Increased expression of collagen-binding heat shock protein 47 in murine bleomycin-induced pneumopathy

Hiroshi Ishii, Hiroshi Mukae, Tomoyuki Kagakawa, Tetsuji Iwashita, Hideyuki Kaida, Takeshi Fujii, Tomayoshi Hayashi, Jun-ichi Kadota, and Shigeru Kohno

Increased expression of collagen-binding heat shock protein 47 in murine bleomycin-induced pneumopathy. Am J Physiol Lung Cell Mol Physiol 285: L957–L963, 2003. First published July 3, 2003; 10.1152/ajplung.00305.2002.—The 47-kDa heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that has been shown to play a major role during the processing and/or secretion of procollagen. Expression of HSP47 has been reported to increase in parallel with expression of collagens during the progression of various fibrosis models. The aim of the present study was to investigate the association between HSP47 expression and collagen accumulation in bleomycin (BLM)-induced murine fibrosis. We investigated the expression of HSP47 protein and mRNA using immunohistochemical analysis and semi-quantitative RT-PCR in murine BLM-induced pulmonary fibrosis. Immunohistochemical analysis showed that higher expression of HSP47 protein was present in BLM-induced pulmonary fibrosis compared with controls. HSP47 was localized predominantly in α-smooth muscle actin-positive myofibroblasts, F4/80 negative, surfactant protein-A-positive type II pneumocytes, and F4/80-positive macrophages. RT-PCR also demonstrated an increase of HSP47 mRNA expression in BLM-treated lungs. Moreover, the relative amounts of HSP47 mRNA correlated significantly with the lung hydroxyproline content as an indicator of pulmonary fibrosis in BLM-treated lungs (r = 0.406, P < 0.05). Our results suggest that these cells may play a role in the fibrotic process of BLM-treated lungs through upregulation of HSP47.

PULMONARY FIBROSIS IS AN ENTITY comprising heterogeneous diseases. Idiopathic pulmonary fibrosis (IPF) is a representative disease with a very poor prognosis that is characterized by patchy fibrotic areas scattered among the normal parenchyma (7, 11). The fibrotic component of IPF is characterized by both a marked increase in the number of fibroblasts and deposition of fibroblast-derived extracellular matrix proteins, notably collagen (14). Increased synthesis and deposition of extracellular matrix are now suggested as an important cause of fibrosis in experimental and human lung diseases (22, 27), but the underlying molecular mechanism responsible for the excessive deposition of collagens in the fibrotic lesions is not fully understood.

The collagen-specific stress protein, 47-kDa heat shock protein (HSP47), localized in the endoplasmic reticulum, is involved in synthesis/assembly of various collagens as a collagen-specific molecular chaperone (5, 13, 25). HSP47 has been reported to play a key role in increased deposition of collagens in allograft renal tissues (1) and the peritoneum of patients on continuous ambulatory peritoneal dialysis (20). With the use of Northern blotting analysis, Masuda and colleagues (12) also showed that the level of HSP47 mRNA correlated with that of collagen type I and type III mRNAs during the progression of rat experimental liver fibrosis. Thus HSP47 has been demonstrated to be involved in fibrotic diseases. In pulmonary fibrosis, it has also been reported that HSP47 is highly expressed on myofibroblasts or fibroblasts in autopsied human pulmonary fibrosis (16) and bleomycin (BLM)-treated rats (15). Also, we previously reported that HSP47 was expressed abundantly on type II pneumocytes in addition to myofibroblasts in active fibrotic areas of usual interstitial pneumonia, which is the most common pathological form of IPF (8). Thus HSP47 seems to be important in pulmonary fibrosis, similar to its role in other fibrotic diseases, but the association between HSP47 and pulmonary fibrosis remains obscure. In the present study, we used a murine BLM-induced fibrosis model to assess the temporal changes in localization of HSP47 proteins and in expression of HSP47 mRNA during development of inflammatory and fibrotic lesions.

