TAK1 Is a Master Regulator of Epidermal Homeostasis Involving Skin Inflammation and Apoptosis

Emily Omori, Kunihiro Matsumoto, Hideki Sanjo, Shintaro Sato, Shizuo Akira, Robert C. Smart, and Jun Ninomiya-Tsuji

Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, North Carolina 27695-7633, Department of Molecular Biology, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan, Solution Oriented Research for Science and Technology (SORST), Japan Science and Technology Agency, Japan, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan, and Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan

Transforming growth factor β-activated kinase 1 (TAK1) functions downstream of inflammatory cytokines to activate c-Jun N-terminal kinase (JNK) as well as NF-κB in several cell types. However, the functional role of TAK1 in an in vivo setting has not been determined. Here we have demonstrated that TAK1 is the major regulator of skin inflammation as well as keratinocyte death in vivo. Epidermal-specific deletion of TAK1 causes a severe inflammatory skin condition by postnatal day 6–8. The mutant skin also exhibits massive keratinocyte death. Analysis of keratinocytes isolated from the mutant skin revealed that TAK1 deficiency results in a striking increase in apoptosis in response to tumor necrosis factor (TNF). TAK1-deficient keratinocytes cannot activate NF-κB or JNK upon TNF treatment. These results suggest that TNF induces TAK1-deficient keratinocyte death because of the lack of NF-κB (and possibly JNK)-mediated cell survival signaling. Finally, we have shown that deletion of the TNF receptor can largely rescue keratinocyte death as well as inflammatory skin condition in epidermal-specific TAK1-deficient mice. Our results demonstrate that TAK1 is a master regulator of TNF signaling in skin and regulates skin inflammation and keratinocyte death.

TAK1 (transforming growth factor β-activated kinase 1) is a member of the mitogen-activated kinase kinase kinase family and is activated by inflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor (TNF) and Toll-like receptor ligands (1, 2). In IL-1, TNF, and Toll-like receptor ligand signaling pathways, TAK1 has been shown to be an essential signaling intermediate that functions upstream of IκB kinase (IKK)-NF-κB and c-Jun N-terminal kinase (JNK) in B cells and some culture cells (3–5). However, the role of TAK1 has not been established in an in vivo context due to embryonic death of TAK1 germ line knock out (3, 4).

Skin homeostasis is maintained through a well balanced interplay of cytokines and growth factors (6). Several cytokines, including TNF, activate JNK and NF-κB pathways (7, 8) that play critical roles in epidermal homeostasis involving skin inflammation and cancer development (9–16). Inactivation of IKKβ or IKKγ, which blocks the so-called canonical NF-κB pathway, produces a severe inflammatory skin condition. Furthermore, NF-κB hypofunction is implicated in epidermal squamous cell carcinoma. In contrast, activation of JNK pathway is involved in epidermal hyperplasia and subsequent cancer development (14, 15). Roles of IKK-NF-κB and JNK in skin have been demonstrated by using genetic and pharmacological inhibitory approaches (12, 14, 15). However, the upstream regulators of NF-κB and JNK pathways in skin have not yet been determined. In this study, we generated and characterized mice with epidermal-specific deletion of TAK1. We found that TAK1 is an essential intermediate in TNF signaling to activate both IKK and JNK in keratinocytes. TAK1 deficiency causes severe dysregulation of skin homeostasis. Our results suggest that the dysregulation in TAK1 mutant skin is mediated by TNF-induced keratinocyte death.

EXPERIMENTAL PROCEDURES

Mutant Mice—To generate K5CreTAK1FL/FL mice, mice carrying a floxed Map3k7 allele (TAK1FL/FL) (mixed background of C57BL/6 and 129/SvJ) (3) were crossed to transgenic mice expressing the Cre recombinase under the control of the keratinocyte-specific keratin 5 promoter (K5Cre) (mixed background of C57BL/6 and DBA/2J) (17). TNFR1-deficient mice C57BL/6 Tnfrsf1a<sup>−/−</sup>Mac (TNFR1<sup>−/−</sup>) (18) were obtained from The Jackson Laboratory.

