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 mJ/cm^2), 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 mJ/cm^2 UVB and mock transfectant control cells irradiated with 50 mJ/cm^2 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. 8*A*, 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 $\alpha^N = 14.91 \pm 0.08$ G and $\alpha_b^H = 2.45 \pm 0.04$ 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. 8*B*, 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

Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with or without 180 mJ/cm^2 UVB. In A, the dorsal skin was removed after 12 h (*mip-2*), 24 h (*il-6*, *mcp-1*), or 48 h (*il-1β*), and total RNA was extracted. Samples were subjected to real-time RT-PCR using a specific primer set for each gene. Values were normalized to the *gapdh* gene, expressed relative to the control sample. In B, the dorsal skin was removed after 48 h and skin homogenates were prepared. The amount of IL-1β and IL-6 was determined by using enzyme-linked immunosorbent assay. Values are mean ± S.E. (n = 6–9).

	UVB (mJ/cm^2)	WT	HSP70 Tg
A) Relative expression			
il-1β	0	1.0 ± 0.11	1.6 ± 0.31
	180	24.7 ± 9.19	3.4 ± 0.10[a]
il-6	0	1.0 ± 0.17	0.48 ± 0.072
	180	10.6 ± 1.66	5.6 ± 0.92[a]
mip-2	0	1.0 ± 0.89	1.8 ± 1.62
	180	16.7 ± 1.13	8.3 ± 0.55[b]
mcp-1	0	1.0 ± 0.46	1.5 ± 0.32
	180	36.7 ± 0.40	15.1 ± 6.00[a]
B) ng/g tissue			
IL-1β	0	0.48 ± 0.12	0.84 ± 0.14
	180	27.9 ± 2.71	8.7 ± 1.00[a]
IL-6	0	1.2 ± 0.37	0.87 ± 0.19
	180	67.4 ± 1.66	36.4 ± 5.93[a]

[a] $p < 0.01$.
[b] $p < 0.05$.

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 therapeutic applicability of 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

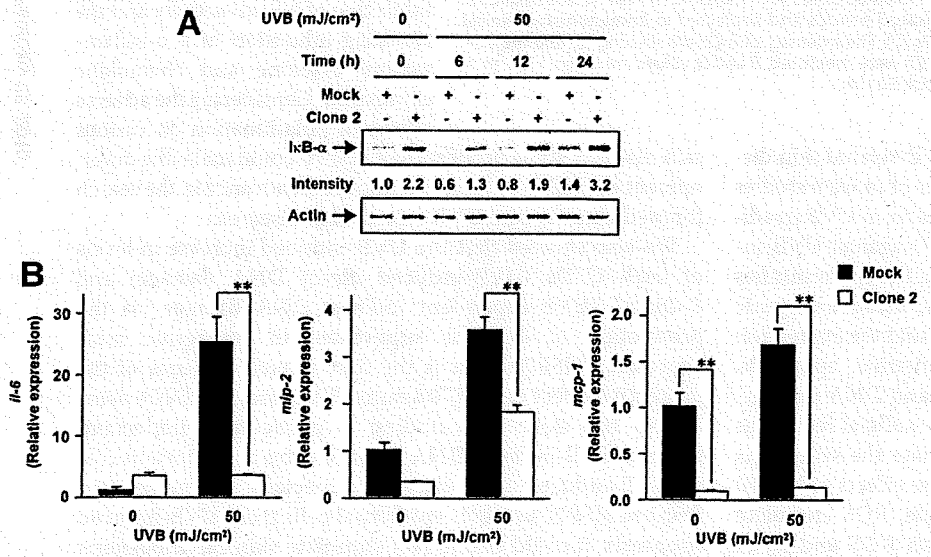

FIGURE 5. **Effect of HSP70 expression on UVB-induced decrease in the level of IκB-α and expression of pro-inflammatory cytokines and chemokines *in vitro*.** HSP70-overexpressing PAM212 cells (*Clone 2*) and mock transfectant control cells (*Mock*) were irradiated with or without 50 mJ/cm^2 UVB and incubated for indicated periods (*A*) or 6 h (*mip-2*), 12 h (*il-6*), or 24 h (*mcp-1*) (*B*). *A*, the expression of IκB-α was estimated by immunoblotting and shown as described in the legend of Fig. 3. *B*, mRNA expression of each gene was monitored as described in the legend of Table 2. Values are mean ± S.E. (n = 3). **, $p < 0.01$.

