Occurrences and phenotypes of RIPK3-positive gastric cells in Helicobacter pylori infected gastritis and atrophic lesions

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Occurrences and phenotypes of RIPK3-positive gastric cells in *Helicobacter pylori* infected gastritis and atrophic lesions

Running head: Necroptosis and gastric precancerous lesions

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List of abbreviations:

DIF: double immunofluorescence

IHC: immunohistochemistry

H. pylori: *Helicobacter pylori*

PCD: programmed cell death

RIPK3: receptor-interacting protein kinase 3

TNF: tumor necrosis factor

Vac A: vacuolating cytotoxin A

WT: wild type

Abstract

Background: Research evidences suggest that diverse forms of programmed cell death (PCD) are involved in the *helicobacter pylori* (*H. pylori*)-induced gastric inflammation and disorders.

Aims: To characterize occurrences and phenotypes of necroptosis in gastric cells in *H. pylori* infected gastritis and atrophic specimens.

Methods: Occurrences and phenotypes of necroptosis in gastric cells were immunohistochemically characterized with receptor-interacting protein kinase 3 (RIPK3) antibody in both human *H. pylori* infected gastric gastritis, atrophic specimens, and transgenic mice.

Results: Increased populations of RIPK3-positive cells were observed in both gastric glands and lamina propria in *H. pylori* infected human oxyntic gastritis and atrophic specimens. Phenotypic analysis revealed that many RIPK3-positive cells were H^+K^+ ATPase-positive parietal cells in the gastric glands and were predominantly CD3-positive T lymphocytes, CD68-positive macrophages, and SMA-alpha-positive stromal cells in the lamina propria. Furthermore, we found an increased expression of RIPK3-positive gastric glandular cells along with the histological process of hyperplasia-atrophy-dysplasia progression in hypergastrinemic INS-GAS mice.

Conclusions: An increased population of RIPK3-positive cells was observed in several types of gastric cells, future studies that define the effects and mechanisms of PCD implicated in the development of *H. pylori induced* gastric disorders are needed.

Key words: Programmed cell death, Necroptosis; *Helicobacter pylori*; Stomach; Atrophy;

Introduction

Although the incidence of gastric cancer is decreased in western countries, it remains as one of the leading causes of cancer mortality worldwide, particularly in Asian countries[1]. It is now well recognized that infection with *Helicobacter pylori* (*H. pylori*) is a major cause of gastric cancer [2]. According to the Correa pathway, the development of gastric cancer is through a multistep histopathologic cascade that now has been labelled as the atrophy-metaplasia-dysplasia-intestinal-type gastric cancer sequence [3]. In which, the induction of parietal cell loss has been known as the key premalignant step of gastric atrophy development [3]. However, the mechanisms by which *H. pylori* leads to gastric parietal cell loss (atrophy) remain as an unresolved issue and becomes a question of great interest currently.

Physiologically, gastric cellular homeostasis is kept by the balance between cell death and renewal. Once this balance is disturbed, it may cause significant morphological and functional damages. For example, resistance to cell death can lead to hyperplasia and dysplasia as seen in the process of gastric carcinogenesis. On the other hand, excessive cell death will result in gastric cell loss as seen in gastric atrophy or ulceration [4].

Considerable amount of *in vitro* and *in vivo* evidence suggest that *H. pylori* is a major pathogen that causes impaired balance of gastric cellular turnover and result in excessive process of programmed cell death (PCD) and atrophy in the gastric mucosa, which accelerates *H. pylori*-induced gastric carcinogenesis [5-10]. Studies have revealed that *H. pylori* infection induced gastric parietal cell loss may be related to an increased rate of PCD [6,9,11]. Several PCD forms such as apoptosis, autophagy and pyroptosis have been shown to be involved in the physiology of gastric mucosal homeostasis [12] and associated with *H. pylori*-induced gastric inflammation and disorders [6,13,14]. Recently, a novel PCD form, necroptosis, was identified. Which can be realized as a programmed necrosis that occurs in an orderly manner [15,16]. In terms of

