

# Restoration of Barrier Function in Injured Intestinal Mucosa

Anthony T. Blikslager, Adam J. Moeser, Jody L. Gookin, Samuel L. Jones and Jack Odle

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# Restoration of Barrier Function in Injured Intestinal Mucosa

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**Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J.** Restoration of Barrier Function in Injured Intestinal Mucosa. *Physiol Rev* 87: 545–564, 2007; 10.1152/physrev.00012.2006.—Mucosal repair is a complex event that immediately follows acute injury induced by ischemia and noxious luminal contents such as bile. In the small intestine, villous contraction is the initial phase of repair and is initiated by myofibroblasts that reside immediately beneath the epithelial basement membrane. Subsequent events include crawling of healthy epithelium adjacent to the wound, referred to as restitution. This is a highly regulated event involving signaling via basement membrane integrins by molecules such as focal adhesion kinase and growth factors. Interestingly, however, *ex vivo* studies of mammalian small intestine have revealed the importance of closure of the interepithelial tight junctions and the paracellular space. The critical role of tight junction closure is underscored by the prominent contribution of the paracellular space to measures of barrier function such as transepithelial electrical resistance. Additional roles are played by subepithelial cell populations, including neutrophils, related to their role in innate immunity. The net result of reparative mechanisms is remarkably rapid closure of mucosal wounds in mammalian tissues to prevent the onset of sepsis.

## I. INTRODUCTION

Epithelial repair has been intensively studied in cultured epithelium (64, 124, 137, 146) but to a lesser extent in mammalian mucosa (21, 67, 125). The former approach allows for intensive molecular-based techniques to be used to study mammalian epithelium, but it has the major shortcoming of not taking into account the natural three-dimensional structure of the mammalian mucosa, particularly in the small intestine where there are villi. Studies

on epithelial cells have largely focused on epithelial crawling (restitution) (146). Some studies have overcome the limitation of the relative simplicity of epithelial monolayers by studying cocultured epithelium and myofibroblasts (147, 148) or by assessing the role of neutrophil transmigration across epithelial monolayers (128). Nonetheless, these cell populations do not fully recapitulate the complex three-dimensional structure that is present in mammalian mucosa (105), and it is the purpose of this review to focus on repair of mammalian mucosa. There

are a number of injurious agents that may be applied to mammalian mucosa *in vivo* or *in vitro*, after which mechanisms of mucosal repair may be studied using standard techniques such as blood-to-lumen clearance of labeled probes or *ex vivo* electrophysiological studies (8, 67, 78). Injury in this review will focus on two major mechanisms of epithelial cell loss: ischemia and bile-induced injury. These mechanisms of injury can be carefully titrated to allow a selected level of injury, and they are relatively uncomplicated methods of injuring epithelium, allowing an emphasis on reparative events. In terms of mucosal reparative mechanisms, perhaps the most surprising finding is that restitution, which was once synonymous with repair itself, appears to be less important than restoration of tight junctions and the subjacent paracellular space, which appears to be the crucial event in recovery of barrier function in mammalian mucosa (66).

## II. IMPORTANCE OF INTESTINAL BARRIER FUNCTION

The intestinal barrier is composed of a single layer of columnar epithelium and interepithelial tight junctions, which reside at the apical-most region of the paracellular space. Tight junctions polarize the cell into apical and basolateral regions (fence function) and regulate passive diffusion of solutes and macromolecules (gate function) (108). Thus the intestinal barrier serves as the first line of defense against a hostile environment within the intestinal lumen (146). There are two components to this innate mucosal defense: mechanisms that reduce the ability of pathogens and their toxins to invade the mucosa and mechanisms that ensure rapid repair of defects in the epithelial monolayer. Regulation of passive diffusion across the intestinal barrier is centered on the ability of tight junctions to allow passage of select solutes that are beneficial to the host, while preventing the passage of antigens, bacterial toxins, and pathogens (146). Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly reform a continuous epithelial monolayer to prevent absorption of bacterial toxins (22). In particular, studies have intensely focused on epithelial restitution, during which epithelium reseals mucosal defects (64, 124). However, it appears that epithelial migration and contact is insufficient alone to restore a functional barrier. Instead, the tight junction must reform and seal before any substantial degree of barrier function can be documented in mammalian mucosa (19, 66, 97).

In addition to contributions to the physical attributes of the mucosal barrier, the epithelium is also a sentinel. In this capacity, the epithelium serves as a sensor, signaling to resident innate immune cells in the mucosa when infection or injury occurs to recruit and ultimately regulate the function of innate and adaptive immune system

elements essential to combat infection and heal wounds. Epithelial cells and innate immune cells in the lamina propria express a number of receptors that recognize conserved moieties on microbial molecules, conferring upon these cells the ability to recognize "microbial non-self" (119). These include complement receptors, scavenger receptors, mannose binding receptors, and members of the toll-like receptor (TLR) family (Table 1). Of these, the biology of the TLR is perhaps the best studied in the intestinal mucosa. Activated TLRs stimulate epithelial cells to elicit alarm signals in the form of a number of proinflammatory mediators that activate and recruit innate immune cells and adhesion molecules that regulate trafficking of inflammatory cells into and even across the epithelium into the intestinal lumen (Table 2). As innate immune cells enter the tissue, they in turn sense the environment for appropriate signals that further activate them to kill microbes. Neutrophils and macrophages have a key role in orchestrating the kinetics and magnitude of the inflammatory response and regulating antigen presenting cells that shape the nature of the adaptive immune response.

While it may be hard to envision how epithelial cells expressing receptors that are activated by microbial molecules are held in check when exposed to the noxious milieu of the intestinal contents, there are a number of mechanisms by which TLR responses are regulated to prevent inappropriate activation of the inflammation. TLRs are spatially distributed to allow some specificity to their responses. For example, some TLRs are principally expressed on the basolateral aspect of epithelial cells (58). Thus they contact ligands only when the tight junction barrier is disrupted by either invading pathogens or epithelial damage. Second, TLR signaling in gut epithelial cells is modulated by as yet poorly defined mechanisms to minimize inappropriate activation of inflammatory gene expression (89). Indeed, TLR signaling induced by commensal organisms has a protective role and is required for

TABLE 1. *Ligands for known mammalian Toll-like receptors*

TLR	Ligand
TLR1	Lipopeptides, modulin
TLR2	Lipoteichoic acid, lipoproteins, peptidoglycan, yeast mannans, lipoarabinomannan
TLR3	dsRNA
TLR4	Lipopolysaccharide
TLR5	Flagellin
TLR6	Modulin
TLR7	ssViral RNA
TLR8	ssViral RNA
TLR9	Microbial CpG-DNA
TLR10	Undefined
TLR11	Toxoplasma gondii profilin-like protein
TLR12	Undefined

TLR, Toll-like receptor.

TABLE 2. *Some genes regulated during inflammation*

Cytokines and Chemokines	Adhesion Molecules	Inflammatory Enzymes
TNF- $\alpha$	ICAM-1	iNOS
IL- $\beta$	VCAM-1	COX-2
IL-6	E- and P-selectin	mPGES-1
IL-8		5-Lipoxygenase
IL-12		12-Lipoxygenase
IL-2		Annexin-1
Rantes		PLA <sub>2</sub>
MIP-2		
Eotaxin		

TNF, tumor necrosis factor; IL, interleukin; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PLA<sub>2</sub>, phospholipase A<sub>2</sub>.

intestinal homeostasis (151). Third, some sensory systems in gut epithelium only respond to intracellular microbial molecules, signaling invasion (36). Finally, the binary signal or “two hit” theory suggests that inflammation is only triggered and propagated when two signals are generated, one indicating infection (signaled by microbial molecules, cytokines, proinflammatory lipids) and one indicating tissue injury (signaled by heat shock proteins, neuropeptides, mitochondrial peptides) (131). Multiple cells in the mucosa, including epithelia, those of the innate immune system such as mast cells, macrophages, and neutrophils, endothelial cells, and even sensory neurons, constantly survey the environment, integrating signals of the two classes until the simultaneous receipt of the two signals occurs. Inflammation is initiated and further propagated and amplified by signals generated by recruited inflammatory cells. Signals generated by the innate immune cells simultaneously trigger and shape the adaptive immune system, resulting in antigen specific responses. As each cell migrates into the site, the environment is sampled to determine if their services are still required. If so, maximal activation occurs. If not, a transition to dampen inflammation and heal wounds begins.

