Role of aquaporin 5 in antigen-induced airway inflammation and mucous hyperproduction in mice

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Abstract

Airway inflammation and mucus hyperproduction play the central role in the development of asthma, although the mechanisms remain unclear. The aquaporin (AQP)-5 may be involved in the process due to its contribution to the volume of liquid secreted from the airways. The present study firstly found the overexpression of AQP5 in the airway epithelium and submucosal glands of asthmatics. Furthermore, we aimed at evaluating the role of AQP5 in airway inflammation and mucous hyperproductions during chronic allergic responses to house dust mite (HDM). Bronchoalveolar lavage levels of interleukin (IL)-2, IL-4, IL-10, interferon-γ and Mucin 5AC (MUC5AC), and number of peribronchial and perivascular cells were measured in AQP5 wild-type and AQP5 knockout (KO) mice. We found that HDM induced airway inflammation, lung Th2 cell accumulation and mucin hypersecretion in C57BL/6 mice rather than AQP5 KO mice. Expression of MUC5AC and MUC5B proteins and genes in the lung tissue was significantly lower in AQP5 KO mice. Thus, our results implicate involvement of AQP5 in the development of airway inflammation and mucous hyperproduction during chronic asthma.

Keywords: aquaporins • mucin • house dust mite • chronic allergy • inflammation

Introduction

Allergic asthma is a complex disorder, which involves a significant contribution of environmental stimuli for the manifestation of symptoms and degree of clinical severity. Chronic asthma is pathologically characterized by airway inflammation, increased mucus production, airway remodelling and airway hyper-responsiveness to bronchoconstrictive agents [1].

The aquaporins (AQPs) are a family of small transmembrane proteins that facilitate osmotically driven water transport, and in some cases the transportation of small solutes such as glycerol [2]. AQP5 is expressed in the apical membrane of type I alveolar epithelial cells, acinar epithelial cells in submucosal glands and large airway epithelia [3, 4]. The volume of liquid secreted from the nasopharynx and the upper airways was lower by 2-fold in AQP5 knockout (KO) mice, resulting in hypertonic fluid with elevated protein concentration [5]. In addition, the previous studies found AQP5 might be involved in the regulation of MUC5AC production [6], airway hyper-responsiveness to cholinergic stimulation and altered lung resistance and dynamic compliance [7], lung infection [8], airway inflammation [9] and acute lung injury [10].

House dust mite (HDM) represents one of the most common allergens in allergy and asthma, influencing approximately 10% of the population. Chronic exposure to HDM extract in mice could lead to persistent airway inflammation, hyper-responsiveness and remodelling, as a accepted model of chronic asthma [11]. In the present study, we tried to validate if AQP5 as the disease-related target could be overexpressed in the asthmatics and furthermore investigate the potential role of AQP5 in airway inflammation and mucin hypersecretion induced by chronic exposure to HDM. We compared the effects of chronic HDM exposure on the development of airway eosinophilia, cytokine production and mucin secretion in AQP5 KO wild-type (WT) mice. Our results firstly showed the overexpression of AQP5 in the airway epithelium and submucosal glands of asthmatics and found that the expression of MUC5AC and MUC5B genes and proteins, number of inflammatory cells and production of Th2 cytokines were
markedly lower in AQP5 KO mice. Thus, our data indicate that AQP5 may act as a novel regulator of the allergic response and a therapeutic target for treating mucin hypersecretion and airway inflammation in chronic asthma.

Materials and methods

Human bronchial tissues

Human bronchial tissues were obtained from patients with lung cancer or with lung cancer and chronic asthma who underwent lung surgery in Zhongshan hospital (Shanghai, China) from 2007 to 2010. The patients (n = 6) were diagnosed as chronic asthma according to the Global Initiative for Asthma (GINA) [13]. Quantitative analysis of pathology was performed by the scoring system, e.g.: 0: normal; 1: low grade of cell influx, diffuse oedema and cell loss; 2: low to middle grade of cell influx, tissue damage, alveolar and bronchial oedema, and cell loss; 3: clear changed grade of cell influx, tissue damage, regional and focal oedema, and cell loss; 4: obvious changed and pronounced grade of cell influx, tissue damage, oedema and cell loss; 5: most obvious changed grade of cell influx, tissue pathology, pneumonic-type oedema, epithelial metaplasia and mucus cell hyperplasia. The results for each lung from the three observers were averaged to obtain a mean value for each parameter. Tissues were sliced and assessed with the periodic acid–Schiff staining to demonstrate the presence of mucin within goblet cells.

