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TOPIC HIGHLIGHT

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Enteric bacterial proteases in inflammatory bowel disease-pathophysiology and clinical implications

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Abstract

Numerous reports have identified a dysbiosis in the intestinal microbiota in patients suffering from inflammatory bowel diseases (IBD), yet the mechanism(s) in which this complex microbial community initiates or perpetuates inflammation remains unclear. The purpose of this review is to present evidence for one such mechanism that implicates enteric microbial derived proteases in the pathogenesis of IBD. We highlight and discuss studies demonstrating that proteases and protease receptors are abundant in the digestive system. Additionally, we investigate studies demonstrating an association between increased luminal protease activity and activation of protease receptors, ultimately resulting in increased intestinal permeability and exacerbation of colitis in animal models as well as in human IBD. Proteases are essential for the normal functioning of bacteria and in some cases can serve as virulence factors for pathogenic bacteria. Although not classified as traditional virulence factors, proteases originating from commensal enteric bacteria also have a potential association with intestinal inflammation *via* increased enteric permeability. Reports of increased protease activity in stools from IBD patients support a possible mechanism for a dysbiotic enteric microbiota in IBD. A better understanding of these pathways and characterization of the enteric bacteria involved, their proteases, and protease receptors may pave the way for new therapeutic approaches for these diseases.

Key words: Protease; Proteinase; Protease associated receptor; Enteric microbiota; Epithelial barrier

Core tip: It is currently accepted that an enteric dysbiosis (alteration of the normal bacterial flora) is involved in the pathophysiology of inflammatory bowel diseases (IBD). One of the suggested mechanisms that ties an intestinal dysbiosis to the pathophysiology of IBD involves the release of enteric bacterial proteases that interact with protease activated receptors on epithelial cells, resulting in intestinal barrier dysfunction and exposure of the enteric immune system to luminal antigens. We have reviewed the literature that examined the role of microbial proteases and their enteric receptors in the pathogenesis of IBD, their suggested pathways of action, and discuss future therapeutic implications.

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INTRODUCTION

Inflammatory bowel diseases (IBD), collectively known as Crohn's disease (CD) and ulcerative colitis (UC), are caused by dysregulated immune responses towards microbial antigens in a genetically predisposed host. The incidence of UC and CD has been increasing worldwide in developed and in developing countries[1,2]. These diseases are highly prevalent in the United States affecting 1.4 million individuals[3] and are associated with reduced quality of life[4,5], and psychological co-morbidity[6]. Current estimates for IBD associated treatment costs in the US are $6.3 billion[7], but the initiating events of IBD and causes of disease exacerbation remain unclear. It is postulated that one potential mechanism involves disruption of the epithelial barrier, and exposure of a genetically defective immune system to enteric microbial antigens. Consistent with this hypothesis are animal models of colitis that use chemical disruption of the epithelial barrier with trinitrobenzene sulphonic acid (TNBS), dextran sodium sulfate or non-steroidal anti-inflammatory drugs (NSAIDs). Additionally, disruption of the intestinal epithelial barrier by exposure of susceptible patients to NSAIDs (blockers of prostaglandins synthesis) is a known risk factor that can trigger intestinal inflammation[8]. In line with this observation, in animal studies, the use of a prostaglandin receptor agonist preserved the intestinal epithelial barrier structure and function, maintained mucous secretion by goblet cells, and prevented the development of colitis[9].

Proteases, peptidases, or proteolytic enzymes, are a class of enzyme that catalyze the cleavage of peptide bonds in other proteins in the presence of H_2O (hydrolysis). Proteases act as both positive and negative effectors of several biological processes either broadly as catalysts of protein degradation or specifically as selective agents that control physiological processes[10]. The importance of proteases is highlighted in the human genome where 2%-4% of genes encompass the *degradome*[11]. In bacteria, proteases are involved in numerous biological processes, such as those associated with metabolism, development, and virulence. Additionally, these enzymes can disrupt mucosal barriers, provide a metabolic advantage, and modulate the host immune response. The high prevalence of proteases in enterobacteria suggests that proteases play important roles in pathogenesis[12]. Both mammalian and bacterial proteases have been implicated in the pathogenesis of IBD, usually through disruption of the epithelial barrier. In pathogenic bacteria, many proteases are virulence factors that aid in bacterial invasion into host cells and cause infectious colitis. However, accumulating evidence shows that commensal enteric microorganisms also produce proteases that possess the ability to disrupt the epithelial barrier[13,14]. These commensal proteases may be involved in the pathogenesis of IBD in the context of a genetically predisposed host and/or when an intestinal microbial dysbiosis occurs. Our aim in this review is to provide an overview of current studies that suggest potential mechanisms in which microbial proteases may play a role in the pathogenesis of IBD.