morphology, unlike cell membrane integrity during apoptosis, the occurrence of necroptosis will destruct the integrity of cell membrane and release a large number of intracellular substances e.g., cytokines and enzymes, these factors could result in a strong immune response and pathological inflammation [17,18]. Therefore, excessive increases in necroptosis are frequently associated with the development of inflammatory diseases [17-23]. For example, recent interest has been focused on the pathological role of necroptosis in intestinal inflammation [16,17,24-26]. Accumulative results suggested that increase in the rate of necroptosis could unbalance epithelial cell turnover and is associated with the development of inflammation in the intestine [16,17,24,25]. Interestingly, previous studies have revealed that chronic infection of *H. pylori* could induce a significantly elevation of tumor necrosis factor (TNF)-α level in both human and mice [27-30], which has been shown to be a factor to promote *H. pylori* induced inflammation and PCD in parietal cell [30]. *H. pylori* vacuolating cytotoxin A (VacA) has been shown to promote progressive vacuolation and induce gastric mucosal injury. More recently, the induction of VacA on necroptosis in gastric epithelial cells has been reported *in vitro* [31]. The findings suggested that *H. pylori* might induce necroptosis and change the PCD rate of gastric cells [31]. This leads to us to hypothesize that necroptosis might be involved in the *H. pylori* infection induced gastric atrophy. On the other hand, hypergastrinemia is a consequence of chronic *H. pylori* infection and involved in the development of hyperplasia-atrophy-dysplasia in the stomach [6,30,32]. However, the change of necroptosis during the hypergastrinemia-induced hyperplasia-atrophy-dysplasia process in the stomach *in vivo* remains unclear.

Here, we immunohistochemically characterized the occurrence and phenotypes of RIPK3, a key element of necroptosis, positive gastric cells in *H. pylori* infected human gastric lesions (gastritis and atrophy). In addition, we also evaluated the dynamic of RIPK3-positive cells in

fundic glands in the pathogenesis of gastric carcinogenesis in a hypergastrinemic transgenic gastric cancer mouse model.

Materials and methods

Human gastric specimens

A total of 40 gastric paraffin blocks of *H. pylori* negative controls (n=10), *H. pylori* infected chronic gastritis (n=18), *H. pylori* infected gastric atrophy (n=12) taken by gastric endoscopy from human oxyntic mucosa (Table 1) retrieved from tissue bank at Department of Pathology, the Second Affiliated Hospital of Zhengzhou University were included in this study. *H. pylori* infection was diagnosed by C13 breath test (Boran Pharmacy, Beijing, China). Human study protocol was approved by the institutional medical ethic review board of the Second Affiliated Hospital, Zhengzhou University,

Transgenic gastric carcinogenesis mouse model

Paraffin blocks of wild type (WT) FVB/N male mice (Charles River Lab., Wilmington, MA) at ages of 3, 6 and 12 months (each group mice N=5), gastric carcinogenesis model of INS-GAS transgenic mice [33] were obtained from Drs. Theodore Koh and Timothy C. Wang (University of Columbia, New York, USA). Animals were euthanized by cervical dislocation under a anesthetized condition with pentobarbital sodium intraperitoneal injection at a dose of 50 mg/kg. Pathological features of INS-GAS mice have been described in previous publications [6,33]. Animal study protocol was approved by the local medical ethic committee of Second Affiliated Hospital of Zhengzhou University.

Stomach immunohistochemistry (IHC) in mice and human specimens

Male WT mice and INS-GAS transgenic mice at the ages of 3-, 6- and 12-month, and human gastric tissues of control, *H. pylori* positive gastritis and *H. pylori* positive atrophy were examined in this study. Midline strips along the lesser curvature of the stomach were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin. Sections were cut at 4 μm, and then stained with hematoxylin and eosin. IHC was performed with avidin-biotin-

peroxidase complex (ABC) *Elicit* kits (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions and our previous published method [34-37]. The following primary antibodies were used: against RIPK3 to stain necroptosis cells (working dilution 1:500, rabbit anti-human, mouse and rat, Thermo Fisher Scientific, Oslo, Norway) and H^+K^+-ATPase β subunit to parietal cells (1:2000, mouse anti-hog, Affinity Bioreagents, Golden, CO, USA). Primary antibodies were incubated at 4 ºC overnight in humidified chamber. 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen and slides were counterstained with Mayer's hematoxylin.

