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Heat shock protein 70 protects against bleomycin-induced pulmonary fibrosis in mice

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) involves infiltration of leucocytes, pulmonary injury, fibrosis and resulting pulmonary dysfunction. Myofibroblasts and transforming growth factor (TGF)-β1 have been suggested to play a major role in the pathology and the myofibroblasts are derived from both lung epithelial cells through epithelial–mesenchymal transition (EMT) and activation of lung fibroblasts. Heat shock protein 70 (HSP70) confers protection against various stressors and has the anti-inflammatory activity. In this study, we examined the effect of expression of HSP70 on bleomycin-induced pulmonary fibrosis in mice, a tentative animal model of IPF. Bleomycin-induced pulmonary injury and inflammatory response were ameliorated in transgenic mice overexpressing HSP70 compared to wild-type mice, even though bleomycin-induced pulmonary fibrosis and dysfunction were also suppressed in the transgenic mice. The production of TGF-β1 and expression of pro-inflammatory cytokines was lower in cells from the transgenic mice than wild-type mice after the administration of bleomycin. *In vitro*, the suppression of HSP70 expression stimulated TGF-β1-induced EMT-like phenotypes of epithelial cells but did not affect the TGF-β1-dependent activation of fibroblasts. Orally administered geranylgeranylacetone (GGA), a clinically used drug with HSP-inducing activity, conferred protection against bleomycin-induced pulmonary injury, as well as against the inflammatory response, fibrosis and dysfunction. These results suggest that HSP70 plays a protective role against bleomycin-induced pulmonary injury, inflammation, fibrosis and dysfunction through cytoprotective effects and by inhibiting the production of TGF-β1, TGF-β1-dependent EMT of epithelial cells and expression of pro-inflammatory cytokines. Results also suggest that HSP70-inducing drugs, such as GGA, could be beneficial in the prophylaxis of IPF.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition with poor prognosis; the reported mean length of survival from the time of diagnosis ranges from 2.8 to 4.2 years. IPF progresses insidiously and slowly, and acute exacerbation of IPF is a highly lethal clinical event [1,2]. As current agents for the treatment of IPF, such as steroids and immunosuppressors, have not been found to improve the prognosis [1,3,4], the development of new types of drugs to treat IPF is therefore required. To evaluate candidate drugs, the bleomycin-induced pulmonary fibrosis animal model provides a convenient option for the study of the disease given that it shares some characteristic features with IPF [5].

Although the etiology of IPF is not yet fully understood, recent studies have suggested that lung injury, inflammation (infiltration of leukocytes and activation of pro-inflammatory cytokines) and transforming growth factor (TGF)-β1 play an important role in IPF. Reactive oxygen species (ROS) that are released from the activated leukocytes cause further lung injury and inflammation. An increase in the number of lung myofibroblasts, an intermediate cell type between fibroblasts and smooth muscle cells, has been suggested to play an important role in the abnormal fibrosis and collagen deposition due to abnormal wound repair and remodelling; to this extent myofibroblasts produce considerable amounts of extracellular matrix components, such as collagen [6,7]. This abnormal process of fibrosis is responsible for the pulmonary dysfunction associated with IPF. It was previously believed that the origin of myofibroblasts was solely peribronchiolar and that perivascular fibroblasts transdifferentiate to myofibroblasts in response to various stimuli, in particular TGF-β1 [8]. However, recently it was revealed that lung epithelial cells undergo epithelial–mesenchymal transition (EMT) to become myofibroblasts after treatment with TGF-β1 *in vitro* [9–11]. EMT of lung epithelial cells also seems to be induced in the lungs of IPF patients and bleomycin-treated animals [9,12–14]. Furthermore, inhibition of EMT by knockout of

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the *integrin-α3* gene has been shown to suppress bleomycin-induced pulmonary fibrosis in mice [15]. Therefore, drugs that inhibit the production of TGF-β1 and/or the EMT of lung epithelial cells could potentially be therapeutically beneficial against IPF.

Different stressors induce cells to express heat shock proteins (HSPs). The expression of HSPs, especially HSP70, in cultured cells protects these cells against a range of stressors, including ROS [16]. Interestingly, geranylgeranylacetone (GGA), a leading anti-ulcer drug on the Japanese market, has been reported to be a non-toxic HSP-inducer [17]. In addition to the cytoprotective effects of HSP70, its anti-inflammatory effects have been identified recently [18]. Thus, it is reasonable to speculate that HSP70 could protect against lung diseases such as acute respiratory distress syndrome (ARDS) and IPF. In fact, induction of the expression of HSP70 at the lung by whole-body heat treatment or by adenovirus with the *hsp70* gene protected against lipopolysaccharide (LPS)-induced or cecal ligation and puncture (CLP)-induced lung damage, respectively, both of which are animal models of ARDS [19–21]. Furthermore, HSP70-deficient mice were reported to be sensitive to CLP-induced lung damage [22]. However, the effect of HSP70 expression on IPF-related fibrosis has not been tested. In this study, we show that bleomycin-induced lung injury, inflammation, fibrosis and dysfunction were suppressed in transgenic mice overexpressing HSP70. Furthermore, orally administered GGA conferred protection against these bleomycin-induced pulmonary alterations, suggesting that HSP70-inducing drugs could be beneficial against prophylaxis of IPF.

2. Materials and methods

2.1. Chemicals and animals

Paraformaldehyde, fetal bovine serum (FBS), 4-(Dimethylamino)-benzaldehyde (DMBA), chloramine T, potassium dichromate, phosphotungstic acid, phosphomolybdic acid, Orange G and acid fuchsin were obtained from Sigma (St. Louis, MO). Antibodies against HSP25, HSP47, HSP60, HSP70 (for immunohistochemical analysis), HSP90 or α-smooth muscle actin (α-SMA) were purchased from Stressgen (Ann Arbor, MI) or Abcam (Cambridge, Cambridgeshire). Antibodies against actin and E-cadherin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An ELISA kit for TGF-β1 and an antibody against HSP70 (for immunoblotting) were from R&D systems, Inc. (Minneapolis, MN). Bleomycin was from Nippon Kayaku (Tokyo, Japan). Novoheparin (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 (TdTase) was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP, Alexa Fluor 594 goat anti-rabbit immunoglobulin G and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). Cytospin® 4 was purchased from Thermo Electron Corporation (MA, USA), while quercetin, TGF-β1, L-hydroxyproline, sodium acetate, trichloroacetic acid (TCA), azophloxin and 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). 4,6-Diamino-2-phenylindole (DAPI) was from Dojindo (Kumamoto, Japan). Transgenic mice overexpressing 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 [23]. Homozygotic transgenic mice overexpressing HSP70 were used in experiments. 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 Institutes of Health, and were approved by the Animal Care Committee of Kumamoto University.

2.2. Administration of bleomycin and preparation of bronchoalveolar lavage fluid (BALF) and cell count

Mice were maintained under anaesthesia with chloral hydrate (500 mg/kg) and were given one intratracheal injection of bleomycin (5 mg/kg) to induce an inflammatory response and fibrosis.

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 200 cells were counted for each sample.

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

Lung tissue samples were fixed in 4% buffered paraformaldehyde and 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 (Tokyo, Japan).

