Breaking Barriers

New Insights into Airway Epithelial Barrier Function in Health and Disease

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Abstract

Epithelial permeability is a hallmark of mucosal inflammation, but the molecular mechanisms involved remain poorly understood. A key component of the epithelial barrier is the apical junctional complex that forms between neighboring cells. Apical junctional complexes are made of tight junctions and adherens junctions and link to the cellular cytoskeleton via numerous adaptor proteins. Although the existence of tight and adherens junctions between epithelial cells has long been recognized, in recent years there have

been significant advances in our understanding of the molecular regulation of junctional complex assembly and disassembly. Here we review the current thinking about the structure and function of the apical junctional complex in airway epithelial cells, emphasizing the translational aspects of relevance to cystic fibrosis and asthma. Most work to date has been conducted using cell culture models, but technical advancements in imaging techniques suggest that we are on the verge of important new breakthroughs in this area in physiological models of airway diseases.

Airway epithelial cells are an important part of the innate immune system. In addition to promoting mucociliary clearance, epithelial cells produce antimicrobial substances as well as chemokines and cytokines that recruit and activate other leukocytes (1). One overlooked component of epithelial defenses are apical junctional complexes (AJC) that form between neighboring cells and consist of the most apical tight junctions (TJ) and the underlying adherens junctions (AJ) (Figure 1). TJ are most apical and regulate paracellular transport of ions and certain molecules, whereas AJ are important for initiation and maintenance of cell–cell adhesion (2, 3). Epithelial TJ and AJ are involved in numerous signal transduction cascades and interact to establish apical–basal epithelial cell polarity (4, 5). Dysfunction of epithelial junctions is increasingly linked to airway diseases and may predispose to infections, but a comprehensive picture of the relationship between junctional integrity and

antimicrobial defenses is lacking. In this review, we focus on the structure and function of airway epithelial TJ and give examples of junctional regulation by environmental exposures. Dysfunction of alveolar TJ contributes to pulmonary edema and lung injury, but this topic is beyond the scope of this review (for reviews see Ref. 6–8). Our understanding of AJC structure and function comes largely from model epithelia and studies of nonpulmonary tissues, and although the basic principles of AJC organization apply to most epithelial cells, we mention examples of lung-specific junctional expression and/or regulation.

Early Studies of TJ and Current Models

Intercellular junctions were first identified using light microscopy, but the advent of electron microscopy (EM), especially freeze-

fracture techniques, was a key step forward. By EM, TJ appear as linear strands just below the apical membrane and run in parallel bands. The first systematic analysis of airway epithelial tight junction morphology by EM was reported in 1992 (9). In this paper, Godfrey and colleagues used freeze-fracture EM with normal human lung tissue and observed approximately 11 strands per epithelial cell, with a similar distribution in main and lobar bronchi (9). Because of the challenges in quantifying junctional morphology systematically in multiple orders of branching airways, it was difficult to determine whether strand number or organization varied based on location within the tracheobronchial tree. In a subsequent paper, Jeffery and colleagues compared airway junctional morphology in lungs from subjects with and without cystic fibrosis (CF) and found no significant difference in strand number but found some differences in junctional complexity

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Figure 1. Cartoon diagram demonstrating organization of junctional structures in the airway epithelium including tight junctions (black) and adherens junctions (blue). Certain environmental exposures lead to dysfunction of epithelial junction, resulting in greater outside/in permeability. The figure demonstrates the potential for barrier dysfunction to lead to cell signaling because it allows apical cytokines and growth factors, which may be constitutively present in epithelial lining fluids (light blue dots), to interact with basolateral receptors (light blue rectangle labeled GFR [growth factor receptor]). In the presence of intact epithelial junctions, these ligand/receptor interactions are prevented. Barrier dysfunction also allows penetration of luminal particles and antigens into the subepithelial space, where they encounter innate immune cells that initiate inflammation and immune reactions. The fate of subepithelial particles is complex; subepithelial particles may, depending on size and shape, translocate via afferent lymphatics to the pleural space, regional lymph nodes, or the blood stream (not shown). The inset shows an enlarged schematic of protein–protein interactions in tight junctions (black text) and adherens junctions (blue text), including ability of zonula occludens (ZO) proteins to interact with intracytoplasmic domains. The inset also indicates that junctional proteins are linked to the actin cytoskeleton (green dashed line) via several potential adaptor proteins (black dashed line). This figure is meant to be illustrative and not comprehensive and does not include many other potential interacting partners or other molecules that contribute to barrier integrity or cell polarity. CAR, coxsackie adenovirus receptor; JAM-A, junctional adhesion molecule.

(e.g., disorganized strands that sometimes extended below the typical apical belt) (10). The functional significance of disorganized tight junction strands was not investigated in that report.

Barrier function is now usually studied by growing epithelial cells *in vitro* on semipermeable membranes. This approach has strengths and weaknesses. Strengths include the ability (1) to analyze junctional structure and function in the same cells, (2) to identify signal transduction cascades involved in junctional regulation, and (3) to conduct gain-of-function or loss-of-function experiments targeting specific AJC components. Two general approaches are used to study junction function: (1) permeability to small ions can be measured

by analyzing transepithelial electrical resistance (TEER), and (2) permeability to probes of different sizes and shapes can be estimated by measuring flux across the monolayer. This is typically expressed normalized to surface area and concentration as apparent permeability: $P_{app} = F/(S \times A_0)$, where F = flow rate, S = surface area, and A_0 = starting concentration (11). Molecules that traverse epithelia paracellularly via TJ should demonstrate linear and nonsaturable permeability. Weaknesses of studying model epithelia *in vitro* include the inability to fully recapitulate the complex structure of surface epithelia including nonepithelial cells in tissue culture. This concern can be mitigated by studying primary epithelial

cells differentiated at air–liquid interface (ALI), which closely resemble epithelial cells *in situ* (12). However, because junctional structure is intimately associated with cell differentiation status, it can be challenging to understand the directionality of a given perturbation *in vitro* (i.e., did junctional structure/function change because of alterations in cell differentiation?). The ability to study intact epithelial sheets or airway explants can circumvent some of these caveats (13).

