NDRG1 is important to maintain the integrity of airway epithelial barrier through claudin-9 expression

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Abstract

Impairment of epithelial barrier integrity caused by environmental triggers is associated with the pathogenesis of airway inflammation. Using human airway epithelial cells, we attempted to identify molecule(s) that promote airway epithelial barrier integrity. Microarray analyses were conducted using the Affimetrix human whole genome gene chip, and we identified the N-myc downstream-regulated gene 1 (NDRG1) gene, which was induced during the development of the epithelial cell barrier. Immunohistochemical analysis revealed strong NDRG1 expression in ciliated epithelial cells in nasal tissues sampled from patients with chronic rhinosinusitis (CRS), and the low expression of NDRG1 was observed in goblet cells or damaged epithelial cells. NDRG1 gene knockdown with its specific siRNA decreased the transepithelial electrical resistance and increased the dextran permeability. Immunocytochemistry revealed that NDRG1 knockdown disrupted tight junctions of airway epithelial cells. Next, we analyzed the effects of NDRG1 knockdown on the expression of tight and adhesion junction molecules. NDRG1 knockdown significantly decreased only claudin-9 expression, but did not decrease other claudin family molecules, such as E-cadherin, and ZO-1, -2, or -3. Knockdown of claudin-9 markedly impaired the barrier function in airway epithelial cells. These results suggest that NDRG1 is important for the barrier integrity in airway epithelial cells.

Keywords: barrier function; chronic rhinosinusitis; claudin-9; NDRG1; tight junction

Introduction

Epithelial cells create a physical barrier to the outside world composed of apical junctional complexes formed between neighboring cells. The apical-junctional complex (AJC) is a selectively permeable barrier composed of tight junctions (TJs) and adherens junctions (AJs) (Tamura and Tsukita, 2014; Capaldo and Nusrat, 2015). The AJCs establish cell–cell contact and cell polarity and biologically limit the passage of foreign substances, including inhaled allergens, into the body. Maintenance of the epithelial barrier function plays an important role in mucosal defense and is an important factor in the pathogenesis of multiple allergic diseases (Schleimer et al., 2007; Georas and Rezaee, 2014; Holtzman et al., 2014). Interestingly, inhaled allergens, pollution particles, and respiratory viruses can disrupt the airway epithelial barrier integrity, which might represent a risk factor for allergen sensitization. The epithelial barrier is increasingly recognized as an important modulator of inflammatory processes. The airway barrier dysfunction might be involved in the initiate inflammatory signal cascades in the airway because of the easier penetration of inhaled allergens and pollutants. Many inhaled allergens degrade the airway epithelial barrier via their proteolytic activity (Wan et al., 1999; Kalaydjieva et al., 2000; Reed and Kita, 2004; Runswick et al., 2007). Inflammatory cytokines such as TNF-α and IFN-γ and Th2 cytokines such as IL-4 and IL-13 can cause barrier dysfunction (Ahdieh et al., 2001; Coyne et al., 2002; Bruewer et al., 2003; Saatian et al., 2013). These might potentially create a positive feedback loop of the barrier dysfunction on airway inflammation of bronchial asthma and chronic rhinosinusitis (CRS).

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Abbreviations: AJC, apical-junctional complex; CRS, chronic rhinosinusitis; NDRG1, N-myc downstream-regulated gene 1; TER, transepithelial electrical resistance; TJs, tight junctions; ZO-1, zonula occludens-1
The airway epithelium of allergic patients with asthma is compromised and presents a defect of the epithelial barrier function, including decreased expression of intercellular junction proteins (Xiao et al., 2011; Soyka et al., 2012; Kozu et al., 2015; Sweerus et al., 2016). The sinus epithelium in the nasal polyps of patients with CRS was also shown to present epithelial dysfunction through decreased expression of tight junction proteins (Soyka et al., 2012; Lee et al., 2016; Steelant et al., 2015; Sweerus et al., 2016). Therefore, the epithelial barrier dysfunction is an important pathogenic factor in the development and progression of bronchial asthma and CRS, but the mechanisms underlying the establishment of the airway epithelial barrier remain largely unknown. The present study was therefore designed to identify the molecule(s) regulating the development of the airway epithelial barrier.