For staining of collagen (Masson's trichrome staining), sections were treated sequentially 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 solution. Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For immunohistochemical analysis, sections were treated with 20 μg/ml Protease K for antigen activation and incubated with 0.3% hydrogen peroxide in methanol for removal of endogenous peroxidase. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with an antibody against HSP70 (1:200 dilution) in the presence of 2.5% bovine serum albumin (BSA) and then incubated for 1 h with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins. Then, 3,3′-diaminobenzidine was applied to the sections and the sections were finally incubated with Mayer's hematoxylin. Samples were mounted with malinol and inspected using a fluorescence microscope (Olympus BX51).

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

2.4. Hydroxyproline determination

Hydroxyproline content was determined as described [24]. Briefly, the right lung was removed and homogenized in 0.5 ml of 5% TCA. After centrifugation, pellets were hydrolysed in 0.5 ml of

10 N HCl for 16 h at 110 °C. Each sample was incubated for 20 min at room temperature after the addition of 0.5 ml of 1.4% (w/v) chloramine T solution and then incubated at 65 °C for 10 min after the addition of 0.5 ml of Ehrlich's reagent (1 M DMBA, 70% (v/v) isopropanol and 30% (v/v) perchloric acid). Absorbance was measured at 550 nm, and the amount of hydroxyproline was determined.

2.5. Real-time RT-PCR analysis

Real-time RT-PCR was performed as previously described [25] with some modifications. Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument (Bio-Rad Laboratories, Hercules, CA)) experiments using iQ SYBR GREEN Supermix, and analyzed with Opticon Monitor Software. 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, actin cDNA was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were (name: forward primer, reverse primer): for human, *col1a1*: 5′-ccctgtctgcttcctgtaaact-3′, 5′-catgttcggttggtcaaagata-3′; *E-cadherin*: 5′-tgcccagaaaatgaaaaagg-3′, 5′-gtgtatgtggcaatgcgttc-3′; *hsp47*: 5′-ccatgttcttcaagccacact-3′, 5′-cgtagtagttgtagaggcctgt-3′; *hsp70*: 5′-aggccaacaagatcaccatc-3′, 5′-tcgtcctccgctttgtactt-3′; *slug*: 5′-gagcatttgcagacaggtca-3′, 5′-acagcagccagattcctcat-3′; *α-sma*: 5′-catcatgcgtctggatctgg-3′, 5′-ggacaatctcacgctcagca-3′; *actin*: 5′-ggacttcgagcaagagatgg-3′, 5′- agcactgtgttggcgtacag-3′. For mouse, *tumor necrosis factor (tnf)-α*: 5′-cgtcagccgatttgctatct-3′, 5′-cggactccgcaaagtctaag-3′; *interleukin (il)-1β*: 5′-gatcccaagcaatacccaaa-3′, 5′-ggggaactctgcagactcaa-3′; *il-6*: 5′-ctggagtcacagaaggagtgg-3′, 5′-ggtttgccgagtagatctcaa-3′; *gapdh*: 5′-aactttggcattgtggaagg-3′, 5′-acacattgggggtaggaaca-3′.

The amount of TGF-β1 in the lung tissue was also measured by ELISA according to the manufacturer's protocol.

2.6. Analysis of lung function

Analysis of lung function was performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ, Montreal, Canada), as described previously [26]. Mice were anesthetized with chloral hydrate (500 mg/kg), tracheotomised with an 8 mm section of metallic tubing, and mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2–3 cm H_2O. The single-compartment model (snap shot) and the constant-phase model (forced oscillation technique (FOT)) were applied to analyse lung function. Total respiratory system compliance or total respiratory system elastance and tissue elastance were measured by snap shot or FOT, respectively. All data were analysed using FlexiVent software (version 5.3).

2.7. Cell culture and immunostaining

A549 cells were cultured in DMEM medium supplemented with 10% FBS in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C.

For immunostaining, A549 cells were grown in the Lab-Tek II chamber slide system (Nalge Nunc International, Rochester, NY). Cells were fixed in 4% buffered paraformaldehyde for 20 min, blocked with goat serum for 15 min, then incubated for 1 h with antibody against E-cadherin or α-SMA in the presence of 2.5% bovine serum albumin, before finally being incubated for 2 h with Alexa Fluor 488 goat anti-mouse IgG. Samples were mounted with VECTASHIELD. Images were captured on a fluorescence microscope (Olympus BX51).

2.8. Statistical analysis

All values are expressed as the mean ± S.E.M. 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. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. Effect of HSP70 on bleomycin-induced pulmonary damage and fibrosis

Pulmonary fibrosis was induced in wild-type mice and transgenic mice overexpressing HSP70 (mice that overexpress human HSP70 under the control of the human β-actin promoter [27]) that had been given a once-only (at day 0) intratracheal administration of bleomycin. First, we monitored the expression of HSPs in lung tissues by immunoblotting. As shown in Fig. 1A and B, the transgenic mice showed higher levels of expression of HSP70 in the lung than did wild-type mice in the presence and absence of bleomycin treatment. There was no clear difference in the expression of HSPs other than HSP70 between transgenic mice and wild-type mice (Fig. 1A and B). We used an antibody against human HSP70 for immunoblotting. Since HSP70 has a high homology (more than 90%) between mouse and human, it may cross-react to mouse HSP70. Results in Fig. 1A support this notion. However, the relative efficiency of recognition for mouse HSP to that for human HP70 has not examined, thus we could not compare the extent of expression of HSP70 between wild-type mice and the transgenic mice. The administration of bleomycin increased the expression of HSP47 as described previously [28], but did not affect the expression of other HSPs, including HSP70 (Fig. 1A and B).

The bleomycin-induced inflammatory response can be monitored as a function of the number of inflammatory cells (alveolar macrophages, lymphocytes and neutrophils) in BALF 3 days after the administration of bleomycin. As shown in Fig. 2A, the total number of inflammatory cells and individual numbers of alveolar macrophages, lymphocytes and neutrophils were all increased by the bleomycin treatment. Compared to wild-type mice, this increase was suppressed in transgenic mice overexpressing HSP70, although differences in the numbers of alveolar macrophages and lymphocytes were not statistically significant between wild-type and transgenic mice in response to bleomycin treatment (Fig. 2A). Histopathological analysis of pulmonary tissues with the aid of hematoxylin and eosin (H & E) staining revealed that the bleomycin-induced pulmonary damage was ameliorated in transgenic mice overexpressing HSP70 compared to wild-type mice (Fig. 2B). Furthermore, the bleomycin-induced increase in the number of TUNEL-positive cells in pulmonary tissues (indicative of apoptosis) was also suppressed in transgenic mice overexpressing HSP70 (Fig. 2C and D). These results suggest that expression of HSP70 suppresses the bleomycin-induced pulmonary inflammatory response and tissue damage.

Bleomycin-induced pulmonary fibrosis can be monitored by histopathological analysis and measurement of hydroxyproline levels (an indicator of collagen levels), 14 days after the administration of bleomycin. Masson's trichrome staining of collagen showed that bleomycin administration induced collagen deposition in wild-type mice and that the extent of this deposition was suppressed in transgenic mice overexpressing HSP70 (Fig. 3A).