Disruption of barrier integrity enhances outside/in translocation of inhaled particles into the subepithelial space, where they encounter innate immune cells and lead to airway inflammation and immune responses (Figure 1). In addition, by affecting the polarized distribution of cell surface receptors and their ligands, AJC dysfunction can activate intracellular signal transduction cascades and lead to secretory activity and cell activation (14–16).

Although our understanding of junction structure has expanded enormously, there is no comprehensive model to fully explain how changes in junction structure affect paracellular permeability. Current thinking is that intercellular junctions are not static resistors but rather dynamic structures with discrete probabilities of being open or closed (3, 17). One recent model proposes that there are two pathways for paracellular movement of molecules (3): (1) the claudin-containing “pore,” which controls movement of ions in a charge and size-selective manner, and (2) the “leak” pathway, which allows limited movement of larger macromolecules. It may seem counterintuitive that macromolecular flux can increase without a decrease in TEER, although this is well documented in the literature (18–21).

TEER measures instantaneous ion flux, whereas permeability to tracers is typically measured over hours. Consequently, if macromolecules move through sequential TJ strand breaks organized in series and if at least one strand blocks the paracellular space during TEER measurement, then instantaneous ion permeability might not be affected, whereas macromolecular permeability increases (the “revolving door model”). Although the molecular basis for this model remains to be determined, it reinforces the notion that AJC are dynamic and potentially compartmentalized structures. Deciphering the structure of AJC in their native membrane context will

help provide clarity in this area, although this will be a challenging task. Because TEER can be dissociated from changes in junctional structure and function, it seems reasonable to conclude that multiple experimental approaches (e.g., TEER, permeability, and microscopy) should be used to provide a complete assessment of AJC function in different models.

Components of the Lung Barrier Including AJC

TJ are comprised of intracellular and extracellular components, many of which were discovered by the late Shoichiro Tsukita (22). There are three major types of transmembrane TJ proteins: (1) members of claudin family, (2) tight junction-associated MARVEL protein family members (i.e., occludin, tricellulin, and MARVELD3), and (3) immunoglobulin-like proteins, such as junctional adhesion molecule (JAM) and coxsackie adenovirus receptor (CAR) (23). E-cadherin and members of the nectin family represent the major transmembrane proteins of epithelial AJ (24). Peripheral membrane proteins form the cytosolic plaque of TJ and AJ, where they cluster and stabilize adhesive components of the AJC, including the zonula occludens (ZO) proteins that link the intracellular domains of TJ components with actin-binding proteins (e.g., cortactin, α -catenin, vinculin, and α -actinin) and the cellular cytoskeleton via mechanisms under active study. Other components of the cytosolic plaque linking TJ and E-cadherin with the cytoskeleton include afadin, cingulin, β -catenin, and p120 catenin.

Claudins

Expression and function in the lung. Since the original discovery of claudin-1 by Tsukita and colleagues in 1998 (25), at least 27 claudin family members have been identified. Although no crystal structures have been reported to date, mutagenesis and domain-swapping experiments have provided insights into claudin structure/function relationships (26, 27). Claudins are predicted to be tetraspanning molecules with short N-terminal and longer C-terminal cytoplasmic domains and two extracellular loops. Homo- and heterotypic

aggregation (the latter especially with claudin-3) between both extracellular loops is thought to be crucial for claudin-claudin interactions in the tight junction, whereas the first extracellular loop may be more important in controlling paracellular ion permeability (28). Claudins are expressed in a tissue-specific manner and can be broadly categorized into two groups. First, some claudins increase paracellular permeability ("pore forming" or "leaky" claudins). For example, in intestinal epithelial cells and models of Th2 inflammation, IL-13, and Stat6 cause epithelial barrier dysfunction in part by inducing expression of the leaky claudin-2 (29). However, we recently showed using 16HBElo— airway epithelial cells that IL-4 and IL-13 induce airway epithelial barrier dysfunction without inducing claudin-2 expression (30). Whether claudin-2 is expressed in the airway is controversial (*see below*). Second, other claudins interact in a homo- or heterotypic manner to promote barrier integrity (e.g., claudin-1).

Different claudins are expressed throughout the respiratory tract, and their cell-type expression and contribution to lung diseases is an active area of research. Excellent recent reviews are available that summarize the expression and potential function of lung claudins in detail (7, 8, 31). There appear to be differences in claudin expression depending on location within the tracheobronchial tree (32–34). This is an emerging area where there is lack of consensus and some discrepancies among published studies, likely reflecting differences in species studied and staining techniques used to detect claudin expression in tissues. In one early study of frozen sections, freshly excised human airways were found to express claudin-1, -3, -4, -5, and -7, which differed in their cellular localization (e.g., apical vs. lateral membrane [32]). Claudin-2 protein was not detected in that report (32), whereas a later study of formalin-fixed tissue detected intense bronchiolar staining of claudin-2 (as well as claudin-1, -3, -4, and -7) (35). Claudin-3 is expressed by ciliated upper airway and by type II alveolar epithelial cells, where it may serve a barrier disruptive function at baseline (36). In the alveolus, up-regulation of claudin-4 has emerged as a potentially protective response to lung injury (reviewed in Ref. 37), but less is known about the role of claudin-4 in the airway. Claudin-3 and -4 are targeted by

clostridium perfringes exotoxin (38), and the ability to disrupt claudins and other AJC components may prove useful for drug delivery or airway gene therapy (39, 40).