Once triggered, immune responses must be tightly regulated and ultimately resolved to heal wounds and prevent chronic inflammation, abscess formation, or fibrosis. Resolution of the immune response is a highly regulated process, and once again, the epithelium has a central role. Epithelial cells are a source of a number of anti-inflammatory mediators that generally inhibit neutrophil responses. These neutrophil responses may be damaging to tissues and propagate inflammation while stimulating functions of other innate immune cells like macrophages necessary for resolution of inflammation and tissue repair. Epithelial cells are an important source of anti-inflammatory mediators derived from fatty acids. For example, anti-inflammatory lipids of the lipoxin family inhibit neutrophil migration and production of microbicidal molecules, stimulate neutrophil apoptosis, stimulate

macrophage scavenging of neutrophil corpses, and promote macrophage-dependent healing of tissues (163). Lipoxins retard neutrophil recruitment into inflamed mucosa by inhibiting epithelial production of the potent neutrophil chemoattractant interleukin (IL)-8 (57). In addition, epithelial cells produce protein anti-inflammatory mediators such as secretory leukocyte protease inhibitor (SLPI) that dampen neutrophil oxidant production and inhibit neutrophil proteases (169). Epithelial cells and macrophages also produce the epithelial cell growth promoting cytokine proepithelin (PEPI), which synergizes with SLPI to dampen neutrophil responses (200). PEPI signals to epithelial cells to induce proliferation, promoting repair. Thus, like initiation and amplification of inflammation, resolution of inflammation in the intestinal mucosa is highly orchestrated with epithelial cells seated firmly in the conductor’s chair.

### III. MEASUREMENT OF INTESTINAL BARRIER FUNCTION

To study mechanisms of intestinal repair and regulation of tight junctions, it is necessary to be able to sensitively and dynamically measure intestinal barrier function. The most sensitive measure of mucosal barrier function is transepithelial electrical resistance (TER), since this measurement reflects the degree to which ions traverse tissue (105). There are two routes ions may passively traverse epithelium: transcellular and paracellular (Fig. 1) (100). In the small intestine, apical epithelial membranes have a resistance to passive flow of ions 1.5–3 log units greater than that of epithelial monolayer as a whole. Thus measurements of TER largely reflect the paracellular resistance, which has been calculated to account for 75–94% of the total passive ion flow across small intestinal epithelium (50, 104, 105, 141, 156). The paracellular pathway can also be assessed using a variety of probes that selectively traverse epithelium via the paracellular space. An example of such a probe is mannitol, which has been used in permeability studies either as mucosal-to-serosal fluxes in tissues placed in Ussing chambers (22, 97, 102) or blood-to-lumen clearance in whole animal studies (78, 90). Paracellular resistance is imparted by the tight junction and lateral intercellular space. Under conditions of tight apposition of the lateral membranes, approximations of intercellular space resistance may exceed the specific tight junction resistance depending on the length of the intercellular space. The latter measurement is difficult to accurately estimate due to extensive folding of the lateral membranes lining the intercellular space (Fig. 2). However, once the intercellular space exceeds the physiological range in width of the tight junction, the contribution of intercellular space to total paracellular resistance approaches zero (31). Thus,



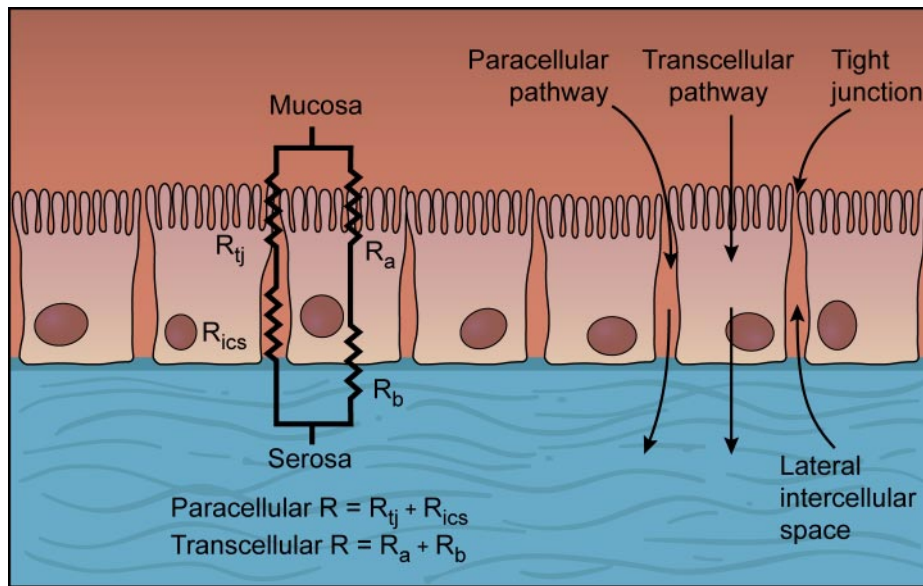


FIG. 1. Diagram of the intestinal epithelium depicting components that contribute to the electrical measurement of transepithelial electrical resistance. Note the critical components of this monolayer that make up the components of transepithelial electrical resistance, including the epithelium itself and the paracellular space.  $R_{tj}$ , tight junction resistance;  $R_{ics}$ , intercellular space resistance;  $R_a$ , apical membrane resistance;  $R_b$ , basolateral membrane resistance.

in leaky epithelium, the tight junction is the most important determination of paracellular pathway resistance. However, the lateral intercellular space is more acutely dynamic in altering TER (54). Important limitations of TER measurements are their inability to distinguish between changes in paracellular permeability and the presence of epithelial cell loss, both of which contribute to the overall surface area of low-resistance paracellular pathway (66). One group was able to distinguish between TER-dependent and TER-independent changes in barrier function by using a series of different-sized fluorescently labeled dextran probes (134). The smaller probes were

shown to traverse the paracellular space and correlated well with TER. However, larger probes could be used to detect cell loss, a process that is largely independent of measurements of TER.

#### IV. EFFECT OF THE MACROSCOPIC ORGANIZATION OF THE MUCOSA ON BARRIER FUNCTION

Measurements of TER and permeability *in vitro* are calculated on the basis of the serosal surface area (i.e., in reference to the luminal aperture of the Ussing chamber) rather than the real surface area of mucosa. The latter can vary dramatically depending on the section of intestine and species under study. In the small intestine, mucosal surface area is greatly amplified by villus projections and undulations (66, 105). In addition, the shape and height of villi vary according to the segment of the small intestine being studied, with the ileum having the shortest villi within the small intestine. The colon has no villi. Accordingly, the surface area of the paracellular pathway available per square centimeter of serosa differs markedly along the intestinal tract. When TER data are corrected for differences in surface area (i.e., expressed on the basis of mucosal surface area), the permeability of small intestinal and colonic epithelium is determined to be virtually identical (32, 105). In repairing tissues, villus contraction results in a reduction in epithelial surface area which tends to elevate measurements of TER (67, 124, 125). Other anatomical structures may also affect the surface area of the mucosa. For example, Peyer's patches in the submucosa of the ileum may project up to the surface epithelium and distort the surface of the mucosa (105). Since these lymphoid aggregates are dispersed through-

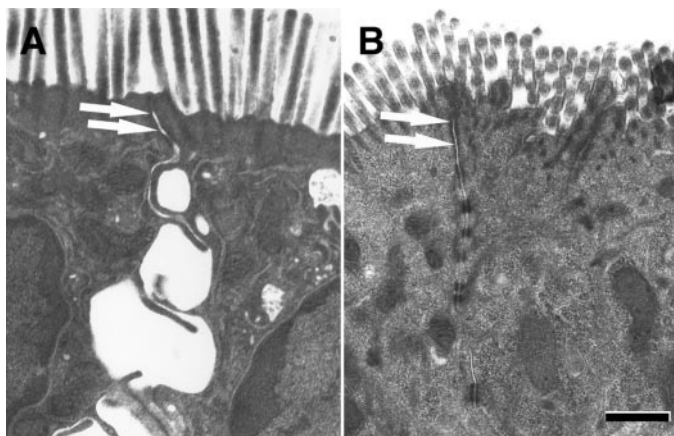


FIG. 2. Transmission electron micrograph of porcine mucosa subjected to ischemic injury and studied *ex vivo* during repair. Note the complexity of the lateral interepithelial membranes, which have extensive folding. *A*: once restitution is complete, epithelium remains excessively permeable because of open tight junctions (arrows) and the paracellular space. *B*: as repair continues, the tight junction is closed (arrows) and the paracellular space is closed, restoring mucosal barrier function. Bar = 4  $\mu\text{m}$ . [From Little et al. (97).]

out the antimesenteric submucosa of the ileum, measurements of resistance may differ according to the segment (mesenteric or antimesenteric) of the ileum being studied. Peyer's patches become particularly enlarged during development of immune competence and tend to greatly affect the structure of the bowel during this developmental period (105).

In addition to differences along the length of the intestine, TER varies dramatically along the crypt-villus axis of small intestinal mucosa. Tight junctions in the crypts are leakier than those in the villus because of fewer and less organized junctional strands (105, 178). There is also a higher linear density of paracellular pathway in the crypt due to the manner in which cells align into the bottom of the crypts. As a result, 63–73% of the total paracellular permeability of small intestinal epithelium can be attributed to the crypts (31, 32, 109).

