Apurinic/Apyrimidinic Endonuclease 1 Is a Key Modulator of Keratinocyte Inflammatory Responses

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Apurinic/Apyrimidinic Endonuclease 1 Is a Key Modulator of Keratinocyte Inflammatory Responses


Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1) functions in both DNA repair and redox signaling, making it an attractive emerging therapeutic target. However, the role of APE1 in cutaneous inflammatory responses is largely unknown. In this study, we report that APE1 is a key upstream regulator in TLR2-dependent keratinocyte inflammatory responses. We found that nuclear expression of APE1 in epidermal layers was markedly up-regulated in psoriatic skin. APE1 was essential for the transcriptional activation and nuclear translocation of hypoxia-inducible factor-1α and NF-κB, both of which are crucial for inflammatory signaling in keratinocytes. Moreover, APE1 played a crucial role in the expression of TLR2-mediated inflammatory mediators, including TNF-α, CXCL8, and LL-37, in HaCaT cells and human primary keratinocytes. Silencing of APE1 attenuated cyclin D1/cyclin-dependent kinase 4 expression and phosphorylation of ERK1/2 and Akt, thereby affecting keratinocyte proliferation. Importantly, TLR2-induced generation of reactive oxygen species contributed to the nuclear translocation and expression of APE1, suggesting an autoregulatory circuit in which the subcellular localization of APE1 is associated with the production of ROS in keratinocytes. The Journal of Immunology, 2009, 183: 6839–6848.
TLR2-dependent cutaneous inflammatory diseases. In the present study, we examined the patterns of APE1 expression in psoriatic skin using immunohistochemical and RT-PCR analyses. Our results showed that APE1 was up-regulated in lesional psoriatic skin, suggesting a pathophysiological role for APE1 in psoriasis. Our results also show that APE1 is a prerequisite for TLR2-dependent transcriptional activation of NF-κB and HIF-1α and for the expression of TNF-α, CXCL8, and LL-37 in human keratinocytes. Furthermore, TLR2-dependent signaling by cyclin D1, ERK1/2, and Akt in keratinocytes also depends on APE1. TLR2-induced generation of ROS is essential for the nuclear translocation and expression of APE1. These findings strongly suggest that APE1 acts as a master regulator of cutaneous inflammatory signaling.

Materials and Methods

Cell culture

Human primary keratinocytes and HaCaT cells were prepared as previously described (20). Briefly, foreskin epidermal keratinocytes were cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Invitrogen). HaCaT cells (a gift from J. H. Lee, Chungnam National University College of Medicine, Daejeon, South Korea) were cultured in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen), 1% l-glutamine, penicillin (50 U/L), streptomycin (50 mg/ml), and gentamicin (50 μg/ml). This study protocol was approved by the Institutional Research Board of Chungnam National University College of Medicine (Daejeon, South Korea). Written informed consent was obtained from each participant.

Reagents, DNA, and Abs

Pam2CSk<sub>2</sub>, a synthetic dipalmitylated lipopeptide (SLP; S-[2,3-bis-(palmitoyloxy)-2R]-propyl-[3]-cysteinyl-[3]-seryl-[3]-lysyl-[3]-lysyl-[3]-lysyl-[3]-lysyl-[3]-lysyl (5 CF3COO H), and zymosan were purchased from InvivoGen. Polynosinic/polycytidylic acid (poly(I:C)) and cobalt chloride were purchased from Sigma-Aldrich. N-Acetylcyesteine (NAC), diphenylene iodonium (DPI), rotenone, and allopurinol were purchased from Calbiochem. DMSO (Sigma-Aldrich) was added to cultures at 0.1% (v/v) as a solvent control. Abs against phospho-Akt (Ser473) and phospho-VEGF and h36B4; 60°C for GAPDH, cyclophilin B, and porphobilinogen deaminase; and 66°C for TNF-α and β-tubulin. The primer sequences are given in supplemental Table I.4

Western blot and ELISA analyses

For Western blot analysis, primary Abs were used at a 1/1000 dilution. The membranes were developed using a chemiluminescence assay (ECL; Amersham/GE Healthcare) and subsequently exposed to chemiluminescence film (Amersham/GE Healthcare). ELISAs were used to detect human TNF-α and CXCL8 (BD Pharmingen) in culture supernatant fractions as described previously (20).

