The Role of Hyaluronan Synthase 3 in Ventilator-Induced Lung Injury

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Online Data Supplement

Gene Targeting

The targeting vector was designed such that insertion of the selectable marker cassette (PGKneo) would delete a large portion of the 3rd intron in addition to the splice acceptor sequence immediately preceding exon 4 plus the first 300 nucleotides, encoding 100 amino acids within the proposed catalytic region for the hyaluronan synthase 3 (Has3) polypeptide. An endogenous KpnI site was used for this purpose. In total, the targeting vector included 7 kilobase pairs of homology, with a 1.5 kilobase pair 5′ arm and a 5.5 kilobase pair 3′ arm. The linearized targeting vector was electroporated into GK129 ES cells as previously described (E1). Resistant clones were expanded and screened by Southern hybridization with a 3′ flanking probe to detect clones that had undergone the desired homologous recombination event. Clones identified in this way were expanded and subjected to Southern analyses with a panel of probes including a neo cassette probe, Has3 internal probe and 5′ and 3′ flanking probes. Correctly targeted clones were determined to have undergone the desired recombination event, with no additional rearrangements or insertions detected.

Two correctly targeted clones were expanded and microinjected into 3.5 day post coitum (dpc) C57BL/6J blastocysts using standard procedures. Microinjected blastocysts were surgically transferred to the uterine horns of 2.5 dpc pseudopregnant CD1 female mice. Chimeric animals were identified on the basis of coat color; agouti and/or white/cream coat color being indicative of ES cell contribution.

Ventilator Protocol

The animals were anesthetized by intraperitoneal ketamine (90 mg/kg) (Abbott Laboratories, N. Chicago, IL) and xylazine (10 mg/kg) (Burns Veterinary Supply Inc. Rockville Centre, NY) while breathing room air. Throughout the experiment, animals were placed in a supine position on a heating blanket and body temperature was monitored with a rectal probe. A 20-guage angiocatheter (Becton Dickinson Infusion Therapy System Inc, Sandy, UT) was inserted into trachea and connected to a Harvard apparatus ventilator, model 55-7058 (Holliston, MA). The mice were then ventilated either at 6 ml/kg with a rate of 120-140 breaths per minute or 30 ml/kg with a rate of 55-65 breaths per minute in room air. End tidal CO_2 was monitored intermittently by a microcapnograph (Columbus Instruments, Columbus, OH) and was kept at 30-40 mmHg by adjusting the respiratory rate of ventilator. The V_T was increased by 5 ml/min to correct the air loss from sample flow adaptor during monitoring of end tidal CO_2. During the period of ventilator use, intraperitoneal ketamine 0.05mg/g and xylazine 0.005 mg/g were administered every 30 minutes, and 0.9% NaCl was infused continuously at a rate of 0.009 ml/g/hr to maintain blood pressure. Harvesting of lung and bronchoalveolar lavage (BAL) were performed after 5 hours of ventilation in the high V_T and low V_T groups, or immediately after the anesthesia in control groups of animals.

Lung Lavage

At the end of the experiments, lungs were lavaged through the tracheal tube three times with 0.6

ml sterile 0.9% NaCl. One ml of the pooled effluents was used for cytospin and subsequent cell differentials, and 100 μl was used for total neutrophil counts. The remaining effluents were centrifuged at 2,000 rpm for 10 minutes at 4°C, after which the supernatants were frozen at -80°C for further measurement of total protein, MIP-2, and HA.

BAL Cell Counts

Neutrophil counts in BAL were used to measure migration of neutrophils into the alveoli and airways (E2). Total cell counts in lavage fluid were performed by use of a hemocytometer (n = 5 in control and low V_T groups, n = 7 in Has3-/- and n = 10 in wild-type of high V_T group). To perform cell differentials, cells in lavage fluid were fixed on glass slides by using cytospin and then stained with a hematologic stain kit (Fisher Diagnostics, Middletown, VA).

Measurement of MIP-2 in Lavage Fluid

MIP-2 protein in BAL was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit containing antibodies that were cross-reactive with rat and mouse MIP-2 (Biosource International, Camarillo, CA) (E3). All the procedures were performed according to the protocol provided by the manufacturer (n = 5 in control and low V_T groups, n = 7 in Has3-/- and n = 10 in wild-type of high V_T group).