Although claudin-5 was detected in freshly excised airway samples (32), it was not detected in human airway epithelial cells differentiated *in vitro* at ALI (which preferentially expressed claudin-1 and -4) (41). In a human CF airway epithelial cell line (IB3.1), overexpression of claudin-5 enhanced airway epithelial permeability to different size dextrans (32). This result was surprising because claudin-5 is an essential component of the endothelial blood-brain barrier (42). Recent studies also demonstrated a crucial protective role for claudin-5 in lung microvascular endothelial cells during influenza and HIV infection (43). Claudin-5 is not expressed by high endothelial venules in lymph nodes, an important site of lymphocyte egress from the circulation, suggesting that these blood vessels may be leaky at baseline (44). Although more research is needed about the role of claudin-5 in the airway, these examples raise the possibility that the same TJ component might have different functions depending on the cellular and tissue context in which it is expressed. The splice variant claudin-18.2 appears relatively specific for the lung, where it has been implicated in maintaining alveolar barrier integrity in models of lung injury (8). In the airway, a preliminary study indicated that claudin-18 was reduced in asthma and inhibited by the Th2 cytokine IL-13 (45).

Lessons from gene-targeted mice.

Although different claudin-deficient mice have been reported, none of the publications to date has reported lung-specific phenotypes. This may reflect redundancy among claudin family members, the possibility that some subtle developmental defects were overlooked, or the possibility that gene-targeted mice were not investigated in models of lung diseases (46). Some examples of gene-targeted mice are shown in Table 1 (42, 47–61).

Evidence from human diseases. Genetic mutations in claudin-1 cause a rare disorder characterized by neonatal ichthyosis and sclerosing cholangitis (62). Other examples of genetic mutations leading to human diseases include familial hypomagnesemia and hypercalcuria with nephrocalcinosis and claudin-16 and -19 (63) and autosomal

Table 1: Lessons from Gene-Targeted and Transgenic Mice

Gene	Phenotype	Reference
Claudin-1	Early lethality, excessive transepidermal water loss	47
Claudin-2	Increased FE_{Na} after saline challenge	48
Claudin-4	Urothelial hyperplasia and lethal hydronephrosis	49
Claudin-5	Increased CNS permeability to small molecules	42
Claudin-14	Cochlear hair degeneration, deafness	50
Claudin-15	Megaintestine; glucose malabsorption	51, 52
Occludin	Normal intestinal barrier function; diverse histologic abnormalities	53
	Knockout T cells with delayed accumulation and migration in intestine	54
ZO-1	Lethal ED 10.5–11.5	55
ZO-2	Disturbed angiogenesis	56, 57
	Lethal ED 7.5	
	Decreased proliferation and increased apoptosis	
	Decreased blood–testis barrier in blastocyst chimeras	
ZO-3	None	56, 58
CAR	Intestinal dilation, trend to decreased barrier function	59
	AV block	
JAM-A	No effect on steady-state or LPS-induced lung permeability	60
	Decrease in LPS-induced inflammation due to inhibited neutrophil extravasation	61
	Increased intestinal permeability	

Definition of abbreviations: CAR, coxsackie adenovirus receptor; CNS, central nervous system; ED, embryonic day; FE_{Na} , fractional excretion of sodium; JAM-A, junctional adhesion molecule; ZO, zonula occludens.

recessive deafness and claudin-14 (64). In skin biopsies of subjects with atopic dermatitis, expression of claudin-1 is reduced in association with increased skin permeability (65), which may confer risk for epicutaneous allergen sensitization (66). The potential role of claudins and other AJC components in airway barrier defects in asthma is considered further below.

Occludin

Structure and function. Occludin was identified before claudin-1 by Furuse and colleagues while characterizing a monoclonal antibody that recognized a tight junction–associated molecule in hepatocytes (67). Together with tricellulin and marvelD3, occludin shares a conserved “marvel” domain, which consists of four hydrophobic segments predicted to form tightly packed transmembrane α -helices. The intracellular N- and C-termini are subjected to numerous posttranslational modifications, which are thought to affect interactions with scaffold components in the AJC (68). For example, the occludin C-terminus can be phosphorylated by protein

tyrosine kinases, serine/threonine kinases, and protein kinase C family members, which regulate association with ZO-1 and perijunctional actin filaments (69–72). The crystal structure of the occludin C-terminus revealed a motif similar to RNA polymerase elongation factors (73). Key studies using photobleaching followed by recovery demonstrated that the cellular pools of claudin-1, ZO-1, and occludin are distinct, with occludin being significantly less stable than claudin (74).

After two decades of investigation, the function of occludin in the AJC remains enigmatic. In cells that normally lack TJ (e.g., fibroblasts), expression of claudin-1 or -2 was sufficient to recapitulate TJ structure and function (75), whereas the same did not hold true for occludin (76). In addition, epithelial barrier function appears to be intact in occludin-null mice (*see below*). Thus, occludin is neither sufficient nor necessary for AJC function, and by interacting with ZO-1 and the actin cytoskeleton, occludin is often suggested to have a regulatory role in the AJC.