PROTEASE CLASSIFICATION

Proteases frequently exist as multi-domain proteins, with catalytic activity restricted to a single structural domain. Although these enzymes appear to have a specific function (*i.e.*, hydrolysis of proteins), they exhibit vast diversity in their action and structure and are not easily categorized by general systems of enzyme nomenclature. Thus, proteases are broadly subdivided into two major groups, exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whilst endopeptidases cleave peptide bonds distant from the termini of the substrate. Proteases are further classified into five distinct groups on the basis of the chemical nature of the groups responsible for their catalytic activity, namely; aspartic, cysteine/thiol, metallo-, serine, and unidentified proteases[15]. In order to generate a comprehensive classification system for proteases, Rawlings and Barrett[16] developed a method to classify this group of enzymes based on the type of reaction they catalyze, the chemical nature of their catalytic site, and their evolutionary structure. This approach is a hierarchical system where classification levels were summarized as peptidases (*i.e.*, serine proteases), families and clans. This system initially recognized 84 families of proteases; however the subsequent massive accumulation of amino acid sequence data and three-dimensional structures of proteases from the scientific community warranted an updated classification system that was easily accessed for academic studies. Thus, based on the system outlined by Rawlings and Barrett[16] the MEROPS database was developed[17]. Along with data regarding protease classification, the MEROPS database also provides information regarding classification of protein inhibitors of peptidases[18], small-molecule inhibitors[19], and a collection of known protease cleavage sites and substrates[20].

Microbial proteases

Proteases are found in all forms of life suggesting that they are vital for the survival of all organisms. Micro-organisms produce a vast array of aspartic, cysteine, metallo-, and serine proteases. Microbial aspartic proteases are specific for aromatic or bulky amino acid residues on both sides of a peptide bond. They are broadly divided into two groups: pepsin- and rennin-like enzymes. Cysteine proteases generally are only active in the presence of reducing agents. Some bacterial cysteine proteases are notable for their role in virulence and the inflammatory response they illicit[21]. Metalloproteases are characterized by the requirement for a divalent metal ion for their activity. These proteases are summarized into neutral and alkaline groups based on their specificity of action[22]. Serine proteases are characterized by the presence of a serine group in their active site and have broad substrate specificity. The complex microbial community in the human gut (referred to as the intestinal microbiota) is a substantial source of serine, cysteine, and metallo-proteases[23-25]. This is exemplified by the reduction of colonic bacteria

densities and protease activity by oral administration of antibiotics to mice[26]. By analyzing the protease activity of representative enteric bacterial strains and human fecal samples it has previously been suggested that the activity of specific classes of proteases present in human feces are likely to originate from *Bacteroides*, *Streptococcus*, and *Clostridium* species[27]. However, to date only one study has reported the correlation between specific groups of proteases and the abundance of enteric bacterial taxa using modern molecular methods. Carroll *et al*[28] used high throughput sequencing of the 16S rRNA gene and correlated the abundances of specific bacterial families with fecal tryptic activity in stool samples from healthy individuals and IBS patients. This study found positive associations between *Lachnospiraceae*, *Streptococcaceae* and *Lactobacillales* with fecal protease activity, and a negative correlation with *Ruminococcaceae*. However, to date microbial proteases have been mainly exploited for commercial purposes. For example, bacterial alkaline proteases are characterized by their high activity at an alkaline pH and their broad substrate specificity, thus, making them ideal for use in the detergent industry[29]. In addition, most academic studies have focused on bacterial proteases as potential virulence factors in pathogenic bacteria[30]. However, little is known regarding the relationship between microbial proteases, found in or on the body, and the health of the host. Examples of such microbial proteases that are produced by enteric commensals are specified in Table 1.

MICROBIAL PROTEASES IN THE PATHOGENESIS OF IBD

The antigenic contents of the intestinal lumen are separated from underlying intestinal tissues by an epithelial barrier that is one cell thick. Pathogenic bacteria have acquired virulence factors, many of which are proteases, that disrupt this barrier and cause infection[31]. For example, the serine protease autotransporter of *Enterobacteriaceae* family are generally secreted into the external milieu and are highly prevalent among enteropathogens, including *Shigella* species and all *Escherichia coli* (*E. coli*) pathotypes[12]. As there is an established genetic component to IBD[32], it is difficult to identify microbial proteases that are potentially involved in the pathogenesis of these diseases as they would not be categorized in the same manner as traditional virulence factors. Indeed, an overproduction of microbial proteases originating for enteric commensal microbes may not have an effect on a healthy individual, but may play a role in the pathogenesis or perturbation of intestinal inflammation in a population with a genetic predisposition to IBD. Here we discuss four potential mechanisms in which microbial proteases from a non-pathogenic source (the intestinal microbiota) could contribute to the pathogenesis of IBD.

