CHEST Online Supplement

Lumican Regulates Ventilation-Induced Epithelial-Mesenchymal Transition Through Extracelluar Signal-Regulated Kinase Pathway

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e-Appendix 1a.

Methods

Generation and maintenance of lumican-null (lumican$^{-/-}$) mice

Briefly, a germ-line chimeric mouse, generated by blastocyst injection of a targeted embryonic stem cell clone from mouse strain J129/sv, was mated with C57BL/6 mice. The offspring were identified by polymerase chain reaction and southern hybridization. All animals used in this study (lumican$^{-/-}$ and their wild-type littermates) were of a C57BL/6 genetic background and were identified by polymerase chain reaction. Mice that are homozygous for the targeted mutation are viable and fertile. The lumican-null mice have serious functional defects including corneal opacity and fragile skin and tendon associated with disorganized and loosely packed collagen fibers. This proteoglycan has also been shown to participate in the regulation of many cellular functions including cell proliferation, migration, adhesion, and gene expression. The lower expressions of the lumican protein in lumican$^{-/-}$ mice were confirmed using Western blot analysis.

Ventilator protocol

In brief, tracheostomy was performed under general anesthesia with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) followed by ketamine (0.1 mg/g/hr) and xylazine (0.01 mg/g/h) at a rate of 0.09 ml/10g/h by a continuous intraperitoneal infusion. The mice were placed in a supine position on a heating blanket and then attached to a Harvard apparatus ventilator, model 55-7058 (Harvard Apparatus, Holliston, MA), set to deliver

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either 6 ml/kg at a rate of 135 breaths per min or 30 ml/kg at a rate of 65 breaths per min, for 2 to 8 h while breathing room air with zero end- expiratory pressure. The tidal volume delivered by the ventilator was checked by fluid displacement from an inverted calibration cylinder. Continuous monitoring of end-tidal CO_2 by a microcapnograph (Columbus Instruments, Columbus, OH) was performed, and respiratory frequencies of 135 breaths per min for 6 ml/kg and 65 breaths per min for 30 ml/kg were chosen in the experiment, with end-tidal CO_2 at 30 to 40 mm Hg. Airway peak inspiratory pressure was measured with a pressure-transducer amplifier (Gould Instrument Systems, Valley View, OH) connected to the tubing at the proximal end of the tracheostomy. Mean arterial pressure was monitored every hour during mechanical ventilation by using the same pressure-transducer amplifier connected to a 0.61-mm outer diameter (0.28-mm inner diameter) polyethylene catheter ending in the common carotid artery. Two hours of mechanical ventilation for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot and 8 h for MIP-2 and TGF-β1 production, free radicals, lung fibrosis and collagen, electron microscopy, and immunolfuorescent analysis were used based on our time-course and previous studies.[1,2] Control, nonventilated mice were anesthetized and sacrificed immediately. At the end of the study period, heparinized blood was taken from the arterial line for analysis of arterial blood gas and the mice were sacrificed.

Analysis of lung water

Lungs were removed *en bloc*, and large airways were removed. Both lungs were weighed and then dried in an oven at 80°C for 48 h. If no changes were found in the dry lung weight at 24 and 48 h, the weight at 48 h was used. Lung wet-to-dry weight ratio was used as an index of pulmonary edema formation.

Evans blue dye analysis

Extravasation of Evans blue dye (Sigma Chemical, St. Louis, MO) into the interstitium was used as a quantitative measure of changes of microvascular permeability in ALI.[3] Thirty minutes before end of mechanical ventilation, 30 mg/kg of Evans blue dye was injected through internal jugular vein. At the time of sacrifice after 8 h of mechanical ventilation, the lungs were perfused free of blood with 1 ml of 0.9% normal saline via the right ventricle and removed *en bloc*. Evans blue was extracted from lung tissue after homogenization for 2 min in 5 ml of formamide

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(Sigma Chemical, St. Louis, MO) and incubated at 37°C overnight. The supernatant was separated by centrifugation at 5,000g for 30 min and the amount was recorded. Evans blue in the plasma and lung tissue was quantitated by dual-wave length spectrophotometric analysis at 620 and 740 nm. We calculated the Evans blue dye amount extracted from lung tissue and divided the amount by weight of lung tissue.