Occludin overexpression in Madin-Darby canine kidney cells (MDCK) cells augments TEER but paradoxically also

enhances permeability to 4-kD dextran (19, 20). Using a tetracycline-inducible system, Van Itallie and colleagues reported that increasing occludin expression by ~ 4 -fold enhanced the barrier-disruptive effects of the cytokines IFN- γ + TNF- α in MDCK II cells, as assessed by monitoring permeability to 3-kD dextran (77). Furthermore, deletion mutants or chimeric constructs lacking the occludin extracellular domains demonstrated reduced paracellular permeability to mannitol (and other tracers) in MDCK cells (78). One interpretation of these studies is that occludin actively promotes the paracellular flux of macromolecules. However, caveats apply to these overexpression studies because ectopically expressed occludin might not integrate similarly in the apical membranes as native protein, and overexpressed occludin might act in a dominant-negative manner. Van Itallie and colleagues suggested that overexpressed occludin disrupted barrier structures by promoting caveolin-dependent endocytosis (77). These apparent barrier disruptive effects of occludin might be cell-type specific because small interfering RNA (siRNA) knockdown of occludin increases paracellular flux of mannitol (and other tracers) in Caco-2 cells, which originated from a human colon carcinoma (79). Furthermore, in human airway epithelial cells, apical application of a synthetic peptide corresponding to the first extracellular loop (a.a. 90–103) reduced TEER and enhanced permeability to 70- and 2,000-kD dextrans (80). This result must be interpreted with caution because occludin peptides were active at high concentrations that also induced cell cytotoxicity (80). In Caco-2 cells, similar peptides enhanced permeability to mannitol without causing significant cell toxicity (81). Future studies of additional cell types, including primary airway epithelial cells at ALI, are needed to help define the precise role of occludin in airway barrier physiology.

Occludin in lung models. Despite the confusing results obtained using occludin gain-of-function and loss-of-function approaches *in vitro*, reduced expression of occludin has been associated with disrupted airway barrier function in several model systems. For example, Olson and colleagues reported that H_2O_2 disrupted barrier function in 16HBElo– cells and primary mouse tracheal epithelial cells in association

with markedly reduced occludin expression (82). This result is in keeping with the ample evidence from other experimental models that occludin is sensitive to cell redox status (*see* Ref. 83 for recent comprehensive review). Airway epithelial H₂O₂-induced barrier dysfunction was prevented by exposure to hypoxia in a HIF-1 α -dependent manner (82). Taken together with results from experimental colitis models (84, 85), these data suggest that HIF-1 α promotes epithelial barrier integrity after diverse insults.

Gene-targeted mice. Two publications describe the phenotype of occludin-null mice, which were generated by homologous recombination targeting exon 3 (the first transmembrane domain to the second extracellular loop) (Table 1). The structure and function of TJ, as analyzed in different tissues including intestine, urinary bladder, and stomach, was found to be indistinguishable from wild-type controls (53, 86). Lung barrier function was not described in these reports. Although epithelial barrier function was apparently intact, genetic deficiency of occludin had significant effects on fertility and postnatal growth and resulted in histological evidence of chronic inflammation in the stomach, calcification in the brain, testicular atrophy, and thinning of the compact bone (53). Thus, the function of occludin in regulating paracellular permeability and epithelial integrity *in vivo* requires further study. Mice in which the expression of occludin can be regulated in a time-dependent and tissue-specific manner should provide more insights into the role of this molecule in the future.

Other functions of occludin. In addition to regulating paracellular permeability, occludin has other functions. In human hepatocytes, occludin (in addition to claudin-1 and other factors) is a hepatitis C viral entry factor (87–89). Yu and colleagues noted that the exclusion of cells from confluent MDCK monolayers was markedly reduced after siRNA-mediated occludin knockdown (i.e., reduced numbers of “floaters” in tissue culture) and concluded that occludin is involved in sensing and excluding apoptotic cells from epithelial monolayers (90). There is an emerging role for occludin in immune cell migration, specifically neutrophil transepithelial migration (91), and intraepithelial migration of $\gamma\delta$ -T cells in the

intestine (54). Huber and colleagues found that the migration of neutrophils across an MDCK monolayer was regulated by the occludin N-terminus independently of effects on TEER or paracellular permeability to mannitol (91). Recently, Edulblum and colleagues used intravital imaging with novel transgenic mice and found that $\gamma\delta$ -intraepithelial lymphocytes, which are involved in immune surveillance, expressed occludin and appeared to migrate within the epithelium via homotypic interactions with occludin-expressing epithelial cells (54). These studies suggest that, in addition to its role in barrier regulation, occludin may act like an adhesion molecule during tissue inflammation.

Zonula Occludens

Structure and function. ZO-1 was identified in 1986 as a TJ-associated protein in several epithelia (92) and is now known to be a member of the membrane-associated guanylate kinase (MAGUK) family (93, 94), together with ZO-2 (95) and ZO-3 (96). The MAGUK family contains PDZ, SH3, and inactive guanylate kinase domains, the latter two of which interact in ZO-1 as a shared module (97, 98). ZO proteins are expressed in a tissue-specific manner and contain numerous domains capable of protein–protein interactions with other TJ components, the actin cytoskeleton, and other signaling molecules (99, 100) (for a recent review *see* Ref. 101). Thus, ZO proteins are key components of the scaffold that is thought to cluster and stabilize TJ in the apical membrane. ZO proteins also contain conserved nuclear localization sequences and translocate in and out of the nucleus in a cell-cycle-dependent manner (102, 103). The function of nuclear ZO is not well understood, but in some contexts it may act as a transcriptional repressor (104).

