Apoptosis Signal-regulating Kinase 1 (ASK1) Is an Intracellular Inducer of Keratinocyte Differentiation*

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Cells differentiate in response to various extracellular stimuli. This cellular response requires intracellular signaling pathways. The mitogen-activated protein (MAP) kinase cascade is a core signal transduction pathway that determines the fate of many kinds of cell. MAP kinase kinase kinase activates MAP kinase kinase, which in turn activates MAP kinase. Apoptosis signal-regulating kinase (ASK1) was identified as a MAP kinase kinase kinase involved in the stress-induced apoptosis-signaling cascade that activates the SEK1-JNK and MKK3/MKK6-p38 MAP kinase cascades. Expression of the constitutively active form of ASK1 (ASK1-ΔN) in keratinocytes induced significant morphological changes and differentiation markers, transglutaminase-1, loricrin, and involucrin. A transient increase in p21WAF1/Cip1-reduced DNA synthesis, and cell cycle analysis verified the differentiation. p38 MAP kinase inhibitors, SB202190 and SB203580, abolished the induction of differentiation markers, transglutaminase-1, loricrin, and involucrin. In turn, the induction of differentiation with ceramide in keratinocytes caused an increase in ASK1 expression and activity. Furthermore, normal human skin expresses ASK1 protein in the upper epidermis, implicating ASK1 in in vivo keratinocyte differentiation. We propose that the ASK1-p38 MAP kinase cascade is a new intracellular regulator of keratinocyte differentiation.

The mitogen-activated protein (MAP)

The epidermis is a self-renewing tissue maintained by the precise regulation of keratinocyte proliferation, differentiation, and cell death. Differentiation is one of the most important ways by which keratinocytes form a multilayered epidermis, and the MAP kinase cascade may be integrated into this process. The ASK1-p38 MAP kinase cascade is a candidate pathway as the regulator. To prove this, the constitutively active form ASK1 (N terminus-deleted mutant, ASK1-ΔN) was introduced into cultured normal human keratinocytes using adenovirus vector (Ad).

EXPERIMENTAL PROCEDURES

Keratinocyte Culture—Normal human keratinocytes were cultured with MCDB153 medium supplemented with insulin (5 μg/ml), hydrocortisone (5 × 10⁻⁷ M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (100 μg/ml), and Ca²⁺ (0.1 mM) as described previously (9).

Western Blotting—The analysis was performed as described previously (9) using a Vistra ECF kit (Amersham Pharmacia Biotech). Rabbit anti-human involucrin (Biomedical Technologies Inc., Stoughton, MA), monoclonal anti-p21WAF1/Cip1 (DB6, PharMingen Co., San Diego, CA), and rabbit anti-ASK1 (DAV, Ref. 10) were used as the first antibodies at a dilution of 1:1000. The intensity of each band was quantified using ImageQuant (Molecular Dynamics Inc.), referring to the control signal as one unit.

Northern Blotting—Total RNA was prepared using Isogen (Nippon Gene Co., Tokyo, Japan). Ten μg of total RNA were separated in a 1% formaldehyde/agarose gel, transferred to a nylon membrane, and probed with a²²P-labeled cDNA corresponding to transglutaminase-1 (11) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard.
Reverse Transcriptase-PCR Analysis—The epidermis was separated from normal human skin by incubation in phosphate-buffered saline at 60 °C for 1 min, immediately followed by immersion in ice-cold phosphate-buffered saline. Total RNA was prepared with Isogen and treated with 50 U of RNase-free DNase 1 (CLONTECH Laboratories, Inc., Palo Alto, CA), 37 °C for 30 min. Specific primers for transglutaminase-1 were synthesized using degenerate oligonucleotides previously published sequences (5). The reverse transcriptase-PCR was performed using RT-PCR High Plus (Toyobo Co., Ltd., Osaka, Japan). The sequences of the primer pair, product size, annealing temperature, and number of cycles were as follows: 5′-TCGACAGTGCTTGTAGGAA-3′ and 5′-ACAAGC- ATTGCAGTTG-3′, 759 base pairs, 25 cycles. The PCR products were sequenced to confirm the mRNA expression.

