ASIP Journal CME Program

The American Journal of Pathology, Vol. 169, No. 6, December 2006
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DOI: 10.2353/ajpath.2006.060681

Amgen Award Lecture

Molecular Basis of Epithelial Barrier Regulation

From Basic Mechanisms to Clinical Application

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The intestinal epithelium is faced with the complex task of providing a barrier while also allowing nutrient and water absorption. The frequency with which these processes are disrupted in disease can be taken as evidence of their importance. It is therefore of interest to define the mechanisms of altered intestinal barrier and transport function and develop means to correct disease-associated defects. Over the past 10 years, some of the molecular events underlying physiological epithelial barrier regulation have been described. Remarkably, recent advances have shown that activation of the same mechanisms is central to barrier dysfunction in both *in vitro* and *in vivo* models of disease. Although the contribution of barrier dysfunction to pathogenesis of chronic disease remains incompletely understood, it is now clear that cytoskeletal regulation of barrier function is both an important pathogenic process and that targeted inhibition of myosin light chain kinase, which affects this cytoskeleton-dependent tight junction dysfunction, is an attractive candidate for therapeutic intervention. *(Am J Pathol 2006, 169:1901–1909; DOI: 10.2353/ajpath.2006.060681)*

The economic and social costs associated with gastrointestinal disease continue to expand. It is estimated that in 2000, the most recent year for which data are available, ulcerative colitis, Crohn's disease, chronic diarrheal disease, and other infectious and inflammatory intestinal diseases in the United States had total costs in excess of $4.7 billion.[1] These diseases are complex and likely involve multiple mechanisms of injury, including immune dysregulation, epithelial apoptosis, and signal transduction events. Many diseases, particularly inflammatory bowel disease, celiac disease, ischemic disease, and graft-versus-host disease, are also associated with loss of intestinal barrier function.[2–15] Although incompletely explored, significant data suggest that tumor necrosis factor (TNF)-induced dysregulation of the intestinal barrier may be a critical pathogenic component of these diseases. The goals of this article are to review current understanding of mechanisms of barrier regulation, consider implications for disease pathogenesis, describe the potential of novel therapeutic interventions, and highlight areas in which further study is needed.

The Epithelial Barrier

One critical function of epithelial-lined surfaces is to define the interface between separate body compartments. Examples include the skin, which maintains a barrier that supports overall homeostasis and prevents systemic infection,[16] and the renal tubule, which forms a barrier that maintains gradients between the renal interstitium and the sterile tubular lumen to allow active and passive transport to regulate urine composition.[17] The intestinal mucosa has a far more difficult charge: it must balance the needs for a barrier against a hostile environment, like the skin, with the necessity of active and passive transport, like the renal tubule. An intact intestinal barrier is, therefore, critical to normal physiological function and prevention of disease.

The intestinal barrier is primarily formed by the epithelium. The individual epithelial cell membranes form the majority of this barrier; they are impermeable to hydrophilic solutes except where specific transporters exist.

Supported by the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases) and the Crohn's & Colitis Foundation of America.

Accepted for publication September 6, 2006.

The ASIP-Amgen Outstanding Investigator Award is given by the American Society for Investigative Pathology to recognize excellence in experimental pathology research. Jerrold R. Turner, a recipient of the 2006 Amgen Outstanding Investigator Award, delivered a lecture entitled "Molecular Basis of Epithelial Barrier Regulation: From Basic Science to Clinical Application" on April 2, 2006 at the annual meeting of the American Society for Investigative Pathology in San Francisco, CA.

