Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma

Kelly Sweerus, MD,[a,b]* Marrah Lachowicz-Scroggins, PhD,[a,c]* Erin Gordon, MD,[a] Michael LaFemina, MD,[a,b] Xiaozhu Huang, MD,[a,c] Mihir Parikh, MD,[a,b] Cindy Kanegai, MD,[a] John V. Fahy, MD,[a,c] and James A. Frank, MD[a,b]

San Francisco, Calif

Background: Epithelial barrier dysfunction and increased permeability may contribute to antigen sensitization and disease progression in asthma. Claudin-18.1 is the only known lung-specific tight junction protein, but its contribution to airway barrier function or asthma is unclear.

Objectives: We sought to test the hypotheses that claudin-18 is a determinant of airway epithelial barrier function that is downregulated by IL-13 and that claudin-18 deficiency results in increased aeroantigen sensitization and airway hyperresponsiveness.

Methods: Claudin-18.1 mRNA levels were measured in airway epithelial brushings from healthy controls and patients with asthma. In patients with asthma, claudin-18 levels were compared with a three-gene-mean marker of T_H2 inflammation. Airway epithelial permeability changes due to claudin-18 deficiency were measured in 16HBE cells and claudin-18 null mice. The effect of IL-13 on claudin expression was determined in primary human airway epithelial cells and in mice. Airway hyperresponsiveness and serum IgE levels were compared in claudin-18 null and wild-type mice following aspergillus sensitization.

Results: Epithelial brushings from patients with asthma (n = 67) had significantly lower claudin-18 mRNA levels than did those from healthy controls (n = 42). Claudin-18 levels were lowest among T_H2-high patients with asthma. Loss of claudin-18 was sufficient to impair epithelial barrier function in 16HBE cells and in mouse airways. IL-13 decreased claudin-18 expression in primary human cells and in mice. Claudin-18 null mice had significantly higher serum IgE levels and increased airway responsiveness following intranasal aspergillus sensitization.

Conclusions: These data support the hypothesis that claudin-18 is an essential contributor to the airway epithelial barrier to aeroantigens. Furthermore, T_H2 inflammation suppresses claudin-18 expression, potentially promoting sensitization and airway hyperresponsiveness. (J Allergy Clin Immunol 2017;139:72-81.)

Key words: *Asthma, epithelium, epithelial barrier function, tight junction, antigen sensitization, airway hyperresponsiveness*

Genetic and environmental factors influence asthma development, progression, and severity. Moreover, recent work has begun to parse the clinical syndrome of asthma into distinct endotypes that may vary in pathogenesis, progression, and response to therapy.[1] Amid this complexity, airway epithelial barrier impairment is a common feature of asthma that has been postulated to contribute to immune and parenchymal cell activation, antigen sensitization, and airway hyperresponsiveness.[2,3] In severe asthma, epithelial cell loss has been reported, but previous work has also demonstrated that more subtle changes in epithelial cell junctions may account for impaired barrier function in mild-moderate asthma.[4] For example, infections, toxins, and environmental proteins, such as Dpr1, modify cell junctions to impair barrier function.[5-7] Moreover, recent work also supports the hypothesis that differences in tight junction protein expression or trafficking account for differences in barrier function in the epithelium of those with asthma. For example, biopsy samples from subjects with asthma exhibit tight junction disruption with reduced expression of occludin and zona occludens-1—key structural components of tight junctions.[8] In parallel, cultured epithelial monolayers derived from airways of subjects with asthma had increased macromolecule permeability compared with monolayers derived from healthy subjects.[8] Because the airway epithelium constitutes a barrier to aeroantigens, these findings raise the possibility that increased epithelial permeability could contribute to allergic inflammation by permitting greater exposure of the subepithelial compartment to inhaled allergens. Alternatively, loss of epithelial compartmentalization and polarity could impact cell signaling through dysregulation of normally segregated receptors and ligands. The mechanisms of airway epithelial tight junction dysfunction in asthma, and the contribution of this abnormality to allergic sensitization and airway hyperresponsiveness, remain incompletely understood.

It is noteworthy that previous studies have established that T_H2-mediated changes in tight junction claudin expression contribute to impaired epithelial barrier function in the gut and

From [a]the Division of Pulmonary, Critical Care, Sleep and Allergy Medicine, University of California, [b]San Francisco VA Medical Center and Northern California Institute for Research and Education, and [c]Sandler Asthma Basic Research Center, University of California San Francisco.

*These authors contributed equally to this work.

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Corresponding author: James A. Frank, MD, San Francisco VA Medical Center, University of California, San Francisco, 4150 Clement St, Box 111D, San Francisco, CA 94121. E-mail: James.frank@ucsf.edu.

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Abbreviations used
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
OVA: Ovalbumin
P_{app}: Apparent permeability
PAS: Periodic acid–Schiff
UCSF: University of California, San Francisco

other organs.[9-11] It is less clear whether this mechanism contributes to airway epithelial barrier abnormalities in asthma; however, a previous report found that IL-4 and IL-13 impaired epithelial barrier function in 16HBE cells.[12] T_H2-high asthma is characterized by higher expression levels of IL-13–dependent genes, greater airway hyperresponsiveness, and higher IgE levels compared with T_H2-low asthma.[13,14] It remains unclear whether tight junction composition and epithelial barrier function differ among subjects with T_H2-high and T_H2-low asthma.

