RESEARCH ARTICLE

Paneth Cell Hyperplasia and Metaplasia in Hirschsprung-associated Enterocolitis in An Aganglionosis Rat Model

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Received date: Jul 26, 2022; Revised date: Sep 8, 2022; Accepted date: Sep 9, 2022

Abstract

BACKGROUND: Many hypotheses regarding the pathophysiology of enterocolitis in aganglionic megacolon or Hirschsprung disease (HSCR) has been proposed. Paneth cells are columnar intestinal epithelial cells that have an important role in maintaining of intestinal homeostasis as a bactericide. Since enterocolitis in HSCR may have association with Paneth cells metaplasia and hyperplasia, current study investigated Paneth cells metaplasia and hyperplasia in the sigmoid colon of HSCR rat model and its products, namely α-defensins and IL-1β, in the sigmoid colon tissues.

METHODS: Aganglionosis-induced and control Sprague-Dawley rats were euthanized on Day (D)-7, -14, -17, -19, -21, -23, -25, and -28. Sigmoid colon tissue was isolated at each time point, and degree of enterocolitis as well as Paneth cells metaplasia and hyperplasia were analyzed by Hematoxylin-eosin staining, then protein levels of α-defensins and interleukin (IL)-1β were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Enterocolitis scores increased with time. The Paneth cells metaplasia and hyperplasia were observed on D14 until D28 (p<0.01 *vs.* control group) followed by an increased in the levels of IL-1β. The levels of α-defensins protein expression were initially increased (D7-D14; p<0.01 *vs.* control group) but then undergo reciprocal changes on D19 until D28 (p<0.01 *vs*. D7 and D14). Positive correlations between the degree of enterocolitis and Paneth cells number were detected in the sigmoid colon (r=0.42).

CONCLUSION: Paneth cells underwent metaplasia and hyperplasia in the sigmoid colon of HSCR rats corresponding to an increase in the degree of enterocolitis, but not followed by an increase in the level of α-defensins as well as IL-1β, suggesting that there is an involvement of Paneth cells in the pathophysiology of enterocolitis due to HSCR.

KEYWORDS: Hirschsprung, enterocolitis, defensins, metaplasia, Paneth cell, animal model

Indones Biomed J. 2022; 14(4): 393-400

Introduction

Enterocolitis due to Hirschsprung disease or Hirschsprung's-associated enterocolitis (HAEC) is the most common and severe complication of children suffering from aganglionic megacolon or Hirschsprung disease (HSCR).(1) To date, HAEC is still the leading cause of morbidity and mortality in HSCR patients and it can occur at any time during the course of HSCR disease, both before and after radical surgery.(2) Although HAEC has been well described for 60 years ago, the pathophysiology of HAEC remains poorly

understood. Several hypotheses have been proposed to explain the pathophysiology of HAEC, including changes in the intestinal mucosa immune system, dysbiosis, and mutation in the integrin β-2 gene, which contribute to the extravasation of leukocytes from the blood to the tissues. Those alterations will lead to the disturbances in the intestinal barrier system.(3-6) One of the cells that play an important role in intestinal barrier homeostasis are Paneth cells, the others are goblet cells, enteroendocrine cells, and absorptive cells.(7)

Paneth cells are intestinal columnar epithelial cells which have eosinophilic granules in their cytoplasm located in crypts of Lieberkühn and contain antimicrobial peptide namely secretory phospholipase A2, lysozyme, interleukin (IL)-17A, IL-1β, and α-defensins (cryptdins in mice) that are released in response to lipoteichoic acid, lipopolysaccharide, and other agonists such as in the response to bacterial, viral, and parasitic gastrointestinal tract infections.(8-11) Paneth cell main location is in the small intestine; in adults, Paneth cells are also found in small numbers in the caecum.

