Coexpression of HSP47 Gene and Type I and Type III Collagen Genes in LPS-Induced Pulmonary Fibrosis in Rats

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Abstract Diffuse alveolar damage is the histopathologic hallmark of acute respiratory distress syndrome (ARDS). A significant proportion of ARDS survivors have residual pulmonary fibrosis and compromised pulmonary function. On the other hand, heat shock protein 47 (HSP47) is a collagen-binding stress protein that is assumed to act as a collagen-specific molecular chaperone during the biosynthesis and secretion of procollagen in living cells. The synthesis of HSP47 has been reported to correlate with that of collagen in several cell lines. We examined the expression of HSP47 mRNA and protein during the progression of lipopolysaccharide (LPS)-induced ARDS in rat lung. Male Wistar rats were randomly divided into two groups: a control group with instillation of 0.9% NaCl solution alone, and a LPS group with instillation of LPS dissolved in 0.9% NaCl solution (10 mg/kg). Histologic changes thereafter appeared in the LPS-treated rats. Northern blot analysis revealed the expression of HSP47 mRNA to be markedly induced during the progression of lung damage in parallel with type I and type III collagen mRNA. These results suggest that the upregulation of HSP47 and collagen may play an important role in the fibrotic process of LPS-induced ARDS lung.

Keywords Acute respiratory distress syndrome (ARDS) · Collagen · Heat shock protein 47 · Lipopolysaccharide · Pulmonary fibrosis

Introduction

Acute respiratory distress syndrome (ARDS) represents a particularly dangerous form of acute respiratory failure, with a persistently high mortality. Fibrosis in the lung is observed in ARDS where the accumulation and disorder of fibrous connective tissue represents a major phenomenon. The ultimate clinical course of ARDS is often determined by the ability of the injured lung to repopulate the alveolar epithelium with functional cells. Death may occur when fibrosis plays a predominant role in the healing response because it results in a worsening lung compliance and oxygenation [1, 2]. An analysis of the lung tissue from ARDS patients showed an abundance of type I and type III collagen [3]. Excessive lung collagen synthesis and accumulation may contribute to the high fatality rates associated with ARDS by promoting progressive respiratory dysfunction.

Collagens constitute a family of extracellular proteins that begin their assembly within the lumen of the endoplasmic reticulum (ER) and then are eventually secreted from the cell. Heat shock protein 47 (HSP47) was found as a collagen-specific binding stress protein [4]. HSP47 was localized in the ER [5] and was also present in the fibroblasts and the connective tissue of various organs [4]. Type I to type V collagens have
been shown to bind to HSP47 [6]. Pulse label and chase experiments combined with in vivo crosslinking and immunoprecipitaion revealed that HSP47 acts as a molecular chaperone during the processing and/or secretion of procollagen in the ER. Collagen is a group of glycoproteins in the extracellular matrix. All cells that express some type of collagen have been found to express HSP47, thus suggesting a functional relevancy to exist between HSP47 and collagen synthesis [7, 8]. Under conditions of stress, HSP47 is part of the quality control system for procollagen, including the prevention of the secretion of procollagen with abnormal conformation. HSP47 does not bind to other proteins such as fibronectin or laminin [9].

HSP47 therefore seems to play an important role in ARDS-related 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 rat LPS-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

All protocols conformed with the National Institutes of Health (NIH) guidelines and the animals received human care in compliance with the Principles of Laboratory Animal Care. Male Wistar rats weighing 250–300 g (Kyudou, Saga, Japan) were used in all experiments. All animals were housed with access to food and water ad libitum.

Experimental Protocols

The animals were randomly assigned to one of two groups: (1) control group (n = 25) in which there was intratracheal administration of 0.9% NaCl solution alone and (2) lipopolysaccharide (LPS) group (n = 25) with intratracheal administration of LPS (Sigma, St. Louis, MO) dissolved in 0.9% NaCl solution (10 mg/kg). All rats were anesthetized with 2% sevoflurane. Under anesthesia the trachea was exposed through a midline anterior neck incision and the agent was injected into the tracheal lumen with a 24-gauge needle. All animals were sacrificed at 4 days, 1, 2, 4, and 8 weeks by clipping the vena cava and transecting the aorta, followed by the intra-atrial injection of normal saline. Lung tissue specimens were then quickly removed and processed as discussed below.

