HSP70 inducers from Chinese herbs and their effect on melanin production

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Skin hyperpigmentation disorders due to abnormal melanin production induced by ultraviolet (UV) irradiation are both a clinical and a cosmetic problem. This melanin production is mediated by tyrosinase whose expression is positively regulated by microphthalmia-associated transcription factor (MITF). We recently found that expression of heat shock protein 70 (HSP70) inhibits melanin production. In this study, we searched for HSP70 inducers from Chinese herbs and selected an ethanol extract of *Eupatorium lindleyanum* (*E. lindleyanum*). Not only melanin production but also the activity and expression of tyrosinase were significantly suppressed in cells treated with *E. lindleyanum* extract as well as in HSP70-overexpressing cells. The expression of MITF was clearly suppressed in cells treated with *E. lindleyanum* extract but not in HSP70-overexpressing cells. These results suggest that *E. lindleyanum* extract suppresses the expression of tyrosinase and melanin production through both HSP70-dependent and HSP70-independent mechanisms.

Key words: melanogenesis, HSP70, *Eupatorium lindleyanum*, MITF

Background

Ultraviolet (UV)-induced skin hyperpigmentation disorders due to abnormal melanin production can cause clinical and cosmetic problems. The melanin production is regulated by a cAMP-dependent pathway, in which exposure of keratinocytes to UV stimulates the release of signal molecules, which stimulate melanin production by elevating intracellular cAMP levels and induction of the expression of microphthalmia-associated transcription factor (MITF). MITF promotes the transcription of the *tyrosinase* gene (1-3). Tyrosinase is a rate-limiting enzyme in melanin synthesis and chemicals that suppress the activity and expression of tyrosinase could be beneficial as hypopigmenting agents (4, 5).

On the other hand, UV-induced modest melanin production plays an important role in protection against UV-dependent skin damage (6). Thus, identification of a mechanism that not only suppresses melanin production but also protects the skin from UV-induced damage is important for developing hypopigmenting agents without worsening UV-induced skin damage.

When cells are exposed to stressors, including UV irradiation, heat shock proteins (HSPs) are induced in order to confer protection against such stressors (7). We recently found that the artificial expression of HSP70 suppresses melanin production in a mouse melanoma cell line (B16) and in the epidermis of mice (unpublished observations). Based on these results, we proposed that HSP70 inducers might be pharmaceutically and cosmetically beneficial as hypopigmenting agents,.

Questions addressed

In this study, we searched for HSP70 inducers from Chinese herbs and selected *Eupatorium lindleyanum* (*E. lindleyanum*).

Experiment design

Melanin content was determined as described previously (8, 9). Tyrosinase activity was assayed as described previously (10).

All values are expressed as the mean ± standard deviation (S.D.). Two-way analysis of variance (ANOVA) followed by the Tukey test or the Student's *t* test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively.

Results

AGS cells were used to search for HSP70-inducers from ethanol (100%) extracts of 400 Chinese herbs was based. We selected four candidate herbs, namely *Inula britannica* (*I. britannica*), *Eupatorium lindleyanum* (*E. lindleyanum*), *Alpinia galanga* (*A. galanga*) and *Amomum xanthioides* (*A. xanthioides*) and examined the dose-response profile of each herbal extract for induction of cell death and induction of HSP70 expression (Fig. S1a-d). To estimate the ability of each herbal extract as a safe HSP70 inducer, we calculated the safe induction (SI) index (cell viability (%) at a concentration that doubles the expression of HSP70) for each herbal extract and found that *E. lindleyanum* extract showed the highest SI index (Fig. S1a-d). Similar results

were observed using B16 cells (Fig. S1e-h). Based on the results, we selected the *E. lindleyanum* extract for further experiments.

