CHAPTER 6

STRUCTURE AND REGULATION OF INTESTINAL EPITHELIAL TIGHT JUNCTIONS

Current Concepts and Unanswered Questions

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Abstract: Intestinal epithelium serves as a key interface between internal body compartments and the gut lumen. The epithelial layer forms a physical barrier that protects the body from the harmful environment of the lumen and also mediates vectorial fluxes of fluids, nutrients and waste. Increased permeability of the epithelial barrier is a common manifestation of different gastrointestinal diseases that enhances body exposure to external pathogens thereby exaggerating mucosal inflammation. Barrier properties of the intestinal epithelium are regulated by specialized adhesive plasma membrane structures known as tight junctions (TJs). It is generally believed that disease-related increase in intestinal permeability is caused by defects in TJ structure and functions. This chapter describes the molecular composition of intestinal epithelial TJs, basic mechanisms that regulate TJ functions in healthy gut mucosa as well as molecular events that contribute to increased mucosal permeability during intestinal inflammation. The chapter outlines our current understanding of TJ structure and dynamics and highlights several unresolved questions regarding regulation of this junctional complex under normal conditions and in gastroenterological diseases.

INTRODUCTION

Epithelial lining of the gut plays a number of vital roles including regulation of water, nutrient and waste fluxes and establishment of the protective barrier between the body interior and noxious content of the gut lumen.[1-3] Differentiated intestinal

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epithelium represents a monolayer of columnar-shaped polarized cells with a free apical pole facing the gut lumen, a basal pole attached to the basement membrane and extended lateral surfaces that form adhesive contacts with adjacent cells. Such architecture provides a physical basis for establishment of the paracellular barrier and regulation of the vectorial transcellular transport of solutes and macromolecules.[1-3]

Integrity and barrier properties of the intestinal epithelium are determined by several types of adhesive structures located along the lateral plasma membrane that are called junctions.[2,4] The most apical tight junctions (TJs) play a key role in formation of the paracellular barrier and establishment of the apico-basal cell polarity. TJs have been initially visualized by transmission electron microscopy (EM) as areas of very close intercellular contacts sealing the paracellular space.[5] Subsequent high resolution freeze-fracture EM revealed an elaborated architecture of this sealing zone that appeared as a honeycomb network of interconnecting strands or fibrils physically linking two opposing plasma membranes.[6] This fibrillar network encircles the entire cell and its complexity (number of strands) is thought to correlate positively with the tightness of the paracellular barrier.[7,8]

The TJ barrier has two major functional properties, permeability and permselectivity, that can be determined experimentally.[2,9] Permeability is measured by transepithelial electrical resistance (TEER), whereas permselectivity is a qualitative characteristic that indicates barrier preferences for either cations or anions and within the particular ion series. Depending on their barrier properties, gastrointestinal epithelia have been classified into three categories: Leaky, with TEER below 200 Ω cm^2, moderately leaky, with TEER in the range of 300-1000 Ω cm^2 and tight with TEER higher than 1,000 Ω cm.[2,9,10] Mammals have leaky epithelium in the small intestine, moderately leaky in the colon and tight epithelial barrier in the gastric fundus and esophagus.[9,10] Likewise, human colonic carcinoma-derived epithelial cell lines that are frequently used to study TJ regulation in vitro, create either moderately leaky (Caco-2 cells) or tight (T84, SK-CO15) barriers.[11-14] Despite the differences in the permeability, leaky and tight intestinal epithelial TJs have similar permselectivity. They are cation selective and show preference for K^+ and Na^+ over Cl anions.[9,10] Such cation selectivity is important for epithelia with apical chloride secretion such as in the small intestine where preferential paracellular passage of Na^+ and limited back diffusion of Cl^- is important for excretion of NaCl and water.

Recent studies provided the first semi-quantitative model of the TJ barrier in simple mammalian epithelia.[15,16] A key feature of this model is the existence of two distinct paracellular pathways. The major pathway that carries most of the ionic fluxes has been described as the pore pathway that is permeable for small solutes with a molecular radius below 4 Å. An additional nonpore pathway is thought to represent temporary breaks in TJ contacts that are permeable for larger than 4 Å molecules. These two pathways have been examined in cultured intestinal and renal epithelial cell monolayers as well as in small intestinal epithelium ex vivo.[10,15,16] The pore and nonpore pathways have different regulatory mechanisms and may play different roles in normal epithelial permeability and during barrier breakdown in diseases.[10,15,17,18] It is noteworthy that such a two-pathway model is based exclusively on the results of permeability profiling experiments and ultrastructural studies are needed to visualize molecular architecture of the paracellular barrier within epithelial TJs.