## V. ANATOMICAL COMPONENTS OF THE INTESTINAL BARRIER

### A. Epithelial Cells

Epithelial cells present a robust barrier to invasion by bacteria and their toxins, negating passive flow of luminal contents by their remarkably high TER compared with the paracellular space. Therefore, the principal pathway by which bacteria, toxins, and antigens can cross the epithelium itself is via facilitated transport mechanisms or via mechanisms that injure the apical membrane. For example, the bacterial *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) can be transported across the epithelium (120). Similar mechanisms may be present for lipopolysaccharide (15, 43). Furthermore, select bacteria preferentially alter the structure of the apical epithelial membrane. For instance, bacteria such as *Salmonella typhimurium* that possess a type III secretory apparatus are able to penetrate the apical membrane and insert bacterial proteins that ultimately result in disruption of barrier function (162). Another example of a bacterial organism with the capability to alter the apical membrane is enteropathogenic *Escherichia coli* (EPEC) that binds to the apical epithelium, forming a so-called cup-and-pedestal lesion, which effaces apical membrane microvilli, and leads to alteration of tight junctional structures (167, 170, 197).

### B. Tight Junctions

Of the junctions that define the paracellular space, the apical-most tight junction is principally responsible for regulating paracellular permeability, although adherens junctions and desmosomes also play critical cellular

functions. The structure of tight junctions was initially defined using electron microscopic (EM) techniques, including transmission EM, which revealed a series of pinpoint contacts between the apical-lateral membranes of adjacent cells, and freeze-fracture EM, which showed that these pinpoint contacts formed a continuous belt of branching fibrils surrounding each cell (4, 121). Recent work has revealed that tight junction fibrils are made up of a complex of integral membrane proteins, which bind to a group of cytoplasmic plaque proteins (51). The latter are responsible for tethering the cytoplasmic component of the tight junction to the cytoskeleton.

Integral membrane proteins include occludin, claudins, and junctional adhesion molecule (JAM). These proteins have similarities in structure despite their wide range in molecular weight, including two extracellular loops that interact in homotypic fashion with integral membrane proteins on neighboring cells. These proteins also have cytoplasmic tails that interact with cytoplasmic plaque proteins via specific binding domains (5, 158). Occludin, a ~65-kDa protein, was the first integral membrane protein to be localized to tight junction fibrils (53), although it was difficult to explain widely differing measurements of TER in a variety of epithelia on the basis of a single fibril protein (28). This dilemma has been partially resolved by the discovery of a group of some 20 integral membrane proteins called claudins (~22 kDa) (52, 126), which are expressed in a tissue-specific manner, and therefore influence regional differences in epithelial permeability. For example, claudin-16 is primarily expressed in the thick ascending loop of Henle, where it is specifically involved in  $Mg^{2+}$  resorption (166). Claudin-specific alteration of junction permeability has also been shown experimentally in Madin-Darby canine kidney (MDCK) cells in which transfection with human claudin-4 resulted in alteration of junction structure and resistance (185). Site-specific claudin expression has been recognized in the gut. For example, claudin-3 and claudin-5 are expressed in both villi and crypts of rat intestine, whereas claudin-2 and claudin-4 are expressed solely in the crypts and villi, respectively (149). An additional integral membrane protein termed JAM (~35 kDa) has recently been characterized (13, 110). The manner in which these integral membrane proteins coalesce to form tight junction fibrils is not presently known, although fibrils are ~10 nm in diameter, which is similar to the diameter of gap junction connexons, which consist of hexamers of integral membrane proteins similar in size to those of tight junctions (121).

The cytoplasmic plaque is composed of a series of proteins (ZO-1, ZO-2, ZO-3) belonging to the membrane-associated guanylate kinase (MAGUK) superfamily and of an array of other molecules, including cingulin, symplekin, and lymphocytic leukemia fusion-6 (AF-6). The ZO proteins are characterized by postsynaptic protein-95/

discs large/zonula occludens-1 (PDZ) domains, a *src* homology (SH-3) domain, and a GUK domain that likely give these proteins the ability to organize integral membrane proteins at the tight junction (121). For example, ZOs bind claudins via their first PDZ domain, and occludin via the GUK domain. Similarly, ZO-1 binds JAM via a putative PDZ domain (14). Other cytoplasmic plaque proteins also directly bind ZOs, including AF-6 and cingulin. The ZO proteins bind in turn to actin (45), whereas cingulin binds myosin II (33), thereby anchoring the tight junction to the cytoskeleton.

The tight junction membrane has distinct differences compared with the composition of the cell membrane at the adjacent cell membrane. In particular, tight junction-associated membrane contains high concentrations of cholesterol and sphingolipids, resulting in the formation of a membrane microdomain that is referred to as a detergent-insoluble glycolipid raft. These rafts may serve to recruit signaling molecules such as the GTP-binding proteins Rab3B, Rab13, and *src* which are modified to interact with cholesterol-enriched lipid membranes (139).

Although permeability of the paracellular pathway correlates to a large extent with the protein composition of tight junction fibrils (106), there are also intricate mechanisms whereby tight junction permeability may be modulated without affecting the fibril structure. For example, tight junctions are selectively opened to facilitate  $\text{Na}^+$  and glucose absorption in response to a signaling cascade initiated by binding of glucose and  $\text{Na}^+$  to SGLT-1 in the apical membrane and completed by cytoskeletal contraction in response to phosphorylation of myosin light-chain kinase (MLCK) (181). Further studies into this mechanism have revealed that the sodium-hydrogen exchanger NHE-3 is critical to alteration of tight junction permeability in response to glucose (180). Interestingly, studies have shown that NHE-3 is regulated in part by binding to regulatory proteins including NHE regulatory factor. This protein binds, in turn, to ezrin, which anchors it to the junctional cytoskeleton (198). This may explain the link between alteration of transport and changes in tight junction permeability. Similarly, the  $\text{Cl}^-$  secretory protein CIC-2 has been localized to the tight junction (75), and we have recently shown that this protein alters tight junction permeability during active transport in ischemic-injured porcine ileum (122).

### C. Lateral Intercellular Space

The lateral intercellular space is composed of the lateral membranes of adjacent epithelial cells. The apical aspect of the space is defined by a series of junctions that have already been mentioned, whereas the basolateral aspect of the lateral intercellular space is open to the lamina propria. Although the lateral intercellular space

may seem to be a simple structure based on alignment of two adjacent relatively straight membranes, this is not the case. Instead, the lateral intercellular space is composed of a complex interdigitation of two remarkably tortuous membranes (Fig. 2). This makes this space difficult to study morphologically because it cannot be measured on a simple linear basis (66), although dilation of the lateral intercellular space can be readily discerned using transmission electron microscopy (Fig. 2). Interestingly, recent studies have identified a novel type of desmosome (type II desmosomes) that is attached to the cytoskeleton just like other junctional structures, but which is distributed along the length of the lateral intercellular membrane (42). This suggests that changes in cytoskeletal tone or rearrangement may alter the apposition of the entire lateral intercellular space (42), thereby providing a mechanism for the intercellular space to mechanistically contribute to TER without simply relying on dilatation or collapse.

## VI. MECHANISMS OF MUCOSAL INJURY

Although infectious agents induce mucosal injury, select mechanisms of injury simplify the study of reparative events. These mechanisms of injury, which in large part result in epithelial loss in the absence of many of the complex signaling events of infectious agent-epithelial interactions, include complete ischemia and application of bile. However, regardless of the mechanism of mucosal injury, acute inflammation plays a role in subacute injury (given the time lapse it takes for neutrophils to infiltrate the site of injury). This is exemplified by reperfusion injury, which has been demonstrated as early as 1 h after ischemic injury in low-flow ischemia in cats (68–70). Aside from this selective mechanism that involves up-regulation of chemoattractant molecules as a result of ischemia, inflammation is an important feature of any mucosal wound regardless of how rapidly it is sealed. For instance, some models of ischemia have a distinct lack of early reperfusion injury but are nonetheless infiltrated by neutrophils within 6 h, as would be expected for any epithelial wound exposed to noxious bacteria, toxins, and antigens.

### A. Ischemia

Ischemia/reperfusion injury is an important mechanism of mucosal injury in people suffering from a number of acute and chronic intestinal ischemic disorders (3). Patients with acute mesenteric ischemia (AMI) account for 1 of 1,000 hospital admissions (173) and 1% of emergency laparotomies (3). AMI may be occlusive, attributable to thrombus formation or occlusion of major mesenteric arteries with emboli, or nonocclusive, associated with shock or vasoconstriction and resultant low flow.



Mucosal injury is typically severe following occlusive AMI, and although nonocclusive AMI results in reduced mucosal injury, this form of AMI may be markedly exacerbated by reperfusion injury (144). Such mucosal injury has resulted in high mortality rates for patients with AMI, ranging between 59 and 93% (3, 96, 173). Despite recent advances in diagnostic procedures and critical care, these mortality rates are similar to those documented more than 70 years ago, most likely because of continued problems with early diagnosis (3). Mucosal ischemia is also thought to play a role in the pathogenesis of necrotizing enterocolitis (NEC) in neonates (94), a disease that affects up to 5% of infants admitted to neonatal intensive care units. Although the pathogenesis of this disease is incompletely understood, mesenteric ischemia, enteral feeding, and enteral infection are all believed to play a role in development of NEC (25). Recent studies indicate that mortality, which may reach rates as high as 40%, has not changed appreciably over the last 30 years (172). It is also becoming increasingly evident that many critically ill patients suffer from multiple organ failure that is initiated by poor splanchnic perfusion, and resultant breaches in intestinal epithelial continuity (37). Multiple-organ failure is the leading cause of death in intensive care unit patients (99).