Reverse Transcription-polymerase Chain Reaction (RT-PCR) for MIP-2 and Has

Lung total cellular RNA was isolated by use of a total RNA isolation kit (Ambion Inc., Austin, TX). After determining the concentration, 1 μg of RNA was reverse-transcribed into cDNA

and amplified with a Gene Amp PCR core kit (PerkinElmer Life Science, Boston, MA) following the manufacturer's instructions (n = 2 per group). The procedure of reverse transcription was performed at 42ºC for 45 min, after which enzyme was inactivated by a 5-min incubation at 99ºC.

Mouse Has sequences were used for this analysis. The primers had the following sequences: Has1 Forward 5′-AGT ATA CCT CGC GCT CCA GA-3′, Has1 Reverse 5′-AGC AGC AGT AGA GCC CAG AG-3′; Has2 Forward 5′-AAC AGG GTG TTG AGT CTG GG-3′, Has2 Reverse 5′-TAA ACC ACA CGG ACA CTG GA-3′; and Has3 Forward 5′-CGG GTG AAG GAG AGA CAG AG-3′, Has3 Reverse 5′-GCA ATG AGG AAG AAT GGG AA-3′. These resulted in reaction products of 481 bp for Has1, 502 bp for Has2, and 712 bp for Has3. The primer sequences for MIP-2 were Forward 5′-GAA CAA AGG CAA GGC TAA CTGA-3′ and Reverse 5′-AAC ATA ACA ACA TCT GGG CAAT-3′, which resulted in a product of 195 bp. PCR for glyceraldehyde-6-phosphate dehydrogenase (GAPDH) was performed as a control, using the following primers: Forward 5′-AAT GCA TCC TGC ACC ACC AA-3′ and Reverse 5′-GTA GCC ATA TTC ATT GTC ATA-3′, which yielded a 516-bp product.

Lung Myeloperoxidase Assay

Lung myeloperoxidase (MPO) activity was used for the quantitative assessment of total pulmonary neutrophil infiltration in the interstitium, alveolar space, and pulmonary vessels as previously described (n = 5 in control and low V_T groups, n = 6 in Has3-/- and n = 9 in wild-type

of high V_T group) (E4-E6). The lungs were homogenized in 5 ml of phosphate buffer (20 mM, pH 7.4) and 1 ml of the homogenate was centrifuged at 10,000 g for 10 minute at 4°C. The resulting pellet was resuspended in 1 ml of phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO). 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 seconds and centrifuged again at 10,000 g for 5 minutes 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) (Sigma Chemical Co.). 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 (Sigma Chemical Co.), and 200 μl of H_2O_2 (final concentration 0.3 mM) dissolved in phosphate buffer (0.08 M, pH 5.4) minutes prior to adding to mixture. The mixture was incubated for 3 minutes at 37°C and the reaction was stopped by adding 1 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.

Peribronchiolar Neutrophil Infiltration

After euthanasia, the heart and lungs were removed en bloc and a polyethylene tube was inserted into the trachea. The lungs were filled with 10% neutral buffered formalin at 30 cm H_2O pressure via the tube and were fixed in formalin solution. After being embedded in paraffin, the

lung tissues were sectioned at 4μm and stained with hematoxylin and eosin. The stained sections were then reviewed in a blinded method by a pathologist. To quantify the infiltration of neutrophils, the number of neutrophils surrounding 10 bronchioles per slide was determined and was expressed as the average number of neutrophils per bronchiole, as previously described (n = 5 in control and low V_T groups, n = 5 in Has3-/- and n = 7 in wild-type of high V_T group) (E6).

Evans Blue Dye Analysis

Extravasation of Evans blue dye (EBD, Sigma Chemical Co.) into the lung interstitium was used as a quantitative measure of changes of microvascular permeability in acute lung injury (n = 5 in control and low V_T groups, n = 5 in Has3-/- and n = 8 in wild-type of high V_T group) (E7-E9). Briefly, 30 mg/kg of EBD was injected through the internal jugular vein 30 minutes before the end of mechanical ventilation. At the end of the experiments, each mouse was euthanized by exsanguination from the abdominal aorta and 1 ml of 0.9% NaCl was injected into the beating right ventricle to flush intrapulmonary vascular blood. The lungs were removed en bloc and homogenized in 5 ml formamide (Sigma Chemical Co.) for 2 minutes. The mixture was incubated at 37°C overnight and then centrifuged at 5,000 g for 30 minutes. EBD in supernatant was quantitated by the dual wavelength (620 and 740 nm) spectrophotometric method as described previously (E9, E10). This method allows for correction of contaminating heme pigments, as determined by the formula: corrected absorbance at 620 nm = measured absorbance

620 nm – (1.426 × absorbance at 740 nm + 0.03). Concentration of EBD was determined from generated EBD standard absorbance curves and expressed as EBD μg / mg wet weight of lung.