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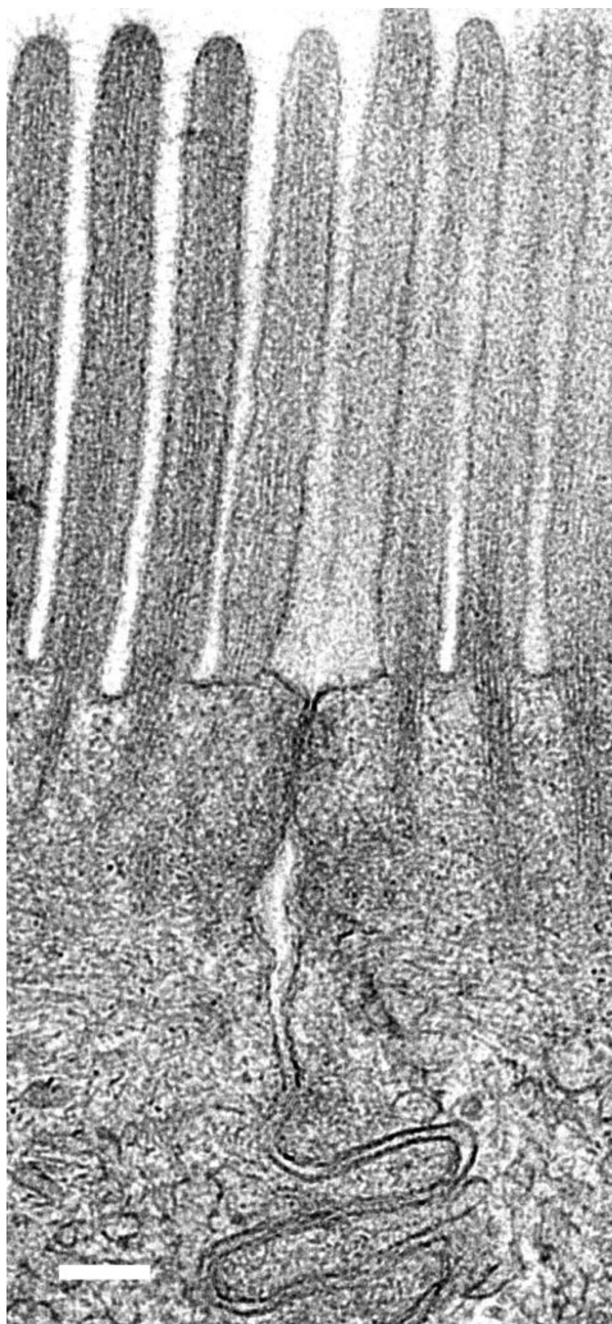

Figure 1. The epithelial tight junction. Electron micrograph of the junction between two adjacent villus absorptive enterocytes. Note the actin core of the microvilli that extends into a filamentous mesh, the cortical actin web, within the apical cytoplasm. Denser filament accumulations are apparent surrounding the apical junction complex. The latter is composed of the tight junction, a zone of close apposition of adjacent plasma membranes just beneath the apical surface, and the adherens junction, which is immediately sub apical to the tight junction. Bar = 200 nm.

However, the space between adjacent cells, the paracellular space, would negate barrier function provided by plasma membranes were it not sealed by intercellular junctions. Of these, the tight junction (Figure 1) is most critical. As the rate-limiting step for paracellular transit, permeability of the tight junction defines the overall barrier function of an intact intestinal epithelium. Of course, the barrier is severely compromised when epithelial cells are lost, as occurs in erosions and ulcerations. Although beyond the scope of this discussion, it should be recognized that intestinal epithelia are programed to rapidly heal such wounds and reseal the barrier within minutes of injury.[18–20] As might be expected, tight junction assembly represents a critical final step in this process.

Tight Junctions Are Intimately Related to the Perijunctional Cytoskeleton

Early morphological studies interpreted the tight junction as a complete paracellular seal, supporting the general consensus that tight junction structure and barrier properties were immalleable. This paradigm was shattered when regulation of *Necturus* gallbladder epithelial tight junction permeability by cyclic adenosine monophosphate (AMP) was reported.[21] This study noted that changes in tight junction permeability were accompanied by reorganization of tight junction ultrastructure, suggesting a link between structural and functional regulation.[21] Despite this remarkable observation, the mechanisms of tight junction regulation remained unexplored. One important clue to these mechanisms came from studies showing the association of thin actin-like filaments within the apical cytoplasm of small intestinal epithelia with tight junctions.[22,23] Moreover, functional studies showed that fungal-derived cytochalasins, which sever actin filaments, were able to disrupt tight junction barrier function and structure.[24] Although one might conclude from these data that tight junctions needed supporting actin filaments to maintain their structural, and therefore functional, integrity, the observation that cytochalasin-induced tight junction disruption was energy-dependent appeared to refute that interpretation.[25] Rather, ultrastructural examination showed that cytochalasin caused the morphological condensation of perijunctional actin,[25] suggesting that cytochalasin-induced severing of actin filaments might cause cytoskeletal contraction that, in turn, led to tight junction disruption.[25]