Animals

AQP5 KO mice (gift from Dr. A. S. Verkmann, California University) were generated as previously described [12] and back-crossed to a C57BL/6 background for 6 to 10 generations. The experiments were performed on litter-matched (age 7–8 weeks, body weight 20–25 g, female) WT (AQP5 WT) and AQP5-null (AQP5 KO) mice. Mice were genotyped from DNA isolated by tail clips with PCR primers to the AQP5 gene [12]. The mice were kept in a 12 : 12 hr night–day rhythm, fed with standard mice chow and provided water ad libitum. This study was approved by the Animal Care Committee of Fudan University Zhongshan Hospital.

Chronic asthma model

Mice were challenged by the intranasal administration of 25 µg of purified HDM extract (Dermatophagoides pteronyssinus; ALK, Hørsholm, Demark) in a total volume of 10 µl of saline for once a day, 5 days a week, for up to five consecutive weeks. Phosphate-buffered solution (PBS) at the same volume without HDM was used in the control group. There were 8–10 mice in each terminate group.

Bronchoalveolar lavage (BAL)

The trachea was cannulated and the bronchoalveolar space was lavaged with 0.5 ml PBS and withdrawn with 0.3 ml twice. BAL samples were centrifuged at 500 × g for 5 min. at 4°C and stored at −70°C until analysis. Levels of interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)-γ were determined using specific ELISA as suggested by the manufacturer manual (ELISA kits, eBioscience, San Diego, CA, USA). The concentrations of cytokine were determined by the comparison of ELISA readings with the standard curve using recombinant cytokine of known concentrations. The amount of MUC5AC in the supernatant of BAL was measured using ELISA (USCN Life Science & Technology Company, Missouri City, TX, USA).

Histological evaluation

Twenty-four hours after the last HDM challenge, lungs were harvested, fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (4 µm) of specimens were put onto 3-aminopropyltriethoxy saline-coated slides. The morphology and leucocyte infiltration in the tissue were assessed using haematoxylin and eosin staining. Inflammatory changes were graded by a scale of 0–5 for perivascular, bronchiolar and submucosal gland eosinophilia [13]. Endogenous peroxidase activity was inhibited by incubating the slides with 3% H2O2 for 30 min., followed by washing in PBS and microwave heating (750 W) for 10 min. in 10 mmol/l citric acid at pH 6.0 for AQP5 and pH 9.0 for MUC5AC and MUC5B. The sections were inactivated with normal goat serum at 37°C for 30 min., stained with freshly prepared DAB (Diaminobenzidine) and counterstained with haematoxylin (Hematoxylin). Morphometric quantification of the stained sections was performed with a customized digital image analysis system (IMAGE-Pro plus 4.5). Analysis of MUC5AC and MUC5B in the airway and capturing images of airway epithelium from which a 20 µm band of tissue was projected in a basal direction. The software then calculated the percentage of each band in the primary antibodies, placed in a humid chamber at 37°C for 1 hr, washed in PBS and incubated at 37°C for 30 min. with biotinylated goat antimouse or rabbit (1:100; Maxin-Bio, Shanghai, China) according to manufacturer’s instructions. The tissue was incubated with Streptavidin Peroxidase (Maxin-Bio) reagents at 37°C for 30 min., stained with freshly prepared DAB (Maxin-Bio) and counterstained with haematoxylin (Maxin-Bio).
periodic acid-Schiff+ cells and counted manually and expressed as cells per millimetre length of airway wall.

Quantitative real-time PCR

The right lung was frozen in liquid nitrogen immediately after harvest and homogenized in TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) using an ULTRA-TURRAX power homogenizer. Total RNA was isolated, as recommended by the manufacturer. The amount of RNA in aqueous solution was determined by absorbance at 260 nm. Equal amounts (1 µg) of total cellular RNA were reverse-transcribed using Moloney murine leukemia virus (MMLV) first-strand synthesis kit (BBI, Madison, WI, USA). Before cDNA synthesis, each RNA sample was treated with DNase I (Invitrogen Life Technologies) to eliminate any potential contamination with genomic DNA. PCRs were carried out in 25 µl containing 2 µl cDNA, 10 µM of each forward and reverse primer. Mouse glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the internal control to quantify initial cellular transcripts. Primer sequences included: mouse GAPDH – sense: 5’TGTGTCGGTGATCTGA3’, antisense: 5’CTTGCTTCAACACTTCTTGAT3’, MUC5AC – sense: 5’AATGCAAGCATCATACACACG3’ antisense: 5’AGGCACAGCCGTCTTCA3’, MUC5B – sense: 5’CTCTGGGAACCC-TAAGGAA3’, antisense: 5’ACCGCAAGACAGTCGATTTA3’. Real-time PCR conditions for amplification of target genes were as follows: The PCR mixture was denatured at 95°C for 10 sec., annealed and extended at 60°C for 1 min., and the above processes was repeated till it totalled 45 cycles. Amplification data measured by fluorescence were collected in real time and analysed by Rotor-Gene 6.0.14 Software.