MOLECULAR COMPOSITION OF INTESTINAL EPITHELIAL TJs

It is generally accepted that TJ fibrils are composed by large complexes of integral and peripheral membrane proteins.[10,19-21] The integral membrane proteins directly mediate cell-cell adhesions and create the paracellular barrier, whereas peripheral membrane components that assemble a so called 'cytosolic plaque' play key roles in regulating TJ stability and remodeling.[10,19-21] The adhesive properties of TJs are determined by three major types of integral proteins that include members of the claudin family, tight junction-associated MARVEL proteins (TAMP) family and immunoglobulin-like proteins such as junctional adhesion molecule (JAM)-A and coxsackievirus and adenovirus receptor (CAR).[10,19-21] The cytosolic plaque of TJ contains a large number of molecular constituents including *Zonula occludens* (ZO)-1 proteins, cingulin and afadin.[20-22] Although numerous studies from different laboratories have examined the contribution of individual TJ proteins in the integrity and functional properties of epithelial barriers, the exact role of many of these junctional components remain elusive and controversial.

Claudins

Claudins consist of a large protein family with approximately 24 members in mammalian epithelia.[20,23] They are small, four transmembrane domain (tetraspan) proteins possessing two extremely hydrophobic extracellular loops that mediate various adhesive interactions at the opposing plasma membranes. Expression of claudins in fibroblastic L cells was shown to generate TJ-like plasma membrane fibrils,[24] whereas genetic or pharmacological removal of claudins from the plasma membrane resulted in TJ disassembly in various model epithelia.[25-27] These experiments highlighted claudins as key structural components of TJ strands. Different types of epithelial cells simultaneously express several claudins and therefore it is not surprising that these proteins can be engaged in homotypical and heterotypical adhesive interactions.[20,23] Interestingly, reconstruction experiments using claudin-expressing L cells revealed certain specificity of such heterotypical interactions. For example TJ strands were formed by mixing claudin-1 and 3 or claudin 2 and 3 expressing cells but not cells bearing claudin 1 and 2.[10,20,23] However, it remains poorly understood how different claudins interact within native TJs in the intestinal epithelium.

Several studies employing overexpression of different claudin isoforms firmly established that claudins control permeability and permselectivity of the paracellular pore pathway.[10,20,23] Based on their functional effects, these proteins can be divided into two groups: Tight claudins, expression of which increases TEER and leaky claudins that decrease barrier properties of model epithelial monolayers. The majority of claudins tested so far (claudin-1, 4, 5, 7, 8, 11, 14, 15, 16, 18, and 19) belong to the tight group whereas only claudins 2 and 10 are leaky.[10,20,23] Expression of 19 claudin isoforms in the intestinal mucosa of small rodents has been examined in several studies. They detected mRNA and protein expression for the majority of claudins except claudin 6, 16 and 19.[28,29] It is noteworthy that different claudin isoforms had distinct localization patterns within the gut or even within the same gut segments. For example, claudins 8 and 13 were predominantly expressed in colon, whereas claudins 12 and 15 had the strongest expression in ileum and jejunum respectively.[28] Furthermore, even co-expressed claudins can be spatially separated along the crypto-villous axis. For example, colonic expression of claudins 2 and 15 was limited to the crypt epithelium, whereas claudin 4 was found exclusively

at the surface.[28,29] Such mosaic expression of different claudins in the gut is likely to determine differences in paracellular ionic fluxes in each segments of the intestinal tract.

Recent pharmacological and genetic studies demonstrated the roles of several claudins in regulating TJ morphology and barrier integrity in cultured colonic epithelial cells and gastrointestinal mucosa of experimental animals (Table 1). For example, synthetic peptides that mimic the first extracellular loop of claudin-1 were shown to induce the decrease in TEER and TJ disassembly in T84 cells and to increase permeability of gastric epithelium in vivo.[26] Furthermore, *Clostridium perfringens* enterotoxin that is known to selectively displace claudins-3 and 4 from TJs[27] increased permeability of Caco-2 monolayers[30] and enhanced absorption of macromolecules in rat intestine.[25] Abnormal development of the intestinal tract has been detected in claudin-15 deficient mice that were characterized by a dramatic expansion of small intestine (duodenum and jejunum) resulting in a megaintestine.[31] This abnormal phenotype was not due to altered epithelial cell-cell adhesions and was associated with increased proliferation of intestinal epithelial cells. Similarly, claudin-15 knockout in zebrafish resulted in abnormal formation of multiple gut lumens which was not accompanied by noticeable changes in intestinal TJ structure and permeability.[32] Claudin-1 and claudin-5 deficient mice died just after birth and morphology and functions of their intestinal epithelium have not been investigated.[33]

