes. Collectively, these results demonstrate that TNF induces pro-cell death events including activation of JNK and caspase-3 in Tak1Δ keratinocytes, which occur concomitantly with accumulation of ROS, resulting in apoptotic and necrotic cell death.

Inhibition of ROS Completely Blocks TNF-induced Cell Death in Tak1Δ/Δ Keratinocytes—It is known that ROS activates JNK resulting in activation of caspases through c-FLIP degradation.
NF-κB. It is known that NF-κB regulates several enzymes and molecules that are involved in ROS metabolism such as manganese superoxide dismutase (MnSOD) and ferritin heavy chain (10, 24). To examine the role of NF-κB in TNF-induced cell death in keratinocytes, we generated wild-type keratinocytes stably expressing the repressor of NF-κB, IκBΔN (IκBΔN keratinocytes). We used Tak1 wild-type keratinocytes isolated from the littermate mice of those we isolated from our Tak1 Δ/Δ keratinocytes. Therefore, the background of Tak1 Δ/Δ and IκBΔN keratinocytes are similar. We confirmed that TNF-induced NF-κB activation was completely abolished in IκBΔN keratinocytes (Fig. 3A). We examined TNF-induced ROS accumulation, apoptosis, and cell death. Tak1 Δ/Δ keratinocytes exhibited a large increase of ROS at 5 h and ROS could not be measured due to cell death at 16 h after TNF stimulation (Fig. 3B). In contrast, ROS was not increased even at 16 h in IκBΔN keratinocytes (Fig. 3B). Annexin V-binding positive cells were also not increased in IκBΔN keratinocytes (Fig. 3C). These results indicate that Tak1 regulates ROS and cell death not through NF-κB. In MEFs, MnSOD is one of the major targets of NF-κB and regulates ROS (10).

Furthermore, the activation of JNK and caspase-3 in Tak1 Δ/Δ keratinocytes occurred concomitantly with ROS accumulation. Therefore, we assume that ROS is the cause of these pro-cell death events. We examined the effect of inhibiting ROS with antioxidants on TNF-induced pro-cell death events. Cells were treated with the antioxidant BHA and the activation of JNK and caspase-3 and c-FLIP degradation examined (Fig. 2A). BHA could abolish all these pro-cell death events. We also measured apoptosis and cell viability. BHA could completely inhibit an increase of apoptotic cells following TNF stimulation in Tak1 Δ/Δ keratinocytes (Fig. 2B). Furthermore, BHA and another antioxidant N-acetylcysteine could totally block TNF-induced cell death (Fig. 2C). These results suggest that ROS is the major mediator of TNF-induced cell death in Tak1 Δ/Δ keratinocytes.

NF-κB Deficiency Does Not Effectively Induce ROS or Apoptosis in TNF-stimulated Keratinocytes—Our results thus far demonstrated that Tak1 deficiency causes ROS accumulation upon TNF stimulation, which results in apoptotic and necrotic cell death. One likely mechanism by which Tak1 deficiency causes ROS accumulation is a lack of activation of NF-κB. It is known that NF-κB regulates several enzymes and molecules that are involved in ROS metabolism such as manganese superoxide dismutase (MnSOD) and ferritin heavy chain (10, 24). To examine the role of NF-κB in TNF-induced cell death in keratinocytes, we generated wild-type keratinocytes stably expressing the repressor of NF-κB, IκBΔN (IκBΔN keratinocytes). We used Tak1 wild-type keratinocytes isolated from the littermate mice of those we isolated from our Tak1 Δ/Δ keratinocytes. Therefore, the background of Tak1 Δ/Δ and IκBΔN keratinocytes are similar. We confirmed that TNF-induced NF-κB activation was completely abolished in IκBΔN keratinocytes (Fig. 3A). We examined TNF-induced ROS accumulation, apoptosis, and cell death. Tak1 Δ/Δ keratinocytes exhibited a large increase of ROS at 5 h and ROS could not be measured due to cell death at 16 h after TNF stimulation (Fig. 3B). In contrast, ROS was not increased even at 16 h in IκBΔN keratinocytes (Fig. 3B). Annexin V-binding positive cells were also not increased in IκBΔN keratinocytes (Fig. 3C). These results indicate that Tak1 regulates ROS and cell death not through NF-κB. In MEFs, MnSOD is one of the major targets of NF-κB and regulates ROS (10). To further examine the involvement of NF-κB in ROS regulation in keratinocytes, we observed the expression levels of MnSOD following TNF stimulation (Fig. 3D). A chemokine MIP2, which is a NF-κB target, was greatly increased by TNF in Tak1 wild-type keratinocytes, indicating that NF-κB was activated under this experimental condition. However, unlike MEFs, MnSOD was not increased by TNF in keratinocytes, indicating that NF-κB was not increased by TNF in keratinocytes. There-
nocytes with tBHP, a prototypical organic oxidant. Tak1Δ/Δ keratinocytes died by 0.3 mM tBHP treatment (Fig. 3E, left panel), whereas Tak1 wild-type and 1kBΔN keratinocytes were resistant to 0.5 mM tBHA and died with 1.0 mM tBHP treatment (Fig. 3F, right panel). These results demonstrate that TAK1 but not NF-κB affects sensitivity to ROS-induced cell death in keratinocytes, suggesting that TAK1 regulates ROS mainly through non-NF-κB mechanisms.

