



Regulation of intestinal permeability: The role of proteases

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Abstract

The gastrointestinal barrier is - with approximately 400 m² - the human body's largest surface separating the external environment from the internal milieu. This barrier serves a dual function: permitting the absorption of nutrients, water and electrolytes on the one hand, while limiting host contact with noxious luminal antigens on the other hand. To maintain this selective barrier, junction protein complexes seal the intercellular space between adjacent epithelial cells and regulate the paracellular transport. Increased intestinal permeability is associated with and suggested as a player in the pathophysiology of various gastrointestinal and extra-intestinal diseases such as inflammatory bowel disease, celiac disease and type 1 diabetes. The gastrointestinal tract is exposed to high levels of endogenous and exogenous proteases, both in the lumen and in the mucosa. There is increasing evidence to suggest that a dysregulation of the protease/antiprotease balance in the gut contributes to epithelial damage and increased permeability. Excessive proteolysis leads to direct cleavage of intercellular junction proteins, or to opening of the junction proteins *via* activation of protease activated receptors. In addition, proteases regulate the activity and availability of cytokines and growth factors, which are also known modulators of intestinal permeability. This review aims at outlining the mechanisms by which proteases alter the intestinal permeability. More knowledge on the role of proteases in mucosal homeostasis and gastrointestinal barrier function will definitely contribute to the identification

of new therapeutic targets for permeability-related diseases.

Key words: Intestinal permeability; Intestinal barrier; Tight junction; Paracellular permeability; Proteases; Proteinase-activated receptor; Protease inhibitor; Antiproteases

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Core tip: Increased intestinal permeability is a novel player in the pathophysiology of various intestinal and extra-intestinal diseases such as inflammatory bowel disease, celiac disease and type 1 diabetes. A dysregulated protease/antiproteases balance is suggested as a cause of intestinal barrier dysfunction, with a subsequent increase in permeability. Immune cells infiltrating in the lamina propria during inflammatory conditions provide a pro-inflammatory environment by the production of cytokines and proteases. Protease inhibition has therapeutic potential but more research is needed to elucidate the exact involvement of specific proteases in gut physiology and intestinal barrier function.

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INTRODUCTION

The intestinal barrier represents the largest interface between the external environment and the internal milieu. Given the enormous intraluminal load of essential and noxious molecules, a selectively permeable barrier is indispensable for maintaining mucosal homeostasis^[1]. The intestinal barrier serves a dual function: on the one hand limiting host contact with pathogens and antigens and on the other hand at the same time allowing the absorption of nutrients and water. Physical, biochemical and immune elements make up the heterogeneous intestinal barrier and collaborate to exert these functions. Firstly, the mucus layer covers the entire epithelial cell surface and consists of gel-forming mucins, produced by the goblet cells. This chemical barrier also contains defensins or antimicrobial peptides that are secreted by Paneth cells within the epithelial cell layer^[2]. Secondly, the epithelial cell layer itself is a physical barrier that consists for 80% of enterocytes, regulating nutrient absorption *via* specific transporters, channels and receptors (transcellular transport)^[3]. Finally, the immunological barrier consists of microfold (M) cells in the epithelial

cell layer and patrolling antigen presenting cells (APC) in the lamina propria. The M cells constantly sample luminal antigens and deliver them to APC such as dendritic cells and macrophages. Innocuous antigens drive the APCs to create a tolerogenic environment with the production of immunosuppressive factors such as IL-10, TGF- β and nitric oxide. Further tolerance is thereby created through the induction of regulatory T cells^[4]. Noxious antigens are also recognized by the APCs and trigger the activation of the inflammatory cascade, starting with T cell activation^[5,6].

The movement of molecules, solutes and ions across the intestinal epithelial cell layer can take place by the trans- or paracellular pathway. Transcellular transport is the main route for nutrient absorption and is facilitated through size- and charge- selective channels and transporters. The paracellular pathway is less selective since it occurs through the intercellular space between neighboring intestinal epithelial cells. The capacity of this paracellular pathway is however low as cells are bound tightly together by junction proteins, with particularly the tight junctions (TJ) regulating transport in response to numerous stimuli^[7].

In the last decade, a barrier defect is suggested as a common factor in the onset of various local and systemic diseases of an inflammatory, autoimmune or functional nature such as inflammatory bowel disease (IBD), celiac disease, irritable bowel syndrome (IBS), type 1 diabetes mellitus and multiple sclerosis^[8-13]. For more detailed information on the relation between intestinal barrier function and these disease pathologies, we refer the readers to Odenwald and Turner who nicely reviewed this topic in 2013^[14] and very recently updated their overview in 2016^[15]. Although the literature data are rather scarce, proteases are believed to regulate the intestinal permeability. They can intervene directly by their proteolytic action on the junction proteins, both intra- and extracellularly, and indirectly through activation of proteinase-activated receptors (PARs). This review provides an overview of the proteases (Table 1) putting emphasis on their role as regulators of the intestinal paracellular permeability.

INTESTINAL TIGHT JUNCTIONS REGULATE PARACELLULAR PERMEABILITY

The intercellular spaces between neighboring intestinal epithelial cells are sealed by the apical junction complex, which contains TJs and adherens junctions, and by the subjacent desmosomes (Figure 1). The selective paracellular permeability is mediated by the TJs, which encircle the apical end of the intercellular spaces^[7]. Various proteins make up TJs, including the adhesive transmembrane proteins occludin, claudins and junctional adhesion molecules as well as cytoplasmic proteins such as zonula occludens