Histologic Examination

The rats were sacrificed after saline or LPS injection by exsanguination under sevofluran anesthesia. The lung was instilled with 10% formalin. Histology paraffin-embedded sections (3 μm) were stained with hematoxylin and eosin. We compared the severity of interstitial fibrosis among the groups using the Ashcroft score [10]. Azan staining was carried out to visualize the collagen fibrils in the tissue.

Immunohistochemical Analysis

The left lobe lungs were obtained from animals under sevofluran anesthesia after intratracheal administration of saline or LPS (10 mg/kg). Tissue samples were then fixed immediately in 4% paraformaldehyde at 4°C overnight, embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan), and cut into 5-μm sections. Immunohistochemistry was performed after blocking endogenous peroxidase activity with 0.3% hydrogen peroxide and adding sodium azide (1 mg/ml) for 10 min. The next step was the blocking of nonspecific protein binding with 10% sheep serum for 10 min. Blocked sections were incubated with anti-HSP47 monoclonal antibody (1:1000 dilution) overnight at 4°C. The sections were then incubated with peroxidase-conjugated anti-mouse IgG. The slides were stained using the LSAB2 kit (Daco, Carpinteria, CA) as the biotin-avidin peroxidase complex system. After development, the slides were then counterstained with Mayer’s hematoxylin and mounted.

Northern Blot Analysis

After washing out the blood by saline perfusion via the right ventricle, the lung was homogenated with a polytron homogenator (IKA Labortechnik, Staufen, Germany) in Isogen reagent (Nippon Gene Co., Tokyo, Japan). After the total cellular RNA was isolated, the precipitated total RNA pellet was washed with 1 ml of 75% ethanol, air-dried, and resuspended in sterile water. The RNA concentration was measured by absorbance at 260 nm.

Total RNA was separated on 1% agarose gels containing formaldehyde and transferred onto nylon membranes (Amersham, Buckinghamshire, England) as recommended by the manufacturer. Blotted filters were prehybridized for 6 h at 42°C in hybrizol (Interogen, Purchase, NY). The filter was hybridized in hybridization solution containing P-labeled DNA at 42°C for 16 h. After washing several times with 2 × SSC at room temperature, 2 × SSC with 0.5% SDS at
65°C, and 2 x SSC with 0.5% SDS at 60°C, the filter was exposed at –80°C overnight using RXO-H film with HR-X intensifying screens (Fuji Photo Film Co., Ltd., Tokyo, Japan). The following four hight-labeled probes (Rediprime II DNA Labelling System, Amersham, Buckinghamshire, England) were used. We used the cDNA as follows: a fragment from mouse HSP47 cDNA, a fragment from mouse type I collagen cDNA, and a fragment from mouse type III collagen cDNA.

Immunoblot Analysis

The right upper lobe lung was obtained from animals after the saline instillation or the LPS (10 mg/kg) instillation. After the washout of blood by saline perfusion via the right ventricle, the lung was homogenated with a polytron homogenator (IKA Labor-technik, Staufen, Germany) in a T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL). A rat lung homogenate was centrifuged at 10,000 rpm for 5 min. Thereafter, the supernatant was collected and the concentration was checked.