Double immunofluorescence (DIF) staining in human gastric sections

We have observed the positive cells for RIPK3 in human stomach in both gastric glands and lamina propria. To show RIP3 in the gastric parietal cells, double DIFs with RIPK3/HK-ATPase antibodies were done according to the protocol described in our previous publication [34,38-40] in human oxyntic mucosa sections. To analyse phenotypes of RIPK3 positive cells in the gastric lamina propria, double DIFs with RIPK3/H^+K^+-ATPase (to show RIPK3 in parietal cells), RIPK3/CD3 (to show RIPK3 in T lymphocytes, mouse anti-CD3 monoclonal antibody, DAKO, Carpinteria, CA, USA), RIPK3/CD68 (to show RIPK3 in macrophages, mouse anti-CD68 monoclonal antibody, DAKO, Carpinteria, CA, USA) and RIPK3/SMA-alpha (to show RIPK3 in stromal myofibroblasts, mouse anti-SMA-α monoclonal antibody, DAKO, Carpinteria, CA, USA) in human *H. pylori* infected gastric lesions including chronic fundic gastritis, atrophy sections. After gastric fundic sections incubated with primary antibodies at 4ºC overnight, RIP3-immunoreactivity (IR) was developed with Texas red-conjugated secondary antibody (Jackson ImmunoRearch Lab., West Grove, PA, USA) and H^+K^+-ATPase -IR, CD3, CD68 and SMA-α were developed with FITC-conjugated secondary antibody (Jackson ImmunoRearch Lab.).

Then, DIF sections were observed and photographed under a confocal microscopy (LSM-700,

Carl Zeiss, Jena, Germany) under 200× magnification.

Morphological evaluation

For mice sections, only RIPK3-labelling glandular cells with appropriate morphology and location in well-oriented sections were counted in at least 10 selected glands with abundant distribution under ×400 magnification (Zeiss, Germany). Since human gastric biopsies are taken by endoscopy and very small, the orientation is therefore difficult to be managed in a well-oriented position as seen in mice gastric biopsies. We have to counter oxyntic glandular cells in 10 selected glands with abundant distribution under ×400 magnification. Densities of RIPK3-positive glandular cells of oxyntic mucosa were expressed as number per gland. The average values of positive cells per glands were used for the statistical analysis.

Statistical analysis

Data were present as mean ± SEM (standard error of the mean) unless otherwise stated. *P* values were evaluated by the Mann–Whitney test. *P* values < 0.05 were considered statistically significant.

Results

Occurrence of RIPK3-positive glandular cells in human *H. pylori* positive gastric oxyntic mucosa

Compared with human control gastric oxyntic mucosa (Fig. 1A). The population of RIPK3-positive glandular cells was increased in *H. pylori* infected gastritis (Fig. 1B) and even higher in *H. pylori* infected atrophy (Fig. 1C). Increased populations of RIPK3 in other types of gastric cells such as surface mucosal cells (Fig. 1B&C) and lamina propria cells (Fig. 1B&C) in gastritis and atrophic tissues were also observed. Further quantitative results of RIPK3-positive glandular cells showed significantly increased densities from control to gastritis and then to atrophy (Fig. 1D).

Phenotypic analysis of RIPK3-positive cells in *H. pylori* positive human gastric lesions

Phenotypic analysis revealed that RIPK3-positive cells in the fundic glands were mostly H^+K^+-ATPase positive parietal cells (Fig. 2A-C). RIPK3-positive cells in the lamina propria were CD3-positive T lymphocytes (Fig. 2D-F) and CD68-positive macrophages (Fig. 2G-I) and occasionally SMA-α-positive stromal cells (Fig. 2J-L). This observation suggested that RIPK3 could be expressed in both gastric glandular cells and many types of cells in the lamina propria.

