Molecular Physiology and Pathophysiology of Tight Junctions
IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells

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Nusrat, A., J. R. Turner, and J. L. Madara. Molecular Physiology and Pathophysiology of Tight Junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am J Physiol Gastrointest Liver Physiol 279: G851–G857, 2000.—The epithelial lining of the gastrointestinal tract forms a regulated, selectively permeable barrier permitting the passive entry of luminal nutrients, ions, and water while restricting pathogen access to underlying tissue compartments. Permeability properties of the epithelium are dynamically regulated by diverse physiological and pathological stimuli (25). Additionally, enhanced paracellular permeability across intestinal epithelium occurs in patients with Crohn’s disease as well as their first-degree relatives, suggesting that altered tight junction (TJ) permeability may be a contributing factor in this process (18). Similarly, altered TJ ultrastructure and increased paracellular permeability have been documented in colonic epithelium from patients with ulcerative colitis, suggesting that the TJ may also be involved in the pathogenesis of ulcerative colitis (28). In the latter case, it is presumed that the TJ defects are secondary to the array of inflammatory signals that characterize this state.

Paracellular permeability is regulated primarily by the most apical epithelial intercellular junction, the TJ or zonula occludens (because this is the rate-limiting barrier in this transport pathway). The TJ forms a regulated, semipermeable barrier and acts as a fence that segregates protein (and partially lipid) components of the apical and basolateral plasma membrane domains. This distinctive barrier and fence function of TJs is vital in regulation of paracellular movement of fluids and solutes, thereby establishing distinct microenvironments on both sides of polarized cells.

The structure of the TJ was the topic of a recent Themes article and can be briefly summarized. By electron microscopy (EM), the TJ appears as a series of discrete contacts or “kisses” between the plasma membranes of adjacent cells. In addition, the TJ appears as a series of anastomosing and branching fibrils within the plane of the membrane that correspond to sites of membrane contact by freeze-fracture EM. Such fibrils appear to have the capacity of dynamic rearrangement within the membrane bilayer and have been shown to harbor proteins. The TJ is composed of a multiprotein complex. It is the combination of the protein complex and membrane lipids that makes this region of the cell membrane unique (22). Moreover, the TJ is linked to the apical perijunctional actomyosin ring. This association regulates the overall permeability and charge-selective properties of the TJ. TJ proteins identified so far include transmembrane proteins occludin and claudin and cytoplasmic plaque proteins ZO-1, ZO-2, ZO-3, cingulin, and 7H6 (19).

The paradigm of protein organization in TJ is analogous to other intercellular junctions, being composed...
of transmembrane proteins that mediate adhesive functions and are linked to underlying plaque proteins that in turn associate with the cytoskeleton. Although the adherens junction (AJ) is spatially distinct and subluminal to the TJ, these junctions form a functional unit referred to as the apical junction complex. Both are structurally related to the perijunctional actomyosin ring. In vascular endothelial cells, the AJ may also play a role in regulation of paracellular permeability and is also involved in diapedesis of circulating leukocytes (10).

The events pertaining to regulation of tight junctions by extracellular stimuli are summarized in Fig. 1 and discussed below.

**PHYSIOLOGICAL REGULATION OF TIGHT JUNCTIONS BY Na\(^+\)-NUTRIENT COTRANSPORT**

Although TJ permeability had been known previously to be regulated by intracellular second messengers, one of the first examples of physiological TJ regulation by an extracellular event was described in 1987 (25). In these studies it was shown that addition of luminal glucose to segments of rodent small intestine mounted in Ussing chambers caused a significant increase in paracellular permeability to small molecules. This was accompanied by simultaneous increases in conductance and decreases in transmucosal resistance. Despite the uncertainties expressed in the literature regarding the in vivo relevance of Na\(^+\)-nutrient cotransport-dependent TJ regulation (34), Na\(^+\)-glucose cotransport-dependent regulation of small intestinal permeability has been demonstrated in vivo in rats and has recently been extended to healthy human subjects as well (32).

EM examination of TJ structure during Na\(^+\)-glucose cotransport demonstrated the formation of intrajunctional TJ dilatations that were penetrated by an oligopeptide tracer applied to the apical (luminal) surface (1). Immunoelectron microscopic evaluation of the TJ protein ZO-1 during glucose-induced regulation of paracellular permeability showed a spatial dissociation between ZO-1 and the morphologically identified TJ, suggesting that the biochemical association between ZO-1 and the junctional fibrils is modified during physiological regulation of TJ permeability.

