

Gut

Leading article

Intestinal zonulin: open sesame!

The paracellular route is the dominant pathway for passive solute flow across the intestinal epithelial barrier, and its permeability depends on the regulation of intercellular tight junctions (TJs), also known as the zonula occludens. As a barrier between apical and basolateral compartments, TJs selectively control the passive diffusion of ions and small water soluble solutes from the intestinal lumen to the bloodstream through the paracellular pathway, thereby counterregulating any gradients generated by transcellular pathways.¹ Variations in transepithelial conductance can usually be attributed to changes in the permeability of the paracellular pathway as the resistance of the plasma membrane of the cell is relatively high.² The TJ represents the major barrier within this paracellular pathway and the electrical resistance of the intestinal mucosa seems to depend on the number of transmembrane protein strands and their complexity within the TJ, as observed by freeze fracture electron microscopy. A century ago, TJs were conceptualised as a secreted extracellular cement forming an absolute and unregulated barrier within the paracellular space.³ Biological studies of the past several decades have shown that TJs are dynamic structures subjected to structural changes that dictate their functional status under a variety of developmental,^{4–6} physiological,^{7–10} and pathological circumstances.^{11–13} To meet the many diverse physiological challenges to which the epithelial and endothelial barriers are subjected, TJs must be capable of rapid and coordinated responses. This requires the presence of a complex regulatory system that orchestrates the state of assembly of the TJ multiprotein network. While our knowledge on TJ ultrastructure and intracellular signalling events have significantly progressed during the past decade, relatively little is known about their pathophysiological regulation secondary to extracellular stimuli. Therefore, the intimate pathogenic mechanisms of diseases in which intestinal permeability is affected and the utility of TJ modulation for drug delivery both have remained unexplored owing to limited understanding of the extracellular signalling involved in TJ regulation.

Discovery of the zonulin system

In recent years much has been discovered about the structure, function, and regulation of TJs. However, the precise mechanism(s) through which they operate is still incompletely understood. Several microorganisms have been shown to exert a cytopathic pathological effect on epithelial cells that involves the cytoskeletal structure and TJ function in an irreversible manner. These bacteria alter the intestinal permeability either directly (that is, EPEC) or through elaboration of toxins (that is, *Clostridium difficile*, *Bacteroides fragilis*).¹⁴ A more physiological mechanism of

regulation of TJ permeability has been proposed for the zonula occludens toxin (Zot) elaborated by *Vibrio cholerae*.^{15, 16} Zot possesses multiple domains that allow a dual function of the protein as a morphogenetic phage peptide for the *Vibrio cholerae* phage CTX ϕ and as an enterotoxin that modulates intestinal TJs.¹⁷ The discovery of Zot has shed some light on the intricate mechanisms involved in the modulation of the intestinal paracellular pathway. Zot action is mediated by a cascade of intracellular events that lead to a protein kinase C (PKC) α dependent polymerisation of actin microfilaments strategically localised to regulate the paracellular pathway (fig 1).¹⁸ The toxin exerts its effect by interacting with a surface intestinal receptor whose distribution varies within the intestine, being detectable in the jejunum and distal ileum but not in the colon, and decreasing along the villous-crypt axis.¹⁹ This receptor distribution coincides with the regional effect of Zot on intestinal permeability¹⁹ and with the preferential F-actin redistribution induced by Zot in the mature cells of the villi.¹⁸ These data also suggest that expression of this receptor(s) is upregulated during enterocyte differentiation. This hypothesis is supported by the observation that human intestinal epithelial CaCo2 cells (that resemble the mature absorptive enteric cell of the villi), but not crypt-like T84 cells, express this receptor(s) on their surface.²⁰ The paucity of Zot binding in the crypt area may also reflect the fact that this region is already leaky compared with the more mature epithelium of the tip of the villi²¹ and thus might not need to express a significant amount of a putative receptor(s) involved in TJ regulation.

Taken together, these data showed that Zot regulates TJs in a rapid, reversible, and reproducible fashion, and probably activates intracellular signals which are operative during the physiological modulation of the paracellular pathway (fig 1).

