Polyinosinic:polycytidylic acid induces protein kinase D–dependent disassembly of apical junctions and barrier dysfunction in airway epithelial cells

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Background: Disruption of the epithelial barrier might be a risk factor for allergen sensitization and asthma. Viral respiratory tract infections are strongly associated with asthma exacerbation, but the effects of respiratory viruses on airway epithelial barrier function are not well understood. Many viruses generate double-stranded RNA, which can lead to airway inflammation and initiate an antiviral immune response.
Objectives: We investigated the effects of the synthetic double-stranded RNA polyinosinic:polycytidylic acid (polyI:C) on the structure and function of the airway epithelial barrier *in vitro*.
Methods: 16HBE14o- human bronchial epithelial cells and primary airway epithelial cells at an air-liquid interface were grown to confluence on Transwell inserts and exposed to polyI:C. We studied epithelial barrier function by measuring transepithelial electrical resistance and paracellular flux of fluorescent markers and structure of epithelial apical junctions by means of immunofluorescence microscopy.
Results: PolyI:C induced a profound decrease in transepithelial electrical resistance and increase in paracellular permeability. Immunofluorescence microscopy revealed markedly reduced junctional localization of zonula occludens-1, occludin, E-cadherin, β-catenin, and disorganization of junction-associated actin filaments. PolyI:C induced protein kinase D (PKD) phosphorylation, and a PKD antagonist attenuated polyI:C-induced disassembly of apical junctions and barrier dysfunction.
Conclusions: PolyI:C has a powerful and previously unsuspected disruptive effect on the airway epithelial barrier. PolyI:C-dependent barrier disruption is mediated by disassembly of epithelial apical junctions, which is dependent on PKD signaling. These findings suggest a new mechanism potentially underlying the associations between viral respiratory tract infections, airway inflammation, and allergen sensitization. (J Allergy Clin Immunol 2011;128:1216-24.)

Key words: *Asthma, polyI:C, Toll-like receptor 3, epithelial permeability, protein kinase C, tight junctions, adherens junctions*

Airway epithelial cells form a tight barrier between the submucosal respiratory immune system and inhaled allergens, particles, and viruses. The airway epithelial barrier consists of surface-lining fluids, mucus, and apical junctional complexes (AJCs), which are specialized adhesive membrane structures that are formed between adjacent cells. AJCs consist of the most apical tight junctions (TJs) and underlying adherens junctions (AJs).[1,2] TJs create a physical barrier that restricts the paracellular movement of ions and uncharged molecules. AJs are important for the initiation and maintenance of epithelial cell-cell adhesions and are thought to be essential for normal TJ structure and function (for reviews see Anderson et al[3] and Shen et al[4]). The adhesive properties of TJs are determined by 3 major types of transmembrane proteins: (1) members of the claudin family; (2) TJ–associated marvel proteins that include occludin, tricellulin, and Marvel D3; and (3) immunoglobulin-like proteins, such as junctional adhesion molecule-A and Coxsackie virus and adenovirus receptor. E-cadherin and nectin family members represent the major transmembrane proteins of epithelial AJs.[5,6] A number of peripheral membrane proteins form the so-called cytosolic plaques of TJs and AJs, which cluster and stabilize transmembrane junctional proteins, thereby enhancing their adhesive properties. Zonula occludens protein 1 (ZO-1) is a key constituent of the TJ cytosolic plaque and is thought to bridge AJC components with the perijunctional cytoskeleton.[7] The cytosolic plaque of AJs includes β-catenin and p120 catenin, which bind to the intracellular domain of E-cadherin and actin-binding proteins and connect AJs to different cytoskeletal structures.[8] Through these interactions, the assembly, maintenance, and disassembly of AJCs are intimately linked to the cell cytoskeleton.[9]

Although emerging evidence points to a role for epithelial barrier dysfunction in inflammatory lung diseases, such as asthma and cystic fibrosis,[10] very little is known about the regulation of airway epithelial junctions in health or disease. In one early study reduced expression of α-catenin, ZO-1, and E-cadherin was reported in bronchial biopsy specimens from asthmatic subjects.[11] Recently, Xiao et al[12] reported that ZO-1 and occludin expression was significantly reduced in bronchial epithelial cells from

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Abbreviations used
AJ: Adherens junction
AJC: Apical junctional complex
dsRNA: Double-stranded RNA
HBSS: Hanks' balanced salt solution
LDH: Lactate dehydrogenase
MDA5: Melanoma differentiation-associated gene 5
MLCK: Myosin light chain kinase
NHBE: Normal human bronchial epithelial
NM II: Nonmuscle myosin II
pAb: Polyclonal antibody
PKC: Protein kinase C
PKD: Protein kinase D
polyI:C: Polyinosinic:polycytidylic acid
PRR: Pattern-recognition receptor
RIG-I: Retinoic acid–inducible gene I
ROCK: Rho-dependent kinase
siRNA: Small interfering RNA
TEER: Transepithelial electrical resistance
TJ: Tight junction
TLR: Toll-like receptor
ZO-1: Zonula occludens protein 1

