Localization of HSP47 mRNA in Murine Bleomycin-Induced Pulmonary Fibrosis

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ABSTRACT

Heat shock protein (HSP) 47 is a collagen-specific molecular chaperone that has been shown to play a major role in the processing and/or secretion of procollagen. However, the knowledge on which cells are actually synthesizing HSP47 in the lung parenchyma in pulmonary fibrosis was only limited. The aim of the present study was to investigate the localization of HSP47 messenger ribonucleic acid (mRNA) in normal lung, and in the lungs of mice in bleomycin-induced pulmonary fibrosis, using *in situ* hybridization. For the purpose, ICR mice were intravenously injected with 10 mg/kg/day of bleomycin for 5 consecutive days. The lung cells expressing HSP47 mRNA were identified in control (saline alone) and bleomycin-treated mice by *in situ* hybridization. The signal for HSP47 mRNA was markedly increased in bleomycin-treated lungs compared with that of controls. HSP47 mRNA was localized in α-smooth muscle actin-positive myofibroblasts, surfactant protein A-positive type II pneumocytes and F4/80 positive macrophages in the active fibrotic areas. These results suggest that these cells may synthesize procollagen in the fibrotic process of bleomycin-treated lungs through upregulation of HSP47 mRNA and play an important role in fibrogenesis.

Key words: pulmonary fibrosis, heat shock protein 47, *in situ* hybridization

Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating disorder characterized by excess deposition of extracellular matrix [1]. Increased synthesis and deposition of extracellular matrix is considered to be an important cause of fibrosis in experimental and human lung diseases [2,3]. However, the molecular mechanisms underlying the excessive deposition of collagen in the fibrotic lesions is not fully understood.

It is of interest to know which cells principally contribute to fibrogenesis in the lung. Although several studies in experimental models emphasized the important role of the alveolar epithelium in normal repair [4,5], the prevailing hypothesis regarding the pathogenesis of interstitial pulmonary fibrosis has revealed that the disease is due to a chronic unsolved inflammatory response, and in this context, roles of the epithelium have been largely neglected. However, there is increasing evidence supporting the notion that epithelial injury in the absence of ongoing inflammation is sufficient to cause the development of pulmonary fibrosis [4-6] and that alveolar epithelial cells may be the primary source of cytokines and growth factors involved in fibroblast migration and proliferation [6]. In addition, many studies have demonstrated that macrophages are also a source of mediators capable of regulating fibroblast proliferation and other functions that are responsible for the fibrogenic outcome [7,8]. In spite of these findings,

the precise biological link among alveolar epithelial injury, macrophage activation and fibrosis represents a challenging puzzle in which several pieces remain to be assembled.

Heat shock protein (HSP) 47 is a collagen-binding, stress-inducible protein localized in the endoplasmic reticulum. HSP47 has a specific role in the intracellular processing of procollagen production as a collagen-specific molecular chaperone [9-12]. HSP47 expression was upregulated in experimental animal models of fibrosis, including murine bleomycin-induced pulmonary fibrosis [13,14], rat peritoneal sclerosis [15] and carbon tetrachloride-induced rat liver cirrhosis [16]. In addition, we previously reported that the expression of human HSP47 was increased in the fibrotic lesions of idiopathic pulmonary fibrosis (IPF) [17,18], fibrotic transplanted kidney [19], and peritoneal sclerosis [20]. These findings suggest HSP47 may play the important role in collagen synthesis in various fibrotic disorders.

We have recently shown in rodent bleomycin-induced pulmonary fibrosis model that type II pneumocytes in addition to myofibroblasts express HSP47 with the progression of fibrosis, while these cells didn't express HSP47 in the control lungs [13,14]. We also previously reported that regenerated type II pneumocytes in usual interstitial pneumonia express HSP47 [17,18]. These findings suggest that type II pneumocytes in addition to myofibroblasts become synthesizing procollagen through the induction of HSP47 and play an important role in the development of fibrosis. However, the previous reports have not shown direct evidence that type II pneumocytes

actually synthesize HSP47 in pulmonary fibrosis, because all the previous findings were based on only protein level using immunohistochemistry.