Fig. 1. Tight junction regulation by extracellular stimuli. This figure depicts epithelial tight junction affiliation with the underlying apical actomyosin ring and documents its regulation by stimuli such as luminal glucose, cytokines, and leukocytes. MLCK, myosin light chain kinase.
Early events in Na\textsuperscript{+}-glucose cotransport-dependent TJ regulation. Further progress in understanding the mechanisms by which TJ permeability is regulated by Na\textsuperscript{+}-glucose cotransport were possible only after the establishment of an in vitro model of physiological Na\textsuperscript{+}-glucose cotransport-dependent TJ regulation (35). In this model, monolayers of Caco-2 cells expressing the Na\textsuperscript{+}-glucose cotransporter SGLT-1 exhibit 30% decreases in transepithelial resistance (TER) after activation of Na\textsuperscript{+}-glucose cotransport (35). The model has been used to characterize early and late events in the signaling pathway linking Na\textsuperscript{+}-glucose cotransport to TJ regulation. For example, it was reported recently that initiation of Na\textsuperscript{+}-glucose cotransport causes a mild cytoplasmic alkalization that is dependent on the activation of the brush border Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform NHE3 (30). Moreover, inhibition of NHE3 causes increases in TER (31). Thus one current working hypothesis is that NHE3 activation may be a critical component of the signaling pathway for Na\textsuperscript{+}-glucose cotransport-dependent TJ regulation. Because NHE3 is a major route of Na\textsuperscript{+} absorption in the small intestine, Na\textsuperscript{+}-glucose cotransport may also trigger a shift from a quiescent to an actively transporting epithelium.

Late events in Na\textsuperscript{+}-glucose cotransport-dependent TJ regulation. In addition to intrajunctional dilatations, EM of intestinal mucosae with active Na\textsuperscript{+}-glucose cotransport identified condensation of the perijunctional cytoskeleton (14). The cultured cell model of Na\textsuperscript{+}-glucose cotransport-dependent TJ regulation has been used to evaluate a biochemical marker of actomyosin contraction—phosphorylation of the myosin II regulatory light chain. Initiation of Na\textsuperscript{+}-glucose cotransport induces an increase in phosphorylation of myosin light chain (35). To determine whether these increases in myosin light chain phosphorylation were mechanistically linked to TJ regulation, the effects of myosin light chain kinase inhibitors were also evaluated (35). In both cultured monolayers and isolated mucosae, these inhibitors prevented Na\textsuperscript{+}-glucose cotransport-dependent increases in TJ permeability (35). Moreover, inhibition of NHE3 exchange also reduced myosin light chain phosphorylation (31), consistent with the hypothesis that Na\textsuperscript{+}-glucose cotransport-dependent activation of NHE3 is linked to increased myosin light chain phosphorylation. Thus one could speculate that initiation of Na\textsuperscript{+}-glucose cotransport leads to activation of NHE3, increased phosphorylation of myosin light chain, contraction of the perijunctional actomyosin ring, and, ultimately, increased permeability of intestinal TJs.

INFLUENCE OF CYTOKINES ON TIGHT JUNCTIONS

A broad array of cytokines, which serve as extracellular signals in a variety of physiological and pathological conditions, influence intercellular associations and permeability across epithelial and endothelial cells. Because a complete review of these cytokines is beyond the scope of this article, we focus on those more extensively studied in this regard.

Interferon-γ. Interferon-γ (IFN-γ) is a 20- to 25-kDa glycoprotein released by activated T cells and natural killer cells in inflammatory states. In vitro models have been used to examine the influence of IFN-γ on intercellular junctions of epithelial and endothelial cells. The initial studies addressing the influence of this cytokine on TJ used the intestinal epithelial cell line T84 (15, 39). These studies demonstrated that IFN-γ induced an increase in paracellular permeability as determined by measurement of TER to passive ion flow and increased paracellular flux of solutes such as mannitol. These functional readouts are supported by morphological and biochemical modification of the TJ protein complex. Decreased expression of the TJ cytoplasmic plaque protein ZO-1 is associated with redistribution of occludin and ZO-2 from the lateral membrane of the TJ under this condition. The IFN-γ-induced disassembly of the TJ protein complex coincides with disruption and disorganization of the apical F-actin pool. Such morphological effects are also accompanied by a change in the differential detergent solubility of ZO-1 and ZO-2. No change in phosphorylation of these proteins was observed. These studies emphasize the link between the actin cytoskeleton and TJ and its critical role in dynamic regulation of paracellular permeability. Nonetheless, the detailed mechanisms by which this cytokine influences epithelial paracellular permeability and the signaling cascade linking IFN-γ and TJ have not yet been defined. One confounding feature of the study of this event is that the response observed occurs days after the original stimulus and requires new protein synthesis; thus the response is not acute phase but paired with a more global remodeling of epithelial architecture.

