REVIEW

Antimicrobial Proteins in Intestine and Inflammatory Bowel Diseases

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Mucosal surface of the intestinal tract is continuously exposed to a large number of microorganisms. To manage the substantial microbial exposure, epithelial surfaces produce a diverse arsenal of antimicrobial proteins (AMPs) that directly kill or inhibit the growth of microorganisms. Thus, AMPs are important components of innate immunity in the gut mucosa. They are frequently expressed in response to colonic inflammation and infection. Expression of many AMPs, including human β-defensin 2–4 and cathelicidin, is induced in response to invasion of pathogens or enteric microbiota into the mucosal barrier. In contrast, some AMPs, including human α-defensin 5–6 and human β-defensin 1, are constitutively expressed without microbial contact or invasion. In addition, specific AMPs are reported to be associated with inflammatory bowel disease (IBD) due to altered expression of AMPs or development of autoantibodies against AMPs. The advanced knowledge for AMPs expression in IBD can lead to its potential use as biomarkers for disease activity. Although the administration of exogenous AMPs as therapeutic strategies against IBD is still at an early stage of development, augmented induction of endogenous AMPs may be another interesting future research direction for the protective and therapeutic purposes. This review discusses new advances in our understanding of how intestinal AMPs protect against pathogens and contribute to pathophysiology of IBD. **(Intest Res 2014;12:20-33)**

Key Words: Antimicrobial protein; Antimicrobial peptide; Colitis; Inflammatory bowel diseases

INTRODUCTION

Human intestines are constantly exposed to the threats of various microorganisms. Healthy intestinal mucosa is characterized by the definite distinction between epithelial surfaces and enteric microbiota, because direct contact between intestinal epithelial cells and enteric microbiota and the colonization of pathogen have negative impacts to the human body.[1] These are commonly discriminated by mucus secreted by goblet cells. Among those, glycocalyx is present in apical surface of intestinal epithelial cells. The glycocalyx of small intestinal epithelium is a single mucus layer which is permeable to bacteria.[2] Glycocalyx of the large intestine is organized into two layers of a firm inner region and an outer layer with permeability. Since the inner layer forms a physical barrier that prevents the contact with enteric microbiota, bacterial colonization is rarely observed. In contrast, bacteria such as *Lactobacillus* spp. and *Bifidobacteria* spp. are usually found to colonize the outer layer.[3] This barrier makes intestinal microbiota to exist about 50 μm apart from intestinal epithelial cells in the colonic mucus of a mouse.[4]

The human body produces antimicrobial proteins (AMPs) as the part of the innate immune response to kill bacteria or prohibit the growth of microorganisms. AMPs are peptide antibiotics that act as an important effector of innate immunity. AMPs are commonly expressed in intestinal mucosa in constant contact with enteric microbiota. Thus, intestinal epithelial cells, paneth cells, and endogenous antimicrobial proteins expressed in different immune cells are expressed naturally

without stimulation or their release is accelerated by external stimulation. Among those, peptides consisting of fewer than 50 amino acids are defined as antimicrobial peptides.[5] Therefore, AMPs include antimicrobial peptides, and peptides are used interchangeably with AMPs in this review paper.

Colitis may occur due to enteric microbiota or the interaction between mucosa of pathogen and host. The induction of AMPs is profoundly related with the function of intestinal barriers. AMPs are involved in immune response, in addition to growth inhibition of pathogenic bacteria. Hence, an increase or decrease in the expression of AMPs during infectious and inflammatory processes can be used as a biomarker for specific diseases. Therefore, AMPs have emerged as new therapeutic agents that kill microorganisms resistant to currently existing antibiotics. This review paper discusses leading AMPs expressed in the intestinal tract and their functions, the association with inflammatory bowel diseases (IBD), and clinical applicability.

TYPES AND FUNCTIONS OF ANTIMICROBIAL PROTEINS

1. Defensin

Defensins are involved in innate immune responses of the gastrointestinal tract and mainly expressed in Paneth cells, epithelial cells, and immune cells. Defensins are cationic proteins abundant with cysteines and they are found in vertebrates, invertebrates and plants. Defensins consist of 18–45 amino acids, and classified into α-defensin and β-defensin according to the distribution of cysteines and disulfide bonds between cysteine residues.[6] Defensins are classified into proteins constitutively expressed without infections or inflammations and inducibly expressed with stimulation.[6]

Defensins are characterized by a strong polarization of charges. Therefore, they easily bind with phospholipids on the surface of microorganisms with negative charges through electrical action. As a result, antimicrobial response is activated by forming pores in the cell membrane.[7] Thus, the outer layer expands and the inner layer tightens in the lipid bilayer of bacteria cell membrane through membrane integration of defensins, and collapse and donut-shaped pores are resulted in bacteria cell membrane.[8] These effects also work on fungi and viruses, in addition to bacteria. Among defensins, human β-defensin 3 (hBD-3) is reported to inhibit the biosynthetic stage of bacteria cell membrane by binding to lipid II, which is the basic composite unit of peptidoglycan similarly with penicillin.[9]

1) Human α-defensin (Human Neutrophil Peptide, HNP)

Human α-defensin is called neutrophil peptides (human neutrophil peptide, HNP) because it is produced mainly by neutrophils.[10] Until now, only four types of human β-defen-

sins (HBD) have been discovered and presented as HNP-1, -2, -3, and -4. Neutrophils which take crucial roles in innate immunity use HNP in destroying the phagocytic pathogens.. Since neutrophils make up the largest percentage of all phagocytes in human body and circulate the entire body, HNPs are widely ranging antimicrobial peptides.