Based on this observation, it was postulated that Zot may mimic the effect of a functionally and immunologically related endogenous modulator of epithelial TJs. The combination of affinity purified anti-Zot antibodies and the Ussing chamber assay allowed the identification of zonulin, an intestinal Zot analogue.^{22, 23} Zonulin has a molecular weight of 47 kDa, an N terminal receptor binding motif that is structurally and functionally similar to the Zot binding motif,²² and a C terminal domain probably involved in the rearrangement of cytoskeletal elements functionally connected to intercellular TJs.

Abbreviations used in this paper: TJ, tight junction; Zot, zonula occludens toxin; PKC, protein kinase C; CD, coeliac disease.

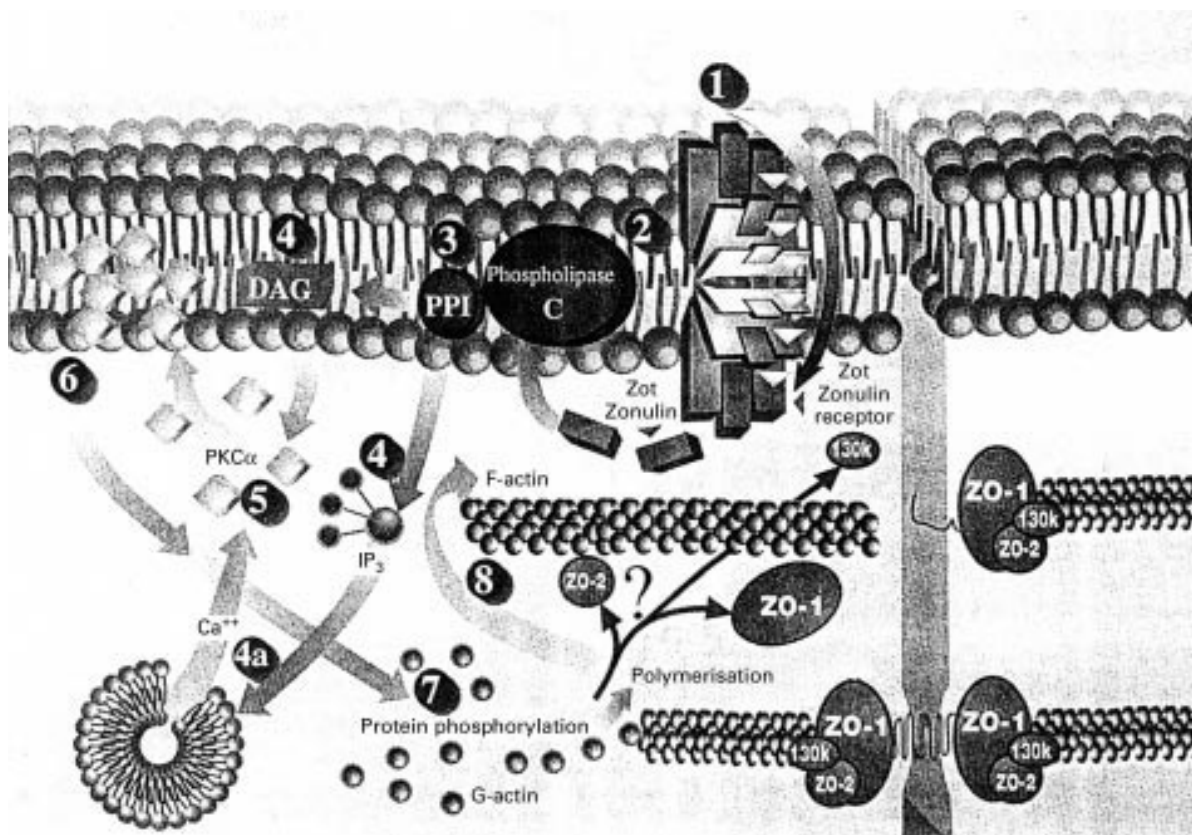


Figure 1 Proposed zonulin/zonula occludens toxin (Zot) intracellular signalling leading to the opening of intestinal tight junctions. Zonulin and Zot interact with the same specific surface receptor (1) whose distribution within the intestine varies. The proteins are then internalised and activate phospholipase C (2) that hydrolyses phosphatidyl inositol (3) to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG) (4). Protein kinase C (PKC) α is then activated (5), either directly (via DAG) (4) or through release of intracellular Ca^{2+} (via PPI-3) (4a). PKC α catalyses the phosphorylation of target protein(s) with subsequent polymerisation of soluble G-actin to F-actin (7). This polymerisation causes the rearrangement of the filaments of actin and the subsequent displacement of proteins (including ZO-1) from the junctional complex (8). As a result, intestinal tight junctions become looser (from Fasano²² with permission, modified).

Physiology of the zonulin system

The physiological role of the zonulin system remains to be established but it is likely that this system is involved in several functions, including TJ regulation responsible for the movement of fluid, macromolecules, and leucocytes between the bloodstream and the intestinal lumen and vice versa. Another possible physiological role of the intestinal zonulin is protection against microorganism colonisation.²⁴ In the absence of enteric infections, the mammalian small intestine is virtually sterile. Colonisation of the proximal gut by enteric microorganisms (even without apparent mucosal damage or elaboration of specific toxins) may lead to a leaky intestine²⁵ but the mechanism(s) by which this disturbed physiological regulation of intestinal TJ permeability secondary to proximal bacterial contamination occurs remains unclear. We have recently provided evidence that both normal enteric bacterial flora isolates (well characterised for not harbouring any known pathogenic traits) and pathogenic bacteria induce alteration of TJ competency, as suggested by changes in epithelial resistance and increased passage of inulin.