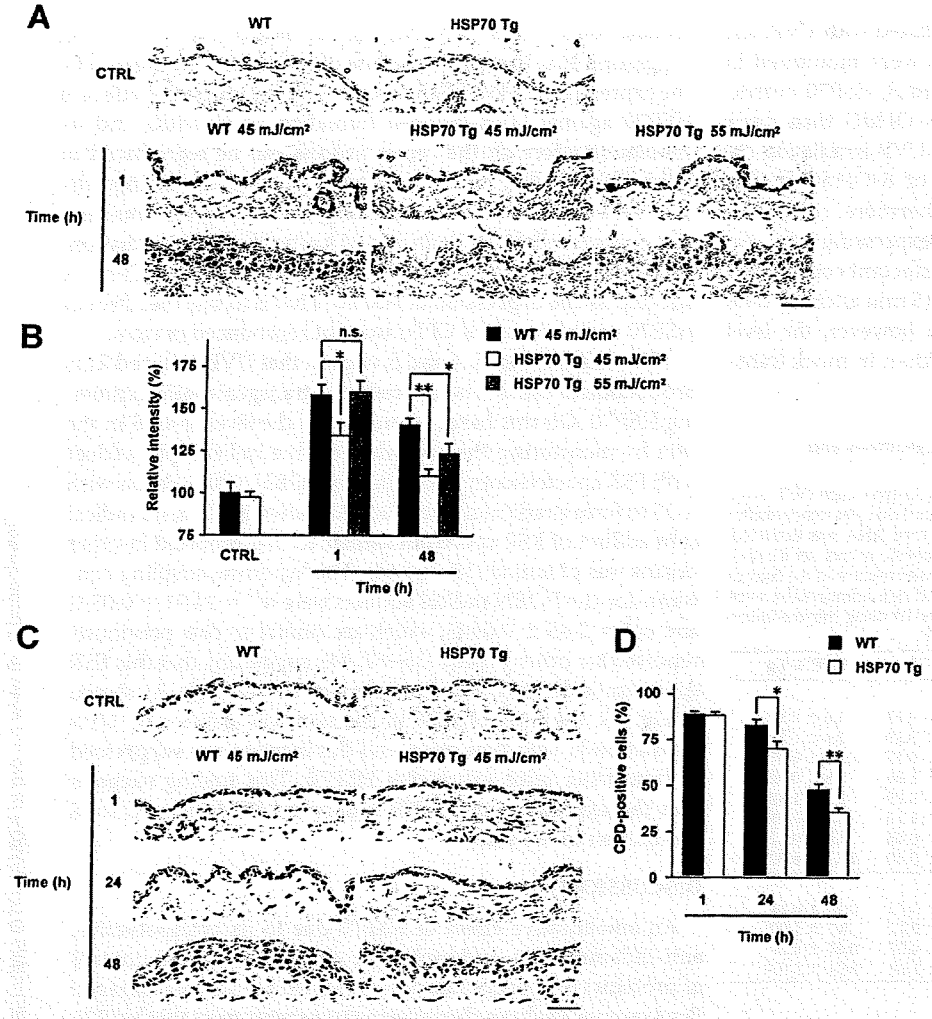

FIGURE 6. **UVB-induced epidermal DNA damage.** Transgenic mice expressing HSP70 (*HSP70 Tg*) and wild-type mice (*WT*) were irradiated with or without (*CTRL*) indicated doses (*A* and *B*) or 45 mJ/cm^2 (*C* and *D*) of UVB. Sections of the dorsal skin were prepared after indicated periods and subjected 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.

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.