The association of human α-defensin with IBD has been investigated . Transcription factor 4 (TCF4), which is one of the transcriptional factors in the Wnt signaling pathway, is known as a gene regulating the expression of α-defensin.[11] Genetic variation in TCF4 promoter region is reported to be associated with Crohn's disease.[12] Moreover, a high rate of genetic mutation of lipoprotein receptor-related protein 6, which is an essential composition in the Wnt pathway, is observed in patients with Crohn's disease.[12] The results suggested that a decrease in α-defensin expression may result in dysbiosis in enteric microbiota and a higher risk of IBD.[13] However, the roles of HNPs on IBD still remain controversial.

HNP-1 and HNP-3 shows inhibition of cytotoxicity and Rho glucosylation in Caco-2 cells exposed to *Clostridium difficile* toxin B. In contrast, no specific action on defensive reaction was detected against *C. difficile* toxin A.[14] Moreover, HNP-1 prohibits the release of interleukin (IL)-1β stimulated by lipopolysaccharide (LPS).[15] The outcome implies that HNP-1 prevents inflammations induced by endotoxin of gram-negative bacteria. When HNP-1 was injected into the peritoneal cavity of a mouse model induced with enteritis using dextran sodium sulfate (DSS), more severe symptoms of colitis and a higher cytokine level in the colon were observed.[16] The expression level of HNP1–3 was high in the mucosa of patients with progressive IBD, suggesting that this could be related with neutrophil infiltration.[17] Plasma HNP 1–3 concentrations were found to be high in patients with IBD. The outcome is assumed to be attributable to the circulation of neutrophils.[18,19] Although the antimicrobial action of HNP-4 is stronger than those of HNP1–3,[20] the effect of HNP-4 on IBD has not been examined yet.

2) Human α-defensin 5 (HD-5) and Human α-defensin 6 (HD-6)

Human α-defensins (HD) 5 and 6 are only expressed in Paneth cells of the small intestine. These antimicrobial proteins are not expressed in the colon since Paneth cells are not present in the colon.[10] Paneth cells are a type of specialized epithelial cells of the gut, and located in the crypt base of the small intestine. However, Paneth cell metaplasia is detected when chronic inflammations exist in the gastrointestinal tract. Under these pathologic conditions, HD-5 and HD-6 could be secreted from Paneth cells present in the other parts of the body besides the small intestine.[21]

These AMPs play various defensive reactions against infections and inflammations. Thus, HD-5 has disinfection ability against all bacterial species. Furthermore, HD-5 induced the expression of IL-8 in intestinal epithelial cells, and the admin-

istration of HD-5 decreased mortality rate in a mouse model with DSS-induced colitis.[22] Transgenic mouse with overexpressed HD-5 showed high resistance against *Salmonella* infections.[23] In addition, HD-5 defenses against cytotoxicity by inhibiting Rho glucosylation by *C. difficile* toxin B.[14]

A decrease in the expression of HD-5 and HD-6 was shown in patients with Crohn's disease in the ileum.[24,25] Although some previous studies proposed that decreased expression of HD-5 and HD-6 is related with nucleotide oligomerization domain 2 (NOD2) mutation,[26] some studies found no association between and α-defensin expression in Paneths cells.[27] The expression of HD-5 detected in metaplastic Paneth cells in the colon of patients with IBD is likely to be a defensive reaction against bacteria during the incidence of enteritis.[28,29]

3) Mouse α-defensin (Cryptdin)

Mouse Paneth cell α-defensins, termed cryptdins, have not been found in human body.[30] Cryptdin precursors are located in the granules of Paneth cells, and formed with disinfection ability through the interaction with matrix metalloproteinase-7 (MMP-7).[30] Since the antimicrobial activity of cryptdin produced in MMP7^{-/-} mice is lowered, the likelihood of developing enteritis is higher.[15] Six cryptdins have been identified currently, and cryptdin-4 has the highest antimicrobial activity.[31,32]

4) Human β-defensin 1 (hBD-1)

Human β-defensin 1 (hBD-1) is an antimicrobial substance constitutively expressed in the human colonic and ileal epithelial cells regardless of enteritis. Therefore, hBD-1 is not expressed even though, stimuli such as IL-1α and others are applied to intestinal epithelial cell lines, Caco-2 and HT-29.[33] The hBD-1 protein is encoded by the *DEFB1* gene and gene expression of *DEFB1* is regulated by peroxisome proliferator-activated receptor gamma (PPARγ). Therefore, the deficiency of PPARγ in the intestinal mucosa of mice leads to a reduction in the expression of *mDefB10* identical to *DEFB1* in humans, and lowered disinfection ability against intestinal microbiota which include *Candida albicans, Bacteroides fragilis, Enterococcus faecalis*, and *Escherichia coli*.[34] Therefore, the expression of hBD-1 by PPARγ is assumed to play important roles in regulating a certain number of intestinal microbes.

Antimicrobial activity of hBD-1 against intestinal microbiota increases after the reduction of three disulfide bonds.[35] Thus, the antimicrobial activity of hBD-1 needs to be evaluated under unique environmental conditions. Thioredoxin is a mediator facilitating reduction of hBD-1 in intestinal epithelia. Thioredoxin expression at very high levels is shown under inflammatory conditions such as tumors or rheumatoid arthritis.[36] On the other hand, a decrease in thioredoxin expression is found at the inflammation site of Crohn's disease.[35] Therefore, reduced thioredoxin expression seems to weaken defense mechanism against enteric microbiota by lowering the antimicrobial activity of hBD-1.