MATERIALS AND METHODS

Animals and BLM treatment. Male 10-wk-old C57BL/6 mice weighing 18–22 g were purchased from Charles River Japan (Yokohama, Japan). The animals were specific patho-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
gen free and maintained under standard conditions with free access to drinking water and pelleted food at the Animal Center of Biomedical Research, Nagasaki University School of Medicine. All animal procedures were reviewed and approved by the Animal Care and Use Committee, Nagasaki University. The mice were divided into two experimental groups: BLM-treated mice (n = 35; n = 5 for each time point of 1, 2, 4, 5, 6, 7, and 8 wk) and controls (n = 35; n = 5 for each time point of 1, 2, 4, 5, 6, 7, and 8 wk). BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in 200 μl of saline and administered intraperitoneally at a dose of 6 mg·kg⁻¹·day⁻¹ for 10 sequential days. For controls, age-matched mice received an identical volume of saline.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was performed (n = 5 in both groups at each time point) at 2, 4, 5, 6, 7, and 8 wk after the BLM or saline administration. The trachea was cannulated with a 25-gauge Teflon needle (Terumo, Tokyo, Japan) under deep anesthesia, an aliquot of saline (0.9% NaCl at room temperature) was injected into the lung, and 5.0 ml of the total volume was recovered. The recovered fluid was centrifuged at 700 g for 10 min to sediment the cells. After being washed twice with PBS solution, cells were suspended with PBS containing 10% heat-inactivated fetal calf serum and counted with a hemocytometer. Differential cell counts were determined from cell suspensions displayed on slides using a cytocentrifuge (Cytospin 2; Shandon, Sewickley, PA). Cells on a slide were dried, fixed, and then stained by the May-Giemsa method. Two hundred cells were identified under a photomicroscope.

Antibodies. Primary antibodies used for the immunohistochemical studies included anti-α-smooth muscle actin (α-SMA; Neomarkers, Fremont, CA), anti-surfactant protein A (SP-A; Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-SMA, SP-A, and F4/80 (Serotec) pretreated with 1% trypsin for 20 min at room temperature before use, and anti-HSP47 (Biotechnology Laboratories, Burlingame, CA) for SP-A and F4/80. Briefly, paraffin sections were deparaffinized with toluene and rinsed thoroughly with ethanol. Sections were then soaked in 0.3% H₂O₂ in methanol for 20 min at room temperature to inactivate endogenous peroxidases. They were incubated with blocking serum for 30 min and then covered with primary antibodies and incubated for 1 h. After being washed in 0.075% Brij (Sigma Chemical, St. Louis, MO) in PBS, sections were processed further using kits according to the protocols provided by the manufacturers and then developed with 3,3’-diaminobenzidine and H₂O₂. The standard Mayer’s hematoxylin staining method was used to localize HSP47 and other proteins in lung sections. For immunoperoxidase labeling, we controlled the development of the peroxidase-based color change, and all sections were developed using the same amount of time.

Measurement of lung hydroxyproline content. To estimate the total amount of collagen deposited in the lung as an indicator of pulmonary fibrosis, we measured the hydroxyproline content of the right lung of mice (n = 5 in both groups at each time point) at 1, 2, 4, 5, 6, 7, and 8 wk after the BLM or saline administration. After measurement of wet weight, a 10-mg aliquot of dried lung homogenate was hydrolyzed in 6 N HCl at 110°C for 24 h. The resulting hydrolysate was fixed by nitroson at 60°C for 20 min after being filtered through a 0.45-μm nylon membrane, dissolved in 12.5 mM sodium borate, and prepared for derivatization using fluorescamine solution. After the resulting solution was centrifuged at 600 g for 10 min, the amount of hydroxyproline in the supernatant was determined with a capillary electrophoresis system (PACE MDQ; Beckman Coulter).

Semiquantification of HSP47 mRNA by RT-PCR. RT-PCR was performed using homogenized lung tissue. For RNA isolation, the whole left lungs, which had been frozen at −80°C immediately after removal from animals in both groups, were homogenized in Isogen (Nippon Gene, Tokyo, Japan). Total RNA was extracted from the lungs (at least 3 mice in both groups at each time point) at 1, 2, 4, 5, 6, 7, and 8 wk after the BLM or saline administration. After measurement of wet weight, a 10-mg aliquot of dried lung homogenate was hydrolyzed in 6 N HCl at 110°C for 24 h. The resulting hydrolysate was fixed by nitroson at 60°C for 20 min after being filtered through a 0.45-μm nylon membrane, dissolved in 12.5 mM sodium borate, and prepared for derivatization using fluorescamine solution. After the resulting solution was centrifuged at 600 g for 10 min, the amount of hydroxyproline in the supernatant was determined with a capillary electrophoresis system (PACE MDQ; Beckman Coulter).