Dynamic of RIPK3-positive cells in fundic glandular cells during the process of gastric atrophy - dysplasia sequence in hypergastrinemic transgenic gastric cancer mouse model

Since hypergastrinemic INS-GAS transgenic gastric cancer mouse model mimics the process of hyperplasia-atrophy (parietal cell loss)-dysplasia sequence in the fundic mucosa of stomach [33], we have therefore examined dynamic of RIPK3-positive cells in fundic glands in this transgenic mouse model at different ages.

As seen in Fig. 2. RIPK3-positive cells were found in both glandular parietal cells and surface mucous cells. Following the age increasing from 3-month, 6-month to 12-month, RIPK3 positive cells in fundic glands of WT FVB/N male mice were shown in a slightly increasing trend (Fig. 3A-C). The intensity of RIPK3-immunoreactivty was stronger in glandular cells in the upper part of fundic glands than those in lower part of glands. The RIPK3-positive cells in hypergastrinemic INS-GAS mice (Fig. 3D-F) were significantly changed as compared with the controls (Fig. 3A-C). The intensity of RIPK3-immunoreactivty was evenly distributed in fundic glandular cells. Counting data showed that densities of RIPK3-positive glandular cells in INS-GAS mice at ages of 3- and 6-month were higher than that in control FVB mice at the same ages (Fig. 3G). However, at the age of 12-month it was lower in the atrophy and dysplasia developed area of INS-GAS mice than control mice (Fig. 3G). But the density of RIPK3-positive cells in the fundic glands adjacent to atrophy and dysplasia (inserted image in Fig. 3F and grey bar in Fig. 3G) was still higher than that in control mice.

Discussion

It has been previously reported that chronic infection of *H. pylori* results in a significantly increased rate of PCD in gastric cells e.g., surface epithelial cells and parietal cells in the stomach [6,7,33,41-43]. In this study, we immunohistochemically characterized the occurrence and phenotypes of RIPK3-positive cells in the gastric glandular cells in *Helicobacter* infection related human specimens and the dynamic of RIPK3-positive cells in hypergastrinemia-induced atrophic and dysplastic lesions, respectively. Our results showed that increased expression of RIPK3-positive cells was demonstrated in diverse types of gastric cells in human specimens. Particularly, many of RIPK3-positive glandular cells were identified as H^+K^+-ATPase-positive parietal cells, which may suggest an potential role of necroptosis in the parietal cell loss and development of atrophy under *H. Pylori* infection condition.

It has been previously demonstrated that PCD plays a fundamental role in tissue homeostasis and disrupted process of PCD is associated with human diseases [44-46]. Numerous studies have shown that chronic *Helicobacter* infection could induce an increased rate of PCD in gastric epithelial and granular cells that is associated with the development of gastric carcinogenesis [9,10,30,47-49]. Rosania et al. previously [9] reported that the number of apoptotic epithelial cells was increased from 2% in normal mucosa to 8-17% in *H. pylori* related gastritis, which suggested a contributing role of PCD to the pathogenesis of *H. pylori* induced gastric lesions. TNF-α has been evident to be a potent inducer of necroptosis through activations of RIPK1 and then mixed lineage kinase domain-like (MLKL) that regulates cell necrosis downstream of RIPK3 [50-54]. Interestingly, previous studies have provided massive evidences to suggest that chronic *H. pylori* infection can induce an remarkably increased expression of TNF-α in the gastric mucosa [28,55,56], which in turn increases the risk of gastroduodenal mucosa injury [57]. Therefore, upregulated expression of TNF-α by *Helicobacter* infection might contribute to the increase in the rate of necroptosis in the gastric mucosal cells. Indeed, a recent *in vitro* study has