Histology and Immunohistochemistry—Sections were stained with hematoxylin and eosin for histological analysis. Immunohistochemical analysis was performed on paraffin. Bound antibodies were visualized by diaminobenzidine, and sections were counterstained with hematoxylin. dUTP nick-end labeling (TUNEL) assay was performed on paraffin sections using an apoptotic cell death detection kit (Promega).
according to the manufacturer’s instructions. Polyclonal antibody against TAK1 described previously (2), polyclonal antibodies against K5, K6, K10, loricrin, filaggrin (Convance), 1k8-α, JNK1 (FL) (Santa Cruz), caspase 3 (Cell Signaling), and phospho-JNK (Thr-183/Tyr-185) (Cell Signaling) or monoclonal antibody against β-actin (Sigma) were used. Immunofluorescence was performed on frozen sections using polyclonal antibody against TAK1 along with monoclonal antibody against nidogen (Chemi-con). Bound antibodies were visualized by Cy2- or Cy3-conjugated secondary antibodies.

Cell Culture—Primary and spontaneously immortalized keratinocytes derived from the skin of P0–2 mice were cultured in Ca2+-free Eagle’s minimal essential medium (Bio-Whittaker) supplemented with 4% Chelex-treated bovine growth serum, 10 ng/ml of human epidermal growth factor (Invitrogen), 0.05 mM calcium chloride, and 1% penicillin-streptomycin at 33 °C in 8% CO2. Murine primary fibroblasts derived from the dermis of P0–2 mice were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine growth serum at 37 °C in 5% CO2.

Cytotoxicity Assays—Keratinocytes were treated with recombinant human TNF-α, mouse IL-1β, human transforming growth factor β1 (TGF-β1) (Roche Diagnostics), or lipopolysaccharide (LPS) (Sigma) for 24 h. The viable adherent cells were stained with 0.1% crystal violet. The stain was solubilized by adding 50% ethanol containing 0.1 M sodium citrate, and the absorbance of each plate was determined at 595 nm.

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

RESULTS

TAK1 is expressed in both the dermis and epidermis (Fig. 1a). To investigate the role of TAK1 in epidermis in vivo, we have generated and characterized mice carrying an epidermal-specific deletion of TAK1. The mice were generated by crossing TAK1 floxed mice (TAK1FL/FL) (3) with a mouse line expressing Cre under the control of the epidermal-specific keratin 5 (K5) promoter (K5Cre transgenic mice) (17). In this system, Cre deletes exon 2 (amino acids 41–77) of mouse TAK1, which contains an ATP binding site, and a truncated/kinase-dead version of TAK1 (TAK1Δ) is generated in K5-expressing cells, while wild-type TAK1 is expressed in other cell types. TAK1Δ was expressed in keratinocytes isolated from K5CreTAK1FL/FL mice, whereas wild-type TAK1 was detected in dermal fibroblasts from the same mice, indicating that TAK1 is specifically deleted in epidermis (Fig. 1b).

K5CreTAK1FL/FL mice were born at the expected Mendelian ratios and were grossly indistinguishable from control genotype littermates from birth until postnatal day 2–3 (P2–3) (Fig. 2a). However, by P7, the K5CreTAK1FL/FL mice showed a strong cutaneous phenotype characterized by hard, inflexible skin and widespread scaling (Fig. 2a). The K5CreTAK1FL/FL mice died between P7 and P8. The control TAK1FL/FL mice as well as mice with epidermal-specific heterozygous deletion of TAK1 (K5CreTAK1FL/+ ) did not show any pathological phenotype. Histological analysis of skin of mice from P0, P1, P3, and
Role of TAK1 in Skin Homeostasis

P7 revealed a progressive epidermal condition involving severe apoptosis, hyperkeratosis, inflammation, and eventually epidermal erosion in K5CreTAK1FL/FL mice (Fig. 2 and Table 1).