Paneth cells are said to be metaplastic if they are found in inappropriate locations, for example in the distal colon (descending colon, sigmoid, and rectum), whereas, Paneth cell hyperplasia or increased Paneth cell number may occur in the proximal colon under pathological conditions.(12) In fact, Paneth cell plays an important role in maintaining intestinal homeostasis, as it shown in previous study that in patients with necrotizing enterocolitis (NEC) there was an absence or decrease in the number of Paneth cells when compared to patients without NEC which in turn will cause a decrease in the expression of α-defensins secreted by Paneth cells.(13,14) Besides, it has also demonstrated that dysfunction or developmental defect in Paneth cells contributed to the pathogenesis of NEC.(15) Paneth cell metaplasia also occurred in the distal colon of inflammatory bowel disease (IBD) including ulcerative colitis and Chron's disease patients.(16) Although Paneth cell hyperplasia and metaplasia as well as decreased α-defensins secretion have been suggested in IBD and NEC, only little information is known about Paneth cell presence and its function in the HAEC. It has been proved that dysbiosis involved in the HAEC and Paneth cell have an important role as a bactericide to maintain the gut homeostasis.(12) Thus, current study demonstrated the involvement of Paneth cell and its secretory products, such as α-defensins and IL-1β, on the occurrence of enterocolitis in the sigmoid colon of the aganglionic megacolon (HSCR) rat model.

Methods

Animals

All the experimental procedures described in the manuscript was conducted in accordance with the guidelines from animal experimentation of Universitas Indonesia, comply with the rules of Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines, and was approved by the Universitas Indonesia Institutional Animal Care and Use Committee (No. 1069/UN2.F1/ETIK/2018).

Surgical Procedures

All surgical procedures were carried out in sterile conditions under ketamine hydroxychloride and xylazine anesthesia. HSCR was induced in rats by applying 0.1% benzalkonium chloride (BAC) (Sigma Aldrich, St. Louis, MO, USA) topically to the sigmoid colon as described previously to induce aganglionosis.(17) After being anesthetized, laparotomy was performed on rats weighing 150-200 g and the 1 cm of sigmoid colon was wrapped with 10 x 20 mm of paper gauze. The gauze was given three drops of 0.1% BAC solution every 5 min to prevent bowel dehydration, and this treatment was carried out every 15 min. The gauze was then released and the colon was washed with 0.9% normal saline. After BAC treatment, the abdomen was closed and all animals were socially housed with a standard diet and tap water *ad libitum*.

Experimental Groups

Male Sprague-Dawley rats were randomized into nine groups (with n=5 in each group) based on the termination time: sham-operation (terminated on the D7 post BAC, Control (C)), and the other groups were terminated on the Day7 (D7), -14 (D14), -17 (D17), -19 (D19), -21 (D21), -23 (D23), -25 (D25), and -28 (D28) after the 0.1% BAC application. Overdose of inhaled isoflurane followed by cervical dislocation was done to terminate the experimental animals.

Tissue Preparation

The isolated BAC-treated sigmoid colon segment and a 1 cm normal descending colon segment were excised from each rat, rinsed three times with saline solution, weighed, and fixed in the 10% neutral buffered formalin pot until analysis. All tissue samples were embedded in paraffin, sliced into 5 μm-thick longitudinal sections and mounted on glass slides before subjected to hematoxylin-eosin (HE) staining.

Enterocolitis Grading Analysis

The enterocolitis degree was observed in 5 high power field of the isolated BAC treated-sigmoid colon segment and 1 cm normal descending colon segment specimens using a light microscope with 100x magnification in each group. To score the degree of enterocolitis, we used a histopathological scoring system explained in previous studies (18,19) and was performed by three independent researchers who did not know the experimental conditions. The enterocolitis degree was assessed as follows: normal and no evidence of injury=0; crypt dilation and mucin retention=1; cryptitis, crypt abscess and macrophage-neutrophil infiltration=2; multiple crypt abscess=3; fibrinopurulent debris and mucosa alteration=4; necrosis and transluminal perforation=5.(17-19)

Histological Studies and Paneth Cells Count

The number of Paneth cells per 10 crypts per tissue section of HE staining were scored by three independent observer who did not know to the experimental conditions using a light microscope at a magnification of 400x and were starting at the lower right region of the tissue section for the length of 10 crypts. Paneth cells were distinguished based on their eosinophilic granules.(20)

Quantitative α-defensins and IL-1β Analysis

Quantitative α-defensin test was assessed using the commercial α-defensin enzyme-linked immunosorbent assay (ELISA) kit (Cat. #ABIN6955302, Antibodies-online, Pottstown, PA, USA). Whereas, for quantitative IL-1β test, the commercial IL-1β ELISA kit (Cat. #ab100768, Abcam, Cambridge, UK) was employed. For this purpose, sigmoid colon tissue was rinsed, weighed, resuspended at 50 mg/mL in 0.01 M PBS pH 7.2, homogenized and analyzed according to each manufacturer protocol. Briefly, specimens of tissue homogenate and conjugate solution, 50 µL and 100 µL, respectively, were incubated in ELISA plates and the levels of α-defensin and IL-1β were measured using an ELISA reader with an optical density of 450 nm. The optical density results obtained will be analyzed using the elisaanalysis.com software. All assays were optimized and performed in duplicate by an experience laboratory technician.

