Prevention of UVB Radiation-induced Epidermal Damage by Expression of Heat Shock Protein 70*§

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Irradiation with UV light, especially UVB, causes epidermal damage via the induction of apoptosis, inflammatory responses, and DNA damage. Various stressors, including UV light, induce heat shock proteins (HSPs) and the induction, particularly that of HSP70, provides cellular resistance to such stressors. The anti-inflammatory activity of HSP70, such as its inhibition of nuclear factor kappa B (NF-κB), was recently revealed. These in vitro results suggest that HSP70 protects against UVB-induced epidermal damage. Here we tested this idea by using transgenic mice expressing HSP70 and cultured keratinocytes. Irradiation of wild-type mice with UVB caused epidermal damage such as induction of apoptosis, which was suppressed in transgenic mice expressing HSP70. UVB-induced apoptosis in cultured keratinocytes was suppressed by overexpression of HSP70. Irradiation of wild-type mice with UVB decreased the cutaneous level of IkB-α (an inhibitor of NF-κB) and increased the infiltration of leukocytes and levels of pro-inflammatory cytokines and chemokines in the epidermis. These inflammatory responses were suppressed in transgenic mice expressing HSP70. In vitro, the overexpression of HSP70 suppressed the expression of pro-inflammatory cytokines and chemokines and increased the level of IkB-α in keratinocytes irradiated with UVB. UVB induced an increase in cutaneous levels of cyclobutane pyrimidine dimers and 8-hydroxy-2'-deoxyguanosine, both of which were suppressed in transgenic mice expressing HSP70. This study provides genetic evidence that HSP70 protects the epidermis from UVB-induced radiation damage. The findings here also suggest that the protective action of HSP70 is mediated by anti-apoptotic, anti-inflammatory, and anti-DNA damage effects.

The skin can be structurally classified into several layers, including the most apical layer, the epidermis, containing large numbers of keratinocytes, and a second layer, immediately under this, the dermis, which has a high fibroblast content (1). Skin provides a major interface between the environment and the body and is constantly exposed to an array of physical and chemical stressors. Therefore, in addition to intrinsic causes, harmful exogenous causes are involved in the process of skin damage. Among exogenous harmful agents, UV irradiation is the most relevant to skin damage (photo-damage). UV light can be separated, based on wavelength, into three categories: UVA (320–400 nm), UVB (290–320 nm), and UVC (100–290 nm). Of these, the cell-damaging effect of UVA is relatively weak, whereas most UVC is absorbed by the ozone layer (2). Thus, UVB seems to play the central role in photo-damage, such as clinical sunburn, hyperpigmentation, erythema, plaque-like thickening, loss of skin tone, deep furrowing, and fine wrinkle formation, all of which constitute both clinical and cosmetic problems. Furthermore, UVB irradiation induces the development of skin cancer (photo-carcinogenesis) (3). UVB-induced photodamage and photo-carcinogenesis both involve epidermal damage (such as induction of apoptosis), immunosuppression, inflammation (activation of pro-inflammatory cytokines and chemokines), and DNA damage (4). Because most UVB radiation is absorbed at the epidermis, keratinocytes become a major target of its deleterious effects. For example, the UVB-induced disruption of collagen and elastin (deep furrowing and fine wrinkle formation in the skin) involves inhibition of their synthesis in fibroblasts and stimulation of their degradation by matrix metalloproteinases and other proteases, both of which are triggered by pro-inflammatory cytokines and chemokines released from UVB-irradiated keratinocytes (4, 5). Therefore, suppression of UVB-induced damage (apoptosis) of keratinocytes is beneficial for the prevention of photo-damage. However, because such protection may actually aid in the survival of DNA-damaged cells, resulting in promotion of photo-carcinogenesis, a mechanism that not only suppresses UVB-induced apoptosis but also UVB-induced DNA damage is important to establish protocols to prevent photo-damage without promoting photo-carcinogenesis.

UVB irradiation damages the epidermis both directly and indirectly. For example, in addition to UVB-induced direct damage of nucleic acids, proteins, and lipids, UVB irradiation stimulates the production of reactive oxygen species.
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When cells are exposed to stressors, a number of so-called stress proteins are induced to confer protection against such stressors. Heat shock proteins (HSPs) are representative of these stress proteins, and their cellular up-regulation of expression, especially that of HSP70, provides resistance given that HSPs re-fold or degrade denatured proteins produced by stressors such as ROS (8, 9). Because stressor-induced tissue damage is involved in various diseases, HSPs and HSP inducers have received much attention for their therapeutic potential. It is known that various HSPs are constitutively expressed in keratinocytes and their expression, especially that of HSP70, is up-regulated by different stressors (10–13). UVB irradiation of keratinocytes induces the expression of HSP70 not only in vitro but also in vivo (11, 13–17). Furthermore, artificial expression of HSP70 in keratinocytes confers protection against UVB and ROS in vitro (8, 16, 18, 19). The protective role of HSP70 against UVB-induced epidermal damage was also suggested by in vivo studies: the whole body hyperthermia of mice prevented UVB-induced sunburn cell formation, and HSP70-null mice showed a sensitive phenotype to UVB-induced epidermal damage (20–22). Protection of the skin against UVB by expression of HSP70 has been suggested to occur in human skin (21). These previous results suggest that HSP70 expression suppresses UVB-induced epidermal damage, although no genetic evidence has been reported showing that overproduction of HSP70 prevents UVB-induced epidermal damage.