In concert with another cytokine, tumor necrosis factor (TNF)-α, IFN-γ enhances paracellular permeability of another cell type, microvascular endothelial cells (2). Subsequent investigations have unveiled another TJ-associated protein termed junction adhesion molecule (JAM) (17) that localizes to TJ and also regulates TJ reassembly (13). Studies in human umbilical vein endothelial cells that form leaky intercellular TJ have documented an influence of IFN-γ and TNF-α on distribution of JAM in intercellular junctions (24). The above-described influences of IFN-γ on TJ, although complex, might have pathophysiological relevance because patients with inflammatory bowel disease have upregulated IFN-γ expression in colonic mucosa and increased paracellular permeability (18, 28).

Hepatocyte growth factor. Hepatocyte growth factor (HGF) is chiefly synthesized by mesenchyme-derived cells and influences epithelial permeability in a paracrine fashion via ligation with its receptor, c-met. It was previously documented that HGF induces a decrease in TER of T84 epithelial monolayers (21) that is maximal at 48 h. The HGF-induced fall in TER is analogous to that induced by IFN-γ. Both cytokines decrease TER from ~1,000 to ~300 Ω·cm\textsuperscript{2}, consistent with a moderate increase in paracellular permeability.
Structural studies to analyze the influence of HGF on intercellular junctions have not yielded unifying results to suggest mechanisms by which this factor influences epithelial permeability. Depending on the origin of epithelial cells, variable effects of HGF on protein organization in the TJ versus its subjacent AJ have been proposed. Initial epithelial cell-cell adhesion is regulated by cadherins in the AJ. Such initial contacts are stabilized by association of the AJ protein complex with the underlying actin cytoskeleton. A renal epithelial cell line, Madin-Darby canine kidney (MDCK) cells, has been extensively used to explore mechanisms by which HGF influences epithelial barrier function. In subconfluent cultures of MDCK cells, HGF inhibited the morphological assembly of AJ and overlying TJ (26). In this study, the authors examined the influence of HGF on junction assembly by investigating the localization, stability, phosphorylation, and detergent solubility of the major AJ proteins. HGF-induced inhibition of junction assembly was associated with an increase in the Triton X-100 insoluble pool of E-cadherin and plakoglobin without influencing the concentration of these proteins. These changes were accompanied by altered phosphorylation patterns of E-cadherin as determined by partial proteolytic peptide mapping. In regard to HGF and TJ, the results are variable and somewhat controversial depending on epithelial cell type, and they range from an HGF effect on distribution of ZO-1 in TJ to no change at all (7, 21). The signal transduction cascade(s) that mediates HGF-induced disassembly of intercellular junctions and cell movement has also not been completely described. Ras-induced activation of both mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinase (PI3-kinase) has been suggested.

**TNF-α.** TNF-α is a 17-kDa proinflammatory cytokine produced mainly by mononuclear cells, and it influences barrier function of epithelial and endothelial cells. A biphasic response of TNF-α on TER has been reported in a porcine renal epithelial cell line, LLC-PK1 (16). In this study, an initial fall in TER and increased paracellular permeability was followed by an increase in TER. The latter phase correlated with decreased relative anion selectivity of TJ (16). This study using pharmacological inhibitors suggested a role for tyrosine kinase and protein kinase A in mediation of the effects of TNF-α on this cell type. However, Schmitz et al. (29) showed that, in the intestinal epithelial cell lines HT29 and Caco-2, TNF-α induces a fall in TER without the subsequent rebound. The only morphological correlate in this study was a decrease in the TJ strand complexity by freeze-fracture EM (29). In HUVEC, TNF-α has been documented to induce increased intercellular gaps, an event associated with dispersion of VE-cadherin from AJ and-mediated at least in part by the Rho family of GTPases (37). In combination with IFN-γ, TNF-α induces a focal loss of cadherin-5 in intercellular associations of endothelial cells and has been implicated in facilitation of passage of blood macromolecules and cells to the interstitium (38).