Until recently, detailed analyses of the mechanisms by which severing or depolymerization of actin filaments leads to tight junction disruption were blocked by the fact that studies of fixed tissues tended to show morphological changes only well after, but not concurrent with, loss of barrier function.[26,27] This impediment was recently overcome by the development of well-validated fluorescent fusion constructs of representative tight junction proteins.[28] These allowed continuous real-time assessment of tight junction structure and function in epithelial monolayers before and during exposure to actin-depolymerizing drugs. Remarkably, this approach demonstrated that one transmembrane protein, occludin (Figure 2), but not another transmembrane protein, claudin-1, nor a cytoplasmic plaque protein, ZO-1, was removed from the tight junction by caveolae-mediated endocytosis at the precise time that barrier function was disrupted.[28] This had not been reported previously after actin depolymerization because the occludin-containing endocytic vesicles were labile under standard fixation techniques. Inhibition of caveolae-mediated endocytosis prevented

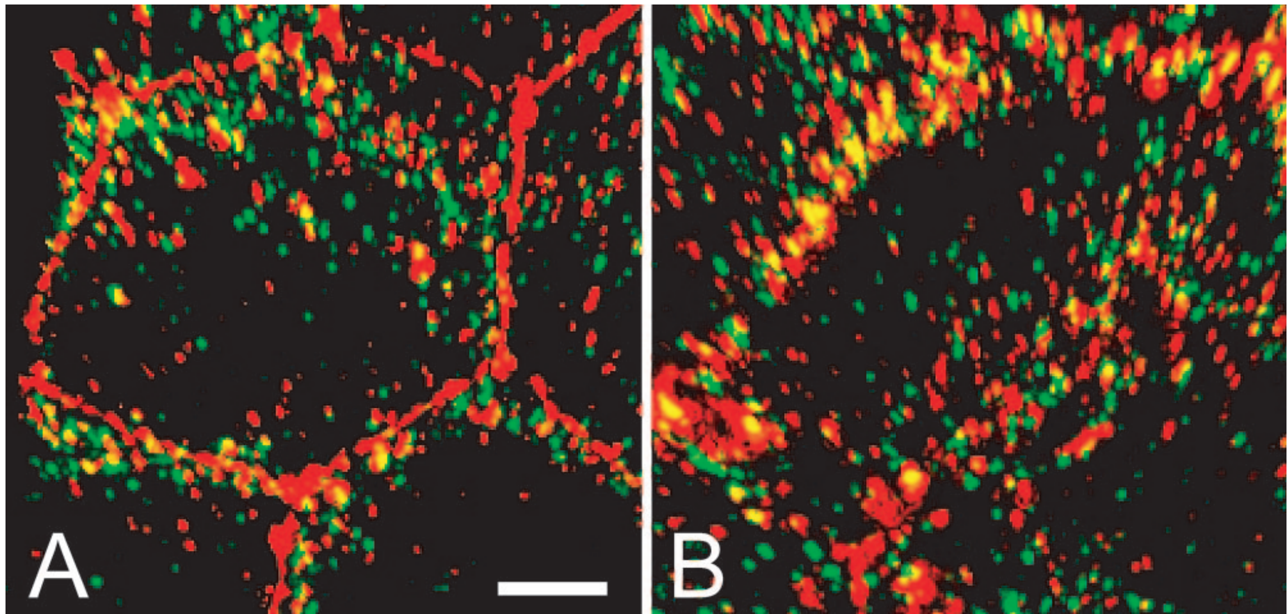

Figure 2. Actin depolymerization induces caveolae-mediated occludin endocytosis. Three-dimensional projections of monolayers labeled for occludin (red) and caveolin-1 (green), a marker of caveolae, are shown. Rather than the ordered appearance of occludin encircling the apical portion of the cell with few intracellular occludin-continuing vesicles in control epithelia (**A**), abundant intracellular occludin-continuing vesicles are evident after actin depolymerization (**B**). Many of these newly formed vesicles also contain caveolin-1, as apparent from the yellow colocalization signal. Bar = 5 μm.

both occludin endocytosis and, more remarkably, barrier dysfunction induced by actin depolymerization.[28] Although these observations do not yet tell us the functional role of occludin or the detailed mechanisms of actin depolymerization-induced tight junction disruption, they do suggest that occludin endocytosis may be an important marker of cytoskeletally mediated tight junction regulation.[29–31]