The potential benefit of HSP70 inducers as medicines for UVB-related skin diseases and cosmetics was also supported by a number of previously reported observations. For example, HSP70 has an anti-inflammatory activity by means of its inhibition of nuclear factor kappa B (NF-κB) and a resulting suppression of pro-inflammatory cytokine and chemokine expression (23–26). HSP70 has been reported to stimulate base excision repair, possibly by activation of human AP endonuclease and DNA polymerase β (27–29). We also recently found that artificial overexpression of HSP70 in mouse melanoma cells suppresses melanin production. Although we showed in that study that the UVB-induced production of melanin in the skin is suppressed in transgenic mice expressing HSP70, the anti-inflammatory and protective effects against DNA damage of HSP70 in UVB-irradiated skin have not been proved genetically. In this study, we examined the protective role of HSP70 against photo-damage by using transgenic mice expressing HSP70. The results obtained here suggest that expression of HSP70 protects the epidermis against UVB-induced damage via anti-inflammatory and anti-apoptotic effects and suppression of DNA damage. Based on these findings, we propose that non-toxic HSP70 inducers could be beneficial for use in cosmetics and medicines for the treatment of UVB-related skin diseases.

EXPERIMENTAL PROCEDURES

Materials and Animals—Parafomaldehyde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), peroxidase standard and fetal bovine serum were obtained from Sigma-Aldrich. Enzyme-linked immunosorbent assay kits for interleukin (IL)-1β and IL-6 were from Pierce. Mayer’s hematoxylin, 1% eosin alcohol solution, and malinol were from Muto Pure Chemicals (Tokyo, Japan). Terminal nucleotidyltransferase was obtained from Toyobo (Osaka, Japan). The Envision kit was from Dako (Carpinteria, CA). Biotin-14-ATP and Alexa Fluor 488-conjugated streptavidin were purchased from Invitrogen (Carlsbad, CA). VECTASHIELD was from Vector Laboratories. 4′,6-Diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). The RNeasy Fibrous Tissue Mini kit was obtained from Qiagen Inc. (Valencia, CA). The first-strand cDNA synthesis kit was from Takara Bio (Ohtsu, Japan), and IQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen. Antibodies against IxB-α and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against HSP70 was from Stressgen (Ann Arbor, MI). Antibody against CPDs was from Kamiya Biomedical Co. (Seattle, WA), whereas another against 8-OHdG was from Nikken SEIL (Shizuoka, Japan). α-(4-Pyril-1-oxide)-N-tert-butyl nitroxide (POBN) was from Alexis (San Diego, CA). Transgenic mice expressing HSP70 and their wild-type counterparts (6–8 weeks old, male) were gifts from Drs. C. E. Angelidis and G. N. Pagoulatos (University of Ioannina, Ioannina, Greece) and were prepared as described previously (30). Homozygotic transgenic mice expressing HSP70 were used in these experiments. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Animal Care Committee of Kumamoto University.

UV Irradiation—Animals and cultured cells were exposed to UVB irradiation with a double bank of UVB lamps (peak emission at 312 nm, VL-2151M lamp, Vilber Lourmat). The UV energy was monitored by a radiometer sensor (UVX-31, UV

2 The abbreviations used are: ROS, reactive oxygen species; CPD, cyclobutane pyrimidine dimer; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; GGA, geranylgeranylanalactone; HSP, heat shock protein; IL, interleukin; IxB-α, an inhibitor of NF-κB; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; MT, 3-(4-(5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; POBN, α-(4-pyril-1-oxide)-N-tert-butyl nitroxide; RT, reverse transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

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Products. Animals were placed under deep anesthesia with chloral hydrate (250 mg/kg), and fur was removed with electric clippers prior to the irradiation.

MPO Activity—Myeloperoxidase (MPO) activity in the skin was measured as described previously (30). Animals were placed under deep ether anesthesia and killed. The skin was dissected, rinsed with cold saline, and cut into small pieces. Samples were homogenized in 50 mM phosphate buffer, freeze-thawed, and centrifuged. The protein concentrations of the supernatants were determined using the Bradford method (31). MPO activity was determined in 10 mM phosphate buffer with 0.5 mM o-dianisidine, 0.00005% (w/v) hydrogen peroxide, and 20 μg of protein. MPO activity was obtained from the slope of the reaction curve, and its specific activity was expressed as the number of hydrogen peroxide molecules converted per minute/mg of protein.