**Other cytokines.** Many other cytokines have been investigated in regards to epithelial/endothelial barrier function. Transforming growth factor (TGF)-β1 has been reported to promote barrier function in human enterocytes (36). Exposure of the epidermal A431 cell line to epidermal growth factor (EGF) promotes TJ assembly. EGF enhances apical redistribution of actin and phosphorylation of TJ cytoplasmic plaque proteins ZO-1 and ZO-2 and intercellular junction assembly of subconfluent monolayers. Other cytokines such as interleukin (IL)-1, IL-4, IL-13, TGF-α, insulin-like growth factor (IGF)-I and -II, and vascular endothelial growth factor (VEGF) have been documented to decrease the barrier properties of epithelial/endothelial cells. As discussed above, mechanisms ranging from redistribution of TJ proteins and altered actin cytoskeleton have been suggested to mediate the cytokine effects on barrier properties of epithelial or endothelial cells.

**MODULATION OF INTERCELLULAR ASSOCIATIONS OF EPithelial AND ENDOTHelial CELLS BY LEUKocytes**

Transmigration of immune cells across vascular endothelium and epithelium is observed during both normal immune surveillance and in pathological conditions associated with an inflammatory response. During a normal immune response, passage of migrating cells is rapid and is not associated with morphological damage to intercellular junctions of endothelial and epithelial cells. Unlike the well-characterized events involved in adhesion of leukocytes to activated endothelium, the mechanisms controlling transmigration of these cells are less well characterized. Moreover, inflammatory states are associated with an influx of leukocytes and release of cytokines that themselves influence TJs and paracellular permeability across endothelial and epithelial cells.

Migration of immune cells from the vasculature into tissues during a normal immune response is rapid. Human polymorphonuclear leukocytes (PMN) can be stimulated to migrate across HUVEC monolayers toward N-formylmethionyl-leucyl-phenylalanine (fMLP). At a low PMN-to-endothelial cell ratio (1:1), no change in barrier function of monolayers was observed, suggesting that intercellular junctions opened to allow passage of migrating cells and then rapidlysealed as determined by measurement of TER and albumin permeability (11). A comparable conclusion was drawn from an earlier study of similarly directed leukocyte migration across epithelia at low density (20). Morphological analysis by EM demonstrated that the plasma membranes of cells in the process of traversing the endothelial monolayers maintained a close association with the lateral membrane of the monolayer. Such physical affiliations might result in maintenance of solute barrier function. However, when the number of PMN stimulated to migrate across endothelial monolayers increased, the paracellular permeability was markedly increased. In addition, Huang et al. (12)

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investigated mechanisms by which transmigrating PMN induced transient opening of intercellular junctions. Rise in intracellular Ca\(^{2+}\) after binding of activated PMN to endothelial cells has been observed in parallel with increase in monolayer permeability, suggesting that Ca\(^{2+}\), acting as a second messenger, can signal to transiently open intercellular junctions (6, 12). The intercellular junction proteins such as VE-cadherin have been implicated in regulation of leukocyte transmigration. Regulation of PMN transendothelial migration by VE-cadherin has been determined with the use of blocking anti-VE-cadherin antibodies that disrupt VE-cadherin cell-cell interactions. In this scenario, enhanced monolayer permeability and PMN transendothelial migration were observed (10). Moreover, intravenous administration of anti-VE-cadherin antibody in mice enhanced migration of PMN to an inflamed peritoneum. It is unclear whether such results were obtained because of antibody-mediated disruption of AJ or because of inhibition of junction resealing after the passage of PMN. Recently, another intercellular junction protein, JAM, that localized to the apical junction complex was reported to influence monocyte transmigration in a mouse model of skin inflammation (17). However, in an in vitro model of PMN transmigration across epithelial monolayers, antibodies to JAM did not influence transmigration of leukocytes (13). Analogous to endothelial cells, migration of low-density PMN across epithelial monolayers in the physiologically relevant (basolateral toward apical) direction is associated with a transient fall in TER that rapidly returns to normal. With increasing PMN-to-epithelial cell ratios, increased damage is observed, resulting in damaged intercellular associations and loss of epithelial cells that manifests as microscopic wounds in the monolayer. However, the initial increase in paracellular permeability is not simply the result of PMN physically impaling intercellular associations of epithelial cells. Addition of PMN to basolateral surfaces of epithelial cells (physiologically relevant) is associated with a fall in TER that is independent of PMN transmigration. The mechanism of this effect is as yet not completely understood and is likely to involve signaling events between PMN and epithelial cells.