As shown in Fig. 1a, treatment of B16 cells with *E. lindleyanum* extract caused a transient increase of HSP70 expression without significantly affecting cell viability. Real-time RT-PCR analysis revealed that the expression of *hsp70* mRNA was also transiently induced by treatment with *E. lindleyanum* extract (Fig. 1b). We also confirmed the induction of HSP70 by *E. lindleyanum* extract in human epidermal melanocytes (Fig. 1c). As shown in Fig. S2a, the expression of HSPs other than HSP70 was not induced by *E. lindleyanum* extract in B16 cells. Similar results were observed at the level of mRNA expression (Fig. S2b).

Treatment of B16 cells with IBMX increased the intracellular melanin content, while treatment of the cells with *E. lindleyanum* extract decreased the melanin content (Fig. 1d). Transfection of cells with siRNA for HSP70 clearly increased the melanin content in the presence of both IBMX and *E. lindleyanum* extract (Fig. 1e), suggesting that HSP70 is involved in *E. lindleyanum* extract-dependent decrease in the melanin content.

Treatment of B16 cells with IBMX increased the tyrosinase activity and this activity was lower in cells treated with *E. lindleyanum* extract than in control cells (Fig. 1f). On the other hand, when *E. lindleyanum* extract was directly added to cell extracts prepared from IBMX-treated B16 cells, tyrosinase activity was not significantly affected (Fig. S3), suggesting that *E. lindleyanum* extract affects the expression of tyrosinase. In fact, treatment of cells with IBMX increased the level of tyrosinase, with the level being lower in cells treated with *E. lindleyanum* extract than in control cells (Fig. 2a). Similar results were observed at the mRNA level; however, the extent of suppression by *E.*

lindleyanum extract at the mRNA level was not so apparent as that at the protein level (Fig. 2b). Since it was reported that the level of tyrosinase is regulated by post-transcriptional mechanisms (11, 12), such regulations may be involved in the *E. lindleyanum* extract-dependent decrease in the level of tyrosinase.

We found that treatment of cells with IBMX increased the level of MITF, while pre-treatment of cells with *E. lindleyanum* extract clearly decreased the level of MITF (Fig. 2c). We recently found that overexpression of HSP70 did not affect the level of MITF (unpublished observations). Those results are confirmed in this study (Fig. S4a). Suppression of MITF expression was observed at a concentration of 0.04 mg/ml *E. lindleyanum* extract; however, induction of HSP70 expression required concentrations higher than 0.08 mg/ml (Fig. S4b), supporting the notion that the inhibitory effect of *E. lindleyanum* extract on the level of MITF is not mediated by the induction of HSP70 expression.

IBMX induced the expression of *mitf* mRNA and treatment of cells with *E. lindleyanum* extract clearly decreased *mitf* mRNA expression (Fig. 2d), suggesting that *E. lindleyanum* extract suppresses the expression of MITF and this suppression in turn plays an important role in the inhibitory effect of *E. lindleyanum* extract on the production of melanin.

Conclusion

E. lindleyanum extract induced HSP70 expression at concentrations that did not significantly affect cell viability. We found that *E. lindleyanum* extract did not affect the expression of p53, which plays an important role in protection against UV irradiation

(Fig. S4c). These results suggest that *E. lindleyanum* extract is a relatively safe HSP-inducer. However, it is necessary to test whether *E. lindleyanum* is beneficial or harmful to human skin in further experiments.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of different herbal extracts on cell viability and expression of HSP70.

Figure S2. Effect of *E. lindleyanum* extract on the expression of various HSPs.

Figure S3. Effect of *E. lindleyanum* extract on activity of tyrosinase.

Figure S4. Effect of *E. lindleyanum* extract on expression of MITF and p53.