Mutant epidermis at P0 resembled normal P0 epidermis; however, by P1 numerous keratinocytes with dark pyknotic nuclei, eosinophilic cytoplasm, and absence of cellular contacts. Asterisks show mislocalized sebocytes.

FIGURE 2. Mice with epidermis-specific deletion of TAK1. a, control (CT) and K5CreTAK1FL/FL (MT) mice at P0, P3, and P7. b, dorsal skin sections from control (CT) and K5CreTAK1FL/FL (MT) mice at P0, P1, P3, and P7 were stained with hematoxylin/eosin. Second panels show representative skin areas, third panels show highly inflamed areas, and bottom panels show highly disorganized areas from K5CreTAK1FL/FL. Open brackets indicate epidermis. Scale bars, 20 μm. Arrows indicate keratinocytes with dark pyknotic nuclei, eosinophilic cytoplasm, and absence of cellular contacts. Asterisks show mislocalized sebocytes.
tacts were present in the mutant epidermis (Fig. 2b, arrow). This staining pattern and cellular morphology is indicative of apoptotic keratinocytes. On P3 there were many more apoptotic basal and suprabasal keratinocytes, and hyperkeratosis was prominent. The mutant epidermis appeared highly disorganized because of keratinocyte hypertrophy, keratinocyte depletion due to apoptosis, and the presence of immune cells in intraepidermal microabscesses as well as occasional sebocytes in the epidermis (Fig. 2b, bottom panel). On P7 the epidermis was hyperplastic, hypertrophic, and severely hyperkeratotic, and areas of complete epidermal erosion accompanied by severe inflammation were common. Immune cells infiltrated into the epidermis and were found in intraepidermal microabscesses as well as embedded in the thickened hyperkeratotic stratum corneum. Sebocytes were also present in the epidermis (Fig. 2b, right column). This inflammatory condition is somehow similar to skin with epidermal-specific deletion of IKKβ or IKKγ (11, 16).

However, this pronounced apoptotic phenotype has not been reported either in mutant skin with deletion of IKKβ or IKKγ (11, 16) or with inactivation of NF-κB (13, 14).

To further characterize the phenotypes of epidermal TAK1 deletion, we conducted histological and molecular analyses of mutant skin at several time points after birth. We examined the expression of epidermal differentiation markers, K5 (basal layer marker), K10 (suprabasal marker), and loricrin (granular layer marker) (Fig. 3a). These marker proteins were expressed with a normal distribution at P0 but became slightly abnormal at P1. At P3, even though the mutant mice showed only minor macroscopic differences from their control littermates (Fig. 2a), expression of the keratinocyte differentiation markers K10 and loricrin was markedly reduced, indicating that the keratinocyte differentiation program was altered. Instead of keratins indicative of normal epidermis, the hyperproliferative/wound-associated keratin 6 (K6) was highly induced in mutant skin at P3 (Fig. 3b, upper panel). This alteration of cytoskeleton composition resembles that in wound edge or skin with inflammatory disease, in which the normal set of keratins are replaced with hyperproliferative/wound-associated keratins. We also conducted a genome-wide gene expression study using microarrays and RNA isolated from K5CreTAK1FL/FL mutant and K5CreTAK1FL/− control epidermis (supplemental Table S1). The expression of many wound-associated keratins, S100 proteins, gap junction proteins, and cytokines was dramatically changed in the epidermal-specific TAK1 deletion mice. Thus, these mice display the hallmarks of wounded and inflamed skin. We quantified the expression of several cytokines and wound-associated proteins by real-time PCR. We found that IL-1, macrophage inflammatory protein-2 (MIP-2; IL-8 in human), S100A9, and wound-associated keratin of K16 were highly overexpressed in mutant skin at P3 (Fig. 3c). These results collectively suggest that by P3 the epidermal TAK1 deletion produces a phenotype in the epidermis that is similar to wounding.