Table 1 Overview of proteases affecting intestinal permeability

Protease	Effect	Model	Mechanism of action	Ref.
Serine proteases				
Matriptase	Protective	ST14 hypomorphic mice	Genetic depletion of matriptase induces an increase in intestinal permeability (decreased TER and increased FITC-dextran flux)	[65, 66]
		Epithelial cell monolayer	Inhibition of matriptase with silencing RNA and the synthetic inhibitor MI-432 increased the intestinal permeability (decreased TER and increased FITC-dextran flux)	[65, 68]
Granzyme M	Protective	GrzM ^{-/-} mice	GrzM ^{-/-} mice display a permeability increase (FITC-dextran method)	[73]
Zonulin, Zonula occludens toxin (Zot)	Harmful	Human epithelial cell monolayer	↑ Permeability after exposure to gliadin (triggers zonulin release; disruption of occludin and ZO-1)	[76]
		Ileal tissue of diabetes prone rats	Zonulin-dependent permeability increase in diabetic rats was abolished after oral treatment with zonulin inhibitor FZI/0 (AT1001/Larazotide)	[82]
PAR₂ activation				
Trypsin, tryptase, chymase, synthetic SLIGRL	Harmful	WT mice, WT rats	↑ Permeability due to PAR ₂ activation (confirmed by selective PAR ₂ agonist SLIGRL; increased ⁵¹ Cr-EDTA flux)	[47, 48, 51]
PAR₁ activation				
Cathepsin G	Harmful	Colonic biopsies from UC and healthy patients	↑ Permeability in response to UC fecal supernatant was abolished by cathepsin G inhibition	[58]
PAR₁ activation				
Thrombin, synthetic TFLLR-NH2	Harmful	WT mice, epithelial cell monolayer	↑ Permeability after PAR ₁ activation (caspase-3 mediated; disruption of ZO-1)	[62]
Endogenous inhibitors				
Elafin	Protective	Gluten sensitive mice	↓ Permeability after elafin delivery by recombinant <i>Lactococcus lactis</i> (⁵¹ Cr-EDTA flux)	[87]
		Human epithelial cell monolayer	Treatment with elafin normalized the TNF-α-induced increase in paracellular permeability (FITC-dextran method)	[88]
Synthetic inhibitors				
Camostat mesilate	Protective	Rat IBS model	Treatment with camostat mesilate normalized the elevated permeability in the rats (⁵¹ Cr-EDTA flux and ZO-1 expression)	[89]
Nafamostat mesilate	Protective	Rectal biopsies from IBS and healthy patients	Nafamostat abolished the trypsin-induced hyperpermeability (macromolecular flux in Ussing chambers)	[94]
		Human epithelial cell monolayer	Treatment with nafamostat normalized the trypsin-induced permeability increase (TER and FITC-dextran method)	[95]
SPI	Protective	IBD mouse model	Treatment with SPI normalized the increased permeability in the T-cell transfer colitis model (FITC-dextran method)	[96]
Metalloproteases				
Mepriin β	Protective	Mep1b ^{-/-} mice	Mepriin β cleaves MUC2 and alters mucus composition	[128, 129]
Matrix metalloproteinases				
MMP-2	Protective	MMP-2 ^{-/-} mice	↑ permeability in MMP-2 ^{-/-} mice (FITC-dextran method)	[111]
MMP-9	Harmful	MMP-9 ^{-/-} mice	= Permeability in MMP-9 ^{-/-} mice after DSS (FITC-dextran method; no increase in MLCK expression)	[114]
		MMP-9 ^{-/-} mice	↑ Goblet cells and MUC2 expression in MMP-9 ^{-/-} mice	[113]
		MMP-9 transgenic mice	↑ Permeability in mice overexpressing MMP-9 (FITC-dextran method)	[112]

MMP-3, MMP-7 ADAM TACE/ADAM17	Harmful	Epithelial cell culture	MMP-7 cleaves E-cadherin	[121]
	Harmful	Human and mouse colon samples Caco-2	↑ TACE activity in IBD; ↑ TNF- α release; ↑ TNF- α -induced permeability increase ↓ Permeability after TACE inhibition (by TAPI-2 and GM6001)	[131, 134, 135] [136]
Cysteine proteases Caspase-3, caspase-8	Harmful	Human epithelial cell monolayer	↓ Cell-cell adhesion (epithelial cell apoptosis; disruption of TJ proteins occludin and claudin-4)	[144]
Endogenous inhibitor Cystatin	No effect	WT mice	No effect on colonic paracellular permeability (51 Cr-EDTA flux)	[51]
Luminal proteases Bacteroides fragilis Fragilysin	Harmful	Human epithelial cell monolayer	↑ Permeability (decreased TER and increase in mannitol flux)	[149, 150]
Entamoeba histolytica Cysteine protease	Harmful	Mice transfected with E. histolytica trophozoites	↑ Permeability (FITC-dextran method)	[151]
Enterococcus faecalis Gelatinases	Harmful	IL10 $^{-/-}$ mice Epithelial cell monolayers	↑ Permeability (E-cadherin splicing) ↑ Permeability (PAR $_2$ signaling)	[156] [155]
Dermatophagoides pteronyssinus Der p 1	Harmful	Human colonic biopsies	↑ Permeability (decreased TER in Ussing chambers; disruption of TJ proteins occludin and ZO-1)	[158]
Kiwifruit cysteine protease Act d1	Harmful	Epithelial cell monolayer	↑ Permeability (disruption of TJ proteins occludin and ZO-1)	[162]
		WT mice	↑ Permeability (FITC-dextran method)	[161]
Aspergillus Amano SD	Protective	WT rat	Improved mucosal homeostasis through alteration of the microbiome composition and SCFA induction	[163]

TJ: Tight junction; PARs: Proteinase-activated receptors; MLC: Myosin light chain; MLCK: Myosin light chain kinase; PKC: Protein kinase C; ROCK: Rho-associated protein kinase; ZO-1: Zonula occludens 1.

(ZO) proteins (Figure 2). The latter act as scaffolding proteins that connect the transmembrane proteins at their cytoplasmic C-terminal strands with F-actin, a filamentous cytoskeleton component^[16]. The adherens junction transmembrane protein, epithelial-cadherin (E-cadherin), is connected to F-actin *via* intracellular proteins of the catenin-family^[17].

The opening of the intercellular spaces is achieved by contraction of the actomyosin microfilaments. Myosin is a motor protein that co-localizes with F-actin and converts chemical energy from adenosine triphosphate into mechanical energy. A crucial step in the induction of this mechanochemical contractile machinery is the phosphorylation of myosin light chain (MLC), the regulatory component of myosin (Figure 2). Myosin light chain kinase (MLCK) mediates the phosphorylation of MLC upon activation in response to Ca²⁺/calmodulin binding. However, there is evidence for other intracellular signaling pathways besides the calmodulin pathway to activate MLCK. The extracellular signal-regulated kinases (ERK1/2) have shown to induce MLCK activation^[18]. Protein kinase C (PKC) on the other hand favors the phosphorylation of MLC by the inhibition of myosin light chain phosphatase (MLCP), the enzyme that dephosphorylates MLC^[19].

Rho-associated protein kinase (ROCK) can increase contractility both by activating MLCK and inactivating MLCP, favoring MLC phosphorylation^[20]. The phosphorylation status of myosin light chain induces a change in myosin tertiary structure causing myosin to “walk” along the actin filaments, increasing the tension in the cytoskeleton resulting in the disruption and cytosolic migration of TJ proteins^[21,22]. This results in an impaired barrier function which is also referred to as a “leaky” barrier. Potentially noxious luminal proteins can now migrate to the underlying mucosal tissue and provoke a pro-inflammatory response. Even whole bacteria can cross the epithelial cells unrestricted at sites of epithelial damage caused by erosions and ulcers in GI disease. A “leaky” gut and epithelial damage often co-exist in disease state^[14].

