Autophagy Activation in Asthma Airways Remodeling

Kielan D McAlinden, Deepak A Deshpande, Saeid Ghavami, Dia Xenaki, Sukhwinder Singh Sohal, Brian G Oliver, Mehra Haghi, and Pawan Sharma

ONLINE DATA SUPPLEMENT

Materials and methods

Acquisition of human lung tissue

Human lung tissue was obtained from surgical resection, explanted lungs and post mortem organ donors with ethical approval from Royal Prince Alfred Hospital (RPAH), Concord Repatriation General Hospital and St Vincent's Hospital (# HREC14-0045, Sydney) [1]. All patients provided consent for their lung tissue to be used for scientific research and in the case of post mortem samples, consent was obtained from the next of kin. Tissue used as non-asthmatic controls were from non-smoking donors with healthy lungs or from macroscopically normal and isolated regions of lungs from patients with non-small cell carcinoma (NSCCa) and free of respiratory or systemic diseases. Non-asthmatic tissue that were deemed to be in close vicinity to cancerous regions were excluded from the study. Patients with a known smoking history were excluded from the non-asthmatic group.

Human subject classification

The mean age of the non-asthmatic subjects was 48 years (SD = 16.41) and in the asthmatic subjects the mean age was 50 years (SD = 21.37) with 100% of the subjects being male. Airways with a diameter of greater than 2mm and identifiable pseudostratified epithelium were deemed to be large airways, whilst airways with columnar and cuboidal epithelium plus a diameter less than 2mm were classified as small airways. Asthmatic subjects were selected on the basis of confirmed clinical diagnosis and associated airway remodeling features determined histologically.

Histology

Human Lung Tissue Processing and Section Preparation

Dissected lung tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, USA). With the use of an automated tissue processor (Excelsior AS Tissue Processor, Thermo Scientific, Waltham, USA), both small and large airway samples underwent dehydration process in ascending grades of ethanol and two periods of xylene. Tissue samples were embedded in paraffin for analyses (HistoStar Embedding Workstation, Thermo Scientific, Waltham, USA) [2]. Tissue sections cut at 4-micron thickness were prepared using a microtome sectioner (Microm HM325 Rotary Microtome, Thermo Scientific, Waltham, USA) and heated water bath. Following mounting on coated slides (PRO-03; Matsunami, Osaka, Japan), sections were deparaffinised in xylene and rehydrated in graded ethanol prior to staining. Haematoxylin and Eosin (H&E) staining was used to assess the structural integrity, inflammation and the absence or presence of additional pathologies.

Trichrome staining

Sections were incubated in Bouin's Solution (Sigma-Aldrich, St. Louis, USA) for 24hours at room temperature and Masson's trichrome staining with Weigert's haematoxylin counterstain (Sigma-Aldrich, St. Louis, USA) was performed to assess features of airway remodeling (HT15-1KT; Sigma-Aldrich, St. Louis, USA).

Morphometric analysis of inflammation and airway remodeling features

Epithelium and reticular basement membrane (RBM) thickness was measured at multiple points of each airway which are averaged and represented as means. Lamina propria depth was measured perpendicularly from the base of the RBM to the outer edge of ASM

bundles at multiple points of each airway. These values are averaged and represented as means. The proportion of ASM in the airway wall of each individual airway (ASM/LP, %) was calculated by measuring the total area of ASM mass per airway and dividing by the total area of the lamina propria. Tissue inflammation in the lung was assessed and scored according to the criteria published previously (0-4 grading for no inflammation to severe inflammation i.e. influx of immune cells into the lung tissue) [3]. Further, nature of tissue inflammation was characterized by counting immune cells at 100x for eosinophils, neutrophils and macrophages per mm^2 of submucosa. Non-asthmatic airways were excluded if they displayed inflammatory infiltrates, if the epithelium was damaged, the RBM was thickened or fragmented, and ASM mass was disproportionate.