Table 1 Commensal enteric microbial protease classification and origin

Protease category	Microbial origin	Protease
Aspartic	*Candida albicans*	Secreted aspartic proteases[119]
	Pseudomonas aeruginosa	Type 4 prepilin peptidase[120]
	Methanococcus voltae	Preflagellin[121]
Cysteine	Gram positive bacteria	Sortases[122]
	Porphyromonas gingivalis	Gingipain[21]
	Staphylococcus aureus	Staphopain[123]
Metalloprotease	*Bacteroides fragilis*	Fragilysin[124]
	Enterococcus faecalis	Gelatinase[87]
	Staphylococcus epidermidis	Elastase[123]
	Clostridium perfringens	Collagenase[13]
Serine	*Helicobacter pylori*	High temperature requirement A[125]
	Bacillus subtilis	Subtilisin[126]

MICROBIAL PROTEASES AND ADHERENCE AND INVASION TO THE INTESTINAL EPITHELIUM

Bacterial adhesion to intestinal epithelial cells is believed to be one of the first steps used in the pathogenicity of many enteric pathogens. Adhesion enables a microbe to colonize the intestinal epithelium and resist exclusion from the intestine by the mechanical movement of the gut. Adherent and invasive *E. coli* (AIEC) are a group of enteric microbes that are capable of adhering to and invading intestinal epithelial cells[33]. AIECs are not classified as enteric pathogens, but exhibit some pathogenic traits in the context of IBD. For example, AIECs isolated from CD patients are able to replicate within macrophages without escaping from the phagosome and without inducing macrophage death[34]. Proteases for pathogenic bacteria play a fundamental role in adherence and invasion virulence traits. For example, enteroaggregative *E. coli* (EAEC) expresses a factor referred to as "protease involved in colonization" or Pic. Pic catalyzes gelatin degradation which can be abolished by disruption of the predicted proteolytic active site. This protease is involved in the early stages of pathogenesis and most probably promotes intestinal colonization[30,35]. Pic is also essential for biofilm formation in EAEC. The first step of biofilm formation is bacterial adherence to a surface and then intercellular aggregation. In general, intercellular aggregation is mediated *via* the proteolytic processing of bacterial aggregation proteins by means of host or bacterial proteases[36,37] ultimately resulting in a biofilm. To date the role of microbial proteases involved in the formation of biofilms in members of the intestinal microbiota have not been investigated in the context of IBD. However, the role of biofilms in AIEC virulence in IBD has begun to emerge. It was reported that biofilm formation indi-

ces were higher amongst AIEC than non-AIEC strains isolated from the intestinal mucosa of CD, UC, and non-IBD controls[38]. Additionally, the adhesion and invasion properties of AIECs correlated positively with higher biofilm formation indices. Furthermore, the σ^E factor, which up-regulates genes that encode proteases, periplasmic foldases, and chaperones in response to environmental stresses, plays a pivotal role in biofilm formation in AIECs in the context of CD[39]. Thus, proteases may be important in biofilm formation and colonization of commensal enteric bacteria and related to IBD pathogenesis.

PROTEASE RECEPTORS

Proteases can mediate their activity on mammalian cells through activation of protease receptors. Protease activated receptors (PARs) are a family of 7 transmembrane domain G-protein-coupled receptors (GPCRs) that mediate multiple responses to external stimuli, such as hemostasis, thrombosis and inflammation, and exist in four isoforms (PARs 1-4)[40-44]. PARs are activated through proteolytic cleavage of the extracellular N-terminal component of the receptor unmasking a tethered peptide ligand residue that binds with another region of the receptor causing a conformational change[45]. The result is an initiation of an intracellular signaling cascade that is diverse and includes calcium mobilization, phospholipase C-dependent production of inositol phosphates and diacylglycerol, Rho and Rac activation, mitogen-activated protein kinase signaling, and gene transcription[46]. Alternatively, PARs can be activated through peptide sequences that are homologues to the intrinsic tethered ligand. These synthetic peptides activate PARs without proteolysis of the N-terminal of the receptor in PAR1, PAR2 and PAR4 but not in PAR3[47]. The outcome of PAR activation is dependent on the type of ligand (*e.g.*, serine protease, matrix metalloprotease, plasmin, coagulation factors *etc.*), receptor type (PAR1, 2, 3 or 4) and on the type of cell which the PAR is expressed (*e.g.*, epithelial cells, platelets, nerve cells, or leukocytes). PAR activation, signaling and degradation are highly regulated by post translational modifications such as phosphorylation, glycosylation and ubiquitination (for review- Grimsey *et al*[48]). In the gastrointestinal (GI) tract, PARs are activated by endogenous proteases secreted by the pancreas (such as trypsin), by cells of the enteric wall (such as mast cells), or by the luminal enteric microbiota. Moreover, PAR expression on the gut epithelium differs between IBD patients and healthy individuals. This may be a result of the type of micro-organisms present in the GI tract and other receptors [such as toll-like receptors (TLRs)] they interact with. For example, on polymorphonuclear (PMN) cells, *Candida albicans* promoted a TLR2-dependent PAR1 activation and expression in contrast to *Aspergillus fumigatus* that suppressed TLR4-dependent PAR2 activation and expression[49]. In this regard it is important to note that endogenous host proteases are also PAR specific, *e.g.*, - thrombin activates PAR1[50], PAR3[40,43] and PAR4[44], while trypsin activates PAR2[51] and PAR4[52].

While the majority of research relating to the relationship between PARs and colitis has examined the role of endogenous activation of PARs by mammalian proteases, the interaction between the enteric microbes, PAR expression and activation and the pathophysiology of colitis have not been extensively studied. The evidence that supports these associations is summarized below.