MUCOSAL IMMUNOLOGY AND BARRIER FUNCTION

In both physiological and pathological conditions, various mediators are able to affect the TJ conformation in order to control the paracellular permeability in epi- and endothelial cell layers throughout the body. Growth factors, cytokines, intestinal bacteria, dietary

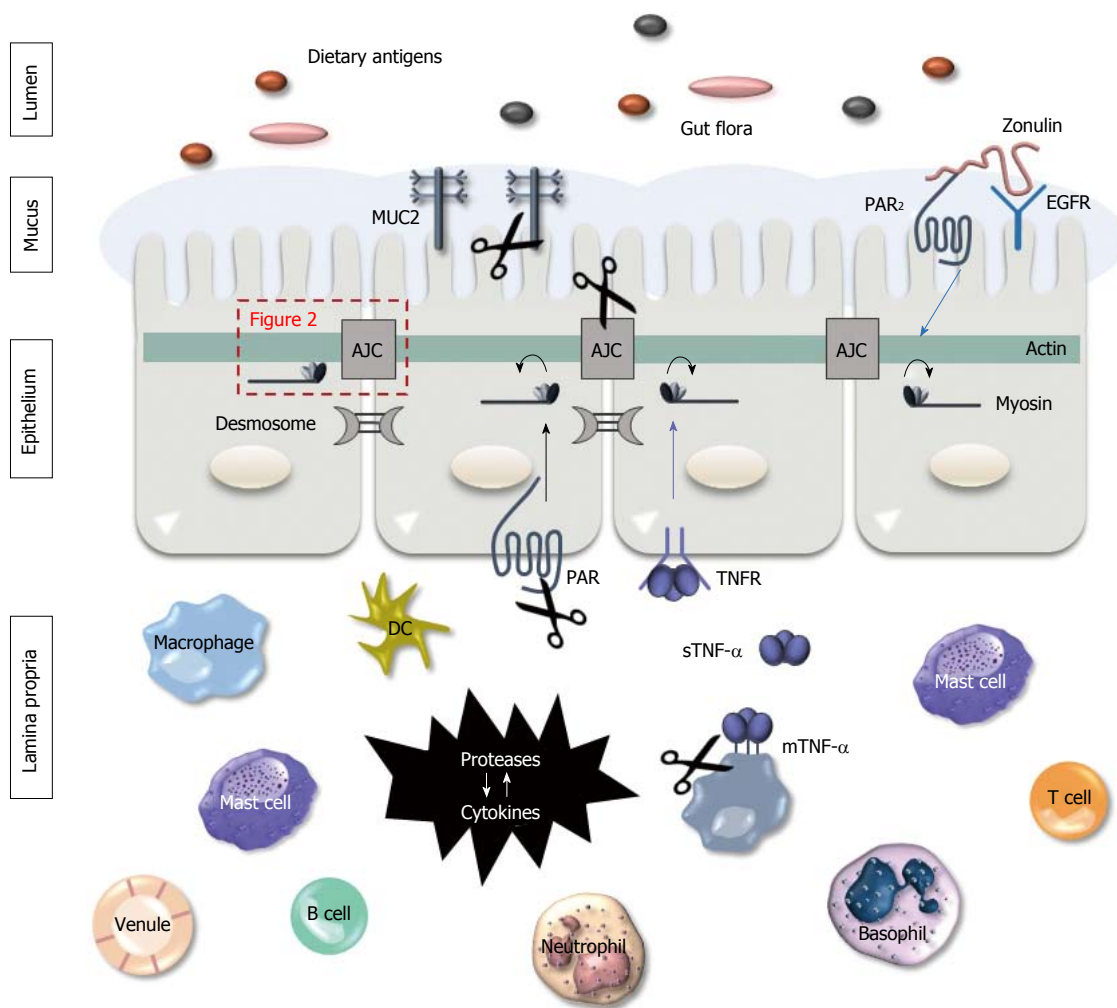


Figure 1 Proteases mediate gut barrier function. Intestinal epithelial cells are constantly exposed to proteases, both on their apical and basolateral side. Luminal proteases can be endogenous (e.g., pancreatic proteases) or can originate from bacteria or food particles present in the lumen. Their proteolytic activity can cause damage to the mucus layer and the junction proteins, affecting the barrier function. In the lamina propria, proteases are produced by various inflammatory cells and by the intestinal epithelial cells. In inflammatory conditions such as inflammatory bowel disease (IBD), immune cells infiltrate in the lamina propria where they produce various cytokines and proteases, contributing to the pro-inflammatory environment. Proteases stimulate immune cells to produce cytokines and vice versa. Besides, they alter the paracellular permeability by direct proteolytic cleaving of the junction proteins and by activation of the proteinase-activated receptors (PARs) on the epithelial cell surface, that induces a contraction of the actomyosin complex and subsequent opening of the apical junction complex (AJC; more in detail in Figure 2).

components and proteases are known to regulate the intestinal TJ opening^[3,23-25]. Though the barrier-regulating capacity of pro-inflammatory cytokines is well studied, the effect of other mediators has received far less attention^[24,26].

An inflamed mucosa -as seen in IBD patients- is characterized by the presence of cytokines amongst which TNF- α and IFN- γ , which are produced by a variety of cells including macrophages, T-cells and natural killer (NK) cells. The binding of these cytokines to specific receptors on the surface of infiltrating immune cells initiates a cascade of events starting with the activation of cell signaling pathways leading to the production of more inflammatory mediators (NF κ B) or apoptosis maintaining on their turn the inflammatory process. Extensive reviews have been published on the regulation of TJs by cytokines^[24,27]. It has been shown in cell culture experiments that

TNF- α and IFN- γ regulate the paracellular permeability through the activation of MLCK, resulting in MLC hyperphosphorylation and opening of the TJs^[28-30], while IL-4 and IL-13 increase paracellular permeability through the induction of the pore-forming claudin-2 and apoptotic pathways^[31-33].

Next to cytokines also proteases are released into the mucosa by inflammatory cells such as macrophages, neutrophils and mast cells to regulate inflammation. On the one hand these proteases degrade the extracellular matrix, mucosal proteins and even live bacteria^[34]. On the other hand, proteases act as signaling molecules *via* specific receptors, which will be discussed in the next section.

Intestinal epithelial cells also express receptors for cytokine and protease signaling. Since the apical and basolateral membranes of the intestinal epithelial cells are constantly exposed to large amounts of bacterial