Luciferase reporter assay

NF-κB luciferase-reporter assays were performed as previously described (22). Briefly, HaCaT cells were transfected with p2xNF-κB-Luc for 24 h or cotransfected with p2xNF-κB-Luc and siAPE1 for 36 h using Lipofectamine 2000. At the indicated lengths of time after transfection, the cells were stimulated with SLP (100 ng/ml) or zymosan (10 μg/ml) for 24 h, washed three times in PBS, and lysed in luciferase lysis buffer (Promega). Luciferase reporter activity was then measured using a luciferase assay system (Promega) according to the manufacturer’s instructions.

Immunofluorescence staining

Expression of LL-37, NF-κB, and APE1 in treated or untreated HaCaT cells was detected using immunofluorescence staining as previously described (7, 20). The cells were fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, permeabilized with 0.01% Triton X-100 in PBS for 12 min, and then treated with 10% BSA for 1 h at 25°C. The cells were then incubated with a diluted (1/400) mouse Ab against human APE1 (Novus Biotechnologies), LL-37 (Santa Cruz Biotechnology), or NF-κB (Santa Cruz Biotechnology) overnight at 4°C. After washing to remove excess Abs, the cultures were incubated for 1 h at room temperature with a fluorescent-conjugated secondary Ab (anti-mouse IgG-Cy2 or anti-mouse IgG-FITC Ab). Excess Ab was removed, and cells were imaged with a confocal microscope (LSM510 META; Carl Zeiss). In some experiments, cells

4 The online version of this article contains supplemental material.
were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to visualize the nuclei and washed three times with PBS before imaging.

**Immunohistochemistry**

The subcellular localization of human APE1 in tissue sections was determined using immunohistochemical staining as previously described (23, 24). The samples were 3-μm-thick, formalin-fixed, paraffin-embedded biopsy sections obtained from 35 psoriasis patient samples and four control specimens. After incubation with primary Ab (anti-human APE1 Ab diluted 1/6400; Novus Biologicals), the samples were incubated with 3,3′-diaminobenzidine (DakoCytomation) to develop the color. The sections were lightly counterstained with Mayer’s hematoxylin and mounted using Immun-Mount (Thermo Shandon). Normal mouse IgG1 (Zymed Laboratories) was used as a negative control Ab. One pathologist examined all of the samples.

**Measurement of ROS**

For intracellular ROS measurement, 5 × 10⁵ cells were incubated for 15 min at 37°C with the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (5 μM) or dihydroethidium (2 μM), followed by FACS Calibur (BD Immunocytometry Systems) analysis, as previously described (7). At least 10,000 cells per sample were analyzed using CellQuest Pro acquisition and analysis software (BD Biosciences) and quantified by FlowJo cytometry analysis software, version 7.5 (Tree Star).

**Statistical analyses**

Data obtained from independent experiments are presented as means ± SD. Statistical analysis was performed using paired Student’s t tests with Bonferroni adjustment or with ANOVA for multiple comparisons. Differences were deemed to be statistically significant at p < 0.05.

**Results**

**APE1 expression is up-regulated in psoriatic epidermis**

To investigate the role of APE1 in inflammatory skin conditions, we first examined the expression of APE1 in epidermal layers of biopsy samples from psoriasis patients and subjects with normal skin. Immunohistochemical analysis revealed the infiltration of high numbers of APE1-expressing cells into the skin of psoriatic patients, whereas far fewer APE1-expressing cells were observed in skin specimens of healthy controls (Fig. 1A). APE1 was expressed in both nuclear and cytoplasmic locations in psoriatic samples but was barely detectable in nuclear or cytoplasmic locations in normal skin samples (Fig. 1A). As shown in Fig. 1B, semiquantitative RT-PCR analysis of mRNA extracted from the skin samples revealed a significantly higher APE1 mRNA level in the psoriatic skin samples than in the normal skin samples (p < 0.001). These results suggest that lesional psoriatic skin is in a primed state in which APE1 nuclear translocation is enhanced and may up-regulate the keratinocyte immune response in human psoriasis.