Materials and methods

Cell culture

Transformed normal human bronchial epithelial cells (16HBE143, abbreviated as 16HBE cells [Cozens et al., 1991; Haws et al., 1992]) were provided by Dr. Dieter C. Gruenert (Department of Laboratory Medicine, University of California, CA, USA), and cultured in the minimum essential medium with 10% (v/v) fetal bovine serum.

Small interfering RNA transfection

16HBE cells were grown in six-well tissue culture plates (IWAKI Glass, Iwaki, Japan) to 50% confluence and transfected individually with either 50 nM Silencer Select Control small interfering RNA (siCtrlRNA; Invitrogen) or human N-myc downstream-regulated gene 1 (NDRG1) (siNDRG1 #1 and #2), or Claudin-8 siRNAs (siCLDN9 #1 and #2) (Sigma–Aldrich) for 24 h using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. After the transfection, the transfected cells were seeded on Transwell chambers (Corning Life Sciences, Corning, NY, USA) before replacing the transfection medium with complete medium.

Transepithelial electrical resistance

Cells were seeded onto Transwell inserts (Costar, New York, NY, USA) at a density of 2 × 10^5 cells/cm². Cell layer barrier integrity was measured by transepithelial electrical resistance (TER) with Millicell-ERS (Millipore Co., Bedford, MA, USA).

Paracellular FITC-dextran fluxes

The permeability of cell monolayers was evaluated by Fluoresceinisothiocyanate (FITC)-dextran fluxes. A FITC-dextran of 4 kDa (1 mg/mL) solution was added to the apical compartment. The supernatant (100 μL) was removed from the basal component at 60 min after addition of FITC-dextran. The fluorescent intensity was measured by a fluorescent microplate reader (Varioskan, Thermo Fisher Scientific, Waltham, MA, USA).

Patients

Tissue samples were obtained during surgical biopsies from 15 patients with CRS, which was defined by the criteria of the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force (Hasan et al., 2015). All ethmoid sinus tissues were collected during surgery to remove nasal polyps. The study was approved by the IRB of the Nippon University Itabashi Hospital, and written informed consent was obtained from all patients.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 30 min at 37°C. Anti-human ZO-1 mAb (Zymed Laboratories Inc., San Francisco, CA), anti-human occludin rabbit mAb (Cell Signaling Technology, MA), or anti-human NDRG1 mAb (Santa Cruz) were used as a primary antibody, and Alexa 488-conjugated anti-mouse IgG was used as a secondary antibody. An FV1000-D laser scanning confocal microscope with a 40× objective lens was used to investigate expression.

Immunohistochemistry

The nasal tissues were paraffin embedded and cut into sections, and then, were deparaffinized and rehydrated. The endogenous peroxidase was inactivated by 3% hydrogen peroxide in methanol after the antigen retrieval procedure. These sections were incubated with anti-NDRG1 ab. (1:500) for 1 h at 30°C, and stained with 3,3′-diaminobenzidine. Counterstaining was performed using hematoxylin. The intensity of cell staining was scored by a semi-quantitative manner. Intensity classification were 0 (no staining), 1+ (weak staining), 2+ (distinct staining), and 3+ (very strong staining).