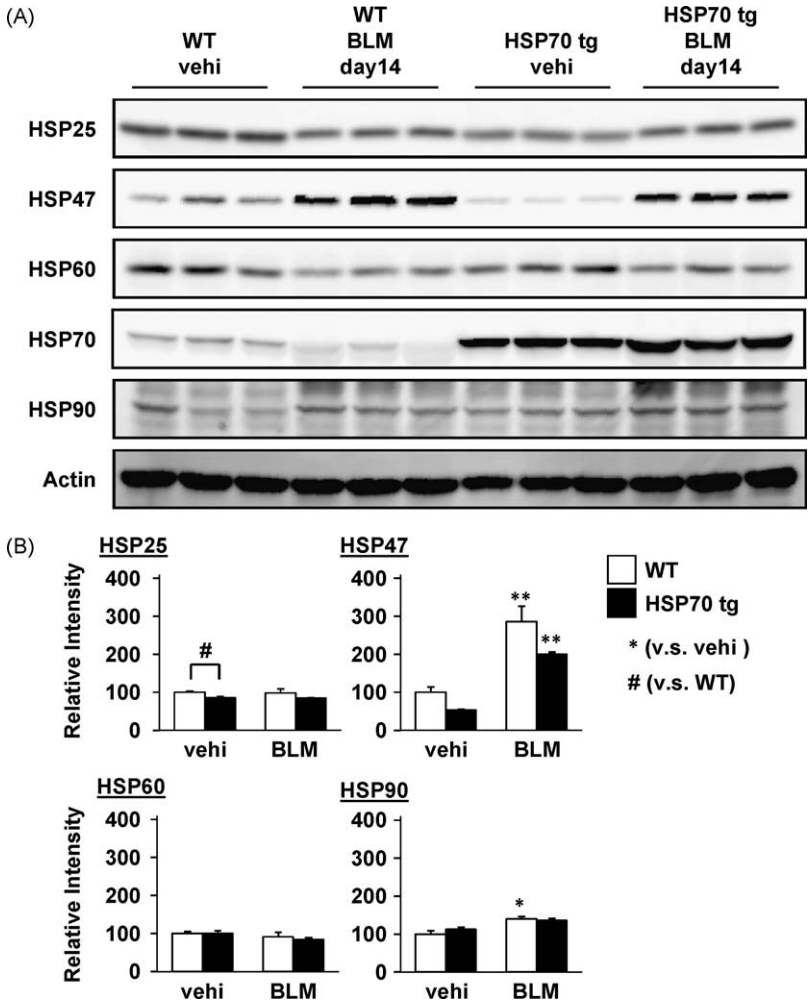

Fig. 1. Expression of HSPs in lung tissues after administration of bleomycin. Transgenic mice overexpressing HSP70 (HSP70 tg) and wild-type (WT) mice were treated with (BLM) or without (vehi) bleomycin (5 mg/kg) once-only at day 0. At day 14, lung tissues were removed and protein was extracted. Samples were analysed by immunoblotting with an antibody against HSP25, HSP47, HSP60, HSP70, HSP90 or actin (A). The band intensity of each HSP (except HSP70) was determined and normalized with its respective actin intensity (B). Values shown are mean ± S.E.M. ($n = 3$). * or $^{\#}P < 0.05$; ** or $^{\#\#}P < 0.01$.

H & E staining revealed that a low level of bleomycin-induced pulmonary damage in transgenic mice was evident 14 days after the administration of bleomycin (Fig. 3A). Bleomycin also increased pulmonary hydroxyproline levels, although to a lesser degree in transgenic mice compared to wild-type mice (Fig. 3B). These results suggest that bleomycin-induced pulmonary fibrosis is decreased by the expression of HSP70.

Myofibroblasts was monitored by immunohistochemical analysis with an antibody against α-SMA, a myofibroblast marker. As shown in Fig. 3C, a bleomycin-dependent increase in the expression of α-SMA was suppressed in transgenic mice overexpressing HSP70 compared to wild-type mice, suggesting that expression of HSP70 also suppresses the bleomycin-dependent increase in pulmonary myofibroblast number.

In humans, pulmonary fibrosis leads to an alteration in lung mechanics characterised by a decrease in compliance and an increase in elastance [26,29]. We thus examined the effect of HSP70 expression on bleomycin-induced alterations to lung mechanics, using a computer-controlled small-animal ventilator. Total respiratory system compliance was decreased as a consequence of bleomycin treatment in wild-type mice and this index was significantly higher in bleomycin-administered transgenic mice overexpressing HSP70 than bleomycin-administered wild-type mice (Fig. 3D). Total respiratory system elastance (elastance of total lung including bronchi, bronchiole and alveoli) and tissue elastance (elastance of alveoli) were increased in response to bleomycin treatment in wild-type mice, and these indexes were significantly lower in similarly treated transgenic mice overexpressing HSP70 (Fig. 3D). These results suggest that bleomycin-induced pulmonary dysfunction is improved under conditions of HSP70 expression.

3.2. Mechanism for the inhibitory effect of HSP70 on bleomycin-induced pulmonary fibrosis

As described above, TGF-β1 plays an important role in bleomycin-induced pulmonary fibrosis. Thus, as a first step to reveal the mechanism underlying the inhibitory effect of HSP70 on bleomycin-induced pulmonary fibrosis, the production of TGF-β1 in cells contained in BALF was compared between bleomycin-administered wild-type mice and transgenic mice overexpressing HSP70. As shown in Fig. 4A, the level of TGF-β1 production was lower in cells prepared from BALF of transgenic mice compared to wild-type mice, both with and without prior bleomycin treatment. As such, bleomycin enhanced the production of TGF-β1 in cells from BALF of wild-type mice but not in those from the transgenic mice (Fig. 4A).

We also compared the bleomycin-induced expression of pro-inflammatory cytokines in cells present in BALF. As shown in Fig. 4B, the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) was clearly induced by the administration of

