Green Tea Polyphenols Reduce Gastric Epithelial Cell Proliferation and Apoptosis Stimulated by *Helicobacter pylori* Infection

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**Summary** Recently the finding of gastric cancer in *Helicobacter pylori* (*H. pylori*)-infected mouse models was reported. Studies of humans and animal models have shown that *H. pylori* infection stimulates gastric epithelial cell proliferation and apoptosis. Polyphenols contained in green tea and related compounds were reported to have a variety anti-tumor effects and bactericidal properties. We studied the effect of green tea polyphenols on gastric cell proliferation and apoptosis in an *H. pylori*-infected mouse model. This model was prepared by inoculating Balb/c mice with 10⁸ cfu of *H. pylori* (NCTC 11637 strain) by gavage. Beginning 18 weeks after inoculation, 0.5% polyphenols were given in drinking water every day for 2 weeks. Mice were sacrificed 1 h after bromodeoxyuridine (BrdU) was given i.p. for preparation of paraffin-embedded specimens. Cell proliferation and apoptosis were examined by the avidin-biotin complex method using anti-BrdU antibody and the TUNEL method, respectively. *H. pylori* infection resulted in increased BrdU-labeled cells in both the antrum and the bodies. Administration of polyphenols suppressed this increased proliferation. *H. pylori* infection increased apoptotic cells in both the antrum and the corpus in comparison with controls. This increase was not seen in *H. pylori*-infected mice given polyphenols. We conclude the administration with polyphenols might suppress gastric carcinogenesis that is in part related to *H. pylori* infection.

**Key Words**: *Helicobacter pylori*, green tea polyphenols, gastric cell proliferation, apoptosis

**Introduction**

*Helicobacter pylori* (*H. pylori*) infection has been proven to cause chronic gastritis [1–5], and is considered to be a factor in gastroduodenal ulceration. Furthermore, gastric cancer was observed in Mongolian gerbils infected with *H. pylori* [6], and *H. pylori* was designated as a class I carcinogen for stomach cancer by the World Health Organization (WHO) [7]. On this basis, attempts have been made worldwide to eradicate *H. pylori*.

Gastric epithelium is continuously injured by a variety of factors, resulting in a loss of epithelial cells. However, gastric mucosa has a remarkable reproductive capacity, and mucosal integrity is maintained by continuous reproduction and migration of new cells from the epithelial proliferative zone. Mucosal integrity is maintained by striking a balance between the number of apoptotic cells, designated as undergoing physiological death, and those reproducing from the proliferative zone. But when cell proliferation exceeds apoptosis, excessive growth of the gastric mucosa occurs, which may induce carcinogenesis. On the other hand, when cell death exceeds reproduction, the decrease in cell numbers results in disintegration of the mucosa, forming
erations and ulceration. In studies on humans and animal models, *H. pylori* infection has been recognized to stimulate cell proliferation in the gastric epithelium [8–12], and also apoptosis is stimulated in the gastric mucosa infected with *H. pylori* [13, 14]. But the mechanisms of the relationship between increased cell proliferation and apoptosis caused by *H. pylori* have not been elucidated well.

Infection with *H. pylori* is known as a factor in chronic gastritis, peptic ulcer and gastric cancer. Chronic inflammation is dominated by neutrophils, macrophages, lymphocytes and plasma cells. Interleukin-8 (IL-8) is a potent neutrophil-activating chemokine and is central to immunopathogenesis of *H. pylori*-induced injury [15].

Green tea polyphenols (GTP), which are compounds in Japanese green tea, are reported to have bactericidal properties against pathogenic bacteria in food and also have anti-tumor effects; attention has been given to their application as a new treatment for infectious diseases and malignant neoplasms [16, 17]. In this study, we examined the effects of GTP on gastric proliferation and apoptosis in an *H. pylori*-infected mouse model. Further, we studied the effect of GTP on the expression of IL-8, in *H. pylori*-infected gastric mucosa and the effect of GTP on *H. pylori* growth on mouse gastric mucosa.

