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ORIGINAL ARTICLE

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Bleomycin-induced pulmonary fibrosis in rat is associated with increased expression of collagen-binding heat shock protein (HSP) 47

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Abstract Increased accumulation of collagens in extracellular matrix (ECM) is mainly responsible for bleomycin-induced pulmonary fibrosis in rats. This study was designed to assess whether increased collagen accumulation in bleomycin-induced pulmonary fibrosis is associated with heat shock protein (HSP) 47, a molecular chaperone for collagen biosynthesis. We investigated the expression of type I and type III collagens and HSP47 in bleomycin-induced pulmonary fibrosis. Fifteen male Wistar rats were divided into two groups; group I: bleomycin-induced pulmonary fibrosis; group II: PBS-treated age-matched control rats. Pulmonary fibrosis was induced by injecting a single dose of bleomycin sulphate (5 U/kg body weight) intratracheally. Three bleomycin-treated rats and two age-matched control rats were sacrificed at the end of each of the 1st, 2nd and 4th weeks of the experiment. In bleomycin-treated rats, histological examination revealed pulmonary fibrosis, which increased with time. Increased type I and type III collagen desposition was observed in the lungs of all the bleomycin-treated rats. Weak immunostaining of HSP47 was noted in the control lungs. In contrast, strong immunostaining for HSP47 was seen in all the bleomycin-treated fibrotic lungs. In addition, increased numbers of phenotypically altered myofibroblasts (α-smooth muscle actin immunopositive) and fibroblast (vimentin immunopositive) were seen in bleomycin-treated lungs and found to express HSP47. Parallel increase of collagens and their molecular chaperone HSP47 expression was found in the bleomycin-treated lungs, and their co-localization could be detected by double immunostaining. Overexpression of HSP47 may play a significant part in the excessive assembly of collagens and could contribute in this way to the fibrosis found in bleomycin-treated rat lungs.

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Introduction

Fibrosis is characterized by increased deposition of various collagens and occurs in virtually every tissue and organ system. However, despite a large literature describing the composition and morphological changes that occur in the fibrotic connective tissue, the underlying mechanisms responsible for excessive deposition of collagens in the fibrotic lesions are still poorly understood.

The newly identified collagen-specific stress protein, HSP47, localized in ER, has an important involvement in the synthesis/assembly of various collagens as a collagen-specific molecular chaperone [11–13, 17, 18]. HSP47 has been found to be involved in the sclerotic/fibrotic process in various experimental fibrotic diseases. A possible role for HSP47 in pulmonary fibrosis has not been demonstrated; nevertheless the potential exists for a pathological role of this HSP47 in modulating fibrotic changes of the lungs, since HSP47 is involved in the fibrotic process in various organs [8, 9, 15]. Although HSP47 has been shown to have a key role in increased deposition of collagens in experimental liver cirrhosis [8, 9] and glomerulosclerosis [15] in rats, it is unclear whether HSP47 has a similar role in the pulmonary fibrosis. The identification and localization of HSP47 may be critical for a better understanding of the mechanism of pulmonary fibrosis.

We investigated a possible role of HSP47 in bleomycin-induced pulmonary fibrosis in rats.

Materials and methods

Male Wistar rats (n = 15) were used for the study. All the rats were kept in an air-conditioned room (21 ± 2°C) lighted for 12 h a day (5:00 A.M. to 5:00 P.M.) at the Animal Centre of Biomedical Research, Nagasaki University School of Medicine. All the rats had free access to pelleted food and drinking water.

