Supplementary information

ASK1 and ASK2 differentially regulate the counteracting roles of apoptosis and inflammation in tumorigenesis

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Supplementary materials and methods

Cell culture

HEK293, HaCaT, and RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 4.5 mg/ml glucose, and 100 units/ml penicillin G. Primary mouse keratinocytes were prepared from newborn mouse skin as described with some modifications (Sayama et al, 2006). Skin samples were incubated with 250 units/ml dispase (Godoshusei) in PBS overnight at 4 °C. After the epidermis was separated from the dermis, epidermal sheets were incubated in 0.25% trypsin solution for 10 min at 37 °C and teased with forceps. Keratinocytes were collected by centrifugation and cultured further in CnT-07 medium (CellnTec) containing 100 units/ml penicillin G. On day 3 of culture, cells were subjected to further analysis. For preparation of mouse skin fibroblasts (MSF), the dermis separated from the epidermis was incubated for 1
h in 0.75% collagenase solution. Cells were collected by centrifugation and cultured in DMEM containing 10% FBS, 4.5 mg/ml glucose, and 100 units/ml penicillin G. A crude population of bone marrow-derived macrophages (BMDMs) was generated in vitro from mouse bone marrow as described (Osaka et al, 2007). Bone marrow cells were cultured in RPMI 1640 medium containing 10% FBS, 100 units/ml penicillin G, 10 ng/ml of recombinant mouse granulocyte-monocyte colony stimulating factor (Strathmann Biotech) and 5 ng/ml of recombinant mouse interleukin-4 (Genzyme-Techne). The medium was replaced on day 2 and day 4, and non-adherent granulocytes and loosely adherent dendritic cells were rinsed away. On day 7 of culture, the firmly adherent cells were obtained and subjected to further analysis. To assess the stimulation-dependent activation of ASK2, we generated HEK293 cells stably expressing Flag-ASK2 and Myc-ASK1-kinase-negative, the latter of which supports basal activity of ASK2 (293-ASK2/1KN cells). Briefly, HEK293 cells stably expressing tetracycline-inducible Flag-ASK2 were first established using the T-Rex System (Invitrogen) according to the manufacturer's instructions. The established clones were further transfected with pcDNA3 (Invitrogen) carrying Myc-ASK1-kinase-negative and then selected with 500 µg/ml G418 (Geneticin, Invitrogen). For induction of Flag-ASK2 expression, cells were treated with 1 µg/ml tetracycline for 24 h.

*Antibodies and reagents*

Antibody to human ASK2 was established as described with some modifications (Yamagoe
et al, 1997). Briefly, 25 µl of a mixture containing recombinant GST-tagged C-terminal fragment of human ASK2 corresponding to amino acids 1075–1288 (2 mg/ml) and complete Freund’s adjuvant (1:1) was injected into the foot pad of BALB/c mice. GST-ASK2 emulsified in incomplete Freund’s adjuvant was further injected into the mouse (15 mg/mouse) at days 4 and 7. Three days after the final injection, lymph node cells were fused with myeloma P3U1 cells using polyethylene glycol 4000 (Merck), and the hybridoma cells were cultured in RPMI1640 medium (Sigma) supplemented with HAT supplement (Gibco). After cloning of the hybridoma cells, the monoclonal antibodies were purified by Protein G Sepharose column chromatography of the peritoneal fluid of mice. Antibodies to mouse ASK2 (Takeda et al, 2007), ASK1 (Saitoh et al, 1998), and phospho-ASK (Tobiume et al, 2002) were described previously. Antibodies to HA tag (3F10) and Flag tag (M2) were purchased from Roche Applied Science and Sigma, respectively. Phospho-specific antibodies to JNK (Thr^{183}/Tyr^{185}) and p38 (Thr^{180}/Tyr^{182}) were purchased from Cell Signaling. Antibodies to p38 (C-20-G) and JNK (JNK1-FL) were purchased from Santa Cruz. Actin (AC-40) mouse monoclonal antibody was purchased from Sigma. 7,12-Dimethylbenz(a)-anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), N-acetyl-L-cysteine (NAC), and Hoechst 33258 were purchased from Sigma. 2’,7’-Dichlorodihydrofluorescein diacetate (DCHF-DA) and propyl gallate (PG) were purchased from Wako and Calbiochem, respectively.
References