Intraepithelial lymphocytes (IELs) constitute one of the largest lymphocyte populations in the body and comprise a distinct T lymphocyte fraction that resides in close proximity with epithelial cells. Increased IELs and a decrease in epithelial barrier function are observed in a number of chronic inflammatory conditions of the gastrointestinal tract. Such disorders include lymphocytic colitis, sprue-like disorders, and chronic idiopathic conditions such as inflammatory bowel disease. In an in vitro model of IEL homing, IELs migrate into intestinal epithelial monolayers from the basolateral side of the epithelium and reside in a subjunctional position. After 4 h of residence, approximately one-half of the IELs were found to exit the monolayer from the basolateral side. In another study, E-cadherin in AJ was found to be a counterreceptor for integrins.αβ7 expressed in IELs and to be important in mediating IEL migration into the epithelial paracellular space (9). Addition of mucosal-derived lymphocytes to T84 monolayers is associated with a decrease in barrier function that requires direct lymphocyte contact with the basolateral membrane of epithelial cells. IFN-γ, IL-4, and IL-10 appear to be the major barrier-disruptive cytokines released by intraepithelial T lymphocytes. These cytokines influence transepithelial migration of PMN. Incubation of T84 monolayers with IFN-γ has been shown to downregulate transepithelial migration of PMN in the basolateral to apical direction (3). The IEL-released IFN-γ, when added to T84 monolayers, was not of a sufficient concentration to explain loss of epithelial barrier function. However, the concentration of cytokines in the microenvironment of lymphocytes and epithelial cells may be significantly higher than cell supernatant values. IL-4, also released by IELs, decreases T84 monolayer TER when applied to the basolateral surface (4). In addition, IL-4 incubation of monolayers results in a decrease in PMN transmigration in addition to enhancing adhesion to the apical surface by a β2-integrin-dependent, intercellular adhesion molecule-1 (ICAM-1)-independent mechanism. Incubation of T84 monolayers with TGF-β1 reduces the barrier-disruptive effects of IFN-γ, IL-4, and IL-10 (27). Thus cytokines released by IELs might play a significant role in regulating epithelial barrier function and in modifying transepithelial migration of inflammatory cells. However, the mechanisms by which IELs modify properties and influence intercellular junctions still remain to be defined.

CYTOSKELETAL REGULATION OF TIGHT JUNCTIONS—A COMMON FINAL END POINT?

A role for myosin light chain phosphorylation in TJ regulation has also been suggested in a variety of epithelial models, including Na\(^+\)-nutrient cotransport-dependent TJ regulation (as discussed above). Physiological Na\(^+\)-glucose cotransport-dependent TJ regulation is rapid, reversible, size selective, and dependent on myosin light chain phosphorylation (35). This association of myosin light chain phosphorylation with TJ regulation has been noted in experimental systems as diverse as hepatocytes exposed to vasopressin and thrombin-stimulated endothelial cells. In addition to soluble mediators, actomyosin contraction occurring as a consequence of the interaction between adherent neutrophils and endothelial cells also appears to be capable of regulating paracellular permeability (5). Thus diverse stimuli such as nutrients, hormones, soluble and cellular inflammatory mediators, and bacterial adherence may all regulate TJ permeability via actomyosin tension/contraction.

Further evidence supporting the role of myosin light chain phosphorylation in TJ regulation comes from studies using epithelial cells transfected with a truncated myosin light chain kinase gene construct that lacks the inhibitory domain necessary for calmodulin dependence (8). The truncated myosin light chain ki-
nase expressed in the transfected cells is continuously active. Hecht et al. (8) found that, when this construct was constitutively expressed in MDCK cells, monolayers developed TER that was <10% of that developed in control monolayers. Expression of a truncated myosin light chain kinase caused approximately threefold increases in myosin light chain phosphorylation and concomitant 20–30% decreases in TER that were reversible on treatment with myosin light chain inhibitors (33).

Further details of the specific molecular interactions that link tension/contraction of the perijunctional actomyosin ring to TJ regulation are not known, but it is tempting to speculate that a multimolecular complex is involved. Likely members of this complex include ZO-1, which is both an actin-binding and cross-linking protein, as well as cingulin, which can interact with both ZO-1 and myosin II heavy chain. Thus it is reasonable to speculate that ZO-1 interactions with cingulin and actin, cingulin interactions with myosin, and actomyosin interactions serve as bridges that allow regulation of TJ by the perijunctional actomyosin ring. Consistent with this hypothesis, we recently observed that TJ proteins are present in specialized membrane microdomains with physical characteristics of detergent-insoluble glycolipid-rich membrane rafts (23) and that regulation of TJ permeability by myosin light chain phosphorylation causes a subtle change in the physical characteristics of these TJ membrane microdomains (33). Thus myosin light chain phosphorylation may cause changes in TJ protein-protein interactions as well as a reorganization of TJ membrane microdomains.

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