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ALTERATIONS IN INTESTINAL PERMEABILITY CAUSE COLONIC INFLAMMATION AND FIBROSIS IN TYPE III PHOSPHATIDYLINOSITOL PHOSPHATE KINASE-DEFICIENT MICE

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Abstract

We recently established mice lacking the type III phosphatidylinositol phosphate kinase gene (*PipkIII*) in enterocytes, which represent a unique model of intestinal inflammation associated with extensive fibrosis that closely resembles human Crohn's disease. These mutant mice develop spontaneous juvenile colitis with marked inflammatory cytokine expression, but the primary pathogenic mechanism remains unclear. In the present study, we found that administration of broad-specific antibiotics effectively ameliorated the colitis of *PipkIII*-deficient mice, suggesting the involvement of intestinal bacterial flora in the onset of colitis. Furthermore, we showed that altered intestinal permeability facilitating commensal bacteria entry putatively caused the intestinal inflammation and fibrosis observed in these mutant mice.

Key words : PipkIII, phosphatidylinositol 3,5-bisphosphate, inflammatory bowel disease

Introduction

The inflammatory bowel diseases (IBD) Crohn's disease and ulcerative colitis are highly heritable[1,2)]. Genome-wide association studies suggested that the gene encoding the phosphoinositide phosphatase myotubularin-related protein 3 (*MTMR3*) is a key genetic factor implicated in the pathogenesis of IBD in addition to the many molecules involved in adaptive and innate immunity[3,4)]. The involvement of *MTMR3* in the pathogenic mechanism underlying IBD has not been directly revealed, although other genes of the family of MTMR phosphatases, such as myotubularin 1 (*MTM1*), *MTMR2*, and *MTMR14*, have been identified as disease-causing genes of myotubular myopathy, Charcot-Marie-Tooth neuropathy, and centronuclear myopathy, respectively[5-9)].

Phosphoinositides consist of phosphatidylinositol and its seven phosphorylated derivatives, which function as lipid second messengers that are implicated in signal transduction and membrane trafficking. Interconversions among the eight phosphoinositides are strictly regulated by a large number of kinases and phosphatases showing distinct substrate specificities[10)]. MTMR family phosphatases, including MTMR3, preferentially hydrolyze the D3 position of phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P_2), or both[10,11)]. These two endosomal phosphoinositides play well-known roles in intracellular vesicle trafficking, but little had been known about the relationship between these lipids and the onset of IBD[9,10,12)].

We recently reported that mice with enterocyte-specific deletion of the type III phosphatidylinositol phosphate kinase gene (*PipkIII*) have short lifespans with

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malnutrition, and exhibited severe chronic intestinal inflammation with fibrosis[13]. These findings suggested the importance of endosomal phosphoinositides metabolism in enterocytes for normal intestinal homeostasis, because PIPKIII/PIKfyve is the sole enzyme that produces PI(3,5)P_2 through phosphorylation at the D-5 position of PI3P[12,13]. In the intestine of enterocyte-specific *PipkIII*-deficient mice, inflammatory genes such as tumor necrosis factor-alpha (*Tnfα*), interleukins (*Il1β, Il-6, Il-12b*), and chemokines (*Ccl2, Cxcl5*) were up-regulated[13], but the primary pathogenic mechanism of inflammation remains unknown. Histologically, the mutant mice displayed vacuolation of enterocytes, lymphocyte infiltration, and decreased Paneth cell granules. The release of antimicrobial proteins by Paneth cells preserves homeostasis of the gut microbiota, suggesting that its breakdown may precipitate the chronic inflammatory pathology observed in IBD.

In this study, we elucidated the participation of bacterial flora in the onset of intestinal inflammation and fibrosis in enterocyte-specific *PipkIII*-deficient mice by treating the mice with antibiotics. The results showed that increased intestinal permeability facilitated bacterial entry in these mutant mice.