Statistical analysis

Data were expressed with means ± S.D. Differences between groups were analysed using software SPSS for windows (version 12.0) by unpaired two-tailed parametric Student’s t-test, after ANOVA test. P-values less than 0.05 were considered statistically significant.

Results

Expression of AQP5 protein in human bronchi

The lower airways without cancer were selected for pathological evaluation. The player of airway epithelial cells in patients with chronic asthma was obviously thicker than those without chronic asthma. Hypertrophy of epithelial cells in the mucosal players and submucosal glands was noticed in asthmatics. Immunohistochemical analysis demonstrated that AQP5 was localized in normal airway epithelium (Fig. 1A) and submucosal glands (Fig. 1C) of the bronchi. The expression of AQP5 proteins was obviously higher in the epithelial cells of airway mucosa (Fig. 1B) and submucosal glands (Fig. 1D) in asthmatics.

Allergic inflammation in HDM-treated mice

Administration of HDM for 5 weeks to C57BL/6 WT mice led to a significant increase in the amount of pulmonary oedema and epithelial hypertrophy in the terminal airway and leucocyte infiltration (Fig. 2B), although not in AQP5 KO animals (Fig. 2D). The total scores of histopathology in HDM-treated AQP5 WT mice were significantly higher than those in PBS-treated animals (Table 1), whereas AQP5 KO mice had significantly less inflammation at the airway as compared with AQP5 WT mice after HDM exposure. To determine whether AQP5 KO mice had reduced airway eosinophilia, we analysed the cell number in the peribronchial and perivascular tissues after HDM exposure. The percentage of airway eosinophils was significantly increased in WT mice with HDM challenge for 5 weeks as compared with those with PBS. HDM-treated AQP5 KO mice had significantly fewer eosinophils as compared with WT animals (Table 1). There was no significant
difference of neutrophil numbers in the lung tissue between WT and AQP5 KO mice (data not shown).

**Role of AQP5 in HDM-induced cytokine production**

BAL levels of IL-4 (Fig. 3A) and IL-10 (Fig. 3B) in animals challenged with HDM were significantly increased as compared with those with PBS ($P < 0.05$ and 0.01, respectively). AQP5 KO mice had significantly lower levels of IL-4 and IL-10 than WT mice after chronic exposure to HDM ($P < 0.05$). BAL levels of IL-2 (Fig. 3C) and IFN-γ (Fig. 3D) in AQP5 KO mice were significantly higher than those in WT mice ($P < 0.05$), whereas levels in both AQP5 KO and WT mice challenged with HDM were significantly lower than those with PBS ($P < 0.01$). There was no significant difference between WT and AQP5 KO after the challenge with PBS.

**Airway epithelial mucin changes after chronic HDM exposure**

Chronic exposure to HDM induced obvious metaplasia (Fig. 4A-2), hyperplasia and hypertrophy of goblet cells with staining positively with periodic acid–Schiff for mucin in WT mice, as compared with animals challenged with PBS (Fig. 4A-1) and AQP5 KO mice with HDM (Fig. 4A-4). There was no significant difference of goblet cell metaplasia between mice with PBS and AQP5 KO mice with PBS. However, the number of goblet cells in the small airway of HDM-challenged WT animals measured by morphometric system was significantly higher than that in PBS- and HDM-challenged AQP5 KO animals ($P < 0.01$ and 0.05, respectively, Fig. 2B). There was no significant difference of goblet cell alterations between PBS-challenged WT and AQP5 KO animals.

