Appendix S1

MATERIALS AND METHODS

Subjects and UV irradiation

Each of 8 volunteers (5 men, 3 women; age range 33–57 years) was irradiated with 3 MEDs UVA and NB-UVB on the abdominal skin, after obtaining informed written consent. Two days after the irradiation, the sites were biopsied, as was a non-irradiated site as a negative control for microarray, real-time PCR, and immunofluorescence analyses. The Institutional Review Board of Dongguk University Ilsan Hospital approved this study, which was conducted according to the principles of the Declaration of Helsinki.

Keratinocyte culture and UV irradiation

Adult skin specimens were obtained from previous Caesarean-section scars and used to establish cell cultures. Epidermis was separated from dermis after treatment with 2.4 U/ml of dispase (Roche, Mannheim, Germany) for 1 h. The epidermal sheets were treated with 0.05% trypsin for 10 min to produce a suspension of individual epidermal cells. The cells were suspended in EpiLife Medium containing 0.06 mM calcium (Invitrogen, Carlsbad, CA, USA) supplemented with bovine pituitary extract 0.2% v/v, bovine insulin 5 μg/ml, hydrocortisone 0.18 μg/ml, human epidermal growth factor 0.2 ng/ml, and bovine transferrin 5 μg/ml (HKGS; Invitrogen). Primary cultured normal human keratinocytes in each 6-well plate were irradiated with corresponding single doses of UVA and NB-UVB. During the irradiation, EpiLife medium was replaced with 2 ml phosphate-buffered saline. The cells were harvested 48 h after the irradiation, and underwent cell viability assay, cytotoxicity test, fluorescence-activated cell sorter (FACS) analysis, real-time PCR, Western blot analysis, immunoprecipitation, and confocal microscopy.

UV light source

The NB-UVB light source was a COSMOLUX N-UVB 1000 U apparatus (Choyang Medical, Seongnam, South Korea) equipped with the UVB Narrowband WL 20W lamps (Royal Philips, Amsterdam, Netherlands; 305–315 nm, peak 311 nm). The UVA light source for volunteers and cultured cells was a COSMOLUX N-UVA 1000 U device (Choyang Medical) and a DBL UVA chamber (Daehan Biolink, Chungbuk, Korea), respectively, equipped with the same TL-K 40W lamps (Royal Philips; 315–380 nm, peak 350 nm).

Pre-elafin overexpression or knockdown

For pre-elafin overexpression, keratinocytes were transfected with a pCMV plasmid containing the skin-derived peptidase inhibitor 3 (*PI3)* gene (OriGene Technologies, Rockville, MD, USA), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After overexpression, the keratinocytes were cultured for 2 days under low or high calcium concentrations (0.06 and 1mM, respectively). For pre-elafin knockdown, cells were transfected with 500 nM small interfering (si) RNA for human pre-elafin or a negative control (On-TARGETplus SMARTpool or Non-targeting siRNA; Thermo Scientific, Rockford, IL, USA) using the TransIT-siQUEST transfection reagent (Mirus Bio, Madison, WI, USA) according to the protocol.

Cell viability test

Cell viability was evaluated by the MTT reduction method. A range of irradiation doses showing approximately 70–80%, 50% and 20–30% of cell viabilities was determined in cultured keratinocytes from 3–4 different donors.

Cytotoxicity test

Cytotoxicity was evaluated with the LDH release method. LDH activity in the culture medium was measured using cytotoxicity detection kit (Roche, Penzberg, Germany) according to the manufacturer's protocol. LDH release to the supernatant of the experimental cultures was determined by measuring optical density at 490 nm subtracted by the optical density at 620 nm. The effect of UVA and NB-UVB irradiation on cytotoxicity was calculated as the percentage of LDH release with either type of UV irradiation compared with LDH release without any irradiation.

FACS analysis

Cells were harvested at 48 h after pre-elafin overexpression and washed in cold phosphate-buffered saline. Cells were treated with 5 μl annexin V-FITC staining solution for 10 min, followed by incubation with 1 μl propidium iodide (PI, 100 μg/ml) for another 5 min in the dark. FACS analysis was performed with Cytomics™ FC500 Flow Cytometry (Beckman Coulter, Indianapolis, IN, USA).