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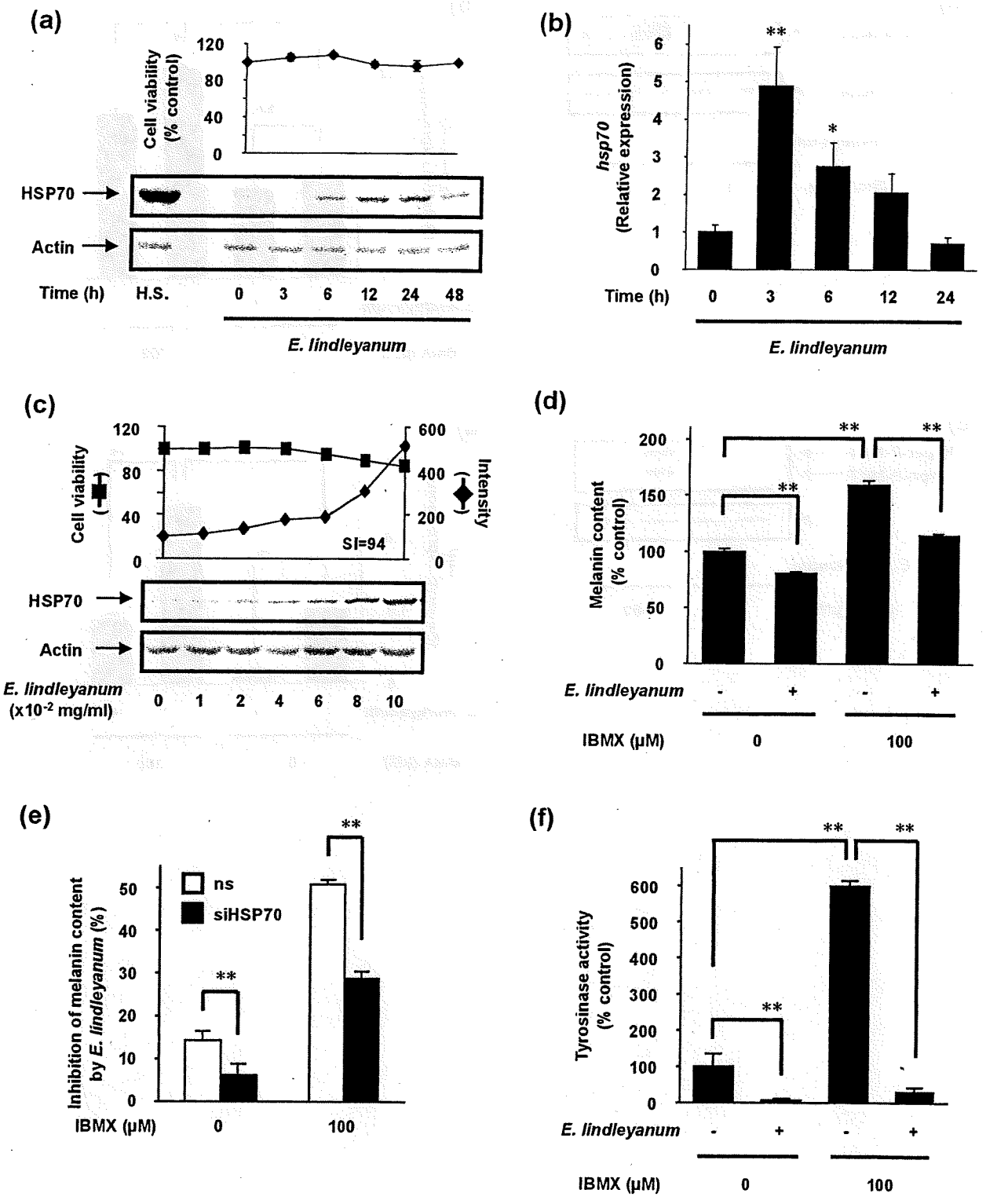
Figure legends