5) Human β-defensin (hBD-2, -3, -4)

Unlike consistently expressed hBD-1, human β-defensins 2–4 (hBD-2, -3, -4) are common AMPs which show an increase in expression levels in response to external stimuli. These are expressed in a very small amount in normal healthy colon. Stimuli accelerating hBD-2 secretion are various types of bacteria and cytokine. Flagellin and *Campylobacter jejuni* obtained from *E. coli* strain Nissle 1917, which is a probiotic for the treatment of ulcerative colitis, could facilitate hBD-2 production.[37,38]

The induction of hBD-2 is mediated by proinflammatory cytokines including IL-1β, tumor necrosis factor (TNF)-α, and IL-17 through nuclear factor-kappaB (NF-κB)- and AP-1-dependent pathways.[38,39] Among all defensins, hBD-3 has the highest net-positive charge. The antimicrobial activities of hBD-1 and hBD-2 are weakened at high salt concentrations, and the activation of hBD-3 is maintained in physiological sodium concentration. Although hBD-1 and hBD-2 exhibit antimicrobial activities against gram-negative bacteria, hBD-3 shows a stronger antimicrobial action.[40]

The expression level of hBD-2 decreased in non-inflamed colonic mucosa of patients with IBD while, it increased in inflamed colonic mucosa.[41] Although hBD-2 secretion increased in the colonic tissues, no significant changes were observed in serum hBD-2 concentration.[18] Moreover, the expression levels of hBD-3 and hBD-4 increased in the colonic crypt of patients with ulcerative colitis while, expression levels remained the same in patients with Crohn's disease.[42] Mouse β-defensin-3, the murine homologue of human hBD-2 greatly increased in the intestinal epithelial cells of a mouse model with DSS-induced colitis.[43]

Differences were found in the expression of hBD-2 mRNA between active Crohn's disease and ulcerative colitis. The expression levels of hBD-2 and hBD-3 increased considerably in patients with active ulcerative colitis while, they were prohibited in patients with Crohn's disease.[44-46] In particular, disinfection ability against intestinal microbiota was relative lower in colonic mucosa of patients with Crohn's disease.[47] However, the mechanism to explain the outcomes still remains unclear. Although the expression level of hBD-2 decreased in a study on European and American patients with IBD,[48] the result was different in a New Zealand patient group.[49] An increase in hBD-2 in patients with IBD has been suggested as one of the possible causes for secondary phenomenon of barrier disruption. Meanwhile, β-defensin has no protective actions against cytotoxicity of *C. difficile* toxin B unlike HD-5.[14]

6) θ-defensin

Humans do not produce θ-defensin protein because human θ-defensin genes contain a premature stop codon.[50] The therapeutic effect of θ-defensin has not been identified yet. Synthetic θ-defensin (retrocyclin) has high antimicrobial activity and strong antiviral activity against human immunode-

ficiency virus.[51] Moreover, modified θ-defensin (RC-1) inhibited the growth of *Listeria monocytogenes* in macrophages more remarkably than α-defensin HNP-1.[52] The results suggest that θ-defensin is applicable to the human body.

2. Cathelicidin (Cathelicidin Anti-microbial Peptide, CAMP)

Cathelicidin antimicrobial peptides (CAMPs) are a family of polypeptides expressed in macrophages, neutrophils, intestinal epithelial cells, and others. They have anti-microbial effects against bacteria, virus, and fungi. CAMPs include human LL-37 and mouse cathelicidin-related antimicrobial peptide (mCRAMP). The mechanism of cathelicidins is very similar to those of defensins. Thus, cationic residues of α-helical peptide present in cathelicidins are inserted to the cell membrane by combining with bacterial plasma membrane. As a result, antimicrobial effect is activated by forming pores in the cell membrane.[53,54] Cathelicidins are released from mucosal surfaces that face the external environment. CAMPs are also found in breast milk and amniotic fluid, and display antimicrobial abilities against *Staphylococcus aureus*, group A *Streptococcus*, invasive strains of *E. coli* O29, and others.[55] Therefore, LL-37 is significantly involved in innate immune response of human newborns.

Cathelicidin secretion is induced by intestinal microbiota. Butyrate, a short-chain fatty acid metabolic substance, is known as a derivative of cathelicidin. Butyrate is an inhibitor of histone deacetylase, and trichostatin A also induces the expression of cathelicidin.[56] PU.1 an E-twenty six family transcription factor induces the expression of *CAMP* genes by binding to *CAMP* promoter.[57] Thus, vitamin D receptor and PU.1 are transferred to *CAMP* promoter and transcription is enhanced in cathelicidin genes of cells stimulated by secondary bile acids such as lithocholic acid, vitamin D, and butyrate.[57] The expression of cathelicidin increased in the colon of patients with ulcerative colitis while, and showed no changes in the colon of patients with Crohn's disease.[58] Symptoms of acute colitis were more severe in *CAMP*-/- mouse with the removal of cathelicidin.[59] These conditions deteriorated with the administration of bacterial DNA, the toll-like receptor 9 (TLR9) ligand. The intestinal expression of cathelicidin decreased in *TLR9*-/- mouse model with DSS-induced enteritis using.[59] The above results reveal that cathelicidins take crucial roles in defending the body against colitis.