Immunohistochemistry

Heated antigen epitope retrieval was performed by placing slides in pre-heated (60-90°C) 0.01M citrate buffer, pH 6.0 for 10 minutes and cooling for 30 minutes. After rinsing in water, sections were identified with a hydrophobic Dako Pen (Agilent, Santa Clara, USA) and endogenous peroxidase activity was quenched with incubation in 3% H_20_2 for 10 minutes [4]. Following rinses with water and TRIS buffer (1X tris-buffered saline (TBS)/0.1% tween-20), sections were incubated in DAKO serum-free Protein Block (Agilent, Santa Clara, USA) for 10 minutes. Sections were then washed in tris-buffered saline with tween-20 (TBST) and incubated for 40 minutes with the following diluted primary antibodies: rabbit polyclonal anti-Beclin1 (1:250, ab62557; Abcam, Cambridge, UK), rabbit monoclonal anti-APGL/ATG5 (1:200, ab109490; Abcam, Cambridge, UK), rabbit polyclonal anti-sequestesome-1/p62 (1:25, ab155686; Abcam, Cambridge, UK), and rabbit polyclonal anti-MAP1LC3B (1:125, L7543; Sigma-Aldrich, St. Louis, USA). Bound antibodies were elaborated with horse-radish peroxidase (HRP)-labelled EnVision+Rabbit secondary

antibody (K4011, Agilent, Santa Clara, USA) incubation for 30 minutes [5]. For each case, the primary antibody was replaced by a species-appropriate isotype-matched immunoglobulin (Rabbit Immunoglobulin Fraction, X0936; Agilent, Santa Clara, USA) for a negative control [6]. The sections were washed firstly in TBST, distilled water, then the antibodies were visualized with the addition of liquid 3,3'-Diaminobenzidine (DAB, Agilent, Santa Clara, USA) and incubated for 10 minutes [7]. The sections were then washed sufficiently in distilled water before nuclei were counter-stained with Mayer's haematoxylin (Fronine, Riverstone, Australia), blued with ammoniated water (Chem-Supply, Gillman, Australia), and dehydrated and cleared through graded ethanol solutions and xylene. Sections were mounted with Permount (Fisher Scientific, Hampton, USA).

Image analysis

Computer-assisted image analysis was performed with a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Hamamatsu City, Japan), Olympus BX51 upright epifluorescence microscope fitted with a DP70 CCD camera (Olympus, Shinjuku, Japan) and ImageJ software. Prior to image analysis observer was blinded to subject and diagnosis. After a minimum of 6-7 images per tissue were taken, out of which four images were randomized per patient to be quantified with the assistance of a random number generator. The percentage staining was quantified in the epithelium and ASM using ImageJ software. Cell staining in the epithelium and airway smooth muscle bundles was separately quantified and presented as per mm^2 in both small and large airways. Data from four random images is presented as means for each corresponding patient.

Human airway smooth muscle cell culture

Human ASM cells were obtained from human lung by a method as described previously [8-10]. Human ASM bundles were microdissected from approximately 4^{th}-6^{th}-order bronchii and were initially cultured in growth medium comprised of DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% antibiotics (Invitrogen, Carlsbad, CA, USA). All the cells tested negative for the presence of mycoplasma before they were set up for experiments and were used between passages 2 and 5. ASM cells were seeded in six-well plates (BD Biosciences, New Jersey, USA) in growth medium and incubated at 37°C/5% CO_2. Cells were starved in quiescing medium consisting of DMEM:F12 media supplemented with 1% ITS (R&D Systems, Minneapolis, MN, USA) for 48h before any treatment.

Mouse models of allergic asthma

All animal procedures were approved by the Institutional Animal Care Committee of Thomas Jefferson University, Philadelphia, USA and University of Technology Sydney. All methods were performed in accordance with the relevant guidelines and regulations of the institutions. All surgeries were performed under tribromoethanol (Avertin, 250 mg/kg) anesthesia, and all efforts were made to minimize suffering. All animals were given standard chow and maintained on a 12 h dark and light cycle within the animal facility.