Measurement of MIP-2 and TGF-β1

At the end of the study period, the lungs were lavaged via tracheostomy with a 20-gauge angiocatheter (sham instillation) 3 times with 0.6 ml of 0.9% normal saline. The effluents were pooled and centrifuged at 2,000 rpm for 10 min. Supernatants were frozen at -80°C for further analysis of the cytokine. MIP-2 with lower detection limit of 1 pg/ml and TGF-β1 with lower detection limit of 4.61 pg/ml were measured in serum and BAL fluid by using a commercially available immunoassay kit containing antibodies that were cross-reactive with rat and mouse MIP-2 and TGF-β1 (Biosource International, Camarillo, CA, USA). Each sample was run in duplicate according to the manufacturer's instructions.

Myeloperoxidase (MPO) assay

The lungs (0.12-0.17 g) were homogenized in five ml of phosphate buffer (20 mM, pH 7.4). One ml of the homogenate was centrifuged at 10,000 g for 10 min at 4°C. The resulting pellet was resuspended in one ml of phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The suspension was then subjected to three cycles of freezing (on dry ice) and thawing (at room temperature), after which it was sonicated for 40 sec and centrifuged again at 10,000 g for five min at 4°C. The supernatant was assayed for MPO activity by measuring the hydrogen peroxide (H_2O_2)-dependent oxidation of 3,3`, 5,5`-tetramethylbenzidine (TMB). In its oxidized form, TMB has a blue color, which was measured spectrophotometrically at 650 nm. The reaction mixture for analysis consisted of 25 μl tissue sample, 25 μl of TMB (final concentration 0.16 mM) dissolved in dimethylsulfoxide, and 200 μl of H_2O_2 (final concentration 0.30 mM) dissolved in phosphate buffer (0.08 M, pH 5.4) minutes prior to adding to mixture. The reaction mixture was incubated for three min at 37°C and the reaction

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was stopped by adding one ml of sodium acetate (0.2 M, pH 3.0) after which absorbance at 650 nm was measured. The absorbance (A650) was reported as units (OD)/g of wet lung weight.

Masson's trichrome stain and fibrosis scoring

The lung tissues from control, nonventilated mice, mice exposed to high or low tidal volume ventilation for 8 h while breathing room air were paraffin embedded, sliced at 4 μm, deparaffinized, stained sequentially with Weigert's iron hematoxylin solution, Biebrish scarlet-acid fuchsin solution, and aniline blue solution according to the manufacturer's instruction of a trichrome kit (Sigma, St. Louis, MO, USA). A blue signal indicated positive staining of collagen. The fibrotic grade of each lung field was assessed using the criteria of Ashcroft, ranging from grade 0 to 5 as follows: grade 0: normal lung; grade 1: minimal fibrous thickening of alveolar or bronchial walls; grade 2: moderate thickening of walls without obvious damage to lung architecture; grade 3: increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 4: severe distortion of structure and large fibrous areas (honeycomb lung); grade 5: total fibrous obliteration in the field.[1] Average number of 10 nonoverlapping fields in Masson's trichrome staining of paraffin lung sections, 5 mice per group, were analyzed for each section by two independent investigator blinded to the mouse genotype.

Collagen assay

The lungs were homogenized and collagen was solubilized in 0.5 M acetic acid. The protein extracts were incubated with Sirius red dye and level of collagen in lung tissues was determined at absorbance of 540 nm by SIRCOL collagen assay kit (Biocolor Ltd., UK) according to manufacturer's instructions. Amount of collagen was expressed in μg/g of wet lung weight.

Measurement of malondialdehyde (MDA)

The lungs were homogenized in phosphate buffered saline containing butylated hydroxytoluene. The MDA in the protein extracts was measured using the Oxiselect TBARS assay kit (Cell Biolabs, San Diego, CA) containing thiobarbituric acid reactive substances. Each sample was run in duplicate and expressed as μmole/g protein according to the manufacturer's instructions.

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Immunoblot analysis

The lungs were homogenized in 1.5 ml of lysis buffer (20 mM HEPES pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, 50 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiotreitol, 400 μM aprotinin, and 400 μM phenylmethylsulfonyl fluoride), transferred to eppendorff tubes and placed on ice for 15 min. Tubes were centrifuged at 14,000 rpm for 10 min at 4°C and supernatant was flash frozen. Crude cell lysates were matched for protein concentration, resolved on a 10% bis-acrylamide gel, and electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). For assay of ERK1/2 phosphorylation, ERK1/2, lumican, and glyceraldehydes-phosphate dehydrogenase (GAPDH) protein expression, western blot analyses were performed with antibodies of phospho-ERK1/2, ERK1/2, lumican, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed by enhanced chemiluminescence (NEN Life Science Products, Boston, MA, USA).