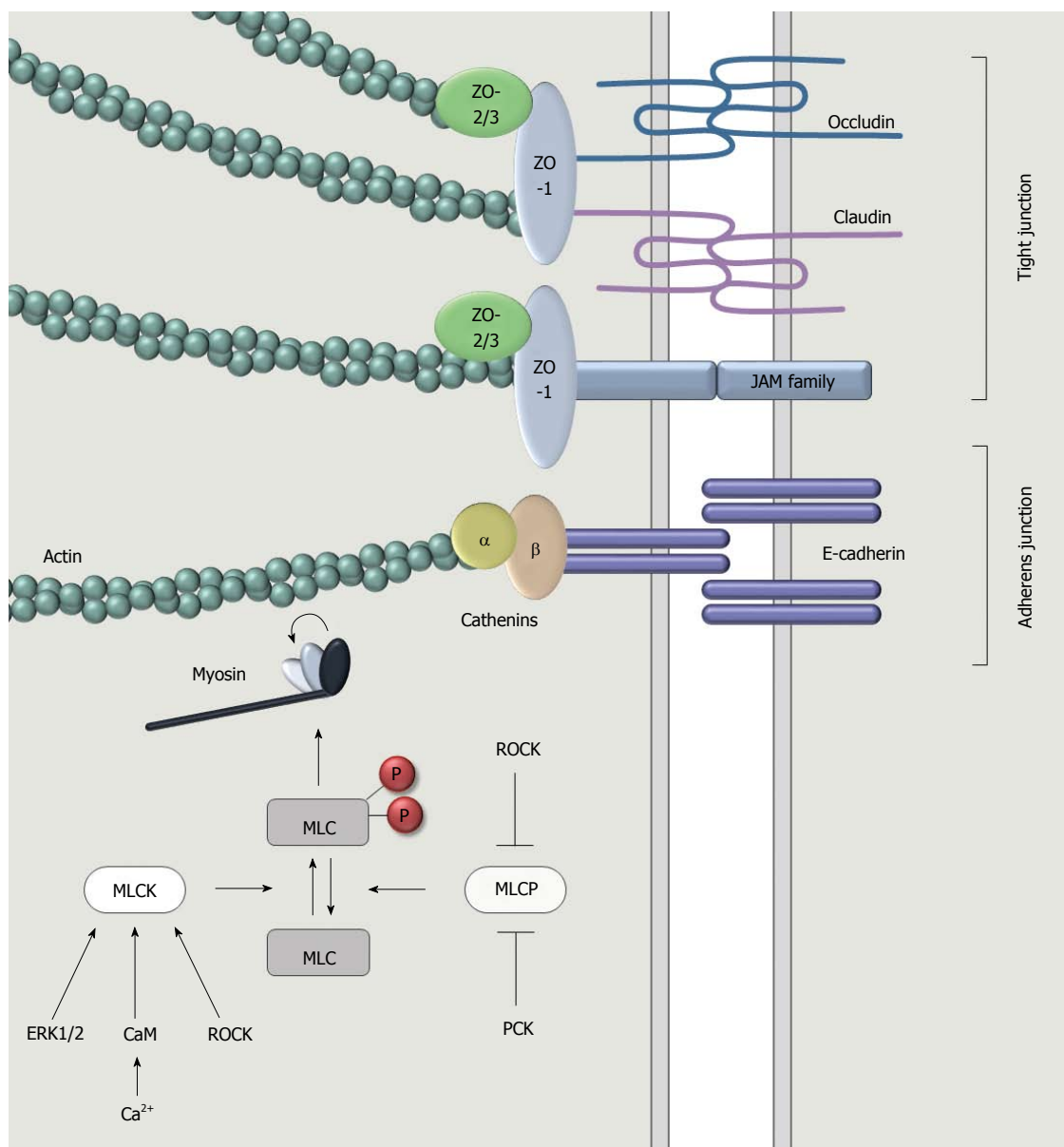


Figure 2 A more detailed representation of the apical junction complex at the intercellular surface between adjacent intestinal epithelial cells. Tight junctions are comprised of three types of transmembrane proteins: occludin, claudins and junctional adhesion molecules (JAMs). Adaptor proteins such as zonula occludens 1 (ZO-1), ZO-2 and ZO-3 connect the transmembrane proteins to filamentous actin. This cytoskeleton component interacts with myosin to induce a contraction, followed by the opening of the intercellular space. Myosin light chain (MLC) is the main regulator of this contractile machinery. Contraction occurs when MLC is phosphorylated. This is regulated through the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), which is on their turn regulated by intracellular signaling pathways involving for instance the extracellular signal-regulated kinases (ERK1/2), calcium, calmodulin, protein kinase C (PKC) or Rho-associated protein kinase (ROCK).

and endogenous proteases, the function of these proteolytic enzymes in intestinal barrier homeostasis should be further elucidated.

PROTEASES AND PROTEINASE-ACTIVATED RECEPTORS

Defined in general manner, proteases are enzymes that hydrolyze a peptide bond and in this respect they are best known for their digestive properties *e.g.*, pancreatic proteases. However, also bacteria, epithelial cells, resident and infiltrating inflamma-

tory cells produce luminal and mucosal proteases exerting various biological functions, both intra- and extracellularly. For instance, proteases are vital in inflammation, apoptosis, coagulation and cell growth and migration^[35,36]. Since excessive proteolysis can cause tissue damage, a tight regulation of protease activity in order to prevent pathology is necessary. There are multiple mechanisms that control the protease activity such as the synthesis as inactive zymogens that require proteolytic cleaving for activation and on the other hand the termination of protease activity by endogenous inhibitors or

antiproteases. A dysregulation in the protease balance with an increased protease activity has been observed in gastrointestinal diseases such as IBD and IBS, making protease inhibition by endogenous or synthetic inhibitors a potential therapeutic intervention^[37]. Proteases are classified based on their mechanism of hydrolysis of the target peptide bond. This implicates that all proteases that belong to the same clan, share the same nucleophilic amino acid in their active site and are more likely to react with the same inhibitors. In mammals, five classes of proteases have been identified: serine-, metallo-, cysteine-, aspartate- and threonine proteases.

Proteases are not merely degrading enzymes. They can also act as signaling molecules by the proteolytic activation of the PARs. Four receptors have been identified in this family (PAR₁, PAR₂, PAR₃, PAR₄)^[38]. Activation of these G-protein coupled receptors occurs after the proteolytic truncation of the N-terminal extracellular tail, releasing a new N-terminus that functions as a tethered ligand. This specific domain binds the second of three extracellular loops on the receptor and thereby generates an intracellular signal. PARs are expressed ubiquitously among tissues and cell types. In the gut, they are present on epithelial cells, endothelial cells, neurons, inflammatory cells, mast cells, smooth muscle cells and fibroblasts. Depending on the cell type, different signaling pathways have been described^[39]. This also implicates that proteases mediate different GI physiological processes such as motility, cell proliferation and apoptosis, immune response, cytokine production, neurogenic inflammation, pain and epithelial barrier function through PAR activation^[40]. For a more detailed description of this topic, we refer the reader to the companion review in (WJG Dec. 2016) from our group, illustrating this topic in detail^[41].

SERINE PROTEASES

Of all proteolytic enzymes, serine proteases are by far the most abundant group^[42,43]. Their successful mechanism of hydrolysis of peptide bonds occurs throughout the entire body in functionally diverse processes including digestion, immune response, blood coagulation, fibrinolysis, apoptosis and pro-hormone processing^[42]. Serine proteases act both directly and indirectly as paracrine signaling effectors on PARs, provoking intracellular signals in order to mediate these vital processes. During the inflammatory cascade for example, proteases are released by infiltrating inflammatory cells and modulate the bioactivity of cytokines and chemokines by proteolytic cleavage^[37]. For instance, the N-terminal truncation of CXCL-8 and CXCL-5 respectively by proteinase 3 and cathepsin G provides an increased chemotactic activity towards neutrophils^[44,45]. Most proteases involved in PAR

activation on the other hand belong to the serine clan of proteases^[8]. In inflammatory cells, the activation of the G-protein coupled pathway leads to downstream activation and nuclear translocation of NFκB. In response, the cell synthesizes pro-inflammatory cytokines boosting inflammation^[46]. In epithelial cells, paracrine signaling of proteases through PARs induces changes in paracellular permeability which will be discussed in the next paragraph.