When mCAMP was administered into the rectum of mouse model with DSS-induced enteritis, the expression of mucin-4 (*MUC-4*) gene increased in the colonic mucosa and restored its thickness. Consequently, colitis was cured.[60] The administration of mCRAMP, n particular, reduced fecal microbiota numbers. According to a previous *in vitro* study, LL-37 inhibited wound healing effect and apoptosis in HT-29 and Caco-2 cells.[61] Another study proposed that promoted cathelicidin induction showed treatment effect in an infec-

tion model. Thus, orally administered butyrate or phenylbutyrate in a rabbit model of *Shigella* increased the expression of cathelicidin in the colonic and rectal mucosa.[62,63] Increased expression level was associated with improvment in clinical symptoms of infections. However, cathelicidin showed insignificant effects on *Entamoeba histolytica* due to the secretion of cysteine proteases which degrade cathelicidins.[64]

3. Protease Inhibitors: Elafin and Secretory Leukocyte Peptidase Inhibitor (SLPI)

The delicate balances between proteases and anti-protease activities partially regulate inflammatory process during colitis progression. Although proteases cause tissue damage during inflammatory states, protease inhibitors facilitate the stabilization and treatment of injured tissues.

Protease inhibitors are elafin and secretory leukocyte peptidase inhibitor (SLPI). Elafin is an elastase-specific inhibitor with anti-microbial action by regulating inflammations.[65] SLPI is expressed mainly in the in the jejunum and colon, and prohibits a wide range of protease.[66] Thus, it is reported to prohibit the function of human leukocyte elastase, cathepsin G, trypsin, neutrophil elastase, and mast cell chymase. Moreover, SLPI has anti-microbial activity against *Salmonella* Typhimurium.[67]

Some studies have reported that protease inhibitors are profoundly associated with the incidence of IBD. For example, elafin expression was considerably high in the intestinal tissues of patients with ulcerative colitis.[68] According to another previous study on the tissues of patients with an ulcerative colitis, elafin was not expressed in inflammation-free colonic mucosa while, the expression of elafin increased in colonic mucosa at the inflammation site.[69] However, an increase in elafin expression still remains unclear in Crohn's disease. SLPI is the dominant protease inhibitor which increases within the intestinal mucosa of inflammation site in patients with ulcerative colitis. On the other hand, SLPI showed no increase in the inflammation-free intestinal mucosa or the large intestine of patients with Crohn's disease.[69] Low expression of elafin and SLPI is related with high expression of MMP in patients with Crohn's disease, this condition poses a potential risk of increasing the incidence of fistula.[69] High expression of elafin and SLPI in patients with ulcerative colitis is assumed to be caused by self-defense mechanism against enteritis.

In DSS- and trinitrobenzenesulfonic acid-induced colitis models, the overexpression of elafin through the introduction of genes by adenovirus alleviated colitis symptoms. During this process, a reduction in protein hydrolysis in the colon was found along with a decrease in cytokine and NF-κB activities.[70] In a laboratory study, the overexpression of elafin significantly reduced TNF-α-induced permeability and IL-8 expression. The outcome indicates that elafin overexpression retains ability to sustain intestinal epithelial integrity and an-

ti-inflammatory effects.[70] Increased SLPI production is likely to be involved in the recovery of tissues during colitis progression. Consequently, the expression of SLPI was reduced in thymic stromal lymphopoietin-deficient mouse model.[71] When enteritis was induced in this mouse model using DSS, the incidence of inflammation was similar to that of control group but, an increase in mortality rate was detected due to slow recovery of colitis. These consequences imply that SLPI plays important roles in the recovery process of damaged mucosa.[71]

4. Bactericidal/Permeability Increasing Protein (BPI)

Bactericidal/permeability increasing protein (BPI) is about 50 kDa glycoprotein that was first discovered in neutrophils. BPI is endogenously expressed in the epithelial cells of intestinal mucosa, and has strong antimicrobial effects on gram-negative bacteria. Thus, BPI kills gram-negative bacteria by binding to LPS, and prevents inflammatory response generated by LPS.[72] GLU216Lys, a single nucleotide polymorphism genotype of BPI is reported to be associated with ulcerative colitis and Crohn's disease.[73] An increase in BPI production was found in the colonic mucosa of patients with ulcerative colitis.[74] Moreover, BPI level in the colonic mucosa is suggested to be correlated with the degree of ulcerative colitis and inflammation.[74] As proven in an experimental study, a decrease in IL-8 expression by *Salmonella* in BPI-overexpressing Caco-2 cells implies that BPIs inhibit inflammation.[75] Anti-neutrophil cytoplasmic auto-antibodies (ANCA) are produced in some patients with IBD, and ANCA is a BPI-targeting auto-antibody.[76] Since IgG from patients with ulcerative colitis and Crohn's disease can neutralize BPI, the antimicrobial ability of BPI is consequently reduced. Auto-antibody ANCA against BPI raises mucosal injury in patients with IBD and the risk of diseases.[77] The above outcomes reveal that ANCA is associated with the development of IBD.