### Table 1 Severity of inflammatory cellular infiltration in lung tissue after immunization

| Groups                        | Perivascular eosinophilia | Peribronchiolar eosinophilia | Oedema         | Epithelial damage |
|-------------------------------|---------------------------|-------------------------------|----------------|------------------|
| Control                       | 0(0–0)                    | 0(0–0)                        | 0(0–0)         | 0(0–1)           |
| WT challenged with HDM        | 4(3–4)*                   | 4(3–4)*                       | 4(3–4)*        | 4(3–5)*          |
| AQP5 KO mice challenged with HDM | 3(2–3)* †                | 4(3–4)*                       | 2(1–2)* †      | 3(2–3)* †        |

Inflammatory changes were graded by histopathological assessment using a semi-quantitative scale of 0–5. Results are presented as median, with range in parenthesis ($n = 8/group$).

* $P < 0.01$ versus animals challenged with PBS, † $P < 0.05$ versus WT mice challenged with HDM.
Fig. 3 Levels of IL-4 (A), IL-10 (B), IL-2 (C) and IFN-γ (D) in BAL fluid harvested from WT and AQP5 KO mice \( (n = 8/\text{group}) \) after intranasal challenges with HDM, once a day, 5 days a week; ‘**’ and ‘***’ stand for the \( P \)-values less than 0.05 and 0.01, respectively, as compared with WT and AQP5 KO mice challenged with PBS. ‘+’ stands for the \( P \)-values less than 0.05, as compared with WT mice challenged with HDM.

Fig. 4 Histological findings (A) of PAS-stained sections of airways of WT mice challenged with PBS (A-1) or HDM (A-2) or AQP5 KO mice with PBS (A-3) or HDM (A-4) after the intranasal challenges, once a day, 5 days a week for 5 weeks. Morphometric quantification of PAS \(^*\) cells (B) in the airway wall of WT or AQP5 KO mice after chronic exposure to PBS or HDM. ‘**’ stands for the \( P \)-values less than 0.01, as compared with PBS-challenged WT mice, and ‘+’ stands for the \( P \)-values less than 0.05, as compared with WT mice with HDM, respectively.
Airway MUC5AC and MUC5B changes after chronic allergen exposure

Figure 5 demonstrates that more cells with positive staining of MUC5AC (Fig. 5A) and MUC5B (Fig. 5B) were observed in the small airway of WT mice after chronic exposure to HDM, as compared with AQP5 KO animals. The number of MUC5AC (Fig. 6A) – and MUC5B (Fig. 6B) – positive epithelial cells in the airway was significantly higher in WT mice as compared with in AQP5 KO mice after chronic HDM challenge (P < 0.01 and 0.05, respectively) and in animals challenged with BPS (P < 0.01, respectively). BAL levels of MUC5AC proteins were significantly increased in HDM-challenged WT (P < 0.01) and AQP5 KO animals (P < 0.05), respectively, as shown in Figure 7A. AQP5 KO mice exhibited a significant decrease about 51.7% in BAL levels of MUC5AC after chronic HDM challenge as compared with WT mice (P < 0.05, Fig. 7A). To confirm the expression of MUC5AC and MUC5B in the lung tissue, mRNA levels of MUC5AC and MUC5B were assessed by quantitative real-time PCR. There was a significant increase in gene expression of MUC5AC (Fig. 7B) and MUC5B (Fig. 7C) in the lung tissue of WT (P < 0.010 and AQP5 KO animals (P < 0.05) challenged with HDM, as compared with those with PBS, respectively. The expression of both MUC5AC and MUC5B in AQP5 KO
Mouse lung tissue was significantly lower than that in WT mice after HDM challenge.

**Discussion**

The AQPs are a family of small (30 kD monomer) integral membrane proteins that function as selective water transporters, and in some cases they also transport glycerol (aquaglyceroporins). Of 13 related AQPs in mammals, at least four are expressed in the lung and airways, *e.g.* AQP1 in microvascular endothelia, AQP3 and AQP4 in airway epithelia and AQP5 in the apical membrane of type I alveolar epithelial cells, acinar epithelial cells in submucosal glands or large airway epithelia [2]. There was no clinical study on the changes of AQP5 expression in the asthmatics, and only one study demonstrated that the gene and protein expressions of AQP5 were altered in patients with chronic obstructive pulmonary disease [14]. The present study firstly examined the expression of AQP5 proteins by immunohistochemistry and found the overexpression of AQP5 protein in the asthmatic airway epithelium and submucosal glands of the bronchi as compared to controls. Due to the lack of reliable, specific and nontoxic inhibitors against AQPs, the significance of AQPs has been examined through the use of gene KO mice or primary cell culture extract from AQP KO mice. In the present study, we investigate potential involvements of AQP5 in chronic HDM-induced mucus hyperproduction by using AQP KO mice.