Hence, this study was undertaken to investigate whether type II pneumocytes actually synthesize HSP47 in fibrotic lung. For this purpose, we employed the technique of nonradioactive *in situ* hybridization with a high resolution to localize the cells and tissue areas expressing HSP47 at mRNA level in normal and bleomycin-induced fibrotic lungs of mice.

Methods

Treatment of animals

Male 10-week-old ICR mice weighing 36-39 g were purchased from Charles River Japan, Inc. (Yokohama, Japan). The animals were specific pathogen-free and maintained under standard conditions with free access to drinking water and pelleted food at the Animal Centre of Biomedical Research, Nagasaki University. The mice were randomly divided into two experimental groups; bleomycin-treated (n = 4) and control (n = 4) groups. The mice were intravenously injected with 10 mg/kg/day of bleomycin (Nippon Kayaku, Tokyo, Japan) dissolved (2 mg/ml) in saline for 5 consecutive days. For controls, age-matched mice received an identical volume of saline alone. At 49 days after the commencement of bleomycin or saline treatment, mice were sacrificed and evaluated in the following manner.

The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Nagasaki University.

Biochemicals and chemicals

Paraformaldehyde was purchased from Merck (Darmstadt, Germany), and 3,3'-diaminobenzidine-4HCl (DAB) was purchased from Dojin Laboratories (Kumamoto, Japan). A mixed bed resin (AG501-X8(D) resin) was purchased from

Bio-Rad Laboratories (Tokyo, Japan). 3-aminopropyltriethoxysilane, proteinase K, bovine serum albumin (BSA, minimum 98%, electrophoresis), yeast transfer RNA (type X-SA), salmon testis DNA, dextran sulfate, polyadenylic acid, heparin and Briji 35 were purchased from Sigma Chemical (St. Louis, MO, USA). Formamide (nuclease and protease-free) was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) and were of analytical grade.

Antibodies

Primary antibodies used for the immunohistochemical studies included anti-α-smooth muscle actin (SMA) (Neomarkers, Fremont, CA), anti-F4/80 (Serotic, Oxford, UK), anti-surfactant protein (SP)-A (Santa Cruz Biotechnology, Santa Cruz, CA). α-SMA, F4/80 and SP-A were used as markers for myofibroblasts, macrophages, and type II pneumocytes respectively. Negative control studies were performed with irrelevant immunoglobulin G with the same subclass of the first antibodies instead of the primary antibodies.

Tissue preparation

At 49 days after the commencement of bleomycin or saline injection, mice from each group were sacrificed by exsanguination under deep anaesthesia (sodium

pentobarbital, 50 mg/kg, intraperitoneally). Lungs were then removed from each mouse via a midline incision and were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed to obtain 4-μm sections for staining with hematoxylin-eosin and Azan-Mallory staining. Sequential lung sections were placed on silane-coated glass slides for *in situ* hybridization and immunohistochemical analyses. In the mirror sections, two consecutive sections were mounted on glass slides with the common cut surface facing upward, so that the same surface of cut cells was compared in different staining ways.

Probes and labelling

Sense and antisense oligo-DNA sequences corresponding to nucleotides No. 564-608 of the murine HSP47 cDNA sequence [21] were synthesized. These 45-base oligo-DNAs were added with two repeats of adenine-thymine-thymine at 5' ends and three repeats of it at 3' ends, respectively, for thymine-thymine (T-T) dimers [22]. We conducted a computer-assisted search (GenBank nucleic acid sequence database Release 129) of the above HSP47 oligo-DNA sequences (without adenine-thymine-thymine repeats) and found appropriate homology only with mouse HSP47 or related mRNA sequences. These HSP47 oligo-DNAs were haptenized by introducing T-T dimers by 12,000 Jm^{-2} UV irradiation, as described previously [22]. We performed immunodetection and dot-blot hybridization using HSP47 probes described previously in detail [22,23], and

these results indicated that antisense probes were specific and had adequate sensitivity to be useful for *in situ* hybridization.