RNase Protection Assay—Analysis was performed using the multiprobe RNase protection assay system (PharMingen Co.) according to the manufacturer's instructions. Oligonucleotide probes were prepared by inserting the PCR-amplified human cDNA corresponding to oligonucleotides 2658–2874 of ASK1 (Genbank TM/EBI accession number D84476, 866–1133 of transglutaminase-1 (Genbank TM/EBI accession number D90287), 966–1176 of loricrin (Genbank TM/EBI accession number M13903) into the EcoRI and HindIII sites of pMPG vector. 5 μg of total RNA were hybridized with 32P-labeled riboprobe and digested with RNase. The hybridization products were separated on a 5% polyacrylamide/8M urea gel and exposed to film. GAPDH is shown as an internal standard. The intensity of each band was quantified using NIH Image, referring to the signal of the control as one unit.

Luciferase Assay—A reporter plasmid containing the involucrin promoter and firefly luciferase was constructed (pIVN-Luc) as follows. The involucrin promoter cassette was a generous gift from Dr. Taichman (12). The coding region of firefly luciferase was digested from pGL3 basic (Promega Co., Madison, WI) and subcloned into the involucrin promoter cassette. The correct insertion and orientation were confirmed by sequencing. To normalize the transfection efficiency, a plasmid containing Renilla luciferase driven by herpes simplex virus thymidine kinase promoter (pRL-TK Promega Co.) was included in the assay. The reporter plasmids were introduced into the keratinocytes using FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. In each transfection, 1 μg of pIVN-Luc and 0.5 μg of pRL-TK were introduced into 2 × 105 keratinocytes in 6-well plates. After 24 h, the cells were infected with the indicated Ad at an MOI of 5 and were incubated for an additional 24 h. Then the cells were harvested with 250 μl of lysis buffer (Promega Co.), and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega Co.), with a luminometer (Luminescencer JNR AB-2100; Atto Co., Osaka, Japan). Transfection was performed in triplicate. The relative luciferase activity was calculated by normalizing to the Renilla luciferase activity.

MAP Kinase Activity—MAP kinase activity was measured with JNK and p38 MAP kinase assay kits (BioLabs Inc., Beverly, MA). The lysate of a 106 keratinocytes was immunoprecipitated with a 1:100 dilution of antibody to JNK and p38 kinases, precipitated, the beads were washed with 20 mM Tris-HCl, pH 7.5, 12 mM EGTA, and 1.5% aprotinin. The lysates of 1 and 1 mM dithiothreitol and was subjected to kinase assay. GST-p38 and GST-JNK were expressed in E. coli and purified by using GST-pull-down method (Amersham Pharmacia Biotech). The resulting immunoprecipitate was then incubated with ATF-2 fusion protein at 30 °C for 30 min in the presence of 200 μM ATP. JNK was precipitated from the cell lysates with c-Jun fusion protein bound to glutathione-Sepharose beads and incubated with 100 μM ATP at 30 °C for 30 min. The phosphorylation of ATF-2 at Thr-63 and c-Jun at Ser-63 was detected by Western blotting using a 1:100 dilution of phosphospecific ATF-2 or c-Jun. To show that equal amounts of JNK and p38 MAP kinase were precipitated, the beads were incubated with SDS sample buffer at 97 °C for 3 min and then subjected to Western blot analysis using a 1:1000 dilution of antibody to JNK and p38 MAP kinase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The amounts of JNK and p38 kinase were precipitated, the beads were washed with 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol and was subjected to kinase assay. GST-MKK6 (0.2 μg) was first incubated with the immune complex for 10 min at 30 °C in a final volume of 10 μl in a solution containing 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, and 100 μM ATP. Thereafter, the activated complex was incubated with 0.3 μCi of [γ-32P]ATP and 1 μg of GST-p38 or in the same solution (final volume 20 μl) for 10 min at room temperature. Kinase reactions were stopped by adding SDS sample buffer and were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Phosphorylation of GST-p38 was analyzed using a Fuji BAS2000 image analyzer.