The majority of the research in the field of intestinal permeability involves PAR₂. In 2002, it was shown for the first time by Coelho *et al.*^[47] and Cenac *et al.*^[48] that PAR₂ activation by the serine proteases trypsin, tryptase and chymase induced an increase in the colonic permeability of Cr⁵¹-EDTA, a marker of paracellular permeability^[49,50]. The selective synthetic PAR₂ agonist SLIGRL (H-serine-leucine-isoleucine-glycine-arginine-leucine-OH) mimicked the effect of endogenous serine proteases, thereby increasing intestinal permeability^[47,51]. Although PAR₂ receptors are expressed on both apical and basolateral membranes of epithelial cells, some authors suggest that only apical administration of PAR₂ agonists - and not intraperitoneal (i.p.) administration - alters the intestinal barrier function^[51]. Other authors however provided proof of direct basolateral PAR₂ activation and a subsequent increase in paracellular permeability^[18,52]. Further investigation into the mechanism of action revealed the involvement of calmodulin and MLCK in the PAR₂-mediated alterations of paracellular permeability as intracolonic injection of SLIGRL increased the MLC phosphorylation on western blot. Pretreatment with ML-7, an MLCK inhibitor, abolished the elevated mucosal permeability caused by contraction of the epithelial cell cytoskeleton after phosphorylation of MLC^[53]. In addition, MLCK was activated by the Ca²⁺-binding messenger protein calmodulin since precipitation of MLCK revealed an increased binding of calmodulin and the inhibition of calmodulin by chlorpromazine reduced the SLIGRL-induced increase in paracellular permeability^[54,55]. Besides the calmodulin pathway, also ERK1/2 can activate MLCK which directly leads to the disruption of TJ composition and function^[18]. The increased permeability induced by tryptase in cultured colonocytes was not only abolished by a tryptase inhibitor but also by the ERK1/2 inhibitor UO126. Finally, incubation of a human intestinal epithelial cell line (SCBN) with SLIGRL induced the disruption and migration into the cytoplasm of the TJ protein ZO-1^[55].

The latest discovered member of the PAR-family, PAR₄, is receiving increasing attention^[56]. This is mainly due to the discovery that cathepsin G is a PAR₄-selective neutrophil serine protease. As cathepsin G is a neutrophil protease (alongside with proteinase 3 and neutrophil elastase) it might represent an inflammatory mediator in conditions such as IBD, where neutrophil accumulation within the submucosa is considered a hallmark^[57]. And indeed, ulcerative colitis (UC) patients

express higher colonic levels of cathepsin G and PAR₄, both involved in the increased paracellular permeability in UC patients^[58]. This was proven by using the PAR₄ inhibitor P4pal-10, a pepducin that selectively blocks PAR₄ signaling^[59]. In Ussing chamber experiments with mice colonic strips, fecal supernatant of UC and Crohn's disease (CD) patients triggered a significant increase in FITC dextran permeability abolished by the pre-treatment with P4pal-10 in strips triggered with UC supernatant but interestingly not for the strips triggered with the supernatant of CD patients^[58]. In addition, the PAR₄ activating peptide AYPGKF-NH₂ was able to induce an increased mucosal permeability with a similar effect as the UC fecal supernatant^[60]. Finally, pre-incubation of UC fecal supernatant with the specific cathepsin G inhibitor completely normalized the elevated permeability effect^[58]. These data suggest that cathepsin G plays a predominant role in the pathophysiology of UC by activating PAR₄, while this is not shown in CD. However there is a lack of studies investigating the expression of cathepsin G and comparing this expression between UC vs CD patients. Other proteases are likely to play a key role in barrier function in CD.

Also PAR₁-signaling induces epithelial barrier dysfunction, but in this case apoptosis seems to mediate the phenomenon. Treatment with PAR₁ agonists (thrombin and selective PAR₁ activating peptide TFLLR-NH₂) increased the paracellular permeability *in vitro* in an epithelial cell line (SCBN) as well as *in vivo* in mice. This permeability increase depends on the disruption of ZO-1. Chromatin condensation and nuclear fragmentation, hallmarks for apoptosis, were induced in a caspase-3-dependent manner. Interestingly, pretreatment with a caspase-3 inhibitor (Z-DEVD-FMK), which irreversibly inhibits apoptosis^[61], completely abolished the effect of the PAR₁ agonists on permeability and apoptosis. Also the MLCK inhibitor ML-9 abolished the abnormalities^[62]. Apart from PARs, also specific serine proteases were studied in the field of intestinal permeability.

A serine protease that is important for the intestinal barrier homeostasis is the transmembrane protein matriptase, or membrane type serine protease-1. A critical role in the epithelial barrier formation and the apical junction complex assembly is attributed to this trypsin-like serine protease^[63,64]. Suppressor of tumorigenicity-14 (ST14) hypomorphic mice, which express less than 1% of the matriptase mRNA levels present in the intestine of control littermates, were found to have an impaired intestinal barrier as measured by transepithelial electrical resistance and FITC-dextran permeability^[65,66]. This could explain the increased susceptibility of ST14 hypomorphic mice to DSS colitis, with a 30% survival rate after 7 d DSS vs 100% in control littermates^[67]. Not only genetic depletion but also pharmacological inhibition

(with MI-432) and RNAi silencing of matriptase modulate the TJ assembly, causing the opening of the paracellular gate^[65,68]. In physiological conditions, matriptase regulates the expression pattern of the "pore-forming" TJ protein claudin-2. Since there is no evidence of direct proteolytic processing by matriptase, it is likely that matriptase enhances the claudin-2 protein turnover *via* activation of protein kinase C-zeta (PKC- ζ)^[65]. Other studies also reported an association of matriptase with other TJ proteins. For instance in ST14 hypomorphic mice, multiple grades of TJ disruption were identified with expression patterns of occludin, ZO-1 and claudin-1 ranging from a decreased protein expression to areas of complete absence, whereas claudin-2 was upregulated^[66,67]. E-cadherin levels however remained unaltered despite co-localisation of matriptase with E-cadherin^[65]. In addition, matriptase expression in inflamed colonic tissues from CD and UC patients is significantly downregulated, making matriptase induction a potential therapeutic strategy^[68]. Recently, Pászti-Gere *et al.*^[69] showed that reinforcement of the intestinal barrier is in fact possible by the induction of matriptase. Incubation of an intestinal epithelial cell line with the matriptase activator sphingosine-1-phosphate increased the TER and resulted in an upregulation of occludin at the apical junction. Also *in vivo* it was shown that matriptase restoration recovers the barrier integrity by decreasing permeability-associated claudin-2 protein levels and thereby protecting against DSS colitis^[67]. Although it should be noted that a tight regulation of matriptase is necessary, since an overexpression could result in malignancies due to its involvement in epithelial proliferation^[70-72].