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 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

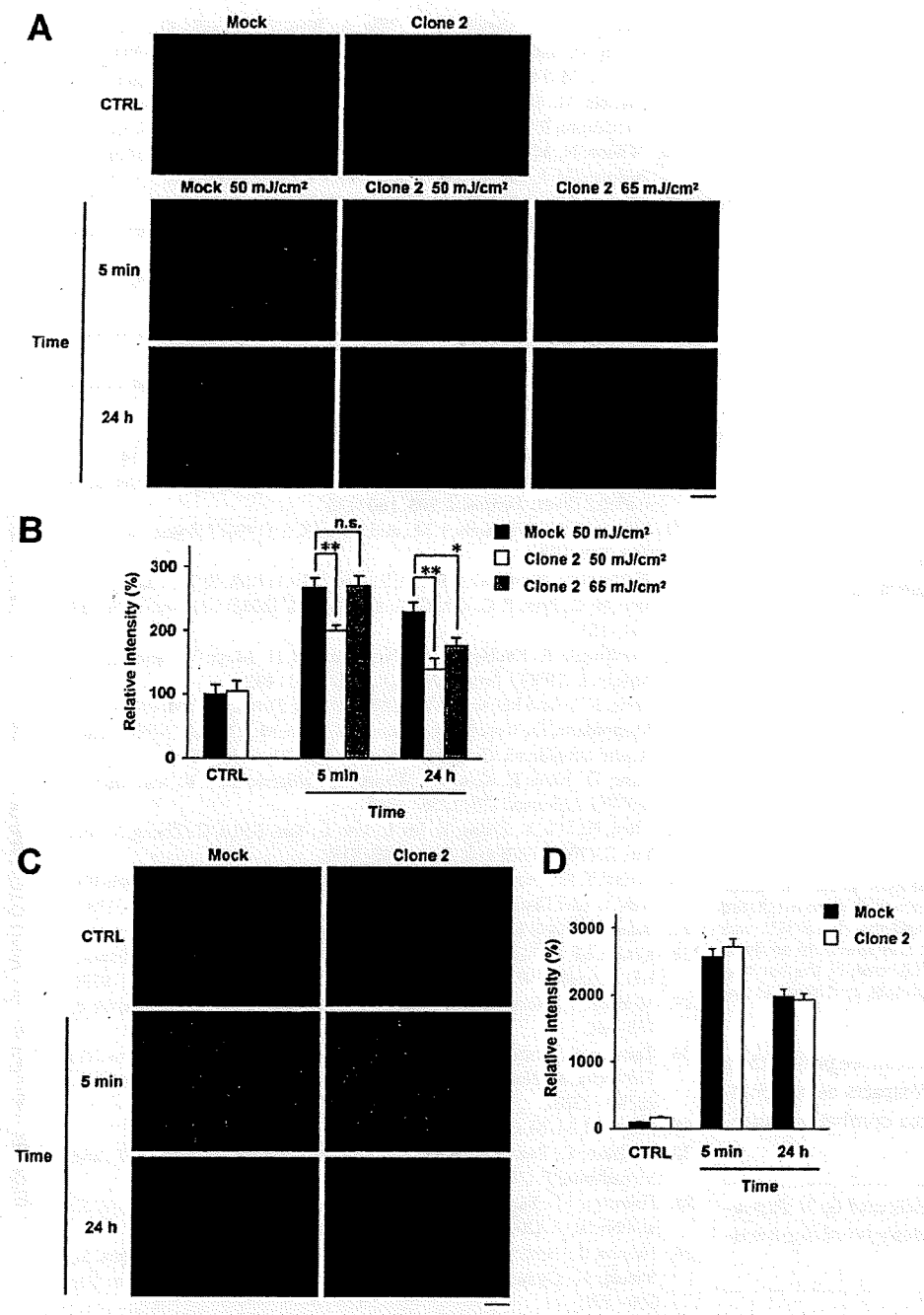

FIGURE 7. **Effect HSP70 expression on UVB-induced DNA damage *in vitro*.** HSP70-overexpressing PAM212 cells (*Clone 2*) and mock transfectant control cells (*Mock*) were irradiated with or without (*CTRL*) indicated doses (*A* and *B*) or 50 mJ/cm² (*C* and *D*) of UVB and cultured for indicated periods. 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.