**APE1 plays a key role in TLR2-dependent transcriptional activation of HIF-1α in human keratinocytes**

TLR2 is expressed at high levels in skin epithelium and particularly in psoriatic skin, suggesting a pathological role for this innate receptor in cutaneous inflammation (18, 19). In a preliminary experiment using real-time RT-PCR and flow cytometric analysis, we found that TLR2 expression in HaCaT cells and human primary keratinocytes was up-regulated by treatment with SLP or zymosan (data not shown). The transcription factor HIF-1α is a major regulator of inflammatory and innate immune functions, not only in myeloid cells but also in skin keratinocytes (25). Our observation that APE1 expression was up-regulated in psoriatic skin led us to investigate whether APE1 is important for SLP- or zymosan-induced transcriptional activation of HIF-1α in human keratinocytes. The cells were treated with the synthetic lipopeptide SLP or with zymosan, a fungal TLR2/6 and dectin-1 agonist (26). The kinetic data showed that both SLP and zymosan led to a rapid, time-dependent, transient induction of HIF-1α gene transcription (Fig. 2, A and B, HaCaT and primary keratinocytes, respectively).

We then examined whether TLR2-dependent HIF-1α activation could be affected by silencing of APE1. To confirm that RNA interference (RNAi) was not cytotoxic to keratinocytes, we transfected HaCaT cells with siAPE1 and transduced primary keratinocytes with shAPE1. Trypan blue staining revealed no significant difference in the viability of RNAi-treated HaCaT cells or primary keratinocytes with shAPE1. Trypan blue staining revealed no significant difference in the viability of RNAi-treated HaCaT cells or primary keratinocytes with shAPE1. Trypan blue staining revealed no significant difference in the viability of RNAi-treated HaCaT cells or primary keratinocytes with shAPE1.

**FIGURE 1.** APE1 expression is up-regulated in psoriatic epidermis. A, Immunohistochemical analysis of APE1 expression in the epidermal cells of normal skin (left panels) and lesional psoriatic skin (right panels). Bars, 200 μm (upper panels) and 100 μm (lower panels). B, Semiquantitative RT-PCR analyses (lower panel) of APE1 mRNA expression in the epidermal layers of normal (n = 16) and psoriatic (n = 34) skin. ***p < 0.001, compared with normal skin samples.
When HaCaT cells were transiently transfected with siAPE1 and incubated with SLP or zymosan, HIF-1α transcription was markedly abrogated (Fig. 2C), suggesting that APE1 may be important in TLR2-mediated activation of HIF-1α. In addition, expression of the HIF-1α target gene for VEGF was markedly down-regulated by siAPE1 transfection, whereas it was not modulated by siNS transfection (Fig. 2C), confirming a role for APE1 in HIF-1α activation. Furthermore, human primary keratinocytes transduced with shAPE1 showed marked down-regulation of APE1 expression (Fig. 2D, upper panel) and a significant reduction in TLR2-dependent activation of HIF-1α transcription (Fig. 2D, lower panel) relative to cells transduced with shNS. These APE1-gene-silencing data demonstrate that TLR2-dependent transcriptional activation of HIF-1α was modulated by APE1 in HaCaT cells and primary keratinocytes.

**APE1 is required for TLR2-dependent NF-κB activation in HaCaT cells and primary keratinocytes**

A recent study demonstrated that a signaling pathway leading from HIF-1α to NF-κB activation enhanced epithelial production of chemokines and cytokines (27). To determine the role of APE1 in TLR2-mediated NF-κB activation in keratinocytes, we transfected HaCaT cells with an NF-κB luciferase reporter gene and then stimulated the cells with SLP or zymosan. As shown in Fig. 3A, NF-κB reporter gene activity in transfected HaCaT cells was enhanced in response to SLP or zymosan, and this response was dose dependent. The NF-κB reporter gene activity evoked by TLR2 ligands was significantly lower in siAPE1-transfected HaCaT cells than in siNS-transfected HaCaT cells (Fig. 3B).