Statistical Analysis

Comparisons between groups were made using One-way ANOVA with a post hoc Tukey t-test for normally distributed data or the Kruskal-Wallis test followed by Dunn's Multiple Comparison test for not normally distributed data. Correlation between Paneth cell abundance and histological features of degree of enterocolitis at each termination time was assessed by calculating Pearson's correlation coefficient. A statistically significant difference was defined at $p<0.05$. All analysis tests were performed using SPSS 20.0 (IBM Corporation, Armonk, NY, USA).

Results

Degree of HAEC

All rats survived until the end of the study. BAC treatment generated HSCR-like manifestations, such as decreased appetite, abdominal distention, and diarrhea which began to appear as early as 14 days after BAC topical application in sigmoid colon. The degree of enterocolitis increased with time with the highest degree reached in rats terminated on D28 ($p<0.01$ vs. C) (Figure 1A). Histopathological appearance of enterocolitis was shown in Figure 1B.

Paneth Cell Metaplasia and Hyperplasia

In normal-control rats, minimal number of Paneth cells (mean Paneth cells number=52) were found in sigmoid colonic tissue as compared to the BAC-treated rats. Unexpectedly, almost all sigmoid colon samples in rats terminated on D14 to D28 showed Paneth cell metaplasia which increased in number per 10 crypts (mean Paneth cells number in D14=199, in D17=138, in D19=174, in D21=164, in D23=172, in D25=186, and in D28=123) with termination time (Figure 2A [D7-D28]). Figure 2B showed the scoring of Paneth cell abundance in the sigmoid colon. Next, analysis on the correlation between the degree of enterocolitis and the abundance of Paneth cells per 10 crypts was also conducted and the results were presented in Figure 2C. There was a positive correlation where an increase in the degree of enterocolitis followed by an increase in the abundance of Paneth cells with r=0.42 (95% CI: 0.14-0.63; $p=0.0045$).

Sigmoid Colon Tissue α-defensins and IL-1β Levels

The α-defensins expression was greatly increased in sigmoid colon rats at the early time point (D7-D14). However, at the late time point (D19-D28) though the Paneth cells still present throughout the study, the level of α-defensins was decreased (Figure 3A). In contrast, IL-1β expression showed an increase until the end of study, although the increase only seemed significant at the late time point (D28) (Figure 3B).