Materials and methods

Mice

The establishment of the enterocyte-specific *PipkIII*-deficient mouse model has been reported previously[13]. Briefly, we intercrossed *PipkIII*$^{flox/flox}$ mice with *VilCre* transgenic mice, which express Cre recombinase under the control of the villin promoter[14,15]. The resulting *VilCrePipkIII*$^{flox/+}$ mice were crossed back to *PipkIII*$^{flox/flox}$ mice to generate *VilCrePipkIII*$^{flox/flox}$, enterocyte-specific *PipkIII*-deficient mice. *PipkIII*$^{flox/+}$ or *PipkIII*$^{flox/flox}$ littermates were used as control mice. All experiments were performed in accordance with the approved institutional animal care guidelines of Akita University Graduate School of Medicine.

Administration of antibiotics

Imipenem and cilastatin (Tienam®, MSD) plus vancomycin hydrochloride (Meiji Seika Pharma) were administrated to mice orally in drinking water throughout pregnancy and weaning. The oral administration was continued until the weaning mice were sacrificed at 4-5 weeks old. Imipenem (50 mg/kg/day) shows antibacterial activities against almost all gram-positive and gram-negative bacterial strains, except for methicillin-resistant staphylococci, enterococci, and some *Pseudomonas* species. To compensate for the antibacterial activity against these species, imipenem was co-administered with vancomycin (50 mg/kg/day)[16].

Histological analysis

The mice were killed, and their intestinal tissues were excised and immersion-fixed in 4% paraformaldehyde (Nacalai) in phosphate-buffered saline overnight. Tissue blocks were embedded in paraffin, and 4-μm sections were cut. Serial sections were stained with hematoxylin-eosin (HE) to determine the extent of leukocyte infiltration and hypertrophy of the submucosa. Azan staining was used to detect fibrosis, and periodic acid-Schiff (PAS) staining was used to identify goblet cells by using standard procedures.

Assessment of intestinal permeability *in vivo*

The cDNA of enhanced green fluorescent protein (EGFP) was introduced into pGEX4T (Takara Bio), a bacterial expression vector. *Escherichia coli* DH5 cells (Takara Bio) were transformed with the pGEX4T-EGFP plasmid. Either 100 μL of the transformants suspension (4×10^9 CFU/mL) or Alexa Fluor 488-labeled 10-kDa dextran (0.5 mg/mL, Molecular Probes®) solution was injected into the mice intrarectally. The colon was dissected one hour after injection, and frozen sections were prepared for confocal fluorescence microscopy (LSM510 META, Carl Zeiss).

Determination of invaded bacteria numbers in the colon lamina propria

The transverse, descending, and sigmoid colons of 3-week-old enterocyte-specific *PipkIII*-deficient or control mice were removed, washed once with sterile phosphate-buffered saline, and then incubated in 167 μg/mL imipenem and 167 μg/mL vancomycin solutions for 5 minutes to remove adherent bacteria. One hundred mil-

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ligrams of colon tissue was added to 600 μL of LB medium and homogenated. To determine the bacterial concentrations, serial 10-fold dilutions of the homogenates were plated on an LB agar plate and bacterial colonies were counted after incubation at 37°C for 24 hours. Results are expressed as the mean concentration of viable bacteria ($\log_{10}$ of CFU per 100 mg of tissue).

Statistical analysis

All data are expressed as the mean±SEM of at least triplicate samples. Data were analyzed by the Mann-Whitney U test. For all experiments, P-values<0.05 were considered significant.