PAR1

PAR1 has been implicated in hemostasis, platelet signaling, systemic pro-inflammatory responses (such as vasodilatation, increased vascular permeability and chemotaxis) and induction of analgesia[53,54]. PAR1 agonists induce apoptosis of intestinal epithelial cells in a caspase-3-dependent manner, with a concomitant loss of the epithelial barrier function and a consequent increase of permeability to macromolecules and bacteria[55]. PAR1 is expressed by enterocytes as well as by other cell types such as endothelial cells, enteric neurons, myocytes and immune cells[52]. The expression of PAR1 on the intestinal epithelium is linked to the presence of enteric microbiota[56], and activation of this receptor in the mouse colon leads to colitis[57,58]. In addition, PAR1 expression has been reported to be increased in colonic biopsies from IBD patients[54]. Altogether, these reports support a role for PAR1 in the pathogenesis of IBD, however it is not clear if the enteric microbiota directly activate PAR1 through release of bacterial proteases. Nonetheless, this mechanism is supported by a study investigating oral epithelial cells, where PAR1 activation by a cysteine protease released by the oral pathogen *Porphyromonas gingivalis* (*P. gingivalis*) caused an up-regulation of pro-inflammatory cytokines[59].

PAR2

The majority of evidence that points towards an association between PARs and intestinal inflammation involves PAR2. This receptor is localized to the apical and basolateral membrane[60-62] of the intestinal epithelium and can be activated by trypsin, tryptase, and bacterial proteases[63]. PAR2 is expressed in immune, stromal, endothelial, and intestinal epithelial cells and thus, PAR2-associated inflammation may be a result of multiple, systemic and local pathways. Systemically, this receptor impacts leukocytes by mediating rolling, adhesion, and extravasation[64]. When activated on sensory neurons PAR2 mediates pain and edema[65]. In the mouse colon, activation of this receptor results in colitis[60] that is significantly ameliorated in PAR2-deficient mice[60,66]. Additionally, antagonism of PAR2 (by GB88) results in amelioration of colitis in rats that is induced by either TNBS or a PAR2 agonist (SLIGRL-NH_2)[67]. Thus, most studies indicate that activation of PAR2 leads to an inflammatory response. However, a single study has reported a protective effect of daily intra colonic administration of PAR2 agonist in a TNBS colitis model in rats[68]. It is not entirely clear why PAR2 exhibits anti-inflammatory properties in this model; however it may be the result of a chronic PAR2 activation and lo-

cal desensitization, or *via* anti-inflammatory effects on macrophages[69]. Additionally, it is not clear which of the various mechanisms that have been implicated in PAR2 activation in the gut is responsible for PAR2-dependent colitis. However, it has been speculated that PAR2-mediated intestinal inflammation is a result of increased levels of PAR2 ligands in the colon of IBD patients. Indeed, in the colon of human IBD patients the natural PAR2 ligands, trypsin[70] and tryptase[71,72] are elevated compared to healthy controls. Moreover, in human IBD, PAR2 is overexpressed on mast cells[73] which have also been implicated in the pathogenesis of PAR2-mediated colitis. In non-IBD patients permeability was found to be proportional to the concentration of tryptase (naturally secreted by mast cells) added to the basolateral surface and not to the mucosal surface of mucosal biopsies[74]. These studies support the importance of mast cells in colitis *via* PAR2 activation, however enteric bacteria may also play a role in PAR2 activation in the colon through release of bacterial proteases in the gut lumen[24]. Róka *et al*[26] demonstrated increased levels of serine proteases in fecal samples from UC patients and hypothesized that these enzymes originated from luminal bacteria as it was reported that increased fecal protease activity was neither of a mast cells nor pancreatic origin. PAR2 can be activated by enteric bacteria either directly by bacterial proteases, as demonstrated in the oral epithelium by proteases of *P. gingivalis*[63] and in infectious colitis by the Toxin A of *Clostridium difficile*[75], or indirectly by bacterial-dependent induction of host proteases[76], as discussed above. Finally, it has been reported that antibiotic treatment directed at the gut microbiota resulted in reduced PAR2 expression suggesting that PAR2 is not only activated by enteric bacteria but its expression is also regulated by the presence of these microbes[77].

PAR3

The biological significance of PAR3 has not been fully delineated. Structurally, this PAR isotype does not have a C-terminal intra cytoplasmatic tail and thus cannot signal through GPCRs. However, PAR3 may serve as a cofactor or co-receptor of other PARs. In mouse platelets, PAR3 functions as a cofactor for PAR4 by presenting thrombin to low-affinity PAR4, thereby resulting in efficient receptor cleavage[78]. On endothelial cells PAR3 can regulate PAR activity by forming a heterodimer with PAR1[79]. Despite evidence of PAR3 mRNA expression in the small intestine, this receptor's relationship with intestinal inflammation and bacterial proteases are unknown[40].