Our histological analysis suggests an increased number of apoptotic keratinocytes in mutant epidermis (Fig. 2b). Therefore, we next examined apoptosis by TUNEL staining and immunohistochemical staining of activated caspase 3 (Fig. 3b, middle and bottom panels). Both demonstrated the presence of massive numbers of apoptotic keratinocytes in the mutant epidermis at P3 and P7. These results demonstrate that TAK1 is essential for skin homeostasis and is involved in both inflammation and keratinocyte survival.

To begin to identify the molecular events that initiate this phenotype, we analyzed keratinocytes isolated from K5CreTAK1FL/FL and TAK1FL/FL (control) mice. We anticipated that the mutant phenotype would be caused by a keratinocyte intrinsic defect. We found that TAK1-deficient keratinocytes could proliferate at a slightly slower rate than the control keratinocytes (Fig. 4a) and were able to terminally differentiate, as demonstrated by expression of involucrin and filaggrin (Fig. 4b). These results demonstrate that TAK1 is not essential for keratinocyte proliferation or differentiation, suggesting that the phenotype of K5CreTAK1FL/FL is not due to a defect in proliferation or differentiation of TAK1-deficient keratinocytes. Expression of IL-1, MIP-2, S100A9, or K16 was not elevated in TAK1-deficient keratinocytes (Fig. 4c). This suggests that the up-regulation of these gene products in mutant skin (Fig. 3c) occurs in other cell types or in TAK1-deficient keratinocytes activated by stimuli from other cell types. Therefore, IL-1, MIP-2, S100A9, or K16 is not directly up-regulated by TAK1 deficiency.

We next examined TNF and IL-1 signaling pathways in TAK1-deficient keratinocytes. In wild-type keratinocytes, TNF and IL-1 induced the activation of JNK and degradation of IkB. However, in TAK1-deficient keratinocytes, TNF- and IL-1-induced activation of JNK and degradation of IkB was largely impaired (Fig. 4d), which is similar to that observed in TAK1-deficient mouse embryonic fibroblasts (3, 4). Because we observed massive apoptosis in TAK1-deficient epidermis (Fig. 3b), we next examined apoptosis in mutant and control keratinocytes in culture. TAK1 plays an important role in TNF, IL-1, TGF-β, and Toll-like receptor signaling pathways, all of which have been reported to mediate cell death in some cell types (19–22). Therefore, we examined cell viability following treatment with TNF, IL-1, TGF-β, and lipopolysaccharide (Fig. 4e). We observed that only TNF could induce cell death in TAK1-deficient keratinocytes, whereas control keratinocytes were absolutely resistant. TNF treatment activated caspase 3 in TAK1-deficient but not in control keratinocytes (Fig. 4f). TNF was expressed in wild-type as well as mutant skin, and the levels of expression were similar in both skin samples (supplemental Fig. S1). These results led us to speculate that TAK1-deficient keratinocytes are killed by low levels of TNF expressed under normal skin conditions, which may underlie the epidermal phenotype caused by TAK1 deletion.

### Table 1

| Epidermis thickness and nucleated cell layers | CF | MT |
|---------------------------------------------|----|----|
| Epidermis thickness (µm)                    | 19 ± 5.6 | 50 ± 11.3 |
| Number of nucleated cell layers             | 1.75 ± 0.46 | 5.33 ± 1.50 |