Immunoblotting Analysis—Whole cell extracts were prepared as described previously (32). The protein concentration of each sample was determined by the Bradford method (31). Samples were applied to 9% (HSP70 and actin) or 12% (IAPP) polyacrylamide SDS gels and subjected to electrophoresis, after which the proteins were immunoblotted with appropriate antibodies.

Real-time Reverse Transcription-PCR Analysis—Total RNA was extracted from skin tissues using the RNeasy Fibrous Tissue Mini kit according to the manufacturer’s protocol. Samples (2.5 μg of RNA) were reverse-transcribed using the first-strand cDNA synthesis kit according to the manufacturer’s instructions. Synthesized cDNA was used in real-time reverse transcription-PCR (Chromo 4 system, Bio-Rad) experiments using iQ SYBR Green Supermix and analyzed with Opticon Monitor software according to the manufacturer’s instructions. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase cDNA was used as an internal standard. The primers used were, hsp70, 5’-tggctgctgacaggtgag-3’ (forward) and 5’-aggtcgagcaggtacgct-3’ (reverse); il-1β, 5’-gatcccaagcaatacccaaa-3’ (forward) and 5’-ggggaaattcctgacatc-3’ (reverse); il-6, 5’-ctggaggtcagctggaag-3’ (forward) and 5’-gtttgccgagtagactc-3’ (reverse); macrocyte chemoattractant protein-1 (mcp-1), 5’-ctcatctctgctatctc-3’ (forward) and 5’-ctggaggtgtggtggtgga-3’ (reverse); monocyte chemotactic protein-2 (mip-2), 5’-acactcattactgctttatc-3’ (forward) and 5’-gcacacatcggtgtagc-3’ (reverse); and gapdh, 5’-aacttggccatgtgggaagg-3’ (forward) and 5’-acatgcggagcctgga-3’ (reverse).

Histological and Immunohistochemical Analyses and TUNEL Assay—Skin samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4-μm-thick sections, which were then deparaffinized and washed in phosphate-buffered saline.

For histological examination (hematoxylin and eosin staining), sections were stained first with Mayer’s hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected using a BX51 microscope (Olympus).

For immunohistochemical analyses, sections were incubated with 0.1% (for 8-OHdG) or 0.3% (for CPDs and HSP70) hydrogen peroxide in methanol for removal of endogenous peroxidase. Sections were incubated with 0.125% trypsin in phosphate-buffered saline for 10 min and then with 1 N HCl for 30 min for DNA denaturation. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against HSP70 (1:200 dilution), 8-OHdG (1:100 dilution), or CPDs (1:500 dilution) in the presence of 2.5% bovine serum albumin, and then incubated for 1 h with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins. 3,3’-Diaminobenzidine was applied to the sections, which were then incubated with Mayer’s hematoxylin (hematoxylin staining was omitted for 8-OHdG). Samples were mounted with malinol and inspected using a BX51 fluorescence microscope (Olympus). The intensity of 8-OHdG staining in the epidermis was measured by LuminaVision (Mitani).

For TUNEL assays, sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with terminal deoxynucleotidyltransferase and biotin-14-ATP for 1 h at 37 °C, and finally with Alexa Fluor 488-conjugated streptavidin and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected using a BX51 fluorescence microscope (Olympus).

Cell Culture and Apoptosis Analysis—PAM212 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air with 5% CO2 at 37 °C. Transfection of PAM212 cells with pcDNA3.1 containing the hsp70 gene (33) was carried out using Lipofectamine (TM) 2000 according to the manufacturer’s protocol. The stable transfectants expressing HSP70 were selected by immunoblotting and real-time reverse transcription-PCR analyses. Positive clones were maintained in the presence of 200 μg/ml G418. Cell viability was determined by the MTT method as previously described (34), and the measurements of caspase-3-like activity and fluorescence-activated cell sorting analysis (for measurement of apoptotic cells in sub-G1) were performed as described previously (34).

Immunostaining of 8-OHdG and CPDs in Cultured Cells—Cells were cultured on 8-well Lab-Tek II Chamber slides (Nunc). They were then fixed in methanol for 20 min after UVB irradiation. Cells were permeabilized with 0.5% Triton X-100 for 5 min, treated in a microwave oven with 0.01 M citric acid buffer for antigen activation, and then treated with 1 N HCl for 20 min for DNA denaturation. Cells were blocked with 5% goat serum for 10 min, incubated for 2 h with antibody against 8-OHdG (1:10 dilution) or CPDs (1:2000 dilution) in the presence of 2.5% bovine serum albumin, and finally incubated with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Cells were simultaneously stained with DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a BX51 fluorescence microscope (Olympus). The fluorescence intensity of 8-OHdG or CPD staining was measured by using LuminaVision.