ZO and the scaffold. ZO family members have been studied extensively in model epithelia, where they show some redundancy in function. Similar to the case of occludin, ZO family members appear to exert cell-type-specific effects on AJC structure and function. For example, in Eph4 cells, double deficiency of ZO-1 and ZO-2 did not affect cell polarity but led to reductions in TJ strand formation and TEER as well as enhanced paracellular flux (105). Using an inducible

small hairpin RNA strategy in MDCK cells, dual ZO-1/ZO-2 knockdown led to markedly increased permeability but no changes in TEER (106). Whereas wild-type MDCK cells excluded probes > 4 angstroms (~ 0.4 nm), size selectivity was lost in ZO1/ZO2 DKO cells (106). These investigators also observed that knockdown of ZO-1/ZO-2 resulted in marked expansion of the perijunctional actin cytoskeleton especially near AJ, reinforcing the notion that TJ/AJ structure is interrelated and suggesting that ZO-1/ZO-2 play a broader role in morphogenesis (106).

Knockout mice. Gene-targeted mice uncovered a unique role for ZO family members during embryogenesis (Table 1). Whereas ZO-1 and ZO-2 deficiency resulted in embryonic lethality, ZO-3-deficient mice were viable and apparently normal (55–58). This may reflect the fact that ZO-1 and ZO-2 are expressed in endothelial and epithelial cells, whereas ZO-3 is restricted to epithelia, where its function may be compensated by other family members.

Little is known about the expression and function of ZO proteins in the airway, although several studies have documented associations between alterations in ZO-1 expression and decreased barrier function in different models. For example, we recently reported that treatment of human bronchial epithelial cells with polyI:C, a double-stranded RNA, or infection with respiratory syncytial virus (RSV) induces marked disassembly of ZO-1-containing AJC from the cell surface (107, 108). Using primary cells and the 16HBElo– cell line, we found that the barrier disruptive effects of polyI:C and RSV were dependent on protein kinase D (PKD) and were not associated with marked changes in ZO-1 expression but rather interfered with its assembly into the AJC. Grumbach and colleagues reported that in 16HBE cells lipoxin A4 enhanced TEER in association with increased ZO-1 expression (109). The potential role of airway epithelial ZO-1 dysfunction in asthma is considered below.

Other Molecules

Other molecules contribute to the macromolecular AJC in the airway epithelium, and it seems likely that new information will be forthcoming as

new models are developed. Here we highlight a few recent observations potentially of relevance to airway biology research.

MARVEL family. As its name implies, tricellulin is enriched at junctions formed by three cells and is a member of the MARVEL family (110). Mutations in the tricellulin gene are associated with nonsyndromic deafness in humans (111). Tricellulin is widely expressed, including in the inner ear, skin, stomach, and nasal epithelial cells (112), but its expression in the lung has not been well studied. Tricellulin is also expressed by nonepithelial myeloid cells (e.g., brain microglia [113]), but whether myeloid cells express this molecule in the lung requires further study. The expression and function of the remaining MARVEL family member MARVELD3 in the lung has not been previously reported to our knowledge. A recent genome-wide association study identified an intergenic single-nucleotide polymorphism in MARVELD3 associated with malaria resistance (114), underscoring the importance of AJC in host–pathogen interactions.

Immunoglobulin family members. It seems likely that further study of JAM and CAR will uncover roles for these molecules in airway diseases. In polarized human airway epithelial cells, JAM-A is expressed on the basolateral but not apical surfaces, where it can promote reovirus entry (115). JAM-A deficiency in mice does not alter steady-state or LPS-induced lung permeability (assessed by bronchoalveolar lavage protein content or wet:dry ratio) (60) but does enhance intestinal permeability (assessed by measuring absorption of 3, 10, and 40 kD dextran into the bloodstream) (61). Although lung permeability was not affected in JAM-A-deficient mice, LPS-induced neutrophil emigration into alveoli was strikingly attenuated (60). The effects of JAM-A deficiency on leukocyte recruitment to proximal airways or on airway epithelial permeability *per se* have not been systematically investigated to our knowledge. Multiple CAR isoforms are expressed by airway epithelial cells that differ in their localization to the apical versus basolateral membrane (116). A seminal early study established that binding of adenovirus to CAR on airway epithelial cells caused AJC disassembly, leading to paracellular leak and escape of virus (117),

which may be a strategy used by viruses to promote infectivity. In the skin, heterotypic interactions between CAR (on keratinocytes) and JAM-L (expressed by a subset of $\gamma\delta$ -T cells) was shown to help initiate immune responses after epidermal injury (118, 119). Whether similar events occur after epithelial injury and barrier disruption in the lung is not known.

AJ molecules. Recent insights into the roles of AJ components in epithelial barrier structure and function were obtained using different mouse models. Targeted deletion of β -catenin in club cells (Clara cells) did not significantly affect epithelial development or repair after naphthalene exposure (120). However, β -catenin may be involved in neutrophil transmigration across alveolar epithelial cells, which by itself is sufficient to cause transient barrier dysfunction (121). Deletion of p120 (exons 3–8) was achieved in the intestine by breeding floxed mice with mice expressing Cre recombinase driven by the villin promoter, which resulted in the disruption of AJ and TJ in a variegated manner (122). This partial loss of p120 inhibited the assembly of AJ and TJ into the AJC and resulted in spontaneous intestinal inflammation probably due to translocation of luminal microbes (122). It will be interesting to determine if p120 has similar roles in the airway, but one prediction is that constitutive deletion in the lung may be better tolerated than in the intestine due to lower microbial burden.