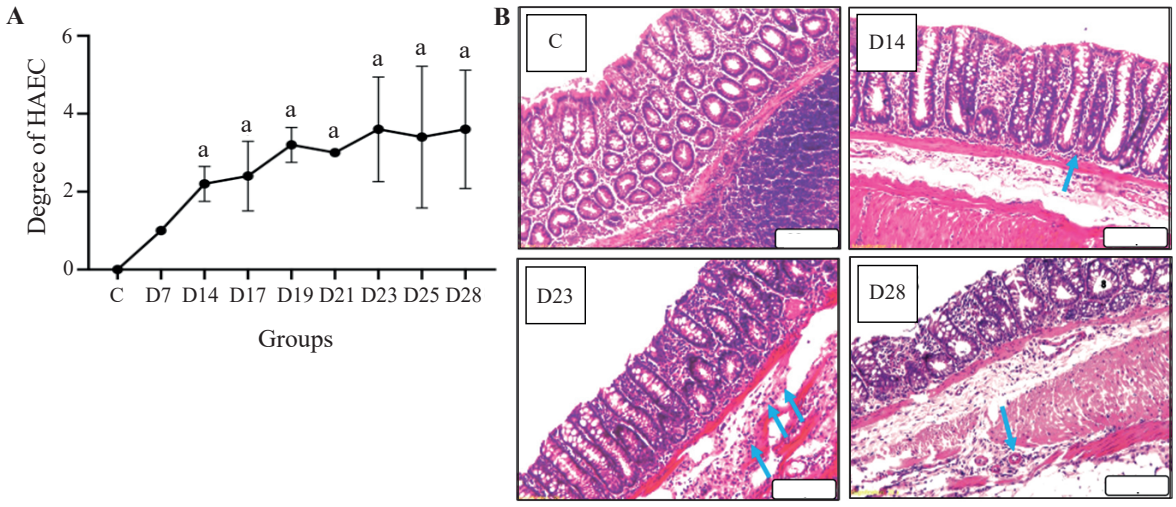

Figure 1. The degree of enterocolitis increases with time in an aganglionosis rat model. A: Degree of HAEC in all animals. The degree of HAEC continued to increase from D14 to D28 which was significantly different compared to the control and D7 groups ($^{a}p<0.01$ *vs.* C). B: Representative images of enterocolitis scores. Total score 0 (normal) (C); Total score 3 (mild inflammatory infiltrates (blue arrow), no necrosis, 1, depth of inflammation, 2) (D14); Total score 5 (mild inflammatory infiltrates (blue arrow), no necrosis, 1, depth of inflammation, 4) (D23); Total score 7 (transmural necrosis (blue arrow), 3, depth of inflammation, 4) (D28). Each figure is a representative sample from 6 rats, white bar: 20 μm.

Discussion

In the present study, we showed Paneth cell metaplasia and hyperplasia in the sigmoid colon tissue of BAC-treated rats and its presence increases according to the degree of enterocolitis. In addition, we demonstrated that Paneth cells products namely, α-defensins reached the lowest secretion on D19 on sigmoid colon rat tissues after BAC application. Decrease in *a*-defensins secretion was followed by an increase in IL-1β secretion which increased very sharply from D21 after BAC application until the end of the study. It has been proved that selective destruction of Paneth cells likely releases TNF-α and other proinflammatory cytokines.(21) This phenomenon showed that Paneth cells dysfunction began to occur around D19 after BAC application.

The role of Paneth cells in the pathophysiology of NEC and IBD has been demonstrated by previous studies (14,16,22), but the role of Paneth cells in the pathophysiology of HAEC is still not clearly understood. To the best of our knowledge, in the present study we demonstrated for the first time that Paneth cells are involved in the development of HAEC. In the sigmoid colon tissues of control rats, only a few of Paneth cells were seen, while in BAC-treated rats, an increase number of Paneth cells were observed. In fact, at an early gestational age, Paneth cells develop very rapidly in both the small intestine and the colon, but after birth, they will move down to the crypts of small intestine and disappear in the colonic mucosa.(23) In the past, several studies conclude that distal obstruction is a causative in the development of HAEC.(18,24,25) However, recent studies have shown that multifactorial etiology are involved in the development of HAEC, including impaired mucosal immunity, impaired mucosal barrier defense, and dysbiosis of the intestinal microbiome, all of which are interrelated.(3,4,26) Various factors involved in the development of HAEC also appear from non-specific HAEC therapy, namely systemic antibiotics, rectal irrigation, and bowel rest.(3) Therefore, by knowing the role of Paneth cells in the development of HAEC, it is hoped that in the future a targeted therapy for HAEC will be established so that mortality can be suppressed and the quality of life can be improved.

In this study, we have demonstrated that all rats with HAEC had disrupt normal Paneth cell function resulting in compromised α-defensins protein expression in the sigmoid colon of rats. In our study, α-defensins protein expression showed its lowest point on D19 after BAC application. Decreased α-defensins protein expression is most likely due to an inflammatory process that triggers the formation of immature Paneth cells. Paneth cells, which reside at the bottom of small intestine crypts, are fundamental for maintaining homeostasis of the intestinal epithelium by augmenting the host defense and supporting constant epithelial renewal. Disrupt normal Paneth cell function will cause significant detrimental effects, such as