Protein Measurement in Lavage Fluid

Total protein concentration of lavage fluid was determined by the Bradford method (n = 5 per group) (E11). The mixture of lavage fluid and Bradford assay reagent (Bio-Rad Laboratories Inc., Hercules, CA) was read at 595 nm with bovine serum albumin (Bio-Rad Laboratories Inc.) as a standard.

Histochemical Stain of Total Hyaluronan

The process for lung harvest and tissue sections for staining was the same as that described for peribronchiolar neutrophil infiltration. The sections were then incubated with a biotinylated fragment of hyaluronan (HA) binding protein (bHABP, Seikagaku Corp., Tokyo, Japan) of aggrecan. After washing, the bHABP was detected using horseradish peroxidase–conjugated streptavidin (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Diaminobenzadine (Sigma Chemical Co.) was then used to develop the color reaction (n = 1 per group).

Extraction and Analysis of Hyaluronan

Approximately 1 g of lung tissues (from five animals) was extracted under dissociative conditions using 4 M guanidium HCl, 50 mM sodium acetate, 9 mM sodium-ethylenediamin tetraacetic acid (Na-EDTA), 1 mM p-chloromercuribenzoate and 1mM phenylmethylsulfonyl

fluoride pH 5.8, for 30 seconds, three times. The homogenate was stirred for 48 hours and then centrifuged at 200,000 g for 30 minutes. The supernatants were diluted to 1:50 and total HA was measured by use of ELISA. Briefly, high bound ELISA plates (Falcon, Lincoln Park, NJ) were coated with HA (Sigma Chemical Co.) overnight. After being washed with PBS, the plates were then blocked with 10% calf serum in PBS for 1 hour, followed by exhaustive washing with water. The 25 μl diluted sample was mixed with 100 μl bHABP (2 mg/ml) for 1 hour at 37°C, then transferred to the HA-coated ELISA plates and shaken for 1hour at 37°C. After washing, the bHABP was detected by use of horseradish peroxidase-conjugated streptavidin. The color reaction was detected using 1,2-phenylene-Diamine Dihydrochloride tablets (Rainbow Inc., Windsor, CT) and read at 490 nm on ELISA reader. The concentration of HA was determined by a standard curve made from 0.01 to 4 μg/ml of HA (2 sets, and n = 5 per group in each set for lung tisssues HA measurement, n = 4 per group for BAL HA measurement).

Size Fractionation of Hyaluronan

Lung extract was precipitated overnight with 80% ethanol at 4°C. The precipitate was centrifuged at 200,000 g for 30 minutes. The residue was dissolved in 4 M guanidium HCl, 50 mM sodium acetate, 9 mM Na-EDTA, 1 mM p-chloromercuribenzoate and 1 mM phenylmethylsulfonyl fluoride at pH 5.8 for further size fractionation. The sample was fractionated on Sepharose CL-4B size exclusion chromatography (150 cm x 2 cm), which was equilibrated in dissociative conditions using 4 M guanidium HCl, 50mM sodium acetate, 9 mM

Na-EDTA, 1 mM p-chloromercuribenzoate and 1 mM phenylmethylsulfonyl fluoride pH 5.8. The column was calibrated in the following manner: void volume (V_o) was measured using dextran blue 2000; total volume (V_t) was measured using uronic acid. HA sizes were determined by relative elution volume (K_{av}) compared with three HA standards of known molecular weight (Sigma Chemical Co. and Biomatrix Inc., Ridgefield, NJ). K_{av} was calculated from the equation: K_{av} = (elution volume- V_o)/ (V_t- V_o). The three HA standards, 1,600 kD HMW HA, 370 kD LMW HA, and 178 kD LMW HA had K_{av} of 0.17; 0.33; and 0.8, respectively (n = 5 per group). HA peaks were detected using HA ELISA as described above.

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