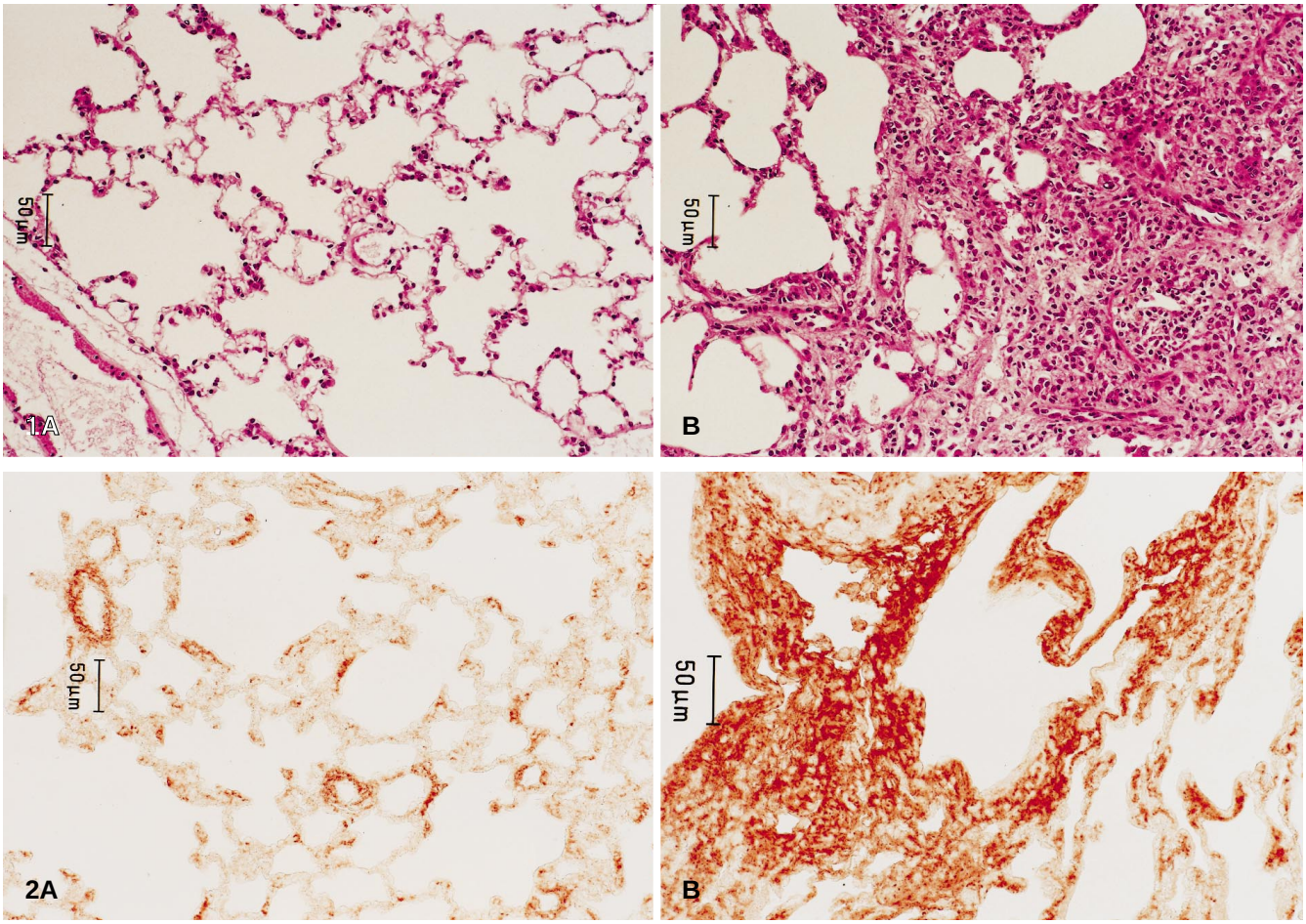

Fig. 1 **A** Lung of a control rat, showing no significant histological changes. **B** Histological features of the lung of a bleomycin-treated rat sacrificed at 2 weeks, showing increased inflammatory cell infiltration and pulmonary fibrosis. The animals were matched for age

Fig. 2 **A** Weak immunostaining of type III collagens is noted in the control lungs. **B** Immunostaining of type III collagen is strong in the fibrotic areas of a bleomycin-treated lung

The rats were divided into two experimental groups. Bleomycin-treated rats (n = 9) were given a single intratracheal injection of bleomycin (5 U/kg of body weight) under anesthesia. Another six rats were given a single intratracheal injection of similar volume of 0.01 M PBS and used as controls. Three bleomycin-treated rats and two age-matched control rats were killed after 1 week, 2 weeks and 4 weeks of the experiment by exsanguination under ether anesthesia. Both lungs were then removed from each rat via a midline incision. A portion of each lung was fixed immediately in Carnoy's solution for 2 h for immunohistochemistry and another portion was fixed in 10% formalin for 24 h for immunohistochemical and histological examination.