PAR4

PAR4 is expressed in the small and large intestine[44] and is localized to colonocytes in rats[80]. It can be proteolytically activated by thrombin, trypsin and by the neutrophil granule protease cathepsin G[81]. Its activation induces leukocyte rolling and adherence, suggesting a pro-inflammatory role for this receptor[45,82-84]. Exposure of mouse colons to PAR4 agonists results in increased paracellular colonic permeability, suggesting that this receptor may be involved in the pathophysiology of IBD[85]. In the human colon, expression of PAR4 on epithelial cells is negligible in non-IBD patients but is significantly higher in UC patients. Interestingly, the activity of cathepsin G was increased in the feces of UC patients compared to controls and inhibition of its activity resulted in ameliorated enteric permeability[85]. Thus, cathepsin G may mediate PAR4-dependent enteric permeability in UC patients. Nevertheless, a direct effect of bacterial proteases was not examined; therefore it is still unknown whether proteases released by the enteric microbiota contribute to enteric permeability and colitis in a PAR4 dependent manner.

PROTEASES AND INTESTINAL BARRIER DISRUPTION

The intestinal epithelial barrier is made up of a single layer of cells that are tethered together *via* tight junctions and cell adhesion molecules. Enteric microbes can circumvent the defense of the intestinal epithelial barrier either directly through proteolytic degradation of cell adhesion molecules (such as E-cadherin) or indirectly by regulation of paracellular permeability *via* tight junctions. Intestinal epithelial tight junctions are composed of different protein complexes which consist of transmembrane and intracellular scaffold proteins (Figure 1).

The trans-membrane proteins include occludin, claudins, and junctional adhesion molecules whose extracellular loops are bound together and intracellular domains interact with scaffold proteins such as zonula occludens (ZO), which in turn are anchored to the actin cytoskeleton. In the intestine the adherence junction protein, E-cadherin, cements epithelial cells together and is a significant factor in maintenance of the epithelial barrier function. The enteric commensal *Enterococcus faecalis* (*E. faecalis*) can induce inflammation in a gnotobiotic *IL-10*$^{-/-}$ mouse[86] and secretes a protease (gelatinase) which has the capacity to degrade collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a[87-92]. The potential of *E. faecalis* gelatinase to damage the intestinal epithelial barrier and cause inflammation in the *IL-10*$^{-/-}$ mouse was recently investigated[14]. Steck and associates created an *E. faecalis* mutant lacking the *gelE* gene (Δ*gelE*). *IL-10*$^{-/-}$ mice mono-associated with *E. faecalis* Δ*gelE* exhibited significantly lower colonic inflammation when compared to mice mono-associated with wild-type *E. faecalis*. The reduction in colonic inflammation was independent of colonization densities of *E. faecalis* strains. Interestingly, the expression of E-cadherin on epithelial cells in *IL-10*$^{-/-}$ mice was reduced in the presence of gelE (wild-type *E. faecalis*) but not when gelE was absent from *E. faecalis* (Δ*gelE*). It was further demonstrated that *E. faecalis* gelE can degrade recombinant mouse E-cadherin. These data strongly suggest a mechanism in which a bacterial protease can disrupt the intestinal barrier function and lead