²⁴ These changes were mirrored by concomitant expression of zonulin in organ culture systems and occurred even when bacteria were killed by gentamicin treatment.²⁴ These results suggest that the presence of enteric microorganisms in the small intestine (but not in the colon, where the zonulin system is not operative^{15 18 19}) induces a host dependent mucosal response that leads to luminal secretion of zonulin. The role of zonulin in bacteria induced impairment of intestinal barrier function is sustained by the observation that zonulin is detected in organ culture supernatants only when exposed to bacteria and by the blocking effect of

zonulin inhibitors on these intestinal barrier changes.²⁴ The fact that the interaction of bacteria with the intestinal mucosa induces zonulin release, irrespective of their pathogenic traits or viability, can be interpreted as a bacteria independent mechanism of defence of the host that reacts to the abnormal presence of microorganisms on the surface of the small intestine. Following zonulin induced opening of TJs, water is secreted into the intestinal lumen following hydrostatic pressure gradients¹⁹ and bacteria are “flushed out” from the small intestine.

Pathology of the zonulin system

Given the complexity of both cell signalling events and intracellular structures involved in the zonulin system, it is not surprising that this pathway may be affected when the physiological state of the intestinal epithelium is dramatically changed as it is in many of the autoimmune diseases in which TJ dysfunction appears to be the primary defect.²⁶ To explore this possibility, we focused our studies on coeliac disease (CD) and type 1 diabetes, two autoimmune conditions in which the finely tuned regulation of intestinal TJ permeability is lost.²⁶

To determine if zonulin is perturbed during the acute phase of CD, intestinal tissues from patients with active CD and non-CD controls were probed for zonulin expression.²⁷ Immunofluorescence analysis of CD tissues revealed increased zonulin expression within the intestinal submucosa with a characteristic reticular pattern that was consistently absent in control tissues.²⁷ Quantitative immunoblotting of intestinal tissue lysates from active CD patients confirmed increased zonulin protein compared

with control tissues.²⁷ The TJ derangements in CD were more pronounced in villous enterocytes²⁷ and therefore coincide with zonulin receptor distribution along the gastrointestinal tract.¹⁹ These findings suggest that this protein contributes to CD pathogenesis by increasing TJ permeability, typical of the early stages of this clinical condition.