Cell Sorter Analysis—Involucrin-positive cells were analyzed with a cell sorter. For keratinocytes, ASK1 was recovered from the dishes with trypsin and fixed with 3.7% formaldehyde at room temperature for 8 min with methanol at −20 °C for 4 min and then with acetone at −20 °C for 2 min (13). After washing with Tris-buffered saline, pH 7.4, containing 0.2% Tween 20, the cells were reacted with a 1:100 dilution of rabbit anti-human involucrin antibody (Biomedical Technologies, Inc.) and a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. The labeled cells were analyzed with a flow cytometer (Becton Dickinson Co.).

The cell cycle distribution was analyzed using a CycleTEST PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems) according to the manufacturer's instructions. Nuclei isolated by trypsinization were stained with propidium iodide and then run on a flow cytometer (Becton Dickinson Co.).

ToT-mediated dUTP Nick End Labeling (TUNEL)—Apoptotic cells were stained on chamber slides with an in situ cell death detection kit (Roche Molecular Biochemicals GmbH) according to the manufacturer’s instructions. After treatment with 4% paraformaldehyde and 0.1% Triton X in sodium citrate, the cells were incubated with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase for 1 h at 37 °C. Fluorescent specimens were observed by fluorescence microscopy.

BrdUrd Incorporation—BrdUrd incorporation was assessed with a cell proliferation kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Cells were incubated with BrdUrd in thymidine-free culture medium for 60 min at 37 °C. BrdUrd incorporated into cellular DNA was stained with monoclonal anti-BrdUrd diluted 1:100, peroxidase anti-mouse IgG diluted 1:70, and 3,3′-diaminobenzidine. Two hundred cells were counted in randomly selected fields to quantify the positive cells. Each experiment was repeated three times.

Immunohistochemical Staining—Paraffin-embedded normal human skin sections were stained immunohistochemically with rabbit anti-ASK1 (DAV, Ref. 10; diluted 1:1000) and normal rabbit IgG, using a streptavidin-biotin-peroxidase staining kit (Nichirei Co., Inc., Tokyo, Japan) according to the manufacturer’s instructions.

RESULTS

Induction of Keratinocyte Differentiation with ASK1-ΔN—Ad carrying ASK1-ΔN (Ad-ASK1-ΔN) was constructed as described previously (14). In this study, Ad expressing a bacterial β-galactosidase gene (Ad-β-gal) and no exogenous gene (Ad-1W) were used as controls to exclude the effect of Ad itself. Gene expression was found in almost all of the keratinocytes with Ad (data not shown). We infected normal human keratinocytes with Ad-ASK1-ΔN or control Ad at an MOI of 5 or 50. As expected from our previous reports, infection of Ad-ASK1-ΔN but not Ad-β-gal at a higher MOI (50) strongly induced apoptosis as determined by morphology and TUNEL staining (Fig. 1). Surprisingly however, infection of Ad-ASK1-ΔN at a lower level (MOI of 5) induced dramatic morphological changes without any sign of apoptotic phenotypes. The cells became enlarged and flattened, showing a differentiated phenotype 48 h after infection with Ad-ASK1-ΔN. There were no morphological changes in keratinocytes infected with Ad-β-gal and Ad-1W at an MOI of 5 compared with no-vector (data not shown). Because, the morphology is apparently different from apoptotic cells, and the TUNEL staining was negative for apoptotic cells, these changes were considered to be that a morphological change induced by the infection of Ad-ASK1-ΔN at an MOI of 5 is not apoptosis. In the following experiments, keratinocytes were infected with Ad at an MOI of 5.