Microarray analysis

Fragmented second-cycle cDNA was labeled using a GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA), according to the manufacturer’s protocol. We confirmed DNA fragmentation using an Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA), according to the manufacturer’s protocol.
Table 1 Sequences of the primers used for quantitative real time polymerase chain reaction.

| Primers | Forward 5’→3’ | Reverse 5’→3’ |
|---------|---------------|---------------|
| E-cadherin | AAGGTGACAGAAGCCTCTGGATAGA | CATCCCGTTGAGTGACACA |
| Occludin | CGAGCAGTGGGTTAAATGTA | ACTGTCACCTTTCACATAGTCAG |
| ZO-1 | CGTGCTCTTGAGCCCTGTAAAG | GGATCTACATGCAGACGACAA |
| ZO-2 | GAGGAGTGGAGCACTGACGA | GGAGCCACATGCAGACGTC |
| JAM-A | CGGTCCTCTGAGCTGTAAGG | TCACCGGTCTCTCATAGGAA |
| JAM-B | GCCTGACCACAACCTCTGA | GAGACACTCCCGACCAGTT |
| CLDN 1 | AGATTTACTCTCTATGCCCGC | CGTGCAGACGAGGACTCCA |
| CLDN 3 | CATCAGTGCAGCAACATCTG | TCATACCTCTGCACATCT |
| CLDN 4 | AGCGTCCAGGCCTCCTCACT | AGCAGGGAAGCTCAGACCT |
| CLDN 5 | CCTGGACCGACACACTGTGA | AGACGAGGAGCTCAGACAG |
| CLDN 6 | ACGGAGGATGGTAGTACGA | CCAGGAAGTACAGACCAG |
| CLDN 7 | AAGGCAATTTCATCTGTG | GAGTGGAGACTAGGTAAGAG |
| CLDN 9 | ATTCGAGTGGCTACGTCGA | ATACGCAAGGTACGAAAG |
| CLDN 11 | CCCGCTTGTGACTGACAG | GATTGGGAGGTACATCCC |
| CLDN 16 | TGGAGGTGCAGCAATGATGC | GAGTGGCAGGAATGATGGA |
| CLDN 18 | TACACATTTGGTGGCTCT | TAAAAGCTCTGGTGAGCG |
| CLDN 19 | GCATTGACAGGTGGTGGT | CAGGCCAAGCTCAGTAT |
| NDRG1 | CCCGCAGCAGCATTGGAAT | GCTGGTAGGCATAGGTAAG |
| GAPDH | GTGGAGTCTCAACGAGAAT | GGCAACAATATCCCTTACCAGAG |

Figure 1 Genome-wide mRNA profiles during the development of the permeability barrier function in 16HBE cells. (A) Time course of transepithelial electrical resistance (TER) in 16HBE cells cultured for 7 days. The results were means ± SD from three independent experiments. (B) Volcano plot showing gene expression levels in 16HBE cells at day 0 versus day 7. (C) Time course of N-myc downstream-regulated gene 1 (NDRG1) mRNA expression in 16HBE cells cultured for 7 days. The results were means ± SD from three independent experiments.
Next, we used Gene Chip HU Gene 1.0 ST Arrays (Affymetrix) for microarray analysis. An array was hybridized using Genechip Fluidics Station 450 and a Genechip Scanner 3000 (Affymetrix) to measure the fluorescence intensity. The obtained gene data were subjected to statistical analysis using Partec software (Partec, Münster, Germany).

Real-time PCR

Total RNA was extracted with the RNAiso Reagent (TaKaRa, Tokyo, Japan). First-strand cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa). The Applied Biosystems 7300 Fast Real-Time PCR System and SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) were followed according to the manufacturer's protocols. The PCR reactions were performed at 95°C for 20 s, followed by 40 cycles each of 95°C for 3 s, and 60°C for 30 s. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The PCR primers used to detect e-cadherin, Zo-1, -2, and -3 and claudin-1, -3, -4, -5, -6, -7, -9, -11, -16, -18, and -19 mRNA are shown in Table 1.

