Lipopolysaccharide induces expression of collagen VI in the rat lung

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Abstract: The involvement of the lung during the septic systemic inflammatory response elicited by administration of lipopolysaccharide (LPS) was investigated. Eight-week-old male Sprague–Dawley rats were injected i.p. with 15 mg/kg LPS. After 24 h, the lungs were excised to evaluate the cellular responses to LPS. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) analysis revealed that type VI collagen (ColVI) was extremely upregulated during sepsis in the rat lung within the first 24 h of LPS administration. Upregulation of ColVI protein and its mRNA was demonstrated by Western blot analysis, real time PCR, and immunohistochemistry. To the best of our knowledge, this is the first report demonstrating the activation of ColVI in the rat lung at the early stage of systemic inflammation. Activation of ColVI might be involved in sepsis-mediated lung fibrosis at an early stage.

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Lung inflammation during sepsis leads to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), which may trigger persistent lung fibrosis. ALI/ARDS is manifested by acute sepsis-related respiratory failure accompanied by a high mortality rate, and is induced by pulmonary edema due to infection, trauma, or allergy. In the late phase of ALI/ARDS, remodeling of the extracellular matrix (ECM) results in accumulation of connective tissues, mainly composed of collagen, and over-activation of this remodeling leads to mortal lung fibrosis. Although the regulatory mechanisms are not well understood, some proteins associated with regulation of acute lung injury have been proposed, including NF-kappaB, neutrophil elastase, vitronectin, and collagens.

The ECM of connective tissues is comprised of a variety of proteins that define structural integrity and various physiological functions. The most abundant family is the collagens comprising >20 different types identified to date. Collagens are centrally involved in the formation of fibrillar and microfibrillar networks of the ECM, such as basement membranes as well as other structures of the connective tissues. In the lung epithelium, the basement membrane contains fibronectin, laminin, and collagen VI (ColVI). ColVI is a widely distributed ECM protein forming microfibrillar networks with proteoglycans as well as other collagen fibers, and is involved in formation of the basement membrane. The triple helix of ColVI is typically composed of alpha1, alpha2 and alpha3 chains and is secreted into the ECM after forming a tetrametric structure.

In humans and other vertebrates, ColVI is present in the vascular and bronchial walls and in the interstitial space in the lungs. In addition, at the early stage of lung fibrosis, ColVI expression is increased and increased mRNA expression is mainly observed in fibroblasts of the myofibroblast type. Recently, we showed that administering 15 mg/kg lipopolysaccharide (LPS) to rats for 24 h induced systemic inflammation and activated lysosome biogenesis and autophagy in the liver and heart. Here we found that systemic inflammation activates ColVI in the rat lung, as an early phase of fibrosis.

The animal experiment protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University. In brief, eight-week-old male Sprague-Dawley rats were injected i.p. with 15 mg/kg LPS (from Escherichia coli obtained from Sigma [L-2630; St Louis, MO, USA], 500,000 Endotoxin Units/mg) dissolved in 0.5 mL isotonic NaCl or vehicle (n = 4/group). LPS was injected i.p. into the rats 24 h prior to sacrifice.

Lungs (0.1 g) were homogenized with 1 mL of ice-cold STE buffer containing 320 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na3VO4, and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Samples were subjected to SDS-PAGE using a 7.5% gel. For MALDI-TOF analysis, proteins were resolved by SDS-PAGE and several proteins were excised, reduced, S-alkylated, and digested.
with trypsin (sequence grade modified; Promega, Madison, WI, USA). The obtained tryptic peptides were subjected to a MALDI-TOF analysis (UltrafleXtreme, Bruker Daltonics, Billerica, MA, USA). The spectra obtained were subjected to a search against the NCBI database using the Mascot search engine (Matrix Science). Western blot analysis was performed with anti-ColVI (Abcam; 1:1,000 dilution), anti-ColI (ab34710, Abcam, Cambridge, MA, USA; 1:1000 dilution), or anti-GAPDH (Millipore) antibodies. Peroxidase-conjugated antirabbit and antimus IgG antibodies were obtained from Promega (Madison, WI, USA). Protein levels were determined using a standard curve constructed by plotting the band densities and was normalized to the GAPDH levels using the CS Analyzer v. 3.0 software (ATTO, Tokyo, Japan).