demonstrated that *H. pylori* toxic factor VacA can significantly induce necroptosis and increase the death rate of gastric cells [31], and suggested a potential role of necroptosis in *H. pylori* induced gastric disorders. RIPK3 is a critical regulator of necroptosis and facilitates the process of necroptosis and cytokine production and then contributes to the process of inflammation [17,18,44,58]. Therefore, the activation of RIPK3 is tightly associated with the induction of inflammation. In this study, we were able to demonstrate increased populations of RIPK3-positive cells in diverse gastric cells including glandular cells in *H. pylori* infected human gastritis and atrophic specimens. Particular interest finding was the identification of RIPK3-positive parietal cells, which are the main acid-producing glandular cells in oxyntic mucosa. This finding may imply that over process of necroptosis in the parietal cells could induce excessive loss of parietal cells and finally result in atrophy in oxyntic glands. Furthermore, we have found that RIPK3-immunoreactivity was observed in lamina propria cells. Phenotypic analysis showed that these positive cells were mostly identified as CD3-positive lymphocytes and some were CD68-positive macrophages and SMA-α-positive myofibroblasts. Since studies have demonstrated that necroptosis can destroy the integrity of cell membrane and then the release of a large number of inflammatory cytokines and contribute to the development of inflammation [17,18], these findings might suggest a potential involvement of necroptosis occurred in the lamina propria immune and stromal cells in the development of gastric inflammation induced by *H. pylori* infection.

Hypergastrinemia induced by chronic *Helicobacter* infection has been considered as a risk factor for the development of premalignant lesion atrophy and gastric cancer [33,42,59]. The mechanisms underlying the carcinogenesis potential of hypergastrinemia are still not fully understood. Previously, an association between gastric cell PCD (apoptosis) and hypergastrinemia has been reported in rodents (*Mastomys*) [60] and INS-GAS transgenic mice [6]. We have also showed that *Helicobacter* infection for 6 months resulted in an increased rate of

apoptosis and accelerated development of gastric atrophy in INS-GAS mice, both was strongly inhibited by the treatment of CCK-2 receptor antagonist YF 476 and H-2 receptor antagonist loxtidine or both [6]. Thus, apoptosis as an PCD manner might contribute to the process of atrophy in the stomach [6]. To evaluate the dynamic of RIPK3-positive gastric glandular cells during the development of gastric carcinogenesis, we examined the occurrence and population changes of RIPK3-positive gastric glandular cells along with hyperplasia-atrophy-dysplasia histological stages in hypergastrinemic transgenic mice. We also found step-up increasingly densities of RIPK3-positive mucosal cells during the process of atrophy-dysplasia in transgenic mice as compared with WT mice, implying an occurrence of necroptosis in this region. RIPK3-immunoreactivity in fundic glands in younger (3-month) INS-GAS mice is located in an even distribution pattern, the density of RIPK3-positive glandular cells was slightly increased and greatly increased at the age of 6-month. After this time point, prolong exposure of mice to hypergastrinemia induced a significant atrophic and dysplastic lesion in the fundic mucosa and the rate of RIPK3-positive glandular cells in fundic atrophy region has decreased after parietal cell lost, but it still remained in a high level in the adjacent region with hyper-parietal cells. Such dynamic of RIPK3-positive glandular cell change along the process of hyperplasia-atrophy-dysplasia sequence in INS-GAS mice may provide a new insight into the role of necroptosis during the development of gastric carcinogenesis.

Despite the limitation by a relatively small size of *H. pylori* infected gastric lesions, we have at first time demonstrated in this study an increased population of RIPK3-positive parietal cells in both human and mice. Our data provide a new insight into the role of PCD in the initiation of *H. pylori* infected-related gastric carcinogenesis. However, to precise the effects and mechanisms of necroptosis in the parietal cell loss and atrophy, there is still a lot of work to do. For example, several cytokines such as interleukin (IL)-11 and IL-17 have been shown to be elevated in patients with *H. pylori* infection and associated with the disease activity and

symptoms [61-64] and recently been demonstrated to participate in the process of parietal cell loss and atrophy through apoptosis in the mouse stomach [32,65]. Therefore, we made a schematic summary of possible effects of necroptosis on the development of gastritis and atrophy under the *H. pylori* infection condition (ref. to Fig. 4). Future studies that focus on the effects and mechanisms of inflammatory mediators on necroptosis process in parietal cells remain to be conducted.