**Materials and Methods**

**Preparation of mouse model**

Four-week-old Balb/C mice (Sankyo Lab, Tokyo, Japan) were inoculated orally with 10⁸ cfu/mouse of *H. pylori* (NCTC 11637 strain) for 2 days as preparation for the infection model. Beginning 18 weeks after inoculation, GTP (Thea-flan, Itoen, Tokyo, Japan) resolved in distilled water at pH 7.5 with the Fab fragment of anti-dioxigenin (Boehringer, Indianapolis, IN) in the reaction containing 100 mM Tris-HCl and 150 mM sodium citrate. Slides were washed with distilled water, and later with 0.1 M acetate buffer at pH 6. After visualization with DAB, cell nuclei were counterstained with hematoxylin.

**Expression of IL-8 and effect of GTP on *H. pylori* growth**

The expression of IL-8 on mouse gastric mucosa was studied by the ABC method with anti-KC goat polyclonal antibody (Santa Cruz, CA). Slides were incubated with anti-KC antibody and 100-fold polyclonal rabbit anti-*H. pylori* antibody (Dako, Copenhagen, Denmark) overnight at 4°C. After washing with PBS, slides were incubated with biotinylated secondary antibody diluted 20-fold (Dako Copenhagen, Denmark). Slides were incubated with ABC solution for 30 min and visualized with DAB.

**Immunohistochemical analysis**

Twenty specimens per mouse from the antrum and body were examined. BrdU-labeled cells were counted from the deepest part to the luminal surface of the crypt. The total number of cells in the proliferative zone (PZ cells) was counted from the BrdU-labeled cell closest to the serosa to the BrdU-labeled cell uppermost on the luminal side. The number of cells in the crypt was counted, and the percentage of cells in the proliferative zone (PZ cells/total cells ratio) was calculated. Apoptosis was expressed as TUNEL-positive cells in 300 crypt cells. The expression of KC was
expressed by the percentage of staining per mm² using Image-pro-plus system (Planetary Inc., Tokyo, Japan). For all parameters, 10 sites were selected per mouse. The number of H. pylori was counted by a blinded pathologist, and scored from 1 to 3. All data was studied by ANOVA test. A p value of <0.05 was considered to be statistically significant.

Results

Number of BrdU-labeled cells

Cells with positive staining for BrdU were counted vertically from the epithelium to the submucosal layer under light microscopy (Fig. 1–3). The number of BrdU labeled cells was increased over the control by H. pylori infection in the antrum (control 2.15 ± 0.15 vs H. pylori 3.74 ± 1.01; p = 0.003). The administration of GTP decreased the number of labeled cells, which was increased by H. pylori infection. (H. pylori + GTP 2.05 ± 0.09 vs H. pylori only 3.74 ± 1.01; p = 0.009). In comparing the number of labeled cells between the antrum of animals infected with H. pylori and those infected with H. pylori and later administered GTP, the number of labeled cells was reduced (H. pylori + GTP 2.05 ± 0.09 vs H. pylori only 3.74 ± 1.01; p = 0.009. In bodies, the number of labeled cells also was greater than in the control in the presence of H. pylori infection (control 2.11 ± 0.09 vs H. pylori only 3.82 ± 0.67; p<0.0001), but this increase was not as great in those administered GTP additionally (H. pylori + GTP 2.14 ± 0.15 vs H. pylori only 3.82 ± 0.67; p<0.0001) (Fig. 4).

Fig. 1. Immunohistochemistry for BrdU staining with the ABC method on gastric mucosal tissue of Balb/c mice (untreated mice): (a), antrum; (b), the body.

Fig. 2. Gastric mucosal tissue of Balb/c mice (untreated mice) stained for apoptotic cells by the TUNEL method.
Number of cells in proliferative zone
In the antrum, the number of PZ cells was increased by *H. pylori* infection (control 2.93 ± 0.37 vs *H. pylori* 5.43 ± 0.96; $p = 0.0001$), but further administration of GTP showed fewer PZ cells than with *H. pylori* infection alone (*H. pylori* + GTP 2.64 ± 0.35 vs *H. pylori* only 5.43 ± 0.96; $p<0.0001$). In the bodies, number of PZ cells was also increased in the presence of *H. pylori* infection (control 3.13 ± 0.56 vs *H. pylori* 7.00 ± 1.70; $p = 0.0003$), but this value was less with the additional administration of GTP (*H. pylori* + GTP 3.14 ± 0.31 vs *H. pylori* only 7.00 ± 1.70, $p<0.0001$) (Fig. 5).