5. C-type Lectin Family

AMPs belonging to the C-type lectin family are human hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP) and mouse regenerating islet-derived protein 3γ (REG3γ). Similar structure of these proteins with C-type lectin exhibits antimicrobial effects by binding to peptidoglycan, a major component of bacterial plasma membrane.[78] HIP/PAP and REG3γ are mainly produced in Paneth cells and endocrine cells around the intestinal epithelial cells of the gut and ascending colon in adults.[79] These cells also secrete chromogranin A and synaptophysin, the biomarkers for endocrine cells. AMPs in this family were very highly expressed in germ-free mice exposed to bacteria and mice with DSS-induced colitis.[80] Moreover, HIP/PAP expression increased in the colonic epithelial cells of patients with IBD.[80] HIP/PAP is reported to be involved in cell proliferation

and differentiation and the expression level was elevated in various types of tumor including colon cancer. Despite its antimicrobial action, the roles of HIP/PAP in the occurrence of colitis still remain unknown.

6. Lysozyme

Lysozyme is a hydrolase breaking the bonds in peptidoglycan layer which consists of an outer membrane of bacterial cell walls. Lysozyme is a leading substance involved in destruction of the cell membrane, and it hydrolyzes the glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan layer in cell walls. Secretory phospholipase A_2 is another antimicrobial enzyme produced in Paneth cells, and displays antimicrobial action by hydrolyzing phospholipids which are major components of bacterial cell walls.[81]

Lysozymes are abundant in mucus, tear, saliva, breast milk, and others, and a massive lysozyme is located in granular cells. Increased expression of lysozyme mRNA was observed in the colonic epithelial cells of patients with ulcerative colitis compare to those of control group.[29] Lysozyme mRNA was produced in the colonic epithelial cells of patients with Crohn's disease, and lysozyme expression is reported to be correlated with the degree of inflammation.[24] However, the use of fecal lysozyme concentration as a diagnostic marker of IBD still remains controversial. A study on the treatment effects of lysozyme on IBD was performed. Egg white is abundant with lysozyme. When lysozymes from egg white were injected to a porcine model with DSS-induced colitis, improvement in colitis symptoms and a decrease in the expression of TNF-α and IL-6 were observed.

7. Lactoferrin (Lactotransferrin)

Lactoferrin, also called as lactotransferrin, is a globular glycoprotein with a molecular mass of about 80 kDa. Lactoferrin is found in human cellular fluids including mucus, tear, saliva, breast milk, and others, and a massive amount is located within neutrophils like lysozyme. Considerably higher levels of lactoferrin are present in colostrum. Therefore, lactoferrin and lysozyme are considered circulating substances making up innate immune response system. The antimicrobial activities of lactoferrin are observed in various aspects. Since lactoferrin with high iron affinity binds iron and makes it unavailable to pathogens.[82] Furthermore, lactoferrin can dissolve cells by enhancing membrane permeability by binding to the LPS layer of bacterial cell wall. In addition, it promotes phagocytosis of immune cells.[83,84]

Fecal lactoferrin concentration increased in patients with IBD, and remained the same in patients with irritable bowel syndrome. Hence, lactoferrin level is suggested to be used in diagnosing IBD.[85] Measurement of lactoferrin is simple and cost-effective, and stability in stool samples is high. Moreover,

a reduction in fecal lactoferrin concentration aligned with therapeutic response to mucosal treatment.[86] Fecal lactoferrin concentration is reported to be higher in toxigenic bacteria of patient with *C. difficile* colitis than non-toxigenic bacteria.[87]

The effectiveness of IBD treatment using lactoferrin is briefly discussed in this section. When lactoferrin was orally administered to a model with DSS-induced colitis, colitis symptoms were alleviated according to doses and cytokine imbalance was resolved.[88] Identical results were observed in a model with trinitrobenzenesulfonic acid-induced colitis.[89] Lactoferricin and lactoferrampin, peptides derived from lactoferrin, are synthetic substances capable of killing *Entamoeba histolytica*. These are anticipated to replace metronidazole, an antibiotic drug.[90]

8. Hepcidin

Hepcidin is predominantly produced in the liver in the mature form of the 25 amino acid peptide derived from a preprohepcidin of 84 amino acids and a prohepcidin of 60 amino acids. Hepcidin, a 25-amino acid peptide hormone, is the principal regulator of plasma iron concentrations by blocking iron efflux from cells into plasma through the binding of hepcidin to its receptor, the iron export channel ferroportin present on the basolateral surface of intestinal epithelial cells and cell membrane of macrophages.[91] Despite iron absorption in the gastrointestinal tract, intestinal iron absorption is reduced because hepcidin inhibits iron uptake from intestinal epithelial cells to hepatic portal system. In addition, hepcidin blocks cellular iron efflux by impeding the roles of ferroportin in macrophages. Through these processes, hepcidin maintains normal blood iron levels. Increased hepcidin level in chronic inflammatory conditions like IBD blocks the movement of iron from macrophages. This results in a reduction of the iron level in blood serum, leading to anemia.[92] Since hepcidin restricts the use of iron by bacteria, the growth of pathogens could be prohibited.[93]

The roles of hepcidin in IBD patients are discussed. Serum hepcidin levels in patient groups of ulcerative colitis and Crohn's disease were significantly higher than those of control group.[94] Serum hepcidin coincided with disease activity and C-reactive protein concentration. Prohepcidin, the precursor of hepcidin, was proportional to hemoglobin level while, hepcidin was inversely proportional to hemoglobin level. The outcome suggests that hepcidin is likely to be associated with IBD-related anemia.[94] Hepcidin expression is dependent on bone morphogenetic proteins. When antibone morphogenetic protein reagent was injected to a colitis model using T cell transfer, an increase in blood iron level and a decrease in inflammatory cytokine expression were resulted by the inhibition of hepcidin expression.[95] Therefore, the inhibition of hepcidin expression is anticipated to cure IBD-related anemia and alleviate colitis symptoms.