It has recently been reported that untreated CD predisposes to autoimmune disorders such as insulin dependent diabetes mellitus, Hashimoto's thyroiditis, autoimmune hepatitis, and connective tissue diseases.²⁸ One could hypothesise that zonulin opens small intestinal TJs during the early stages of CD and permits entry of putative allergens into the intestinal submucosa where an autoimmune response is elicited.

Alterations in intestinal TJ permeability have also been shown to be one of the preceding pathophysiological changes associated with the onset of type 1 diabetes.²⁷ To establish whether zonulin may be responsible, at least in part, for these early changes, we used an established rat model of type 1 diabetes.²⁹ Two genetic breeds (that is, BB/Wor diabetic prone and diabetic resistant rats) were studied to determine if they exhibited significant changes in intraluminal secretion of zonulin and intestinal permeability. No difference in intraluminal zonulin concentration was observed in the two groups of animals up to age 40 days.³⁰ Thereafter, a fourfold increase in intraluminal zonulin was observed in diabetic prone rats while no significance increment was detected in diabetic resistant animals.³⁰ This increase in intraluminal zonulin was found: (1) to be age related; (2) to be detectable only in the small intestine (that is, the jejunum and ileum) but not in the colon; (3) to correlate with an increase in intestinal permeability of the intestine; (4) to precede the onset of diabetes by at least 3–4 weeks; (5) to remain high in these diabetic prone rats; and (6) to correlate with progression towards full blown diabetes.³⁰ Thus these results suggest that zonulin can be responsible for the early permeability changes (and therefore the pathogenesis of type 1 diabetes) already described in this animal model³¹ and confirmed by our studies. This hypothesis is further supported by the observation from both us³⁰ and other investigators³¹ that intestinal permeability changes in these diabetic rates are confined to the small intestine, paralleling the regional distribution of the zonulin intestinal receptor¹⁹ and therefore of the site in which the zonulin system is operative.

Therapeutical use of the zonulin system

Considering the limitations of the TJ modulators currently tested for drug delivery,³² it was reasonable to explore whether findings from basic research on the zonulin system could be applied to developing new approaches to enhancement of drug absorption via regulation of intercellular TJs. To date, the main source of zonulin is biochemical purification from human cadavers. Therefore, Zot was used as a valid alternative to utilise the zonulin system for drug delivery as both proteins share the same binding motif to engage to the zonulin receptor³³ and therefore activate the same intracellular signalling, leading to modulation of intercellular TJs.^{15 18 19 22 29}

To establish the efficacy of Zot as an intestinal absorption enhancer, insulin and immunoglobulin G were selected as drugs to be delivered orally. This choice was based on the relative size, structure, biological activities, and therapeutic relevance of these proteins. In vitro experiments in the rabbit ileum demonstrated that Zot reversibly increased intestinal absorption of both insulin (by 72%) and immunoglobulin G (by 52%) in a time dependent manner.²⁹ When tested in the intact host using the rabbit in

vivo perfusion assay, Zot increased the passage of insulin across both the jejunum and distal ileum 10-fold whereas no substantial changes were observed in the colon.²⁹

To evaluate the bioactivity of insulin after enteric coadministration with Zot, the hormone was orally administered to acute type 1 diabetic male rats with or without Zot, and blood glucose levels of rats were serially measured. After oral administration of insulin alone, given in doses of 5–30 international units (IU), blood glucose levels of treated animals were not appreciably lowered.²⁹ In contrast, when insulin (at doses as low as 10 IU) was orally coadministered with Zot, a significant reduction in blood glucose concentration was observed.²⁹ This decrement was comparable with that seen with a conventional dose of insulin subcutaneously administered (range 1.2–2.4 IU); blood glucose levels returned to normal within six hours after administration. None of the animals treated with insulin in combination with Zot experienced diarrhoea, fever, or other systemic symptoms, and no structural changes were demonstrated in the small intestine on histological examination.²⁹

Both in vitro and in vivo experiments demonstrated that the zonulin system is also operative in primates.²² Coadministration of Zot significantly increased the apparent permeation coefficients of desmopressin in the ileum and, to a lesser degree, in the jejunum in vitro (Lundin S, Fasano, unpublished). Similarly, Zot increased monkey intestinal absorption of insulin compared with untreated controls in a time dependent manner both in the jejunum and ileum.³⁴