Determination of ROS Production in Vivo by ESR Analysis—In vivo ESR analysis was performed as described (35) with some modifications. Immediately after UVB exposure, animals were placed under deep anesthesia with chloral hydrate (250 mg/kg) and injected with POBN (a spin trap reagent) (36, 37) intraperitoneally (4 mmol/kg). After 1 h, mice were sacrificed, the skins
were dissected, and the lipid phase was extracted. After evaporating the sample, ESR spectra were immediately recorded at room temperature using a quartz flat cell (160 μl) in a JES-TE200 spectrometer (JEOL). The operating conditions of the ESR apparatus were: 9.43 GHz, field 335.2 ± 5 milliteslas, 40-milliwatt microwave power, 100-kHz modulation frequency, 0.25-field modulation width, 0.3-s time count, and sweep time of 2 min.

Statistical Analysis—All values are expressed as the means ± S.E. Two-way analysis of variance followed by the Tukey test was used to evaluate differences between more than three groups. Differences were considered to be significant for values of p < 0.05.

RESULTS
Effect of Expression of HSP70 on UVB-induced Epidermal Apoptosis—Overexpression of HSP70 in the transgenic mice that we used in this study has been shown in various organs (9, 30, 38–40). We examined HSP70 expression in the skin of these animals as this has not been determined to date. Transgenic mice expressing HSP70 and wild-type mice were irradiated or not with 180 mJ/cm² UVB. The dorsal skin was removed 24 h after completion of the irradiation and subjected to immunoblotting analysis. As shown in Fig. 1 (A and B), the level of HSP70 was significantly higher in transgenic mice than in wild-type mice in both the presence and absence of UVB irradiation. However, under these conditions, UVB irradiation did not up-regulate the expression of HSP70 in either type of mice (Fig. 1, A and B), a finding that differs from previous reports (20). Although we examined the effect of UVB on expression of HSP70 under various conditions (various doses of UVB and time course of the induction periods), we could not detect the UVB-dependent up-regulation of expression of HSP70 under any conditions by immunoblotting analysis (supplemental Fig. S1). We consider that this is due to the UVB-dependent increase in total amount of proteins (we applied the same amount of proteins in each lane in immunoblotting analysis).

Supporting this notion, immunohistochemical analysis with an antibody against HSP70 demonstrated that the expression of HSP70 was induced by UVB irradiation at the skin (the top panels in supplemental Fig. S2). Immunohistochemical analysis also demonstrated that the expression of HSP70 is higher in the epidermis than in the dermis, as described previously (11), and that expression in the epidermis is further heightened in transgenic mice (Fig. 1C). The results in Fig. 1 suggest that these transgenic mice could be useful for examining the protective role of HSP70 against UVB-induced epidermal damage.

Histological observations revealed extensive infiltration of leukocytes and epidermal disruption in skin sections prepared from UVB-irradiated wild-type mice, whereas the extent of cutaneous damage was not so apparent in transgenic mice expressing HSP70 (Fig. 2A). MPO activity, an indicator of the inflammatory infiltration of leukocytes, was increased in wild-type mice in response to the UVB irradiation. This activity was lower in UVB-irradiated transgenic mice expressing HSP70 compared with wild-type mice (Fig. 2B). The overexpression of HSP70 in transgenic mice did not affect the background level of MPO activity (Fig. 2B). These results show that UVB-induced epidermal damage and the resulting infiltration of leukocytes are suppressed in transgenic mice expressing HSP70.

The extent of epidermal cell apoptosis was determined by TUNEL assay. An increase of TUNEL-positive (apoptotic) cells in the epidermis of wild-type mice was observed after the UVB irradiation, and this increase was clearly suppressed in transgenic mice expressing HSP70 (Fig. 2, C and D). The overexpression of HSP70 in transgenic mice did not affect the background level of epidermal apoptosis (Fig. 2, C and D). These results suggest that the expression of HSP70 protects epidermal cells (keratinocytes) from UVB-induced apoptosis.