Complementary DNA was synthesized from lung tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qPCR) (StepOnePlus, Applied Biosystems, Foster City, CA, USA) was performed using SYBR green as a fluorescent dye. The primers used were as follows: 5′-GTACATCAGCCAAACCCCA-3′ and 5′-CAGGAT-GCG-ACC-CTT-CGC-CC-3′ for Coll-alpha1; 5′-CCC-TGG-TGG-ACA-AGG-TGA-AA-3′ and 5′-CGC-ATG-AGC-CTC-TGA-AT-3′ for CollI-alpha1; 5′-TGCT-GAG-ACG-TCC-TTT-GTC-CG-3′ and 5′-GTA-GAA-GTT-CTG-CTC-GCC-CA-3′ for CollVI-alpha1; 5′-GGG-ACA-CAC-GTC-TTC-AGG-TT-3′ and 5′-CCA-TGA-GTT-GTT-GGG-3′ for CollVI-alpha2; 5′-GGG-ACA-CAC-GTC-TTC-AGG-TT-3′ and 5′-CAAT-TCT-GCT-GCC-GAG-GTT-CC-3′ for CollVI-alpha3; and 5′-CAT-CCG-TTC-TCT-ACC-CAG-CC-3′ and 5′-AAT-TCT-GAG-GCT-GGA-GTT-CC-3′ for 18S rRNA. The conditions for the PCR reactions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s.

Lung tissues fixed in paraformaldehyde were embedded in paraffin, sliced in 3-μM-thick sections and stained with hematoxylin-eosin (HE). In addition, in order to identify the location of ColVI and myofibroblasts, samples were subjected to immunohistochemical analysis as previously described. In brief, sections were incubated at 4°C overnight with 1/100 diluted anti-ColVI antibodies (ab6588, Abcam, Cambridge, MA, USA) and alpha smooth muscle actin (α-SMA) antibodies (A2547, Sigma), and subsequently incubated with peroxidase-conjugated secondary antibody (Nichirei Bioscience, Tokyo, Japan). Diaminobenzidine was used as a substrate to visualize antigens. The samples were eventually analyzed using a light microscope (Olympus AX80).

To evaluate the cellular responses to LPS in the rat lung, we first examined any major changes in the composition of lung proteins using conventional electrophoresis and protein staining with Coomassie Brilliant Blue. A protein with an apparent molecular weight of 108 kDa was clearly upregulated at 24 h following LPS treatment (Fig. 1a). MALDI-TOF analysis revealed that the protein was type VI collagen (ColVI), a widely distributed ECM protein (Table 1). Western blot analysis confirmed a significant upregulation of ColVI in the LPS-treated rat lung compared with the

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**Fig. 1.** Increase of ColVI protein in LPS-treated rat lungs. (a) Major changes in the protein composition of the rat lung treated with LPS (15 mg/kg for 24 h) as shown by conventional electrophoresis (SDS-PAGE) and protein staining with Coomassie Brilliant Blue. The arrow indicates the major LPS-responsive protein. (b) Western blot analysis of the rat lung treated with LPS (15 mg/kg for 24 h) to determine the levels of ColVI and Coll. GAPDH was used as a loading control. (c) Ratios of ColVI, Coll, and GAPDH determined using densitometry analysis. Each bar represents the mean ± SE (n = 4). **, p < 0.01 versus control by Student’s t-test.
untreated control, whereas the cytoplasmic protein GAPDH was unaltered (Fig. 1b, c). Coll, one of the major matrix proteins, was unchanged (Fig. 1b, c). Therefore, ColVI may be activated at an early stage in the process of lung fibrosis, prior to activation of Coll.

We next performed qPCR analysis to determine whether or not LPS induces the expression of ColVI genes. As shown in Fig. 2, a 2.3- to 9.9-fold induction of the ColVI genes (ColVI alpha 1-3 chains) was observed 24 h after LPS administration. In contrast, the Coll alpha 1 gene showed no induction after LPS treatment. A ribosomal RNA (18S rRNA) was used as the internal control.