Barrier Function Can Be Regulated by Physiological Stimuli

Although studies using nonphysiological stimuli such as mucosally applied cyclic AMP analogs or actin-depolymerizing drugs have shown us that the tight junction is not a simple static structure with fixed structural and functional characteristics, these data do not show that tight junctions can be regulated in response to physiological, or even pathophysiological, stimuli. Nonetheless, it is clear that epithelial barrier properties are commonly modified in intestinal disease. Based on the hypothesis that pathophysiological stimuli regulate tight junctions by mechanisms similar to physiological stimuli, a well-defined example of physiological tight junction regulation was sought. Only one had been described: the increased intestinal epithelial tight junction permeability that followed initiation of Na^+-coupled nutrient absorption.[32] The ultrastructural sequelae of this regulation were explored in rodent small intestinal mucosae studied *ex vivo*[33] and included condensation of perijunctional actin similar to that induced by cytochalasin.[25] This suggested a potential role of actomyosin contraction as a mechanism of physiological barrier regulation. Unfortunately, the structural, cellular, and biochemical heterogeneity of small intestine as well as the ischemic damage that limits *ex vivo* studies of this tissue prevented further mechanistic analyses. To circumvent this obstacle, an *in vitro* model of Na^+-glucose cotransport-induced tight junction regulation was developed using a transformed intestinal epithelia cell line.[34] This model, composed of a single homogenous cell type, showed that myosin II regulatory light chain (MLC) phosphorylation accompanied Na^+-glucose cotransport-induced tight junction regulation. Moreover, pharmacological inhibition of myosin light chain kinase (MLCK) prevented both MLC phosphorylation and regulation of tight junction barrier function in cultured intestinal epithelia cells as well as tight junction regulation in isolated rodent mucosa.[34] These data, therefore, showed that MLCK-mediated MLC phosphorylation was necessary for Na^+-glucose cotransport-induced tight junction regulation. However, as subsequent work has identified a complex cascade of kinases that are activated by Na^+-glucose cotransport,[35–38] it was not clear whether MLCK-mediated MLC phosphorylation alone was sufficient to regulate tight junction permeability.

To determine whether tight junction permeability could be regulated by MLCK-mediated MLC phosphorylation in the absence of upstream signaling events, a means to express a constitutively active MLCK under the control of an inducible promoter was developed.[39] This system allowed model epithelial monolayers to form normally; constitutively active MLCK expression was not induced until after tight junctions were completely assembled. Constitutively active MLCK expression led to rapid MLC phosphorylation and increases in tight junction permeability that were quantitatively similar to those following Na^+-glucose cotransport.[39] Tight junction regulation required MLCK enzymatic activity and was accompanied

by morphologically—and biochemically—evident redistribution of occludin and ZO-1.[39] Thus, MLCK-mediated MLC phosphorylation is both necessary and sufficient to affect *in vitro* tight junction regulation by physiologically relevant stimuli. As will be discussed below, actin and myosin have also been suggested to participate in more extensive tight junction disruption following a variety of pharmacological[28,39,40] and pathophysiological[9,31,41,42] stimuli.

Barrier Function Is Compromised in Intestinal Disease

Barrier function can be compromised in intestinal disease. This was initially recognized *in vivo* in patients with small intestinal Crohn's disease[11,43] but has subsequently been reported in patients and experimental models of a spectrum of inflammatory, immune-mediated, and infectious intestinal diseases.[2] In the case of Crohn's disease, some data suggest that barrier defects may even be related to disease pathogenesis. For example, a subset of healthy relatives of Crohn's disease patients have increased small intestinal permeability.[44] This may have an environmental as well as genetic component, since some spouses of Crohn's disease patients also have increased small intestinal permeability.[45] One anecdotal case study reported that the healthy daughter of a Crohn's disease patient who was found to have increased intestinal permeability at age 13 developed ileocolonic Crohn's disease at age 21.[46] Although circumstantial, these clinical data support the hypothesis that increased intestinal paracellular permeability is an early step in the initial pathogenesis of Crohn's disease.

Further data suggest that barrier dysfunction may contribute to activation of Crohn's disease. For example, peripheral immune activation correlates with increased small intestinal permeability in Crohn's disease patients.[47] In addition, several studies have established that, among patients with clinically inactive Crohn's disease, those with increased small intestinal permeability have an elevated risk of clinical relapse.[48,49] Finally, TNF-neutralizing antibodies restore barrier function in Crohn's disease patients.[5] Although TNF-neutralizing antibodies likely exert their powerful therapeutic effect through multiple mechanisms, their effect on barrier function may result from reduced TNF signaling to intestinal epithelia.