TAMP Family

The TAMP family of transmembrane TJ proteins is composed of occludin, tricellulin and marvelD3.[36,39] These tetraspan proteins possess structural domains that mediate cell-cell adhesion as well as intracellular trafficking and protein targeting to membrane rafts.[36] In cultured intestinal epithelial cells and tissue sections of intestinal mucosa, occludin and marvelD3 were uniformly localized in all TJs whereas tricellulin selectively accumulated at tricellular junctions.[36,48] Despite the fact that TAMP represent the first identified transmembrane components of TJs, their exact physiological roles remain unclear. For example, a peptide or a monoclonal antibody that inhibit interactions with the second extracellular loop of occludin were shown to attenuate TJ re-assembly and barrier recovery in calcium-switched T84 cells,[34,35] whereas siRNA-mediated knockdown of occludin delayed development of the paracellular barrier in Caco-2 monolayers.[36] On the other hand, overexpression of occludin in L cells did not lead to assembly of TJ-like fibrils,[49] while occludin-knockout mice did not show obvious abnormalities in intestinal epithelial barrier architecture and permeability[38] and did not develop spontaneous gut diseases.[37] Similarly, siRNA-mediated downregulation of other members of the TAMP family failed to prevent establishment of the paracellular barrier in Caco-2 cells.[36,39] Overall, this apparent dispensability of individual TAMP for proper functioning of the intestinal epithelial barrier can be explained by either high redundancy of these homologous proteins or that they play other cellular roles, which are unrelated to regulation of epithelial cell-cell adhesions.

JAM-A and CAR

Immunoglobulin-like proteins JAM-A and CAR represent another type of integral membrane constituents of intestinal epithelial TJs. These proteins have a single transmembrane domain and two extracellular Ig-like domains which can be engaged in either

Table 1. Effects of individual TJ protein inhibition on structure and permeability of the intestinal epithelial barrier

TJ protein	Method of Inhibition	Effect on the Barrier	Effect on TJ Structure	Reference
Claudin-1	Inhibitory peptides	Blocked TEER recovery after calcium switch	Disassembled mature TJs	26
Claudins 3 & 4	*Clostridium perfringens* enterotoxin	Increased steady-state permeability of cultured cell monolayers and rat jejunum		25,30
Claudin 15	(a) Claudin-15 null mice	(a) Decreased ion permeability of jejunum	(a) Decreased complexity of TJ strands	(a) 31
	(b) Zebrafish mutant lacking claudin-15		(b) Normal TJs but multiple lumens in the gut	(b) 32
Occludin	(a) Inhibitory peptide	(a) Attenuated TEER recovery after calcium switch	(a) Attenuated TJ re-assembly	(a) 34
	(b) Inhibitory antibody	(b) Impaired fence function of TJ and apico-basal cell polarity	(b) Attenuated TJ re-assembly	(b) 35
	(c) siRNA mediated gene knockdown	(c) Attenuated TEER recovery after calcium switch		(c) 36
	(d) Occludin null mice	(d) Did not affect intestinal permeability	(d) Did not affect structure of intestinal TJs	(d) 37,38
Tricellulin	siRNA mediated gene knockdown	Attenuates TEER recovery after calcium switch		36
MarvelD3	siRNA mediated gene knockdown	Attenuates TEER recovery after calcium switch	Did not affect TJ architecture and assembly	36,39
ZO-1	(a) siRNA-mediated gene knockdown	(a) Attenuated TEER recovery after calcium switch	(a) Attenuated TJ re-assembly	(a) 13
	(b) *Zonula occludens* toxin	(b) Increased permeability of rabbit ileum		(b) 40
	(c) ZO-1 null mice	(c) Embryonic lethality		(c) 41
ZO-2	ZO-2 null mice	Embryonic lethality		42
ZO-3	ZO-3 null mice		Did not affect structure of intestinal TJs	42
JAM-A	(a) Inhibitory antibody or siRNA knockdown in vitro	(a) Attenuated TEER recovery after calcium switch	(a) Attenuated TJ re-assembly	(a) 43,44
	(b) JAM-A null mice	(b) Increased permeability of healthy gut	(b) Did not alter TJ structure in healthy gut	(b) 45,46
CAR	Extracellular CAR-Fc protein	(a) Attenuated TEER recovery after calcium switch		47