To confirm the localization of HSP47, immunohistochemistry was performed with the conventional avidin-biotin-peroxidase histochemical technique using Histomouse-Plus kits (Zymed Laboratories, South San Francisco, CA) for HSP47 and α-SMA and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) for SP-A and F4/80. Briefly, paraffin sections were deparaffinized with toluene and rinsed thoroughly with ethanol. Sections were then soaked in 0.3% H₂O₂ in methanol for 20 min at room temperature to inactivate endogenous peroxidases. They were incubated with blocking serum for 30 min and then covered with primary antibodies and incubated for 1 h. After being washed in 0.075% Brij (Sigma Chemical, St. Louis, MO) in PBS, sections were processed further using kits according to the protocols provided by the manufacturers and then developed with 3,3’-diaminobenzidine and H₂O₂. The standard Mayer’s hematoxylin staining method was used to localize HSP47 and other proteins in lung sections. For immunoperoxidase labeling, we controlled the development of the peroxidase-based color change, and all sections were developed using the same amount of time.

Measurement of lung hydroxyproline content. To estimate the total amount of collagen deposited in the lung as an indicator of pulmonary fibrosis, we measured the hydroxyproline content of the right lung of mice (n = 5 in both groups at each time point) at 1, 2, 4, 5, 6, 7, and 8 wk after the BLM or saline administration. After measurement of wet weight, a 10-mg aliquot of dried lung homogenate was hydrolyzed in 6 N HCl at 110°C for 24 h. The resulting hydrolysate was fixed by nitroson at 60°C for 20 min after being filtered through a 0.45-μm nylon membrane, dissolved in 12.5 mM sodium borate, and prepared for derivatization using fluorescamine solution. After the resulting solution was centrifuged at 600 g for 10 min, the amount of hydroxyproline in the supernatant was determined with a capillary electrophoresis system (PACE MDQ; Beckman Coulter).

Semiquantification of HSP47 mRNA by RT-PCR. RT-PCR was performed using homogenized lung tissue. For RNA isolation, the whole left lungs, which had been frozen at −80°C immediately after removal from animals in both groups, were homogenized in Isogen (Nippon Gene, Tokyo, Japan). Total RNA was extracted from the lungs (at least 3 mice in both groups at each time point) at 1, 2, 4, 5, 6, 7, and 8 wk after the BLM or saline administration. After measurement of wet weight, a 10-mg aliquot of dried lung homogenate was hydrolyzed in 6 N HCl at 110°C for 24 h. The resulting hydrolysate was fixed by nitroson at 60°C for 20 min after being filtered through a 0.45-μm nylon membrane, dissolved in 12.5 mM sodium borate, and prepared for derivatization using fluorescamine solution. After the resulting solution was centrifuged at 600 g for 10 min, the amount of hydroxyproline in the supernatant was determined with a capillary electrophoresis system (PACE MDQ; Beckman Coulter).

Statistical analysis. All values were expressed as means ± SD. The Mann-Whitney U-test was used to examine differences between unpaired samples. The correlation coefficient was tested for statistical significance with the Spearman’s rank test. Statistical analysis was performed using StatView-J 4.5 software (Abacus Concepts, Berkeley, CA). Significance was defined by a P value of <0.05.
RESULTS

Total and differential cell counts of BAL fluid and histological findings. We first analyzed the total cell and differential cell counts in BAL fluid between 2 and 8 wk. The mean total cell counts obtained from BLM-treated mice in overall time course \( (5.5 \pm 2.2 \times 10^5/ml, P < 0.0001) \) were significantly higher than in control mice \( (3.1 \pm 1.8 \times 10^5/ml) \). However, there was no significant difference in the differential count between BLM-treated mice \( (93 \pm 4\% \text{ macrophages and } 7 \pm 4\% \text{ lymphocytes}) \) and control mice \( (94 \pm 6\% \text{ macrophages and } 6 \pm 5\% \text{ lymphocytes}) \). In the BLM-treated group, however, both total cell counts and percentages of lymphocytes gradually decreased with time from 2 to 6 wk after BLM administration (data not shown).

Histological examination revealed no significant differences in the differential count between BLM-treated mice \( (93 \pm 4\% \text{ macrophages and } 7 \pm 4\% \text{ lymphocytes}) \) and control mice \( (94 \pm 6\% \text{ macrophages and } 6 \pm 5\% \text{ lymphocytes}) \). In the BLM-treated group, however, both total cell counts and percentages of lymphocytes gradually decreased with time from 2 to 6 wk after BLM administration (data not shown).