Tissues were processed to paraffin, and 4 μm of each lung section was stained with haematoxylin-eosin (HE) and Masson's trichome. The extent of pulmonary fibrosis was determined by light microscopic examination.

Immunohistochemistry was performed as described earlier [14, 16]. Briefly, paraffin sections (4 μm) were deparaffinized with xylene, and rinsed thoroughly with ethanol. The sections were then soaked in 0.3% hydrogen peroxide in methanol for 30 min at room temperature to inactivate endogenous peroxidase activity. After mild treatment with trypsin (15 min), the sections were incubated with either 10% goat serum or 10% rabbit serum (30 min), then covered with primary antibodies and incubated for 1 h. As immunohistochemical controls, the antibodies were replaced with 0.01% PBS or with a similar concentration of either mouse or rabbit IgG. After washing with PBS, the sections were processed further using Histofine SAB-PO kit as directed by the manufacturer, and the sections were developed with 3,3′-diaminobenzidine and H_2O_2. A conventional alkaline phosphatase staining method was used to localize HSP47 in the lung sections (Histostain-AP kit; Zymed Laboratories, USA).

The staining intensity of α-smooth muscle actin (anti-α-smooth muscle actin: Dako Corp, Denmark), vimentin (anti-vimentin: Dako Corp, Denmark), type I collagen (anti-type I collagen: Cosmo Bio, Japan), type III collagen (anti-type III collagen: Chemicon, USA) and HSP47 (anti-HSP47: Biotechnologies Corp, Canada) was graded semiquantitatively according to the following scale: (0) = no staining, (+) = weak staining, (++) = moderate staining, (+++) = strong staining.

Double immunostaining was performed to localize HSP47/type I collagen, HSP47/type III collagen, HSP47/α-smooth muscle actin and HSP47/vimentin in the same lung sections as described earlier [15]. Briefly, paraffin sections (4 μm) were deparaffinized, denatured with 0.3% hydrogen peroxide in methanol (30 min), reacted with 10% nonimmune goat serum (10 min) and incubated with monoclonal antibody against HSP47 for 1 h. The sections were treated further with biotinylated second antibody (10 min) and streptavidin-alkaline phosphatase (10 min) successively and developed with BCIP/NBT, which produces a dark purple stain. Then the sections were further stained for type I collagen, type III collagen, α-smooth muscle actin or vimentin by streptavidin-biotin-peroxidase (HRP) method and the antigen–antibody complex was visualized by aminoethyl carbazole (AEC)/H_2O_2, which produces an intense red stain. As immunohistochemical control, primary antibodies were replaced with either 0.01 M PBS or mouse/rabbit IgG diluted with PBS (similar concentration to that of primary antibody).

Results

The histological study exhibited no significant histological changes in the age-matched control lungs (Fig. 1A), while marked pulmonary fibrosis and inflammatory cell infiltration were always noted in the lungs obtained from bleomycin-treated rats (Fig. 1B). The extent of the fibrosis increased gradually with time (2 weeks and 4 weeks).

Weak interstitial immunostaining (+) for type I (data not shown) and type III (Fig. 2A) collagens was noted in the lungs of control rats. In contrast, strong immunostaining (+++) with increased deposition of type I collagen (data not shown) and type III collagen (Fig. 2B) was always noted in the fibrotic lesions in bleomycin-treated rats. Although the intensity of collagen immunostaining was same in all bleomycin-treated rats lungs, the level of collagen deposition increased with time.

Immunoreactive HSP47 expression was weakly detected in the interstitial cells (+) and endothelial cells (+) of the blood vessels in the control lungs (Fig. 3A). In contrast, markedly increased HSP47 immunostaining was noted predominantly in interstitial cells (+++) in and around the pulmonary fibrosis in bleomycin-challenged lungs (Fig. 3B, C). When monoclonal HSP47 was replaced with a similar concentration of mouse IgG, no specific staining was noted (data not shown). Although the intensity of HSP47 immunostaining was same in all bleomycin-treated rat lungs, the level of HSP47 expression (in terms of area) increased with time.

To examine whether the increased expression of HSP47 in bleomycin-challenged lungs is associated with increased accumulation of collagens, double immunostaining for HSP47 and collagens was performed in the lung sections. Co-expression of HSP47 and type I collagen (data not shown) and HSP47 and type III collagen (Fig. 4A, B) was noted in the fibrotic areas induced by bleomycin injection. A similar pattern of immunostaining was seen in all bleomycin-challenged lungs.