Figure 1. Induction of expression of HSP70 and inhibition of melanin production by *E. lindleyanum* extract. B16 cells were transfected with siRNA for HSP70 (siHSP70) or non-silencing siRNA (ns) and incubated for 48 h (e). B16 cells (a, b, d-f) or human epidermal melanocytes (c) were incubated with 0.08 mg/ml (a,b, d-f) or indicated concentrations (c) of *E. lindleyanum* extract for indicated period (a, b) or 24 h (c-f) or heat-shocked for 1 h at 43°C (H.S., heat shock). Cells were further incubated with or without 100 μM IBMX for 72 h (d, e) or 48 h (f). Cell viability was determined by the MTT method and whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin (a, c). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for the *hsp70* gene. Values were normalized to *gapdh* gene expression and expressed relative to the control sample (b). The amounts of melanin in the cell extract were determined and are expressed relative to the control (d, e). Tyrosinase activity was determined and expressed relative to the control (f). Values are given as mean ± S.D. (n=3). $^{**}P<0.01$; $^{*}P<0.05$.

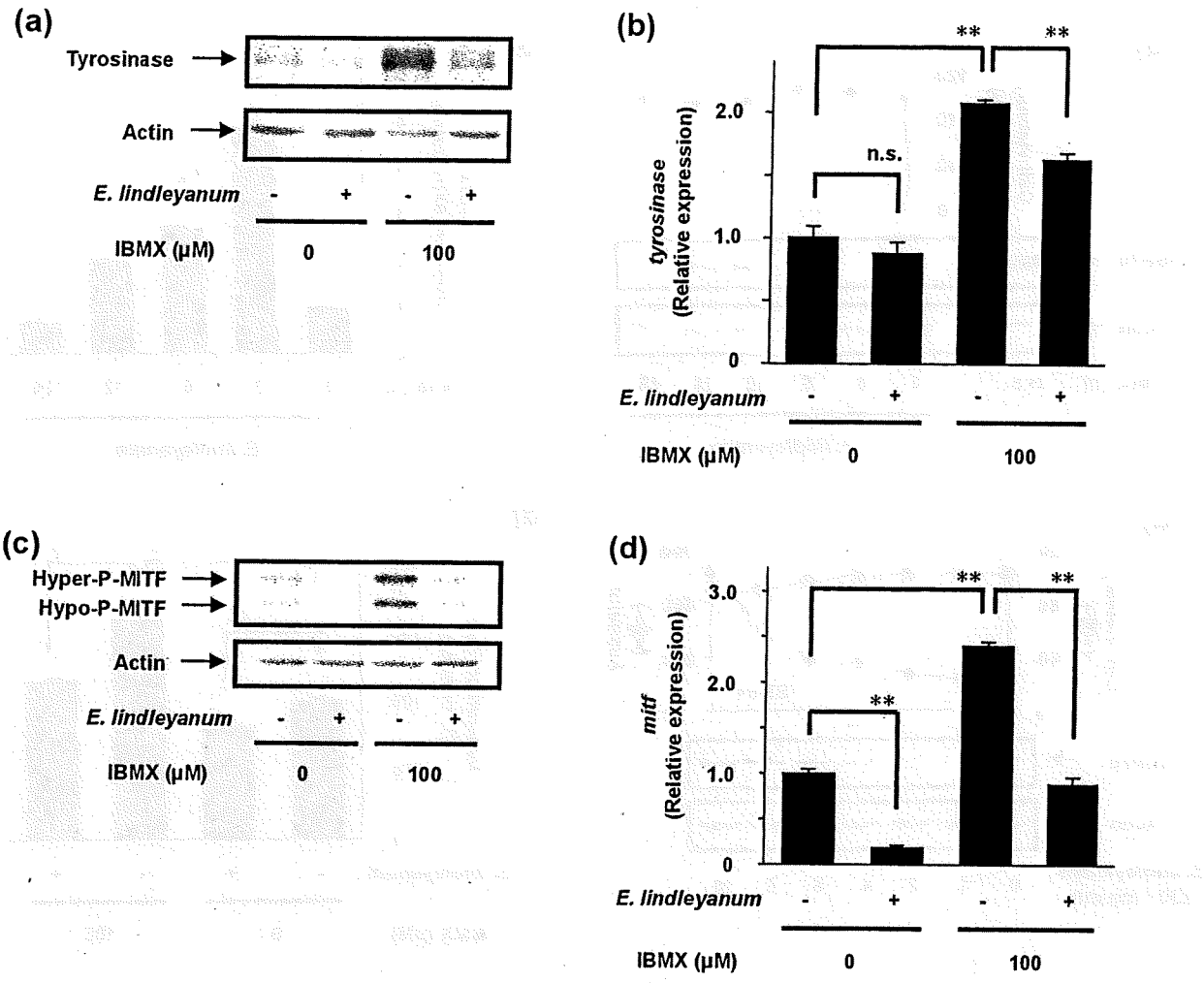
Figure 2. Effect of *E. lindleyanum* extract on expression of tyrosinase and MITF. B16 cells were incubated for 24 h with or without 0.08 mg/ml *E. lindleyanum* extract and further incubated for 48 h (a, b), 3 h (c) or 1.5 h (d) with or without 100 μM IBMX. Whole cell extracts were analyzed by immunoblotting with an antibody against tyrosinase, MITF or actin (a, c). Bands representing the hyper-phosphorylated (Hyper-P) and hypo-phosphorylated (Hypo-P) forms of MITF are shown (c). The *tyrosinase* and *mitf* mRNA expression was monitored as described in the legend of Fig.