Ischemia/reperfusion injury has been modeled in a number of animal species, most notably cats, rodents, and pigs, with a number of distinct forms of ischemia. For example, in some seminal feline investigations, low-flow ischemia (mesenteric blood flow reduced to 20% of baseline) was used to prime tissues for reperfusion injury while inflicting relatively little mucosal injury itself (92, 144, 161). Mucosal reperfusion injury was attributed largely to activation of neutrophils located within the mucosa. In other studies assessing microvascular injury

in similar feline models, reperfusion injury was clearly linked to elevations in xanthine oxidase and subsequent increases in neutrophil infiltration. Pharmacological studies suggested that xanthine oxidase-elaborated superoxide was largely responsible for initiating reperfusion injury (70). More recently, mechanisms of mucosal injury have been more definitively assessed using mutant mice. For example, in mice genetically engineered to overexpress superoxide dismutase, reperfusion injury was blunted, providing support for the role of superoxide suggested by prior studies (38). The nature of the ischemia appears to have an important role in the extent of injury that develops during ischemia compared with reperfusion. Thus complete ischemia results in rapid mucosal degeneration during ischemia, with relatively little injury during reperfusion compared with the marked reperfusion injury that occurs following low-flow ischemia (142).

Regardless of the nature of the ischemic event, mucosal injury appears similar: villus contraction and epithelial sloughing (Fig. 3). Sloughing of epithelium commences at the tip of the villus and continues toward the base of the villus and ultimately the crypt depending on the degree of ischemia (complete or low-flow) and the duration of ischemia. The reason for this pattern of sloughing is likely the distance of the epithelium from the arterial blood supply. In addition, species-specific anatomical variations in blood supply may play an important role in epithelial ischemic injury. For example, in many species, including humans and important animal models such as rodents, cats, and pigs, villi in the small intestine have a central arteriole that arborizes near the tip of the villus, draining into peripheral venules. This vascular architecture sets up a countercurrent exchange mechanism in which oxygen diffuses from the arteriole into the tissue

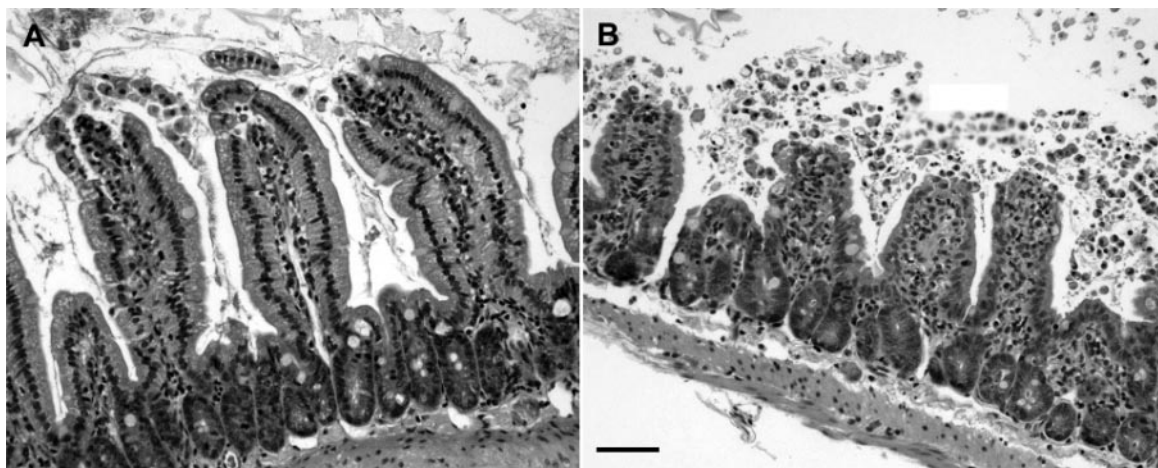


FIG. 3. Appearance of mucosal injury in mouse jejunum subjected to 45 min of complete ischemia. *A*: initial injury is marked by lifting of epithelium at the tip of the villus, followed by sloughing of epithelium along the lateral aspects of the villi toward the crypts. *B*: appearance of ischemic mucosa in *ex vivo* conditions during the early phases of recovery. Note evidence of villus contraction and flattening of epithelium adjacent to the mucosal wound in preparation for restitution. Bar = 100  $\mu\text{m}$ .



and adjacent peripheral venules prior to reaching the tip of the villus, resulting in a relatively hypoxic villus tip. When arterial blood flow is reduced, the villus tip becomes progressively hypoxic, resulting in epithelial injury. Therefore, ischemic mucosal injury results in time-dependent sloughing of epithelium from the tip of the villus toward the crypts (164). For instance, occluding the small intestinal mesenteric vasculature for 60 min results in loss of epithelium from the upper third of the villus, whereas ischemia for 120 min results in near-complete loss of epithelium (20). Although there are no villi in the large intestine, surface epithelium is lost initially during ischemia, followed by crypt epithelial injury as the duration of ischemia becomes more prolonged, following a similar pattern as the small intestine.

## B. Bile

Increases in luminal bile salt concentrations appear to have two main deleterious effects on the gastrointestinal epithelium. The first relates to an increase in mucosal permeability and the second to altered electrolyte transport. Both effects contribute to the mucosal injury and diarrhea which accompany bile salt malabsorption, ileal resection, bacterial overgrowth, and diseases associated with bile reflux. The injurious effects of bile salts make these solutions attractive for use to initiate mucosal damage in models designed to study the protection and repair mechanisms of gastrointestinal mucosa.

Evidence suggests that bile salts reversibly increase epithelial permeability by altering the integrity of tight junctions (49). These effects occur at physiological concentrations and are associated with widening of the intercellular spaces and a decrease in tight junctional strand number and continuity (46). With increasing bile salt concentrations, dose-dependent epithelial cell degeneration and sloughing occurs (78). The cytotoxic mechanisms of bile salts are poorly understood. Bile salts increase fragility of membrane vesicles and increase influx of extracellular  $\text{Ca}^{2+}$  and  $\text{H}^{+}$  into enterocytes (48, 199). These effects are presumably related to amphipathic solubilization of lipid membranes and generation of reactive oxygen metabolites, which arise from the metabolism of arachidonic acid liberated by bile salt activation of membrane phospholipase (34, 35).

Bile salt-induced alterations in electrolyte transport include decreases in  $\text{Na}^{+}$  absorption and stimulation of electrogenic  $\text{Cl}^{-}$  secretion (39, 48). Chloride secretion appears to require bile salt interaction with the basolateral aspect of the enterocyte and therefore only occurs once bile salts have altered barrier function and crossed the epithelial layer (39, 48).

## VII. ROLE OF ACUTE INFLAMMATION IN BARRIER REPAIR

As noted previously, inflammation is essential for wound repair. Of the cells that are involved in acute inflammation in the intestinal mucosa, neutrophils are the most abundant, accumulating in the lamina propria and subepithelium (Fig. 4). Although many elements of the acute inflammatory response influence wound repair, particular attention is often paid to neutrophils because of the perception that they are detrimental to the process. In contrast to this view, neutrophils in fact have key roles in promoting wound repair (168). The first is to control infection by sterilizing the wound. Wounds heal poorly and may even be fatal in individuals with insufficient neutrophils (23), in those with genetic defects that prevent migration of neutrophils into inflamed tissues, or in those with genetic deficiencies that prevent the activation of microbicidal functions (157).

A second mechanism by which neutrophils can promote wound healing is by production of proreparative signals. Neutrophils are a rich source of prostaglandin  $\text{E}_2$ , which has been shown to facilitate mucosal repair in the intestine (19, 21). Neutrophils also produce a number of protein mediators such as  $\text{IL-1}\beta$  that promote repair. In a model of ischemic injury, neutrophil-derived  $\text{IL-1}\beta$  stimulated repair by a cyclooxygenase (COX)-2-dependent mechanism (165), illustrating the importance of well-controlled inflammation on tissue healing.

