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Original Article

The number of Foxp3-positive regulatory T cells is increased in *Helicobacter pylori* gastritis and gastric cancer

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ABSTRACT

Helicobacter pylori (*H. pylori*) colonization induces vigorous innate and specific immune responses; however, the infection is not removed, a state of chronic active gastritis persists for life if untreated. Recent studies have shown that $CD4^+$ $CD25^+$ Foxp3-positive regulatory T cells (Tregs) suppress the immune response to *H. pylori*. Persistent *H. pylori*-associated gastritis is closely associated with gastric carcinogenesis. We investigated the number of Tregs in the context of *H. pylori* colonization in chronic gastritis, examined the relationship between it and histopathological findings and compared it with that of gastric dysplasia and adenocarcinoma. This study was based on the analysis of gastric biopsy specimens from 126 cases of *H. pylori*-associated gastritis, 16 cases of *H. pylori*-negative gastritis, 17 cases of gastric dysplasia, and 25 cases of gastric adenocarcinoma. The number of Tregs was elevated in *H. pylori*-associated gastritis, where it was positively correlated with the grade of chronic inflammation and the number of lymphoid follicles. It was significantly elevated in adenocarcinomas compared to chronic gastritis and gastric dysplasia. In summary, the number of Tregs is increased in *H. pylori*-associated gastritis and gastric cancer.

Introduction

Helicobacter pylori (*H. pylori*) is closely associated with chronic gastritis, gastroduodenal ulceration, and gastric malignancy such as adenocarcinoma and lymphoma of the mucosa-associated lymphoid tissue [27]. Although mucosal *H. pylori* colonization induces vigorous immune responses involving both innate immune cells and *H. pylori*-specific T- and B-cells, the infection is not removed, and a state of chronic active gastritis persists for life if untreated [6]. During *H. pylori* infection, T cells are generally hyporesponsive and infected gastric tissues have also shown the presence of transforming growth factor β (TGF-β), which has a suppressive effect on T cells [14–16]. Recent studies have shown that $CD4^+$ $CD25^+$ regulatory T cells (Tregs) suppress the immune response to *H. pylori* [5,18,23,24]. *In vivo* depletion of Tregs in infected mice leads to increased gastric inflammation and reduced colonization of *H. pylori* [23]. Gastric Tregs are able to suppress *H. pylori*-induced T-cell proliferation and interferon-γ production [5]. Moreover, *H. pylori* infection is an established risk factor for gastric cancer. In fact, the population of Tregs in tumor-infiltrating lymphocytes is significantly larger than in normal tissue in several malignancies, including gastric cancer [5,10,17,19,21,22,29]. These findings suggest that Tregs contribute to the persistence of *H. pylori* infection, which may be closely related to gastric carcinogenesis.

Tregs are thought to be a functionally unique population of T cells, and function to maintain immune homeostasis [12,20,25]. Tregs have a functionally immunosuppressive property that prevents effector cells from acting against themselves in autoimmune diseases or a tumor. Naturally arising $CD4^+CD25^+$ Tregs characteristically express CD25, cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene, TGF-β, and Foxp3. However, CD25 is not a marker suitable for defining Tregs, because activated T cells generally express CD25. Compelling studies have revealed that CTLA-4 and TGF-β play roles in the suppressive activity of $CD4^+CD25^+$ Tregs against $CD4^+$ or $CD8^+$ T cells, although they are not expressed exclusively in Tregs. Experiments with *Foxp3*-overexpressing transgenic or *Foxp3* gene-depleted mice and other studies have shown that *Foxp3* is a master control gene for the development and function of natural $CD4^+CD25^+$ Tregs [7,8]. Thus, Foxp3 is thought to be a single marker suitable for detecting $CD4^+CD25^+$ Tregs.

The aim of this study was to investigate the number of Tregs in the context of *H. pylori* colonization in chronic gastritis, to examine the relationship between it and histopathologic findings and to compare it with that of gastric dysplasia and adenocarcinoma.

Materials and methods

Patients

This study was based on the analysis of gastric biopsy specimens from 126 cases of *H. pylori*-associated gastritis,

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16 cases of *H. pylori*-negative gastritis, 17 cases of gastric dysplasia (16 low-grade dysplasias, 1 high-grade dysplasia), and 25 cases of gastric adenocarcinoma referred for upper gastrointestinal endoscopy at Dongguk University Kyongju Hospital between March 2005 and March 2008. Tumor differentiation was as follows: well ($n = 5$), moderate ($n = 7$), and poor ($n = 13$). Informed written consent was obtained for upper endoscopy and biopsy procedures. None of the patients had a history of gastric surgery, nor had they taken antibiotics or bismuth preparations in the preceding 6months or histamine-2 receptor antagonists, omeprazole, or nonsteroidal antiinflammatory drugs 14 days before gastric biopsy. They had not been treated for *H. pylori*. Gender ratio and age distribution in each group were characterized as follows: *H. pylori*-associated gastritis, including 12 gastric ulcers (66 males and 60 females, age range 9–85 years), *H. pylori*-negative gastritis (7 males and 9 females, age range 23–69 years), gastric dysplasia (13 males and 4 females, age range 40–79 years), and gastric adenocarcinoma (16 males and 9 females, age range 24–83 years). *H. pylori* infection was assessed by rapid urease test and an immunohistochemical method using anti-*H. pylori* polyclonal antibody. Patients were considered to be *H. pylori*-positive if the result of one or both diagnostic methods was positive and *H. pylori*-negative if both methods revealed negative results.