We also examined the role of APE1 in TLR2-induced translocation of NF-κB into the keratinocyte nucleus. Immunofluorescent staining for NF-κB p65 in HaCaT cells revealed a pronounced cytoplasm-to-nucleus shift in the subcellular localization of p65 after SLP or zymosan treatment (Fig. 3C and data not shown). We confirmed the role of APE1 in NF-κB p65 nuclear translocation using siAPE1-transfected HaCaT cells (Fig. 3D). These results reflect the critical involvement of APE1 in the activation and nuclear translocation of NF-κB in human keratinocytes.

**APE1 is essential for the expression of inflammatory cytokines and chemokines in HaCaT cells and primary keratinocytes**

Proinflammatory cytokines and chemokines play important roles in inflammatory and immunological responses in human skin. Keratinocyte activation by TLR ligands results in massive release of proinflammatory and immunological responses in human skin. Keratinocyte activation by TLR ligands results in massive release of proinflammatory chemokines and cytokines (27). To determine the role of APE1 in TLR2-mediated NF-κB activation in keratinocytes, we transfected HaCaT cells with an NF-κB luciferase reporter construct (A) or cotransfected with an NF-κB reporter construct and either siNS or siAPE1 B. Cells were stimulated for 24 h with SLP (10, 100, or 1000 ng/ml for A; 100 ng/ml for B), zymosan (1, 10, or 100 μg/ml for A; 10 μg/ml for B), or poly(I:C) (P; 20 μg/ml), and assayed for luciferase reporter activity 36 h after transfection. Luciferase activities are shown as the means ± SD of three independent experiments. C and D, Immunofluorescence analysis of NF-κB localization. HaCaT cells were stimulated with SLP (100 ng/ml) for 24 h (C). Either siNS- or siAPE1-transfected HaCaT cells were stimulated with SLP (100 ng/ml) or zymosan (10 μg/ml) for the indicated lengths of time. The cells were fixed, stained with DAPI to visualize the nuclei (blue), and immunolabeled with anti-NF-κB p65 Ab and Cy2-conjugated goat anti-mouse IgG (green). Representative immunofluorescence images (upper panels) and the percentages of cells exhibiting NF-κB nuclear translocation (lower panels) are shown (D). Results shown are from at least three separate experiments. ***, p < 0.01, compared with control cultures. U, Untreated; S, SLP; Z or Zym, zymosan; P, poly(I:C) stimulation for 6 h.
and CXCL8 mRNA expression after as little as 3 h of incubation with TLR2 ligands; expression peaked at 6 h and remained elevated at 18 h (Fig. 4A).

We next examined whether APE1 silencing would modulate TLR2-dependent expression of TNF-α and CXCL8 by measuring SLP- or zymosan-induced expression of these mRNAs and proteins in siRNA- and shRNA-mediated knockdown experiments. Levels of SLP- or zymosan-induced expression of TNF-α and CXCL8 mRNA (Fig. 4B and 4C) were markedly lower in siAPE1-transfected HaCaT cells than in siNS-transfected cells. Additionally, levels of TNF-α and CXCL8 mRNA induction in response to SLP or zymosan stimulation were much lower in shAPE1-transduced primary keratinocytes than in shNS-transduced control keratinocytes (Fig. 4D). These data suggest that APE1 is required for TLR2-dependent expression of proinflammatory cytokines and chemokines in human keratinocytes.

**APE1 is required for cathelicidin LL-37 expression in human keratinocytes**

We next investigated the role of APE1 in the modulation of keratinocyte expression of cathelicidin LL-37, a human antimicrobial peptide important for the production of proinflammatory cytokines and a crucial component of the innate host defense system against cutaneous infection (29). RT-PCR analysis indicated that cathelicidin mRNA expression in human keratinocytes peaked after 6 h of incubation with TLR2 ligands and returned to the baseline level within 18 h (Fig. 5, A and C, for HaCaT cells and primary keratinocytes, using semiquantitative and real-time RT-PCR analyses, respectively). The role of APE1 in this TLR2-dependent cathelicidin expression was then examined using siAPE1- or siNS-transfected HaCaT cells stimulated with SLP or zymosan for 18 h. Images shown are representative of three independent experiments with similar results. The data shown are means ± SD of three experiments. **APE1 silencing significantly attenuated LL-37 mRNA and protein expression.**