Role of TAK1 in Skin Homeostasis
To verify the idea that TNF-induced cell death is the cause of the phenotype of TAK1-deficient epidermis, we generated double-mutant deletions of the TNF receptor I (TNFRI) and K5CreTAK1Fl/Fl (MT) mice were immunostained with the indicated keratinocyte differentiation markers, K5 (basal layer marker), K10 (suprabasal marker), and Loricrin (granular layer marker). b, immunostaining with anti-keratin 6 (top) and anti-cleaved caspase 3 (bottom) and TUNEL staining (middle) were performed on the same sections. Scale bars, 20 μm. c, RNA was isolated from TAK1Fl/Fl, K5CreTAK1Fl/Fl (control, open bars), and K5CreTAK1Fl/Fl (filled bars) skin at P1 or P3. Real-time PCR analysis was performed to quantify the indicated gene expression. Results were analyzed using the comparative Ct Method. Relative mRNA levels were calculated using control mRNAs as a base line. Data show the means ± S.E. (n, 6–12 for P1; n, 5–6 for P3).

FIGURE 3. Severe inflammation and apoptosis in epidermis-specific TAK1 deletion of TAK1 mice. a, dorsal skin sections from control (CT) and K5CreTAK1Fl/Fl (MT) mice were immunostained with the indicated keratinocyte differentiation markers, K5 (basal layer marker), K10 (suprabasal marker), and Loricrin (granular layer marker). b, immunostaining with anti-keratin 6 (top) and anti-cleaved caspase 3 (bottom) and TUNEL staining (middle) were performed on the same sections. Scale bars, 20 μm. c, RNA was isolated from TAK1Fl/Fl, K5CreTAK1Fl/Fl (control, open bars), and K5CreTAK1Fl/Fl (filled bars) skin at P1 or P3. Real-time PCR analysis was performed to quantify the indicated gene expression. Results were analyzed using the comparative Ct Method. Relative mRNA levels were calculated using control mRNAs as a base line. Data show the means ± S.E. (n, 6–12 for P1; n, 5–6 for P3).
for those plaques (Fig. 5, b and c). The inflamed small plaques in some double-mutant mice showed the same dysregulation as that in epidermal-specific TAK1 mutants at P5 (Fig. 5, b and c). Expression of IL-1, MIP-1, S100A9, and K16 was increased in these affected mice at P5 (Fig. 5, d). TNF family cytokines such as TNF-related apoptosis-inducing ligand (TRAIL) may be involved in this minor dysregulation. The inflamed plaques and expression of inflammation markers disappeared shortly, and the double-mutant mice were grossly normal by P25 (Fig. 5, a and d). From these results, we conclude that TNF causes cell death in TAK1-deficient keratinocytes, which in turn stimulates inflammatory responses. Because TNF always resides at some levels in the skin, cell death is induced constitutively in TAK1-deficient epidermis; this causes the prolonged activation of inflammatory responses, which results in a skin condition similar to chronic inflammatory diseases.