To identify cells expressing HSP70 in transgenic mice and wild-type mice irradiated with UVB, we performed co-immunostaining assay. As shown in supplemental Fig. S2, strong co-staining of HSP70 with CD11b (a marker of macrophage) and pan cytokeratin (a marker of keratinocyte) was observed at the skin of transgenic mice expressing HSP70 or wild-type mice irradiated with UVB. A relatively weak co-staining of HSP70 with MPO (a maker of neutrophil) and vimentin (a maker of fibroblast) was also observed (supplemental Fig. S2). These
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To test the idea that the expression of HSP70 protects epidermal cells (keratinocytes) from UVB-induced apoptosis in vitro, we constructed a stable transfection of a mouse keratinocyte cell line (PAM212) that continuously overexpresses HSP70 (Clone 2). As shown in Fig. 3A, the level of HSP70 in Clone 2 was higher than mock transfectant control cells in both the presence and absence of UVB irradiation. We also found that UVB irradiation up-regulated the expression of HSP70 in both types of cells (Fig. 3A). Exposure of cells to UVB irradiation decreased cell viability in a dose-dependent manner; this effect was suppressed in HSP70-overexpressing cells (Fig. 3B). To detect UVB-induced apoptosis, we counted cells in sub-G1 (apoptotic cells) by fluorescence-activated cell sorting analysis. UVB irradiation increased the number of apoptotic cells, and this increase was suppressed in HSP70-overexpressing cells (Table 1). We also monitored apoptosis by measuring caspase-3-like activity using fluorogenic peptide substrates and obtained similar results to those for the fluorescence-activated cell sorting analysis (Table 1). Overexpression of HSP70 did not affect the background level of apoptosis (Table 1). The results in Fig. 3 and Table 1 suggest that the expression of HSP70 helps to protect keratinocytes from UVB-induced apoptosis.

Effect of HSP70 Expression on UVB-induced Epidermal Inflammation—As described above, HSP70 was reported to suppress the activation of NF-κB through various mechanisms such as suppression of the inflammatory stimuli-induced degradation of IκB-α (an inhibitor of NF-κB) (26). We therefore examined the effect of UVB irradiation and/or expression of HSP70 on the level of IκB-α both in vivo and in vitro. As shown in Fig. 4 (A and B), UVB irradiation decreased the cutaneous level of IκB-α both in wild-type mice and in transgenic mice expressing HSP70, although the level remained significantly higher in the latter. We also compared the mRNA expression of pro-inflammatory cytokines (IL-1β and IL-6) and chemokines (MIP-2 and MCP-1) between UVB-irradiated transgenic mice expressing HSP70 and wild-type mice. The mRNA expression of il-1β, il-6, mip-2, and mcp-1 was increased by UVB irradiation, but this increase was much lower in skin samples prepared from transgenic mice expressing HSP70 compared with samples from wild-type mice (Table 2A). Table 2B shows that the expression of HSP70 in transgenic mice did not affect the background levels of mRNA expression. Similar results were observed for the protein levels of cytokines (IL-1β and IL-6) determined by enzyme-linked immunosorbent assay (Table 2B).

results suggest that the transgenic mice and wild-type mice irradiated with UVB express HSP70 in various types of cells at the skin.

We also tried to examine the effect of expression of HSP70 induced by geranylgeranyacetone (GGA), a leading anti-ulcer drug on the Japanese market and an HSP inducer (41). However, as shown in supplemental Fig. S3, GGA did not induce expression of HSP70 by any route of administration (oral, intraperitoneal, and percutaneous administrations). Thus, we used heat treatment to induce expression of HSP70. As shown in supplemental Figs. S3 and S4, heat treatment induced the expression of HSP70 at the skin, and we found that this heat treatment protects the skin from UVB-induced damage (epidermal disruption, increase in MPO activity, and epidermal apoptosis).

FIGURE 2. UVB-induced skin damage and apoptosis in wild-type mice and transgenic mice expressing HSP70. Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with or without the indicated doses of UVB, and the dorsal skin was removed after 48 h (A), 24 h (B), or 12 h (C and D). A, sections of dorsal skin were prepared and subjected to hematoxylin and eosin staining. B, MPO activity was measured as described under “Experimental Procedures.” Values are mean ± S.E. (n = 8–12). **, p < 0.01. C, sections of dorsal skin were subjected to TUNEL assay and DAPI staining. D, the ratio of TUNEL-positive cells in the epidermis was counted (400–1000 cells in total). Values are mean ± S.E. (n = 3). ***, p < 0.01; *, p < 0.05. Scale bar, 50 μm.
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To test this notion in vitro, we examined the effect of UVB irradiation and/or expression of HSP70 in cultured keratinocytes on the degradation of IκB-α and expression of just one pro-inflammatory cytokine (IL-6, it has been reported that IL-1β is not expressed in mouse keratinocytes (42, 43)) and chemokines (MIP-2 and MCP-1). As shown in Fig. 5A, UVB irradiation transiently (at 6–12 h after the UVB irradiation) decreased the level of IκB-α, and this level was higher in HSP70-overexpressing cells than in mock transfectant control cells at any period after the UVB irradiation. Expression of HSP70 increased the background level of IκB-α (Fig. 5A), these results being different from those observed in vivo (Fig. 4A). Furthermore, expression of the pro-inflammatory cytokine and chemokine genes tested (il-6, mip-2, and mcp-1) was up-regulated by the UVB irradiation, although the expression was suppressed in UVB-irradiated HSP70-overexpressing cells compared with mock transfectant control cells (Fig. 5B). Overexpression of HSP70 suppressed the background expression of mcp-1 but not il-6 and mip-2 genes (Fig. 5B). The results in Fig. 5 support the notion that the expression of HSP70 in keratinocytes suppresses the UVB-induced expression of cytokines and chemokines via the inhibition of IκB-α degradation and the resulting suppression of NF-κB activity.