homotypical or heterotypical interactions.[19,21,50] Strong evidence implicated JAM-A and CAR in regulation of TJ structure and functions. For example, treatment with anti-JAM-A antibodies or siRNA-mediated knock-down of JAM-A increased permeability of cultured intestinal epithelial cell monolayers and attenuated TJ re-assembly.[43,44] Furthermore, JAM-A knockout mice were characterized by increased baseline permeability of the gut and by exaggerated intestinal inflammation during experimental colitis.[45,46] Likewise, exposure of T84 cells to soluble extracellular fragments of CAR attenuated development of the paracellular barrier whereas overexpression of CAR resulted in the barrier enhancement.[47] On the other hand, knockdown of CAR in mice or zebrafish caused cardiac and renal abnormalities without disrupting TJs in these organs.[51,52] Effects of CAR deletion on TJ structure and integrity of the gut epithelial barrier in vivo remain to be investigated.

Cytosolic Plaque TJ Proteins

The cytosolic plaque of epithelial TJs contains a number of scaffolding, signaling, polarity, and cytoskeletal proteins. These proteins are responsible for correct TJ assembly and remodeling and they act by clustering transmembrane junctional components and by regulating their trafficking and association with the cytoskeleton.[20,22,53] Members of ZO protein (ZO-1, 2, and 3) are prototypical constituents of the cytosolic TJ plaque. They possess key protein-protein interacting PDZ and SH3 domains and are able to associate with claudins, occludin, JAM-A and CAR.[20,22,53] Experiments with expressional downregulation of different ZO isoforms in cultured epithelial cells demonstrated distinct roles of these scaffolds in the regulation of the epithelial barrier. For example, knock-down of ZO-2 or ZO-3 did not affect TJ formation,[33,42] whereas depletion of ZO-1 delayed TJ re-assembly and establishment of the paracellular barrier in renal and intestinal epithelial cells.[13,54] A simultaneous knock-down of all three ZO isoforms resulted in a complete loss of TJs and a severe impairment of barrier properties in mammary epithelial cell monolayers.[55] These experiments established a key role of ZO scaffolds in formation of TJs and indicated some functional redundancy of their isoforms. Little is known about functions of mammalian ZO proteins in vivo since ZO-1 or ZO-2 null animals exhibited embryonic lethality whereas ZO-3 null mice did not show any adverse phenotype.[41,42] Interestingly, a *Vibrio cholerae* protein toxin that is known to specifically interact with ZO-1 increased permeability and disrupted TJ both in Caco-2 monolayers and in rabbit ileum, thereby supporting roles of ZO-1 in regulating the gut barrier in vitro and in vivo.[40,56] Another abundant scaffold at cytosolic TJ plaque is a myosin II- and Rho-A interacting protein, cingulin.[22] However, its importance for intercellular junctions remains unclear since knockdown of cingulin in renal epithelial cell monolayers did not affect TJ structure and epithelial permeability.[57]

DYNAMICS OF EPITHELIAL TJs IN NORMAL CONDITIONS AND DISEASES

Epithelial TJs are characterized by an intrinsic plasticity, which is manifested as the ability to partially or completely remodel (disassemble and re-assemble) their structure. For example, live imaging of cells expressing fluorescently-labeled claudins showed a rapid break-down and re-assembly of TJ strands,[58] as well as a continuous internalization of claudin-containing vesicles from intact TJs in confluent cell monolayers.[59] Furthermore, a

recent study involving time-resolved microscopy of different TJ proteins has revealed their extensive intramembrane mobility even after incorporation into mature TJs.[60] At physiological conditions, the dynamics of apical junctions is likely to be essential for fine modulation of the paracellular barrier by various physiological stimuli, including nutrients and hormones.[1,61,62] Additionally, a steady-state junctional plasticity is essential for re-organization of cell-cell contacts during tissue morphogenesis and normal rejuvenation of epithelial layers.[63,64] However in disease conditions, the accelerated junctional dynamics results in TJ disassembly and leakiness of epithelial barriers.[62,65] Indeed, increased epithelial permeability is a known consequence of mucosal inflammation that contributes to the pathophysiology of different gastroenteropathies and particularly to inflammatory bowel disease (IBD) that includes Crohn's disease (CD) and ulcerative colitis (UC).[62,66,67] This notion is based on clinical data demonstrating that the decline in barrier function of the intestinal epithelium positively correlates with the degree of mucosal inflammation in CD and UC patients,[68] and that the increased epithelial permeability can precede clinical relapse of CD.[69]