1 (b, d). Values are given as mean ± S.D. (n=3). **P<0.01; n.s., not significant (b,d).

Yamashita *et al.*, Figure 1



Yamashita *et al.*, Figure 2



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Prevention of UVB Radiation-induced Epidermal Damage by Expression of Heat Shock Protein 70*[S]

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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 IκB-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 IκB-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 photo-damage 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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(ROS),[2] which also damages these molecules by oxidization. In this way, direct absorption of UVB by DNA causes DNA damage through the formation of covalent linkages, resulting in products such as cyclobutane pyrimidine dimers (CPDs). On the other hand, UVB-produced ROS also damage DNA by producing damaged nucleotides such as 8-hydroxy-2′-deoxyguanosine (8-OHdG) (6). Supporting this notion, it was reported that anti-oxidant molecules prevent UVB-induced epidermal DNA damage (7). Thus, mechanisms that protect the epidermis from both UVB and ROS are important to establish ways in which to suppress photodamage efficiently.

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.[3] 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—Paraformaldehyde, 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 IκB-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-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (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-215LM 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, geranylgeranylacetone; HSP, heat shock protein; IL, interleukin; IκB-α, an inhibitor of NF-κB; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; MTT, 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-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; RT, reverse transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

[3] T. Hoshino, M. Matsuda, Y. Yamashita, M. Takehara, M. Fukuya, K. Mineda, D. Maji, H. Ihn, Y. Funasaka, and T. Mizushima, unpublished data.

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% (IκB-α) 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′-tggtgctgacgaagatgaag-3′ (forward) and 5′-aggtcgaagatgagcacgtt-3′ (reverse); *il-1β*, 5′-gatcccaagcaatacccaaa-3′ (forward) and 5′-ggggaactctgcagactcaa-3′ (reverse); *il-6*, 5′-ctggagtcacagaaggagtgg-3′ (forward) and 5′-ggtttgccgagtagatctcaa-3′ (reverse); monocyte chemoattractant protein-1 (*mcp-1*), 5′-ctcacctgctgctactcattc-3′ (forward) and 5′-gcttgaggtggttgtggaaaa-3′ (reverse); macrophage inflammatory protein-2 (*mip-2*), 5′-accctgccaagggttgacttc-3′ (forward) and 5′-ggcacatcaggtacgatccag-3′ (reverse); and *gapdh*, 5′-aactttggcattgtggaagg-3′ (forward) and 5′-acacattgggggtaggaaca-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 microscope (Olympus). The intensity of 8-OHdG staining in the epidermis was measured by LuminaVision (Mitani).

