Protective role of 17β-estradiol against the development of *Helicobacter pylori*-induced gastric cancer in INS-GAS mice

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The incidence of gastric cancer is higher in men than women. Epidemiological studies suggest that female hormones reduce gastric cancer risk. We examined the effect of ovarian-dependent female hormones on *Helicobacter pylori*-induced gastric cancer in hypergastrinemic INS-GAS mice. Male and female sexually intact or ovariectomized (OVX) mice were inoculated with *H. pylori* SS1 or vehicle-only at 10 weeks of age, and tissues were evaluated at 16 or 28 weeks post-infection (WPI). A subset of OVX females were supplemented with 17β-estradiol (E2), beginning at 16 WPI. Stomachs were evaluated by histopathology, Ki-67 proliferation index, *H. pylori* quantitative culture and quantitative polymerase chain reaction for messenger RNA expression of inducible nitric oxide synthase (iNOS) and inflammatory cytokines. Infected OVX females developed significantly more severe gastritis (P < 0.05) than infected intact females at both time points. E2 treatment in infected OVX females attenuated the severity of gastritis. Gastrointestinal intraepithelial neoplasia (GIN) developed in 42% of infected males and 10% of infected OVX females by 28 WPI, whereas infected intact females and E2-treated OVX females did not develop GIN. Infected OVX females showed significantly increased iNOS expression and epithelial cell proliferation when compared with intact, infected females. Likewise, interferon-gamma, tumor necrosis factor-alpha and interleukin-1β (IL-1β) expression in infected OVX females were significantly increased at 28 WPI when compared with intact counterparts. E2 treatment in infected OVX females significantly decreased IL-1β expression, increased IL-10 expression and reduced epithelial cell proliferation. These results demonstrate a protective effect of E2 in *H. pylori*-induced gastric cancer in a mouse model.

Introduction

Although the incidence of gastric cancer has decreased in the last decade, it remains the second most common cause of cancer-related mortality worldwide (1). The incidence rate of gastric cancer in males is approximately two times that recorded in females (1). This male predominance is an unexplained aspect of gastric cancer. Interestingly, the age-specific male-to-female ratio of annual gastric cancer incidence decreases after a peak at ~60 years of age (2). This unique pattern of the male/female ratio is globally consistent, similar in populations with low and high incidence of gastric cancer (2), and suggests that post-menopausal women have an increased risk of gastric cancer. Studies have also reported that a delay of menopause is associated with decreased risk of gastric cancer (3–5); however, these data remain controversial (6–8). Correspondingly, hormone replacement therapy for menopausal symptoms decreased gastric cancer risk in post-menopausal women (9).

*N*-Methyl-∗N*-nitro-*N*-nitrosoguanidine-induced gastric cancer in rats is predominantly observed in males (10), and 17β-estradiol (E2) treatment in male rats decreased the frequency of gastric adenocarcinoma (11) and grade of dysplasia (12). Progesterone treatment also had a promoting effect on the development of gastric tumors (13). Although these results suggested that E2 had a protective role in chemically induced gastric carcinogenesis, the molecular mechanism of protection has not been elucidated.

One potential target of hormonal modulation may be the inducible isoform of nitric oxide synthase (iNOS). Nitric oxide has been shown to stimulate mucosal inflammation and epithelial cell growth, and *Helicobacter pylori* infection in humans leads to increased iNOS expression in the gastric mucosal epithelium, endothelium and immune cells (14). In studies using iNOS gene-deleted mice, iNOS deficiency was associated with amelioration of gastric lesions seen with chronic *Helicobacter felis* infection (15). On the other hand, it has been reported that ovariectomy increased gastric iNOS expression and attenuated indomethacin-induced acute gastric mucosal injury in the rat (16). The modulation of iNOS expression by female hormones in *H. pylori*-associated gastritis has not been evaluated.