9. Lipocalin-2 (Lcn-2, Neutrophil Gelatinase-associated Lipocalin [NGAL], siderocalin)

Microorganisms use siderophores, iron chelating compounds to obtain iron.[96] Iron-bound siderophores are ferric siderophore complex, and common examples are siderophore enterobactin (Ent) of gram-negative bacteria such as *E. coli, Salmonella* Enterica, and *Klebsiella pneumoniae*.[97,98] In the human body, lipocalin-2 (Lcn-2) or siderocalin hampers bacterial growth by binding to ferric siderophore complex of bacteria.[99]

Lcn-2, also known as neutrophil gelatinase-associated lipocalin, is a 25 kDa antimicrobial protein abundant in neutrophil granules. Lcn-2 is produced in intestinal epithelial cells infected with enterotoxigenic *B. fragilis* (ETBF).[100] ETBF infection also increases hBD-2 expression.[33] However, the expression patterns manifested by ETBF infection in two antimicrobial proteins vary. Thus, hBD-2 is mainly released from basolateral surface in the early stage of infection while, Lcn-2 is secreted at both basolateral and apical cell surfaces in the late stage of infection. The expression of hBD-2 is regulated by NF-κB while, Lcn-2 is regulated by AP-1, a transcription factor. *B. fragilis* uses outer membrane proteins instead of siderophores for iron absorption.[101-103] Lcn-2 produced from apical surface is assumed to act on other bacteria in addition to *B. fragilis*.[100]

RESISTANCE TO ANTIMICROBIAL PROTEINS

Antibiotic resistance of bacteria is gradually increasing. Theoretically, there is the possibility of resistance in endogenous AMPs. However, endogenous AMPs have maintained the effectiveness on intestinal bacteria for a long period of time. The mechanism has not been clarified yet. Distribution of diverse antimicrobial protein families in the colon and consistently maintained cell wall or membrane structure targeting AMPs are suggested as possible mechanism.[104] Pathogens including *S. aureus, Salmonella* spp. and *Legionella pneumophila* block attack from cation AMPs such as defensins by replacing anions in cell walls with cations.[105-108] Moreover, *S. aureus* and group A *Streptococcus* could be deactivated through protein hydrolysis of AMPs.[109,110] In *Neisseria* spp., AMP resistance was manifested through efflux pump.[111] In *Shigella* spp., anti-micorbial activities against cathelicidin and β-defensin were reduced by inhibiting the synthesis of intrinsic AMPs in the intestines.[112] Therefore, AMPs are anticipated to prohibit the response to decrease intestinal microbe numbers to below normal level, in addition to the prevention of excessive growth of intestinal bacteria.

REGULATION OF IMMUNE RESPONSE BY ANTIMICROBIAL PROTEINS

Antimicrobial proteins regulate innate immune response

through diverse methods in addition to directly destroying microorganisms. Cathelicidins and defensins constitute major AMP families with immune regulatory function.

1. Chemoattraction of Immune Cells by Antimicrobial Proteins

Cathelicidins and defensins directly regulate inflammatory response through chemotactic activities. The human cathelicidin LL-37 can migrate neutrophils, monocytes, and CD4 T cells to the inflammatory sites. The chemotactic ability is regulated by G protein-coupled formyl peptide receptor-like 1 (FPRL1), also known as formyl peptide receptor 2.[113] Although the mouse cathelicidin (mCRAMP) has considerably different amino acid sequences of LL-37, FPRL1 or FPRL2 (also known as formyl peptide receptor 3) attracts the human and mouse cells dependently.[114] Moreover, human α-defensin, hBD-3, and hBD-4 manifest chemotaxis towards monocytes and macrophages.[115] LL-37 and hBD-2 attract mast cells.[116] LL-37, in particular, stimulates mast cells to release histamine and induces the formation of new blood vessels.[117]

AMPs different in structures displays various activities in chemotaxis. For example, human α-defensin selectively induced the movement of naïve CD4$^+$CD45RA$^+$ and CD8$^+$ T cells but did not move CD4$^+$CD45RO$^+$ memory T cells.[118] However, β-defensin exhibited chemotactic response to immature dendritic cells and CD4$^+$CD45RO$^+$ memory T cells. The chemotactic effect of human defensin was inhibited by antibody against CC-chemokine receptor 6.[119] These results indicate that AMPs can regulate inflammatory response by exhibiting chemokine action.

2. Regulation of Toll-like Receptor (TLR) Responses

TLR is a molecular structure existing on human plasma membrane or endosome surface and binds to pathogen-associated molecular patterns present on bacterial surface. Therefore, TLR molecule is a type of membrane-bound pattern recognition receptors (PRRs). As a result, NF-κB and others are activated to induce or inhibit inflammatory response through intracellular signal transmission in the human body.[81] Cathelicidins, a leading example, are reported to act directly on bacterial LPS or TLR ligands such as self DNA.[104] Cathelicidins influence TLR signaling by affecting the internalization of TLR ligands or changing cell surface membrane microdomain function.[120,121] The human cathelicidin LL-37 increases the production of type I interferon by activating TLR-9 through the interaction with DNA. Consequently, cathelicidins influence the maturation of Th17 cells.[121] AMPs expressed by pathogen infection can destroy the pathogen itself and neutralize excessive immune response at the same time. However, abnormal expression of antimicrobial proteins could elevate the risk of inflammation regulation disturbances and autoimmune disease. This is thought to be attributable to changes in sensitivity against self DNA.[104] Therefore, the mechanism preventing excessive expression of AMPs exists in the human body.