Polarity proteins. Emerging evidence points to an important role for polarity proteins in the proper assembly of tight and AJ (123). Most research to date has been conducted in model organisms (e.g., *Caenorhabditis elegans* and *Drosophila melanogaster*), where different families of proteins have been identified including the PAR complex (containing six *par* genes and protein kinase C3), the Crumbs complex (composed of Crumbs, PALS1, and PATJ), and the Scribble complex (containing scribble, Dlg, and Lgl). Polarity proteins are essential for proper morphogenesis and maintenance of apical versus basolateral polarization. The relationship between polarity proteins and AJC structure/function is a rapidly expanding area of research (124), but very little is known about their role in the lung (125, 126). Crumbs3 was recently shown to be important for epithelial development and

viability in mice. Crumbs3 gene-targeted mice died at birth with lungs filled with proteinaceous debris, although alveolar TJ structure appeared intact (127). More research into the expression and function of polarity proteins in the lung is needed and may provide insights into epithelial barrier regulation in disease states.

Inducible Airway Epithelial Barrier Dysfunction: Role of Air Pollution and Viruses

Increased epithelial permeability is a long-recognized hallmark of mucosal inflammation, although the causes and molecular mechanisms involved have been studied only recently. Loss of junctional components such as E-cadherin occurs in epithelial–mesenchymal transition, but the relationship between this complicated process and inflammation-induced AJC disassembly is beyond the scope of this review (for review see Ref. 128 and 129). Three general mechanisms of inducible AJC dysfunction have emerged, which are not mutually exclusive: (1) apical junctions can be degraded by extracellular proteases that are released by inflammatory cells, (2) the expression of AJC components can be silenced at the transcriptional or posttranscriptional level, and (3) junction complexes can be disrupted without affecting gene expression *per se* via internalization or shedding of AJC components. In models of inflammatory bowel disease, junctional endocytosis has emerged as an important mechanism of intestinal epithelial barrier dysfunction. The molecular mechanisms of junctional endocytosis are complex and involve caveolin-1-dependent pathways, (macro)pinocytosis, and clathrin-dependent mechanisms as well as cytoskeletal remodeling (for reviews see Ref. 130 and 131). In intestinal epithelial cells, caveolin-1-dependent endocytosis involving myosin motors underlies TJ disruption in response to inflammatory cytokines and other insults (132–134).

Several environmental exposures have been shown to enhance airway epithelial permeability without inducing cytotoxicity. These include air pollution components, respiratory viruses, allergens, and cigarette smoke. Schamberger and colleagues recently reported that cigarette smoke extract

reduced barrier integrity in human airway epithelial cells by dissociating ZO proteins from the AJC (135). In this section, we highlight examples of air pollution- and virus-induced airway barrier dysfunction but caution that some effects were observed at very high concentrations that likely overestimate real-world exposures (Table 2) (108, 136–143).

Air Pollution

In 1994, Yu and colleagues documented the barrier-disruptive effects of ozone exposure (0.5–0.8 ppm) on canine bronchial epithelial cells (136) (Table 2). A subsequent study showed similar effects of lower ozone concentrations (0.1–0.4 ppm) and showed that primary bronchial epithelial cells obtained from subjects with asthma were more sensitive than those from control subjects (137). High concentrations of diesel exhaust particles and carbon nanotubes were shown to reduce TEER and/or to increase permeability in different lung epithelial cell lines (Table 2). Recently, Thevenot

and colleagues exposed neonatal mouse airway epithelial at ALI to a synthetic mixture of ultrafine particles and observed loss of barrier integrity in association with decreased E-cadherin expression (144). The authors extended their observations to *in vivo* exposures and, using a lineage tracing strategy, concluded that particulate exposure induced epithelial–mesenchymal transition. However, exposure of healthy volunteers to particulate matter (PM)-rich air did not increase “outside/in” permeability as assessed by clearance of 99mTc-labeled DTPA (145).

Repeated exposure to PM may be needed to induce barrier disruption. Sidhaye and colleagues found that two applications of PM were required to enhance normal human bronchial epithelial cell permeability *in vitro* (16). Thus, more research is needed to determine how inhaled pollutants affect epithelial barrier integrity. In future studies, it will be important to look for interactions between pollution components and other environmental exposures and to study

vulnerable subjects who may be at more risk of adverse effects from air pollution.

Respiratory Viruses

Infections with some viruses leads to barrier dysfunction by causing epithelial cell death due to direct cytopathic effect or indirectly via immune cell cytotoxicity (e.g., severe influenza). However, a body of research has uncovered novel roles for respiratory viruses in causing apical junctional disruption without inhibiting cell viability. These studies complement the established notion that junctional components can serve as receptors for viruses and promote cell entry (e.g., Coxsackie B, adenoviruses, and reoviruses) (146–148). Because junctional dysfunction can facilitate virus escape and expose TJ and AJ molecules on the basolateral epithelial surface, virus-induced AJC disruption probably represents an evolutionary strategy that facilitates viral replication (117, 149). The molecular mechanisms by which respiratory viruses decrease junctional integrity have been studied only recently. In Caco-2 cells,

Table 2: Inducible Airway Epithelial Barrier Disruption by Air Pollutants and Respiratory Viruses

Stimulus	Cells	TEER	Permeability (Tracer)	AJC expression	Notes	Reference
Air pollution						
Ozone (0.5–0.8 ppm)	CBE	Decreased	Increased (mannitol)	ND		136
Ozone (0.1–0.4 ppm)	PBEC	Decreased	Increased (BSA)	ND	More in asthmatics	137
MWCNT (100 µg/ml)	Calu-3	Decreased	Increased (mannitol)	ND		138
DEP (125 µg/ml)	16HBElo–	Decreased	ND	Slight decrease membrane occludin		139
Ambient PM ₁₀ (50 µg/cm ²) DEP	A549 Rat AlveEC	Decreased	Increased	Decreased membrane occludin by WB and IF	Possible cytotoxicity; mitochondrial ROS-dependent	140
Ambient PM (150 µg/ml)	NHBEC	ND	Increased (4 kD dextran)	No change in β-catenin	Required 2 applications, unless septin-2 knocked-down	16
Respiratory viruses						
Rhinovirus (RV39, RV1B, MOI = 1)	PTEC 16HBElo–	Decreased	Increased (FITC-inulin)	ZO-1 dissociation from AJC	Independent of IL-1β, TNF-α, and IFN-γ; increased translocation of apical bacteria; blocked by DPI	141, 142
RSV (A2, 2.5–5)	PBEC A549	Decreased	ND	Cytoskeletal rearrangement	Dependent on MAPK	143
RSV (A2, MOI = 0.05–1)	16HBElo–	Decreased	Increased (3 kD dextran)	Expression-independent AJC disassembly	Dependent on sustained PKD activation	108

