Development of chronic bronchitis and emphysema in βENaC overexpressing mice

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Online Data Supplement

Methods

Experimental animals. All animal studies were approved by the animal care and use committees of the relevant institutions. The generation of transgenic mice with airway specific overexpression of βENaC has been described previously (1). The colony of βENaC overexpressing mice (line 6608) was maintained on a mixed genetic background (C3H/HeN x C57BL/6N) by breeding of βENaC overexpressing mice with C3B6 F1 mice (Charles River Laboratories, Wilmington, MA). Transgene positive animals were identified by PCR of genomic DNA, as previously described (1). We studied embryonic (E 18.5), newborn (PN 0.5), neonatal (first week), juvenile (2 to 3 weeks) and adult (6 to 8 weeks) βENaC overexpressing mice, and wild-type littermates served as controls in all experiments. Experimental mice were bred in two colonies, at UNC-CH and at the University of Heidelberg, were housed in specific pathogen-free animal facilities and had free access to chow and water.

BAL cell counts and cytokine measurements. Mice were deeply anesthetized via intra-peritoneal injection of a combination of ketamin/xylazin (120 mg/kg and 16 mg/kg, respectively), or Avertin (2,2,2 tribromoethanol, 250 mg/kg), the trachea cannulated, and the total lung (5 day old mice) or right lung (all other age groups) lavaged with PBS. Samples were centrifuged and the cell-free bronchoalveolar lavage (BAL) fluid was stored at –80°C. Total cell counts were determined and differential cell counts performed on cytospin preparations, as previously described (1). Macrophage size was determined by measuring their surface area using Analysis B image analysis software (Olympus, Hamburg, Germany). Concentrations of TNF-α, KC and IL-13 were measured in lung homogenates (fetal and newborn mice) or BAL (5 day and older mice) using ELISA (R&D Systems, Minneapolis, MN) or with a Cytometric Bead Array Mouse Inflammation Kit (BD, Biosciences, San Diego,

CA) according to manufacturer's instructions using a FACScalibur and BD CBA Analysis Software (BD Bioscience, San Jose, CA).

Morphology. Anesthetized mice were killed via exsanguination by cutting the renal arteries. Lungs and tracheae were removed through a median sternotomy, fixed in 4% buffered formalin, and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin (H&E) or alcian blue periodic acid-Schiff (AB-PAS) as previously described (1). For transmission electron microscopy (TEM) studies, lungs were fixed with 4% glutaraldehyde (Ted Pella, Inc. Redding, CA) via intratracheal instillation or by immersion (newborn mice) and stored for at least 24h prior to further processing. Fixed tissues were postfixed in 1% phosphate-buffered osmium tetroxide, dehydrated through a series of ethanol and propylene oxide, and embedded in Poly/Bed-Araldite resin (Polysciences, Inc., Warrington, PA). Sections (1 micron thick) were cut and stained with toluidine blue for light microscopic identification of tissue sites for TEM. Ultrathin tissue sections for TEM were cut approximately 75 nm with a diamond knife, mounted on copper grids, and stained with lead citrate and uranyl acetate. Sectioning was done with LKB Ultramicrotome III (LKB Instruments, Inc., Rockville, MD). Ultrastructural tissue examination and photography were performed with a JEOL JEM 100CXII electron microscope (JEOL Ltd., Tokyo, Japan).

Airway morphometry. Morphometric analyses of airway mucus obstruction were performed in non-inflated, immersion fixed trachea and left lungs. Tracheae were sectioned longitudinally, and lungs were sectioned transversally at the level of the proximal intra-pulmonary main axial airway near the hilus, and at the distal intra-pulmonary axial airway, at 1000 μm (2 week old mice) and 1500 μm (6 week old mice) distal to the hilus. For quantitative assessment of airway mucus obstruction, we used Analysis B image analysis

software (Olympus, Hamburg, Germany) to determine mucus volume density as previously described (2). In brief, images of airway sections were taken with an Olympus IX-71 microscope (Olympus, Hamburg, Germany) at a magnification of 10x. The length of the airway boundary, as defined by the epithelial basement membrane, was measured by this interactive image measurement tool, and the AB-PAS positive surface area within this boundary was measured by phase analysis according to the automatic threshold settings of the software. The volume density of airway mucus, representing the volume of airway mucus content per surface area of the basement membrane (nl/mm^2), was determined from the surface area of AB-PAS positive mucus and the basement membrane length, as previously described (2). Glycogen volume density in airway epithelia of neonatal mice was determined in a similar fashion by measuring the surface area of intra-epithelial PAS positive material. Goblet cells were identified by the presence of intra-cellular AB-PAS positive material, and degenerative airway epithelial cells were identified by morphologic criteria (i.e., cell swelling with cytoplasmic vacuolization). Numeric cell densities of airway epithelial cells were quantitated by counting epithelial nuclei per mm of the basement membrane. Epithelial height was measured at defined sample sites along the airway circumference, as previously described (3). All morphometric measurements were performed by an investigator blinded to the genotype of the mice.