DISCUSSION

In this study, we found that mice carrying epidermal-specific deletion of TAK1 gene develop severe skin inflammation, and
we attempted to determine the cause of this phenotype. We have demonstrated that (i) TAK1-deficient keratinocytes are normal in proliferation and differentiation; (ii) TAK1 deficiency causes impaired activation of NF-κB and JNK in response to TNF; (iii) TNF induces apoptosis in TAK1-deficient keratinocytes, whereas wild-type keratinocytes are abso-
B. B. (2003) Nat. Rev. Immunol. 4, 499–511
2. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) Nat. Cell Biol. 2, 491–497
3. Sato, T., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. (2005) Nat. Immunol. 6, 1087–1095
4. Shim, J. H., Xiao, C., Paschal, A. E., Bailey, S. T., Rao, P., Hayden, M. S., Lee, K. Y., Bussey, C., Steckel, M., Tanaka, N., Yamada, G., Akira, S., Matsumoto, K., and Ghosh, S. (2005) Genes Dev. 19, 2668–2681
5. Takaesu, G., Surabhi, R. M., Park, K. J., Ninomiya-Tsuji, J., Matsumoto, K., and Gaynor, R. B. (2003) J. Biol. 326, 105–115
6. Fuchs, E., and Raghavan, S. (2002) Nat. Rev. Genet. 3, 199–209
7. Ghosh, S., and Karin, M. (2002) Cell 109, (suppl.) S81–S96
8. Varfolomeev, E. E., and Ashkenazi, A. (2004) Cell 116, 491–497
9. Makris, C., Godfrey, V. L., Krah-Senfelder, G., Takahashi, T., Roberts, J. L., Schwarz, T., Feng, L., Johnson, R. S., and Karin, M. (2000) Mol. Cell 5, 969–979
10. Schmidt-Supprian, M., Bloch, W., Courtois, G., Addicks, K., Israel, A., Rajewsky, K., and Pasparakis, M. (2000) Mol. Cell 5, 981–992
11. Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeiler, M., Gillitzer, R., Israel, A., Krieg, T., Rajewsky, K., and Haase, I. (2002) Nature 417, 861–866
12. Lind, M. H., Rozell, B., Wallin, R. P., van Hogerlinden, M., Ljunggren, H. G., Tofgfrd, R., and Sur, I. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4972–4977
13. van Hogerlinden, M., Rozell, B. L., Tofgfrd, R., and Sundberg, J. P. (2004) J. Invest. Dermatol. 123, 101–108
14. Zhang, J. Y., Tao, S., Kimmel, R., and Khavari, P. A. (2005) J. Cell Biol. 168, 561–566
15. Zhang, J. Y., Green, C. L., Tao, S., and Khavari, P. A. (2004) Genes Dev. 18, 17–22
16. Nenci, A., Huth, M., Funteh, A., Schmidt-Supprian, M., Bloch, W., Metzger, D., Chambon, P., Rajewsky, K., Krieg, T., Haase, I., and Pasparakis, M. (2006) Hum. Mol. Genet. 15, 531–542
17. Ramirez, A., Page, A., Gandarillas, A., Zanet, J., Pibire, S., Vidal, M., Tussell, L., Genesca, A., Whitaker, D. A., Melton, D. W., and Jorcano, J. L. (2004) Genesis 39, 52–57
18. Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, O., Ohashi, P. S., Kronke, M., and Mak, T. W. (1999) Cell 73, 457–467
19. Aggarwal, B. B. (2003) Nat. Rev. Immunol. 3, 745–756
20. Piek, E., Heldin, C. H., and Ten Dijke, P. (1999) FASEB J. 13, 2105–2124
21. Wright, K., Kolios, G., Westwick, J., and Ward, S. G. (1999) J. Biol. Chem. 274, 17193–17201
22. Liew, F. Y., Xu, D., Brint, E. K., and O’Neill, L. A. (2005) Nat. Rev. Immunol. 5, 446–458
23. Rudolph, D., Yeh, W. C., Wakeham, A., Rudolph, B., Nallainathan, D., Potter, J., Elia, A. J., and Mak, T. W. (2000) Genes Dev. 14, 854–862
24. Tanaka, M., Fuentes, M. E., Yamaguchi, K., Durnin, M. H., Dalrymple, S. A., Hardy, K. L., and Goeddel, D. V. (1999) Immunity 10, 421–429
25. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F., and Verma, I. M. (1999) Science 284, 321–325
26. Lamb, J. A., Ventura, J. J., Hess, P., Flavell, R. A., and Davis, R. J. (2003) Mol. Cell 11, 1479–1489
27. Arnott, C. H., Scott, K. A., Moore, R. J., Robinson, S. C., Thompson, R. G., and Balkwill, F. R. (2004) Oncogene 23, 1902–1910
28. Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M., Kollias, G., and Balkwill, F. (1999) Nat. Med. 5, 828–831

Acknowledgments—We thank S. Zhu, M. Inagaki, S. Kishida, H. Hanafusa, T. Yamamoto, Y. Mishina, Y. Komatsu, R. Tennant, C. Trempus, M. Mattmuller, and K. Linder for discussion and support.