Declaration of Competing Interest: None declared.

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Table 1. Human gastric oxyntic biopsies

	N	Male/Female	Average age (range)	Lesion position	C13 breath test
Control	10	1/9	50.8 (31~67)	oxyntic	negative
Gastritis	18	12/6	57.89 (34~84)	oxyntic	positive
Atrophy	12	7/5	57 (36~85)	oxyntic	positive

Legends to Figures 1-4

Fig. 1. Immunohistochemical examination for RIPK3-positive cells in the fundic glands of *H. pylori* infected human gastric gastritis and atrophy.

As compared with the *H. pylori* negative controls (Fig. 1A), RIPK3-positive cells in the oxyntic glands were shown in an increasing trend in *H. pylori* infected gastritis (Fig. 1B) and atrophic gastritis (Fig. 1C).

The counting data showed that as compared with the controls (*white bar* in Fig. 1D), the number of RIPK3-positive cells in fundic glands was significantly increased in *H. pylori* infected gastritis (*grey bar* in Fig. 1D). It was slightly decreased in *H. pylori* infected atrophy (*black bar* in Fig. 1D), but still higher than that in the controls.

(A-F, IHC images, counterstained with Hematoxylin, original magnification 400×).

Fig. 2. Double immunofluorescence examination of RIPK3-positive cell phenotypes in human *H. pylori* infected gastric lesions

Phenotypic analysis revealed that some RIPK3-positive cells (Fig. 2A) in oxyntic glands of human *H. pylori* infected gastric lesions were H^+, K^+, ATPase (Fig. 2B) positive parietal cells (merged image, Fig. 2C). RIPK3-positive cells (Fig. 2D, G, J) in oxyntic glands of human *H. pylori* infected gastric lesions were CD3-positive (Fig. 3E) lymphocytes (merged image, Fig. 2F), CD68-positive (Fig. 2H) macrophages (merged image, Fig. 2I) and SMA-α-positive (Fig. 2K) stromal cells (merged image, Fig. 2L).

(A-L, double immunofluorescence-stained confocal images, original magnification 200×).

Fig. 3. Immunohistochemical examination for RIPK3-positive fundic glandular cells in INS-GAS mice along the process of hyperplasia-atrophy-dysplasia sequence.

RIPK3-psotive cells were shown in control wide-type (WT) FVB mice at different ages (Fig. 3A-C), some of them were located in the upper part of glands. RIPK3 positive cells in the fundic glands of INS-GAS mice were evenly distributed in the fundic glands and showed a gradual increasing trend from 3- (Fig. 3D) to 6-month ages (Fig. 3E) but decreased in atrophic region and remained in a high peak in adjacent region with hyper-parietal cells in 12-month age (Fig. 3F, insert image shows PRIPK3-positive glandular cells in normal region). Throughout this period a hyperplasia-atrophy-dysplasia sequence was established.

Counting data showed that density changes of RIPK3-positive cells/gland (Fig. 3G) in the fundic mucosa were increased from 3- to 6- and 12-month along the process of hyperplasia-atrophy-dysplasia sequence.

(A-F, IHC images, counterstained with Hematoxylin, original magnification 400×).

Fig. 4. Schematic summary of possible effects of necroptosis on the development of gastritis and atrophy under the *H. pylori* infection condition incorporating integrated analysis of current and literature data.

Chronic *H. pylori* infection resulted in the recruitment of diverse immune cells into the infected site and *H. pylori* toxicity factors, such as vacuolating cytotoxin A (VacA), induced necroptosis in immune cells and release high amounts of inflammatory mediators e.g., tumor necrosis factor (TNF) that triggered inflammatory cascade and inflammation in gastric mucosa. Persisted production of inflammatory mediators further induced increased necroptosis in parietal cells and parietal cell lost, and finally atrophy is developed.