α-Smooth muscle actin was present mainly in the vessel walls and the bronchial wall in the control lungs (Fig. 5A), while increased numbers of α-smooth muscle actin-positive myofibroblasts was noted in bleomycin-treated rat lungs, revealing phenotypic modulation of these cells (Fig. 5B). Similarly, compared with the control lung (Fig. 5C), increased numbers of vimentin-positive mesenchymal origin cells (fibroblasts) were also seen in bleomycin-treated rat lungs (Fig. 5D).

To determine the cells expressing HSP47 in bleomycin-challenged lungs, double immunostaining for HSP47 and α-smooth muscle actin or vimentin was performed in the lung sections. Co-expression of HSP47/α-smooth muscle actin (Fig. 6A, B) and HSP47/vimentin (Fig. 6C, D) was noted in the bleomycin-treated lungs. A similar pattern of immunostaining was seen in all bleomycin-challenged lungs, suggesting that HSP47-expressing cells are mostly vimentin-positive fibroblasts and/or α-smooth muscle actin-positive myofibroblasts.

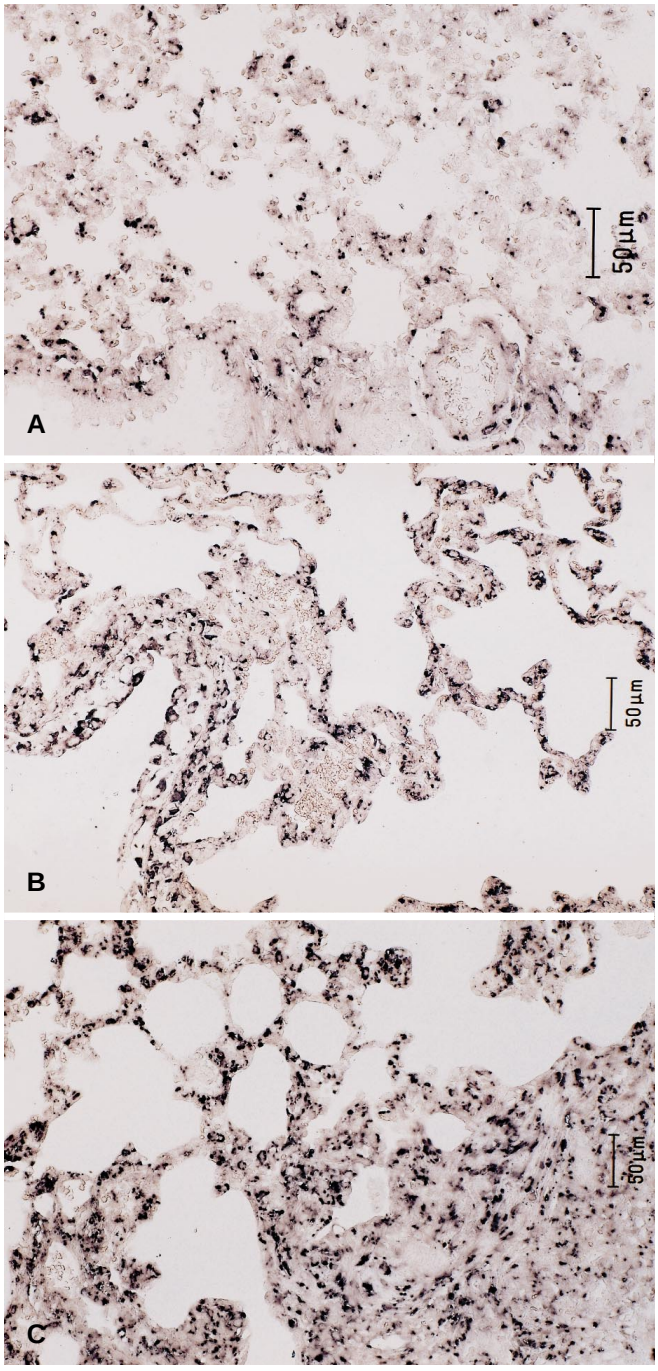

Fig. 3 **A** Immunohistochemistry for heat shock protein (HSP) 47 in a control lung, showing weak staining for HSP47, mainly in the interstitial cells. **B, C** In contrast, markedly increased HSP47 immunostaining is noted, mainly in the stromal interstitial cells, in bleomycin-treated lungs