Zot was also tested in an in vivo primate model of diabetes mellitus. Insulin was intragastrically administered to diabetic monkeys either alone or in combination with increasing amounts of Zot. Oral coadministration of Zot and insulin decreased blood glucose levels in a dose dependent manner. Measurements of blood insulin levels revealed that insulin bioavailability increased from 5.4% in controls to 10.7% and 18% when Zot 2 µg/kg and 4 µg/kg were coadministered, respectively.³⁴ Taken together, these results demonstrate that the permeating effect of the zonulin system also occurs in primates and that the kinetics and regional effects of this system are similar to those observed in the rabbit model.

Conclusions

The paracellular pathway was once considered to be exclusively the route for passive unregulated passage of water, electrolytes, and small molecules. Its contribution to the general economy of transepithelial transports was therefore judged to be simply secondary to the active transcellular transport processes. It is now becoming apparent that the elements that govern this pathway (that is, TJs) are extremely dynamic structures involved in developmental, physiological, and pathological circumstances. An increased number of autoimmune diseases are now described whose pathogenesis is associated with a primary dysfunction of intestinal intercellular TJs. These same structures however are used to develop innovative strategies for the delivery of macromolecules normally not absorbed through the intestine. The discovery of the zonulin system has shed some light on the intricate pathophysiological regulation of intercellular TJs that, however, remains far from being completely addressed. It is conceivable that zonulin participates in the physiological regulation of intercellular TJs of the small intestine. Dysregulation of this conceptual zonulin model may contribute to disease states that involve disordered intercellular communication, including developmental and intestinal disorders leading to autoimmune disease (that is, CD and type 1 diabetes), tissue inflammation, malignant transformation,

and metastasis. This same system can offer the opportunity of targeted tissue specific delivery of macromolecules and drugs currently engineered by recombinant DNA techniques or that will become available through the human genome project.