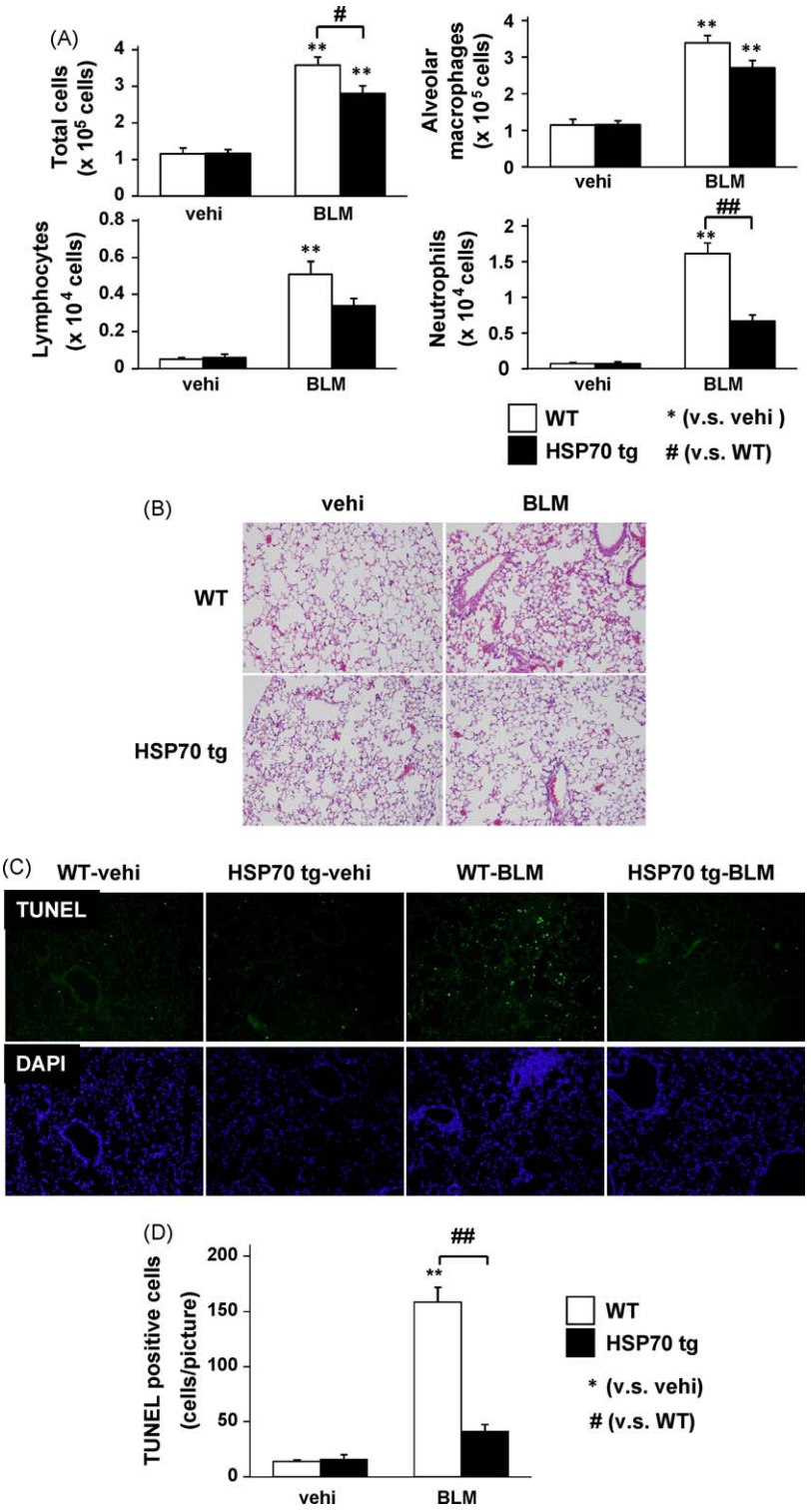

Fig. 2. Bleomycin-induced pulmonary inflammatory response and damage in transgenic mice overexpressing HSP70. Transgenic mice overexpressing HSP70 (HSP70 tg) and wild-type (WT) mice were treated with (BLM) or without (vehi) bleomycin (5 mg/kg) once-only at day 0. At day 3, total cell number, and individual numbers of alveolar macrophages, lymphocytes and neutrophils in BALF were determined as described in Section 2 (A). Sections of pulmonary tissue were prepared at day 3 and subjected to histopathological examination (H & E staining) (B) or TUNEL assay and DAPI staining (C). TUNEL-positive cells in the three sections were counted (D). Values shown are mean ± S.E.M. (n = 3–13). $^{\#}P < 0.05$; ** or $^{\#\#}P < 0.01$.

bleomycin and this expression was lower in cells in BALF prepared from the transgenic mice compared to wild-type mice. The results in Fig. 4 suggest that the decreased production of TGF-β1 and expression of pro-inflammatory cytokines in bleomycin-treated transgenic mice overexpressing HSP70 is responsible for their resistance to bleomycin-induced pulmonary fibrosis.

As described in Section 1, an increase in the number of pulmonary myofibroblasts associated with fibrosis is due to the stimulation of EMT of epithelial cells and the activation of fibroblasts. Thus, the results in Fig. 3C suggest that the TGF-β1-dependent EMT of lung epithelial cells and/or activation of fibroblasts are/is suppressed by the expression of HSP70. To test

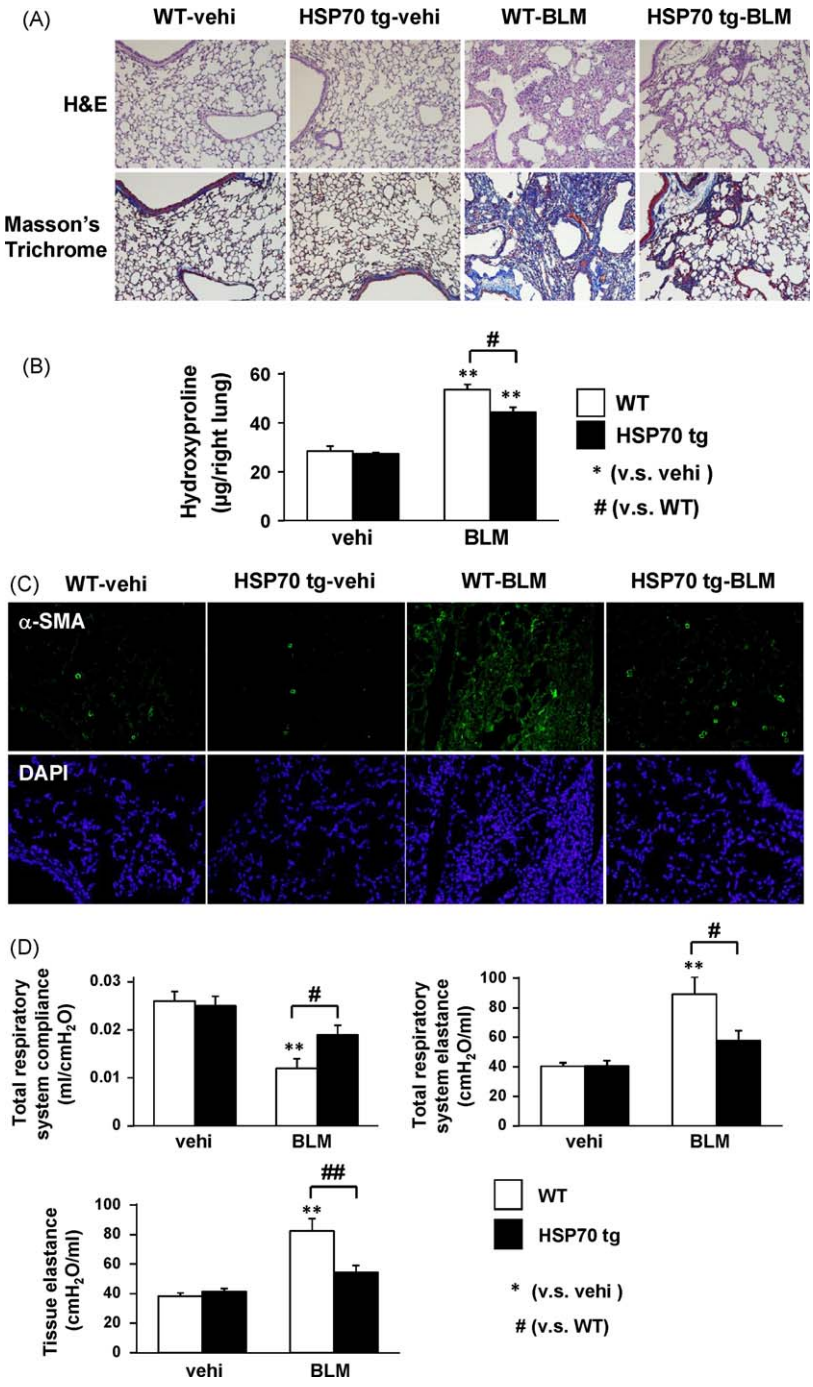

Fig. 3. Bleomycin-induced pulmonary fibrosis and dysfunction in transgenic mice overexpressing HSP70. Transgenic mice overexpressing HSP70 (HSP70 tg) and wild-type (WT) mice were treated with (BLM) or without (vehi) bleomycin (5 mg/kg) once-only at day 0. At day 14, sections of pulmonary tissue were prepared (A and C). The sections were subjected to histopathological examination (H & E staining and Masson's trichrome staining) (A). The pulmonary hydroxyproline level was determined at day 14 as described in Section 2 (B). The sections were subjected to immunohistochemical analysis with an antibody against α-SMA (C). At day 14, total respiratory system compliance, total respiratory system elastance and tissue elastance were determined as described in Section 2 (D). Values shown are mean ± S.E.M. (n = 3–7). $^{\#}P < 0.05$; ** or $^{\#\#}P < 0.01$.