c-Jun Is Partially Involved in TAK1 Regulation of ROS—We next attempted to determine the mechanism by which TAK1 regulates ROS. TAK1 can activate two groups of transcription factors, namely NF-κB and AP1. As described above, we found that ROS is regulated through non-NF-κB mechanisms. Therefore, we examined the levels of AP1 family transcription factors including the Jun family and c-Fos. We also examined CREB. Among these, we found that the levels of
c-Jun were reduced in TAK1-deficient keratinocytes under the basal condition (Fig. 4, A and B). The level of c-Jun was not significantly altered by TNF stimulation both in wild-type and Tak1Δ/Δ keratinocytes. Thus, only the basal level of c-Jun is regulated by TAK1. TAK1 is activated by the number of stimuli including cytokines and stresses (13, 25, 26), which presumably are constitutively present at low levels even in unstimulated conditions. We speculate that TAK1 may be constitutively activated at a low level under the normal culture conditions, and this constitutive activity of TAK1 is important to maintain the basal level of c-Jun. We hypothesized that TAK1-dependent basal expression of c-Jun is involved in ROS regulation and cell survival. To examine this possibility, we generated Tak1Δ/Δ keratinocytes that ectopically express c-Jun (Fig. 4C), and examined accumulation of ROS, apoptosis, and cell death following TNF stimulation.

We found that the levels of ROS were reduced by c-Jun expression in TNF-treated Tak1Δ/Δ keratinocytes (Fig. 4D). Concomitantly, TNF-induced apoptosis was significantly reduced in c-Jun expressing keratinocytes (Fig. 4E). However, cell viability at 24 h after TNF stimulation was not greatly rescued by c-Jun expression (Fig. 4F). We note that higher levels of c-Jun expression did not increase the level of rescuing the TNF-induced cell death (data not shown). These results suggest that the TAK1-c-Jun pathway participates in ROS regulation but is not sufficient to rescue cell death at later time points.

Antioxidants Rescue the Skin Disorder in Mice Caused by Epidermal-specific Deletion of TAK1—Our results in cultured keratinocytes indicate that TAK1-dependent ROS regulation is important for cell survival. Finally, we asked whether ROS are the cause of keratinocyte death and subsequent skin disorder.
How Does TAK1 Regulate ROS?

—ROS are regulated by a number of enzymes including NF-κB target gene products such as MnSOD and ferritin heavy chain. However, we found that deletion of TAK1 did not affect their expression (Fig. 3D and data not shown). Moreover, we found that NF-κB-deficiency did not induce ROS following TNF stimulation (Fig. 3B). Thus, NF-κB does not regulate the level of ROS in keratinocytes, and the mechanisms mediated by other than NF-κB should be important for ROS regulation. We found that TAK1-dependent c-Jun expression may partially contribute to ROS regulation in keratinocytes. However, we could not totally block either TNF-induced ROS accumulation or cell death by ectopic expression of c-Jun.
**TAK1 Regulates ROS and Cell Death**

Therefore, TAK1 activates several mediators including c-Jun, and unidentified factors that may cooperatively regulate the levels of ROS in keratinocytes. We have recently found that intestinal epithelial specific Tak1 mutant mice die immediately after birth and they show massive epithelial cell death (19). In contrast, intestinal epithelial specific NF-κB-deficient (NEMO deletion) mice slowly develop intestinal inflammation by 6 weeks of age (20). Our current results raise the possibility that Tak1 deletion increases ROS, thereby inducing cell death and tissue damage in intestinal epithelial specific Tak1 deletion mice more severely compared with the epithelial specific NEMO deletion mice.

**TAK1, ROS, and Psoriasis**—ROS has been associated with psoriasis; however, it has not been determined whether ROS is the cause or the consequence of inflammation (28). We have shown here that Tak1 deletion causes dysregulation of ROS in keratinocytes, which is causally associated with skin inflammation (Ref. 17 and Fig. 5). TAK1 can be activated by cytokines and innate immune stimuli including interleukin-1, TNF, and microbial components and stress conditions (13, 25, 26). The levels of those TAK1 stimuli in the epithelial tissues may be fluctuated in vivo. For example, reduction of commensal bacteria decreases TAK1 activity, thereby increasing the accumulation of ROS. Such dysregulation in TAK1 activity may contribute to ROS-mediated epithelial inflammation. Our results warrant further studies on the relationship of the TAK1 signaling pathway, ROS metabolism, and psoriasis.

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