Dysfunction of the epithelial barrier during intestinal inflammation is likely to be mediated by perturbations of normal TJ structure. This conclusion is supported by extensive immunocytochemical studies that documented the loss of the characteristic labeling pattern for different TJ proteins after exposure of model epithelial monolayers to pro-inflammatory agents such as cytokines, free radicals and microbial products.[62,70-72] In cultured epithelia, proinflammatory mediators are known to disrupt barrier integrity by various mechanisms involving expressional down-regulation or post-translational modification of TJ proteins, endocytosis of apical junctions and remodeling of the perijunctional cytoskeleton.[8,62,65,66,73,74]

Defects in the organization of intestinal epithelial junctions have also been observed in animal models of inflammation and tissue biopsies from IBD patients. For example, internalization of occludin and JAM-A from TJs has been shown to occur in the small intestine of mice with experimental T-cell dependent intestinal inflammation,[75] whereas loss of junctional localization of ZO-1 was detected in the colonic epithelium of mice with dextran sulfate sodium-induced colitis.[76] Furthermore, lipopolysaccharide-dependent sepsis in rats was found to induce rapid disorganizations of TJs in colonic epithelium.[77] The animal model data are in good agreement with several clinical studies, which demonstrated substantial loss of occludin, ZO-1, JAM-A, and claudin-1 from TJs in intestinal mucosa of CD and UC patients.[78,79] Such a redistribution of junctional proteins in the intestinal epithelium of IBD patients is consistent with the decreased complexity of TJ strands as identified by freeze-fracture EM.[80] Together, these data strongly suggest that TJ disassembly represents a key mechanism of epithelial barrier dysfunction observed in inflamed intestinal mucosa in vivo.

Studies in knockout animals have provided a strong causal link between disruption of the epithelial barrier and exaggerated gut inflammation. For example, two recent studies revealed increased colonic epithelial permeability in JAM-A knockout mice[45,46] that was accompanied by signs of chronic intestinal mucosal inflammation.[45] Additionally, JAM-A-null animals demonstrated dramatically exaggerated inflammatory response and higher mortality during experimental colitis compared to wild-type controls.[45,46] Finally, a recent report has demonstrated that pharmacological enhancement of the intestinal epithelial barrier function significantly ameliorated mucosal inflammation in spontaneous colitis-prone mice.[81] Together, these studies provided the first direct evidence that specific defects in epithelial junctional structure are sufficient to disrupt the intestinal epithelial barrier and accelerate mucosal inflammation in vivo.

UNANSWERED QUESTIONS ABOUT REGULATION OF INTESTINAL EPITHELIAL TJs

Despite the enormous progress achieved during last two decades in understanding the organization and functioning of epithelial barriers, our knowledge of structure and regulation of epithelial TJs remain very fragmented and incomplete. One can still compose an endless list of questions to address unknown molecular architecture of TJs, exact physiological roles of their protein constituents or the hierarchy and interplay of intracellular signaling cascades that regulate junctional dynamics. Below, I outline a handful of questions or controversial points that may provide food for thought for the future studies of these fascinating cellular structures in intestinal epithelium.

Question 1: What do we know about the diversity and regulation of TJs in the gastrointestinal tract in vivo?

The majority of our knowledge about structure and functions of TJs in the gastrointestinal tract has been generated by in vitro studies that used intestinal epithelial cell lines. The most popular cell lines represent transformed cells originating from colorectal tumors, which display morphological characteristics of either human enterocytes (Caco-2BBE cells) or colonocytes (T84, SK-CO15, HT29-Cl.19A cells). These cultured epithelial cells form morphologically distinct TJs and develop a measurable paracellular barrier, however it remains unclear how well their TJs resemble analogous adhesive structures formed by human intestinal epithelial cells in vivo. Extensive morphological and biochemical studies indicate a close similarity in the ultrastructure and molecular composition of TJs in cultured cell monolayers and in the gut.[82,83] However the regulation of TJs in model cell lines and in the mammalian gut is likely to be different. Evidence suggests that compared to normal intestinal mucosa transformed intestinal epithelial cells form more stable TJs and much tighter paracellular barrier, which can be resistant to modulation by a number of physiological and pathophysiological stimuli. For example, well-studied T84 and SK-CO15 colonic epithelial cell monolayers develop TEER values in the range of 1,500-3,000 $\Omega \times cm^2$,[11-13] which is significantly higher than 350-730 $\Omega \times cm^2$ of TEER reported for rodent colon.[9] Such a tight barrier can be difficult to disrupt. Indeed, T84 and SK-CO15 cells did not respond to TNFα with junctional disassembly even after prolonged (72 h) cytokine treatment.[13,84] By contrast, TNFα administration induced rapid (within 1 h) and massive disruption of TJs in mouse colon.[85] The hyporesponsiveness of transformed epithelial cells is likely to be explained by their frequent chromosomal deletions or genetic mutations that may inactivate important intracellular signaling pathways. A comparative analysis of TJ regulation in transformed and nontransformed primary intestinal epithelial cell lines is required to fully understand physiological implications of the results obtained in commonly used intestinal epithelial cell lines.