The conditions of electrophoresis were as described using the discontinuous Tris-glycin buffer system. The proteins were separated on gel; 10% for HSP47. The same amount of immunoblotting samples for gel electrophoresis were suspended in SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) buffer and boiled for 1 min before loading. Proteins run on SDS-PAGE were immediately electrotransferred to PVDF (polyvinylidene difluoride) (Millipore, Bedford, MA) membrane at 60 V for 3 h in a wet transfer system (Transfer buffer; 20 mM Tris-HCl, 0.2 M glycine, 20% MeOH). The membrane was blocked with 5% nonfat dry milk in TBS/Tween [25 mM Tris-HCl, 0.14 M NaCl, 2% Tween 20 (Bio-Rad, Hercules, CA)] overnight at 4°C. Preimmune serum was diluted 1:1000 in the 1% nonfat dry milk and incubated with gentle shaking for 1 h. The nitrocellulose was then rinsed three times for 10 min in TBS/Tween. The second antibody was diluted with the 1% nonfat dry milk and incubated with the membrane for 1 h. Washing between steps was performed three times for 10 min with TBS/Tween. The enhanced chemiluminescence (ECL) reagent (Amersham, Buckinghamshire, England) was used. The ECL-treated membrane was then exposed further to film. Monoclonal anti-HSP47 antibody was purchased from Stressgen Biotechnology (Temecula, CA). The film was scanned. The band concentration was calculated by the quantification of the integrated optical density of the appropriate band using the NIH Image software program (National Institutes of Health, Bethesda, MD).

Statistical Analysis

All data were presented as the mean ± standard error of the mean (SEM). The data were analyzed by analysis of variance and Scheffe’s post-hoc test (for multiple comparisons) and by the unpaired t test (for single comparisons). When p < 0.05, the result was considered to be statistically significant.

Results

Histology of the Lung of Rats Treated with Lipopolysaccharide

In the normal lung, fine fibrous connective tissue was observed in the alveolar wall (Fig. 1). After 14 days of LPS treatment, pulmonary fibrosis was observed. Fibrotic lung sections show inflammatory infiltrates and proliferated alveolar macrophage with alveolar edema around the fibrotic area (Fig. 2). The Ashcroft scores in the LPS group were significantly reduced compared with the control group (Fig. 3). Azan-stained sections show an increased amount of connective tissue in the lung of the LPS group compared with the control lung (Fig. 4).

Immunohistochemistry

To determine the effects of LPS on HSP47 protein production, LPS was injected into the tracheal of Wistar rats. They were treated simultaneously with an LPS that induces pulmonary fibrosis. Immunohistochemical analysis strongly stained for HSP47 antibody in the LPS group compared with the control group (Fig. 5).

Expression of HSP47 and Type I and Type III Collagen mRNA

HSP47 and type I and type III collagen mRNA levels in the lung tissue samples harvested at 4, 7, and 14 days after treatment were analyzed using Northern blot analysis and then were compared between the two experimental groups. The mRNA level of HSP47 was significantly higher in the LPS-treated lung than in the control lung after 7 days. In addition, the expression of HSP47 increased in the LPS-treated lung over time. The mRNA levels of collagen increased in parallel with those of HSP47 in the LPS-treated lung after 7
days (Fig. 6). However, the expression of HSP47 and type I and III collagen decreased in the LPS-treated lung after 4 weeks and then it was the same level as the control lung after 8 weeks (data not shown).

Expression of HSP47 Collagen Protein

The HSP47 protein levels in the lung tissue samples harvested at 1, 2, 4, and 8 weeks after LPS treatment were analyzed using immunoblot analysis. The protein level of HSP47 was significantly higher in the LPS-treated lung than in the control lung after 1 and 2 weeks. However, the expression of HSP47 decreased in the LPS-treated lung after 4 weeks and it demonstrated the same level as that of the control lung after 8 weeks (Fig. 7).

Discussion

Acute respiratory distress syndrome (ARDS) represents a sudden onset of hypoxemia and bilateral lung infiltrates in critically ill patients. The prognosis for ARDS patients is still poor, with motility rates ranging from 50% to 70% [11]. One of the reasons for the poor prognosis is the excessive deposition of collagen during the development of fibrosis [1–3]. The expression of HSP47 has been observed in various fibrotic diseases of clinical and basic studies [12–14]. The areas of diffuse alveolar damage and pulmonary fibrosis showed an increased deposition of collagen in the areas of pulmonary fibrosis, which was associated with an overexpression of HSP47. Razzaque et al. [15] demonstrated that the colocalization of collagen and HSP47 was noted in the regions of pulmonary fibrosis. Regarding pulmonary fibrosis, HSP47 plays an important role in the progression of fibrosis. The overexpression of HSP47 may contribute to the fibrosis found in pulmonary fibrotic lung diseases.
In this study we showed that LPS-induced ARDS rat lung also expressed HSP47. Northern blot analysis of lung after a single administration of LPS showed that the expression of HSP47 mRNA was maximal 14 days after treatment. Type I and type III collagen mRNAs were also induced in parallel with HSP47 mRNA. The expression levels of HSP47 mRNA were proportional to that of collagen mRNAs during the progression of pulmonary fibrosis. The expression of these three mRNAs and of the total collagen and HSP47 protein also closely correlated with the progression of pulmonary fibrosis. An immunoblot analysis of the lung after a single administration of LPS showed HSP47 protein to be the same as that of mRNA. These observations suggest that HSP47 might thus play an important role in the synthesis, processing, or secretion of procollagen.