Results and Discussion

Oral administration of antibiotics reduces the severity of colitis in enterocyte-specific *PipkIII*-deficient mice

Resident bacteria are implicated in the pathogenesis of experimental colitis and IBD[17,18]. In both spontaneous and induced animal colitis models, broad-spectrum antibiotics (imipenem plus vancomycin) effectively prevent colitis[16]. To examine the involvement of microbiota in the pathogenesis of intestinal inflammation and fibrosis in enterocyte-specific *PipkIII*-deficient mice, imipenem and vancomycin were orally administrated to the mother of mutant mice in drinking water throughout pregnancy and weaning. At 1-2 weeks after weaning, colitis was assessed histologically. The administration of antibiotics

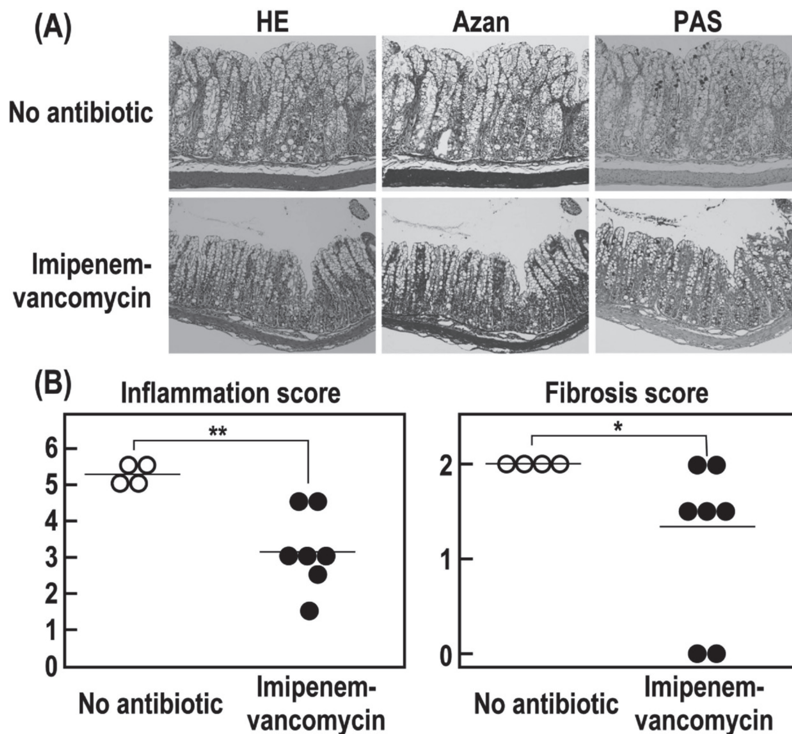

Fig. 1. Antibiotics administration reduced inflammation and fibrosis of the colons in the mutant mice. (A) Representative histology of the colons in enterocyte-specific *PipkIII*-deficient mice that were given normal drinking water (No antibiotic) or water containing selected antibiotics (Imipenem-vancomycin). Inflammatory cell infiltration, hypertrophy of the submucosa (HE), fibrosis (Azan), and elimination of goblet cells (PAS) were recovered by administration of antibiotics. (B) Summary of the colon inflammation and fibrosis histological scores. *P<0.05, **P<0.01. The horizontal lines indicate the mean values.

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Table 1. Histologic inflammation and fibrosis scoring system

Group	Description	Score
Inflammation score (0-7)		
Leukocyte infiltration	No infiltration	0
	Leukocytes infiltrated in the lamina propria	1
	Leukocytes infiltrated in the lamina propria and submucosa	2
Goblet cells elimination	No elimination	0
	Mild (<10%)	1
	Moderate (10-25%)	2
	Severe (25-50%)	3
	Profound (>50%)	4
Hypertrophy of the submucosa	Normal	0
	Thickened	1
Fibrosis score (0-2)		
Fibrosis	No fibrosis	0
	Localized fibrosis	1
	Extensive fibrosis	2

recovered inflammatory cell infiltration, thickening of the mucosa (evaluated by HE staining), fibrosis (Azan staining), and the loss of goblet cells (PAS staining) in the colon compared to mutant mice that did not receive antibiotics (Fig. 1A). Both the inflammation and fibrosis scores (Table 1) were statistically significantly decreased by antibiotics treatment (Fig. 1B). These data indicate that the elimination of microbiota by antibiotics treatment could effectively prevent colitis and colonic fibrosis of enterocyte-specific *PipkIII*-deficient mice, suggesting that intestinal commensal bacteria were the main cause of these symptoms.