There were no histological abnormalities and a faint positive stain of ColVI in control lung tissue (Fig. 3a, c). In contrast, lung tissue treated with LPS revealed prominent hemorrhage and thickening of the alveolar walls, and the presence of neutrophils indicating early inflammation (Fig. 3b). In addition, LPS treatment showed a higher number of ColVI-positive lung cells compared with the control (Fig. 3d). In contrast, there was no increase in α-SMA-positive cells in the LPS-treated rat lung compared with the untreated rats (Fig. 3e, f), suggesting that the upregulation of ColVI occurs before the increase in myofibroblasts during lung fibrosis. Although previous studies indicated that tumor-associated macrophages induce ColVI and promote tumor inflammation14, only the increase in neutrophils in the LPS-treated rat lung was observed, suggesting that the influence of macrophages may be negligible.

In this study, we demonstrated upregulation of ColVI protein and mRNA during the systemic inflammatory response in the rat lung within the first 24 h. This result reinforces a previous study evidencing ColVI deposition at the early stage of fibrosis and suggests that ColVI is being highly expressed in the lung from the time of injury until fibrosis becomes obvious11. Histologically, ColVI is upregulated mainly in the pulmonary interstitial space as previously described10.

ColVI has been reported to induce myofibroblast differentiation in heart tissue, which promotes fibrosis after infarction15. However, it is likely that other triggers, such as untreated control, whereas the cytoplasmic protein GAPDH was unaltered (Fig. 1b, c). Coll, one of the major matrix proteins, was unchanged (Fig. 1b, c). Therefore, ColVI may be activated at an early stage in the process of lung fibrosis, prior to activation of Coll.

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**Table 1.** Tryptic Peptides of the Rat ColVI Observed by MALDI-TOF Analysis

| Observed (m/z) | Theoretical (m/z) | Error (ppm) | Start–end | Amino acid sequence |
|---------------|------------------|-------------|-----------|-------------------|
| 943.5153      | 942.4771         | 32.9        | 871–878   | EDPTQVYR          |
| 1052.5936     | 1051.5774        | 8.47        | 722–730   | LLPPTQNNR         |
| 1073.6187     | 1072.6029        | 8.42        | 1012–1020 | VPNYQALLR         |
| 1289.6851     | 1288.6775        | 0.26        | 997–1007  | TAEYDVAFGER       |
| 1289.6851     | 1288.6775        | 0.26        | 208–218   | LSIAHTDHTRY       |
| 1580.8339     | 1579.8358        | −5.84       | 194–207   | VFSVAITPDHLEPR    |
| 1701.8555     | 1700.8595        | −6.61       | 879–894   | VAVVQYGSGQQQPGR   |
| 1985.8093     | 1984.8190        | −8.51       | 249–264   | NNVEQCVCTFECQAAR  |
| 2029.9623     | 2028.9727        | −8.74       | 702–719   | LQWMAGGTFGEALQYTR |
| 2199.9664     | 2198.9120        | −10.39      | 91–109    | NLYVNAGALHYSDEVIEIR |
| 2713.3218     | 2712.3469        | −11.95      | 36–59     | AIAFQDCPVDLFFVLDTSESVALR |

* a The m/z values of observed ions by MALDI-TOF analysis. b The theoretical m/z values of tryptic peptides from rat ColVI. c The differences of m/z between observed and theoretical peptide ions. d The numbers of start and end positions relative to the first amino acid of rat ColVI. e Amino acid sequences in rat ColVI.

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**Fig. 2.** Activation of ColVI genes. Relative mRNA levels of Coll alpha1 (a), ColVI alpha1 (b), ColVI alpha2 (c), and ColVI alpha3 (d) to GAPDH were analyzed by qPCR. Each graph represents the mean ± SE (n = 4). **, p < 0.05 versus control by Student’s t-test.
collagen III deficiency, are also involved. Myofibroblasts produce collagens that mainly make up the ECM in pulmonary fibrosis, and so ColVI may have a fundamental role in fibrosis.

Several reports have indicated that tumor-associated macrophages induce ColVI and promote tumor inflammation; however, we did not observe any increase in macrophages under our experimental conditions (Fig. 3b). No induction of Coll proteins and genes was observed after LPS treatment (24 h) (Figs. 1 and 2).

It has been reported that ColVI may be an early phenomenon rather than a late phenomenon in lung fibrosis, and to the best of our knowledge, this is the first report on ColVI activation during early stage systemic inflammation in the rat lung. Activation of ColVI appears to be involved in sepsis-mediated lung fibrosis at an early stage.

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