Effect of HSP70 Expression on UVB-induced Epidermal DNA Damage—As described in the introduction, UVB irradiation damages DNA (formation of photo-products) directly (formation of products such as CPDs) and indirectly via the production of ROS (formation of products such as 8-OHdG). To examine the effect of HSP70 expression on UVB-induced DNA damage in the epidermis, we compared the time-course profile of the level of CPDs and 8-OHdG after irradiation with UVB between transgenic mice expressing HSP70 and wild-type mice. As shown in Fig. 6 (A and B), the level of 8-OHdG, judged from the intensity of immunohistochemical staining, was significantly lower in the epidermis of UVB-irradiated transgenic mice expressing HSP70 than in wild-type mice 1 h after the UVB irradiation (45 ml/cm²), suggesting that the UVB-induced formation of 8-OHdG is suppressed in the transgenic mice. Although the level of 8-OHdG 1 h after the irradiation was similar between wild-type mice irradiated with 45 ml/cm² UVB and transgenic mice irradiated with 55 ml/cm² UVB, the level was significantly lower in transgenic mice than in wild-type mice 48 h after the irradiation (Fig. 6, A and B), suggesting that the repair process of 8-OHdG is stimulated in transgenic mice expressing HSP70.

We also measured the level of CPDs in a similar manner. As shown in Fig. 6 (C and D), the number of CPD-positive cells was similar between wild-type mice and transgenic mice 1 h after the UVB irradiation. On the other hand, the number was significantly lower in transgenic mice than in wild-type mice 24 or 48 h after the UVB irradiation (Fig. 6, C and D). The results suggest that the repair process rather than the formation of CPDs is affected by the expression of HSP70.

We then tested whether or not the effect of HSP70 expression on the formation and repair of 8-OHdG and CPDs can be reproduced in vitro. HSP70-overexpressing PAM212 cells and...
mock transfectant control cells were irradiated with UVB, and the nuclear levels of 8-OHdG and CPDs were monitored by immunostaining. As shown in Fig. 7, A and B, HSP70-overexpressing cells showed a lower level of 8-OHdG than mock transfectant control cells 5 min after the UVB irradiation (50 ml/cm²), suggesting that the formation of 8-OHdG is suppressed by the expression of HSP70. Furthermore, comparing the level of 8-OHdG between HSP70-overexpressing cells irradiated with 65 ml/cm² UVB and mock transfectant control cells irradiated with 50 ml/cm² UVB, the initial (5 min after the UVB irradiation) levels were indistinguishable; however, the level was lower in HSP70-overexpressing cells than in mock transfectant control cells 24 h after the irradiation (Fig. 7, A and B), suggesting that the repair process of 8-OHdG is stimulated by the expression of HSP70. In other words, the protective effect of HSP70 against UVB-induced formation of 8-OHdG and its simulative effect on the repair process can be reproduced in vitro. On the other hand, the level of CPDs was indistinguishable between HSP70-overexpressing cells and mock transfectant control cells both 5 min and 24 h after the UVB irradiation, suggesting that neither the formation nor repair of CPDs is affected by the expression of HSP70. That is to say, the effect of HSP70 on the repair of CPDs was not reproduced in vitro.

The results in Fig. 6, A and B, suggest that UVB-induced ROS production in the skin is suppressed in transgenic mice expressing HSP70. On this basis, we measured the level of ROS in the skin by monitoring the lipid-derived free radical spin adduct with ESR spectroscopy and spin trap POBN, which reacts with ROS to form a radical spin adduct. As shown in Fig. 8A, a radical spin adduct of ESR spectrum similar to that reported in other organs was obtained (35–37, 44). The hyperfine coupling constants for the POBN radical adducts were αN = 14.91 ± 0.08 G and αH = 2.45 ± 0.04 G, which are similar to data previously reported for other organs (35–37, 44), suggesting that this ESR spectrum is derived from lipid-derived free radicals. As shown in Fig. 8B, the level of ROS in the skin was elevated by UVB irradiation in wild-type mice, and this increase was suppressed in transgenic mice expressing HSP70. This finding suggests that the expression of HSP70 suppresses UVB-induced ROS production in the skin.