TNF Regulates Barrier Function in Vitro

Further evidence for a potential role of increased paracellular permeability, or epithelial barrier dysfunction, in intestinal bowel disease comes from the observation that exposure of cultured epithelial monolayers to TNF *in vitro* can cause barrier loss.[50,51] There have been many hypotheses as to the mechanism of TNF-induced barrier dysfunction, including single cell epithelial apoptosis,[52] nonapoptotic mechanisms,[53] and reduced transcription of tight junction proteins.[54] Although a role for each of these processes cannot be excluded, a series of recent reports suggests that TNF regulates tight junction permeability via cytoskeletal contraction. Exposure of cultured epithelial monolayers to TNF caused marked increases in MLC phosphorylation that could be prevented by specific MLCK inhibition.[9] More importantly, such MLC inhibition also restored barrier function in TNF-treated monolayers.[9] These data show that correction of a single biochemical alteration is sufficient to reverse the effects of TNF on intestinal epithelial barrier function *in vitro*, suggesting that this is the primary mechanism by which TNF regulates intestinal permeability. Moreover, these data suggest that TNF regulates epithelial tight junctions by processes similar to those that regulate tight junctions in response to physiological stimuli, such as Na^+-glucose cotransport.

TNF Up-Regulates MLCK Expression in Vitro *and* in Vivo

To define the mechanisms by which TNF augments MLC phosphorylation, MLCK expression was examined in TNF-treated cultured epithelial monolayers.[42] TNF enhanced MLCK protein expression, and prevention of TNF-induced MLCK up-regulation blocked TNF-induced barrier dysfunction.[42] Thus, one mechanism by which TNF causes MLC phosphorylation and tight junction regulation *in vitro* is via increased MLCK expression.[42,55] Studies using cultured epithelia have also shown that the TNF signal is transduced through intestinal epithelial TNF receptor 2.[56]

Although epithelial MLCK is transcribed from the same gene as smooth muscle MLCK, transcription begins at upstream start sites, resulting in a larger MLCK, termed long MLCK,[57] with an amino-terminal extension.[58,59] Alternative splicing of long MLCK transcripts leads to translation of two different MLCK isoforms in intestinal epithelia,[58] of which one can be regulated by Src kinase as well as Ca^{+2}/calmodulin.[60] Thus, whereas the mechanisms by which TNF activates MLCK activity in intestinal epithelia are not yet described, both Src kinase and Ca^{+2}/calmodulin are potential signaling intermediates.

Although the relationship between TNF-induced MLCK expression and disease are currently undefined, the observations that TNF increases long MLCK mRNA transcription in cultured human intestinal epithelial cells *in vitro* and in murine intestinal epithelial cells *in vivo*[56] suggest that this transcriptional upregulation may be critical to disease-related barrier loss. Consistent with this, intestinal epithelial MLCK protein expression is increased *in vivo* in chronic immune-mediated intestinal disease models[56] and in human inflammatory bowel disease patients.[61] Strikingly, in inflammatory bowel disease patients, the extent of increased MLCK expression and MLC phosphorylation correlates directly with the magnitude of active inflammation, suggesting a relationship between MLCK expression and disease activity.[61]

To understand better the means by which TNF increases MLCK expression, the transcriptional start site of long MLCK in cultured human intestinal epithelia was identified.[59] Two novel exons that encode alternative transcriptional start sites were found, and the long MLCK promoter upstream of one of these sites was cloned. It

identified functional response elements for both activator protein-1 (AP-1) and nuclear factor-κB. However, only the AP-1 sites appeared to be used in well-differentiated intestinal epithelial monolayers.[59] Thus, MLCK transcriptional regulation is likely to be both tissue and differentiation specific.