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,[3] 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

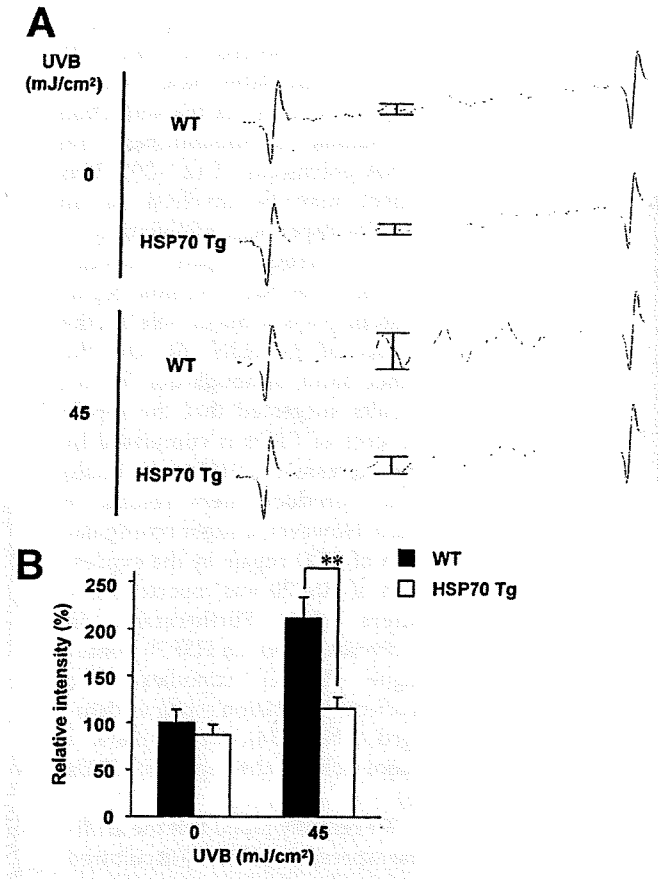

FIGURE 8. **UVB-induced increase in the epidermal ROS level.** Transgenic mice expressing HSP70 (*HSP70 Tg*) and wild-type mice (*WT*) were irradiated with 45 mJ/cm² UVB. *A*, POBN was administered, and the dorsal skin was removed after 1 h and subjected to radical adduct ESR spectrum analysis. *B*, 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. ($n = 6–10$). **, $p < 0.01$.

found that their HSP70-inducing activities were more potent than GGA.[4] We hope to develop some of these extracts as hypopigmenting (whitening) cosmetics or as drugs to combat melanin-related diseases.

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Therapeutic effect of lecithinized superoxide dismutase (PC-SOD) on bleomycin-induced pulmonary fibrosis

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Running head: PC-SOD and Bleomycin–induced Fibrosis

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is thought to involve inflammatory infiltration of leukocytes, lung injury induced by reactive oxygen species (ROS), in particular superoxide anion, and fibrosis (collagen deposition). No treatment has been shown to improve definitively the prognosis for IPF patients. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified by catalase. Lecithinized SOD (PC-SOD) has overcome clinical limitations of SOD, including low tissue affinity and low stability in plasma. In this study, we examined the effect of PC-SOD on bleomycin-induced pulmonary fibrosis. Severity of the bleomycin-induced fibrosis in mice was assessed by various methods, including determination of hydroxyproline levels in lung tissue. Intravenous administration of PC-SOD suppressed the bleomycin-induced increase in the number of leukocytes in bronchoalveolar lavage fluid. Bleomycin-induced collagen deposition and increased hydroxyproline levels in the lung were also suppressed in animals treated with PC-SOD, suggesting that PC-SOD suppresses bleomycin-induced pulmonary fibrosis. The dose-response profile of PC-SOD was bell-shaped, but concurrent

administration of catalase restored the ameliorative effect at high doses of PC-SOD. Intratracheal administration or inhalation of PC-SOD also attenuated the bleomycin-induced inflammatory response and fibrosis. The bell-shaped dose-response profile of PC-SOD was not observed for these routes of administration. We consider that, compared to intravenous administration, inhalation of PC-SOD may be a more therapeutically beneficial route of administration due to the higher safety and quality of life of the patient treated with this drug.

Keywords: PC-SOD, bleomycin, idiopathic pulmonary fibrosis, reactive oxygen species, superoxide dismutase

Abbreviations: BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; DMBA, 4-(Dimethylamino)-benzaldehyde; EMT, epithelial-mesenchymal transition; H & E, hematoxylin and eosin; IPF, idiopathic pulmonary fibrosis; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; PC, phosphatidylcholine; PC-SOD, lecithinized superoxide dismutase; QOL, quality of life; ROS, reactive oxygen species; SOD, superoxide dismutase; SP-A, surfactant protein-A; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UC, ulcerative colitis.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition with poor prognosis; the mean length of survival from the time of diagnosis is 2.8-4.2 years. IPF progresses insidiously and slowly, and acute exacerbation of IPF is a highly lethal clinical event (1, 4, 21, 36). Current agents for the treatment of IPF, such as steroids and immunosuppressors have not been found to improve the prognosis (1, 2, 26, 47), thus requiring the development of new types of drugs to treat IPF. To evaluate candidate drugs, the bleomycin-induced pulmonary fibrosis animal model provides a convenient option for the study (33).