In situ hybridization

In situ hybridization was performed as described previously [24]. Briefly, lung paraffin sections were deparaffinized with toluene and rehydrated by serially graded ethanol solutions. The slides were treated with 0.2N HCl for 20 minutes and proteinase K (50 μg/ml, 37 ℃ for 15 minutes), successively. After the slides were post fixed with 4% paraformaldehyde in PBS, the slides were immersed twice with 2 mg/ml glycine in PBS for 15 minutes. Hybridization was carried out at 37 ℃ for HSP47 overnight in a medium containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution, 250 μg/ml yeast transfer RNA, 125□μg/ml salmon testis DNA, 10% dextran sulphate, 200 U/ml heparin, 10 μg/ml polyadenylic acid (potassium salt), 40% deionized formamide, and 2.0 μg/ml T-T dimerized HSP47 probe. After hybridization, the slides were washed 5 times with 2×SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0)/50% formamide at 37℃ for 1 hour each and finally washed twice with 2 ×SSC at room temperature for 15 minutes. After successive treatment with ethanol and acetone to dilapidate the sections, the slides were subjected to enzyme-immunohistochemistry. After incubation with blocking solution (5% BSA, 100 μg/ml salmon testis DNA, 100 μg/ml yeast transfer RNA, and 500 μg/ml mouse IgG in

PBS) for 1 hour at room temperature, the slides were reacted overnight with HRP-mouse anti-T-T antibody (1:80) at room temperature. After washing with 0.075% Brij 35 in PBS, the visualization of HRP sites was performed with DAB, Ni, Co, and H_2O_2 according to the method of Adams [25]. To confirm the specificity of mRNA signals, a variety of control experiments were conducted. In every experimental run, sense probe was used as a negative control. To evaluate the level of hybridizable RNAs in tissue sections, a 28S rRNA probe was used as a positive control in every case [23].

Immunohistochemistry

To confirm the localization of HSP47 mRNA, immunohistochemistry was performed with the conventional avidin-biotin-peroxidase histochemical technique using Histomouse^{TM}-Plus Kits (Zymed Laboratories, South San Francisco, CA) for α-SMA, and the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) for F4/80 and SP-A. Briefly, sequential paraffin sections (4-μm-thick) were deparaffinized with toluene and were rinsed thoroughly with ethanol. Sections were then soaked in 0.3% H_2O_2 with absolute methanol for 20 minutes at room temperature to inactivate the endogenous peroxidase activity. They were incubated with blocking serum for 30 minutes, and then covered with primary antibodies and incubated for 1 hour. After washing in phosphate-buffered saline, sections were processed further using kits according to the instructions provided by the manufacturer, and then developed with

3,3'-DAB and H_2O_2. Finally, the sections were counterstained with methyl green and mounted.

Results

Histological findings

As shown in Fig. 1, marked interstitial pneumonitis and fibrosis was noted in the lungs of bleomycin-treated mice (Fig. 1A), while no significant change was noted in the lungs of control mice (Fig. 1B). The pneumonitis and fibrosis was found mainly in the subpleural regions. With the Azan-Mallory staining, collagen deposition was seen in the lung of bleomycin-treated mice (Fig. 1C), while almost no collagen deposition was seen in the lung of control mice (Fig. 1D).

In situ hybridization for HSP47 mRNA

HSP47 mRNA was not found in control lung (Fig. 2A). However, there was a significant increase in the number of cells expressing HSP47 mRNA in the lungs of bleomycin-treated mice (Fig. 2B). When an adjacent section was hybridized with HSP47 sense probes, the staining intensity was markedly reduced compared to that with HSP47 antisense probe (Fig. 2C).