MLCK Activation Is Necessary for Acute Immune-Mediated Diarrhea in Vivo

The data presented above show that MLCK-mediated MLC phosphorylation is an important mediator of *in vitro* tight junction regulation in response to physiological and pathophysiological stimuli. Similar MLCK-dependent tight junction regulation has also been reported in *in vitro* models of enteropathogenic *Escherichia coli* and *Giardia lamblia* infection.[62,63] Thus, it has been proposed that MLCK may be a shared component that is used by diverse stimuli and signaling pathways to regulate tight junction permeability.[12] These hypotheses were based on *in vitro* studies; the role of MLCK in epithelial function *in vivo* had not been explored. Although they have provided critical mechanistic insight, *in vitro* studies of cultured monolayers do not recreate the complex interactions between epithelia and other cell types present *in vivo* nor do they provide insight into the development of disease symptoms. To address this, an *in vivo* model of acute immune-mediated intestinal disease was studied. Systemic T cell activation induced by administration of anti-CD3 antibodies causes acute self-limited diarrhea in humans and mice.[64] This diarrhea is TNF-dependent because it can be blocked by TNF-neutralizing antibodies.[65] Thus, although this represents an acute disease model, the mechanisms are relevant to graft-versus-host disease and inflammatory bowel disease. It was of particular interest to determine whether epithelial barrier dysfunction was involved in disease pathogenesis and if preventing barrier dysfunction could lessen disease severity.[41]

To characterize changes in intestinal transport induced by systemic T-cell activation, an *in vivo* perfusion system that allowed simultaneous analysis of epithelial barrier function and water absorption in a loop of jejunum with an intact neurovascular supply was developed.[41] These studies showed that *in vivo* T-cell activation results in acute diarrhea, as defined by net water secretion and barrier dysfunction. Neither ulceration nor epithelial apoptosis occurred, thereby excluding these as potential mechanisms of barrier loss. In contrast, TNF-neutralizing antibodies restored water absorption and reduced the extent of barrier dysfunction, indicating that this cytokine is a critical mediator of immune-mediated diarrhea. Systemic T-cell activation also caused morphological changes in tight junction structure, including occludin internalization (Figure 3) and perijunctional actin condensation.[41] Both immunofluorescent and biochemical analyses of jejunal epithelia showed that T-cell activation caused TNF-dependent MLC phosphorylation within the perijunctional cytoskeleton. Moreover, the extent of MLC phosphorylation paralleled the development and resolution of the acute self-limited diarrhea induced by anti-CD3 antibody injection.[41] These data therefore suggested that MLC phosphorylation could be related to the *in vivo* barrier dysfunction observed after systemic T-cell activation.

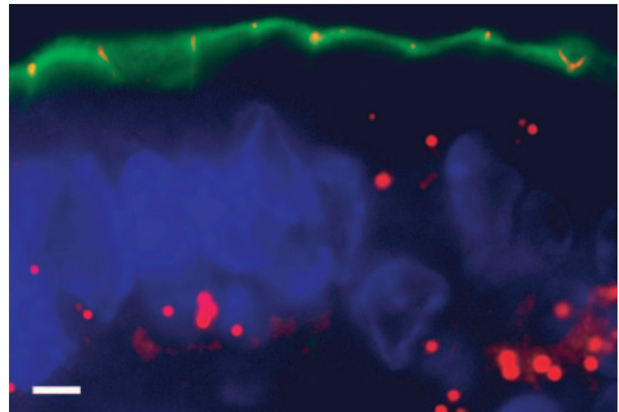

Figure 3. *In vivo* T-cell activation causes occludin endocytosis. Three hours following systemic T-cell activation, mouse jejunum was snap frozen and fluorescently labeled for occludin (red), F-actin (green), and nuclei (blue). In addition to some residual tight junction-localized occludin, which is associated with the perijunctional actomyosin ring, bright vesicular occludin deposits are present within the cytoplasm of these villus enterocytes. Intracellular occludin deposits were not seen within the cytoplasm of villus enterocytes from control animals. Bar = 5 μm.

A combination of genetic and pharmacological approaches to inhibit MLCK were used to directly assess the role of MLCK-dependent MLC phosphorylation in the pathogenesis of acute immune-mediated diarrhea. Although MLCK knockout mice die within 5 hours of birth,[66] the observation that intestinal epithelia only express long MLCK allowed use of mice specifically lacking long MLCK.[67] These mice grow and develop normally, despite the absence of long MLCK in many tissues. However, when stressed by anti-CD3 injection, long MLCK knockout mice failed to develop intestinal barrier dysfunction or diarrhea; water absorption continued.[41] In addition, epithelial MLC phosphorylation was not increased by anti-CD3 injection in long MLCK knockout mice. This nonresponsiveness was not due to deficient immune activation, as mucosal cytokine transcripts increased normally in long MLCK knockout mice. Thus, long MLCK expression is necessary for T cell-induced barrier dysfunction and diarrhea to occur.[41]