Microscopic examination and immunohistochemistry

For histopathological analysis, biopsy specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Five-micrometer-thick sections were cut from each paraffin block. Sections were initially stained with hematoxylin and eosin for routine histology. A pathologist blinded to the clinical information of subjects assessed the histopathological change. The grades of activity, chronic inflammation, intestinal metaplasia, and glandular atrophy were determined in biopsy tissue fragments as suggested by the Updated Sydney system: 0, absent; 1, mild; 2, moderate; and 3, marked [4]. For immunohistochemical analysis, 4-μm serial sections were made and spread on poly-L-lysine-coated slides. Paraffin sections were immersed in three changes of xylene, and were hydrated using a graded series of ethanol solutions. Antigen retrieval was performed routinely by immersing the sections in 0.01 M of citrate buffer (pH 6.0) in a pressure cooker by autoclaving for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min and then incubated with a primary antibody overnight in a humidified chamber at 4 °C. The primary antibodies were polyclonal rabbit anti-*H. pylori* antibody (Dako Corp., Carpinteria, CA) and monoclonal mouse anti-Foxp3 antibody (Abcam, Cambridge, UK). Staining was performed using EnVision kit labeled with peroxidase (DAKO) and developed with 3,3′-diaminobenzidine tetrahydrochloride (Zymed Laboratories, Inc., San Francisco, CA). Sections were counterstained for 5 min with Meyer's hematoxylin and then mounted. Tonsil tissue was used as a positive control for Foxp3. As a negative control, rabbit and mouse IgG isotypes were used instead of primary antibody.

The density of *H. pylori* was analyzed as suggested by the Updated Sydney system: 0, absent; 1, mild; 2, moderate; and 3, marked [4]. The number of Tregs was calculated by counting nuclear positive lymphocytes throughout the entire area of tissue section at 10 high power fields. The proportion of Tregs was defined as the number of Tregs divided by the degree of chronic inflammatory cells.

Statistical analysis

Pearson's correlation, the *t*-test, and one-way ANOVA were used. Statistical significance was assumed if a *p*-value was less than 0.05. Data were expressed as mean ± standard error.

Results

Our previous study confirmed that Foxp3-positive cells were CD4(+) T lymphocytes in human tonsil by double immunohistochemistry [11]. As shown in Fig. 1, Foxp3-positive Tregs were located adjacent to the foveolar epithelium and frequently aggregated close to lymphoid follicle. The number of Tregs was 16.2 ± 1.2 in *H. pylori*-associated gastritis and 0.8 ± 0.2 in *H. pylori*-negative gastritis. As shown in Fig. 2a, it was 4.5 ± 1.9 in mild chronic inflammation and 16.8 ± 1.2 in moderate and marked inflammation. Therefore, the number of Tregs was significantly correlated with chronic inflammation ($p = 0.03$) and colonization of *H. pylori* ($p = 0.00$). To investigate whether the increase in Tregs reflects the general infiltration of chronic inflammatory cells due to gastritis or whether it is specifically caused by the colonization of *H. pylori*, the proportion of Tregs was used. The proportion of Tregs, in the context of the density of *H. pylori,* was examined in chronic gastritis. It was 0.8 ± 0.2 in the absence of *H. pylori*, 5.5 ± 1.0 in mild density, and 7.3 ± 0.6 in moderate and marked density. As shown in Fig. 2b, there was a statistical significance between it and colonization of *H. pylori* ($p = 0.01$); however, in *H. pylori*-associated gastritis, it was not significantly different between mild density and moderate to marked density ($p = 0.20$). The relationship between the proportion of Tregs and the histopathological findings in *H. pylori*-associated gastritis was examined. As shown in Fig. 2c, a significant, positive correlation between it and the number of lymphoid follicle was present ($r = 0.70$, $p = 0.00$). However, there was no significant relationship between it and the grades of other pathological parameters such as activity, intestinal metaplasia, and glandular atrophy (data not shown). In addition, it was similar between gastric ulcer and non-ulcer (data not shown). A recent study has shown that the number of Tregs was elevated in children compared to adults [9]. Therefore, the relationship between age and the proportion of Tregs was examined. It was inversely

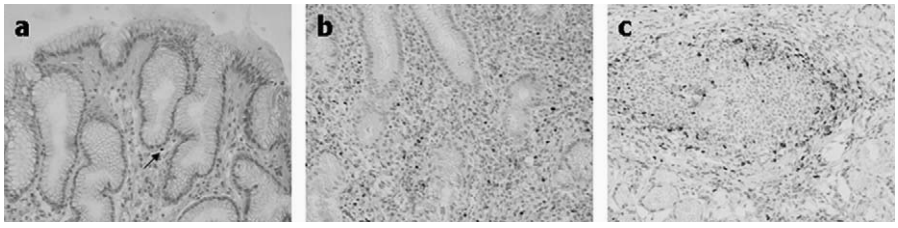

Fig. 1. Immunohistochemistry of Foxp3 in chronic gastritis (400 ×). (a) Foxp3-positive T-regulatory cell is present near the foveolar epithelium in *H. pylori*-negative gastritis (arrow). (b, c) The number of Foxp3-positive T-regulatory cells (Tregs) is elevated in *H. pylori*-associated gastritis (b, c) compared with *H. pylori* negative gastritis (a). Tregs are located adjacent to the foveolar epithelium (b) and are frequently aggregated close to lymphoid follicle (c).