Localization of HSP47 mRNA

To determine the type of cells that expressed HSP47 mRNA, we performed hematoxylin and eosin staining and immunohistochemistry for α-SMA, SP-A and

F4/80, in addition to in situ hybridization for HSP47 mRNA using adjacent sections and mirror sections in the bleomycin-treated mice. The majority of HSP47 mRNA positive interstitial spindle-shaped cells (Fig. 3A and 3B) were also positive for α-SMA (Fig. 3C) indicating that these cells were myofibroblasts. And most of the HSP47 mRNA positive cuboidal cells located in alveolar walls (Fig. 3D and 3E) were also positive for SP-A (Fig. 3F), indicating that these cells were type II pneumocytes. Furthermore, HSP47 mRNA positive intra-alveolar mononuclear cells (Fig. 3G and 3H) were identified similarly as macrophages, based upon the F4/80 co-staining (Fig. 3I).

Discussion

The present study confirmed the expression and localization of HSP47 at mRNA levels in bleomycin-treated lungs. HSP47 mRNA was localized in SP-A positive type II pneumocytes, F4/80-positive macrophages and α-SMA-positive myofibroblasts. HSP47 binds to the nascent types I-V procollagen immediately after procollagen is translated into the endoplasmic reticulum [9,26]. HSP47 is thought to prevent the incorrect unfolding and aggregation of nascent procollagen polypeptide chains until the correct carboxy-terminal association has been made [27]. Recent reports have demonstrated that HSP47 expression is restricted to phenotypically altered collagen-producing cells, and its expression correlates well with that of collagen [15,17,19,20]. Clarke *et al.* [28] demonstrated that cell lines that did not produce collagen mRNA lacked the capacity to produce HSP47 mRNA. The present findings together with these previous studies imply that type II pneumocytes and macrophages in addition to myofibroblasts might become synthesizing collagen in the process of pulmonary fibrosis. However, the exact mechanism of intracellular processing of HSP47 and procollagen in these cells remains unclear.

While many neoteric cell lineages migrate during embryogenesis to new locations using a fate pathway that involves epithelial-mesenchymal transition [29,30], such transitions in mature tissues are less well appreciated. However, transitions do

occur particularly during oncogenesis [31] and fibrogenesis [32,33]. This process has been particularly studied in renal fibrosis. It has been hypothesized that when basement membrane is injured by matrix metaroproteinase, epithelial cells that detach from their basement membrane may enter into epithelial-mesenchymal transition, express HSP47 and other mesenchymal markers and divide as fibroblasts [33-35]. Whether this process takes place in the lung in IPF or in other forms of pulmonary fibrosis is largely unknown, but urgent evaluation in *vitro* and in *vivo* would be necessary. The present findings that type II pneumocytes express HSP47 mRNA imply that the similar process might take place in pulmonary fibrosis. Further studies are warranted in order to elucidate the precise mechanism(s) involved in this process.

We previously reported that the treatment with pirfenidone, an anti-fibrotic agent, decreased the expression of HSP47 at protein level in bleomycin-induced pulmonary fibrosis in mice [14]. Furthermore, it was demonstrated that inhibition of HSP47 by antisense oligodeoxynucleotides markedly suppressed the production of collagen in 3T6 cells [12], in experimental proliferative glomerulonephritis [36] and in experimental peritoneal fibrosis [15]. These findings suggest that HSP47 might be a promising target for the treatment of pulmonary fibrosis. Further studies using an antisense oligonucleotide against HSP47 are underway in our laboratories to examine whether this antisense suppresses collagen accumulation in an experimental pulmonary fibrosis induced by bleomycin.

In summary, we have successfully identified the localization of HSP47 mRNA in pulmonary fibrosis in bleomycin-treated lungs. Our results suggest that type II pneumocytes and macrophages, in addition to myofibroblasts, might synthesize procollagen in the fibrotic process of bleomycin-treated lungs, through upregulation of HSP47 mRNA. Further studies are required to investigate the exact mechanism of intracellular processing of HSP47 and procollagen in these cells.

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Conflict of interest statement

We declare that we have no conflict of interest.

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FIGURE LEGENDS

Figure 1.

Photomicrographs of representative lung specimens in bleomycin-treated (A, C) and control (B, D) groups. A, B; hematoxylin and eosin staining and C, D; Azan-Mallory staining. Calibration bar = 200 μm.