MLCK Inhibitors May Represent a Class of Novel Therapeutic Agents

Commonly used MLCK inhibitors, such as ML-7, have a narrow window of utility and limited specificity because they target the adenosine 5′-triphosphate-binding pocket. To develop a highly specific MLCK inhibitor, several groups have designed peptides based on the autoinhibitory regulatory domain within MLCK.[68,69] These peptides inhibit MLCK without appreciable effects on related non-MLCK protein kinases. One of these peptides was able to access the cytoplasm of cultured intestinal epithelia due to the presence of an HIV-1 TAT-like protein

transduction domain.[9,70] Once within the cytoplasm, the peptide inhibits MLCK. The peptide was therefore named PIK, membrane permeant inhibitor of MLC kinase. PIK effectively reduced MLC phosphorylation and restored barrier function in TNF-treated cultured intestinal epithelial monolayers.[9] Unfortunately, preliminary studies showed that PIK was not stable in the harsh environment of the intestinal lumen.[71] It was thus necessary to develop a stabilized form of PIK suitable for *in vivo* use. This stable PIK variant retained the ability to inhibit cytoplasmic MLCK as well as the high degree of specificity for MLCK that made the parent compound so useful *in vitro*.[71]

When stable PIK was perfused into the intestinal lumen, a method of delivery topologically equivalent to oral administration, the peptide readily entered epithelial cells but did not cross the epithelium in detectable quantities.[41] Consistent with this, even high-dose stable PIK administration was not associated with systemic toxicity. However, stable PIK completely prevented T-cell activation-induced increases in MLC phosphorylation.[41] Stable PIK also restored tight junction structure and barrier function. Finally, stable PIK reversed the direction of net water movement, from secretion to absorption, in anti-CD3-treated animals.[41] These observations lead to two critical conclusions. First, because PIK only accessed the epithelial cells, these data demonstrate that the protection from diarrhea seen in the long MLCK knockout mice is due to the absence of epithelial long MLCK. Second, and perhaps more importantly, the data suggest that oral administration of stable PIK or a similar molecule might be an effective nonimmunosuppressive means of preventing immune-mediated diarrhea. This would be of great clinical significance, as available immunosuppressive and immunomodulatory agents are associated with increased risk of systemic infection and other significant complications.[72,73]

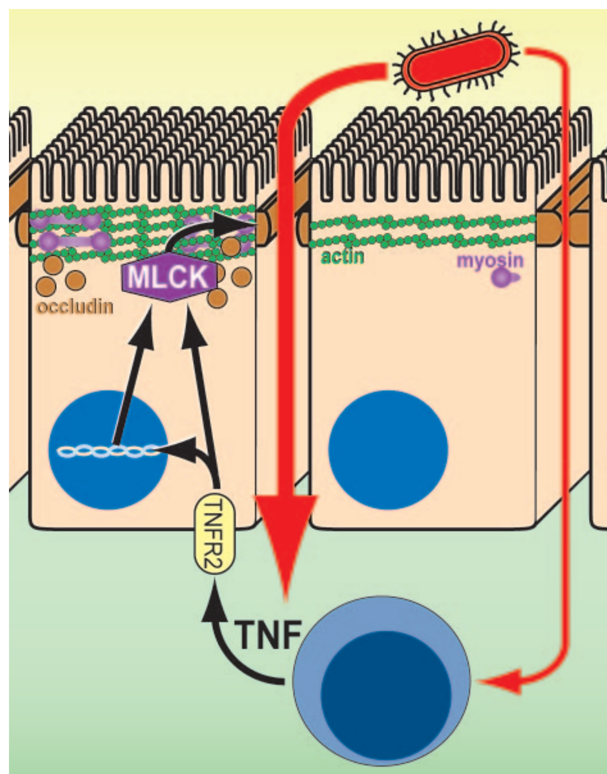

Figure 4. A mechanism-based model of intestinal disease. This model proposes a self-amplifying pathway where a small amount of luminal, eg, bacterial-derived, molecules cross the epithelium to activate lamina propria immune cells. This results in secretion of proinflammatory cytokines, eg, TNF, that signal to intestinal epithelial. This TNF receptor 2 (TNFR2)-mediated signal increases both MLCK transcription and enzymatic activity, resulting in occludin endocytosis and epithelial barrier dysfunction. The barrier loss results in greater access leakage of luminal material, greater immune activation, and even greater barrier defects. In the absence of appropriate regulatory cues, this triggers a self-amplifying cascade. Although described here as being initiated by luminal molecules activating immune cells, it should be apparent that the primary event could as easily be inappropriate immune activation or epithelial barrier dysfunction.