There is another reason to ask if our current knowledge about TJs in cultured enterocytes or colonocytes is sufficient enough to understand the complexity of the intestinal epithelial barrier in vivo. Although absorptive epithelial cells are the most abundant cell-type in the intestinal mucosa, this tissue also contains exocrine goblet and Paneth cells.[86,87] Since exocrine and absorptive cells have clearly different morphological features, one can suggest that these cell types may also have differences in structure and regulation of TJs. This suggestion is supported by a study that used freeze-fracture

EM to directly compare enterocyte and goblet cell junctions in the rat ileum.[88] While absorptive epithelial cells showed complex TJs with uniform number and depth of junctional strands, goblet cells TJs appeared to be variable and somewhat abnormal. Such abnormalities included consisting of few strands junctions, strands fragmentation and poor cross-linking.[88] Furthermore, lanthanum and barium tracers easily penetrated goblet cell TJs in contrast to absorptive enterocyte junctions.[88] Secretory activity of goblet and Paneth cells may explain structural and functional peculiarities of their TJs. Indeed, extensive remodeling of the apical surface that accompanies granule secretion in exocrine cells is incompatible with formation of a rigid apical actin cytoskeleton that is essential for stabilization of TJs and enhancements of barrier properties in absorptive enterocytes.[61,65,74] Furthermore, TJs appear to be a 'hot spots' for docking and fusion of exocytic vesicles with the plasma membrane.[89,90] Even apically-targeted proteins can be initially delivered to perijunctional areas of the lateral plasma membrane from which they are transcytosed to the final destination at the apical surface.[91] Whether or not similar events happen in intestinal goblet and Paneth cells remains to be investigated, but it is tempting to speculate that intensive perijunctional vesicle trafficking may destabilize TJ structure and weaken the paracellular barrier in exocrine epithelial cells.

Question 2: What mechanisms regulate integrity and remodeling of the TJ-associated actomyosin cytoskeleton?

Association with the apical actin cytoskeleton plays key roles in the integrity and remodeling of epithelial TJs.[61,65,74] This conclusion is based on EM studies of absorptive intestinal epithelia that showed a close association of TJs with a meshwork of actin filaments lining the interior part of the plasma membrane.[92,93] Furthermore, many studies have shown that specific actin-depolymerizing drugs disrupted integrity of the epithelial barrier and impaired TJ structure and remodeling.[12,94-96] TJ-associated actin filaments are enriched in nonmuscle myosin II (NM II), a molecular motor that converts chemical energy of ATP hydrolysis into mechanical forces thereby mediating tension and contractility of the actin cytoskeleton.[97] This motor protein works as a molecular ensemble of two heavy chains, two essential, and two regulatory myosin light chains (RMLC).[97] The NM II heavy chain has a globular head, which binds to actin filaments and hydrolyzes ATP, and an extended tail that coils together with another heavy chain tail to form a rigid rod-like structure. The tails of multiple NM II molecules readily undergo a side-by side self-association, creating myosin filaments. Such a high-order organization of NM II is critical for two major functions of this protein. One function is the sliding of actin filaments against each other, which mediates the myosin II-dependent contractility, whereas the other is the cross-linking (bundling) of actin filaments thereby producing thick actomyosin fibrils.[97] Intestinal epithelial cells express three different NM II heavy chains, IIA, IIB and IIC that have different tissue distribution and may play unique roles in regulating cell shape, cell-cell and cell-matrix adhesions and cell motility.[11,98]

Several recent studies that used either pharmacological or siRNA approaches to block NM II activity have demonstrated a critical role of this actin motor in regulating epithelial TJs. For example, inhibition of NM II with blebbistatin prevented TJ disassembly caused by IFNγ in T84 cells and by protein kinase C-activating tumor promoters in HPAF II pancreatic epithelial cells.[99,100] On the other hand, blebbistatin treatment dramatically attenuated calcium-dependent reformation of TJs in vitro.[95] Similarly, genetic depletion