TABLE 2
UVB-induced expression of pro-inflammatory cytokines and chemokines

<table>
<thead>
<tr>
<th>UVB (ml/cm²)</th>
<th>WT</th>
<th>HSP70 Tg</th>
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</thead>
<tbody>
<tr>
<td>A) Relative expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>il-1β</td>
<td>0</td>
<td>1.0 ± 0.11</td>
</tr>
<tr>
<td>180</td>
<td>247.7 ± 9.19</td>
<td>3.4 ± 0.10</td>
</tr>
<tr>
<td>il-6</td>
<td>0</td>
<td>1.0 ± 0.17</td>
</tr>
<tr>
<td>180</td>
<td>106.7 ± 1.66</td>
<td>5.6 ± 0.92</td>
</tr>
<tr>
<td>mip-2</td>
<td>0</td>
<td>1.0 ± 0.89</td>
</tr>
<tr>
<td>180</td>
<td>167.7 ± 1.13</td>
<td>8.3 ± 0.55</td>
</tr>
<tr>
<td>mcp-1</td>
<td>0</td>
<td>1.0 ± 0.46</td>
</tr>
<tr>
<td>180</td>
<td>36.7 ± 0.40</td>
<td>15.1 ± 6.00</td>
</tr>
</tbody>
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| B) ng/g tissue | | | |
| --- | --- | --- |
| IL-1β | 0 | 0.48 ± 0.12 | 0.84 ± 0.14 |
| 180 | 27.9 ± 2.71 | 8.7 ± 1.00 |
| IL-6 | 0 | 1.2 ± 0.37 | 0.87 ± 0.19 |
| 180 | 67.4 ± 1.66 | 36.4 ± 5.93 |

DISCUSSION

An ameliorative effect of HSP70 due to its cytoprotective, anti-inflammatory, and molecular chaperone (quality control of proteins) properties has been reported for various diseases. For example, we have shown using transgenic mice that HSP70 protects against irritant-produced lesions in the stomach and small intestine and inflammatory bowel disease-related experimental colitis (30, 38–40). The potential therapeutically applicable to HSP70 for use in other diseases, such as neurodegenerative diseases, ischemia-reperfusion damage, and diabetes has also been suggested (9, 45). Interestingly, GGA, a leading anti-ulcer drug on the Japanese market, has been reported to be an HSP inducer, up-regulating various HSPs not only in cultured gastric mucosal cells but also in various tissues in vivo (41). It was reported that GGA suppresses not only gastric lesions but also lesions of the small intestine, inflammatory bowel disease-related experimental colitis, and neurodegenerative diseases (39, 40, 46, 47). On the other hand, the use of HSP70 inducers in cosmetics
and medicines to aid in the treatment of UVB-related skin diseases has not been fully evaluated. A number of in vitro studies suggested that HSP70 protects keratinocytes from UVB irradiation; however, the protective role of HSP70 against UVB-induced functional and structural alterations of the epidermis has not been proved genetically. In this study, using transgenic mice overexpressing HSP70, we have shown that the expression of HSP70 suppresses UVB-induced epidermal apoptosis, inflammatory responses, ROS production, and DNA damage, suggesting that HSP70 inducers could be beneficial for use as agents in medicines and cosmetics to alleviate the symptoms and/or cure UVB-related skin diseases. These effects of HSP70 should be mutually dependent. For example, ROS stimulate NF-κB activity and DNA damage, and both ROS and DNA damage induce apoptosis (48, 49). Our results also suggest that the high level of constitutive expression of HSP70 in keratinocytes could play an important role in protecting the skin against UVB irradiation.

We showed here that UVB-induced apoptosis was suppressed in the epidermis of transgenic mice and in cultured keratinocytes overexpressing HSP70. These results are basically consistent with previous results (8, 16, 18, 20–22). It was suggested that HSP70 suppresses various steps in the molecular pathways governing apoptosis, including p53 activation, which plays an important role in UVB-induced apoptosis (50, 51). In addition to this anti-apoptotic (cytoprotective) effect of HSP70, an anti-inflammatory effect (suppression of NF-κB activity) was recently revealed and thought to be important for HSP70 function (24–26). However, it was not clear whether HSP70 suppresses the activity of NF-κB in vivo. In this study, we confirmed that expression of HSP70 increases the level of IκB-α (an inhibitor of NF-κB) in vitro and found that a UVB-induced decrease in the level of IκB-α in the skin is suppressed in transgenic mice expressing HSP70. We also showed that the UVB-induced expression of pro-inflammatory cytokines and chemokines is suppressed in transgenic mice expressing HSP70. These results suggest that HSP70 expression in the skin suppresses inflammation via the inhibition of NF-κB activity and the resulting inhibition of pro-inflammatory cytokine and chemokine expression. Considering the adverse effects of inflammation on various skin diseases, these properties of HSP70 could make the development of HSP70 inducers an important advance in the search for medicines to cure UVB-related skin diseases.