Lung volume and mean linear intercepts. For assessment of lung volume, the total lung (5 day old mice) or right lung (all other age groups) was inflated with 4% buffered formalin to 25 cm of fixative pressure, and lung volume determined by the volume displacement method (4). Subsequently, lungs were processed for histology, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Histologic images were digitally captured with an Olympus IX 71 microscope, using Analysis B image analysis software (Olympus, Hamburg, Germany)

with a line counting tool at a magnification of 16x beginning at a randomly selected point. Mean linear intercepts were determined by dividing the sum of the lengths of all lines in all frames by the number of intercepts between alveolar septi and counting lines, as previously described (5). For each animal, we measured a minimum of 200 intercepts sampled in 10 fields in different lobes.

Immunohistochemistry. Immunohistochemical staining for Ym1 was performed on formalin-fixed, paraffin embedded lung sections cut at a thickness of 5 µm. Unstained and hydrated paraffin sections were pretreated with 3% hydrogen peroxide in methanol followed by incubation with a nonspecific protein-blocking solution containing normal sera (Vector Laboratories Inc., Burlingame, CA). Tissue sections were incubated for one hour with rabbit polyclonal anti-Ym1 antibody at a dilution of 1:16,000. This antibody was a generous gift from Dr. Shioko Kimura (National Cancer Institute, Bethesda, MD) and was prepared by immunizing rabbits with 2 peptide sequences derived from eosinophilic crystals in gastric epithelium of CYP1A2 deficient mice (6). Secondary antibody was a biotinylated goat anti-rabbit IgG. Immunoreactivity of Ym1 was visualized with Vector R.T.U. Elite ABC-Peroxidase Reagent followed by Nova Red (Vector Laboratories Inc., Burlingame, CA). After immunohistochemistry, slides were lightly counterstained with hematoxylin.

Hypoxia detection in the lung. Pimonidazole hydrochloride (Hypoxyprobe^{TM}-1®, Chemicon, Temecula, CA) was solubilized in 50 µl of sterile saline solution and administered via i.p. injection (100 mg/ kg body weight) to 3 day old mice. After 90 min, animals were sacrificed and lungs quickly removed and frozen in OCT compound (Sakura Finetek, Torrace, CA). In hypoxic tissues, pimonidazole binds to protein thiol groups, producing stable complexes that can be detected using FITC labeled MAb 1 (Hypoxyprobe^{TM}-1 Plus Kit,

Chemicon, per manufacturer's instructions). Littermates without pimonidazole hydrochloride treatment were used as negative controls. Esophagus, which was harvested with lungs, was used as a hypoxia positive tissue. Immunostained tissues were analyzed in a Leica SP5 confocal microscope with a 488 nm Ar laser and a 20x lens.

Blood gas analyses. Mixed arterio-venous blood samples were collected immediately after decapitation of neonatal mice (PN 3.5 to 5.5) using heparin coated plastic capillaries (Klinika Medical, Unsingen, Germany), and analysed for pH, CO_2 pressure, O_2 pressure, HCO_3^- concentration, base excess, and O_2 saturation using a blood gas analyzer (Radiometer ABL 500, Diamond Diagnostics, Holliston, MA) according to manufacturer's instructions.

Western blotting: Total protein content in cell-free BAL fluid from βENaC overexpressing mice and wild-type littermates was measured with Pierce BCA (bicinchoninic acid) Protein Assay (Pierce Biotechnology, Rockford, IL), using BSA in PBS as standard. 2 µg of total proteins were loaded on 4-12% PAGE gels, run and transferred onto PVDF membranes. Membranes were blocked in 5% non-fat dry milk in PBS and probed with anti-AMCase rabbit serum (7)(kind gift from Dr. Jack A. Elias, Yale University) diluted 1:250 in PBS/0.1% Tween-20. Secondary antibody was an anti-rabbit HRP-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:10,000 in PBS/0.1% Tween-20. AMCase specific signal was detected using ECL chemoluminescence.

Real-time RT-PCR. Mice were killed by decapitation or with 100% CO_2 and lungs were immediately removed and stored in RNAlater (Applied Biosystems, Darmstadt, Germany). RNA was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany), RNA integrity verified by agarose gel electrophoresis, and cDNA obtained by reverse transcription of 2 µg

of total RNA (Superscript III RT; Invitrogen, Karlsruhe, Germany). Real-time PCR for Muc5ac, Muc5b, Muc4, Gob5, Eotaxin-1, IL-13, IFN-γ, Ym1, Ym2, AMCase and β-actin was performed on an Applied Biosystems 7500 Real Time PCR System using TaqMan universal PCR master mix and inventored TaqMan gene expression assays according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). Relative fold changes in target gene expression were calculated from the efficiency of the PCR reaction and the crossing point deviation between samples from βENaC overexpressing mice and wild-type littermate controls, and determined by normalization to expression of the reference gene β-actin, as previously described (8).

Pulmonary function studies. Invasive measurements of lung mechanics were performed in anaesthetized and paralyzed 8 week old adult mice using a computer-controlled small animal ventilator (Flexi Vent system, Scireq, Montreal, Canada) to determine dynamic resistance (R_L), dynamic compliance (C_{dyn}), pressure volume curves, and static compliance (C_{stat}) of the lung, as previously described (9; 10)**.**

Statistics. All data were analyzed with SigmaStat version 3.1 (Systat Software, Erkrath, Germany) and are reported as mean ± S.E.M. Statistical analyses were performed using Student's t-test, Mann-Whitney Rank Sum test, One Way Analysis of Variance (ANOVA), Kruskal-Wallis ANOVA on Ranks, or Chi-Square test as appropriate, and $P < 0.05$ was accepted to indicate statistical significance.

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