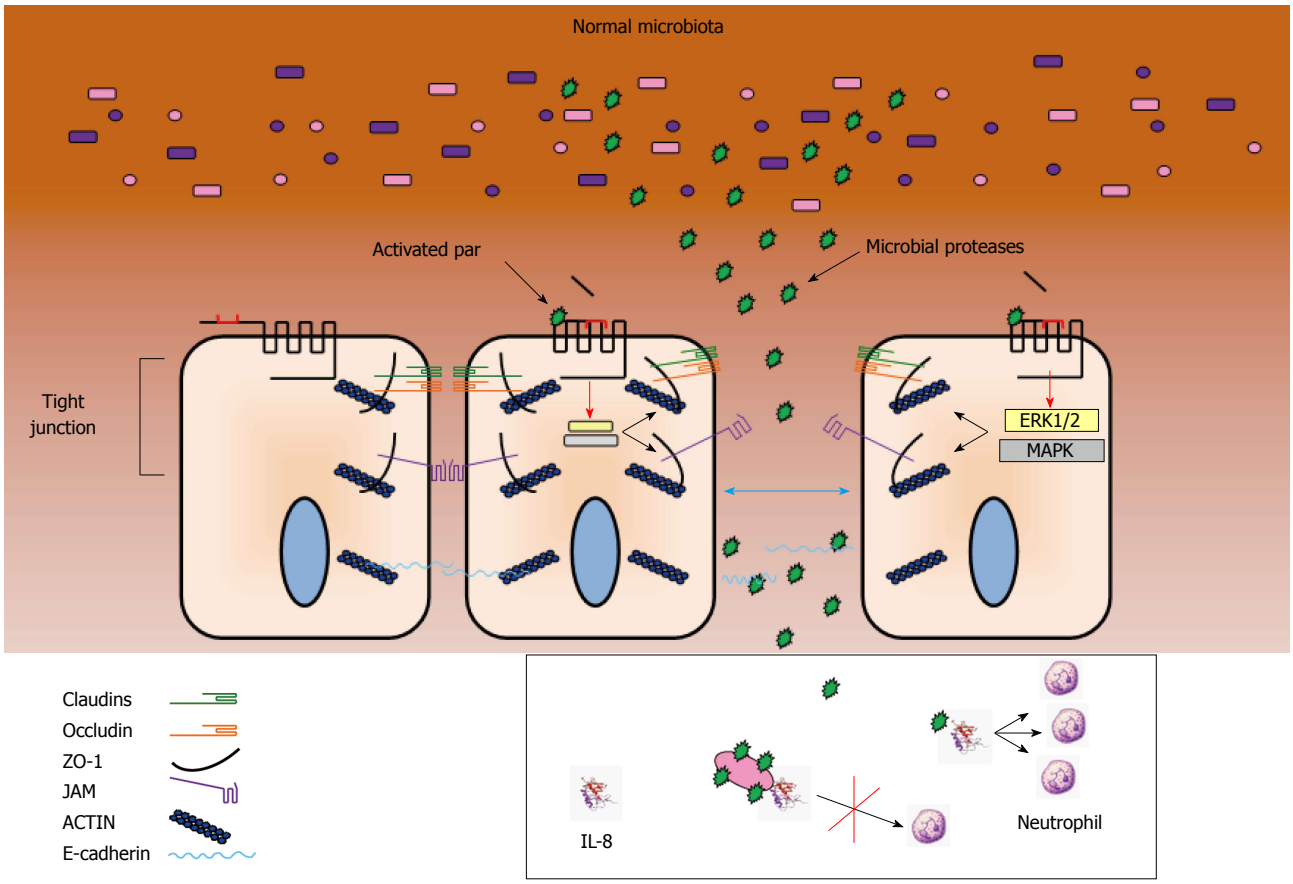

Figure 1 Model for enteric microbial protease-dependent increased intestinal permeability. Enteric microbial proteases activate epithelial protease activated receptors (PARs) through release of the tethered ligand. This results in intra-cellular signal transduction and activation of ERK 1, 2 and MAPK. These signaling molecules mediate disruption of tight junctions and consequently cause increased intestinal permeability that enables penetration of microbes and their proteases which can act upon cytokines. Further possible effects of bacterial proteases on the immune response are illustrated in the black box. These mechanisms have been demonstrated for *Porphyromonas gingivalis* in the oral cavity (and not in the gut) where gingipain proteases can enhance Interleukin (IL)-8-dependent attraction of neutrophils (when in their soluble forms) by partially degrading the N-terminal of this cytokine, or inhibit neutrophil activity via complete degradation of IL-8 when associated with the microbial membrane.

to inflammation. This finding is specifically significant to CD pathogenesis where a greater diversity of microbes with gelatinolytic activity was reported when compared to healthy controls[13].

The intestinal microbiota has long been thought of as a significant contributor to the proteolytic activity of stool[24,27]. Specifically, Macfarlane *et al*[24] found that the proteolytic activity in the stool from a patient that had undergone a pancreatectomy was comparable to that of the protease activities in stools from individuals that had not undergone surgery to remove their pancreas. This indicates that a source other than the pancreas (*i.e.*, enteric microbes) significantly contributes to the protease activity of the intestine. These observations have been more recently demonstrated by the reduction of colonic bacteria densities and protease activity by oral administration of antibiotics to mice[26]. As previously mentioned, increased protease activity has been reported in fecal samples obtained from subgroups of patients suffering from irritable bowel syndrome (IBS) and IBD[25,93,94]. Róka *et al*[26] initially saw a four-fold increase in trypsin-like activity in diarrhea-predominant IBS (D-IBS) and UC patients. Subsequently, it was found that fecal supernatants from D-IBS patients could increase colonic paracellular permeability in the mouse gut[94]. The application of D-IBS supernatants to the mouse colon resulted in an increase in phosphorylation of myosin light chain kinase and delayed redistribution of the tight junction-associated molecule ZO-1. Further investigations demonstrated that fecal supernatants from UC patients can affect visceral sensitivity and colonic permeability in mice that was mediated *via* differing protease receptors (see protease receptors in this review). Together these studies suggest a mechanism in which microbial proteases can alter intestinal barrier function by regulating tight-junctions.

ENTERIC MICROBIAL PROTEASES AND IMMUNE CELL REGULATION

Once the intestinal epithelial barrier has been breached microbes or microbial antigens can potentially traverse into the underlying tissues of the intestine and interact with immune cells, ultimately leading to inflammation. Although enteric microbes are essential environmental