The permeability of the intestinal epithelium increased in enterocyte-specific *PipkIII*-deficient mice

The intestinal epithelium is comprised of a single layer of columnar epithelial cells that separates the intestinal lumen from the underlying lamina propria. The space between epithelial cells is sealed by tight junctions, which regulate the paracellular permeability and are crucial for maintaining the integrity of the epithelial barrier. Importantly, the epithelial barrier prevents paracellular diffusion of microorganisms from entering the mucosal tissues. Tight junctions have been linked to a wide range of pathological conditions, and a leaky intestinal barrier has been shown to cause IBD[19,20]. Treatment with the PIPKIII inhibitor YM201636 was reported to result in delayed formation of a functional tight junction permeability barrier in cultured renal epithelial Madin-Darby canine kidney cells[21]. Given this context, we examined whether the intestinal permeability of enterocyte-specific *PipkIII*-deficient mice was functionally maintained. The fluorescent-labeled *E. coli* cells or dextrans were injected into mutant and littermate control mice intrarectally. After one hour, live bacteria had clearly invaded into the mucosa of the mutant mice but not the control mice (Fig. 2, upper panels). Macromolecules were also more extensively diffused into the mucosa of mutant mice compared to control mice (Fig. 2, lower panels).

Bacterial entry into the colon lamina propria was increased in enterocyte-specific *PipkIII*-deficient mice

The enhanced intestinal paracellular permeability of enterocyte-specific *PipkIII*-deficient mice was confirmed by the intrusion of exogenous fluorescent molecules.

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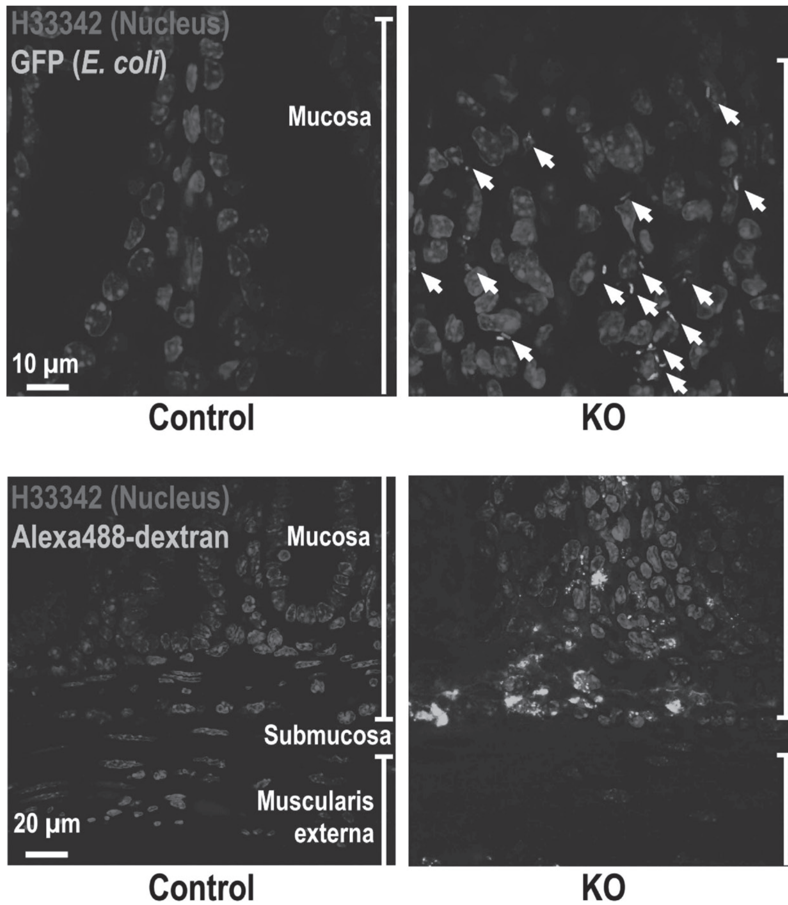