For TUNEL assay, sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with terminal nucleotidyltransferase 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% CO_2 at 37 °C. Transfection of PAM212 cells with pcDNA3.1 containing the *hsp70* gene (33) was carried out using Lipofectamine (TM2000) 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-G_1) 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/cm^2 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. 1*C*). 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. 2*A*). 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. 2*B*). The overexpression of HSP70 in transgenic mice did not affect the background level of MPO activity (Fig. 2*B*). These results show that UVB-induced

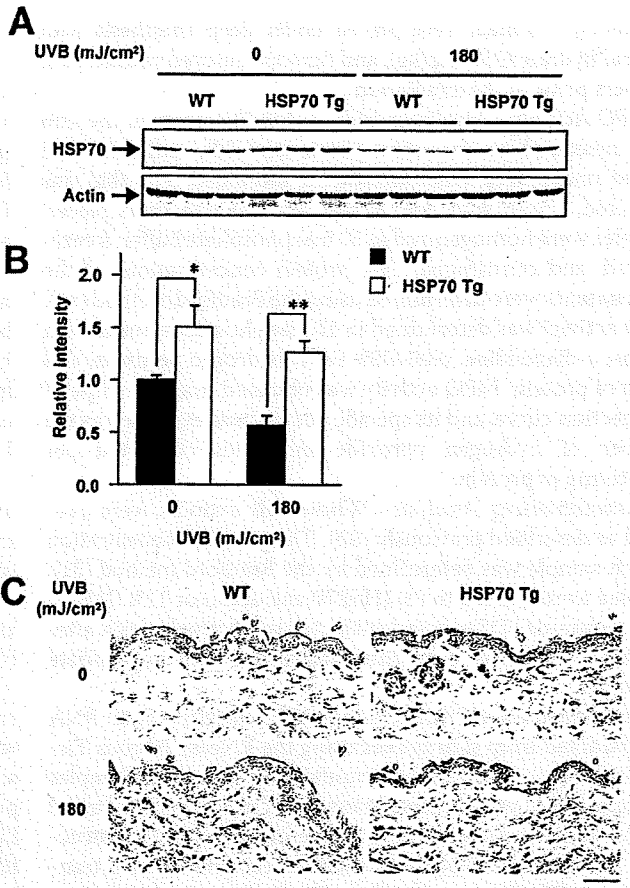

FIGURE 1. **Expression of HSP70 in the dorsal skin after UVB irradiation.** Transgenic mice expressing HSP70 (*HSP70 Tg*) and wild-type mice (*WT*) were irradiated with or without 180 mJ/cm^2 UVB, and the dorsal skin was removed after 24 h. *A*, whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin. *B*, the band intensity of HSP70 was determined and expressed relative to the control sample ($n = 6$) (one of two gels is shown in *panel A*). Values are mean ± S.E. **, $p < 0.01$; *, $p < 0.05$. *C*, sections of dorsal skin were prepared and subjected to immunohistochemical analysis with an antibody against HSP70. *Brown* staining indicates HSP70 expression. *Scale bar*, 50 μm.