Neutrophils are also a source of a number of mediators that retard their own accumulation, inhibit their own activation, promote their own death, and stimulate anti-inflammatory and proreparative functions of macrophages. Arachidonic acid-derived anti-inflammatory li-

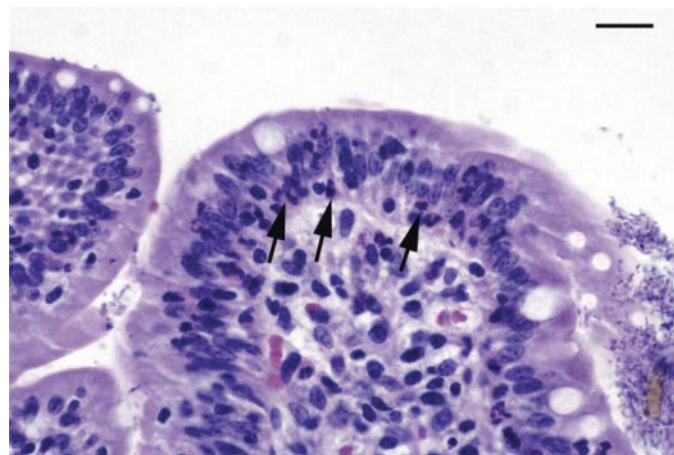


FIG. 4. Histological appearance of porcine ileal mucosa 18 h following ischemic injury. Note that despite evidence of complete reepithelialization, neutrophils have accumulated immediately beneath the epithelium (arrows), after which they will traverse the epithelium on their way to the lumen. Bar = 5  $\mu\text{m}$ .

poxins are examples of such mediators (163). Elegant mechanisms of lipoxin synthesis occur in *trans* between platelets and neutrophils at the endothelial luminal surface and between neutrophils and tissue cells in the mucosa, whereby neutrophils supply precursor lipids essential for lipoxin synthesis. In addition to being a source of anti-inflammatory lipids, neutrophils are an important source of AMP, which is converted to adenosine. Adenosine binds A<sub>2A</sub> receptors on neutrophils, inhibiting several functions including migration, degranulation, and production of reactive oxygen intermediates (77). Neutrophil-derived adenosine is also a potent secretagogue, inducing epithelial Cl<sup>-</sup> secretion, providing an epithelial flush at the apical surface, and being an important step in the process of tight junction repair in wounded intestinal mucosa (21, 107). Neutrophils also have a passive role in switching off inflammation once the work is done. Ingestion of apoptotic neutrophils stimulates macrophage production of resolvins and protectins that inhibit further neutrophil accumulation (163). Thus neutrophils are key cells regulating the switch from proinflammatory to anti-inflammatory, prorepair conditions.

Despite the beneficial effects of neutrophils in host defense and wound repair, the nature of the neutrophil's microbicidal mechanisms produces bystander damage, destroying tissue. If activated neutrophils do not quickly encounter their microbial targets, they respond by releasing all of the weapons in their antimicrobial arsenal into the extracellular environment (132). Thus, if the inflammatory response is not well controlled, or if the infection or cellular injury is extensive, neutrophils account for a good deal of the tissue damage that ensues (192). Indeed, neutrophils account for much of the damage in a number of intestinal diseases. This is illustrated by the fact that neutrophil depletion or prevention of neutrophil migration into intestinal mucosa by blocking or genetically deleting adhesion receptors necessary for trafficking or by inhibiting signals that recruit neutrophils reduces the pathophysiology associated with intestinal ischemic damage (55, 81), nonsteroidal anti-inflammatory drug-induced ulceration (16, 188), dextran sodium sulfate- and trinitrobenzene-induced colitis (56, 133, 187), and *Clostridium difficile* toxin A-induced intestinal secretion, mucosal mannitol permeability, and histological damage (88).

Neutrophils can damage intestinal tissue during inflammation in several ways. As neutrophils migrate into and through tissue, they progressively discharge granule contents containing a number of potent proteases, including elastase, protease-3, and metalloproteinases-8, -9, and -25. Liquifaction of matrix proteins by these proteases aids migration to the site of infection. However, the collateral damage can be considerable. Accumulations of neutrophils below the epithelium (Fig. 4) can disrupt the integrity of the monolayer by protease-mediated cleavage of proteins integral to epithelial support structures and

attachments (47, 192). Elastase also induces epithelial apoptosis, hastening epithelial loss (61). Other toxic substances released by neutrophils can also be quite toxic to cells and proteins, particularly reactive oxygen intermediates (ROM). Not only do ROM damage structural and attachment proteins and induce cellular necrosis (80), but they are important signals that regulate the recruitment of neutrophils into the site of inflammation (130, 201).

Migration of neutrophils across epithelium in response to infection or damage is an important component of host defense at the intestinal epithelial surface. Neutrophils migrate in response to microbial chemoattractant peptides that leak across damaged epithelium (29) or are transported by the specialized transporter hPepT1 (120). Neutrophil recruitment is also regulated by signals generated by epithelial cells invaded by pathogens (116). Once neutrophils reach the apical surface of the epithelium, they are poised to battle invading pathogens (127). Indeed, transepithelial migration of neutrophils is so substantial in some inflammatory diseases of the gut that the appearance of neutrophils in the stool is a hallmark of inflammatory bowel disease and invasive infectious diseases (76). In sufficient numbers, neutrophils migrating across the epithelium disrupt the monolayer and increase paracellular permeability (128, 138).

Neutrophils affect paracellular permeability in several ways. They physically alter the paracellular space as they migrate through (Fig. 5) and increase paracellular permeability (55, 128, 129). Blockade of CD11b/CD18, a necessary leukocyte integrin in the process of transepithelial migration, abrogates the effects on paracellular permeability (55, 143). While the physical process of migrating across epithelial tight junctions is important in this increase in paracellular permeability, tight junctions have the capacity to close quickly following transmigration. Neutrophil-derived proteases (such as elastase) cleave structural proteins, resulting in more permanent damage to the tight junction barrier (60). Sustained neutrophil transmigration may cause more substantial loss of the intestinal barrier by inducing epithelial apoptosis (95).

Neutrophils also deliver signals that loosen tight junctions. Proteases that trigger epithelial protease-activated receptor-2 (mast cell tryptase, neutrophil protease-3, and neutrophil elastase) increase paracellular permeability (84). Neutrophils do not produce the same concentrations of proinflammatory cytokines as macrophages, mast cells, or lymphocytes on a per cell basis. However, they outnumber these cells in acute inflammation by several orders of magnitude and are thus important sources of proinflammatory mediators such as tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  (131). TNF- $\alpha$  increases intestinal epithelial tight junction permeability by a mechanism that involves activation of the myosin contractile apparatus (195). The importance of TNF- $\alpha$  in the pathophysiology of intestinal inflammation

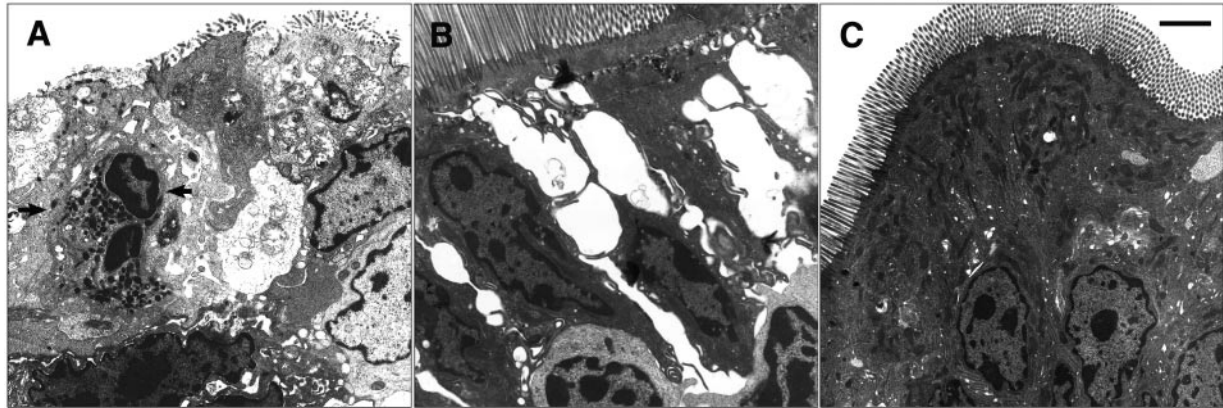


FIG. 5. Electron microscopic view of porcine epithelium 18 h following ischemic injury. *A*: note a neutrophil penetrating between neighboring epithelial cells (arrows), damaging the epithelium during transmigration. *B*: in similar repairing sections of epithelium, note widened interepithelial spaces as neutrophils (seen at *bottom* of the figure) migrate along the paracellular spaces toward the lumen. *C*: in animals systemically pretreated with anti-CD11/ CD18 antibody, note the lack of neutrophil infiltration and the close proximity of epithelial cells at the level of the paracellular spaces. Bar = 1.5  $\mu\text{m}$ . [Modified from Gayle et al. (55).]

is illustrated by the efficacy of anti-TNF- $\alpha$  therapy in patients with active inflammatory bowel disease (160). IFN- $\gamma$  also disrupts epithelial tight junctions by engaging the myosin contractile apparatus (103), resulting in internalization of a number of tight junction structural proteins (24, 184). In fact, IFN- $\gamma$  and TNF- $\alpha$  may act synergistically through the myosin contractile mechanism to increase paracellular permeability (189). Interestingly, the ability of IFN- $\gamma$  to alter tight junction integrity is associated with serine protease-dependent cleavage of the tight junction protein claudin-2 (193). This suggests an interesting interplay between neutrophil-derived IFN- $\gamma$  and serine proteases (such as elastase) in regulating epithelial tight junctions during intestinal inflammation (24).