Recently, serine protease granzyme M was also shown to be essential for normal barrier function^[73]. In this study, mice deficient of granzyme M were more susceptible to DSS colitis and showed an elevated paracellular permeability compared to wild type (WT) mice. Furthermore, granzyme M expression was upregulated in the inflamed colon tissue samples from UC patients, suggesting that granzyme M acts to induce colonic protection during active disease^[73].

In 2000, Fasano *et al.*^[74] discovered the serine protease analogue zonulin, the first known endogenous physiologic modulator of TJ proteins regulating the paracellular permeability. The human protein zonulin is similar to Zonula occludens toxin (Zot) that was discovered earlier in *Vibrio cholerae*. It increased intestinal paracellular permeability in a similar fashion^[75]. Luminal exposure to bacteria and the gluten component gliadin are identified as the two most powerful triggers for zonulin release in the gut^[76,77]. In addition, gliadin can cause celiac disease in genetically susceptible individuals, which is associated with increased paracellular permeability. The expression level of zonulin was shown to be increased in the intestinal submucosa of celiac disease patients^[74]. Also in CD

patients, serum zonulin levels are higher compared to their relatives and to healthy control subjects. Interestingly, 50% of the first degree relatives had serum zonulin levels that were increased tremendously (more than two times the standard deviation above the mean) whereas this large increase could only be observed in 4.9% of controls^[78]. For both zonulin and Zot, the mechanism of opening intercellular TJs is likely to resemble the effect of certain serine proteases (*cf. supra*) although it is not fully elucidated yet. It is suggested that zonulin and Zot cause TJ disassembly by activating the epidermal growth factor receptor (EGFR) on the epithelial cell through transactivation of PAR₂ (Figure 1)^[78,79]. The involvement of PAR₂ in EGFR activation was confirmed by the findings that zonulin could not reduce the TER in ileal strips from PAR₂^{-/-} mice in contrast to strips of WT mice. Subsequently, intracellular signaling involves protein kinase C- α (PKC- α)-activation that leads to polymerization of F-actin, TJ disassembly and opening of the paracellular space^[80]. Meanwhile, a synthetic octapeptide resembling the receptor-binding domain of zonulin was developed. This molecule, named Larazotide acetate or AT-1001, prevents the opening of TJs in response to zonulin by competitively antagonizing the zonulin receptors (EGFR and PAR₂)^[81,82]. Clinical trials are currently ongoing to assess its efficacy as a therapy for celiac disease, but there also is evidence for therapeutic potential in other pathologies such as IBD^[83,84].

Serine protease inhibitors

Two families of endogenous protease inhibitors, the Serpins and Chelonianins, tightly regulate the activity of the serine proteases by binding to the target protease and largely adjusting its confirmation leading to an irreversible disruption of the active site^[85]. A defect in the protease/antiprotease balance leads to tissue damage due to excessive proteolytic capacity causing gastrointestinal diseases.

Serpins are most studied in their role in controlling the coagulation cascade. However, in the gastrointestinal tract, secretory leucocyte protease inhibitor (SLPI) and elafin are of importance. Both are produced by intestinal epithelial cells or leucocytes and inhibit neutrophil elastase and proteinase-3. Besides, SLPI is also a potent inhibitor of trypsin, trypsin, chymotrypsin, chymase and cathepsin G^[37]. Although elafin, or skin-derived antileukoproteinase, and SLPI are poorly investigated when it comes to barrier function disturbances, an elevated expression of these antiproteases was reported in "leaky gut"-related gastrointestinal diseases. For instance in CD and UC patients it was shown that the levels of elafin and SLPI are significantly higher in inflamed colonic tissue vs noninflamed and control tissues. Interestingly, the upregulation was less pronounced in inflamed CD samples vs inflamed UC samples^[86]. In contrast, the elafin expression in small intestinal samples was lower in patients with active celiac disease compared

to control patients. Local treatment of gluten-sensitive mice with elafin using a recombinant *Lactococcus lactis* vector, restored the intestinal barrier function and normalized the ZO-1 expression, which was disrupted in a mouse model of celiac disease^[87]. *In vitro* experiments in a Caco-2 cell line confirm these barrier-protective effects of elafin. Cells treated with TNF- α to induce a barrier defect, showed a complete restoration in paracellular permeability after simultaneous treatment with elafin^[88].

Restoring an impaired barrier by pharmacological protease inhibition has been proposed as a promising therapeutic treatment option for IBD, functional GI disorders such as IBS and for colorectal cancer^[37] because epithelial barrier dysfunction is a common factor in these diverse pathologies. Studies with the synthetic broad specificity serine protease inhibitors nafamostat mesilate and camostat mesilate revealed positive effects on paracellular permeability. In an animal model for IBS, where the colonic permeability towards ⁵¹Cr-EDTA was elevated and ZO-1 disrupted, treatment with camostat mesilate not only normalized the fecal protease activity but also the colonic permeability significantly improved and the ZO-1 protein levels were restored^[89]. Nafamostat mesilate, that has shown beneficial effects on disease outcome in animal models of colitis^[90], IBS^[91], acute pancreatitis^[92] and colorectal cancer^[93], also seems to restore the intestinal barrier function. The addition of trypsin to the basolateral side of human colonic strips mounted in Ussing chambers increased the permeability proportional to the trypsin concentration. This was abolished after simultaneous addition of nafamostat mesilate^[94]. In an *in vitro* co-culture model, Caco-2 cells responded to mast cell degranulation with a disruption of epithelial integrity shown by a decrease in TER, an increase of FITC-dextran flux and a decrease in the expression of the TJ proteins claudin-1 and ZO-1. These effects were prevented by trypsin inhibition using nafamostat mesilate^[95]. These positive findings of nafamostat on intestinal permeability could however not be confirmed *in vivo* in a chronic animal model of T-cell transfer colitis (unpublished results). However, in our own lab, a beneficial effect of a novel serine protease inhibitor (Di-(4-acetamidophenyl) 1-(benzyloxycarbonylamino)-2-[(4-guanidino)phenyl]ethanephosphonate trifluoroacetate; abbreviated as SPI in Table 1) was shown in a T-cell transfer colitis model. A curative i.p. treatment with this novel protease inhibitor abolished the elevated intestinal permeability that was seen in the vehicle-treated colitis animals while also exerting anti-inflammatory effects whereas nafamostat only showed the anti-inflammatory effects^[96].

METALLOPROTEASES

Matrix metalloproteases

Matrix metalloproteases (MMPs) are generally

known for their ability to degrade and remodel the extracellular matrix (ECM). But in addition to their role in ECM turnover, MMPs degrade or proteolytically activate a wide range of molecules such as chemokines, cytokines, growth factors, membrane receptors, cytoskeleton proteins and junctional proteins^[97,98]. Under normal physiological conditions, their activity is tightly regulated by the tissue inhibitors of metalloproteases (TIMP-1-4). A dysregulation of the balance between MMPs and TIMPs is associated with inflammation and tissue damage^[99,100]. Indeed, various studies have reported an upregulation of MMPs in inflamed IBD epithelium, suggesting that the inhibition of MMPs could be an interesting therapeutic intervention in IBD^[99,101-104]. However, the failure of MMP inhibitors in cancer trials has led to rethink the clinical potential of these compounds^[105,106]. Indirect inhibition of the effects of MMPs or intervening in the signal transduction pathways influenced by MMPs seems more likely to be successful. In this respect, the *new* physiological and pathological roles of MMPs in specific diseases are being further investigated, such as their effect on the epithelial barrier integrity, discussed below.