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

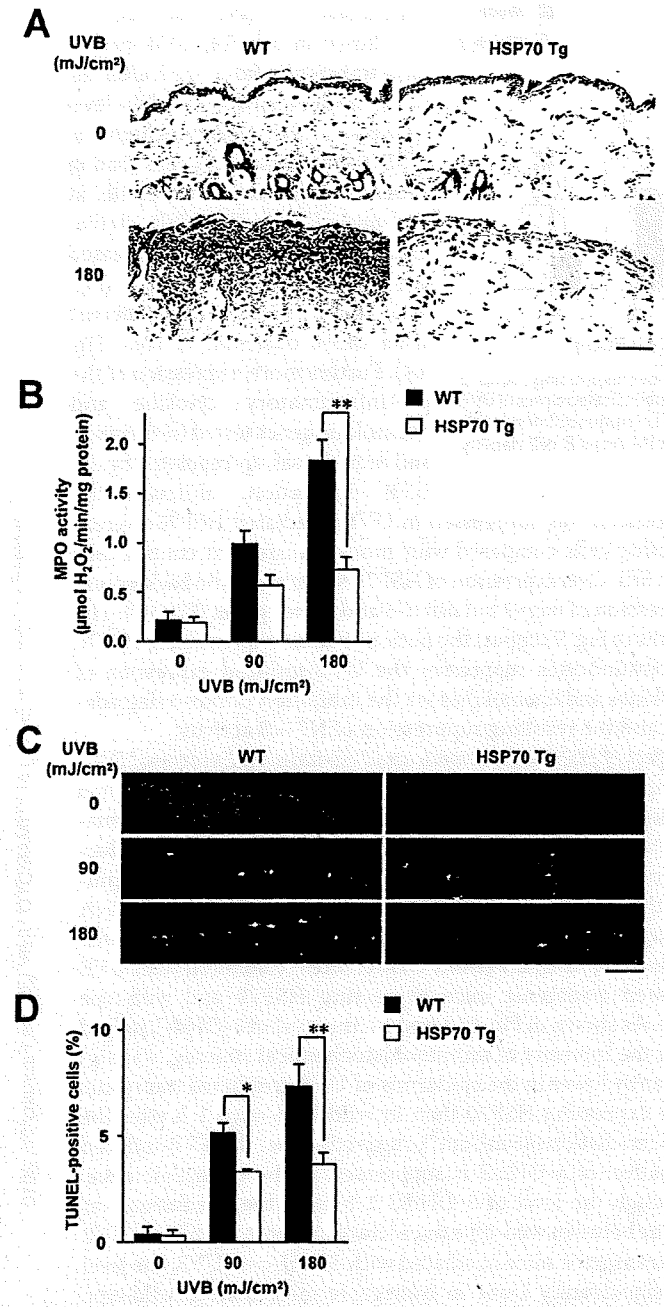

FIGURE 2. **UVB-induced skin damage and apoptosis in wild-type mice and transgenic mice expressing HSP70.** Transgenic mice expressing HSP70 (*HSP70 Tg*) and wild-type mice (*WT*) were irradiated with or without the indicated doses of UVB, and the dorsal skin was removed after 48 h (*A*), 24 h (*B*), 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.

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 geranylgeranylacetone (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).

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. 3*A*, 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. 3*A*). Exposure of cells to UVB irradiation decreased cell viability in a dose-dependent manner; this effect was suppressed in HSP70-overexpressing cells (Fig. 3*B*). To detect UVB-induced apoptosis, we counted cells in sub-G_1 (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 2*A*). The expression of HSP70 in transgenic mice did not affect the background levels of mRNA expression (Table 2*A*). Similar results were observed for the protein levels of cytokines (IL-1β and IL-6) determined by enzyme-linked immunosorbent assay (Table 2*B*). The results in Fig. 4 and Table 2 suggest that expression of HSP70 in the skin suppresses the UVB-induced expression of cytokines and chemokines via the inhibition of IκB-α degradation and the resulting suppression of NF-κB activity.