**TLR2-dependent expression of cyclin D1 and phosphorylation of ERK1/2 and Akt are modulated by APE1 in human keratinocytes**

Coordinated control of keratinocyte differentiation and proliferation is vital for skin homeostasis and the prevention of skin diseases such as psoriasis (30). In cell proliferation the D-type cyclins (D1, D2, and D3), together with their specific partners (Cdk4 and Cdk6), control the G1/S phase transition via phosphorylation of the
FIGURE 6. APE1 is required for TLR2-dependent expression of cyclin D1 and phosphorylation of Cdk4 and Akt in keratinocytes. A–D, Western blot analysis of phosphorylated (p)-Akt, cyclin D1, Cdk4, p-ERK, and APE1 levels in HaCaT cells stimulated with SLP (100 ng/ml) or zymosan (10 µg/ml) for the indicated lengths of time (A and C) and in siNS- and siAPE1-transfected HaCaT cells stimulated, after 24 h, with SLP (100 ng/ml) or zymosan (Z, 10 µg/ml) (B and D). In B, cells were stimulated for 6 h for p-Akt analysis and for 12 h for cyclin D1/Cdk4, analysis. In D, cells were stimulated for 30 min for p-ERK analysis. After blotting, the membranes were stripped and reprobed for β-actin as a loading control. Data shown are representative of three independent experiments with similar results. E, HaCaT cells were stimulated with SLP (100 ng/ml) or zymosan to activate TLR2-dependent cell proliferation and ROS generation in human keratinocytes. As shown in Fig. 6A, stimulation with SLP or zymosan enhanced cyclin D1/Cdk4 expression in these cells in a time-dependent manner. Similarly, stimulation with SLP or zymosan also enhanced Akt phosphorylation in HaCaT cells. When we examined the effects of APE1 on TLR2-dependent cyclin D1/Cdk4 expression and Akt phosphorylation in HaCaT cells using RNAi experiments, we found that transfection of HaCaT cells with siAPE1 efficiently reduced the levels of cyclin D1/Cdk4 as well as Akt phosphorylation (Fig. 6B), suggesting a role for APE1 in cell proliferation and cell cycle progression.

Recently, epithelial cell cycle progression through G2/M phase was reported to require ERK1/2 activity (34). Additionally, the Ras-Raf-MEK-ERK signaling cascade has been well studied in terms of cell proliferation in a variety of human cancers (35). We therefore tested whether TLR2 stimulation would induce ERK1/2 activation in human keratinocytes and assessed the effects of APE1 on ERK1/2 phosphorylation. We detected ERK1/2 phosphorylation in SLP- or zymosan-treated HaCaT cells (Fig. 6C) and primary keratinocytes (data not shown) within 5 min of SLP or zymosan addition, and a peak effect was detected within 30 min (Fig. 6C). The phosphorylation of ERK1/2 was significantly abrogated by APE1 knockdown in siAPE1-transfected HaCaT cells (Fig. 6D).

We further investigated the effects of APE1 on TLR2-dependent proliferation of keratinocytes using the TLR2 ligands SLP and zymosan to activate TLR2-dependent cell proliferation and APE1 knockdown to inhibit TLR2-dependent cell proliferation (Fig. 6E). Cell proliferation was measured using a Dojindo cell counting kit-8 assay. As shown in Fig. 6E, SLP and zymosan both caused substantial increases in cell proliferation. APE1-knockdowned cells had a significantly reduced proliferation (by 43 and 38% after 96 h of stimulation with SLP and zymosan, respectively; Fig. 6E), when compared with control cells, without reducing cell viability (supplemental Fig. S1A). These data show that by regulating ERK1/2 and Akt activation and cyclin D1/Cdk4 expression, APE1 plays an essential role in human keratinocyte proliferation.

TLR2 stimulation causes ROS-dependent translocation of APE1 into the nucleus in human keratinocytes

Because our previous study showed that APE1 is translocated into the macrophage nucleus after HMGB1 stimulation (7), we examined whether TLR2 stimulation also causes nuclear translocation of APE1 in human keratinocytes. In agreement with published data (6, 7), APE1 began to translocate into the nucleus of HaCaT cells within 10 min of treatment with SLP or zymosan and was completely translocated to the nucleus within 1 h (Fig. 7A).