Discussion

Fibrotic lung diseases are one of the major chronic problems of clinical practice. The mechanism by which pulmonary fibrosis occurs in various disease conditions is unknown. Although several reports have documented increased synthesis of ECM, including various collagens,

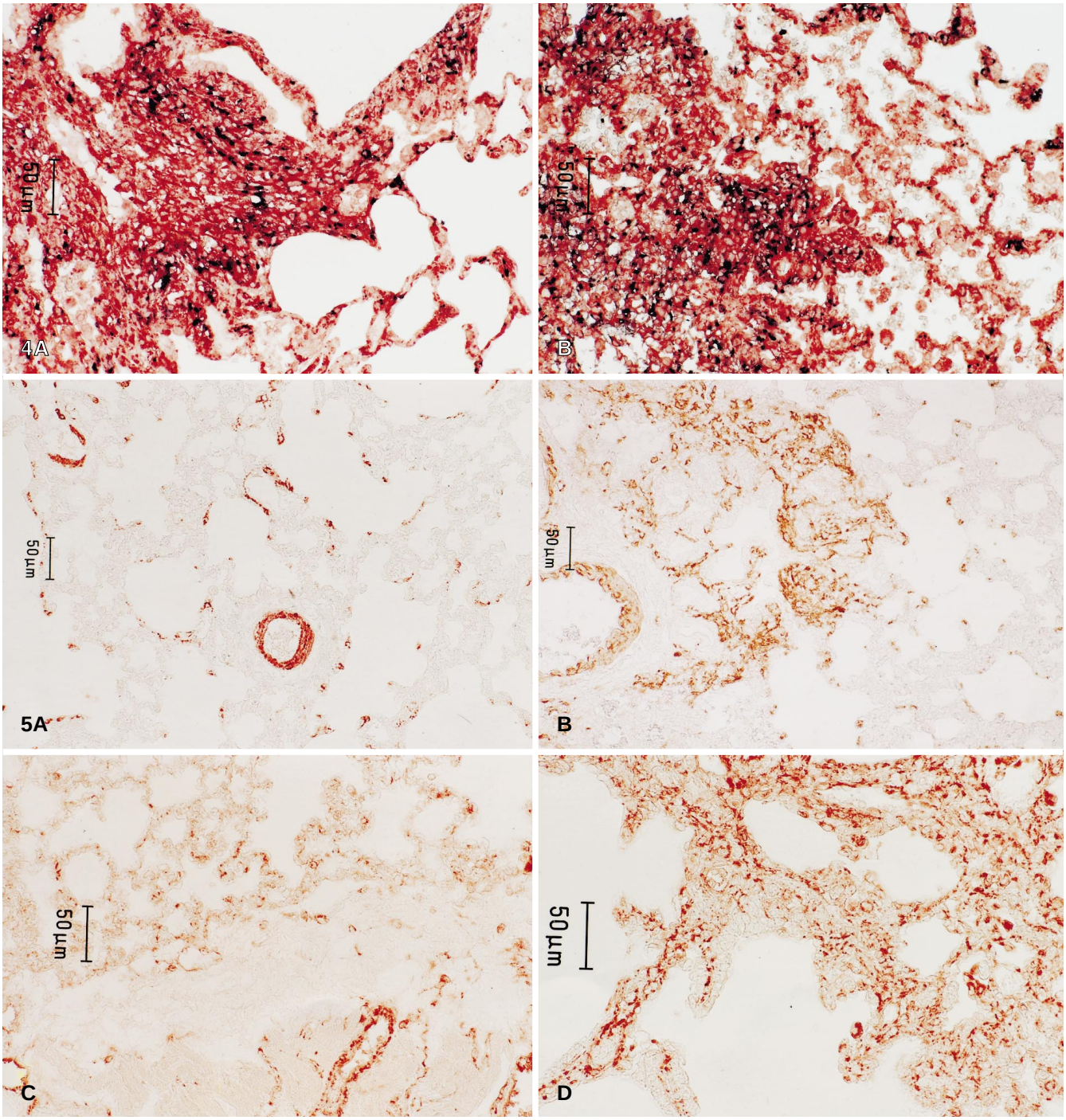

Fig. 4A, B Double staining on a paraffin section of bleomycin-treated lungs using antibodies against HSP47 and type III collagen. HSP47 is stained dark purple and collagen is stained intense red. Note that increased expression of HSP47 is associated with increased expression of type III collagen in the fibrotic areas of bleomycin-treated rat lungs