In addition to the morphological changes, ASK1-ΔN expression induced differentiation markers including involucrin protein and transglutaminase-1 mRNA (Fig. 2A) as seen with 10%
FCS, a potent inducer of keratinocyte differentiation (15). Transglutaminase-1 enzymatically cross-links its substrate proteins including involucrin and loricrin, forming a cornified envelope in terminally differentiated keratinocytes (16). Similar results were obtained with three keratinocyte strains (data not shown).

To further confirm that Ad-ASK1-ΔN infection at an MOI of 5 induces keratinocyte differentiation, the time course for protein and mRNA expression, the percentage of involucrin-positive cells, 5-bromo-2′-deoxyuridine (BrdUrd) incorporation and the cell cycle were analyzed (Figs. 2 and 3). ASK1-ΔN protein appeared within 3 h of infection with Ad-ASK1-ΔN, reaching a maximum level at 48 h, which lasted until 72 h (Fig. 2B). The expression of mRNA of differentiation markers including transglutaminase-1, loricrin, and involucrin significantly increased at 24 h (Fig. 2C). The increase of p21\(^{\text{Cip1/WAF1}}\), the cyclin-dependent kinase inhibitor, occurred 6 h after infection, which was earlier than the transglutaminase-1, loricrin, and involucrin induction (Fig. 2, B and C), and reached a maximum at 24 h. The transient increase of p21\(^{\text{Cip1/WAF1}}\) with differentiation is consistent with a previous report (17, 18); p21\(^{\text{Cip1/WAF1}}\) expression is up-regulated in the early stage of keratinocyte differentiation and then declines in the late stages of differentiation. ASK1-ΔN expression increased the percentage of involucrin-positive cells from 5.5 to 29.1% (Fig. 2D), indicating that ASK1-ΔN differentiates keratinocytes 48 h after Ad infection (Fig. 3B) indicates that ASK1-ΔN expression causes G0/G1 and G2/M arrest. Differentiated epidermal keratinocytes in vivo are in G0/G1 arrest (19). However, in cultured keratinocytes the induction of differentiation by suspension culture and FCS results in G0/G1 and G2/M arrest (15), which is consistent with our results. G0/G1 and G2/M arrest by differentiation stimuli suggests that in cultured keratinocytes differentiation signals are not restricted to the cell cycle stage.

To investigate the role of ASK1 in the regulation of involucrin expression, a luciferase assay was performed. Keratinocytes transfected with an involucrin promoter-luciferase reporter plasmid (pINV-Luc) were infected with Ad at an MOI of 5. The reporter activity increased 5.5-fold with Ad-ASK1-ΔN, whereas Ad-β-gal had no effect (Fig. 4), suggesting a positive role of ASK1 in the regulation of involucrin expression. All of these data (Figs. 2–4) verify that ASK1-ΔN differentiates keratinocytes.

Involvement of p38 MAP Kinase in ASK1-induced Keratinocyte Differentiation—ASK1 is a MAPKKK that activates SEK1-JNK and MKK3/MKK6-p38 MAP kinase cascades. Therefore, we examined whether the expression of ASK1-ΔN enhanced the JNK and p38 MAP kinase activities in keratinocytes. The activities of both JNK and p38 MAP kinase started to increase 6–12 h after Ad-ASK1-ΔN infection (Fig. 5A). This increase paralleled the level of ASK1-ΔN protein shown in Fig. 2A, suggesting that ASK1-ΔN activates the SEK1-JNK and MKK3/MKK6-p38 MAP kinase cascades in keratinocytes.