Previous studies have shown that APE1 is actively translocated from the cytoplasm to the nucleus upon exposure to ROS (36). Thus, we investigated whether TLR2 stimulation induces ROS generation in human keratinocytes. As shown in Fig. 7B, SLP or zymosan treatment profoundly up-regulated ROS levels in HaCaT cells, as determined by flow cytometric analysis. Because previous studies suggested that intracellular ROS production or Ca2+ mobilization is involved in APE1 translocation (37), we examined the mechanism of APE1 translocation in TLR2-stimulated cells using inhibitors of ROS generation. As shown in Fig. 7C, pretreatment with various antioxidants, such as the general ROS scavenger NAC, the NADPH oxidase inhibitor DPI, or the mitochondrial electron transfer chain subunit I inhibitor rotenone (but not the xanthine oxidase inhibitor allopurinol), dose-dependently decreased the TLR2-stimulated nuclear translocation of APE1 in HaCaT cells. Similar results were obtained in primary keratinocytes (data not shown), reinforcing the physiological importance of ROS generation in APE1 trafficking in keratinocytes.

APE1 expression is up-regulated in a ROS-dependent manner upon TLR2 stimulation in human keratinocytes

Because human APE1 is a multifunctional protein regulated at the levels of both transcription and translation (37), we more closely examined the modulation of APE1 mRNA and protein expression in response to TLR2 stimulation in human keratinocytes. Real-time RT-PCR analysis showed that the APE1 mRNA level in human primary keratinocytes peaked 6 h after cellular treatment with SLP or zymosan (Fig. 8A). The APE1 protein was constitutively expressed and actively induced in HaCaT cells within 3 h of SLP or zymosan treatment, reaching a peak at 24 h (Fig. 8B).

The hAPE1 gene is activated by sublethal levels of ROS and ROS generators, including ionizing radiation (38). Thus, we next...
investigated whether blockade of ROS generation would affect hAPE1 expression in keratinocytes. As shown in Fig. 8C, a 45-min pretreatment with NAC, DPI, or rotenone, but not allopurinol, dose-dependently attenuated SLP- or zymosan-induced expression of APE1 mRNA. These results suggest that NADPH oxidase-derived intracellular ROS play a major role in transcriptional regulation of TLR2-induced expression of APE1, as well as nuclear translocation of APE1, in human keratinocytes.

Discussion

APE1 plays a central role in the repair of oxidative DNA damage (1, 2) and is involved in transcription factor activation (1, 3, 4). Although these functions are essential for mammalian cell survival (39), host defense against infectious stimuli (5), and inflammatory responses (6, 7), the roles of APE1 in human keratinocytes and in cutaneous inflammatory responses or disease conditions have not been previously reported. In the present study, we showed that APE1 is up-regulated in psoriatic skin lesions from psoriasis patients. Using specific RNAi technology, we also found evidence that APE1 is a master regulator of cutaneous inflammatory responses, affecting transcription factor activation, secretion of inflammatory cytokines and chemokines, cathelicidin expression, and cell proliferative signaling in human keratinocytes.
Psoriasis is characterized by hyperproliferation of the epidermis. Although the pathogenesis of psoriasis is closely associated with altered regulation of T cell activation, it is probably induced by an intrinsic alteration in epidermal keratinocytes (40). In this study, we showed that epidermal APE1 expression is significantly higher in psoriatic lesions than in a normal epidermis. Our data indicate that APE1 not only is required for keratinocyte inflammatory responses but also plays a significant role in psoriasis as a key regulator of epidermal hyperplasia and inflammation, two distinctive features of this disease. Previous immunohistochemical studies have demonstrated that HIF-1α is also expressed at a much higher level in the epidermis of human psoriatic skin than in that of normal skin (41). We found that the proinflammatory cytokines TNF-α (42) and CXCL-8 (43) were over-expressed at the mRNA level in psoriatic lesions from psoriasis patients. Moreover, cathelicidin LL-37 mRNA expression was increased in human psoriatic skin but was not detected in the skin of healthy subjects (17). TLR2 protein expression was also strongly induced in the epidermis of lesional psoriatic skin (18). Thus, APE1 may be a crucial element in the pathogenesis of psoriasis, acting to up-regulate inflammatory mediators in the epidermis of psoriatic skin.