Fig. 5 A Immunohistochemistry for α-smooth muscle actin, showing immunostaining in the vessel wall in the control lung. **B** Compared with the control lung, markedly increased immunostaining for α-smooth muscle actin is noted in the bleomycin-treated rat lung. **C** Immunohistochemistry for vimentin, showing weak immunostaining in the control lung. **D** An increased number of vimentin-positive mesenchymal origin cells are noted in the bleomycin-treated rat lung

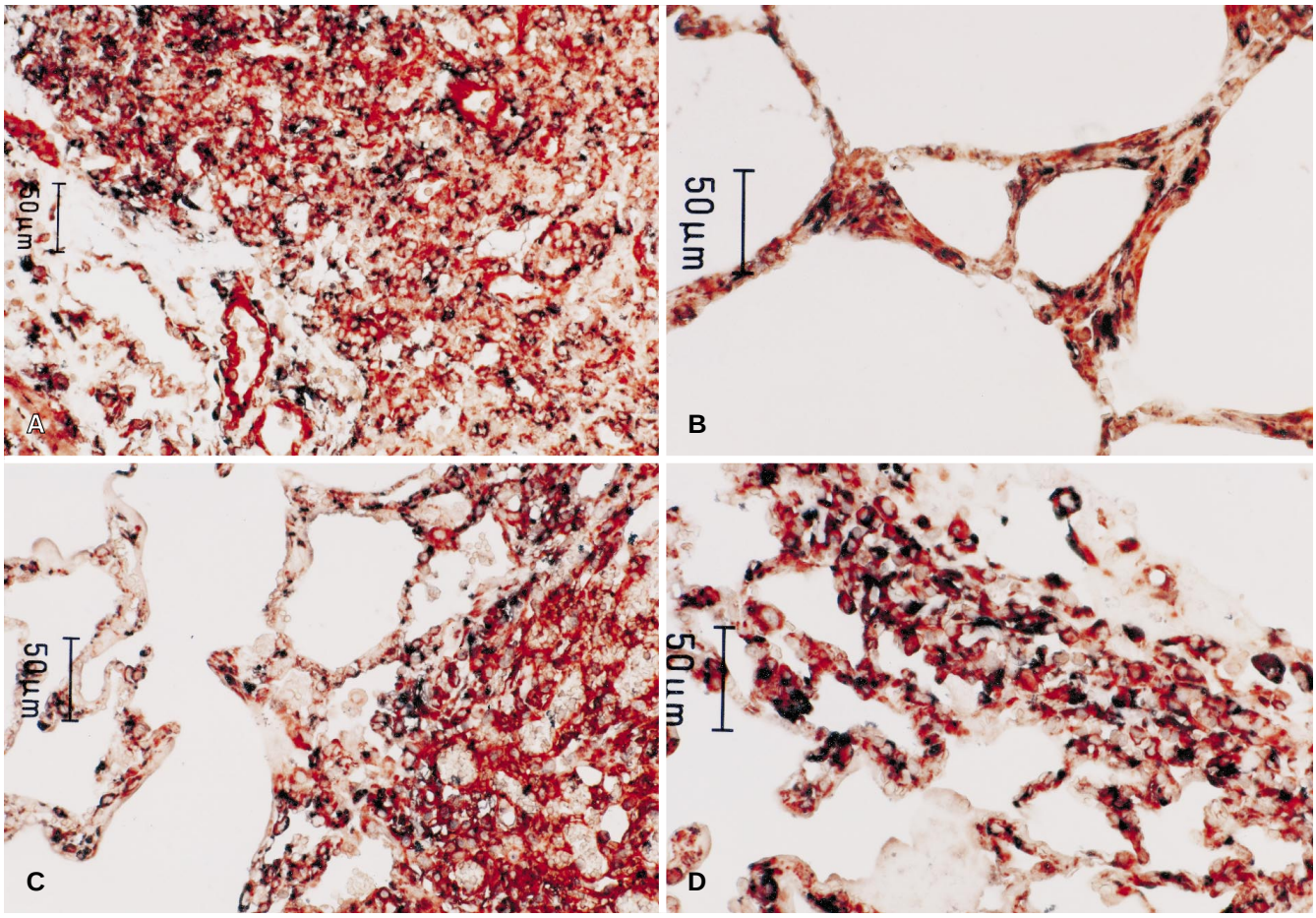

Fig. 6 Double staining on a paraffin section of bleomycin-treated lungs using **A**, **B** antibodies against HSP47/α-smooth muscle actin and **C**, **D** HSP47/vimentin. HSP47 is stained purple and α-smooth muscle actin or vimentin is stained red. HSP47-expressing cells are co-expressed with either α-smooth muscle actin (**A, B**) or vimentin (**C, D**) in bleomycin-treated rat lungs

during the development of pulmonary fibrosis in experimental and human lung diseases [2, 20, 22], very little is known about the intracellular processing of the collagen molecules during fibrosis.

Bleomycin sulfate has been used to induce fibrosis in rodents, and is associated with increased inflammatory cell infiltration, fibroblast proliferation and collagen accumulation [1, 21]. The histological changes in the bleomycin-challenged lungs in the experimental animals are similar to those seen in human idiopathic pulmonary fibrosis [5]. Structural changes in bleomycin-challenged lungs include thickening of alveolar septa and loss of lung elasticity, which subsequently leads to morbidity in the animals. In this preliminary study, we examined the possible role of HSP47 in pulmonary fibrosis using bleomycin-injected rats.

Recently a 47-kD heat shock protein has been identified as a collagen-binding stress protein, and several studies have suggested that HSP47 plays (an) important part(s) in the synthesis/assembly of various collagens as a collagen-specific molecular chaperone [3, 4, 11–13]. From recent reports, it appears that overexpression of HSP47 has an important role in sclerotic/fibrotic changes. Masuda et al. [9] demonstrated that the expression of HSP47 mRNA was markedly induced during the progression of fibrosis in parallel with alpha 1(I) and alpha 1(III) collagen mRNAs in carbon tetrachloride-induced liver fibrosis in rats. In rats, the expression of HSP47 was also