There is also evidence to suggest that IFN- $\gamma$ -induced reductions in epithelial barrier function are linked to decreases in the expression of tight junction proteins such as occludin and ZO-1. Recent studies indicate that the mechanism for this process is cytoplasmic internalization (24). Research indicates there is no role for clathrin- and caveolar-mediated endocytosis, but inhibitors of macropinocytosis blocked IFN- $\gamma$ -induced internalization of tight junction proteins. More specifically, investigators have documented colocalization with markers of macropinocytosis, including phosphatidylinositol 3,4,5-trisphosphate. In addition to the above-mentioned mechanisms of inflammatory mediator-induced alterations in barrier function, IFN- $\gamma$  has an alternate effect on barrier function as a result of inhibition and ultimately downregulation of epithelial basolateral Na<sup>+</sup>-K<sup>+</sup>-ATPase. IFN- $\gamma$  acutely downregulates Na<sup>+</sup>-K<sup>+</sup>-ATPase, resulting in increases in intracellular Na<sup>+</sup> concentration and subsequent cell swelling (175). This signaling event ultimately results in epithelial dysfunction and increases in epithelial permeability. Furthermore, Na<sup>+</sup>-K<sup>+</sup>-ATPase appears to have a distinct

role in formation of tight junctions, desmosomes, and development of cell polarity (150). Although the mechanism linking Na<sup>+</sup>-K<sup>+</sup>-ATPase to formation of paracellular structures is unclear, this response is clearly linked to increased intracellular Na<sup>+</sup>. TNF- $\alpha$  also appears to have alternate mechanisms of reducing barrier function. In particular, TNF- $\alpha$  induction of apoptosis has been shown to reduce epithelial barrier function (62).

### VIII. MECHANISMS OF REPAIR OF INTESTINAL BARRIER FUNCTION

After acute mucosal injury, three local events culminate in restoration of epithelial continuity and normal permeability: 1) villus contraction, which reduces the total and denuded surface area for repair; 2) migration of epithelial cells to seal the exposed basement membrane; and 3) closure of leaky epithelial intercellular spaces and tight junctions. These events are initiated within minutes after injury and are locally regulated by mediators arising from a complex network of nerves, immune effector cells, structural and contractile fibroblasts, endothelial cells, and extracellular matrix within the underlying lamina propria. A critical role for these lamina propria mediators has only begun to be fully appreciated. These acute events are followed 18–24 h later by increased crypt cell proliferation, which culminates in replacement of lost cells and restoration of villus architecture as well as digestive and absorptive function. Experimental models of epithelial repair that integrate reparative events within the context of the native lamina propria and its overlying epithelium are complex and infrequently reported (21, 66, 67, 97, 122, 124, 125).



## A. Villus Contraction

In small intestinal mucosa, villus contraction aids in restoration of barrier function by reducing the surface area of basement membrane requiring resealing by migrating epithelium (44, 125). Villus contraction is believed to arise from an underlying network of communicating smooth muscle cells and contractile fibroblasts within the villus lamina propria (44, 86, 125). This network consists of myofibroblasts subjacent to the epithelium, a three-dimensional reticular network of stellate cells within the lamina propria, and smooth muscle cells that extend from the muscularis mucosa along the central lacteal. Intercommunication among these cells is suggested by the presence of gap junctions, focal contacts, and enteric innervation (86). Villus contraction can be separated temporally and functionally into two phases. The first is an immediate contraction, which is commensurate with mucosal injury, energy dependent, mediated by enteric nerves, and necessary for optimal rate of epithelial repair (125). This event likely involves selective contraction of myofibroblasts subtending the epithelial basement membrane, which results in a rapid decrease in size of the epithelial defect. The second phase is an ongoing contraction that progresses throughout the initial hours following injury and is dependent on endogenous synthesis of prostaglandins but does appear to be of insufficient magnitude to influence the reparative capacity of the migrating epithelium (44, 67, 125). Ongoing villus contraction likely results from longitudinal contraction of smooth muscle cells lining the villus core, which mediate an overall reduction in total villus surface area (44).

## B. Epithelial Restitution

Epithelial cells are attached to the underlying basement membrane by transmembrane integrins, which serve to link the cytoskeleton to the extracellular matrix at specialized regions called focal contacts. In response to loss of epithelial continuity, cells shouldering the wound are mobilized within minutes of injury and extend membrane projections across the epithelial defect (Fig. 6). This reparative response is termed restitution and does not depend on cellular regeneration, but instead arises from a highly coordinated response of the remaining epithelium (6). Mechanistically, epithelial cell migration involves extension of plasma membrane in the direction of cell migration, assembly of new focal contacts at the leading edge, generation of traction to facilitate forward motion, and detachment of focal contacts at the trailing edge of the cell (10, 31, 41). Evidence suggests that plasma membrane extensions (lamellipodia) arise from the disassembly of the microvillus brush border, which provides apical and lateral membrane to the leading edge

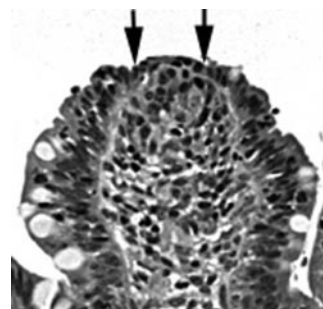


FIG. 6. Histologic appearance of restituted mucosa following ischemic injury. Notice thinning and extension of cells across the defect (arrows) created by loss of epithelial cells. In this series of experiments in porcine ileal mucosa, this process was complete within 60 min immediately following 45 min of ischemia. Bar = 20  $\mu$ m.

of the migrating cell (2). Lamellipodial extensions are in turn driven by the actin-myosin cytoskeleton which functions as a treadmill by repeatedly moving actin monomers from the rear to the leading edge of the lamellipodia (Fig. 7).

The focal contact between restituting epithelium and the basement membrane appears to be a key regulatory site of cell adhesion and kinesis. The adhesive function is served by transmembrane integrins, which are bound on the cytoplasmic surface to a plaque of proteins, many of which are linked to cytoskeletal filaments. Numerous signaling proteins colocalize with integrins at sites of attachment and initiate diverse cellular signaling pathways. In particular, focal adhesion kinase (FAK) is purported to initiate a highly complex series of tyrosine phosphorylation events and biochemical signals affecting cell migration anchorage-dependent survival, proliferation, and changes in gene expression (71). FAK is a nonreceptor protein tyrosine kinase whose activation results from binding to the cytoplasmic domain of activated  $\beta_1$ -integrins.  $\beta_1$ -Integrins are activated by specific interaction with extracellular matrix ligands. FAK activity can also be regulated by a variety of growth factors, neuropeptides, and other stimuli. While several studies have failed to detect any direct effect of FAK expression on cell adhesion, others have demonstrated a key regulatory role for FAK in cell migration (26, 59, 83). The mechanism by which FAK stimulates cell migration is not clear. Remodeling of the cytoskeleton and turnover of focal adhesions are critical processes during cell migration, and activation of FAK may regulate the rate of attachment, or more likely the release of focal adhesions rather than their de novo formation.

In addition to the importance of FAK, numerous growth factors found in the intestine are potent stimulators of epithelial cell migration including transforming growth factor (TGF)- $\beta$ , insulin-like growth factor I, hepatocyte growth factor, platelet-derived growth factor, epidermal growth factor, TGF- $\alpha$ , fibroblast growth factor,



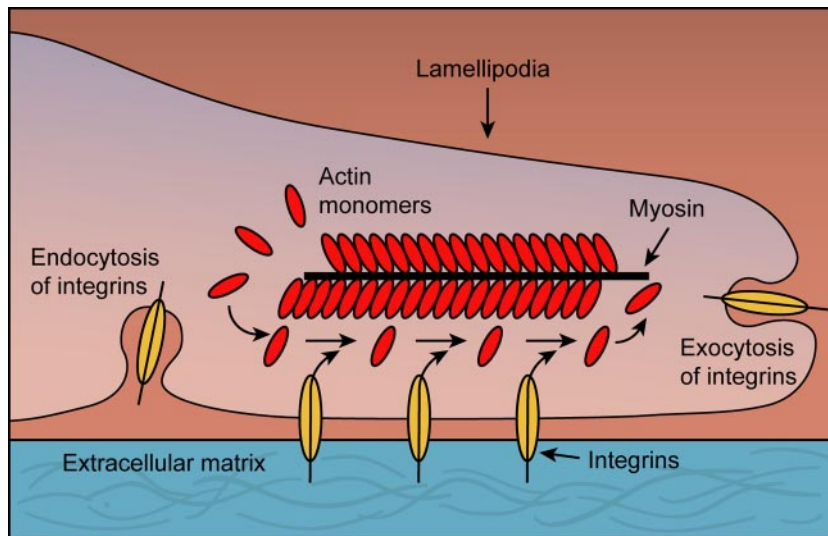


FIG. 7. Diagram of the cellular events that result in epithelial restitution. Note the attachment of the cell to the basement membrane by integrins, which presumably also convey signals to the cell to commence formation of lamellipodia. Note also the intracellular mechanism utilizing the cytoskeleton whereby the cell is propelled across denuded basement membrane.

and inflammatory cytokines such as IL-1 $\beta$  and IFN- $\gamma$  (6, 41, 63, 153, 155, 194). Many of these factors appear to converge on a TGF- $\beta$ -dependent pathway by either increasing TGF- $\beta$  expression or its extracellular bioactivation, illustrating a central role for this peptide in mediating enhanced migration (41, 174). Subepithelial myofibroblasts are known to orchestrate several important epithelial functions, including enhancing epithelial cell migration via release of bioactive TGF- $\beta$  (117). TGF- $\beta$  is also expressed by epithelial cells in response to wounding, resulting in autocrine stimulation of cell migration (30, 41). Reported actions of TGF- $\beta$  on repair of the epithelium after injury include enhanced epithelial cell migration (117), expression and turnover of extracellular matrix proteins and cell surface integrin (64), and decreases in epithelial paracellular permeability (17).