HSP47 is an ER-resident stress protein that can specifically bind to newly synthesized procollagen. HSP47 binds to types I–V collagens but it does not bind to other extracellular matrix proteins such as fibronectin and laminin [6, 16]. Satoh et al. [17] showed that HSP47 transiently binds to procollagen in the ER. When abnormal procollagens caused by heat shock, treatment with dipyridyl, or an iron chelator that inhibits the triple helix formation of procollagens occurred, HSP47 was not dissociated from abnormal procollagens and thus inhibited procollagen secretion [7, 17]. In addition, HSP47 is essential as a molecular chaperone for the development of triple helix formation in collagen. In the absence of HSP47, collagen microfibril formation is impaired [18]. The expression of HSP47 always correlates with that of collagens in vitro [19, 20].

In this study we showed that the mRNA and protein of HSP47, a collagen-binding stress protein, both increased in the LPS-treated lungs. However, mRNA of HSP47 did not increase at 4 days. Thereafter, HSP47 was markedly induced in parallel with type I and type III collagen mRNAs during pulmonary fibrosis in the rats induced by the administration of LPS. In the normal rat lung, only trace amounts of collagen are synthesized and the expression of HSP47 mRNA is only slightly detectable. This suggests that increasing the expression of HSP47 was delayed in type I and type III collagens and afterward it increases in the same way as type I and type III collagens. Finally, type I and type III collagens are coexpressed with HSP47 in the LPS-induced lung disease.

The transcription of heat shock genes is regulated by cis-acting heat shock elements (HSE) in the promoter.

**Fig. 4** Collagen changes in LPS-administered rat lung. A: The rat lung was treated with saline and 14 days later was stained with Azan to visualize the collagen fibrils (×100). B: The rat lung was treated with intratracheally administered LPS and after 14 days was stained with Azan. There was an increase in the collagen content of the lung parenchyma in the control group (×100).

**Fig. 5** Immunohistochemical staining of lung tissues from rat without and with LPS treatment. A: Immunohistopathologic examination of the saline-administered lung for the detection of HSP47 in an animal killed 28 days later; × 400. B: Immunohistopathologic examination of the LPS-administered lung for the detection of HSP47 in an animal killed 28 days later; × 400.
region and by trans-acting heat shock factors [21]. HSP47 is induced by several stressors inducing heat shock through the regulation of the HSE/heat shock factor system, because there is an HSE in the HSP47 promoter [22]. In the current study the mRNA levels of HSP47 did not change until after 4 days of LPS administration and increased after 7 days of LPS administration. Thus it was suggested that the HSP47 overexpression was not caused by LPS toxicity directly; it was induced in parallel with the progression of fibrosis. These results suggested that the induction of HSP47 is different from that of other HSPs and that HSP47 protein, which was observed during the progression of pulmonary fibrosis, is therefore probably caused by a different mechanism regarding heat shock stress.

In summary, the increased expression of HSP47 in the LPS-treated lung may possibly lead to progressive pulmonary fibrosis by regulating the increased synthesis/assembly of various collagens. Based on the above results, we feel that the therapeutic intervention directed against HSP47, such as the administration of HSP47 antisense oligonucleotides, could possibly alter the pulmonary fibrotic process in ARDS, thereby providing a clinically positive effect.

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References