Figure 2  N-myc downstream-regulated gene 1 (NDRG1) expression in the nasal tissues of patients with chronic rhinosinusitis (CRS). (A) A representative image of NDRG1 expression in the nasal tissue from a patient with CRS (a–c). (a) Ciliated airway epithelial cells and basal cells in the nasal tissue of a patient with CRS. (b) Goblet cells and basal cells in the nasal tissue of a patient with CRS. (c) Damaged epithelial cells in the nasal tissue of a patient with CRS. (B) Immunohistochemistry score for NDRG1 expression in ciliated airway epithelial cells, basal cells, goblet, and damaged epithelial cells of patients with CRS (n = 15). The horizontal bars represent the mean values of the expression score. *P ≤ 0.05.
from 16HBE cells cultured for 1 or 7 days. The total RNA was analyzed by a DNA microarray. Expression profiles were normalized to those of the cells at day 1 after culture. We performed a fold-change analysis using a cut-off value of 1.5-fold increase ($P < 0.05$). Within the significantly increased genes in the microarray data, we focused on a gene, NDRG1 because NDRG1 has been reported to be involved in the regulation of E-cadherin (Kachhap et al., 2007) (Figure 1B). Next, we examined NDRG1 mRNA expression by real time-PCR during the development of AJC in 16HBE monolayers. The expression of NDRG1 significantly increased for 7 days (Figure 1C).

Expression of NDRG1 protein in nasal mucosal tissues

To study the localization of NDRG1 protein in the airway, we performed immunohistochemical staining with anti-NDRG1 antibodies in the nasal tissues of 15 patients with CRS. As a pathological feature of airway remodeling in CRS, morphological changes of the epithelium such as desquamation and goblet cell metaplasia were observed. We studied the expression levels of NDRG1 on various types of airway epithelial cells such as ciliated epithelial cells, basal cells, damaged epithelial cells, and goblet cells (Figure 2A). In the nasal tissues of CRS, NDRG1 expression was mainly observed in ciliated epithelial cells and basal cells in an apparently normal region of the nasal airway mucosa, as reflected by a significantly higher mean staining score (Figure 2B). On the other hand, very low or absence of NDRG1 was observed in goblet cells and damaged epithelial cells in the inflamed region of the nasal mucosa in CRS (Figure 2B).

NDRG1 knockdown impairs airway epithelial barrier formation

To study the roles of NDRG1 in epithelial barrier formation, we depleted NDRG1 by its specific siRNA-mediated silencing. For the experiments, 16HBE cells were transfected with siRNAs and then seeded onto Transwell filters at a higher density to minimize the influence of cell growth and to accelerate epithelial differentiation by transfection. Transfection of NDRG1-specific siRNA into 16HBE cells revealed that NDRG1 mRNA was reduced by over 70% at

![Figure 3](image-url)
24 h after addition to the Transwell filters (Figure 3A). Over the 3-day period, TER in the NDRG1 knocked down cell monolayers was significantly lower than that in the control cells (Figure 3B).

We determined whether the reduced TER in NDRG1 knocked down 16HBE monolayers was caused by changes in cell growth or viability. Cell growth assays showed that there were similar cell numbers in NDRG1 knocked down and control 16HBE monolayers (data not shown). Cell viability evaluated by trypan-blue staining revealed that NDRG1 knockdown did not lead to change in cell viability (data not shown). These results indicated that neither decreased cell growth nor reduced cell viability accounted for the defective epithelial barrier integrity in NDRG1-depleted cell monolayers.

To evaluate the role of NDRG1 on epithelial barrier development, we assessed the formation of AJCs in NDRG1 knocked down and control 16HBE cell monolayers by confocal immunofluorescence microscopy. After 3 days, cells were fixed with paraformaldehyde and stained for AJs and TJs with anti-occludin-specific antibodies (left row) and anti-zonula occludens-1 (ZO-1) antibodies (right row), respectively. ZO-1 was strongly expressed and localized as a junction protein at the cell–cell contact sites in the control cell monolayers (Figure 3C, upper panel). In contrast, in NDRG1 knocked down cell monolayers, most cells showed a reduction of occludin and ZO-1 expression at the apical surface of cell–cell contact sites (Figure 3C, lower panel).