elevated in anti-thymocyte serum-induced glomerulosclerosis, in parallel with increased expression of various collagens [15]. Consistent with the earlier reports [8, 9, 15], in the present study, we found that the expression of HSP47 was substantially increased in the bleomycin-treated lungs, along with an increased deposition of collagens in the fibrotic areas.

Increased numbers of α-smooth muscle actin-positive myofibroblasts and vimentin-positive fibroblasts was noted in bleomycin-treated lungs. These phenotypically altered myofibroblasts and fibroblasts were shown to be the predominant cell type responsible for procollagen mRNA expression in this model [21]. In bleomycin-challenged lungs all these phenotypically altered cells were found to express HSP47, which might contribute significantly to the development of fibrosis in the lung, possibly by regulating increased synthesis of various collagens. Further studies using Northern blot hybridization and in situ hybridization techniques are under way to ex-

amine the relative expression and cellular origin of HSP47 mRNA in bleomycin-treated lungs.

Using a double immunostaining technique, we have clearly shown that increased expression of HSP47 in the fibrotic lung is associated with increased deposition of various collagens. However, the exact nature of the association between increased expression of HSP47 and collagens in bleomycin-treated lungs has yet to be defined. In view of the fact that HSP47 plays an important role(s) in the synthesis, processing and assembly of various collagens [6, 7, 10, 18, 19], elevated levels of HSP47 in bleomycin-treated lungs may have a significant role in the subsequent manifestation of pulmonary fibrosis. However, the factors regulating increased expression of HSP47 in bleomycin-treated lungs need further study. Therapeutic intervention directed against HSP47 might alter the fibrotic process, which might be of clinical value. Experimental studies have shown that phosphorothioate antisense oligodeoxynucleotides to HSP47 inhibited both HSP47 production and consequently diminished the production of $\alpha(1)$ chains of type I pro-collagen [18].

In summary, increased expression of HSP47 in bleomycin-treated lungs may lead to progressive pulmonary fibrosis by regulating increase synthesis/assembly of various collagens.

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