Total number of crypt cells
There was no significant difference in the number of total crypt cells between the *H. pylori*-inoculated group and the control. Even with GTP administration the total cell numbers were not very different (antrum: control 20.40 ± 3.66, *H. pylori* 20.93 ± 2.93, *H. pylori* + GTP 18.83 ± 2.66, bodies: control 35.08 ± 7.64, *H. pylori* 40.16 ± 3.88, *H. pylori* + GTP 36.77 ± 4.14) (Fig. 6).

Number of apoptotic cells
TUNEL-positive cells were counted microscopically, with the specimen without a mucosal defect as a control (Fig. 2). The number of apoptotic cells was greater than in the control in the presence of *H. pylori* infection (control 0.0.26 ± 0.065 vs *H. pylori* 0.414 ± 0.266, $p = 0.005$) in the antrum. With the additional administration of GTP the number of apoptotic cells was less than with *H. pylori* alone (*H. pylori* + GTP 0.092 ± 0.042 vs *H. pylori* 0.414 ± 0.266; $p = 0.008$) Also, in the bodies, the number of apoptosis cells
was greater by infection with *H. pylori* than in controls (control 0.067 ± 0.090 vs *H. pylori* 0.198 ± 0.163; *p* = 0.005), but apoptosis appeared to be suppressed by GTP administration (*H. pylori* + GTP 0.048 ± 0.027 vs *H. pylori* 0.198 ± 0.163; *p* = 0.03) (Fig. 7).

**H. pylori growth**

The administration of GTP suppressed *H. pylori* growth on Balb/C mouse gastric mucosa (*H. pylori* + GTP 1.47 ± 0.71 vs. *H. pylori* only 2.81 ± 0.26; *p* = 0.0015) (Fig. 8).

**Expression of KC**

Expressions of KC were increased by *H. pylori* infection (control 0.4 ± 0.3% vs *H. pylori* 6.3 ± 1.5%, *p*<0.0001).

However, with the administration of GTP the expression of KC was reduced compared with values for *H. pylori* infection alone (*H. pylori* + GTP 0.6 ± 0.3% vs *H. pylori* 6.3 ± 1.5%; *p*<0.0001) (Fig. 9).

**Discussion**

It is widely known that tumors appear due to the disruption of the integrity maintained by two mechanisms, cell proliferation and cell death [18, 19]. The integrity of normal gastric mucosa is maintained by the balance in cell numbers between apoptosis, defined as cell suicide, and cell proliferation. However, excessive cell proliferation over cell death results in the occurrence of malignant neoplasm [20].
Otherwise, excessive cell death over proliferation results in gastric ulceration or erosions. *H. pylori* is related to the occurrence of gastric cancer and was designated as a class I carcinogen by WHO in 1994 [7]. It has been confirmed that Mongolian gerbils developed gastric cancer after long-term infection with *H. pylori* [6]. Clinical studies indicated that *H. pylori* infection increased cell proliferation in the gastric epithelium, but the increase was suppressed by *H. pylori* eradication [18, 21]. Our results demonstrated that cell proliferation and apoptosis were both stimulated from 18 weeks after the *H. pylori* infection.

We previously reported the relationship between cell turnover and apoptosis in the gastric mucosa over time in mice infected with *H. pylori* [19]. We showed that apoptosis was increased from 6 weeks after infection, but that cell proliferation was stimulated from 12 weeks after incubation with *H. pylori* [22]. Apoptosis is increased in the gastric mucosa infected with *H. pylori* and subsequently the phenomenon of increased cell proliferation was observed.
In the present study, *H. pylori* infection did not increase the total number of cells in gastric mucosa in our mouse model. The reason may be that we only studied gastric epithelial cell proliferation until 18 weeks after *H. pylori* infection. In human patients who had been infected with *H. pylori* since childhood it took many years to develop cancer. Long-term infection with *H. pylori* may cause an increase in the total number of cells in gastric mucosa over 18 weeks. It has been speculated that one reason why *H. pylori* stimulates cell proliferation in gastric mucosa is that the infiltration of inflammatory cells into the mucosa by the infection induces cell proliferation [10]. It is considered that the stimulated cell proliferation is a secondary reaction to the inflammation caused by infection. Histologically, inflammatory cells, including polymuclear neutrophils and lymphocytes, are present in *H. pylori*-infected gastric mucosa.