Iriyama_Supplementary data

Figure S1. Generation of ASK2 knockout mice by gene targeting. (A) Structures of mouse ASK2 gene, the targeting vector, and predicted mutated locus. Homologous regions for recombination are indicated by thick lines in the restriction map. Coding and non-coding regions of the ASK2 gene are represented by filled and hatched boxes, respectively. The genomic fragment used as a probe for Southern blotting is indicated below the “Wild-type allele”. NLS-LacZ, coding sequence for β-galactosidase containing nuclear localization signal (NLS) sequence followed by poly A signal; PGK-neo, coding sequence for neomycin phosphotransferase flanked by the phosphoglycerate kinase promoter and poly A signal; DT-A, diphtheria toxin A gene driven by MC1 promoter. (B) Genomic DNA from mouse tail with the indicated genotypes was digested with Hind III and analyzed by Southern blotting using the probe indicated in (A). (C) Immunoblotting of mouse embryonic fibroblasts (MEF) and primary keratinocytes from mice with the indicated genotypes.
Figure S2. Proliferation of basal keratinocytes in the skin treated with DMBA and/or TPA. (A) The dorsal skin of WT and ASK2<sup>−/−</sup> mice were first treated with acetone or DMBA (100 µg each), and seven days later it was treated twice with acetone or TPA (10 µg each) with a 24-h interval. Mice were sacrificed at 48 h after the last treatment. Eight µm sections of the fresh-frozen skin were immunostained with Ki67 antibody (TEC-3; DAKO) according to the manufacturer's instructions. Scale bar = 100 µm. (B) Quantification of Ki-67-positive cells. The number of Ki-67-positive cells in the interfollicular epidermis was counted over a linear distance of approximately 15 mm, and averaged for each 1-mm interval. Values of two independent mice per genotype and treatment are shown.
Figure S3. (A) Knockdown of ASK2 in HaCat cells. HaCaT cells seeded in 12-well plates were transfected with 20 pmol/well of the indicated small interference RNA (siRNA) using 2 µl/well of Lipofectamine RNAiMAX (Invitrogen). After 48 h, cells were treated with 100 µM DMBA or DMSO for the indicated periods. Cell lysates were then subjected to immunoblotting. siRNA for human ASK2 and control was purchased from Invitrogen (Stealth Select RNAi, HSS 113398 and Stealth RNAi Negative CTL, respectively). (B) Reduced expression of ASK2 does not affect DMBA-induced ROS production. HaCaT cells transfected with siRNA for human ASK2 or control were pretreated with 2 µM DCHF-DA and then stimulated with DMSO or 100 µM DMBA for 30min. ROS production was measured by FACS analysis and the percentage of ROS-producing cells was calculated. Values are the mean ± SEM (n=3).
Figure S4. Requirement of ASK1 and ASK2 for ROS-induced activation of JNK and p38 in primary keratinocytes.

Immunoblotting of primary keratinocytes treated with 0.3 mM H$_2$O$_2$ for the indicated periods.
Figure S5. (A) UVA induces intracellular ROS production. HaCaT cells were pretreated with 2 µM DCHF-DA and then treated with 80 kJ/m² UVA or 250 J/m² UVC for 30 min. Cells treated with 5 mM H₂O₂ for 15 min were used as a positive control. Fluorescence intensity was measured by flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 580 nm. Fluorescent cells were detected by FACScalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). Shown is representative results of three independent experiments are shown. (B) ASK2 is activated by UVA but to a lesser extent by UVB and UVC. 293-ASK2/1KN cells were treated with the indicated doses of UVA (1 h), UVC (1 h), and UVB (0.5 h) (upper panels) and with 80 kJ/m² UVA, 2 kJ/m² UVB, and 250 J/m² UVC for the indicated periods (lower panels). Cell lysates were subjected to immunoblotting.
Figure S6. Proliferation of basal keratinocytes in TPA-treated mouse skin. The dorsal skin of WT, \( ASK2^{-/-} \), \( ASK1^{-/-} \), and \( ASK1^{-/-}; ASK2^{-/-} \) mice was treated twice with acetone (TPA (–)) or TPA (10 µg each) with a 24-h interval. Mice were sacrificed at 48 h after the latter treatment. Eight µm sections of the fresh-frozen skin were immunostained with Ki67 antibody (TEC-3; DAKO) according to the manufacturer’s instructions. Scale bar = 100 µm.
Figure S7. Expression of TNF-α and IL-6 is attenuated in papillomas of ASK1–/– mice. RNA was extracted from three to five papillomas, depending on their size, each from WT and ASK1–/– mice. mRNA expression of TNF–α and IL-6 was quantified using real-time RT-PCR. Expression of each gene was normalized to GAPDH. Data are mean ± SEM of 4 mice. *, P < 0.05, **, P < 0.01, compared with WT mice.