The involvement of p38 MAP kinase in ASK1-ΔN-induced differentiation was shown using p38 MAP kinase inhibitors: SB202190 and SB203580 (Fig. 5B). They significantly reduced the induction levels of transglutaminase-1, loricrin, and involucrin mRNA mediated by ASK1-ΔN but not the negative control SB202474. In SB202190-treated cells, the levels of transglutaminase-1, loricrin, and involucrin mRNA declined to 0.13, 0.02, and 0.38-fold compared with the controls, respectively. Because the inhibitors did not affect the level of ASK1-ΔN protein expression, the suppressive effect of p38 MAP kinase inhibitors on the induction of transglutaminase-1 and involucrin mRNA was caused by the blockade of p38 MAP kinase. Therefore, p38 MAP kinase is necessary for the downstream signal transduction of ASK1-induced differentiation.

Induction of ASK1 with Differentiation and ASK1 Localization in Vivo—We next studied whether the induction of differentiation caused ASK1 expression. Inducing differentiation with C2 ceramide, a potent differentiation inducer (22), enhanced ASK1 mRNA expression (Fig. 6A) and activity (Fig. 6B). This experiment was repeated more than three times with essentially identical results. In vivo, normal human epidermis expressed ASK1 mRNA (Fig. 7A). Moreover, an immunohistochemical study revealed that the expression of ASK1 in the upper epidermis paralleled keratinocyte differentiation (Fig. 7B), implicating ASK1 in in vivo differentiation.

**DISCUSSION**

The regulation of keratinocyte differentiation by extracellular stimuli has been studied primarily; studies include suspension culture, high Ca\(^{2+}\), FCS, 1α-25-dihydroxyvitamin D₃, 12-O-tetradecanoylphorbol-13-acetate (TPA), and ceramide (15, 22–27). However, the intracellular signaling mechanisms of differentiation are poorly understood. We propose that the ASK1-p38 MAP kinase cascade is a newly discovered signaling cascade for keratinocyte differentiation.

Protein kinase C (PKC) is an intracellular signal transduction molecule that regulates keratinocyte differentiation (20, 21), and epidermal keratinocytes express α, δ, ε, η, and ζ isoforms of PKC (20, 28–31). We examined whether PKC isoforms were involved in the ASK1 signaling cascade. Activation of PKC isoforms was determined by analyzing the subcellular distribution of PKC isoforms using Western blotting. The re-

**Fig. 1. The morphological change of keratinocytes with Ad-ASK1-ΔN.** After infection with Ad-ASK1-ΔN or Ad-β-gal at an MOI of 5 or 50, normal human keratinocytes were cultured for 48 h. The morphological change was observed under phase-contrast microscopy. Apoptotic cells were stained with TUNEL.
distribution of PKC from the soluble fraction to a particulate fraction is a useful indicator of PKC activation (32). However, the expression of ASK1-DN did not affect the subcellular distribution of PKCs (data not shown), indicating that PKCs were not activated by ASK1-DN. Therefore, PKCs are not localized in the downstream signaling of the ASK1-p38 MAP kinase cascade. A recent study showed that PKC regulates involucrin.

**Fig. 2. Induction of differentiation markers with Ad-ASK1-ΔN.** Keratinocytes were infected with Ad-ASK1-ΔN or Ad-β-gal at MOI of 5. Ad-β-gal is a control. **A**, induction of involucrin protein and transglutaminase-1 mRNA 48 h after Ad infection was analyzed by Western and Northern blotting, respectively. The treatment with 10% FCS is a positive control for differentiation. **B**, time course for the expression of ASK1-ΔN, involucrin, and p21<sub>Cip</sub>/WAF1 protein after Ad infection, analyzed by Western blotting. The BrdUrd-positive cells were stained and counted under a microscope. The dotted line indicates control IgG.