Definition of abbreviations: AJC, apical junctional complexes; CBE, canine bronchial epithelial cells; DEP, diesel exhaust particles; DPI, diphenylene iodonium; IF, immunofluorescence; MAPK, mitogen-associated protein kinases; MOI, multiplicity of infection; MWCNT, multivalued carbon nanotubes; ND, not determined; NHBEC, normal human bronchial epithelial cells; PKD, protein kinase D; PM, particulate matter; PM₁₀, particulate matter < 10 µm in diameter; PBEC, primary bronchial epithelial cells; PTEC, primary tracheal epithelial cells; ROS, reactive oxygen species; RSV, respiratory syncytial virus; WB, Western blot.

Coxsackie virus causes occludin internalization via macropinocytosis, dependent on the Rab GTPases (149).

Pioneering studies by Hershenson and colleagues showed that infection with rhinovirus (RV) leads to epithelial barrier dysfunction in the absence of cell death (141). The apical/basal translocation of bacteria was significantly enhanced after RV infection, suggesting a mechanism whereby respiratory viral infection predisposes to bacterial superinfection (141). In 16HBElo[−] cells, RV infection induced expression and activity of NADPH oxidases, and RV-induced barrier dysfunction was inhibited by the NADPH oxidase antagonist diphenylene iodonium (142). We recently showed that RSV also infects 16HBElo[−] and primary airway epithelial cells, resulting in sustained decreases in TEER and increased paracellular permeability (108). Barrier disruptive effects of RSV were not associated with cell cytotoxicity but were dependent on sustained activation of PKD. Originally known as PKC μ , this molecule was renamed because it has different structure and substrate specificity than other PKC family members (150). PKD regulates cell shape and motility in part by controlling actin dynamics, and in support of a role for cytoskeletal remodeling we found that RSV infection induces phosphorylation of the actin binding protein cortactin (108). Additional evidence that cytoskeletal remodeling contributes to RSV-induced barrier dysfunction comes from the studies of Singh and colleagues (143). In a separate study, we found that the double-stranded RNA poly(I:C) also induced barrier dysfunction and AJC disassembly in a PKD-dependent manner (107). This was not dependent on production of soluble factors but rather involved a cell-intrinsic signaling mechanism dependent in part on toll-like receptor 3 (107). Taken together, these studies suggest that PKD could be more broadly involved in barrier dysfunction caused by respiratory viruses, which will be an interesting area for future studies. Although potentially an evolutionary strategy co-opted by viruses to promote their infectivity, virus-induced epithelial permeability may facilitate the translocation of inhaled aeroallergens and may help explain the association between respiratory viral infection and allergen sensitization in asthma (see below).

Airway Epithelial Barrier Dysfunction in CF and Asthma

CF

CFTR protein regulates chloride and sodium movement across the epithelial membrane and is mutated in most cases of CF. In lung epithelium, CFTR facilitates transport of chloride to the apical surface of the epithelium, thus controlling transcellular and paracellular trafficking of ions. In cell lines defective in CFTR, TEER is decreased and permeability to mannitol is increased, due to defective localization and function of TJ in a Rho/Rock-dependent manner (151). In polarized epithelial cells, CFTR is tethered to apical actin cytoskeleton through association with PDZ and ezrin protein, suggesting a mechanism whereby CFTR may regulate other apical membrane proteins, although junctional proteins were not specifically investigated in that report (152). Exposure to cAMP analogs improves barrier function in CFTR-deficient cells (151). In one study, induction of CFTR in CFBE41o[−] cells increased the transepithelial resistance and decreased the paracellular permeability of mannitol (153). These authors suggested that CFTR regulated the depth of TJ at least in part in a tyrosine phosphorylation-dependent manner. Taken together, these studies indicate that normal trafficking and function of CFTR may contribute to airway epithelial tight junction assembly and barrier function, although more research is needed in this area. Another area worthy of study is the role of metalloproteases such as meprin, which are elevated in the lung in CF (154) and have the potential to cleave occludin (155).

Dysfunction of airway barrier function in patients with CF could result in paracellular invasion of pathogenic bacteria, leading to chronic infection and inflammation. One common pathogen in CF is *Pseudomonas aeruginosa*, which can colonize airway epithelia and lead to substantial morbidity. *Pseudomonas* species possess virulence factors that disrupt TJ during bacterial invasion without affecting cell viability, which allows cells to reside in the intercellular space of epithelial cells (156). The ability of epithelial cells to internalize *P. aeruginosa* was correlated with cell polarity and assembly of ZO-1

into junctional complexes but was not affected by cell surface CFTR expression (157). However, the precise role of CFTR in regulating epithelial responses to *P. aeruginosa* is controversial (158). Pretreatment of the bronchial epithelium with the antibiotic azithromycin attenuated the disruptive effect of *P. aeruginosa* on airway epithelial cells and facilitated epithelial recovery independent of antimicrobial activity (159). This was likely due to the fact that azithromycin can augment junctional integrity of airway epithelial cells by promoting the integration of claudin-1, claudin-4, occludin, and JAM-A into the AJC (160). These studies highlight the potential for a widely used antibiotic to enhance airway epithelial barrier function and suggest that future studies of barrier restoration in CF may be worthwhile.