Immunofluorescence labeling

The lung tissues were paraffin embedded, sliced at 4 μm, deparaffinized, and stained according to the manufacturer's instruction for an immunohistochemical kit (Santa Cruz Biotechnology, Santa Cruz, CA). The lung sections were incubated with primary rabbit anti-mouse antibodies of E-cadherin, S100A4 and α-SMA (1:100; New England BioLabs, Beverly, MA) and fluorescent Cy3-conjugated anti-rabbit secondary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear staining was performed with Hoechst solution (0.5 μg/ml; Sigma, St. Louis, MO). The fluorescence-labeled slides were then examined with the Leica TCS 4D confocal laser scanning microscopy system (Leica, Wetzlar, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

For isolating total RNA, the lung tissues were homogenized in TRIzol reagents (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed by using a GeneAmp PCR system 9600 (PerkinElmer, Life Sciences, Inc., Boston, MA), as previously described.[3] The following primers

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were used for PCR: lumican, forward primer 5'-GTCACAGACCTGCAGTGGCTCAT-3' and reverse primer 5'-ATC TTGGAGTAAGACAGTGGTCC-3' and GAPDH as internal control, forward primer 5'-AATGCATCCTGCA CCACCAA-3' and reverse primer 5'-GTAGCCATATTCATTGTCATA-3' (Integrated DNA Technologies, Inc., Coralville, IA).[4]

Transmission electron microscopy assay

The lungs were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, (pH 7.4) for 1 hr at 4 °C. The diaphragms were then postfixed in 1% osmium tetroxide (pH 7.4), dehydrated in a graded series of ethanol, and embedded in EPON-812. Thin sections (70 nm) were cut, stained with uranyl acetate and lead citrate, and examined on a Hitachi H-7500 EM transmission electron microscope.

References

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e-Appendix 1b.

We demonstrated that level of lumican increased after 2 h of normal tidal volume mechanical ventilation (10 ml/kg) but decreased after 4 and 8 h of mechanical ventilation as compared to that of control, nonventilated mice (arbitrary units of lumican: Control=1.0±0.1, V_T 10 ml/kg, 2h =1.4±0.3·, V_T 10 ml/kg, 4h =1.1±0.1; V_T 10 ml/kg, 8h =1.0±0.2, ·P<0.05 versus Control). The levels of EBD (Control= 24.1±3.4 ng/mg lung weight, V_T 10 ml/kg, 8h =

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47.3±2.9 ng/mg lung weight·, ·P<0.05 versus Control), wet-to-dry weight ratio (Control= 4.3±0.5, V_T 10 ml/kg, 8h = 5.5±0.2 ·, ·P<0.05 versus Control), and MPO (Control= 0.42±0.19 optical density (OD)/g lung weight, V_T 10 ml/kg, 8h = 0.65±0.12 OD/g lung weight·, ·P<0.05 versus Control) were also increased in mice ventilated at V_T 10 ml/kg compared with that of control, nonventilated mice. Increased production of MIP-2 (Control= 10.7±1.3 pg/ml, V_T 10 ml/kg, 8h = 23.5±1.6 pg/ml·, ·P<0.05 versus Control) but no significant elevation of TGF-β1 (Control= 8.7±0.8 pg/ml, V_T 10 ml/kg, 8h = 9.3±0.9 pg/ml, P = 0.14) versus Control) were observed after 8 h of normal tidal volume mechanical ventilation (10 ml/kg) as compared to that of control, nonventilated mice. Only transient elevation of lumican expression and no increase of the crucial pro-fibrogenic cytokine TGF-β1 suggested that lumican played a less significant role in the normal tidal volume mechanical ventilation-induced lung injury.

Because different animals have different respiratory mechanics, the physiologically valid magnitudes of stretch for the developments of microvascular leak differ depending on the species of animals. A previous murine study of stretch-induced injury revealed that normal tidal volume mechanical ventilation (10 ml/kg) induces lung inflammation because of repetitive alveolar collapse and reopening but are unlikely to cause substantial lung overstretch.[1] This may explain the discrepancy between the levels of MIP-2 and TGF-β1. While MIP-2 was involved in the induction of acute lung inflammation, the secretion of TGF-β1 was associated with epithelial-mesenchymal transition (EMT).