**Fig. 3. Suppression of cell growth with Ad-ASK1-ΔN.** A, incorporation of BrdUrd into keratinocytes after Ad-ASK1-ΔN infection. Keratinocytes were studied 0, 24, and 48 h after infection with Ad-ASK1-ΔN (triangle), Ad-β-gal (circle), Ad-1W (diamond), and no-vector (square). Values are the mean ± S.D. of triplicate determinations. Two hundred cells were counted in randomly selected fields to quantify the positive cells. **B**, cell cycle distribution of keratinocytes 48 h after infection with Ad-ASK1-ΔN and Ad-β-gal. The solid line indicates anti-involucrin. The dotted line indicates control IgG.

**Fig. 4. Activation of the involucrin promoter by ASK1-ΔN.** Keratinocytes cultured to 2 × 10<sup>5</sup> in 6-well plates were transfected with 1 μg of involucrin reporter plasmid (pINV-Luc) and 0.5 μg of pRL-TK (Renilla luciferase) as a standard, using FuGENE6. After 24 h, the cells were infected with Ad-ASK1-ΔN or Ad-β-gal at an MOI of 5 and cultured for an additional 24 h. Luciferase activity was measured using the Dual-Luciferase reporter assay system. Transfection was performed in triplicate. The relative luciferase activity was calculated by normalizing to the Renilla luciferase activity. Statistical analysis was performed using Student's t test. * statistically significant (p < 0.0001).
NF-κB is another candidate for a molecular regulator of keratinocyte differentiation (34–37). The epidermis of mice lacking the inhibitor of κB kinase α (IKKα) shows abnormal differentiation (35). In normal epidermis, NF-κB is localized in the cytoplasm of basal cells and then translocates to the nucleus in suprabasal cells, suggesting a possible role for commitment to differentiate (34). In contrast to NF-κB, ASK1 protein is found in the upper epidermis and has a different distribution from that of NF-κB. Furthermore, the expression of ASK1-ΔN does not activate NF-κB as analyzed with a gel shift mobility assay (data not shown), indicating that NF-κB is not involved in the ASK1-induced keratinocyte differentiation. One suggested role for NF-κB is that it prevents premature apoptosis before the final step by regulating the expression of the antiapoptotic molecules TRAF1, TRAF2, c-IAP1, and c-IAP2 (37).

On the other hand, ASK1 is an apoptosis inducer and appears in the late stages of differentiation in vivo. Therefore, NF-κB and ASK1 have distinct roles in the regulation of keratinocyte differentiation.

Although, ASK1 has been identified as an apoptosis inducer (5), we have shown that ASK1 induces keratinocyte differentiation. We also found that introducing ASK1 induced apoptosis. However, it required 10-fold higher (MOI of 50) levels of ASK1-ΔN expression than those required for differentiation (MOI of 5). These results suggest that ASK1 has dual physiological functions in keratinocytes; weak or strong activation of ASK1 may lead to differentiation or apoptosis of keratinocytes.
respectively. The biological activity of ASK1 depends on the cell type and conditions. In the pheochromocytoma cell line PC12, moderate expression of ASK1-ΔN induces neuronal differentiation and survival rather than apoptosis (38). Thus, ASK1 is not only an apoptosis inducer but also mediates a wide range of cellular functions.

In continuously self-renewing tissues, such as the gastrointestinal tract, epithelial cells are shed by terminal differentiation and apoptosis. Epidermal keratinocytes also differentiate and are ultimately shed from the epidermis after cell death. However, the morphology of cells dying in terminal differentiation is different from that of cells undergoing pathological apoptosis induced by ultraviolet light and Fas. This physiological cell death is suggested to be a specialized form of apoptosis (39). In ASK1-induced apoptosis, involucrin and transglutaminase-1 are strongly induced before apoptosis (MOI of 50, data not shown), whereas ultraviolet B irradiation does not enhance the expression of transglutaminase-1 mRNA (data not shown). Therefore, the two apoptosis mechanisms are different. Because ASK1 induces apoptosis with differentiation markers, and its expression is localized in the upper epidermis, ASK1 may be involved in the mechanism of apoptosis in terminal differentiation.

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