Asthma

Until recently, the evidence for epithelial barrier defects in asthma was circumstantial (161), but several new studies have documented defects in barrier structure and function in airway biopsies or epithelial cells propagated *in vitro* from subjects with asthma. De Boer and colleagues studied junctional protein expression by immunohistochemistry in bronchial biopsies (6- μ m frozen sections) obtained from subjects with asthma or control subjects (162). Twenty-two subjects with relatively mild asthma who were not on inhaled corticosteroids were enrolled (average age, 30 yr; mean FEV₁, 98 $\pm$ 16%) and compared with 14 nonatopic nonasthmatic control subjects and 25 atopic subjects without asthma (determined by positive skin prick test and negative methacholine). Airway biopsies were analyzed by immunohistochemistry for intensity of α - and β -catenin, E-cadherin, and ZO-1 expression using a semiquantitative scoring system in a blinded manner. The main finding was a small but statistically significant reduction in expression of α -catenin, E-cadherin, and ZO-1 in superficial epithelial cells in asthmatic versus control biopsies. A slight reduction in α -catenin was also detected in biopsies from nonasthmatic atopic subjects (162). The functional significance of these findings was not determined in this report. Subsequent studies have compared

primary airway epithelial cells from subjects with asthma versus healthy subjects and reported variable results ranging from no change (163, 164) to slight decreases in baseline TEER (165, 166). The numbers of subjects analyzed in these studies was generally small. Fujita and colleagues recently reported that intracellular claudin-1 expression is increased in airway smooth muscle cells in asthma, where it appears to promote proliferation. The molecular mechanisms underlying this unusual effect of claudin-1 require further study (167).

The most comprehensive study to date was reported by Xiao and colleagues in 2011 (168). This important paper analyzed junctional structure and function using different approaches in bronchial biopsies and in bronchial epithelial cells propagated *in vitro* at ALI. Bronchial biopsies from subjects with asthma with varying asthma severity versus healthy control subjects were embedded in methacrylate, and 2- μ m sections were analyzed by immunohistochemistry in a semiquantitative and blinded manner. Epithelial brushings were obtained from healthy control subjects and from patients with moderate to severe asthma. Several important observations were contained in this report (168). First, the expression of ZO-1 was significantly reduced in epithelial biopsies (using immunohistochemistry) and in epithelial whole mounts (using confocal imaging), with a trend toward reduced occludin expression. Second, TEER was significantly reduced in epithelial cells after 21 days of culture *in vitro* and correlated with asthma severity. Cells from subjects with asthma and control subjects responded equally to submersion in tissue culture medium with increased TEER (an expected response indicating

barrier tightening), demonstrating that reduced TEER in asthmatic epithelial cells was potentially reversible. Third, reduction in TEER in airway epithelial cells from subjects with asthma was accompanied by increased permeability to 4- and 20-kD dextrans. Finally, treatment with EGF restored asthmatic epithelial TEER values into the normal range (168). The fact that epithelial cells from subjects with asthma demonstrate defective barrier function after several weeks of culture *in vitro* indicates that this is a stable property of these cells (165, 166, 168). The molecular basis for this apparent stability in epithelial barrier dysfunction is unknown, but this may involve changes in cellular composition, epigenetic alterations, or other mechanisms that are resistant to environmental reprogramming.

Very recently, Hackett and colleagues obtained primary bronchial epithelial cells from cadaveric donor lungs from subjects with and without asthma ($n = 6$ each) and obtained bronchial brushings from five subjects with asthma (average age, 42 yr; average FEV₁, 93%) and six healthy control subjects (169). After propagation at ALI, confluent monolayers were analyzed by immunofluorescence and confocal microscopy, and TEER was analyzed. The key finding was that cells obtained from asthmatic airways demonstrated reduced expression of E-cadherin, β -catenin, and caveolin compared with control airways, although baseline TEER was unchanged (169). A causal role for reduced caveolin-1 was inferred because caveolin-1 knockdown in 16HBE10– cells reduced TEER. The authors speculated that caveolin-1 was required to recruit and stabilize E-cadherin in the AJC, which would be distinct from the barrier-disruptive role for caveolin-1 in junctional endocytosis in the intestine (132–134).

Conclusions

AJC are complex and dynamic structures and are a key part of epithelial defense mechanisms. Defects in junctional integrity are associated with asthma and CF and may predispose to allergen-induced mucosal inflammation and microbial persistence. Although we have a growing knowledge of the mechanisms involved, the precise molecular basis for airway epithelial junctional dysfunction in disease states requires further study. Most studies to date have been conducted with model epithelia *in vitro*, but future studies that investigate junctional structure and function in primary cell types *in vivo* should be especially revealing. Advances in real-time imaging, including multiphoton microscopy, may allow molecular analysis of lung epithelial barrier structures *in situ* in the near future. The development of noninvasive assays of junctional integrity should accelerate clinical research in this area. Persistence of a dysfunctional barrier in airway epithelial cells from subjects with asthma propagated *in vitro* indicates that this is a stable cellular phenotype, but there are hints that junctional integrity could be restored. Further delineation of the molecular pathways that promote epithelial barrier function seems particularly important because this will have immediate therapeutic relevance. Perhaps in the not too distant future we will be able to study epithelial barrier function and use targeted therapeutics to restore junctional integrity in the clinic. This would represent an exciting example of translational research in respiratory cell and molecular biology. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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