Prophylactic model

BALB/c mice (female) at 8 weeks were intranasally challenged 5-days/week for three consecutive weeks with house dust mite (HDM) extract (Dermatophagoides pteronyssinus, Greer Labs, USA) (25μg/35μL) to develop a sub-chronic model of allergic asthma as shown in Fig 8A [11]. Thirty min prior to the HDM challenges, a select set of mice were administered either chloroquine (50mg/kg) or saline vehicle. Twenty-four hours after the last HDM challenge, lung function measurements were performed using a small animal ventilator

(flexiVent, Scireq, Montreal, Canada). Bronchoalveolar lavage (BAL (2x0.5 ml)) fluid was collected to assess cellular influx into the airways and BAL supernatants were frozen for cytokines and chemokine, and lungs were formalin-fixed or flash frozen and stored at -80°C for histopathology and biochemical analysis.

Treatment model

BALB/c mice (female) at 8 weeks were intranasally challenged 5-days/week for five consecutive weeks with house dust mite (HDM) extract (Dermatophagoides pteronyssinus, Greer Labs, USA) (25µg/35µL) to develop a chronic model of allergic asthma as shown in Fig 9A. From week four onwards, thirty min prior to the HDM challenges, a select set of mice were administered either chloroquine (50mg/kg) or saline vehicle for the remaining two weeks of the model. Twenty-four hours after the last HDM challenge, lung function measurements were performed using a small animal ventilator (flexiVent, Scireq, Montreal, Canada). Bronchoalveolar lavage (BAL (2x0.5 ml)) fluid was collected to assess cellular influx into the airways and BAL supernatants were frozen for cytokines and chemokine, and lungs were formalin-fixed or flash frozen and stored at -80°C for histopathology and biochemical analysis.

Mouse BAL Immune Cell Staining, Lung H&E, PAS and Masson's trichrome staining

BAL samples were subjected to centrifugation and cell pellet resuspended in 1 ml PBS. The cells were stained with Hema-3 staining kit (Fisher Scientific, Hampton, USA). The lung tissues fixed in 10% formalin, embedded in paraffin were cut and stained with H&E, PAS and masson's trichrome staining using a standard histological protocol as described previously [11-14], using 5-µm sections mounted on Superfrost Plus slides. Image acquisition and analysis was performed using a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Hamamatsu City, Japan), a brightfield microscope and ImageJ software.

Mouse immunohistochemistry staining

Embedded lung tissues from the chronic 5-week HDM model were also cut and stained immunohistochemically for markers of autophagy (Beclin1, ATG5, LC3B, and p62, sm-a-actin) as described previously [5-7], using 5-μm sections mounted on coated slides (PRO-03; Matsunami, Osaka, Japan). Image acquisition and analysis was performed using a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Hamamatsu City, Japan) and ImageJ software. Images were analysed and quantified using the same method as employed with the human tissue samples analysed within this paper.

Measurement of TGFβ1

The content of TGFβ1 in BAL fluid was measured by Multiplexing LASER Bead Technology (Eve Technologies, Calgary, Canada) using a custom TGF-beta 3-Plex Cytokine Array.

Preparation of Lung Lysates

Mouse lungs were cut into small pieces and approximately half of the lungs were preserved in 250 μl of lysis buffer (composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0) and stored at –80˚C for protein analysis. Frozen lung tissues in the lysis buffer were slowly thawed in ice and were transferred into 5 ml tubes for homogenization using a polytron. The lysate was transferred to 1.5 ml plastic tube, centrifuged (760 × g, 5 min) and the supernatant stored at -80°C for subsequent protein assay and immunoblot analyses.

Immunoblotting

Protein concentration was determined by Pierce BCA assay kit (Thermo Scientific, Rockford, USA) and subjected to immunoblot analysis using protocols described previously [11, 13, 15] with primary antibodies noted above (1:1000), followed by incubation with respective secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000). Immunoblots were visualized and bands quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, USA).