Tight junction and adherence junction gene expression profile in NDRG1 knocked down cells

We used RT-PCR to analyze the effects of NDRG1 expression on the expression of tight junction and adherence junction molecules. A previous report suggested that airway epithelial cells express claudin-1, -3, -4, -5, -6, -7, -9, -11, -16, -18, and -19 (Frank, 2012). The cells transfected with two different NDRG1-specific siRNAs (NDRG1-siRNA #1 and #2) showed a significant reduction in the expression of claudin-9 (Figure 4), but no change was detected for the other claudin family members. In contrast, the expression of the adherence junction protein, E-cadherin, and tight junction molecules, occludin and ZO-1, -2, and -3 did not decrease. These results suggest that NDRG1 expression is involved in the maintenance of the epithelial barrier integrity through the expression of claudin-9.

Next, we examined the effect of claudin-9 reduction on the epithelial barrier integrity of 16HBE cells. We specifically depleted claudin-9 by siRNA-mediated silencing. Transfection of two different claudin-9-specific siRNAs into 16HBE cells revealed that claudin-9 mRNA expression was reduced by over 70% at 24 h (Figure 5A). These claudin-9-specific siRNAs did not reduce the expression of claudin-1 which has been known to be important to barrier function (Figure 5A). Over the 3-day period, TER in the claudin-9 depleted 16HBE cell monolayers was significantly lower than that in the control cell monolayers (Figure 5B). FITC-dextran permeability was significantly increased in the claudin-9 knocked down 16HBE cell monolayers (Figure 5C).

Discussion

In the present study, we identified a regulatory molecule of the epithelial barrier function, NDRG1, which might be involved in the stabilization of the airway epithelial barrier integrity. In this study, we examined global gene expression analyzed by DNA microarray. As shown in Figure 1B, the microarray data revealed that the induction of several genes was observed during the development of AJC in 16HBE cells. The gene with the highly changed mRNA expression was NDRG1. NDRG1 has been reported to be initially identified as a gene that is upregulated in N-myc knockout mouse...
embryos, and is repressed by N-myc and c-myc (Kalaydjieva et al., 2000), after it was isolated by numerous laboratories under various pathophysiological conditions (Ellen et al., 2008; Bae et al., 2013). NDRG1 is a 43 kDa protein, which is composed of 394 amino acids and is highly conserved among species. NDRG1 is predominantly cytosol and upregulated in response to cellular stress signals (Yao et al., 2008). It has been recently demonstrated that NDRG1 interacts with a Wnt receptor, LRP6, leading to the blockade of the Wnt signaling pathway (Liu et al., 2012; Sun et al., 2013). The biological function of NDRG1 is still largely unknown. The roles of NDRG1 in the lung physiology or pathobiology have not been previously studied, except in lung cancer (Kovacevic et al., 2011; Fan et al., 2012; Liu et al., 2014). To the best of our knowledge, the present study is the first study designed to investigate the roles of NDRG1 in airway epithelial barrier function.

We demonstrated that NDRG1 plays a role in the maintenance of the integrity of the airway epithelial barrier. The epithelial barrier is maintained by AJC through formation of TJs and AJs. TJs connect cells to each other by OCLN and claudin family proteins. TJs are connected to the actin cytoskeleton through protein complexes containing various intracellular proteins such as ZO-1 and -2. We showed that NDRG1 knockdown reduced AJ formation in immortalized normal human airway epithelial cell lines through the reduction of claudin-9 expression. Unfortunately we were not able to study the expression of NDRG1 in the nasal tissues of healthy subjects, which would be a valuable future study. Future study would be required for the expression levels and distribution of NDRG1 in the nasal epithelial tissues of healthy subjects.

Consistently, proliferation, viability, or E-cadherin levels of the cells were not affected by NDRG1 knockdown.