Polyamines (putrescine, spermidine, spermine) are an additional group of compounds that have been extensively studied because of their role in restitution. Polyamines are highly charged, multivalent cations that play a well-established role in epithelial restitution (113). Polyamines are hypothesized to interact electrostatically with DNA to regulate gene transcription and are substrates for transglutaminase, an enzyme which catalyzes the formation of cross-links between polyamines and protein (115). Numerous studies have linked polyamines to reorganization of the cytoskeleton during the process of cell migration including the organization of F-actin and tropomyosin (12, 114), expression of nonmuscle myosin II (152, 191), polymerization of tubulin (11), and distribution, activity, and actin binding of epidermal growth factor receptor (112). A large number of the effects of polyamines may be secondary to stimulation of TGF- $\beta$  synthesis. Depletion of intracellular polyamines inhibits increased expression of the TGF- $\beta$  gene and enhanced migration rate of epithelium observed after wounding, whereas exogenous

TGF- $\beta$  in the presence of depleted polyamines restores migration (190). Ornithine decarboxylase, the rate-limiting enzyme for polyamine synthesis, can be rapidly and strikingly induced within the cytosol of multiple epithelial cell lines by a variety of insults, luminal nutrients, and growth factor stimuli (87, 177). In addition to de novo synthesis, the luminal surface of the gastrointestinal tract is exposed to polyamines at relatively high concentrations, whereas concentrations in serum are low (12). These extracellular polyamines are derived from food, synthesis by intraluminal bacteria, gastrointestinal secretions, and sloughed epithelial cells (85).

Trefoil peptides have received a great deal of attention because of their role in restitution. These peptides are protease-resistant factors secreted by goblet cells into the lumen of the small and large intestine. Their production is markedly increased in response to a variety of intestinal insults, and their presence both protects against intestinal epithelial injury and promotes repair (10, 145). Trefoil peptides promote restitution of wounded epithelial monolayers in vitro (40), and mice deficient in trefoil factors (by means of targeted gene deletion) are highly susceptible to intestinal mucosal injury and show no evidence of epithelial repair (111). Much remains to be understood regarding the intracellular signaling events resulting from the actions of trefoil factors. One downstream effect of trefoil factor appears to be the redistribution of E-cadherin away from intercellular adherens junctions that may facilitate cell movement by detaching cells from one another during the process of migration (146).

Studies both in vitro and in vivo suggest that nitric oxide exerts permissive effects on growth factor-stimulated epithelial cell migration. Numerous growth factors (e.g., vascular endothelial growth factor, hepatocyte growth factor, endothelin, insulin-like growth factor I,

IL-1 $\beta$ , substance P) mediate chemotactic migration of cultured cells while simultaneously stimulating nitric oxide (NO) formation in a dose-dependent manner (72, 136). Growth factor-stimulated cell migration is blocked by treatment with NO synthesis inhibitors, while the addition of Arg, NO donors, or cGMP (an NO second messenger) enhances migration (135, 136). NO synthase is induced by epithelial cells in response to wounding and appears to be the source of NO for promoting epithelial migration in a number of studies (67, 153). The exact site of NO action in the process of cell migration remains unclear. Recent studies have shown that NO may interfere with cellular adhesion events by decreasing the number of adhesion molecules expressed, the affinity of integrins for extracellular matrix, or the assembly of focal adhesions (65, 93, 135, 136). The antiadhesive effects of NO can be mimicked by cGMP analogs and blocked by inhibitors of soluble guanylate cyclase, indicating that they involve the generation and action of cGMP. Because attachment and release of focal adhesions are critical processes during cell migration, it is possible that they are regulated by regional differences in intracellular NO concentration.

### C. Closure of the Paracellular Space

To date, the basic events leading to reassembly and closure of tight junctions (TJs) remain largely unknown. However, cell culture systems utilizing the Ca<sup>2+</sup> switch model and ATP depletion/repletion models (28, 179), events which model ischemia/reperfusion injury, have been used to elucidate these events. The key events defining TJ closure include recruitment of TJ proteins to the apical lateral membrane and closure of the subjacent paracellular space. However, although reassembly of TJ appears to be paramount in restoring barrier function, there are critical cellular events at the level of the adherens junction (AJ) that must take place before TJ protein recruitment and assembly. It has been shown that the ultimate formation of the TJ in epithelial cells is largely dependent on AJ formation (73, 74). The AJs form a continuous belt below the TJs and play a role in stabilizing the TJs and serving as regulatory sites for recruitment of signaling molecules and TJ proteins to the apical lateral membrane. The initial formation of AJs is initiated during cell migration when neighboring cells recognize each other and a primordial junction forms initiated by interactions of a variety of cytoskeletal proteins including nectins,  $\beta$ - and  $\alpha$ -catenins, and E-cadherin (1, 186, 196). The primordial junctions fuse with each other and develop into mature AJs. Once the AJs have formed, TJ reassembly begins with initial ZO-1 localization at the apical lateral membrane followed by recruitment of JAMS, claudins, and occludin to the apical side of the AJ leading to establishment of TER (5, 9, 159). The initial

ZO-1 localization appears to be a critical event in the recruitment of integral membrane proteins to the TJ (179). For example, recruitment of TJ proteins (claudins and occludin) to the TJ and polarization are significantly delayed in mouse epithelial cells lacking ZO-1 expression in Ca<sup>2+</sup> switch experiments (183). Likewise, McNeil et al. (118) showed that gene silencing of ZO-1 by RNA interference in MDCK cells resulted in a pronounced delay in TJ reformation and recovery of TER in response to Ca<sup>2+</sup> switch and that SH3 binding site of ZO-1 is the key element mediating TJ assembly (118). In T84 cells, monoclonal antibodies directed against the extracellular domain of JAM prevented redistribution of occludin from the cytoplasm to the TJ membrane, corresponding with inhibition of the recovery of TER. These experiments suggest that integral membrane proteins may also serve to recruit occludin to the TJ (98).

Although TJ repair has been studied predominantly in cell culture systems, the mechanisms of TJ repair in whole animal tissue have not been previously well studied. However, utilizing an ex vivo porcine model of acute intestinal injury, we have shown that recovery of barrier function (in terms of TER) coincides with the assembly of TJ proteins such as ZO-1 and occludin to the TJs, confirming findings observed in epithelial cell culture systems (97). We have accumulated convincing evidence for a role of prostaglandins in TJ protein recruitment and repair of intestinal barrier function in this model. For example, treatment of ischemia-injured intestine with the nonselective nonsteroidal anti-inflammatory drug indomethacin significantly impaired the ability of the intestine to recover barrier function (21). Furthermore, we showed that application of exogenous prostaglandins (PGE<sub>2</sub> and PGI<sub>2</sub>) to injured mucosa rapidly restored TER to control levels (19), an effect associated with the recruitment of ZO-1 and occludin to the TJ and closure of the paracellular space (97).

The mechanism by which PGs trigger recovery of barrier function and TJ structure have not been fully characterized but appear to involve their ability to modulate ion transport. For example, it was shown that recovery of TER in injured mucosa treated with prostaglandins was preceded by marked elevations in short-circuit current ( $I_{sc}$ ) that were attributable to Cl<sup>-</sup> secretion as determined by radiolabeled <sup>36</sup>Cl<sup>-</sup> flux experiments. Prostaglandin-stimulated elevations in TER in these tissues were inhibited with pretreatment with the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> transport inhibitor bumetanide, thus confirming the importance of Cl<sup>-</sup> secretion in this response. The concept that Cl<sup>-</sup> secretion is associated with elevations in TER in this model is somewhat counterintuitive, since secretory events are associated with opening of apical Cl<sup>-</sup> channels, which in turn might be expected to reduce, not increase, TER. However, mammalian small intestine is relatively leaky so that measurement of TER is essentially

a measure of paracellular resistance. Thus alteration of apical membrane conductance has relatively little effect on TER in these tissues.