REGULATION OF ANTIMICROBIAL PROTEIN EXPRESSION

The expression and release of AMPs are systematically controlled in the human body, since toxicity of AMPs can affect the human cell membrane. Excessive immune responses by AMPs expression need to be controlled.

1. Developmental Regulation

The human intestinal microbiota changes with age from the birth. Obligate anaerobes, mainly comprising *Bifidobacterium* spp. and *Bacteroides* spp. settle at birth and then, intestinal microbiota stabilizes gradually after weaning period.[84] Intestinal AMPs are highly expressed at birth and in the early post-birth period. Control of AMPs expression is assumed to take roles in maintaining immune homeostasis during the developmental period of infant intestinal tissues.[104] In a mouse model, antimicrobial proteins angiogenin 4 (Ang4) and REG3γ were strongly induced in the small intestine during the early post-birth period. The expression level of Ang4 increases by about 20 times higher during weaning period (about 17–21 days post birth) in conventionally raised mice and then, matured state is maintained.[122] The expression level of REG3γ increases by about 3,000 times.[78] To sum up the above results, the overexpression of AMPs is an important mechanism for host defense during periods of microbiota settlement and disappearance of passive immunity maintained by breastfeeding.

Cathelicidin expression shows inverse switching phenomenon. The expression level of cathelicidin is high during infant period and then, gradually decreases right before stopping breastfeeding.[123] The expression of cathelicidin varies according to anatomical locations in adults. Cathelicidin expression is ceased in the small intestine and maintained at a high level in the colon. Likewise, cathelicidins highly expressed in the colon provide defensive activity against pathogen infection.[124] The physiological association of a decrease in cathelicidin expression according to the maturation of the gut has not yet been fully verified.

2. Transcriptional Regulation by Microbiota

The expression of α-defensin mostly requires transcriptional factor TCF-4 in the Wnt pathway but, α-defensin is produced regardless of intestinal bacteria.[125] Lysozymes, Secretory phospholipase A$_2$, and some β-defensins do not require normal intestinal bacteria signaling.[122,126,127] According to a study on germ-free mouse, bacterial signals are critical in the expression of some intestinal AMPs. For instance, the expres-

sion of human β-defensin (hBD-2) is regulated by intestinal bacteria.[126] Moreover, AMPs such as Ang4 and REG3γ were deficient in germ-free mouse and generally up-regulated by microbiota.[78,122]

The PRR of the host can also regulate the expression of AMPs. For example, the stimulation of TLR is essential for REG3γ mRNA expression in intestinal epithelial cells. The expression level of antimicrobial protein REG3γ was lowered in myeloid differentiation primary response protein 88 (MyD88) knockout mice. The outcome demonstrates that TLR is crucial in AMPs expression.[4,128-130] Moreover, both intestinal epithelial and Paneth cells recognize bacteria using TLRs and then, REG3γ expression is up-regulated.[4,128,129] LPS binding to TLR4[128,129] and flagellin binding to TLR5[131] induce REG3γ expression. This process can be carried out without the aid of enteric microbiota. For instance, butyrate, a short-chain fatty acid produced through the fermentation of dietary fibers by intestinal microbes in the colon is important in the expression of cathelicidin LL-37.[132]

NOD2 can also control the expression of intestinal AMPs. NOD2 is a cytoplasmic PRR produced in Paneth cells, macrophages, and intestinal epithelial cells. NOD2 can activate NF-κB by recognizing muramyl dipeptide, a substructure of peptidoglycan.[80] muramyl dipeptide facilitates the antimicrobial activity of crypts distributed in Paneth cells. Therefore, the composition of the intestinal microbiota changed noticeably in $Nod2^{-/-}$ mice.[133] These results suggest that some AMPs are regulated intestinal microbiota.

3. Post-translational Regulation

Host cells secrete AMPs that are toxic to cell membranes and these proteins are potential factors to damage cell membranes. To prevent this problem, the activities of AMPs must be tightly regulated during storage in membrane-bound secretory granules. For instance, α-defensins are stored in Paneth cell granules as inactive pro-peptides. Mouse α-defensins (cryptdin) have to be processed in terminus of the pro-peptide by MMP7 to produce active peptides with antibacterial ability.[134] In humans, trypsin cleaves α-defensins to their mature active peptides.[135] REG3γ also requires N-terminal proteolytic process by trypsin to yield an active protein with antibacterial ability. After this process, proteins with antimicrobial activities are secreted into the gut lumen.[136] Cathelicidins are synthesized as pro-peptides, in which signaling peptides called "cathelin pro-sequence" are located at N-terminal, and C-terminal area showing antimicrobial characteristics consists of cation area. Pro-peptides are converted to cathelicidins with antimicrobial activity by serine proteases.[137]

A recent study reported interesting results on the regulation of HD-6. Adequate antimicrobial activities are not detected with the presence of HD-6 alone. Dimer shaped HD-6 is processed to tetramer by binding to proteins present on the surface of bacteria and then, they are self-assembled into large oligomers. As a result, antimicrobial effect is finally exhibited as HD-6 undergoes self-assembly to form fibrils and nanonets that surround bacteria.[138]