Figure 5 siRNA knockdown of claudin (CLDN)-9 impairs the epithelial barrier formation. (A) Quantification of CLDN9 (upper) and CLDN1 (lower) mRNA by real-time PCR. mRNA was purified from the cells harvested at 24 h after transfection of the control (siCtl) or two CLDN9-specific siRNAs (si CLDN9 #1 and #2). Results are expressed relative to the control value (siCtl-treated cells) and are mean ± SD values. *P ≤ 0.05 (B) Effect of NDRG1 knockdown on TER. Results are expressed as a percentage of the control value (siCtl-treated cells) and are mean ± SD values. *P ≤ 0.05 (C) CLDN9 knockdown effects on paracellular permeability of FITC-dextran. Results are expressed as apparent permeability coefficients (Paap) of FITC-dextran and are means ± SDs. *P ≤ 0.05.
Claudins are a family of tetraspan transmembrane proteins that form the basal structure for TJ permeability (Tsukita and Furuse, 2002; Schlingmann et al., 2015). The classic claudin family members show a high degree of structural similarity in the second extracellular domain and generally have a short C-terminal cytosolic domain. In contrast, the second extracellular domain of non-classic claudins presents more heterogeneity (Krause et al., 2008). Claudin-9 belongs to the classic claudins (Krause et al., 2008). Claudin-1 expression in the human lung is enriched in the large airway epithelium, but not in the small airways, unlike claudin-4 and -7, which are expressed throughout the airway (Kaatteenaho-Wiik and Soiini, 2009; Kaatteenaho et al., 2010). Other classic claudins are also likely to be present (Ohta et al., 2012; Rezaee and Georas, 2014). Claudin-9 is expressed in endothelial cells and functions as an important tight junction protein (Nelson et al., 2007; Wessells et al., 2009). A study using claudin-9 mutant mice revealed that the subapical tight-junction strands have biologically important ion barrier functions (Nakanoto et al., 2009). However, the functional role of claudin-9 on airway epithelial cells has not been determined. In the present study, we showed that the knockdown of claudin-9 resulted in the decrease of epithelial barrier integrity in a normal airway epithelial cell line. Several previous studies have shown that NDRG1 appears to prevent epithelial mesenchymal transition-induced metastasis through the inhibition of the down-regulation of E-cadherin expression in cancer cells (Chen et al., 2012; Liu et al., 2014). In the present study, although we did not investigate the roles of NDRG1 on EMT of normal epithelial cells, we did not observe a change in E-cadherin expression. In the differentiated normal epithelial cells, NDRG1 would be important for the stability of tight junctions through the expression of claudin-9, but not E-cadherin. However, we could not clarify any detailed mechanism by which NDRG1 regulates claudin-9 expression. Further study is required to determine how NDRG1 regulates claudin-9 expression in the cells.

We studied the distribution of NDRG1 expression in the nasal tissue obtained from patients with CRS. Both the nasal and bronchial mucosa are characterized by basal cells and ciliated epithelial cells in normal conditions. The histological appearance of these epithelia is similar between BA and CRS (Hellings and Prokopakis, 2010). NDRG1 was mainly expressed in ciliated epithelial cells in the nasal epithelium. Considering that the biological barrier integrity is especially important in the airway, which is constantly in contact with environmental stimuli, it is not surprising that NDRG1 is highly expressed in ciliated epithelial cells, which have established AJCs. In addition, we demonstrated very low and absent expression of NDRG1 in damaged epithelial cells, which are partially shed or separated cell junctions in inflamed regions. This observation suggests that loss of NDRG1 might be important for maintaining the integrity of the barrier function in highly differentiated airway epithelial cells. These results have indicated that restoration of NDRG1 expression levels and/or function should be a potential therapeutic strategy for the treatment of airway diseases, such as CRS and asthma. Thus, our study results may contribute to the development of new treatment methods for airway epithelial injury. Further study is required to fully understand the pathological roles of NDRG1 on asthma and CRS.

Conclusions

These results suggest that NDRG1 is increased during tight junction development, and is important for airway barrier integrity through the regulation of claudin-9 expression. Down regulation of NDRG1 might be involved in the pathogenesis of chronic airway inflammation.

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Author contributions

YG, YK, SM, KK, and AS performed in vitro cell experiments, data analysis, drafted the paper, and approved its final version. YK, YG, HK, YN, MI, and SH contributed to the study design and clinical study, drafted the paper, and approved its final version.

Conflicts of interest

The author(s) declare that they have no competing interest.

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