References

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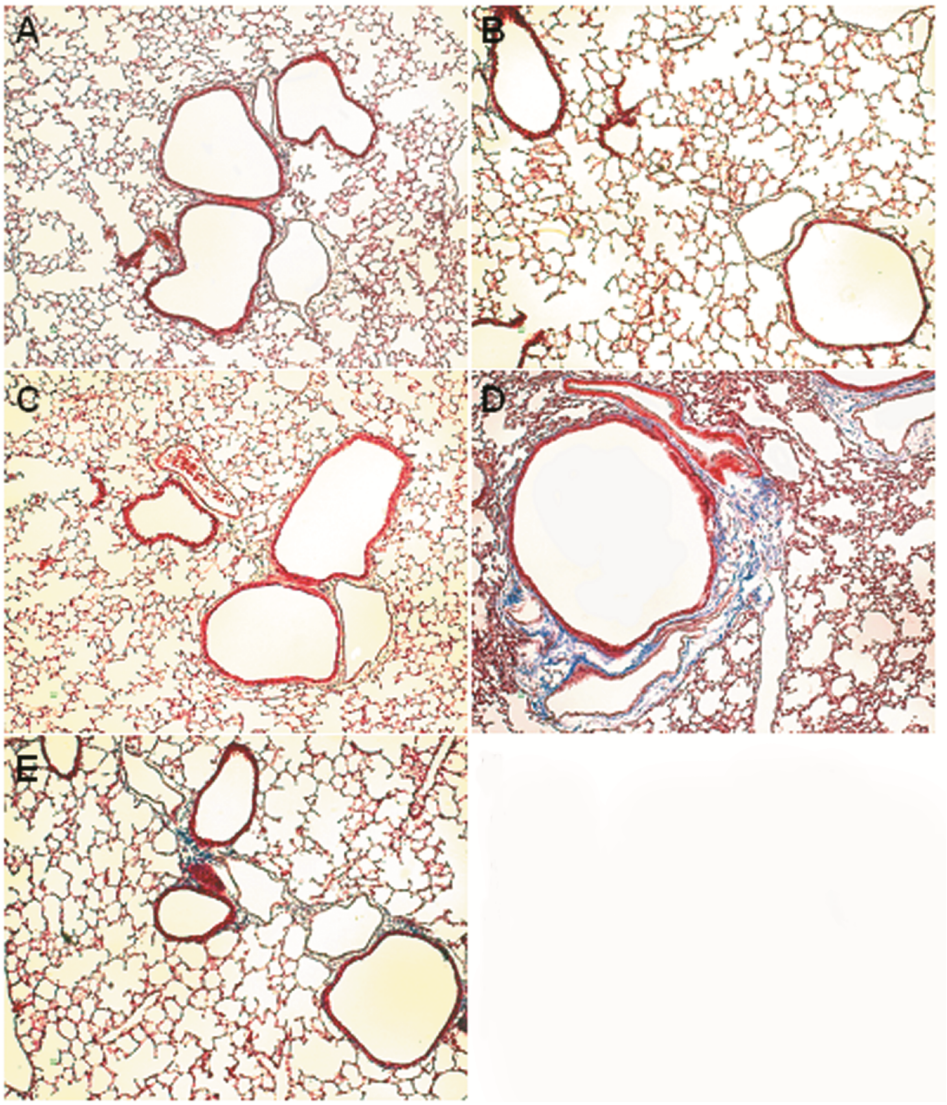
e-Figure 1. Lumican deficient mice reduced lung stretch-induced lung fibrogenesis. Representative photomicrographs (x100) with Masson's trichrome staining of paraffin lung sections were from control, nonventilated mice and mice ventilated at V_T 6 ml/kg or V_T 30 ml/kg for 8 h with room air (n = 5 per group). (A) Control wild-type mice; (B) Control lumican deficient mice; (C) V_T 6 ml/kg wild-type mice; (D) V_T 30 ml/kg wild-type mice; (E) V_T 30 ml/kg lumican deficient mice. Peribronchiolar and parenchymal blue staining indicates positive staining for lung fibrosis.



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