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- 1 Diamond JM. The epithelial junction: bridge, gate and fence. *Physiologist* 1977;20:10-18.
- 2 Madara JL. Loosing tight junctions: lessons from the intestine. *J Clin Invest* 1989;83:1089-94.
- 3 Cerejido M. Evolution of ideas on the tight junction. In: Cerejido M, ed. *Tight junction*. Boca Raton, Florida: CRC Press Inc, 1992:1.
- 4 Magnuson T, Jacobson JB, Stackpole CW. Relationship between intercellular permeability and junction organization in the preimplantation mouse embryo. *Dev Biol* 1978;67:214-24.
- 5 Revel JP, Brown SS. Cell junctions in development with particular reference to the neural tube, Cold Spring Harbor Symposium. *Quant Biol* 1976;40:443-55.
- 6 Schneeberger EE, Walters DV, Olver RE. Development of intercellular junctions in the pulmonary epithelium of the foetal lamb. *J Cell Sci* 1978;32:307-24.
- 7 Gilula NB, Fawcett DW, Aoki A. The sertoli cell occluding junctions and gap junctions in mature and developing mammalian testis. *Dev Biol* 1976;50:142-68.
- 8 Madara JL, Pappenheimer JR. Structural basis for physiological regulations of paracellular pathways in intestinal epithelia. *J Membr Biol* 1987;100:149-64.
- 9 Mazariegos MR, Tice LW, Hand AR. Alteration of tight junctional permeability in the rat parotid gland after isoproterenol stimulation. *J Cell Biol* 1984;98:1865-77.
- 10 Sardet C, Pisam M, Maetz J. The surface epithelium of teleostean fish gills: Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. *J Cell Biol* 1979;80:96-117.
- 11 Milks LC, Conyers GP, Cramer EB. The effect of neutrophil migration on epithelial permeability. *J Cell Biol* 1986;103:2729-38.
- 12 Nash S, Stafford J, Madara JL. The selective and superoxide-independent disruption of intestinal epithelial tight junctions during leukocyte transmigration. *Lab Invest* 1988;59:531-7.
- 13 Shasby DM, Winter M, Shasby SS. Oxidants and conductance of cultured epithelial cell monolayers: inositol phospholipid hydrolysis. *Am J Physiol* 1988;255(Cell Physiol 24):C781-8.
- 14 Fasano A. Cellular microbiology: can we learn cell physiology from microorganisms? *Am J Physiol* 1999;276:C765-76.
- 15 Fasano A, Baudry B, Pumplun DW, et al. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA* 1991;88:5242-6.
- 16 Baudry B, Fasano A, Ketley J, et al. Cloning of a gene (ZOT) encoding a new toxin produced by *vibrio cholerae*. *Infect Immun* 1991;60:428-34.
- 17 Uzzau S, Cappuccinelli P, Fasano A. Expression of *vibrio cholerae* zonula occludens toxin and analysis of its subcellular localization. *Microb Pathog* 1999;27:377-85.
- 18 Fasano A, Fiorentini C, Donelli G, et al. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. *J Clin Invest* 1995;96:710-20.
- 19 Fasano A, Uzzau S, Fiore C, et al. The enterotoxic effect of zonula occludens toxin (Zot) on rabbit small intestine involves the paracellular pathway. *Gastroenterology* 1997;112:839-46.
- 20 Uzzau S, Lu R, Wang W, et al. Purification and preliminary characterization of the zonula occludens toxin receptor from human (CaCo2) and murine (IEC6) intestinal cell lines. *FEMS Microbiol Lett* 2001;194:1-5.
- 21 Marcial MA, Carlson SL, Madara JL. Partitioning of paracellular conductance along the ileal crypt-villus axis: a hypothesis based on structural analysis with detailed consideration of tight junction structure-function relationships. *J Membr Biol* 1984;80:59-70.
- 22 Wang W, Uzzau S, Goldblum SE, et al. Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci* 2000;113:4435-40.
- 23 Fasano A. Regulation of intercellular tight junctions by zonula occludens toxin (Zot) and its eukaryotic analogue zonulin. *Ann N Y Acad Sci* 2001;915:214-22.
- 24 El Asmar R, Panigrahi P, Bamford P, et al. Zonulin is involved in the impairment of the gut barrier function following small intestinal bacterial colonization. *Pediatr Res* 2000;47:960.
- 25 Isolauri E, Kaila M, Arvola T, et al. Diet during rotavirus enteritis affects jejunal permeability to macromolecules in suckling rats. *Pediatr Res* 1993;33:548-53.
- 26 Fasano A. Pathological and therapeutical implications of macromolecules passage through the tight junction. In: Cerejido M, Anderson J, eds. *Tight junctions*. Boca Raton, Florida: CRC Press (in press).
- 27 Fasano A, Not T, Wang W, et al. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* 2000;358:1518-19.
- 28 Ventura A, Magazzu G, Greco L. Duration of exposure to gluten and risk for autoimmune disorders in celiac patients. *Gastroenterology* 1999;117:297-303.
- 29 Fasano A, Uzzau S. Modulation of intestinal tight junctions by zonula occludens toxin permits enteral administration of insulin and other macromolecules in an animal model. *J Clin Invest* 1997;99:1158-64.
- 30 Watts T, Not T, Berti I, et al. Alterations in zonulin secretion parallels changes in intestinal permeability and precede the onset of diabetes in BB/Wor diabetic rats. *J Invest Med* 2000;48:184.
- 31 Meddings JB, Jarand J, Urbanski SJ, et al. Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. *Am J Physiol* 1999;276:G951-7.
- 32 Fasano A. Innovative strategies for the oral delivery of drugs and macromolecules. *Trends Biotechnol* 1998;16:152-7.
- 33 Di Piero M, Lu R, Uzzau S, et al. Zonulin occludens toxin structure-function analysis: identification of active fragments and the receptor binding domain. *J Invest Med* 2000;48:202.
- 34 Watts TL, Alexander T, Hansen B, et al. Utilizing the paracellular pathway: a novel approach for the delivery of oral insulin in diabetic rhesus macaques. *J Invest Med* 2000;48:185.