Although the etiology of IPF is not yet fully understood, recent studies have suggested that it is triggered by lung injury and inflammation (infiltration of leukocytes (such as alveolar macrophages, lymphocytes and neutrophils) and activation of cytokines). Reactive oxygen species (ROS) that are released from the activated leukocytes cause further lung injury and inflammation. On the other hand, ROS and activated cytokines, especially TGF-β1, stimulate abnormal fibrosis (abnormal wound repair and remodelling), that is characterized by collagen deposition (22, 40). TGF-β1

seems to stimulate the production of interstitial collagen through both activation of fibroblasts and transformation of epithelial cells to fibroblasts (epithelial-mesenchymal transition, EMT) (3, 6, 48). This abnormal process of fibrosis is responsible for the pulmonary dysfunction associated with IPF. Supporting this idea, genetic inhibition of neutrophil elastase, of the TGF-β1-dependent signal transduction pathway, or of collagen synthesis was reported to suppress the progress of bleomycin-induced pulmonary fibrosis (5, 9, 14, 52). However, it is not clear whether pharmacological inhibition of these factors can improve the prognosis for IPF in humans.

A number of previous studies have suggested that the cellular redox state, determined by the balance between ROS (such as the superoxide anion) and antioxidant molecules (such as superoxide dismutase (SOD) and glutathione), plays an important role in the pathogenesis of IPF. Pulmonary inflammatory cells prepared from IPF patients generated higher level of ROS than those from controls (25, 45). An increase in the level of ROS was reported in pulmonary tissues, blood and bronchoalveolar lavage fluid (BALF) of IPF patients and bleomycin-administered animals (8, 18, 38, 41). Genetic modulation that increases or decreases the pulmonary level of ROS resulted in

stimulation or suppression, respectively, of bleomycin-induced pulmonary fibrosis (11, 29). Thus, antioxidant molecules have attracted considerable attention as therapeutic candidates for the treatment of IPF. In fact, administration of N-acetylcysteine (NAC), which stimulates the synthesis of glutathione, exhibited therapeutic effects on IPF patients and bleomycin-induced pulmonary fibrosis in animals (10, 30, 31, 39).

SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified to oxygen and water by catalase or glutathione peroxidase (23). A decreased level of SOD was observed both in IPF patients and in animals with bleomycin-induced pulmonary fibrosis (37, 53), thus suggesting that increasing SOD could be of therapeutic benefit in the treatment of IPF. However, the low affinity of SOD to the cell membrane where superoxide anion is produced, and its low stability in plasma, with a half-life of only a few minutes, were obstacles to the application of SOD in a clinical setting (13, 16, 17, 46). As a result of this, various SOD drug delivery systems have been devised to help overcome these limitations (16, 17, 20, 51).

Among these applications, lecithinized SOD (PC-SOD) has potentially beneficial effects for the treatment of IPF. PC-SOD is lecithinized human Cu/Zn-SOD

in which four phosphatidylcholine (PC) derivative molecules are covalently bound to each SOD dimer (17). *In vitro* experiments with cultured cells have shown that this modification drastically improves the cell membrane affinity of SOD without decreasing its activity (16, 17), while *in vivo* experiments have demonstrated that it also greatly improves plasma stability (17). In phase I clinical study, intravenously administered PC-SOD (40-160 mg) had a terminal half-life of more than 24 h, with good safety and tolerability (7, 42) and recently published results of a phase II clinical study have shown that intravenously administered PC-SOD (40 or 80 mg) significantly improved the symptoms of patients of ulcerative colitis (UC), which also involves ROS-induced tissue damage (43). Furthermore, intravenously administered PC-SOD ameliorated bleomycin-induced pulmonary fibrosis in mouse (44, 50), suggesting that PC-SOD could be effective in the treatment of IPF patients. However, a bell-shaped dose-response profile of PC-SOD has been reported for its ameliorative effect against bleomycin-induced pulmonary fibrosis (44, 50). Furthermore, when considering the quality of life (QOL) of patients, the present clinical protocol of PC-SOD administration (daily intravenous infusion for 4 weeks) is expected to be improved. In this study, we

provide evidence that the ineffectiveness of higher doses of PC-SOD is due to the accumulation of hydrogen peroxide. Furthermore, based on the results obtained here, we propose that administration of PC-SOD by inhalation is a clinically viable option to improve the QOL of IPF patients treated with this drug.