of NM II motor was shown to diminish barrier functions of mature TJs and to attenuate their disassembly and re-assembly triggered by various external stimuli.[11,99] Despite strong evidence supporting a key role of NM II in junctional dynamics, mechanisms that regulate activity of this motor at TJs remain poorly understood. There is a common believe that NM II-dependent remodeling of epithelial junctions is driven by the increased phosphorylation of RMLC, which is mediated by either Rho-associated kinase (ROCK) or myosin light chain kinase (MLCK).[53,62,65,101] This concept is based on two lines on evidence. First, RMLC phosphorylation is a classical activation mechanism that enhances the ATPase activity and promotes the self-assembly of myosin II heavy chains.[97,102] Second, many studies have demonstrated that either pharmacological or genetic inhibition of ROCK and MLCK activities disrupted the integrity of epithelial barriers and impaired junctional structure/remodeling.[53,62,65,103] However, recent data suggest that RMLC phosphorylation may not be essential for the activity of NM II motor, which is selectively associated with epithelial TJs. Indeed, effects of phospho-RMLC on NM II functions appeared to be heavy chain isoform specific and limited to NM IIB, while the assembly and activity of NM IIA was found to be independent on the level of RMLC phosphorylation.[104,105] On the other hand, we have recently identified NM IIA isoform is a unique regulator of TJ structure and dynamics in well-differentiated epithelia. For example, NM IIA comprised a majority (65-85%) of all NM II heavy chains in high-resistance T84 and HPAF II epithelial cell monolayers,[98] where expression of NM IIB protein was undetectable.[11,98] Furthermore, NM IIA is abundantly expressed in well-differentiated surface epithelium of normal human colon whereas NM IIB expression appears to be restricted to the less-differentiated crypt epithelium (A.I. Ivanov, unpublished observation). Finally, selective downregulation of NM-IIA was shown to attenuate remodeling (disassembly and re-assembly) of TJs in SK-CO15 cells whereas depletion of NM IIB did not affect such a TJ dynamics.[11] Since NM IIA functioning is poorly sensitive to the level of RMLC phosphorylation, alternative mechanisms should regulate self-assembly and motor activity of the TJ-associated NM IIA in epithelial cells. These mechanisms should specifically target NM IIA heavy chains and may involve either heavy chain phosphorylation or their binding to various accessory proteins such as Mts1, septins and Shroom. An important remaining question is how to explain effects of ROCK and MLCK inhibition of TJ remodeling if such remodeling is independent of RMLC phosphorylation? It is likely that TJ can be regulated by alternative molecular targets of these kinases. For example, ROCK is known to mediate F-actin turnover by controlling cofilin-dependent filament disassembly,[106] and F-actin turnover is essential for TJ dynamics.[12,94] On the other hand, MLCK was recently shown to regulate integrin functions,[107] and thereby may have indirect integrin-mediated effects on apical junctions and the paracellular barrier.[108] Further studies will clarify the signaling pathways that link NM II, ROCK, MLCK activities and remodeling of epithelial TJs.

Question 3: Which endocytic pathways mediate TJ disassembly and how these pathways become activated in disease conditions?

Endocytosis is an emerging mechanism that mediates rapid disassembly of epithelial TJs under physiological conditions and in diseases.[62,64,73,74] This process is not only essential for reversible opening of the paracellular barrier but it also contributes to the loss of epithelial cell phenotype during epithelial to mesenchymal transition in invasive tumors.[73] Generally, plasma membrane components can be internalized *via*

multiple endocytosis pathways, and at least three such pathways have been implicated in TJ internalization. Examples include clathrin-dependent endocytosis of TJ proteins in calcium-depleted[109] or transforming growth factor-treated[110] epithelial cells, as well as lipid raft/caveolae-mediated endocytosis[96] or macropinocytosis[111] of TJs in cells treated with a F-actin-depolymerizing drug and IFNγ respectively. Additionally, caveolar-mediated endocytosis was shown to mediate TNFα-induced disruption of the intestinal epithelial barrier in vivo.[85] Such a multiplicity of endocytic pathways involved in TJ disassembly can be explained by the diversity of external stimuli that trigger junctional internalization as well as by a predominance of the particular endocytosis machinery in different types of epithelia. Despite of the fact that virtually all stimuli that trigger a sustained junctional disassembly result in internalization of TJ proteins, a little is known about mechanisms that activate this process. Two possible scenarios can be envisioned. First, it is known that the perijunctional actin cytoskeleton stabilizes TJs and antagonizes their internalization in stationary, well-differentiated epithelia.[96] On the other hand, many stimuli that induce junctional disassembly also trigger cytoskeletal re-arrangements, and thereby may simply relieve the cytoskeletal inhibition of TJ endocytosis.[61,65] An alternative mechanism involves specific signaling that stimulates interactions between various endocytosis regulators and TJ proteins that triggers junctional internalization. This mechanism has been recently highlighted by the findings of increased associations between occludin and clathrin adaptors epsin-1 and Eps15 during vascular growth factor-induced disassembly of endothelial junctions.[112] Post-translational modification of TJ proteins is likely to be important for the junctional recruitment of endocytic adaptors. For example, occludin and claudin-1 can be ubiquitinated by different ubiquitin ligases and such modification is sufficient to promote TJ disassembly and internalization.[112-114] Virtually nothing is known about signals/mechanisms that stimulate lipid raft/caveolae-mediated endocytosis of TJs. Since this pathway is regulated by intracellular level of caveolin-1,[115] the acute modulation of caveolin-1 expression may dramatically affect stability and internalization of epithelial junctions. Additionally, tyrosine phosphorylation of caveolin-1 by Src kinases that promotes caveolae formation[116] may accelerate TJ endocytosis especially in growth-factor treated epithelial cells. Occludin appears to link epithelial TJs to lipid raft/caveolae domains. Indeed, occludin and caveolin-1 were found to colocalize and physically interact at normal and internalized TJs.[115,117] Furthermore, occludin was shown to regulate caveolin-1 partitioning in lipid rafts,[17] whereas caveolin-1 knockdown inhibited cytokine-stimulated occludin endocytosis.[85]