We also showed that the UVB-induced increase in levels of both CPDs (UVB-induced direct DNA damage) and 8-OHdG (UVB-dependent indirect DNA damage via the production of ROS) is suppressed in transgenic mice expressing HSP70. This is the first in vivo evidence of the protective effect of HSP70 against UVB-induced DNA damage to the skin. This finding is particularly important, because UVB-induced DNA damage plays an important role in UVB-induced skin diseases, especially carcinogenesis. Because HSP70 protects epidermal cells from UVB-induced apoptosis, it could also in fact stimulate skin carcinogenesis by aiding the survival of DNA-damaged cells. However, a further beneficial effect of HSP70 (suppression of UVB-induced DNA damage) may circumvent this problem. The formation and repair of 8-OHdG have been suggested to be

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**FIGURE 6. UVB-induced epidermal DNA damage.** Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with or without (CTRL) indicated doses (A and B) or 45 mJ/cm² (C and D) of UVB. Sections of the dorsal skin were prepared after indicated periods and subjected to immunohistochemical analysis with an antibody against 8-OHdG (A) or CPDs (C). The intensity of 8-OHdG-staining (B) and the percentage of CPD-positive cells (400–600 cells in total) (D) were measured. B and D, values are mean ± S.E. (n = 6). **, p < 0.01; *, p < 0.05; n.s., not significant. Scale bar, 50 μm.
suppressed and stimulated, respectively, in transgenic mice expressing HSP70. We also reproduced those findings in cultured keratinocytes, suggesting that HSP70 expressed in these cells is directly responsible for these phenomena. HSP70 seems to suppress the formation of 8-OHdG by decreasing the level of ROS, because the UVB-induced increase in the level of ROS was suppressed in transgenic mice expressing HSP70. A decrease in the level of ROS due to the increased expression of HSP70 was also reported in vitro (52). HSP70 stimulates base excision repair, possibly via the activation of human AP endonuclease and DNA polymerase β (27–29). This effect may be involved in an HSP70-dependent stimulation of the 8-OHdG repair process, because the base excision repair system plays a major role in the repair of 8-OHdG (6). On the other hand, although our in vivo results suggested that the repair process of CPDs is stimulated by the expression of HSP70, we could not reproduce these results in vitro. However, a slight up-regulation of CPD repair by the expression of HSP70 was reported elsewhere (53). Furthermore, in Escherichia coli, an HSP70 homologue (DnaK) stimulates the nucleotide excision repair of damaged DNA (54), which plays a major role in the repair of CPDs (6).

We recently found that the artificial expression of HSP70 in cultured melanoma cells suppresses melanin production, suggesting that HSP70 inducers could be beneficial for use as hypopigmenting cosmetics and medicines. A number of compounds that inhibit melanin production have been discovered, however most of their cosmetic and pharmaceutical applications have not been successful due to the occurrence of skin irritation (55), which is caused by the fact that UV-induced mild melanogenesis has a protective role against UVB-induced skin damage, especially DNA damage. Melanin also acts as a scavenger of the UVB-induced production of ROS (56). Therefore, the findings in this study that HSP70 expression suppresses both UVB-induced epidermal DNA damage and the increase in the cutaneous level of ROS are important for the development HSP70 inducers as hypopigmenting cosmetics and medicines. The anti-inflammatory effects of HSP70 may help in this manner, because UVB-induced inflammation actually stimulates pigmentation (57). Based on these results, we propose that HSP70 inducers could have numerous cosmetically and pharmaceutically beneficial applications. We have already screened for HSP70 inducers from Chinese herbal extracts and

![FIGURE 7. Effect HSP70 expression on UVB-induced DNA damage in vitro. HSP70-overexpressing PAM212 cells (Clone 2) and mock transfector control cells (Mock) were irradiated with or without (CTRL) indicated doses (A and B) or 50 mJ/cm² (C and D) of UVB and cultured for indicated periods. Cells were subjected to immunostaining analysis with an antibody against 8-OHdG (A) or CPDs (C). The fluorescence intensity of 8-OHdG (B) and CPDs (D) staining was measured. B and D, values are mean ± S.E. (n = 6), **, p < 0.01; *, p < 0.05; n.s., not significant. Scale bar, 100 μm.](http://www.jbc.org/)

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found that their HSP70-inducing activities were more potent than GGA. To develop some of these extracts as hypopigmenting (whitening) cosmetics or as drugs to combat melanin-related diseases.

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FIGURE 8. UVB-induced increase in the epidermal ROS level. Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with 45 mJ/cm² UVB. A, POBN was administered, and the dorsal skin was removed after 1 h and subjected to radical adduct ESR spectrum analysis. B, the intensity of the ESR signal of the radical adduct (shown by the bar in A) was determined, expressed relative to the control sample, and given as the mean ± S.E. (n = 6–10). **, p < 0.01.

Prevention of Epidermal Damage by HSP70

Prevention of UVB Radiation-induced Epidermal Damage by Expression of Heat Shock Protein 70
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