Skin plays an essential role in mediating systemic responses to environmental stimuli (oxygen) through epidermal HIF-1α (44), which is regulated by hypoxic stimuli and up-regulated by bacterial and viral compounds even under normoxic conditions (45). HIF-1α coordinates an appropriate and effective antimicrobial immune response and is a global regulator of myeloid cell-mediated inflammation (46). NF-κB has been linked to proinflammatory cytokine release (both HIF-1α dependent and independent) in macrophages (19, 47). Proinflammatory mediators activate HIF-1α in certain cell culture systems (48, 49), suggesting that the induction of HIF-1α expression is closely associated with inflammatory responses. Our results show that APE1 is essential for TLR2-dependent activation of HIF-1α and NF-κB gene promoter activity and for nuclear translocation of HIF-1α and NF-κB in human keratinocytes. APE1 protein stimulates the DNA binding activity of numerous transcription factors such as AP-1, p53, and HIF-1α and is thus important for the control of gene expression, contributing to cell survival and cancer progression (50). Additionally, APE1 functions as an important repressor factor, maintaining transcription factors in their active reduced states (50). Taken together, these data suggest an important role for APE1 in the activation of keratinocyte inflammatory signaling.

TLRs are important pattern recognition receptors that activate NF-κB pathways. Human keratinocytes constitutively express TLR2, TLR3, TLR5, and TLR10 (14), but TLR2 is strongly over-expressed in the epidermis of psoriatic skin, suggesting a role for TLR2 in the pathogenesis of psoriasis (18). Additionally, keratinocyte activation by TLR2 ligands leads to the nuclear translocation of NF-κB and the secretion of proinflammatory cytokines, including TNF-α and CXCL8 (18). Keratinocytes secrete specific cytokines and chemotactic factors that play crucial roles in mobilizing leukocytes from the blood and in initiating signaling in other cutaneous cells (43). The results of our present experiments show that APE1 modulates the production of inflammatory cytokines in response to TLR2 stimulation.

Our data also indicate that APE1 is required for LL-37 mRNA and protein expression in keratinocytes. In human PBMC, LL-37 is important for the production of proinflammatory cytokines and chemokines in synergy with the endogenous inflammatory mediator IL-1β, indicating a reinforcing role in the innate immune response (29). Recently, LL-37 was shown to stimulate the expression of cyclooxygenase-2 and prostaglandin E2 and to contribute to reduced keratinocyte apoptosis in pathological conditions such as psoriasis (51). Other recent studies have shown that LL-37 significantly enhances keratinocyte migration on collagen type I or fibronectin and induces the expression of the Slug and Snail transcription factors, which are closely associated with keratinocyte migratory activity during wound healing (52). Furthermore, LL-37 induces keratinocyte migration by transactivating the epidermal growth factor receptor (53). Our finding that APE1 is also required for LL-37 expression suggests that APE1 should be investigated for its possible role in wound healing.

Because the chronic skin inflammation of psoriasis is associated with unregulated cell growth, we hypothesize that TLR2 engagement increases expression of the cyclin D1/Cdk4 complex and phosphorylation of Akt and ERK1/2 in human keratinocytes. The present findings support this hypothesis in HaCaT cells and primary keratinocytes. The evidence suggests that cyclin D/Cdk4/6 complexes play a key role in the control of cell cycle progression and growth through the integration of multiple mitogenic and antimitogenic stimuli (54). In addition, the PI3K/Akt pathway is important for the induction of cyclin D expression, which activates cell proliferation (33). In keratinocytes, chronic activation of the epidermal growth factor receptor by UV irradiation has been shown to result in cyclin D expression and deregulation of cell cycle progression, thus contributing to keratinocyte proliferation and epidermal hyperplasia (55). Because cyclin D1 expression is regulated and markedly influenced by the activation of AP-1 and NF-κB (56, 57), we speculate that APE1 may affect cyclin D1 expression by regulating the transcriptional activity of AP-1 or NF-κB.