In 1994, *H. pylori* was classified as group I gastric carcinogen by the World Health Organization (17). Chronic infection with *H. pylori* and the host immune response to the infection plays a role in initiation and promotion of gastric cancer (18). Transgenic INS-GAS male mice are hypergastrinemic and develop gastric cancer by 7 months following *H. pylori* or *H. felis* infection (19,20). There are two main histological types of gastric cancer in humans: intestinal type and diffuse type. Intestinal-type gastric cancer predominates in males, occurs at a late age and progresses through relatively well-defined histological steps including: chronic atrophic gastritis, intestinal metaplasia and dysplasia (21,22). Gastric cancer observed in *H. pylori*-infected INS-GAS mice is also male predominant and follows a similar carcinogenic sequence (19,20). In this study, we investigated the effect of ovariectomy and E2 supplementation on *H. pylori*-induced gastric carcinogenesis using INS-GAS mice (23).

Material and methods

**Animals and study design**

One hundred and nine (80 females, 29 male) specific pathogen-free (including *Helicobacter spp.*) INS-GAS mice on FVB background were used in this study. The mice were housed on hardwood bedding in microisolator, solid-bottom polycarbonate cages and fed regular chow diet (ProLab RMH3000, LabDiet, Richmond, IN) and water *ad libitum*. The mice were divided into four groups: (i) males (n = 29), (ii) OVX females (n = 35), (iii) sham OVX (intact) females (n = 29), (iv) E2-treated OVX females (n = 16). The numbers of mice in each group are listed in Table 1. All the mice from respective groups were analyzed for histopathological parameters, *H. pylori* colonization levels and messenger RNA (mRNA) levels of selected cytokines and iNOS. Ovariectomy and sham surgeries were performed at 8 weeks of age. Two weeks after surgery, mice were either infected with *H. pylori* or dosed with broth only. E2 treatment in OVX females was performed at 16 weeks post-infection (WPI). The mice were euthanized by CO2 inhalation at 16 and 28 WPI. All protocols were approved by the MIT Committee on Animal Care.

**Ovariectomy and administration of E2**

Ovariectomy was performed using sterile techniques under general anesthesia with isoflurane. Ovaries were removed through a dorsal bilateral approach. The peritoneal incisions were closed using 5-0 absorbable sutures (Vicryl, Ethicon, Inc.)
Table I. Body weights, uterine weights and serum E2 levels in female mice at 28 WPI with Helicobacter pylori

<table>
<thead>
<tr>
<th></th>
<th>Intact females, n = 7</th>
<th>OVX females, n = 10</th>
<th>E2-treated OVX females, n = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 level (pg/ml)</td>
<td>24.6 ± 3.9*</td>
<td>13.0 ± 1.2*</td>
<td>49.2 ± 8.9*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>31.3 ± 1.9*</td>
<td>39.0 ± 1.3*</td>
<td>28.5 ± 0.6*</td>
</tr>
<tr>
<td>Uterus weight (mg/g body wt)</td>
<td>5.1 ± 0.5</td>
<td>0.4 ± 0.1*</td>
<td>5.9 ± 0.3*</td>
</tr>
</tbody>
</table>

*P < 0.001 compared with intact females.
*P < 0.001 compared with OVX females.

Somerville, NJ. The skin incisions were closed using 9 mm surgical clips (MikRon Autoclip, Clay Adams, Parsippany, NJ) and tissue adhesive (3M Vetbond, 3M Animal Care Products, St Paul, MN). For sham surgery, a similar approach to access the peritoneal cavity was performed to identify both ovaries. Incisions were closed using the same procedure described above. All the mice received subcutaneous injections of buprenorphine (0.1 mg/kg) and 0.5 ml of warm lactated Ringer’s solution after surgery for post-operative management.

E2 treatment under anesthesia was performed by subcutaneous placement of a time-release E2 pellet (Innovative Research of America, Sarasota, FL) at 10 days after surgery. For E2-treatment, female mice were divided into four groups: a) intact females, b) OVX females, c) E2-treated OVX females, and d) OVX females treated with E2 since 16 WPI.