this idea *in vitro*, we examined the effect of siRNA for HSP70 (we could not overexpress HSP70 by plasmids under the conditions) on the TGF-β1-dependent alteration of expression of EMT-related genes in cultured human type II alveolar (A549) cells. Treatment of cells with TGF-β1 down-regulated the expression of a marker of epithelial cells (*E-cadherin*) and up-regulated the expression of a marker of myofibroblasts (*col1a1* (one of the genes for collagen I)) (Fig. 5A), suggesting that TGF-β1-induced the EMT of A549 cells. The down-regulation of expression of E-cadherin by TGF-β1 was also observed at protein level (Fig. 5B). TGF-β1 did not alter the expression of *α-sma* mRNA under these experimental conditions (Fig. 5A). Transfection of cells with siRNA for HSP70 not only suppressed the expression of *hsp70* mRNA and HSP70 protein in the presence and absence of TGF-β1 but also suppressed the expression of *E-cadherin* mRNA and E-cadherin protein and induced the expression of *α-sma* and *col1a1* mRNAs in the presence of TGF-β1 (Fig. 5A and B). The siRNA also affected the background expression of *E-cadherin* and *α-sma* mRNAs and E-cadherin protein (Fig. 5A and B). Suppression or induction of expression of E-cadherin and α-SMA, respectively, by either treatment with TGF-β1 or transfection with siRNA for HSP70 was also confirmed by immunostaining analysis (Fig. 5C and D). As

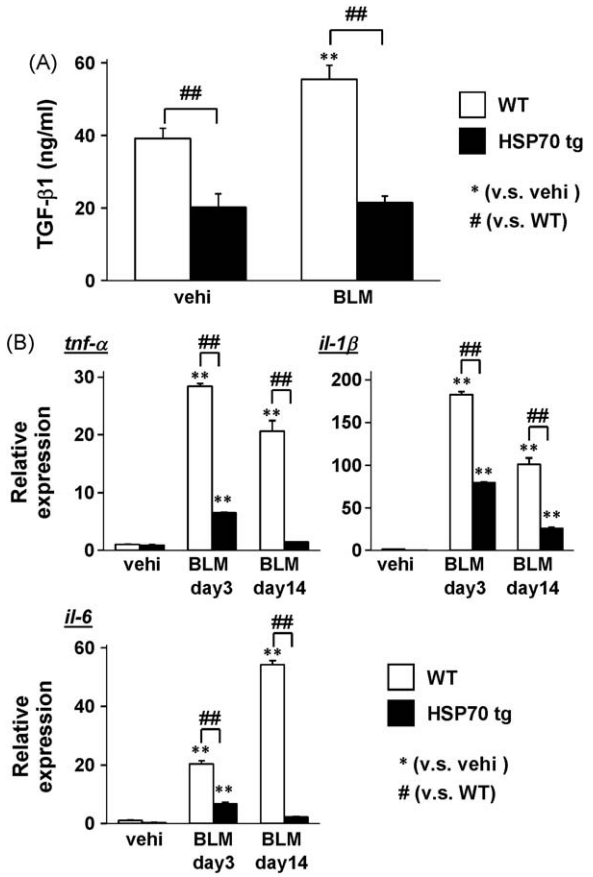

Fig. 4. Effect of expression of HSP70 on production of TGF-β1 and expression of pro-inflammatory cytokines. Transgenic mice overexpressing HSP70 (HSP70 tg) and wild-type (WT) mice were treated with (BLM) or without (vehi) bleomycin (5 mg/kg) once-only at day 0 and cells in BALF were collected at day 3 (A and B). Cells were incubated for 24 h and the level of TGF-β1 in the culture medium was determined by ELISA (A). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer set for each gene. Values were normalized to the *gapdh* gene and expressed relative to the control sample (B). Values shown are mean ± S.E.M. (n = 3–6). ** or ## $P < 0.01$.

shown in Fig. 5E, either treatment with TGF-β1 or transfection with siRNA for HSP70 induced morphological change (from a cobble-stone-like epithelial monolayer to dispersed spindle-shaped mesenchymal cells with reduced cell-cell contact), one of characteristic features of induction of EMT.

A number of transcription factors (such as Slug) are involved in the induction of EMT [30,31]. We found that the transfection of cells with siRNA for HSP70 enhanced the expression of *slug* mRNA in the presence and absence of TGF-β1 (Fig. 5A). The results in Fig. 5 suggest that the expression of HSP70 suppresses the bleomycin-induced EMT of A549 cells by suppressing the expression of Slug.

We also examined the effect of HSP70 expression on the TGF-β1-dependent activation of lung fibroblasts *in vitro*. Treatment of HFL-I cells (human embryonic lung fibroblasts) with TGF-β1-induced the expression of *col1a1*, *α-sma* and *hsp47* mRNAs and collagen I protein (Fig. 6A and B), suggesting that TGF-β1 activated fibroblasts to myofibroblasts. The transfection of cells with siRNA for HSP70 did not affect the TGF-β1-dependent alteration in expression of these genes (Fig. 6A and B), suggesting that the expression of HSP70 did not affect the TGF-β1-dependent activation of fibroblasts.

3.3. Effect of GGA on bleomycin-induced pulmonary fibrosis

We subsequently examined the effect of GGA on bleomycin-induced pulmonary damage, inflammatory response, fibrosis and dysfunction. Oral administration of GGA suppressed the bleomycin-induced increase in the number of inflammatory cells present in BALF (Fig. 7A), and decreased pulmonary damage (Fig. 7B) and epithelial apoptosis (Fig. 7C and D) 3 days after the administration of bleomycin. Immunohistochemical analysis with an antibody against HSP70 revealed that treatment with bleomycin slightly induced the pulmonary expression of HSP70, and the simultaneous administration of GGA enhanced this expression (Fig. 7E). The extent of bleomycin-induced pulmonary fibrosis 14 days after the administration of bleomycin was also reduced by the administration of GGA; GGA suppressed both the bleomycin-induced collagen deposition and the increase in pulmonary hydroxyproline content (Fig. 7F and G).

We also examined the effect of GGA on bleomycin-induced alterations to lung mechanics. As shown in Fig. 7H, the bleomycin-induced decrease in total respiratory system compliance and the increase in total respiratory system elastance and tissue elastance were significantly suppressed by the administration of GGA, suggesting that GGA exerts a protective effect against bleomycin-induced lung dysfunction. Taking these findings with the results shown in Figs. 2 and 3, it is likely that GGA suppresses the negative effects of bleomycin and improves lung function by inducing HSP70 expression.

In order to test this idea, we examined the effect of quercetin, an inhibitor for HSP70, on the ameliorative effect of GGA for bleomycin-induced pulmonary fibrosis and dysfunction. As shown in Fig. 7F–H, the ameliorative effect of GGA on bleomycin-induced pulmonary fibrosis and dysfunction was not observed in the presence of simultaneous administration of quercetin, suggesting that GGA suppresses bleomycin-induced pulmonary fibrosis and dysfunction by inducing HSP70 expression.