Question 4: Does programmed cell death play a role in disruption of epithelial TJs during intestinal inflammation?

Intestinal epithelium is a very dynamic tissue that undergoes a continuous self-renovation. This process is characterized by a constant appearance of new enterocytes from stem cells that are located in gut crypts with their subsequent differentiation and migration up the crypt axis.[87] In the small intestine, terminally-differentiated enterocytes reach tips of the villi where they undergo apoptosis and are shed into the lumen. In the large intestine, which is devoid of villi, apoptotic enterocytes are shed from the flat colonic surface. Shedding of epithelial cells is an intensive process with estimated normal loss of approximately 1400 cells from each villus every 24 h.[118] Despite the fact that extrusion of apoptotic cells from epithelial monolayers breaks the continuity

of their TJs, cell shading in normal gut does not compromise integrity of the mucosal barrier.[119] This can be explained by a so called 'purse-string' mechanism of apoptotic cell extrusion that involves formation and rapid contraction of actomyosin rings around apoptotic cells by their neighbors.[120] Contraction of such rings results not only in forceful squeezing out of shedding cells from the monolayer but also leads to a rapid resealing of the focal TJ breaks.

Apoptosis is known to be dramatically exaggerated in model epithelial monolayers treated with various proinflammatory agents[121] as well as in intestinal mucosa of IBD patients.[8] Since excessive apoptosis in vitro was shown to disrupt the epithelial barrier[122] it has been proposed that enhanced cell death and shedding can be responsible for increased TJ disassembly and barrier leakiness in the inflamed intestinal mucosa. However, this mechanism remains controversial and dependent on employed experimental models. For example, apoptosis appeared to be a major mediator of increased epithelial permeability caused by IL-13,[123] whereas this mechanism has been repeatedly dismissed by studies of IFNγ-induced disruptions of the mucosal barrier.[84,124] Conflicted results were obtained for the role of apoptosis in TJ disassembly and barrier breakdown caused by another important proinflammatory cytokine TNFα.[8,125] It is noteworthy, that dismissal of cell death as a barrier-disruptive mechanism is often based on the failure of caspase inhibitors to prevent cytokine-induced disruption of TJs and increase in epithelial permeability.[84,124] However, caspase inhibitors, while blocking apoptosis, can induce alternative modes of cell death such as necrosis or autophagy.[126] Furthermore, autophagy itself is likely to be an abundant alternative cell death mechanism in inflamed intestinal mucosa where epithelial cells are exposed to pro-auhophagic conditions such as nutrient deprivation and oxidative stress. Additional studies are needed to explore a diversity of epithelial cell death pathways and their contribution to disruption of the mucosal barrier during intestinal inflammation.

CONCLUSION

Tight junctions represent key cellular structures that control the architecture and functions of epithelial layers. These structures are especially important in the gut where TJs mediate formation of the protective barrier that dramatically limits body exposure to pathogens and their toxins. Recent studies convincingly demonstrated that defects in TJ structure exaggerate mucosal inflammation, thereby highlighting intestinal epithelium as an active player in the mucosal immune responses. Two major features of epithelial TJs became obvious in recent years: Their enormous complexity and their dynamic nature. Future research in the field will be focusing on these features aiming to reconstruct a precise molecular organization of TJs and to understand mechanisms of junctional plasticity. Such knowledge is critical for the development of new therapeutic strategies that will prevent disruption of the intestinal epithelial in various diseases.

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