Soluble Collagen Assay

Total soluble collagen content in the lung lysates was assessed using Sircol collagen assay (Biocolor, Carrickfergus, UK) according to the manufacturer's protocol as per [11, 16]. Collagen assay was performed by mixing lung homogenates with Sircol Dye reagent and measuring absorbance using a plate reader. Collagen content was quantified using a standard curve generated by reference standards and was normalized to the total lung protein content in each sample.

Statistical analysis

Morphometric data was analyzed using unpaired t-tests assuming Gaussian distribution and is presented as mean ± SD. Immunohistochemistry data was analyzed using unpaired t-tests with Welch's correction assuming Gaussian distribution and is presented as mean ± SD with normal distribution. One-way or two-way ANOVA was used appropriately with Bonferroni's multiple comparisons test and data expressed as ±SEM. PRISM V7.04 software was used for analysis (GraphPad, La Jolla, USA) and $p<0.05$ was considered statistically significant.

1. Faiz, A., et al., *The expression and activity of cathepsins D, H and K in asthmatic airways.* PLoS One, 2013. **8**(3): p. e57245.

2. Eapen, M.S., et al., *Profiling cellular and inflammatory changes in the airway wall of mild to moderate COPD.* Respirology, 2017. **22**(6): p. 1125-1132.
3. Sharma, P., et al., *Bitter Taste Receptor Agonists Mitigate Features of Allergic Asthma in Mice.* Sci Rep, 2017. **7**: p. 46166.
4. Eapen, M.S., et al., *Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD).* Sci Rep, 2017. **7**.
5. Sohal, S.S., et al., *Reticular basement membrane fragmentation and potential epithelial mesenchymal transition is exaggerated in the airways of smokers with chronic obstructive pulmonary disease.* Respirology, 2010. **15**(6): p. 930-8.
6. Sharma, P., et al., *Characterization of the dystrophin-glycoprotein complex in airway smooth muscle: role of delta-sarcoglycan in airway responsiveness.* Can J Physiol Pharmacol, 2015. **93**(3): p. 195-202.
7. Mahmood, M.Q., et al., *beta-catenin, Twist and Snail: Transcriptional regulation of EMT in smokers and COPD, and relation to airflow obstruction.* Sci Rep, 2017. **7**(1): p. 10832.
8. Chen, L., et al., *Differential regulation of extracellular matrix and soluble fibulin-1 levels by TGF-beta(1) in airway smooth muscle cells.* PLoS One, 2013. **8**(6): p. e65544.
9. Panettieri, R.A., et al., *A human airway smooth muscle cell line that retains physiological responsiveness.* Am J Physiol, 1989. **256**(2 Pt 1): p. C329-35.
10. Sharma, P., et al., *Expression of the dystrophin-glycoprotein complex is a marker for human airway smooth muscle phenotype maturation.* Am J Physiol Lung Cell Mol Physiol, 2008. **294**(1): p. L57-68.
11. Sharma, P., R. Yi, and D.A. Deshpande, *Bitter taste receptor agonists mitigate asthma phenotype in murine models.* Association for Chemoreception Sciences, 2015.
12. Sharma, P., et al., *Epithelium-dependent modulation of responsiveness of airways from caveolin-1 knockout mice is mediated through cyclooxygenase-2 and 5-lipoxygenase.* Br J Pharmacol, 2012. **167**(3): p. 548-60.
13. Sharma, P., et al., *beta-Dystroglycan binds caveolin-1 in smooth muscle: a functional role in caveolae distribution and Ca2+ release.* J Cell Sci, 2010. **123**(Pt 18): p. 3061-70.
14. Sharma, P., et al., *Role of dystrophin in airway smooth muscle phenotype, contraction and lung function.* PLoS One, 2014. **9**(7): p. e102737.
15. Deshpande, D.A., et al., *Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction.* Nat Med, 2010. **16**(11): p. 1299-304.
16. Schaafsma, D., et al., *The mevalonate cascade as a target to suppress extracellular matrix synthesis by human airway smooth muscle.* Am J Respir Cell Mol Biol, 2011. **44**(3): p. 394-403.