asthmatic subjects. Interestingly, this was apparent both in bronchial biopsy specimens and epithelial cells propagated *in vitro* and was associated with significantly attenuated barrier function.[12] In other studies E-cadherin was depleted from epithelial cell-cell contacts and accumulated in the cytoplasm in biopsy specimens obtained from asthmatic subjects.[13,14] Additionally, E-cadherin shedding from the cell surface into bronchoalveolar lavage fluid has been detected after antigen challenge[15] and soluble E-cadherin levels in induced sputum correlated with asthma severity.[16] Although these findings suggest that disruption of the epithelial AJC is an important feature of the airway epithelium in asthmatic subjects, the molecular mechanisms involved in this process are not well understood.

Airway epithelial cells express a variety of pattern-recognition receptors (PRRs), including members of the Toll-like receptor (TLR) family.[17] These receptors sense and respond to microbes, viruses, and fungi and induce epithelial cells to secrete cytokines and chemokines that initiate lung inflammation and immune responses by recruiting and activating antigen-presenting dendritic cells and other cell types. Double-stranded RNA (dsRNA), produced either as an intermediate of viral replication or as a part of the viral RNA genome, is now recognized as a powerful adjuvant that drives antiviral immune responses, and dsRNA derivatives have demonstrated marked efficacy in both systemic and mucosal vaccine strategies.[18-20] The molecular mechanisms underlying the adjuvant properties of dsRNA are under active investigation. Potential molecular sensors of dsRNA include protein kinase R, TLR3, and the more recently identified cytoplasmic helicases (eg, retinoic acid-inducible gene I [RIG-I], melanoma differentiation-associated gene 5 [MDA5], and LPG2). TLR3 is thought to recognize dsRNA oligonucleotides in an acidified lysosomal compartment,[21,22] whereas the helicases recognize "free" RNA in the cytoplasm through mechanisms still being worked out.[23-25]

Very little is known about how different environmental exposures affect airway epithelial barrier structure and function. We undertook the present study to address this gap in our current knowledge by using model epithelia grown *in vitro*. Here we report that the synthetic dsRNA polyinosinic:polycytidylic acid (polyI:C) induces marked disruption of airway epithelial AJC function and structure. This does not appear to involve autocrine or paracrine effects of a secreted mediator or mediators but rather direct effects of polyI:C acting in part in a protein kinase D (PKD)–dependent manner. Our description of a previously unsuspected barrier-disruptive effect of polyI:C provides new insights into how dsRNA might act as a mucosal adjuvant and suggests a pathway potentially explaining the strong associations between viral respiratory tract infections, allergen sensitization, and asthma.

METHODS

Epithelial cell culture

16HBE14o- human bronchial epithelial cells (a gift from Dr D. C. Gruenert, University of California San Francisco, San Francisco, Calif)[26] were cultured in minimum essential medium supplemented with 10 mmol/L HEPES, 10% FBS, and glutamine. Normal human bronchial epithelial (NHBE; Lonza, Basal, Switzerland) cells were grown in defined media and differentiated at the air-liquid interface. For immunolabeling and permeability studies, epithelial cells were grown on collagen-coated, permeable polycarbonate filters of 0.4 μm in pore size (Costar, Cambridge, Mass). For biochemical experiments, cells were cultured on 6-well plastic plates. Detailed methods can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Transepithelial electrical resistance and paracellular flux measurements

Transepithelial electrical resistance (TEER) was measured with an EVOMX voltohmmeter (World Precision Instruments, Sarasota, Fla). The resistance of cell-free collagen-coated filters was subtracted from each experimental point, and the data were presented either as absolute values ($\Omega \times cm^2$) or changes relative to the control group. Paracellular flux of fluorescent markers was investigated by measuring passage of apically added markers across epithelial monolayers, as detailed in the Methods section in this article's Online Repository at www.jacionline.org.

Immunofluorescence staining, immunoblot analysis, and cytotoxicity assays of junctional proteins

After the indicated treatments, cell monolayers were fixed for immunofluorescence or lysed to extract protein for immunoblot analysis, and the supernatant was used for cytotoxicity assay by measuring LDH release. For more information on immunofluorescence staining, immunoblot analysis, and cytotoxicity assays of junctional proteins, detailed methods are described in the Methods section in this article's Online Repository at www.jacionline.org.

RNA interference

We transfected 16HBE cells with 100 nmol/L small interfering RNA (siRNA) oligonucleotides targeted to TLR3, RIG-I, and MDA5 (SMART Pool; Dharmacon, Lafayette, Colo) and nontargeted control siRNA with DharmaFECT 1 (Dharmacon), as per the manufacturer's protocol, and monitored knockdown efficiency by using Western blotting and real-time PCR.

Statistical analysis

Results are expressed as means ± SEMs, unless otherwise specified. The data were evaluated statistically with ANOVA and the Student *t* test, with