4. Discussion

An ameliorative effect of HSP70 due to its cytoprotective, anti-inflammatory and molecular chaperone (quality control of proteins) properties has been reported for animal models of various diseases. For example, using transgenic mice over-expressing HSP70, we have reported that HSP70 protects against irritant-produced lesions in the stomach and small intestine, inflammatory bowel disease-related experimental colitis and ultraviolet (UV)-induced skin damage [23,32–35]. The potential therapeutic applicability of HSP70 for use in other diseases, such as neurodegenerative diseases, ischemia-reperfusion damage and diabetes has also been suggested [36–38]. Interestingly, GGA, an anti-ulcer drug and HSP-inducer has been reported to suppress not only gastric lesions but also lesions of the small intestine, inflammatory bowel disease-related experimental colitis and neurodegenerative diseases [32,34,37,39]. On the other hand, the role of HSP70 in IPF has not been fully evaluated. It was recently reported that oral administration of GGA suppresses bleomycin-induced pulmonary fibrosis [40]. However, because GGA induces expression of HSPs other than HSP70, and has various pharmacological activities other than induction of HSPs (such as an increase in blood flow, stimulation of surface mucus production and direct protection of cell membranes [41–43]), it remains unclear whether GGA achieves its anti-fibrotic activity through up-regulation of expression of HSP70. In this study, we used transgenic mice overexpressing HSP70 to identify the role of HSP70 in bleomycin-induced pulmonary fibrosis and found that the transgenic mice showed a phenotypic resistance to this treatment. This is the first genetic evidence of the protective role of HSP70 against IPF-related fibrosis. The results presented here also suggest that GGA achieves its anti-fibrotic activity via the up-regulation of HSP70 expression.

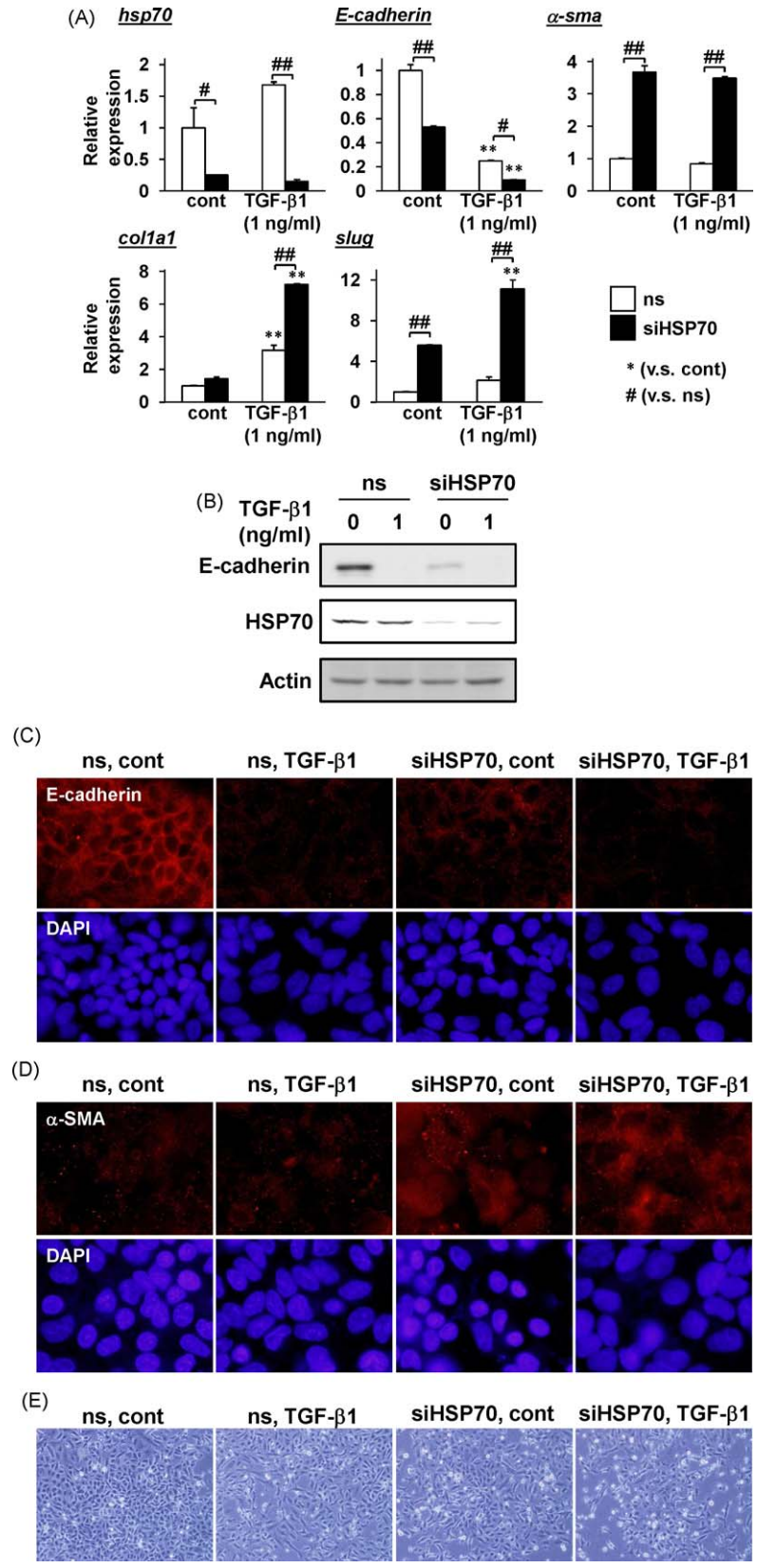

Fig. 5. Effect of siRNA for HSP70 on TGF-β1-induced EMT-like phenotypes. A549 cells were transfected with 1.2 μg of siRNA for HSP70 (siHSP70) or non-silencing (ns) siRNA and incubated for 24 h. Cells were then incubated with the indicated concentration of TGF-β1 for 48 h. Expression of each gene was examined by real-time RT-PCR as described in the legend of Fig. 4 (A). Expression of each protein was examined by immunoblotting as described in the legend of Fig. 1 (B). Immunostaining with an antibody against E-cadherin (C) or α-SMA (D) was done as described in Section 2. Cell morphology was examined by phase-contrast microscopic observation (E). Values shown are mean ± S.E.M. ($n = 3$). $^{\#}$ $P < 0.05$; ** or $^{\#\#}$ $P < 0.01$.