Fig. 2. The integrity of the intestinal epithelium is impaired in enterocyte-specific *PipkIII*-deficient mice. *E. coli* cells (4×10^8 CFU) transformed with the pGEX4T-EGFP plasmid (upper panels, arrowheads) and Alexa Fluor 488-labeled 10-kDa dextran (green, lower panels) were injected into the rectum of enterocyte-specific *PipkIII*-deficient (KO) and control mice. Nuclei (blue) were counterstained with H33342.

We next examined whether such permeability would facilitate the spontaneous penetration of commensal bacteria into the mucosa of mutant mice. The colons of enterocyte-specific *PipkIII*-deficient or control mice were resected, incubated in an antimicrobial solution to remove any adherent bacteria, and homogenized in LB medium. The numbers of invaded bacteria in the colon were determined after incubation for 24 hours on an LB agar plate. Figure 3 shows that bacterial invasion into the colon lamina propria was significantly more frequent in the mutant mice than in the control mice ($P<0.05$). This increase of commensal bacteria entry into the colon is a plausible mechanism for the pathogenesis of colitis and colonic fibrosis observed in enterocyte-specific *PipkIII*-deficient mice.

Conclusions

In this report, we demonstrate that alterations of intestinal permeability owing to PIPKIII deficiency allow for invasion of commensal bacteria, which likely cause the intestinal inflammation associated with fibrosis observed in enterocyte-specific *PipkIII*-deficient mice. No genetic association of *PIPKIII* to human inflammatory or fi-

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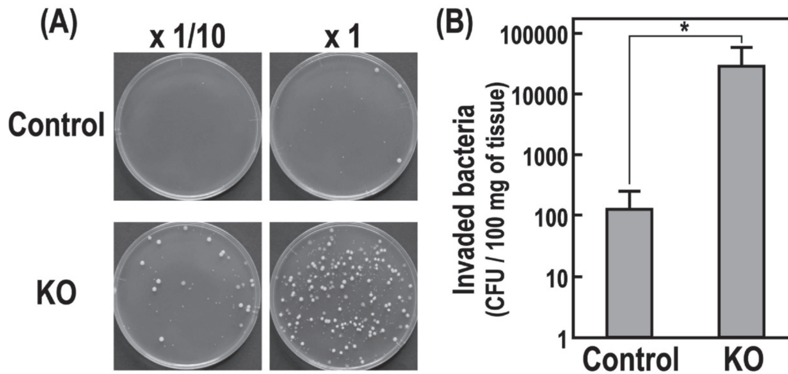

Fig. 3. The entry of commensal bacteria into the colon is augmented in enterocyte-specific *PipkIII*-deficient mice. (A) Aliquots of the colon tissue homogenates from the mutant (KO) and control mice were spread on agar plates to assess the invasion of the colon microbiota. (B) Bacterial invasion is presented as the number of colony-forming units (CFU) per 100 mg of tissue weight. $^{*}P<0.05$.

brotic diseases has been reported to date, although mutations in the *PIPKIII* gene were found in patients with Francois-Neetens Fleck Corneal Dystrophy (CFD, MIM 121850)[22,23]. Further investigation will be necessary to elucidate the involvement of PIPKIII signaling in the pathogenesis of human IBD pathogenesis. Our findings suggest a previously unknown relationship between IBD and the metabolism of endosomal phosphoinositides such as PI3P and PI(3,5)P_2, thereby shedding light on the associations between IBD pathogenesis and MTMR3, which catalyzes the reverse reaction of PI(3,5)P_2 synthesis by PIPKIII. These findings also suggest potential for the development of new therapeutic strategies for IBD patients, although further studies are required.

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