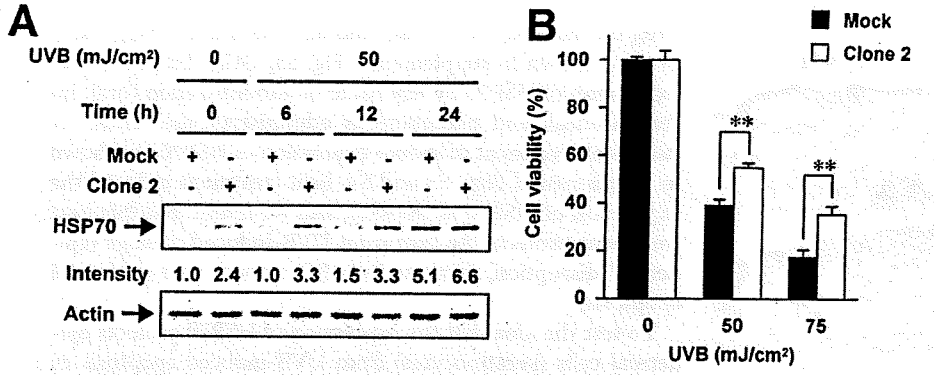

FIGURE 3. **Effect of HSP70 expression on UVB-induced apoptosis *in vitro*.** HSP70-overexpressing PAM212 cells (*Clone 2*) and mock transfectant control cells (*Mock*) were irradiated with or without indicated doses of UVB and cultured for indicated periods (*A*) or 24 h (*B*). *A*, whole cell extracts were subjected to immunoblotting with an antibody against HSP70 or actin. The relative HSP70 band intensity is shown *under the band*. *B*, cell viability was determined by the MTT method. Values are mean ± S.E. (n = 3). **, $p < 0.01$.

TABLE 1

Effect of HSP70 expression on UVB-induced apoptosis *in vitro*

HSP70-overexpressing PAM212 cells (Clone 2 in Fig. 3) and mock transfectant control cells (Mock) were irradiated with or without indicated doses of UVB and cultured for 24 h. Apoptotic cells (cells in sub-G_1) were counted by fluorescence-activated cell sorting. Caspase-3-like activity was measured. Values are mean ± S.E. (n = 3).

UVB	Cells in sub-G_1		Caspase-3-like activity	
	Mock	Clone 2	Mock	Clone 2
mJ/cm²	*%*		*Units/mg protein*	
0	2.8 ± 0.37	1.6 ± 0.24	14.2 ± 2.5	27.6 ± 5.7
50	19.2 ± 0.26	10.3 ± 0.18[a]	762.9 ± 19.0	601.7 ± 29.3[a]
75	65.2 ± 1.40	24.4 ± 0.28[a]	1316.9 ± 7.4	700.7 ± 12.6[a]

[a] $p < 0.01$.

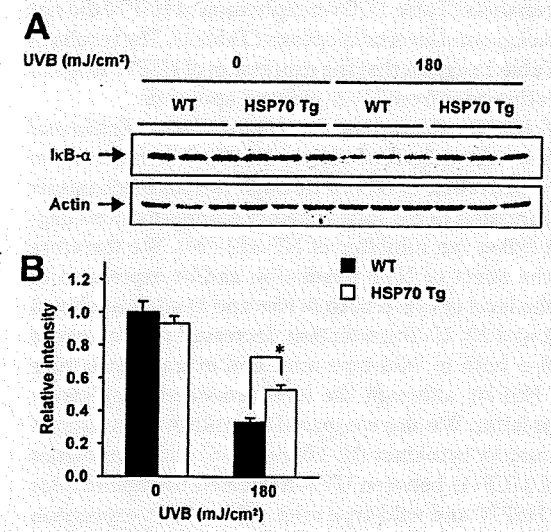

FIGURE 4. **UVB-induced decrease in the level of IκB-α.** Transgenic mice expressing HSP70 (*HSP70 Tg*) and wild-type mice (*WT*) were irradiated with or without 180 mJ/cm² UVB. *A*, the dorsal skin was removed after 48 h, and whole cell extracts were analyzed by immunoblotting with an antibody against IκB-α or actin. *B*, the band intensity of IκB-α was determined and expressed relative to the control sample (one of two gels is shown in *panel A*). Values are mean ± S.E. (n = 6). *, $p < 0.05$.

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. 5*A*, 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. 5*A*), these results being different from those observed *in vivo* (Fig. 4*A*). 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. 5*B*). Overexpression of HSP70 suppressed the background expression of *mcp-1* but not *il-6* and *mip-2* genes (Fig. 5*B*). 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 mJ/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 mJ/cm² UVB and transgenic mice irradiated with 55 mJ/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 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