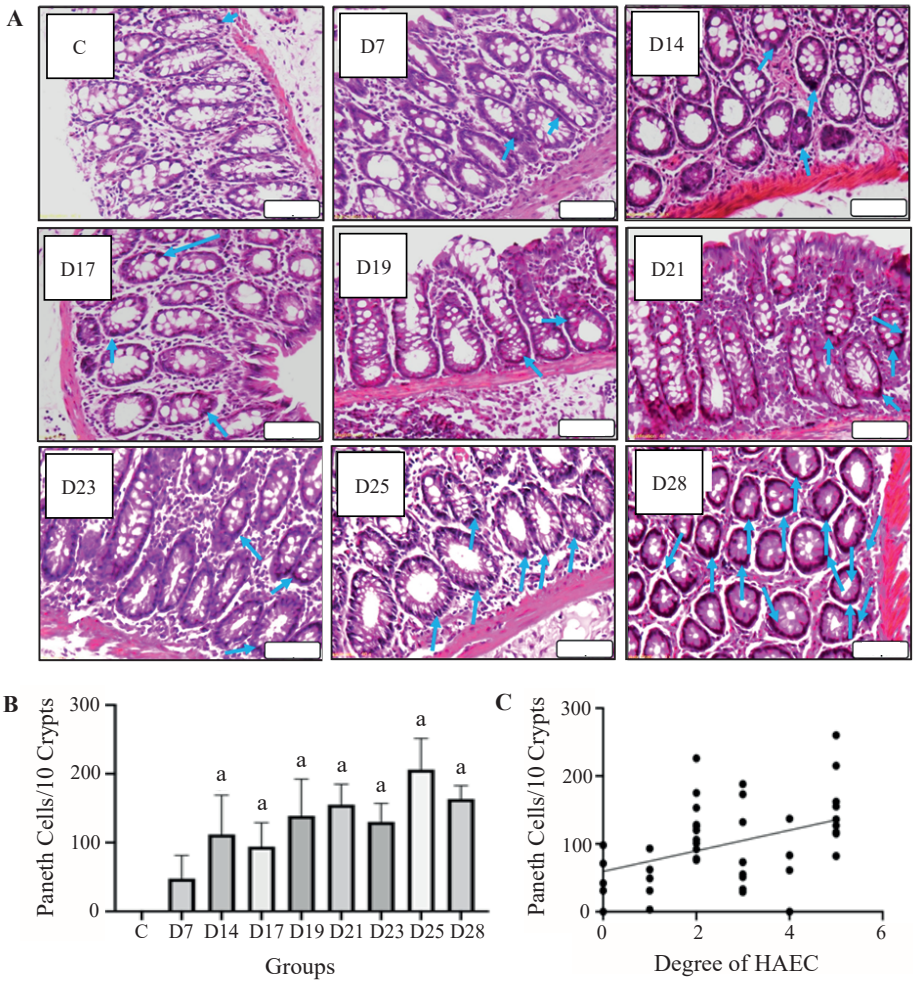

Figure 2. Paneth cell hyperplasia and metaplasia in sigmoid colon of rat. A: HE staining of Paneth cells. Paneth cells showed as an eosinophilic cytoplasm (blue arrows) at each stage of termination time. It appears that the abundance of Paneth cells increases with the increase in termination time. Each group is a representative sample from 6 rats, white bar: 20 µm. B: Quantitative analysis of Paneth cell abundance in all animals. The mean abundance of Paneth cells per 10 crypts began to increase significantly as compared to that of control group. The increase in their abundance corresponds to the increase in termination time. Data presented in mean±SD (per group, n=5), analyzed using One-way ANOVA followed by Tukey's post-hoc analysis, $^{a}p<0.01$ *vs.* C. C: Correlation analysis between degree of HAEC and Paneth cells per 10 crypts. The graph shows that an increase in the degree of HAEC will be followed by an increase in the abundance of Paneth cells per 10 crypts. Data were analyzed using Pearson correlation with r=0.42.

reduced clearance of pathogens, impaired intestinal stem cell function, and the development of intestinal mucosal inflammation.(27,28) In fact, Paneth cells are the only intestinal epithelial cells that secretes α-defensins onto the intestinal luminal surface in response to certain stimuli. (8,29) Similar finding of decrease expression of Paneth cell α-defensins is also reported in ileal Chron's disease.(30)

It has been reported that in addition to α-defensins, Paneth cells also secrete IL-1β.(31) However, it is possible that some components of the granules in Paneth cells are produced elsewhere before being finally collected and added to the Paneth cell granules. IL-1β is one of these components produced by cells involved in the innate immune system, namely macrophages and monocytes.(32) IL-1β is an important proinflammatory cytokine that plays a role in innate immunity and in the occurrence of tissue damage in IBD.(33) Recently, previous study has reported that the proinflammatory cytokine IL-23 increased with increasing degree of HAEC, indicating that there was an inflammatory process underlying the condition.(34) It has also been demonstrated that the inflammatory process occurs in ulcerative colitis, which was indicated by an increase in the levels of TNF-α and IL-6.(35) Low-grade inflammation and elevated levels of TNF-α, IL-1β, and IL-6 have also been reported in patients with type 2 diabetes mellitus and obese, both of which can trigger ulcerative colitis.(36,37) In addition, increased levels of TNF-α also play a role in the pathophysiology of other diseases that increase the