Figure 2.

In situ detection of HSP47 mRNA in lung specimens of bleomycin-treated and control mice. HSP47 mRNA was not found in control lung (A). There was a significant increase in the number of cells expressing HSP47 mRNA in the lungs of bleomycin-treated mice (B). When an adjacent section was hybridized with HSP47 sense probe, the staining intensity was markedly reduced compared to that with HSP47 antisense probe (C). Calibration bar = 100 μm.

Figure 3.

Identification of cell types of HSP47 mRNA positive cells in the lung tissues of bleomycin-treated mice. A, D, G; hematoxylin and eosin staining. B, E, H; HSP47 mRNA staining by *in situ* hybridization. C, F, I; immunohistochemistry for α-SMA,

SP-A and F4/80, respectively. Each set of photos (A-C, D-F, and G-I) was taken from adjacent and mirror sections. Calibration bar = 50 μm.

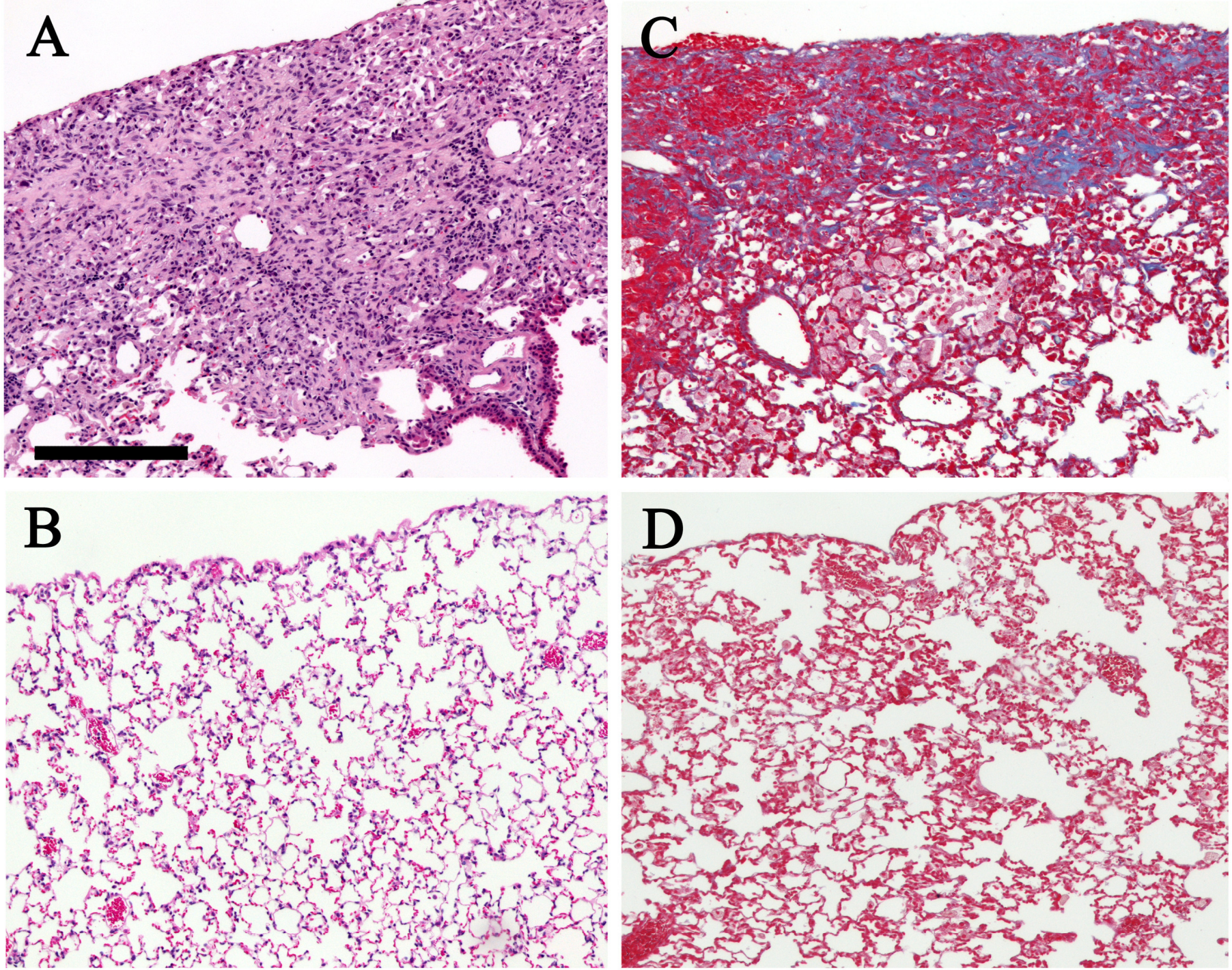

Figure 1

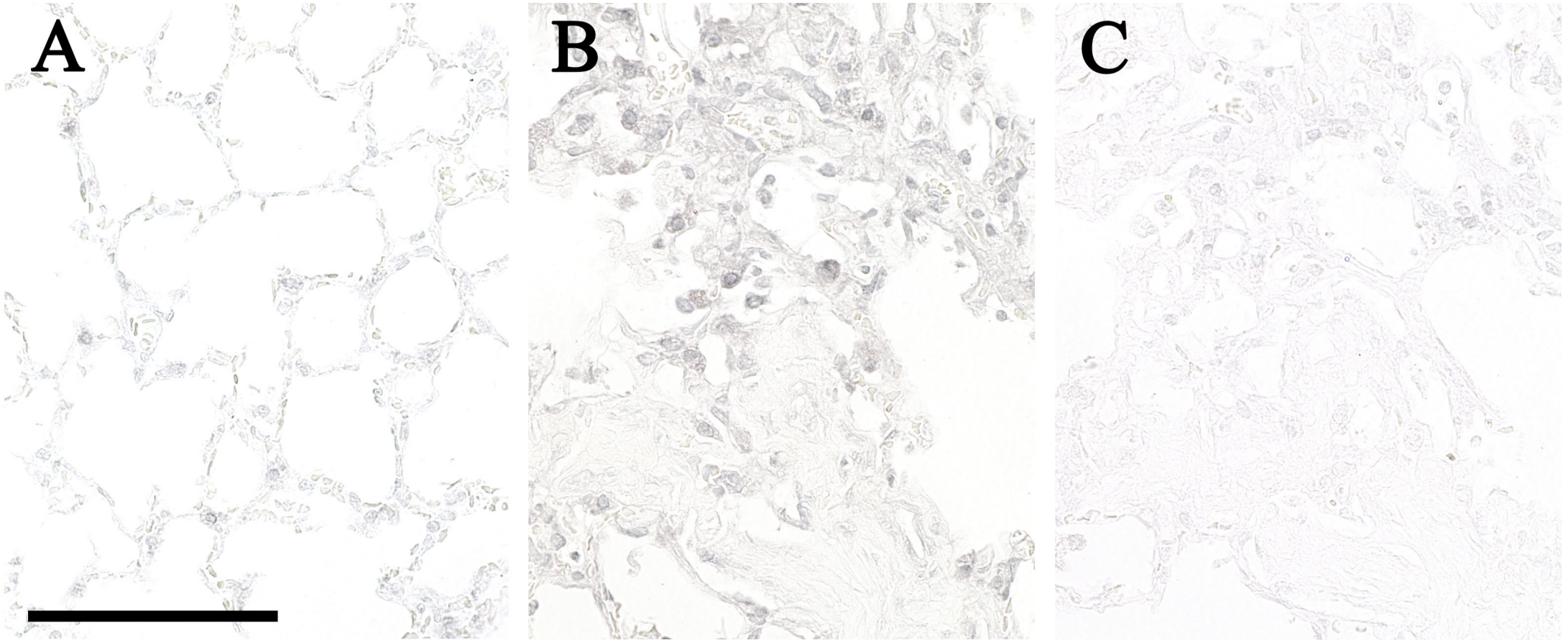

Figure 2

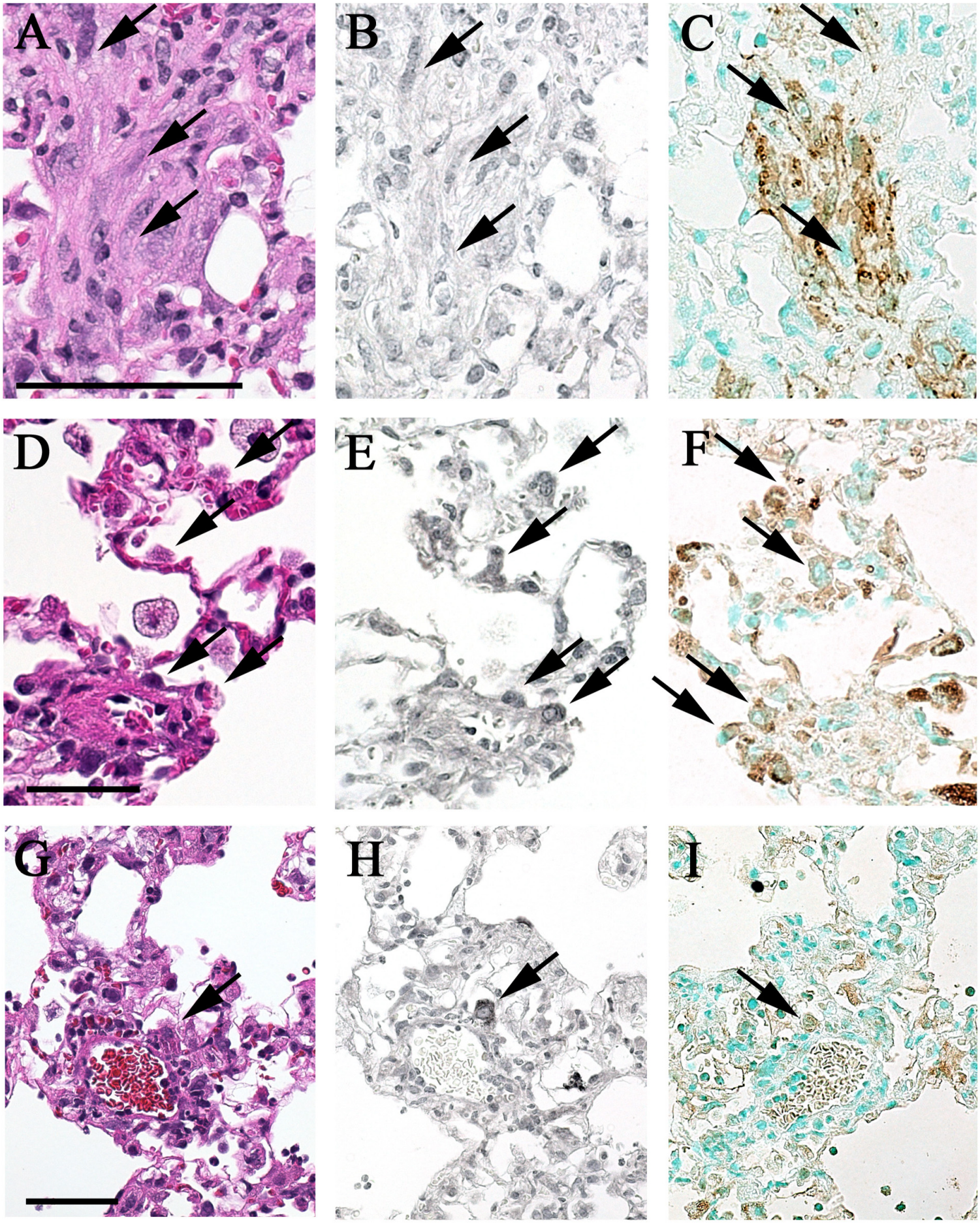

Figure 3