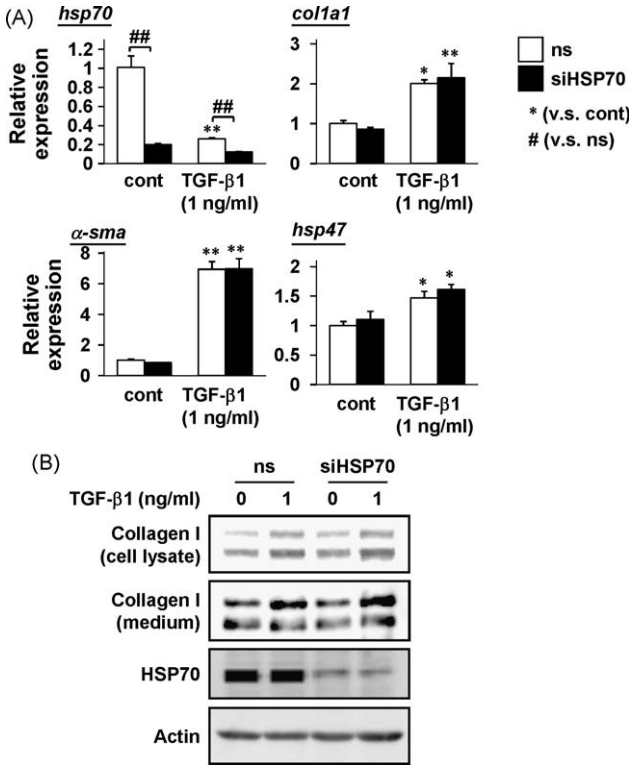

Fig. 6. Effect of siRNA for HSP70 on the TGF-β1-dependent activation of fibroblasts. HFL-I cells were transfected with 1.2 μg of siRNA for HSP70 (siHSP70) or non-silencing (ns) siRNA and incubated for 24 h. Cells were then incubated with the indicated concentrations of TGF-β1 for 24 h. Expression of each gene was examined by real-time RT-PCR as described in the legend of Fig. 4 (A). The levels of collagen I and HSP70 in cell lysate and collagen I in medium were examined by immunoblotting as described in the legend of Fig. 1 (B). Values shown are mean ± S.E.M. ($n = 3$). $^{*}P < 0.05$; ** or $^{\#\#}P < 0.01$.

Given that an increase in alveolar epithelial cell apoptosis has been observed in human IPF [44], the apoptotic process is believed to play an important role in the development of IPF. We here showed that bleomycin-induced pulmonary damage and epithelial cell apoptosis were suppressed in transgenic mice overexpressing HSP70. It is well known that HSP70 has anti-apoptotic effects through various mechanisms such as binding to apoptotic protease activating factor (Apaf)-1 to prevent the activation of caspase-9, suppression of the apoptotic pathway downstream of caspase-3 activation, and suppression of apoptosis-inducing factor-induced chromatin condensation [45–48]. We recently reported that HSP70 inhibits the activation of bcl-2-associated X protein (BAX), which is important for apoptosis-related mitochondrial dysfunction [32]. We consider that these mechanisms are involved in the HSP70-dependent suppression of bleomycin-induced lung epithelial cell apoptosis, because the activation of BAX and of caspases were reported to play an important role in bleomycin-induced apoptosis in lung epithelial cells [49,50].

In addition to this anti-apoptotic (cytoprotective) effect of HSP70, its anti-inflammatory effect was recently revealed and is thought to be important for its protective role against various diseases. HSP70 suppresses the activation of nuclear factor kappa B (NF-κB; an inflammation-inducing transcription factor) through various mechanisms such as suppression of inflammatory stimuli-induced degradation of IκB-a (an inhibitor of NF-κB) [18,51]. We previously reported that inflammatory responses (such as expression of pro-inflammatory cytokines and infiltration of leucocytes) in the stomach, colon and skin were suppressed in transgenic mice overexpressing HSP70 and that this suppression is mediated by the inhibition of NF-κB [23,32,34,35]. In this study, we showed that a bleomycin-induced increase in leucocytes in BALF and the pulmonary expression of pro-inflammatory cytokines were suppressed in transgenic mice overexpressing HSP70. We also showed that production of TGF-β1 was suppressed in bleomycin-treated transgenic mice overexpressing HSP70 compared to corresponding wild-type mice. This is the first demonstration of the inhibitory effect of HSP70 on the production of TGF-β1. It is known that TNF-α induces the expression of TGF-β1 [52] suggesting that the inhibitory effect of HSP70 on NF-κB and the resulting decrease in the level of TNF-α are responsible for this inhibitory effect of HSP70 on TGF-β1 production. Since TGF-β1 plays a major role in bleomycin-induced pulmonary fibrosis through various mechanisms such as activation of fibroblasts and stimulation of EMT of epithelial cells, the inhibitory effect of HSP70 on the production of TGF-β1 should be responsible for its protective effect against bleomycin-induced pulmonary fibrosis.

We also examined the effect of HSP70 expression on TGF-β1-dependent cellular responses involved in pulmonary fibrosis. We found that the TGF-β1-dependent induction of EMT-like phenotypes in lung epithelial cells (up-regulation of expression of markers of myofibroblasts and down-regulation of expression of markers of epithelial cells) and up-regulation of the expression of Slug (a transcription factor inducing EMT) were stimulated by the suppression of HSP70 expression. On the other hand, the TGF-β1-dependent activation of fibroblasts was not affected by the suppression of HSP70 expression. These results suggest that the expression of HSP70 suppresses the bleomycin-induced increase in lung myofibroblast number via suppression of the TGF-β1-dependent EMT of epithelial cells rather than via the activation of fibroblasts. It was recently reported that expression of HSP70 in cultured rat kidney proximal tubular epithelial cells inhibited TGF-β1-induced EMT, although the mechanism governing this inhibition is unknown [53]. Thus, it seems that expression of HSP70 generally suppresses EMT of epithelial cells. It is possible that the inhibitory effect of extracellular HSP70 on mitogen-activated protein kinases (MAPKs) [54] is involved in the inhibitory effect of HSP70 on the TGF-β1-induced EMT of epithelial cells, because MAPKs are involved in TGF-β1-dependent signal transduction pathways [55]. Furthermore, it is also possible that HSP70 achieves its inhibitory effect on EMT via the inhibition of NF-κB, because it was recently reported that NF-κB stimulates EMT via a mechanism that is independent on TGF-β1 [56].

While bleomycin-induced pulmonary fibrosis has been used as an animal model of IPF, this model does however has some limitations, such as the spontaneous resolution of fibrosis, which is rare in human IPF [57]. Furthermore, although assessment tools used in bleomycin-induced pulmonary fibrosis are primarily based on histology and quantitative collagen analysis, clinical management of IPF relies on lung function analysis. Therefore, in this study, we used a computer-controlled small-animal ventilator to monitor the bleomycin-induced decrease in compliance and increase in elastance, which are known to be associated with human IPF [58]. We found that these bleomycin-induced alterations in lung mechanics were ameliorated in transgenic mice overexpressing HSP70, suggesting that an increased expression of HSP70 could potentially improve the pulmonary dysfunction associated with human IPF.