factors for immune cell development, as evidenced by an under established immune system found in germ-free mice[95], the immune system can also be subverted by enteric microorganisms *via* microbial proteases. Bacterial proteases capable of disrupting cytokine signaling can potentially affect the pathogenesis of disease. For example, cysteine protease gingipains K (Kgp) and R (RgpA and RgpB) are produced by *P. gingivalis* and are significant factors in this oral microbe's pathogenesis[96]. Soluble gingipains secreted by *P. gingivalis* are capable of cleaving the N-terminus of IL-8 and enhancing this cytokine's activity of attracting neutrophils[97]. Additionally, Kgp, RgpA, and RpgB can also instantly degrade IL-8 when these enzymes are associated with membrane vesicles of *P. gingivalis*. This dual role of enhancing and inhibiting immune cell activity by the soluble and membrane-bound forms of these microbial proteases, respectively, may explain the pro- and anti-inflammatory sites found in periodontitis infections. The massive infiltration of neutrophils at periodontitis sites without the elimination of infection may also be explained by the dual roles of these microbial proteases. Another example is that of necrotizing fasciitis caused by *Streptococcus pyogenes* (*S. pyogenes*) that is characterized by an absence of neutrophils within lesions. It has been reported that the relative absence of neutrophils in necrotizing fasciitis lesions were due to restricted proteolysis of the C-terminal of IL-8 by the *S. pyogenes* protease *Sly*CEP[98]. Further investigations revealed that cleavage of the IL-8 C-terminal by *Sly*CEP from *S. pyogenes* is sufficient to reduce neutrophil endothelial trans-migration and is fundamental in the promotion of resistance of this microbe to neutrophil killing[99]. Given that a homologue of *Sly*CEP has been found in another *Streptococcus* species and no substrates other than cytokines have been identified, it is likely that this microbial protease is an effective weapon used by streptococci to impair bacterial clearance by neutrophils. Enteric microbial proteases can not only affect cytokines that are responsible for attracting the cellular branch of the innate immune system, but can also directly act upon neutrophils, macrophages, monocytes, and natural killer cells. SpeB from *S. pyogenes* has been shown to cause mitochondrial damage and prevent phagocytosis by granulocytes[100]. Additionally, a cysteine protease from *Staphylococcus aureus* (SspB) has been shown to selectively cleave CD11b on phagocytes which undergo apoptosis and are subsequently cleared by macrophages[101]. Taken together these studies identify microbial proteases from pathogenic and potentially commensal sources important molecules that have the ability to regulate the host immune system *via* specific mechanisms.

FUTURE FOR MICROBIAL PROTEASES AND IBD

The importance of the enteric microbiota in IBD has been established during the last decade[102]. Currently, efforts are being made to decipher the pathways through which bacteria and their products cross-talk with various cell types in the digestive tract that can potentially mediate inflammatory responses, pain or protection from chronic inflammation. The diversity of bacterial proteases and their effect on the intestinal epithelial, immune cells, and the enteric nervous system through various receptors open new avenues for research and potential therapeutic targets. Characterization of pathogenic proteases in IBD, the bacterial species that produce them and their mechanism of action are required to enhance our capability to understand the pathogenesis of these diseases and therapeutically intervene. Potential targets for therapeutic intervention include the following.

Specific bacterial groups that carry potentially pathogenic bacterial proteases

The list of specific enteric bacteria that carry bacterial proteases that can disrupt epithelial barrier function and cause colitis in animal models is small and has been discussed earlier in this review. In humans there is even less information. However, the beneficial effects of antibiotics and probiotics in pouchitis[103,104] and IBS[105-107], and antibiotics in CD[108] are well established. Although the proposed mechanisms for antibiotic and probiotic action are beyond the scope of this review, it is conceivable that one of the mechanisms involves action against protease-producing bacteria that cause increased permeability, pain and activation of the immune response. Future research characterizing these bacteria using high throughput sequencing, proteomics and metabolomics will potentially identify microbial targets for treatment of IBD.

Bacterial proteases

Production of proteases is not restricted to bacteria. Host derived proteases have an important role in normal physiology of the digestion, immune response, signaling *etc.* Therefore, strategies that target bacterial derived, intraluminal, colonic proteases without harming the host may prove to be beneficial. Novel drugs for IBD could potentially target bacterial protease production or secretion, such as the serine protease autotransporters from *Enterobacteriaceae*[12]. This approach was recently demonstrated by Löwer *et al*[109] who investigated a specific inhibitor for the *Helicobacter pylori* serine protease. High temperature requirement A (HtrA) is a secreted serine protease that cleaves E-cadherin on the surface of host cells and disrupts the epithelial barrier. Through a receptor-based virtual screening method, they found a specific inhibitor of HtrA activity that was able to prevent *in vitro* cleavage of E-cadherin, without cross reactivity to mammalian proteases. HtrA is a virulence factor for other enteric bacteria, such as *E. coli*, *Shigella flexneri* and *Campylobacter jejuni*[110]. Thus, examining the ability of this inhibitor to reduce HtrA activity and its effect on intestinal inflammation and permeability in models of colitis is warranted.

An alternative approach is to use probiotics that can be beneficial through various mechanisms such as favorable metabolic effects on the epithelial cells, anti-bacterial activity or directly through production of protease inhibi-

tors. For example, *Bifidobacterium longum* and *Bifidobacterium breve* produce serine protease inhibitors (serpins)[111,112] that may antagonize potentially pathogenic bacteria proteases and may exert at least part of its favorable effects on the colon through this mechanism. Another probiotic micro-organism, the yeast *Saccharomyces boulardii*, produces a serine protease that is beneficial to the host through it activity against *Clostridium difficile* adherence to the gut wall and against its toxin, and thus suppresses bacterial overgrowth and infectious colitis[113]. These examples demonstrate that the potential favorable effects of the enteric microbiota on gut inflammation are vast and involve multiple mechanisms that are not yet fully understood.