Claudins are essential to tight junction formation and are a primary determinant of paracellular permeability through intact tight junctions. Differential claudin expression accounts for the differences in epithelial permeability in diverse epithelia, and mutations in claudin genes and changes in claudin expression result in clinical disease.[15,16] The claudin-18 gene encodes 2 variants that differ in the first exon. Claudin-18.1 expression requires the transcription factor NKX2-1 and is exclusive to lung epithelia.[17] At present, claudin-18.1 is the only known lung-specific tight junction gene product. The expression of specific claudin family members may vary in different airway epithelial cell types, but claudins-1, -4, and -7 are expressed at high levels in airway epithelium.[18,19] Although claudin-18 is also normally expressed in airway epithelial cells, its contribution to the permeability barrier in the airways has not been fully defined.

This study was undertaken to determine the contribution of claudin-18 to airway epithelial barrier function and whether claudin-18 expression is regulated by T_H2 inflammation. In addition, the consequences of claudin-18 deficiency to aeroantigen sensitization and airway hyperresponsiveness were examined. The presented data substantiate the hypothesis that claudin-18 is a central barrier-forming component of tight junctions and show that IL-13 downregulates claudin-18. These data also suggest that the loss of claudin-18 is associated with increased sensitization to aeroantigens and airway responsiveness.

METHODS

Airway mRNA expression studies

Aliquots of RNA extracted from airway epithelial brushings and stored in the Airway Tissue Bank at the University of California, San Francisco (UCSF) were analyzed by quantitative PCR as previously described.[20] The UCSF Committee on Human Research approved the policies and procedures of the UCSF Airway Tissue Bank and use of samples for this study. These epithelial brushings had been collected during research bronchoscopy from 67 nonsmoking subjects with asthma and 42 healthy nonsmoking control subjects (Table I). All subjects signed an Airway Tissue Bank informed consent form approved by the UCSF Committee on Human Research. Subjects with asthma had a prior physician's diagnosis of asthma, a PC_{20} methacholine value of less than 8 mg/mL, and were using only inhaled β-agonist medications for therapy. RNA was reverse transcribed with random hexamer primers and then amplified in a multiplex reaction with custom primers (see Table E1 in this article's Online Repository at www.jacionline.org). The amplified cDNA was then used for quantitative PCR with custom primers and Taqman-based probes run in separate batches.[13,21] Normalization was done using the geometric mean of the value of 3 housekeeping genes as previously described.[21,22] Log2 transformed, normalized, relative expression is reported. The three-gene-mean marker of T_H2 inflammation was determined as described previously.[13,14] This measure is based on the geometric mean of mRNA expression levels of CLCA1, SERPINB2, and POSTN. Claudin-18 mRNA expression levels were compared with serum IgE levels and blood eosinophil counts obtained from subjects with asthma at the time of bronchoscopy.

Primary cell culture and immunostaining

Cadaveric airway tissues from lungs rejected for transplantation were obtained in accordance with UCSF Committee on Human Research approval from the Northern California Organ Donor Network. Normal airway surface epithelial cells were harvested from 8 individual donors and expanded and cultured at an air-liquid interface as described previously.[23,24] Treatment with IL-13 (10 ng/mL) was begun at 2 weeks, once air-liquid interface cultures had established confluence, and continued for 7 days. Recombinant human IL-13 (R&D Systems, Minneapolis, Minn) was reconstituted in sterile PBS containing 0.1% BSA. For primary human airway epithelial cells, quantitative PCR was done without preamplification. Normalization was done as above using EEF1A1 and PPIA. Data are reported as relative mRNA expression normalized to the housekeeping genes. Claudin-18 protein levels were compared in cell lysates using immunoblot densitometry normalized to tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Blots were incubated with claudin-18 and tubulin primary antibodies in series and images developed such that both bands could be visualized on the same blot. In addition, separate, equally loaded blots were probed for either claudin-18 or GAPDH. Data normalized to tubulin or GAPDH were similar and the GAPDH data are shown. For immunostaining, tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were stained for beta catenin (G10, Santa Cruz Biotechnology, Santa Cruz, Calif) and claudin-18 (ZMD.385, Life Technologies, Waltham, Mass) and counterstained with 4′-6-diamidino-2-phenylindole, dihydrochloride.

16HBE cell culture

16HBE cells (a gift from Dr Dieter Gruenert, UCSF) were cultured in minimum essential medium Eagle's with Earle's basic salt solution medium supplemented with 10% BSA, 1% penicillin/streptomycin, and 1% glutamine. A coating medium comprising LHC basal media, 0.1% type 1 collagen, 0.1% BSA, and 0.1% fibronectin was used to coat the culture plates. Cells were cultured until they reached 80% confluence, and 250,000 cells/cm^2 were then passaged on to 1.13-cm^2 Transwell polycarbonate inserts (3407, Corning Costar, Sigma Aldrich, St Louis, Mo) treated with coating medium. Claudin-18 and tubulin protein expression levels were measured using Western blotting, and densitometry data were analyzed using ImageJ (National Institutes of Health, Bethesda, Md). Data are reported as claudin-18/tubulin. Transepithelial electrical resistance was measured using a voltohm meter (World Precision Instruments, Sarasota, Fla). Permeability to the 500 Dalton fluorescent tracer pyranine was measured by adding 10 μg/mL pyranine to the apical chamber of transwells and measuring fluorescence recovery in the basolateral chamber.[25] Data are reported as apparent permeability (P_{app}).

shRNA studies

Claudin 18 shRNA sequences (TRCN0000116737 to TRCN 000011673741) were cloned into the third-generation lentiviral vector pLKO.1-puro (Sigma Mission shRNA library) and transfer plasmids were cotransfected with packaging plasmids and VSV-G–expressing envelope plasmid into human 293 cells at low passage and 30% confluence. When the cells became confluent (36-48 hours), the culture medium was collected and filtered. Supernatants were aliquoted and kept at −80°C. After confirmation of posttransduction knock down by immunoblot,