From these studies, it appears that the initial rapid phase of prostaglandin-induced recovery of TER is associated with  $\text{Cl}^-$  secretory processes, whereas later phases of recovery were  $\text{Cl}^-$  independent and were attributable to prostaglandin's ability to inhibit apical electroneutral  $\text{Na}^+/\text{H}^+$  exchangers (NHE). For example, ischemic intestinal tissues bathed in indomethacin can be partially stimulated to recover TER by treatment of tissues with the NHE inhibitor amiloride (19). Thus essentially all of the recovery response stimulated by prostaglandins can be accounted for by  $\text{Cl}^-$  secretion, and substantial contributions are made by inhibition of NHE.

The mechanism by which activation of  $\text{Cl}^-$  secretion and inhibition of NHE restores TER in injured tissues is not entirely understood. Initially, we reasoned that  $\text{Cl}^-$  secretion and inhibition of  $\text{Na}^+$  absorption could result in the luminal accumulation of  $\text{Cl}^-$  and  $\text{Na}^+$ , resulting in a lumenally directed osmotic load pulling extracellular fluid from the paracellular space. This might lead, in turn, to closure of the paracellular space. Osmotic loads have been previously shown to increase barrier function in mammalian mucosa, lending some credence to this theory (19, 97, 101). An additional interesting finding was that secretory events could be uncoupled from recovery of barrier function by treating tissues with selective inhibitors of phosphatidylinositol 3-kinase (PI3K). In other words, inhibition of PI3K did not alter the magnitude of  $\text{Cl}^-$  secretion, but did fully inhibit recovery of TER and restoration of normal mucosal-to-serosal fluxes of mannitol and inulin. This same finding was demonstrated following application of mucosal osmotic loads (97). However, given the marked disruption of the TJ protein architecture that occurs during ischemia in our model, it seems unlikely that an osmotic gradient, resulting from luminal  $\text{Cl}^-$  accumulation, would form without some degree of TJ integrity. Alternatively, there is mounting evidence for a role of targeted activation of select ion channels in this process. For example, we have shown that the  $\text{Cl}^-$  secretory event responsible for the phases of TER elevations is mediated predominantly through CIC-2  $\text{Cl}^-$  channels expressed in the TJs, with a more minor role for  $\text{Cl}^-$  transporters such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Fig. 8) (122). More recently, we have shown that inhibition of NHE2 appears to have an important role in mucosal barrier repair and restoration of TER, whereas NHE3, the predominant isoform mediating electroneutral  $\text{Na}^+$  absorption in the intestine, did not influence TER (123). The mechanistic link between CIC-2 and NHE2 and regulation of the TJ remains to be elucidated but may involve signaling events via the link of the COOH-terminal region of CIC-2 and NHE2 to the actin cytoskeleton, serving to recruit proteins such as

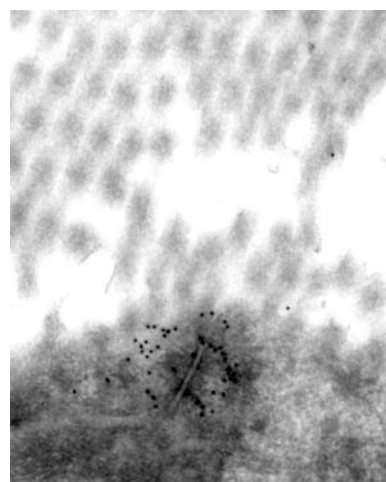


FIG. 8. Electron micrograph of porcine ileal mucosa showing immunogold labeling of CIC-2. Note the localization of this secretory  $\text{Cl}^-$  channel to the tight junction. This likely explains, in part, the role of this channel in recovery of the tight junction following injury. Bar = 3  $\mu\text{m}$ .

occludin and claudin to the TJ. However, more studies are required to understand this link.

#### D. Relative Importance of Paracellular Structures

During studies of recovery of barrier function, it is apparent that epithelial coverage (restitution) and concurrent villous contraction initiate the process of recovery of restoration of barrier function. However, these events are insufficient to reform a fully functional barrier (66). One theory that has arisen from this observation is that closure of the lateral intercellular space is ultimately the event that restores barrier function (19), although it could not occur without epithelial restitution because cellular crawling to cover denuded basement membrane places the cells in close proximity. However, it is not clear which component of the paracellular space (including TJs and the lateral epithelial membranes) is most important in restoring barrier function. Studies on the resistance of paracellular structures have been performed predominantly in normal tissues. The relative percentage contribution of the lateral intercellular space, compared with the TJ, to overall measurements of TER in normal tissues is in the range of 20–30% (91, 171). In the small intestine, stimulation of anion secretion results in increased TER, associated with concurrent collapse of the lateral intercellular space in both normal tissues (54) and acutely injured tissues (19). Although one series of studies has identified CIC-2 as a potentially important anion channel in mucosal recovery (122), anion secretion-induced collapse of the lateral intercellular space has been shown to be dependent on CFTR, since no such collapse occurred in CFTR knockout mice (54). To further demonstrate the



importance of the lateral intercellular space, increased serosal hydrostatic pressure prevents increases in TER in secreting small intestinal tissues by blocking collapse of the lateral intercellular space (19, 54). However, in injured mucosa, this maneuver also opens TJs so that the TJ and lateral intercellular space appear to act in concert. Similar experiments have evaluated osmotic loads placed on the luminal surface of injured mucosa and have shown an increase in TER associated with collapse of the lateral intercellular space (19, 97). However, experiments on normal rabbit mucosa have shown that luminal osmotic loads rapidly alter the structure of the TJ (101). Overall, it is likely that both the TJ and lateral intercellular space play a role in recovery of mucosal barrier function. The percentage contribution of each structure has only been tested in normal mucosa, but there is almost certainly a role for each structure in recovery of barrier function in acutely injured mucosa given the appearance of the lateral intercellular space and TJ during recovery (Fig. 2). The lateral intercellular space likely requires some degree of TJ integrity to collapse. However, one unifying hypothesis is that early recovery of TER is largely attributable to collapse of the lateral intercellular space driven by anion secretion principally through the CFTR. Subacute maintenance of TER is more likely due to continued restructuring of the TJs, as this becomes a permanent barrier that is not dependent on the degree to which the lateral intercellular space is open or collapsed. This junctional restructuring also appears to be driven initially by anion secretion, but via CIC-2 rather than CFTR (122). Restructuring is then continued by mechanisms linked to NHE2 independent of anion secretion (123).

### IX. ROLE OF LAMINA PROPRIA CELLS IN MUCOSAL RECOVERY

There is extensive communication between epithelial cells and subepithelial lamina propria cells, including myofibroblasts (147, 148), enteric nerves (125), and neutrophils (140) that may play a key role in recovery of epithelial barrier function. For example, myofibroblasts have been shown to markedly alter the secretory activity of epithelial cells to a range of secretagogues when juxtaposed in cell culture (18). Furthermore, myofibroblasts have been shown to express COX-2 in response to inflammatory mediators such as IL-1 $\beta$ , which may well be relevant to postischemic injury (82). Enteric nerves have been shown to relay signals from enterochromaffin cells to epithelial cells in response to exposure to bacterial toxins such as cholera toxin (27, 182), and nerves have been shown to be important signaling conduits for prostanoids. For example, in porcine cryptosporidiosis, much of the secretory response was relayed via PGI<sub>2</sub>-stimulated cholinergic and vasoactive intestinal polypeptidergic nerves

(7), and we have previously shown an important role for cholinergic nerves in prostaglandin-stimulated mucosal recovery (21). Subepithelial neutrophils have also been shown to play a critical role in both epithelial secretory and reparative events. For instance, neutrophils release a host of mediators, including reactive oxygen metabolites, that alter secretory and barrier epithelial functions, and may alter epithelial recovery. In particular, a series of studies performed by Nusrat et al. (138) have shown that large numbers of neutrophils traversing the epithelium can reduce epithelial barrier function by damaging interepithelial tight junctions, a mechanism that we have confirmed in postischemic porcine ileal mucosa (55).

### X. RELATIVE CONTRIBUTION OF EPITHELIAL REPAIR MECHANISMS TO BARRIER FUNCTION

Restitution is considered to be a major determinant of early recovery of gastrointestinal barrier function following an acute injury. This conclusion derives from numerous *in vitro* studies demonstrating that barrier function recovers in parallel with morphological restitution after acute mucosal injury (124, 154, 176). Consequently, studies of gastrointestinal repair using intact mucosa have largely been replaced by migration assays of wounded epithelial monolayers. Regrettably, these latter studies fail to account for the simultaneous roles played by villus contraction and TJ permeability in recovery of small intestinal barrier function. More recent studies of epithelial repair in intact mucosa have demonstrated that restoration of paracellular resistance, and not restitution or villus contraction, can account for the majority of barrier recovery after epithelial injury (19, 21, 66). This is because 63–73% of the total paracellular conductance of small intestinal epithelium is via a high linear density of TJs residing in the crypts (32, 109), and after villus contraction, crypt epithelium accounts for the majority of surface area remaining after acute mucosal injury. Failure to close the intercellular spaces created by migration of epithelium can additionally explain why restitution is incapable of promoting recovery of barrier function in the absence of paracellular space closure.

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