Inflammatory cytokines have been found to be involved in overproduction and overexpression of mucus proteins and genes in the airway epithelium [15, 16]. For example, Th2 cytokines like IL-4 were suggested to regulate mucin gene expression [17] and were down-regulated in the AQP5 KO mouse, suggesting that AQP5 may be involved in robust Th2-driven responses to allergens. The fact that AQP5 deletion resulted in increased levels of IFN-γ and IL-2 suggests that there may be a switch from Th2 towards Th1 responses where AQP5 plays a critical role. Th2 cytokines were found to be important in eosinophil recruitment, airway hyper-responsiveness and mucus hypersecretion [18, 19]. It is possible that AQP5 may be involved in the development of HDM-induced chronic airway inflammation and mucus hyperproduction through the overproduction of those inflammatory mediators. We found the formation of metaplasia, hyperplasia and hypertrophy of airway goblet cells and peri-bronchial accumulation of inflammatory cells in WT mice following chronic HDM exposure, although obviously less in AQP5 KO mice. Although there is a potential that animals with the genetic modification may have physiological difference and changes, the present study did not find the significant alternations of any measured parameters between WT and AQP5 KO mice challenged with PBS.

Of mucin genes in adult human lung [20], MUC5AC and MUC5B appear to be the predominant genes expressed and the most abundant in mucus secretions [21]. Our data provide solid evidence to support the involvement of AQP5 in overproduction of...
MUC5AC and MUC5B during chronic lung inflammation. We investigated metaplasia, hyperplasia and hypertrophy of goblet cells by immunochemical staining, super-resolution and hypersecretion of mucins by both morphometry and BAL levels measured and hyperexpression of MUC5AC and MUC5B proteins and genes in the lung tissue. AQP5 KO mice had significantly less alterations in those measurements after chronic challenge with HDM, as compared with WT mice, but still higher than in those challenged with PBS. It indicates that the AQP5 is, at least, partially, involved in development of mucus hyperproduction in chronic asthma. The previous results demonstrated that AQP5 KO mice had low inflammatory responses to ovalbumin, including leucocyte infiltration in lung tissues and cytokine expression [22]. Like acute allergic reaction, the role of AQP5 in chronic airway inflammation and mucosal hyperproduction may be involved in down-regulation of antigen presenting cells, because dendritic cells in AQP5 KO mice had less antigen-presenting function [22–24].

Mucins, cystic fibrosis transmembrane conductance regulator protein (CFTR) and AQP5 are involved in the formation and composition of the airway surface liquid, essential for proper mucociliary clearance. Expressions of AQP5 and CFTR were found in the airway [25], of which CFTR expression was enhanced whereas AQP5 expression was completely abolished after IL-13 treatment. However, amiloride administration did not inhibit water permeability of airway AOPs [26]. IL-13 did not affect AQP3 or AQP4 expression, whereas it completely inhibited the expression of both AQP5 mRNA and protein [25].

Our findings are supported by a number of previous studies, e.g. pilocarpine-induced fluid secretion [5] and AOP-deletion-impaired osmotic equilibration [27]. However, another study showed that mice deficient in AQP5 were hyper-responsive to cholinergic stimulation and had high lung resistance and low dynamic compliance [7]. The differences between present and previous study [7] include the genetic background, e.g. 129svj mice in the previous and CD1/C57 in the present, and the models, e.g. bronchostimulator-induced fluid secretion in the previous and HDM-induced chronic asthma in the present. Previous studies showed that a bronchodilating β-adrenergic agonist increased AQP5 abundance and redistribution of AQP5 to the apical membrane of mouse lung epithelial cells (MLE-12) through an adenosine 3’, 5’- monophosphate (cAMP)-protein kinase A (PKA) dependent pathway [28]. However, other studies demonstrated that siRNA-induced AQP5 down-regulation was associated with MUC5AC over-expression in SPC-1 cell line [29]. We believe the discrepancy between our findings and those of other investigators may be due to the differences between the in vitro and animal models. There is a great need to investigate and understand the role of AQP5 in acute and chronic airway diseases.

In conclusion, our data demonstrated that the chronic challenge with HDM resulted in the development of airway inflammation and mucus hyperproduction in WT mice, although significantly less in AQP5 KO mice. Levels of leucocyte recruitment, cytokine production and mucus hyperproduction in AQP5 KO mice after HDM challenge were still higher than those with PBS challenge. Thus, our data indicate that AQP5 may act as one of new and novel therapeutic targets to treat chronic airway diseases.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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