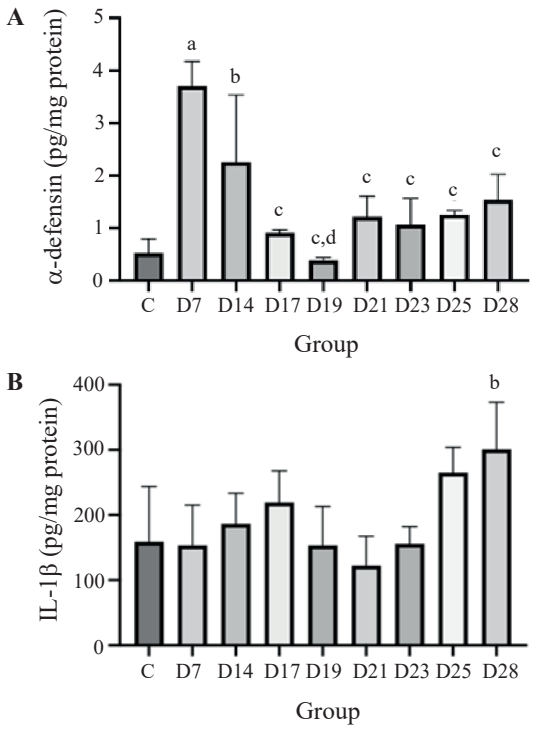

Figure 3 Fluctuating levels of α-defensins and IL-1β in the sigmoid colon of rat. A: Protein expression of α-defensins in sigmoid colon tissue of HAEC rats. The graph shows a change in the protein expression of α-defensins which is initially an increase followed by a decrease in its protein expression, respectively at D7-D14 and D19-D28. B: Protein expression of IL-1β in sigmoid colon tissue of HAEC rats. The graph shows an increase of IL-1β protein expression over time with the highest expression reached on D28. Data presented in mean±SD (per group, n=5), analyzed using One-way ANOVA followed by Tukey post-hoc analysis, [a]$p<0.01$ *vs.* C; [b]$p<0.05$ *vs.* C; [c]$p<0.01$ *vs.* D7; [d]$p<0.05$ *vs.* D14.

inflammatory process.(38,39) We found that, in contrast to α-defensins protein expression which was decreased, IL-1β protein expression was increased during the study and the increase was significant at the end of the study as compared to that of normal-control rats. This indicates that the inflammatory process in HAEC will continue as a result of a decrease in the protein expression of α-defensins, the antimicrobial peptide. It has been reported that important secretory products by Paneth cells are antimicrobial peptides that are necessary for normal function of gastrointestinal tract.(40)

Furthermore, in this study we also showed that Paneth cells number increased with the degree of enterocolitis, though the correlation between them was weak. In contrast, in preterm acute NEC infants there was an increased in the number of Paneth cells accompanied by an increased in HD5 mRNA levels significantly compared to term control infants in line with the increases in the degree of NEC.(41) In our study, we did not quantify Paneth cells-specific expression of HD5, as we quantify Paneth cells based on its eosinophilic granules in cytoplasm. Paneth cells count were carried out by three researchers who were unaware of the conditions of our study. Eventhough manual calculations might differ greatly from one researcher to another; However, our study is the first to report involvement of Paneth cells and HAEC. We recommend conducting a longer duration study such as until 166 days post BAC application (42) to evaluate further development of Paneth cells in HAEC.

Conclusion

Considering all these findings, it is suggested that Paneth cells contributes to the development of HAEC. There might be Paneth cell dysfunction on producing the α-defensins despite of the increasing Paneth cell number in reaction of the inflammation. With the knowledge that Paneth cells have a role in the pathogenesis of HAEC, we believe these findings hold promise for the future development of targeted therapy for HAEC.

Acknowledgments

We thank Yulvian Sani, PhD for the technical assistance. This study was supported by the Director of Research and Development, Universitas Indonesia with the grant number NKB-491/UN2.RST/HKP.05.00/2021.

Authors Contribution

IRB, AF, YM, VS were involved in planning and supervised the work, IRB and VS performed the measurements, IRB, AAJ and VS processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. IRB and VS performed the statistical analysis. IRB, AF, YM, AAJ and VS aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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