Our data also highlight the role of APE1 in the regulation of ERK1/2 phosphorylation in keratinocytes. Earlier reports demonstrated that sustained activation of ERK1/2 caused continued expression of cyclin D1 and G1 progression (58). Moreover, ERK1/2 depletion in epithelial cells inhibited cyclin B1 and c-Fos expression and induced G2/M arrest, suggesting that ERK1/2 is necessary for tissue self-renewal and cell division (34). We observed no profound effect of APE1 knockdown on cell viability or apoptosis (see supplemental Fig. S1). Together, our data strongly suggest that APE1 plays a critical role in keratinocyte proliferation and growth through modulation of cyclin D1/Cdk4 expression and Akt and ERK1/2 phosphorylation.

Our results indicate that TLR2 stimulation mediates the generation of ROS, which up-regulates APE1 transcription and nuclear translocation in human keratinocytes. Accumulating evidence suggests that the expression and subcellular localization of APE1 are finely tuned at both the transcriptional and posttranslational levels (37). Our data are consistent with previous findings indicating that oxidative agents and ROS generation promote transient induction of APE1 expression (36). Additionally, APE1 undergoes active cytoplasm-to-nucleus translocation upon ROS exposure (36) or purinergic stimulation by extracellular ATP (37). Furthermore, in LPS-stimulated macrophages, blockade of NADPH oxidases or transfection of p47phox siRNA significantly reduced nuclear translocation of APE1 (6). These observations and our data suggest that the shuttling of APE1 between the cytoplasm and nucleus affects the redox status of the subcellular compartments. The involvement of TLR2-dependent ROS generation in the nuclear translocation and transcriptional activation of APE1 suggests an autoregulatory positive feedback model for APE1 expression (Fig. 9). We propose that APE1 translocation and expression are interrelated, indicating a functional autoregulation of APE1 production per se in response to TLR2 stimulation. During this process, ROS generation may play an essential role as an initial mediator for the regulation of subcellular localization and transcription of APE1, which plays a
TLR2-dependent Akt activation promotes keratinocyte proliferation by en-
and that it acts by modulating the activities of NF-
HIF-1
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key role in keratinocyte inflammatory signaling through redox reg-
ulation of multiple transcriptional factors, including NF-kB and HIF-1α (see Fig. 9).
In conclusion, we have demonstrated that APE1 is a master regula-
tor of TLR2-dependent keratinocyte inflammatory responses and that it acts by modulating the activities of NF-kB and HIF-1α. Exacerbated expression and nuclear translocation of APE1 may contribute to the immunopathogenesis of psoriasis, presumably modulated through ROS-dependent signaling. Further studies are necessary to investigate whether the modulation of APE1 activity or trafficking will provide a therapeutic modality for psoriasis.

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Disclosures
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induced iNOS expression in murine macrophage cell line RAW 264.7. Immuno-
ium and endothelial progenitor cells: therapeutic implications in tumor angio-
laric/apyrimidinic endonuclease/HIF-1α through redox regulation, thus increasing the expression of keratinocyte inflamma-
tory mediators, including TNF-α, CXCL8, and LL-37. Additionally, TLR2-dependent Akt activation promotes keratinocyte proliferation by en-
hancing cyclin D1/Cdk4 expression, thereby contributing to cell cycle progression.

FIGURE 9. Schematic model for roles of APE1 protein and APE1-mediated regulatory pathways in keratinocytes. Engagement of receptors by TLR ligands initiates ROS generation, presumably through the activation of NADPH oxidase. TLR2-dependent ROS generation is involved in the nuclear translocation and transcriptional activation of APE1, suggesting the existence of an autoregulatory circuit for APE1 expression. TLR2-depend-
tent ROS generation is required for the regulation of subcellular localiza-
tion and the transcription of APE1. Once endogenous APE1 reaches the nucleus, it contributes to the activation of NF-κB and HIF-1α through redox regulation, thus increasing the expression of keratinocyte inflamma-
tory mediators, including TNF-α, CXCL8, and LL-37. Additionally, TLR2-dependent Akt activation promotes keratinocyte proliferation by en-
hancing cyclin D1/Cdk4 expression, thereby contributing to cell cycle progression.

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