Oxidation-reduction environment involves the activation of AMPs. In human cells abundant with oxygen, the antimicrobial effect of hBD-1 is low under this condition. On the other hand, the condition changes to the gut where oxygen is insufficient, and the antimicrobial effect of hBD-1 is remarkably strong. The outcome is assumed to be caused by the reduction of disulphide bridges and structural changes under conditions of limiting oxygen. The disulphide bridges of hBD-1 are reduced and antimicrobial activity increases drastically as an amphipathic arrangement of cationic and hydrophobic residues is broken up.[139] Antimicrobial activities against *Bifidobacteria* spp., *Lactobacillus* spp., and *Candida albicans* are more effective when hBD-1 is reduced to linear peptides. Not reduced hBD-1 does not act on those bacteria.[35]

4. Secretion Regulation

The secretion of AMPs seems to be regulated by intestinal microbiota. For example, Paneth cells lead the production of most AMPs including α-defensin and lysozyme. Paneth cells secrete the intracellular content of granules to the intestinal tract when they are exposed to living bacteria or bacterial molecules such as LPS.[140] The release of AMPs is anticipated to be accurately regulated in response to microorganisms which pose potential threats to human health. However, the detection mechanisms of microorganisms regulating the secretion of AMPs by Paneth cells have not been clarified yet. The expression and production of AMPs are assumed to be controlled by complex networks formed by interactions between microorganisms and nutritional signals.[104] More efforts need to be exerted to further investigate these regulation networks. Successful studies will enable the implementation of new therapeutic strategies aiming for the enhancement of endogenous AMPs production.

ARTIFICIAL ANTIMICROBIAL PEPTIDE

Attempts have been made to synthesize small-sized antimicrobial peptides using endogenous AMPs expressed in the body.[30] One of difficulties in the development is the lack of knowledge on peptide structures involved in antimicrobial or anti-inflammatory activities. AMPs listed above greatly vary in amino acid arrangement , molecular weight, and molecular structure.[5] Peptide structures can be easily identified by measuring antimicrobial activities of synthetic peptides manufactured with some changes in amino acid sequence in AMPs with short peptide sequences. For example, cathelicidins are short, linear peptides with 37 amino acids, and many studies have reported synthetic peptides with artificially changed structures.[141,142] These artificial approaches are difficult in AMPs with high molecular masses.

The development of synthetic peptides is in progress using natural AMPs normally present in nature. For example, coprisin is a 9-amino acid peptide (LLCIALRKK) derived from an insect called Korean dung beetle. This peptide exhibits antimicrobial activities by destructing bacterial cell wall. In an animal testing performed on mice, coprisins were found to prevent inflammation and mucosal damage caused by *C. difficile* infection.[143] In addition, telavacin and dalbavacin, semi-synthetic glycopeptides showed antimicrobial activities against vancomycin-resistant Gram-positive bacteria.[144] According to a clinical trial, orally administered peptides are reported to be effective in improving *C. difficile* colitis.[145] Newly introduced synthetic antimicrobial peptides are expected to be used as candidate materials for preventing and treating IBD and intestinal bacterial infections.

CONCLUSIONS

AMPs, enteric microbiota, and the interaction of immune regulation in the intestine are being actively investigated. An increase in some AMPs expression is observed in IBD and intestinal infections. Specific antimicrobial materials can be used as biomarkers for estimating disease activity. Moreover, the development of therapeutic method is currently in progress applying the fact that decreased secretion of AMPs exists in IBD. An abnormal expression of defensin was detected in patients with Crohn's disease in the ileum, and assessing the applicability of recombinant antimicrobial proteins HD-5 in these patients is a representative example. The treatment effects of several AMPs have been proved in animal testing. Although new groups of small-sized AMPs have been developed, this technology is still at an early-stage of development. For example, only a few studies have been performed on the stability of administered peptides or proteins, transfer methods to inflammation sites, efficacy and safety. Orally administered AMPs have to reach the intestines by avoiding the attack from gastric acid. Moreover, the comparison of the effectiveness between AMPs and antibiotics, and the effects of AMPs in already colonized enteric microbiota need to be further investigated. In addition, the chemotactic characteristics of AMPs have to be taken into consideration.

Despite the above difficulties, attempts have been made to resolve those problems. For example, orally administered AMPs have been encapsulated against gastric juice. Since the stability of most AMPs is low in the blood, the expression of AMP genes has been attempted using virus. However, problems on the stability and transfer method of administered AMPs still restrict clinical trials. As an alternative measure, enhancing innate immunity by increasing the induction of naturally expressed AMPs in the body has been proposed. For instance, calcipotriol ointment (vitamin D analogue used for the treatment of psoriasis) used on healthy men's skin increases the production of cathelicidin.[146] Furthermore, 1,25-dihydroxyvitamin is known to induce the expression

of cathelicidin,[147] and phenylbutyrate promotes the expression of cathelicidin in various cell lines.[148] *E. coli* Nissle 1917 (Mutaflor®, Mutaflor capsules have been proven to heal mild UC) and other probiotics including *E. coli* and *Lactobacillus reuteri* are known to increase the expression of hBD-2.[37,38,149] Moreover, the expression of hBD-2 is also elevated by 1,25-dihydroxyvitamin D3.[147] The induction of AMP expression is currently realizable in laboratory and clinical trials.[150] From host's perspective, knowledge expansion on AMPs is anticipated to contribute in clarifying the pathogenic mechanism of IBD and finding cure methods.

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