Previous studies have demonstrated the upregulation of the gelatinases MMP-2 and MMP-9 in IBD patients^[103,107,108] as well as in animal models of colitis^[109-111]. It was found that a specific overexpression of MMP-9 in the intestinal epithelium is associated with a defective barrier function and mucin production due to a decrease in goblet cell differentiation^[112]. Supporting their role in barrier function, MMP-9^{-/-} mice have an increased number of goblet cells and MUC-2 expression compared to WT mice^[113]. Recently, Nighot *et al.*^[114] showed an attenuation of the DSS-induced increase in colonic permeability in MMP-9^{-/-} mice. The protein levels of tight junctional occludin were elevated in MMP-9^{-/-} mice, both DSS- and vehicle-treated, vs WT mice. It was also found that the protein expression of MLCK was upregulated in DSS colitis in WT mice but not in MMP-9^{-/-} mice. Interestingly, MLCK is a key regulator of TJ permeability as described above^[115], suggesting that MMP-9 is a key regulator of TJ permeability *via* MLC phosphorylation. The other gelatinase, MMP-2, plays a barrier protective role in contrast to MMP-9. Mice deficient from MMP-2 have an impaired intestinal barrier function, making them more susceptible to develop experimental colitis compared to their WT counterparts^[111]. The authors suggest that MMP-2 exerts its barrier protective function by associating with TJ proteins, since multiple studies have shown close interaction between MMP-2 and claudins^[116,117].

Besides MMP-2 and MMP-9, transcript and protein levels of MMP-7 (matrilysin) and MMP-3 (stromelysin-1) are shown to be upregulated in the mucosal tissues of active IBD patients vs healthy control tissue^[118-120]. These MMPs are linked to the ectodomain shedding of

E-cadherin and thereby releasing soluble E-cadherin in the interstitium and loss of E-cadherin function in cell-cell adhesion^[121]. Although the majority of the research revolves around cancer, it is relevant to include the proteolytic splicing of E-cad in this review since loss of E-cadherin is associated with disturbances in barrier function during intestinal inflammatory diseases^[122,123]. In cancerous cell lines the ability of matrilysin and stromelysin-1 to release soluble E-cadherin was proven with a loss of E-cad function in the cell-cell adhesion and a facilitation of tumor cell invasion as a consequence^[121,124,125]. The released sE-cadherin works as a paracrine/autocrine signaling molecule, promoting MMP production and thereby worsening the disease progression^[126,127].

Not all metalloproteases are however harmful. The transmembrane endopeptidase meprin β is found to be essential in mucus homeostasis. A loosely organized, nonattached mucus layer covers the epithelial cells on the luminal side, protecting them from digestive proteases thereby forming an important part of the physical barrier against infiltration of harmful substances. The mucin MUC2 is produced by goblet cells and detached by meprin β splicing. Mice lacking the gene encoding for meprin β (Mep1b^{-/-}) display a more dense mucus phenotype^[128]. In addition, the detached MUC2 is an important luminal factor promoting oral tolerance. The outer mucus layer inhabits commensal bacteria which bind MUC2 and are picked up by patrolling dendritic cells in the intestinal mucosa. After binding, MUC2 induces immunoregulatory signals such as IL-10 and retinoic acid, which are important co-stimulatory factors helping CD103⁺ dendritic cells to induce regulatory T cells, and thus oral tolerance^[129].

A disintegrin and metalloproteinase

Another family of metalloproteases are A Disintegrin and Metalloproteinase (ADAM) involved in the release of the ectodomain from transmembrane proteins, a process that is referred to as "shedding".

TNF- α converting enzyme (TACE), or ADAM17, generates the biologically active, soluble form of TNF- α by cleaving the transmembrane bound precursor at the cell surface^[28,130,131]. Since TNF- α is a major contributor of the increased intestinal permeability in inflammatory conditions^[28,131,132], TACE is a potential key player in the regulation of paracellular permeability. In the past, TACE inhibition (by the endogenous inhibitor TIMP-3 or synthetic inhibitors) has been investigated in diseases that benefit from anti-TNF- α therapy, such as IBD, showing promising results^[133-135]. Recently, Al-Sadi *et al.*^[132] investigated the signaling pathways that mediate TNF- α -induced modulation of intestinal paracellular permeability showing MAP kinase ERK1/2 activation, on their turn phosphorylating and activating Elk-1 which subsequently leads to an increase in MLCK and opening of TJs.

The effect on intestinal permeability of pharmacological TACE-inhibition was investigated on a Caco-2 monolayer. Pretreatment with two synthetic inhibitors, the broad MMP-TACE inhibitor GM6001 or the TACE-specific inhibitor TAPI-2, suppressed the permeability increase induced by TACE^[136]. In contrast, Fréour *et al.*^[137] demonstrated that TACE inhibition, by TIMP-3 or by TAPI-2, amplified the TNF- α -mediated increase in paracellular permeability *in vitro*. The authors suggest that this might be due to an autocrine effect of TIMP-3, triggering the release of pro-inflammatory cytokines that contribute to a hyperpermeable intestinal barrier. It should be noted however that no hard evidence to prove this statement was provided.

CYSTEINE PROTEASES

The vast majority of cysteine proteases reside intracellularly, where they mediate distinct signaling pathways affecting programmed cell death and inflammation^[37,138]. Particularly the caspase family of cysteine proteases is well known in the field of cell death. For instance, caspase 1 and 5 take part in inflammasome activation, promoting IL1- β and IL-18 maturation. Caspase 8 plays a central role in both apoptotic and inflammatory pathways, activating respectively pro-apoptotic proteins and NF- κ B^[138]. Hence, cysteine proteases affect the epithelial barrier integrity for the most part indirectly *via* their effect on inflammation and cell death.

Intestinal epithelial cells undergo apoptosis in a tightly regulated fashion in order to renew the entire cell population every 72 to 96 h^[139]. Under inflammatory conditions, such as during IBD, the apoptosis rate increases significantly, inducing morphologic changes in the intestinal barrier^[140,141]. During apoptosis of the intestinal epithelial cells, TJ proteins undergo proteolytic cleavage and dislocate from the lateral cell surface^[142]. In human colonic HT-29/B6 cells, induction of apoptosis resulted in a significant decrease of TER and an increase in macromolecular tracer permeability^[143]. Caspases cleave the transmembrane protein occludin (at the cytoplasmic tail) and adaptor proteins ZO-1 and ZO-2. Although the chronologic sequence of events may not always be clear, there is evidence that a disruption of TJ proteins, caused by for instance bacterial infection or inflammation, activates caspase-8 and -3 and thus initiating cell death^[144]. In an *in vivo* permeability assay however, no effect on Cr51-EDTA flux over the colonic barrier was measured after intraluminal administration of cystatin, a cysteine protease inhibitor, whereas serine- and metalloprotease inhibitors lowered the flux significantly^[51].