MATERIALS AND METHODS

Chemicals and animals.

Paraformaldehyde, FBS, catalase from bovine liver (1340 U/mg), an antibody against human Cu/Zn-SOD, 4-(Dimethylamino)-benzaldehyde (DMBA), chloramine T, potassium dichromate, phosphotungstic acid, phosphomolybdic acid, Orange G and acid fuchsin were obtained from Sigma (St. Louis, MO). Bleomycin was from Nippon Kayaku (Tokyo, Japan). Novo-Heparin (5000 units) for injection was from Mochida Pharmaceutical Co. (Tokyo, Japan). Chloral hydrate was from Nacalai Tesque (Kyoto, Japan). Diff-Quik was from the Sysmex Corporation (Kobe, Japan). Terminal deoxynucleotidyl transferase was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). An ELISA kit for TGF-β1 was from R&D systems, Inc. (Minneapolis, MN). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). Cytospin® 4 was purchased from Thermo Electron Corporation (Massachusetts, USA), while L-hydroxyproline, sodium acetate, TCA, azophloxin, aniline blue were from WAKO

Pure Chemicals (Tokyo, Japan). Xylidine ponceau was from WALDECK GmbH & Co. KG, DIVISION CHROMA (Muenster, Germany), and Mayer's hematoxylin, 1% eosin alcohol solution, mounting medium for histological examination (malinol) and Weigert's iron hematoxylin were from MUTO Pure Chemicals (Tokyo, Japan). PC-SOD (3000 U/mg) was from our laboratory stock (17). DAPI was from Dojindo (Kumamoto, Japan). Wild-type mice (6-8 weeks old, ICR, male) were used. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

Administration of bleomycin, PC-SOD and catalase.

ICR mice maintained under anesthesia with chloral hydrate (500 mg/kg) were given one intratracheal injection of bleomycin (5 mg/kg) in PBS (1 ml/kg) by use of micropipette (p200) to induce an inflammatory response and fibrosis. PC-SOD and catalase were dissolved in 5% xylitol and administered intravenously (tail vein) or intratracheally.

For control mice, 5% xylitol solution was administered. The first administration of PC-SOD was performed just before the bleomycin administration.

For administration of PC-SOD by inhalation, 5 mice were placed in a chamber (volume, 45 L) and maintained under normoxic and normocapnic conditions. PC-SOD was dissolved in 10 ml of 5% xylitol and an ultrasonic nebulizer (NE-U17 from Omron, Tokyo, Japan) that was connected to the chamber, was used to nebulize the entire volume of the PC-SOD solution in 30 min. For control mice, 5% xylitol solution was subjected to nebulizer. Mice were kept in the chamber for a further 10 min after the 30 min of nebulizing.

Preparation of BALF and cell count.

BALF was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 units/ml heparin (two times). About 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a hemocytometer. Cells were stained with Diff-Quik reagents and the ratios of alveolar

macrophages, lymphocytes and neutrophils to total cells were determined. More than 100 cells were counted for each sample.

Histological and immunohistochemical analyses and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

Lung tissue samples were fixed in 4% buffered paraformaldehyde, then embedded in paraffin before being cut into 4 μm-thick sections.

For histological examination, sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For staining of collagen (Masson's trichrome staining), sections were sequentially treated with solution A (5% (w/v) potassium dichromate and 5% (w/v) trichloroacetic acid), Weigert's iron hematoxylin, solution B (1.25% (w/v) phosphotungstic acid and 1.25% (w/v) phosphomolybdic acid), 0.75% (w/v) Orange G solution, solution C (0.12% (w/v) xylidine ponceau, 0.04% (w/v) acid fuchsin and 0.02% (w/v) azophloxin), 2.5% (w/v) phosphotungstic acid, and finally Aniline Blue