Histological examination revealed no significant changes in control lungs (Fig. 1A), whereas marked interstitial and intra-alveolar pneumonia and/or fibrosis were noted in the lungs between 2 and 8 wks after the BLM administration (Fig. 1B). The extent of pneumonitis/fibrosis, localized mainly in the subpleural regions, increased gradually with time from 2 to 6 wk after BLM administration. The mean fibrosis score in BLM-treated mice \( (2.28 \pm 0.96) \) was significantly higher than in control mice \( (0.94 \pm 0.73, P = 0.0002\) (Fig. 2). Collagen content was also assessed by measuring hydroxyproline in the right lung in both groups at 1, 2, 4, 5, 6, 7, and 8 wk after BLM or saline administration. The mean hydroxyproline contents for the BLM-treated mice and control mice were \( 66.0 \pm 10.7 \mu \text{mol/g} \) and \( 58.2 \pm 4.8 \mu \text{mol/g} \), respectively \( (P = 0.0003) \). There were significant differences between these two groups at 5, 6, and 7 wk after BLM or saline administration (Fig. 3).

Immunohistochemistry. We next examined the localization of HSP47 in BLM-treated lungs. Weak immunoreactive HSP47 expression was detected in airway epithelial cells and interstitial cells in control lungs (Fig. 4A). In contrast, markedly increased HSP47 immunostaining was noted in BLM-treated lungs (Fig. 4B), and the level of HSP47 expression increased microscopically with time from 2 until 6 wk after BLM administration. This increment of HSP47 paralleled the changes in fibrosis score and hydroxyproline content (Figs. 2 and 3). To determine cells expressing HSP47 in BLM-treated lungs, immunostaining for HSP47 and SP-A, F4/80, or \( \alpha\)-SMA was performed in sequential or pairs of mirror image lung sections. Around 4 wk after BLM administration, SP-A-positive cuboidal cells located in alveolar walls began to increase in fibrotic areas, and some of them were HSP47 positive. At 5 wk after treatment, the alveolar walls were frequently lined by abundant SP-A-positive type II pneumocytes (Fig. 5A), and some of them were also HSP47 positive (Fig. 5B). At the same time interval, we also noted increases of F4/80- (Fig. 5C) and HSP47- (Fig. 5D) positive macrophages in fibrotic areas. Inte-
stitial cells expressing α-SMA (Fig. 5E) and HSP47 (Fig. 5F) further increased at 6 wk after the treatment. After that period, these changes of pneumonitis/fibrosis appeared to diminish (data not shown).

Semiquantification of HSP47 mRNA. HSP47 mRNA levels in the lung tissue samples harvested at 1, 2, 4, 5, 6, 7, and 8 wk after treatment were analyzed using RT-PCR and compared between the two experimental groups. At 6 and 7 wk after treatment, the relative amounts of HSP47 mRNA were significantly higher in the BLM-treated lungs than in control lungs (Fig. 6). Moreover, the relative expression of HSP47 mRNA correlated significantly with the hydroxyproline content in BLM-treated lungs ($r = 0.406$, $P < 0.05$; Fig. 7).

DISCUSSION

Several investigators have demonstrated increased synthesis of extracellular matrix, including various collagens, during development of pulmonary fibrosis in experimental and human lung diseases (22, 27). Another morphological characteristic of fibrotic lung disorders is the presence of remodeled fibrotic alveolar walls frequently lined by abundant type II pneumo...
cytes, cuboidal and squamous metaplastic cells (9, 10). Regenerated type II pneumocytes are also thought to be associated with the fibrotic process through their acquired ability for production of cytokines, such as interleukin-8, monocyte chemoattractant protein-1, and granulocyte/macrophage colony-stimulating factor (21). In addition, many studies have demonstrated that monocytes/macrophages are also a source of mediators capable of regulating fibroblast proliferation and other functions that are responsible for the fibrogenic outcome (19). However, very little is known about the intracellular processing of collagen in those cells, and no direct evidence has been provided for the contribution of either type II pneumocytes or alveolar macrophages to pulmonary fibrosis.