A Model of Disease Pathogenesis

These data suggest a testable model that can serve as a basis for future studies of inflammatory bowel disease pathogenesis.[2] This model incorporates the critical clinical observations that intestinal barrier function is disrupted in Crohn's disease patients and a subset of their unaffected relatives,[11] that barrier dysfunction is a marker of impending disease reactivation,[49] that the degree of barrier dysfunction correlates with immune activation,[47] and that TNF neutralization corrects barrier dysfunction.[5] The model also includes the experimental observation that the absence of normal gut flora prevents the development of disease in multiple animal models of inflammatory bowel disease.[74] Finally, the model considers data showing that acute TNF-mediated barrier dysfunction requires MLCK activation *in vitro* and *in vivo*[9,41,42] and that intestinal epithelial MLCK expression and activity correlate with disease activity in inflammatory bowel disease patients.[61] This model proposes a self-amplifying pathway where leakage of luminal bacterial-derived molecules across the epithelium causes activation of lamina propria immune cells (Figure 4). This immune activation then leads to secretion of proinflammatory cytokines such as TNF. In turn, TNF signals to intestinal epithelial TNF receptor 2 to activate MLCK and cause barrier dysfunction. This allows further leakage of luminal material, resulting in greater immune activation and triggering a self-amplifying cascade. One attractive feature of this model is that it anticipates that patients with similar disease phenotypes may have differing underlying defects. For example, self-amplification of the cycle might be limited in a normal host by regulatory elements such as IL-10. In contrast, a susceptible individual might have functional IL-10 deficiency, exaggerated TNF or TNF receptor 2-mediated signaling, or hyperresponsive MLCK-induced barrier dysfunction. Thus, it could be possible for a variety of defects to cause disease with similar features.

A Future for Rational Mechanism-Based Therapy?

Although such a model is conceptually attractive and could successfully explain the data from studies by ge-

neticists, microbiologist, immunologists, and epithelial biologists, it is, at present, only hypothetical. No data exist to show that regulated barrier dysfunction, as opposed to massive epithelial disruption,[75] can cause or even exacerbate clinical or experimental inflammatory bowel disease. In fact, available data suggest that barrier dysfunction alone is not sufficient to cause intestinal disease; many healthy subjects with increased paracellular permeability do not develop Crohn's disease. Thus, barrier dysfunction can only be one piece of the complex puzzle that is inflammatory bowel disease pathogenesis. Therefore, although it is tempting to speculate that therapeutic MLCK inhibition might be able to serve as maintenance therapy for quiescent Crohn's disease, that conclusion must await experimental confirmation.

Conclusion

In the relatively short time since tight junction permeability was discovered to be regulated by physiological and pharmacological stimuli, defects in such regulation have been detected in human disease. Moreover, some of the mechanisms of this defective regulation have been identified. This has led to enhanced understanding of disease pathogenesis as well as the development of novel opportunities for mechanism-based therapeutic approaches. However, much work remains if we are to fully comprehend and better treat intestinal disease.

Acknowledgments

First, I thank my wife, Judith A.B. Turner, for her continuous support throughout the successes and failures that are a necessary part of any scientific endeavor. I am also indebted to James L. Madara, John P. Atkinson, Alan M. Tartakoff, Steven N. Emancipator, Ramzi S. Cotran, and Vinay Kumar for the important roles each of them has played in my career. Finally, I am grateful to the members of my laboratory and collaborators whose participation was critical to the studies described here including, alphabetically, Eric D. Black, Stephanie A. Blair, Daniel R. Clayburgh, W. Vallen Graham, Gail Hecht, Wayne I. Lencer, Randall J. Mrsny, Brad Schwarz, Le Shen, Fengjun Wang, Yingmin Wang, and Yevgeny Zolotarevsky. Figures 2 and 3 were prepared by Le Shen and Daniel R. Clayburgh, respectively.

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