Yoshino *et al.* [23] reported that *H. pylori* infection induces Reg protein expression in human gastric mucosa, which is significantly up regulated by IL-8. IL-8 can be a stimulator of gastric cell proliferation by *H. pylori* infection through expression of Reg protein. We also demonstrated that *H. pylori* infection increased cell proliferation, and also that *H. pylori* infection increased the expression of IL-8. From these findings, the expression of IL-8 can be considered a mediator for cell proliferation in our model.

Green tea, which has polyphenols as a principal constituent, has been drunk widely in Japan and China for more than 5000 years [24]. It has been reported that the polyphenols in green tea have anti-tumor effects [16, 17], and epidemiologically those who drink green tea regularly have a lower risk of cancer. Those who drink 10 cups or more of green tea every day have a lower risk of gastric cancer compared with those having a lesser intake[25]. Also, those who live in Shizuoka prefecture in Japan, where green tea leaves are grown, have lower risks of all cancer, including gastric cancer [26]. Polyphenols are elements also found in red wine and cacao, and a variety of their properties have been reported recently. Briviba *et al.* [25] reported that the polyphenols in red wine suppress proliferation of colon cancer cells.

In our study, a 14-day administration of GTP significantly suppressed cell proliferation and apoptosis stimulated by *H. pylori* infection. This suppression was at the same level as in mice without infection. Mabe *et al.* [21] reported that green tea itself has antibacterial and bactericidal properties, and its benefit in eradication of *H. pylori* in mouse models has been reported. We demonstrated that the number of *H. pylori* organisms was reduced by GTP administration. The eradication effect of GTP on *H. pylori* seemed to be one reason for the decrease in the gastric cell proliferation and apoptosis that were stimulated by infection. GTP suppressed the expression of EGF in JB6 mouse epithelial cells [27], and it can be speculated that GTP administration suppressed the increased cell proliferation caused by *H. pylori* infection through activity to suppress the induction of growth factors in the gastric mucosa.

IL-8 is a potent neutrophil-activating chemokine, central to the immunopathogenesis of *H. pylori*. In this study, we measured mouse KC as a marker for inflammation. Mouse KC is a neutrophil-attracting chemokine and has a function corresponding to human IL-8 as well as MIP-2 in mice. We showed that the expression of KC protein was stimulated by *H. pylori* infection and that the administration of GTP reduced the stimulated KC expression. Naito *et al.* [28] reported that oxidative and nitrosative stress associated with inflammation plays an important role in gastric carcinogenesis as a mediator of carcinogenic compound formation, DNA damage, and cell proliferation. IL-8 expression is reported as the most important factor for inflammation and/or the occurrence of gastric cancer. Joh *et al.* [29] reported that *H. pylori* and IL-8 significantly stimulate EGFR phosphorylation. The inhibition of EGFR phosphorylation by *H. pylori* and also IL-8 reduced cell growth of gastric cells. Administration with GTP reduced IL-8 expression and also cell proliferation. From these data, the effect of GTP was a reduction of the expression of IL-8 on gastric mucosa, and IL-8 may reduce cell proliferation through the phosphorylation of growth factors and these receptors. Likewise, in the gastric mucosa, GTP administration may suppress the increased cell proliferation caused by *H. pylori* infection by inhibiting cytokine production and preventing the spread of inflammation in the gastric mucosa.

The effect of GTP on apoptosis has been examined in various organs, and most results indicate that the effect is proapoptotic. However, in our present study, administration of GTP tended to suppress the apoptosis stimulated by *H. pylori* infection. This difference, first of all, may be ascribed to the existence of a mechanism in the gastric mucosa to maintain its integrity, and when GTP administration suppresses proliferation excessively, injury to the epithelium may appear. However, the mechanism to maintain integrity may affect the degree of apoptosis induced by GTP administration. This possibility is conceivable because our result showed that the total cell number in gastric mucosa was not affected.

We presented data that *H. pylori* infection stimulated cell proliferation and apoptosis in a mouse model, which was in turn inhibited by GTP administration. This indicates that administration of GTP may inhibit carcinogenesis in the stomach, which is partially related to *H. pylori* infection.

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