As described above, it was recently reported that administration of GGA ameliorates bleomycin-induced pulmonary fibrosis [40], a result that we confirmed in this study. We also found that the administration of GGA improved lung function in the presence of bleomycin treatment. As described in Section 1, current agents for the treatment of IPF have not been found to improve the

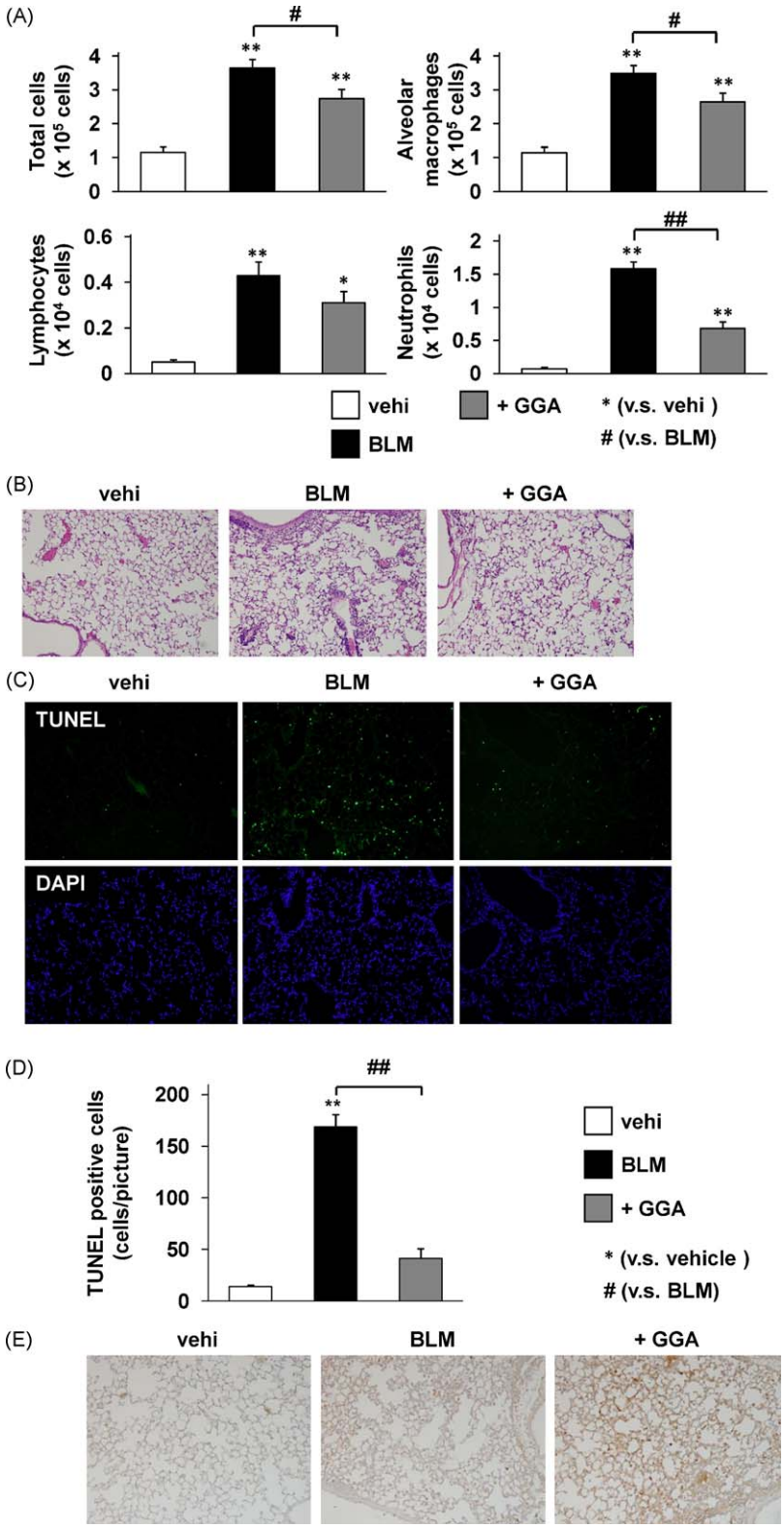

Fig. 7. Effect of oral administration of GGA on bleomycin-induced pulmonary damage, inflammatory response, fibrosis and dysfunction. C57/BL6 mice were orally administered with GGA (200 mg/kg) once per day for 3 days (A–E) or 14 days (F–H). Quercetin (200 mg/kg) was orally administered into mice 30 min before each administration of GGA (F–H). Mice were treated with (BLM) or without (vehi) bleomycin (5 mg/kg) once-only at day 0 and cells in BALF (A), pulmonary damage (B–D), pulmonary fibrosis (F and G) and lung mechanics (H) were assessed as described in the legends of Figs. 2 and 3. Immunohistochemical analysis with an antibody against HSP70 was performed as described in Section 2 (E). Values shown are mean ± S.E.M. (n = 3–12). *, $^{\#}$ or $^{\$}$ < 0.05; **, $^{\#\#}$ or $^{\$\$}P < 0.01$.

prognosis [1,3,4,59]. We consider that HSP70-inducers such as GGA could be beneficial for the prophylaxis of IPF. The development of new molecules as candidate drugs to treat this disease must pass through the clinical trials process and may encounter unanticipated side effects. Thus, based on the results of this study, we propose that clinical studies should be performed to prove the effectiveness of GGA for treating IPF given that the safety of GGA has already been shown clinically.

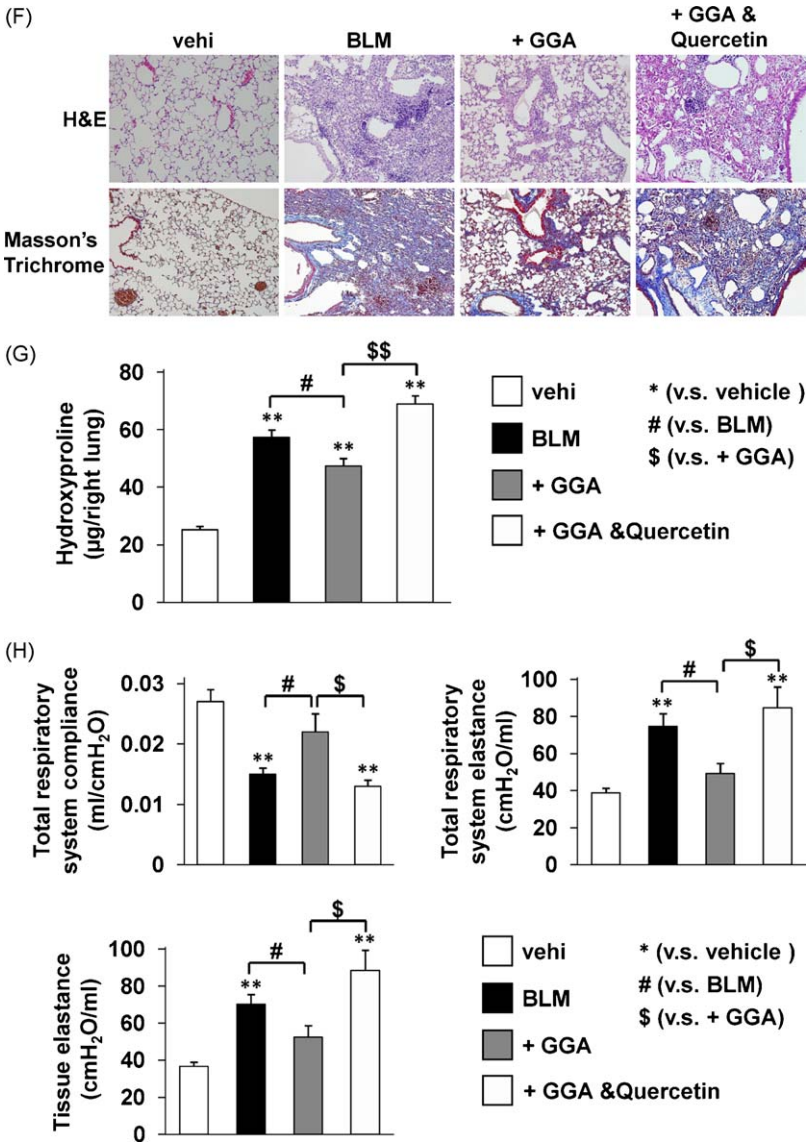

Fig. 7. (*Continued*).

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