Protease activated receptors

PARs can be activated or antagonized by synthetic peptides that are analogous to the tethered ligand, irrespective of proteolytic cleavage of the receptor. Design of new, selective and potent drugs that correspond to the tethered ligand but also contain non-peptidic moieties may become useful in selective activation or inhibition of specific PARs. Activation of PAR2 is associated with colitis in animal models and has been used as a colitis model in rats[67] while oral administration of PAR2 antagonist resulted in amelioration of colitis. Although it is not clear if this action antagonizes host or bacterial derived proteases, the advantage of this approach is that it targets the final common receptor of the proteases, regardless of their source (bacterial or mammalian).

An additional approach would be to block PAR associated receptors. There is evidence that PAR signaling by *Candida* or *Aspergilus* on PMNs depends on the presence of TLR 2 and 4[49]. Although, similar studies regarding enteric epithelial cells is lacking, it is conceivable that such mechanisms are also required to induce PAR signaling on epithelial cells, and thus may be serve as additional potential targets against activation by microbial proteases.

Inhibitors of downstream molecular pathways

Activation of PARs by bacterial proteases results in diverse and complex signaling pathways. Characterization of specific pathways that may be inhibited to block the pathogenic effect of bacterial proteases without harming host homeostatic pathways, are required. Pepducins are such an evolving therapy[114]. Peducins are lipoprotein molecules, composed of a synthetic peptide sequence (10-20 amino acids) that relates to the GPCR intracellular sequences and of a lipid hydrophobic moiety. The lipid component tethers the pepducin in the lipid bilayer membrane of the cells and enables these molecules to interact with specific and stabilize GPCRs (for review, Dimond *et al*[115]). Specific pepducins that act as antagonists of PAR1 GPCR (P1pal-7) have shown favorable results in pre-clinical trials for lung cancer[116]. Additionally, PAR2 GPCR specific pepducin (P2pal-18S) ameliorates experimental pancreatitis through inhibition of PAR2 action that is expressed on pancreatic acinar cells[117] and ameliorates inflammation in additional mouse models[118].

CONCLUSION

In this review we have discussed the putative role and evidence of microbial proteases in inflammatory bowel disease pathogenesis. Proteases are essential for normal physiological development and are involved in numerous processes in our body. They are secreted by various cell types and their receptors are abundant in the gut wall, on immune cells, epithelial cells, and on neuronal cells. A growing amount of evidence supports a role for proteases and their receptors to IBD pathophysiology. The understanding that the enteric microbiota are crucial to disease initiation, and the fact that proteases are secreted by most bacteria and are considered virulence factors in infectious colitis, suggest that perhaps commensal bacterial proteases can also damage epithelial barrier function and may be involved in the initiation and perpetuation of IBD in genetically predisposed patients. Indeed, in this review we have summarized the current evidence that support this notion, the mechanisms through which bacterial proteases can impact the mucosal barrier function (through activation of PAR receptors), and the downstream signal pathways that result in increased epithelial permeability and perhaps in colitis.

However, it is not clear whether the proteolytic activity found in the gut lumen is exclusively of mammalian or bacterial origin. This is complicated by the fact that mammalian proteases, such as pancreatic digestive enzymes, are abundant in the gut lumen, and proteases secreted within the gut wall by leukocytes, such as neutrophils (cathepsin G) or mast cells (tryptase), "spill" into the inflamed gut. These factors may account for some of the discrepancies found between various studies investigating the origin of luminal proteolytic activity and the receptors they activate. Moreover, it is currently difficult to characterize luminal proteolytic activity, and while some research studies examine tryptic activity or gelatinase activity (each of which represents only a portion of the total luminal proteolytic activity) other studies have sought to characterize total luminal protease activity *via* functional assays and through inhibition of specific protease activity. These challenges may also explain why it is not fully clear which PAR isotype mediates increased enteric permeability and inflammation. For example, PAR1 and PAR2 have been implicated in mediating enteric inflammation or permeability by bacterial proteases or mammalian proteases while activation of PAR4 can equally result in increased enteric permeability. It is not improbable to hypothesize that for increased intestinal permeability and colitis to occur there is a multi-factorial hit process that results in activation of multiple PARs simultaneously by different proteases.

Only now we begin to unravel the effects of alterations in the normal enteric microbiota (dysbiosis), and how these "normal" bacteria can potentially induce colitis. The current challenge is to explore which commensal bacteria can secrete proteases that result in damage of the mucosal barrier. Additionally, we need to understand

which microbes are associated with colitis and what the genetically predisposing factors are that "allow" these events to happen. For example, genetic mutations associated with the reduction of mucus production and increased mucosal bacterial adherence, immune abnormalities that result in dysbiosis, and innate immune response defects that cause dysregulated immune responses once the mucosal barrier is breached.

Investigating these aspects through cell lines, mono-associated gnotobiotic animals, Ussing chambers, high-throughput sequencing of microbial DNA, metabolomics and genome wide association studies will enable us to understand the role of enteric microbial proteases in the pathogenesis of IBD and to develop effective targeted therapies that will involve specific enteric bacteria, PARs, and the downstream regulation and host immune response.

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