Real-time PCR

The amount of target mRNA in synthesized cDNA was quantified by real-time PCR using Light Cycler real-time PCR machine (Roche, Penzberg, Germany). The relative amount of mRNA was calculated as the ratio of target relative to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used were pre-elafin 5'-TGATCGTGGTGGTGTTCCT-3' (Forward) and 5'-ACGGCCTTTGACAGTGTCTT-3' (Reverse); GAPDH 5'-TCCACTGGCGTCTTCACC-3' (Forward) and 5'-GGCAGAGATGATGACCCTTT-3' (Reverse).

Western blot analysis

Equal amounts of extracted proteins on nitrocellulose membranes were incubated with rabbit polyclonal antibodies to pro and cleaved forms of caspase-9, cleaved caspase-3, nuclear factor kappa B (NF-κB) p65, phosphatidyl inositol-4,5-bisphosphate 3-kinase (PI3K), AKT, extracellular signal-regulated kinase (ERK), phospho-NF-κB p65, phospho-PI3K, phospho-AKT, phospho-ERK (Cell Signaling Technology, Danvers, MA, USA) and pre-elafin (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubating with appropriate anti-rabbit horseradish peroxidase-conjugated antibodies (Thermo Scientific), an enhanced chemiluminescence solution (Thermo Scientific) was applied and the signals were captured on a LAS-3000 Image Reader (Fuji Photo Film, Tokyo, Japan). To monitor the amount of protein loaded in each lane, the membranes were re-probed with a mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, Saint Louis, MO, USA) and were processed as described above. The protein bands were then analysed by densitometry.

Histological analysis

Human skin biopsy specimens were embedded in paraffin. Five-μm sections were stained with haematoxylin and eosin (H&E). The number of cells with pyknotic nuclei and eosinophilic cytoplasm was counted in 6 microscopic fields (×400) using light microscopy (DM LB Microscope; Leica Microsystems, Wetzla, Germany) equipped with a camera (DC300F; Leica Microsystems, Heerbrugg, Switzerland).

Immunofluorescence

Five-μm sections were prepared from human skin biopsy specimens embedded in paraffin. After deparaffinization and blocking,

the sections were incubated with an anti-pre-elafin antibody (rabbit polyclonal; Santa Cruz) and stained with an Alexa Fluor®488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). The stained specimens were observed using a Dp Manager 2.1 fluorescence microscope (Olympus Optical, Tokyo, Japan).

Immunoprecipitation

Supernatants of cell lysates were incubated with anti-cleaved caspase-3, pro and cleaved forms of caspase-3 (rabbit polyclonal; Cell Signaling Technology), or pre-elafin antibody and resin in Pierce™ Direct IP kit (Thermo Scientific, Waltham, MA, USA) at 4°C. The eluted resin-bound proteins were analysed by immunoblotting with anti-pre-elafin and anti-cystatin-A antibodies (mouse monoclonal; Santa Cruz).

Confocal microscopy

Cells fixed in paraformaldehyde were double-stained using pre-elafin and cleaved caspase-3, pre-elafin and TG1 (goat polyclonal; Santa Cruz), or pre-elafin and TG2 (mouse monoclonal; Santa Cruz). antibodies, and then stained with Alexa Fluor® 488 donkey anti-Goat IgG, Alexa Fluor® 594 goat anti-Rabbit IgG, or Alexa Fluor® 594 goat anti-Mouse IgG (Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with Hoechst 33258 (Sigma, St Louis, MO, USA). The stained specimens were imaged using a C1 confocal laser scanning microscope (C1; Nikon, Tokyo, Japan). To analyse the localization of each antigen in double-stained cells, different images obtained from the same area were merged using EZ-C1 software (EZ-C1; Nikon, Tokyo, Japan) and measured using NIS-Elements AR 3.2 software (Nikon Instruments, Melvillie, NY, USA). Overlap coefficient (R) to show co-localization efficiency was calculated using Wright Cell Imaging Facility (WCIF) ImageJ software (http://www.uhnresearch.ca/facilities/wcif/imagej).

Statistical analysis

Statistical analysis was performed using Student's *t*-test. The results are expressed as mean ± SD. A p-value < 0.05 was considered significant.