LUMINAL PROTEASES

When it comes to research into the pathogenesis of

IBD and IBS, both the gut microbiome and proteases are receiving increasing attention^[37,145,146]. Surprisingly, the current knowledge on proteases focusses mostly on host-derived proteases while bacteria-derived proteases have been largely ignored^[147]. As previously mentioned, IBD and IBS patients have elevated fecal protease levels. In IBS-D patients it was shown that the majority of the fecal protease activity is most likely due to human pancreatic enzymes and not bacteria. However, an oral antibiotic treatment in mice resulted in decreased fecal activity, supporting the hypothesis that bacteria contribute to the luminal protease content^[148].

In the 1990s it was discovered that the metallo-proteinase fragilysin, which is produced by the colonic commensal *bacteroides fragilis*, alters the intestinal permeability. Experiments with the purified fragilysin on a human colon cell line (HT-29) revealed the increase in permeability towards ions (shown by a decrease in TER) as well as towards macromolecules (increase in mannitol passage across the cell monolayer)^[149,150]. Later, the role of *Entamoeba histolytica* (*Eh*)-derived cysteine proteases in the pathogenesis of amoebic colitis was investigated. The amoebic cysteine proteases induce inflammation by activating IL-1 β in a way similar to the human caspases (*cf. supra*), and damage to the intestinal epithelial barrier resulting in an increased paracellular permeability^[151,152]. Two specific proteases mediating TJ disruption were identified; *Eh* cysteine protease A5 (*EhCPA5*) and *EhCP112*^[152,153]. Recently, bacterial-derived gelatinases were investigated. Pruteanu *et al.*^[154] screened bacterial colonies in fecal samples of healthy controls and IBD patients for gelatinolytic activity. The researchers could link *Clostridium perfringens* to the majority of the proteolytic activity in the fecal samples. In addition, *C. perfringens* culture supernatant reduced the TER in Ussing chamber experiments performed on rat distal colon. Gelatinase (GelE) produced by the enteric microbe *Enterococcus faecalis* also has shown to induce a barrier defect in epithelial cell monolayers and *in vivo* in mice^[155,156]. Originally, the degradation of E-cadherin by proteolytic activity of GelE was considered to be the cause of the permeability increase^[156]. Later on, Maharshak *et al.*^[155] discovered the involvement of PAR₂ activation in the GelE-induced permeability increase. Purified *E. faecalis* GelE failed to induce a permeability increase in PAR₂^{-/-} mice as well as in epithelial monolayers treated with a PAR₂ antagonist.

The feces of the house dust mite (HDM; *Dermatophagoides pteronyssinus*) contains a cysteine protease, Der p 1, which is known to disrupt the lung epithelial TJ proteins occludin and ZO-1 and thereby facilitating allergen delivery and eventually provoking asthma^[157]. Recently, Der p 1 was shown to be present in the human gut, affecting barrier function. Exposure of colonic biopsies to a HDM extract reduced the

expression of TJ proteins occludin and ZO-1, reduced the mucus layer thickness, increased TNF- α and increased the paracellular permeability. Pre-incubation of the HDM-extract with the cysteine protease inhibitor E64 abolished the HDM-induced damage to the intestinal barrier^[158].

Furthermore, there is an established link between food allergens and the degradation of the epithelial barrier^[159,160]. In the digestion process, food particles are broken down by pancreatic and brush border proteases into tripeptides, dipeptides and single amino acids. When the intestinal barrier function is not impaired, oral tolerance is induced against these soluble peptides that can cross the epithelium in a selective and regulated manner. But in other -possibly genetically susceptible- individuals, partially or undigested proteins can still reach the mucosa where they provoke an inflammatory signal instead of tolerance. Plasma cells produce allergen-specific IgE which causes mast cell degranulation at contact. The secreted mast cell proteases and cytokines both contribute to the increased barrier dysfunction *via* their effect on the TJ configuration, leading to an opening of the paracellular route. The difficulty lies however in the "chicken and egg" paradigm. Until today there is evidence for only one food protease to affect the intestinal barrier directly. The kiwifruit cysteine protease actinidin (Act d1) has shown to induce a loss of barrier function in epithelial cell monolayers by the disruption of the occludin and ZO-1 network^[161,162]. This effect could be confirmed *in vivo*. Mice gavaged with actinidin exerted an elevated permeability towards FITC-dextran compared to mice gavaged with the vehicle^[161].

Dietary proteases can also contribute to intestinal health. Addition of *Aspergillus*-derived proteases (Amano SD) to the diet of rats improved intestinal health *via* the expansion of commensal colonic bacteria of the *Bifidobacterium* and *Lactobacillus* species. The altered microbiota composition enhanced the formation of short chain fatty (SCFA) acids such as butyrate, propionate and lactate^[163]. SCFA with butyrate in particular promote mucosal homeostasis among other things by enhancing the intestinal barrier function through the upregulation of tight junction proteins^[164].

CONCLUSION

The epithelial cell layer lining the gastrointestinal tract is the body's largest surface area in contact with environmental antigens. The role of these intestinal epithelial cells in the continuous maintenance of intestinal homeostasis is indispensable, providing a physical and biochemical barrier against noxious luminal antigens as well as allowing nutrient absorption. The selective opening of the intercellular spaces, allowing paracellular transport of macromolecules, is regulated by the interplay between TJ proteins

and the actomyosin contraction upon activation of intracellular signaling pathways. An increased intestinal permeability is suggested as an important player in the pathophysiology of various intestinal and extra-intestinal pathologies. Targeting the epithelial barrier is a tempting therapeutic approach, but so far no therapies have succeeded. Larazotide acetate showed promising results in preclinical trials, restoring the intestinal barrier function. But clinical trials failed to mirror these effects. This shows that more research is needed to define epithelial barrier function and dysfunction, underlying different pathologies and diseases. Proteases are important signaling molecules in this regard. With their proteolytic capacity they can cleave proteinase-activated receptors on the cell surface of intestinal epithelial cells, influencing the cytoskeleton contractile machinery and paracellular permeability. Also extracellular proteolytic cleavage of the junction proteins occurs, leading directly to epithelial damage and increased intestinal permeability. In homeostasis, the proteolytic activity is tightly regulated by antiproteases, but this balance is dysregulated in organic and functional GI disorders. As a result, protease inhibition has become a "hot topic" in a therapeutic point of view, mainly focusing on inflammation and hypersensitivity, ignoring the effect on permeability. But since a large array of proteases is involved and for many proteases no specific inhibitors are available yet, more research is needed to elucidate the exact involvement of specific proteases in gut physiology in general and intestinal permeability in particular, in order to become a therapeutic target.

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