In the present study, we have shown that both HSP47 immunoreactivity and mRNA were markedly induced during lung fibrosis in mice treated with BLM. The relative expression of HSP47 mRNA also correlated significantly with the hydroxyproline content, which reflects total collagen content. HSP47, which has a molecular chaperone-like function under stress conditions, is involved in the processing and transporting of procollagen in the endoplasmic reticulum (5, 13, 25). Recent reports demonstrating that expression of HSP47 correlates well with collagen expression (1, 8, 21),
12, 15–17, 20, 23) indicate that HSP47 possibly plays a key role in increased deposition of collagens during the progression of human fibrotic diseases, including pulmonary fibrosis. Previous studies have shown overexpression of HSP47 in myofibroblasts and fibroblasts in BLM-treated rats and autopsy examination of cases with various pulmonary fibrosis, along with increased deposition of types I and III collagen in fibrotic areas (15, 16). The present findings confirmed and extended these early findings by demonstrating overexpression of HSP47 at both the protein and mRNA levels. In addition, the relative expression of HSP47 mRNA correlated significantly with hydroxyproline content. These findings suggest that HSP47 may play an important role in the pathogenesis of pulmonary fibrosis, similar to other fibrotic diseases (1, 12, 20).

It is of interest to know which cells contribute to fibrogenesis in the lung. Razzaque and colleagues (15, 16) noted colocalization of collagens and HSP47 in regions of pulmonary fibrosis and that HSP47-expressing cells were found to be mainly α-SMA-positive interstitial cells (myofibroblasts) and vimentin-positive cells (fibroblasts) by double immunostaining of lung sections from autopsies of patients with various pulmonary fibrotic diseases and BLM-induced pulmonary fibrosis in rats (15, 16). In addition, we recently demonstrated that HSP47 was coexpressed with type I procollagen on both myofibroblasts and the abundant type II pneumocytes in active fibrotic areas of lung biopsy specimens from patients with IPF. We speculated that these cells play an important role in pulmonary fibrosis through HSP47-associated regulation of type I procollagen (8). In the present study using an animal model, HSP47 was localized predominantly in α-SMA-positive myofibroblasts, intra-alveolar F4/80-positive macrophages (Fig. 5), and F4/80-negative, SP-A-positive type II pneumocytes, similar to the pattern observed in human pulmonary fibrosis (8). Therefore, alveolar macrophages and type II pneumocytes, in addition to fibroblasts and myofibroblasts, may play an important role in the fibrotic process in BLM-treated lungs through the induction of HSP47. The exact mechanism of intracellular processing of collagen in those cells remains unclear. Furthermore, there is no evidence that type II pneumocytes and alveolar macrophages synthesize type I procollagen in BLM-treated lungs. Further studies are necessary to elucidate whether these cells can synthesize HSP47 and procollagen.

Pulmonary fibrosis remains a devastating clinical disorder for which there are limited therapeutic options. A number of experimental approaches have been investigated in animal models, including the inhibition of key cytokines and growth factors (3, 4, 6, 24, 26, 28), but to date none of these approaches has come to fruition in the clinic. Therapeutic intervention directed against HSP47 might alter the fibrotic process, which might be of clinical value. Experimental studies have shown that an antisense oligodeoxynucleotide against HSP47 inhibited both HSP47 production and consequently diminished the levels of α1(I) procollagen chains in vitro (18). Furthermore, in a recent in vivo study, Sunamoto et al. (23) reported that inhibition of HSP47 by antisense oligodeoxynucleotides markedly suppressed collagen accumulation and subsequently attenuated the histological manifestations in experimental glomerulonephritis. Further studies using an antisense oligonucleotide against HSP47 are under way in our laboratories to examine whether this antisense suppresses collagen accumulation in an experimental pulmonary fibrosis/pneumonitis induced by BLM-sulfate. However, it is possible that the protective effect of antisense-HSP47 is specific to BLM-induced pulmonary fibrosis, since BLM-induced pulmonary fibrosis and IPF are not the same disease.

In summary, we have demonstrated, in the present study, the role of increased expression of HSP47 mRNA and protein levels in the progression of pneumo-
monitis/fibrosis in BLM-treated lungs. Our results suggest that alveolar macrophages and type II pneumocytes, in addition to myofibroblasts, may play an important role in the fibrotic process of BLM-treated lungs through upregulation of HSP47. Further studies are required to investigate the therapeutic effects of antisense against HSP47 in BLM-treated lungs.

The authors thank Atsushi Yokoyama for excellent technical support.

REFERENCES