Table I.

Real-time reverse transcription polymerase chain reaction assay of iNOS and cytokines

Total RNA was extracted from the corpus of the stomach using TRI Reagent (Sigma-Aldrich) following the manufacturer’s instructions. Two microgram of total RNA was converted into cDNA using high capacity cDNA Archive Kit (Applied Biosystems, Foster city, CA) according to manufacturer’s protocols. The real-time quantitative polymerase chain reaction was performed in an ABI PRISM Sequence Detector 7700 (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. cDNA was amplified using TaqMan Universal Master Mix (Applied Biosystems). Commercially available primer and probe mix used in these experiments included interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-10, iNOS and glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems). For relative quantification of gene expression, a standard curve was generated for each gene assay using cDNA of a selected calibrator sample. The relative expression of target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase. All data were expressed as fold change when compared with an average value of uninfected males at 16 WPI.

Statistical analysis

For analyzing non-parametric data including five histological parameters and gene expression assays, the Kruskal–Wallis test was performed to determine differences among the infected groups. Direct comparisons between two groups were made using Mann–Whitney U-test. For analyzing the parametric data including body and uterus weights, serum E2 level, H. pylori colonization level and Ki-67 LI, statistical differences were determined by one-way analysis of variance followed by Student’s t-test (Fisher’s protected least significant difference) or non-parametric analysis described above depending on whether the data were normally distributed with Kolomogorov–Smirnov test. Statistical analyses were performed using Prism 4 software for windows (GraphPad, San Diego, CA). All data were presented as mean ± SEM, and statistical significance was defined as a P value of <0.05.

Results

Changes in body weight, uterus weight and plasma E2 concentrations

Ovariectomy was confirmed by the histopathological findings of the uterus. Both endometrium and myometrium were completely atrophied in OVX females. In contrast, by 28 WPI, the endometrium was hypertrophic with cystic dilated endometrial glands in OVX females treated with E2 since 16 WPI.

Ovariectomy significantly reduced serum E2 levels (13.0 ± 1.2 pg/ml) when compared with intact females (24.6 ± 3.9 pg/ml) (P < 0.01). By 28 WPI, E2 treatment elevated serum E2 level (49.2 ± 8.9 pg/ml), which is slightly in the supraphysiological range (Table I). To evaluate the in vivo effect of ovariectomy and E2 treatment, we recorded body and uterus weights (Table I). The body weight of OVX females was significantly higher than those of intact females at both 16 and 28 WPI (all P < 0.01). E2 treatments in OVX females gradually decreased body weight to the same level as intact females. The uterine weight of OVX females (0.4 ± 0.1 mg/g body wt) was significantly lower than that of intact females (5.1 ± 0.5 mg/g body wt) at 28 WPI (P < 0.01). The uterine weight of E2-treated OVX females (5.9 ± 0.3 mg/g) was slightly higher than that of intact females, and a statistically significant difference was not observed. There was a significant inverse correlation between body weight and uterine weight adjusted for body weight (P < 0.001, R² = 0.54).

Ovariectomy exacerbated H. pylori gastritis

At 16 WPI, all uninfected mice had developed only minimal to mild corpus gastritis, although epithelial defects, oxyntic atrophy, hyperplasia and intestinal metaplasia were evident, especially in male mice (Figure 1a and b), in agreement with the known phenotype of hypergastrinemic INS-GAS mouse (20,21). Ovariectomy in uninfected mice exacerbated atrophy, hyperplasia and intestinal metaplasia, resembling the male phenotype (Figure 1c). This gender effect and influence of ovariectomy was more pronounced in infected mice (Figure 1d–f). Median histological scores of inflammation, atrophy, hyperplasia, intestinal metaplasia and dysplasia in infected males and OVX females were significantly higher than those in infected, intact
females (Figure 2). These results demonstrated that ovariectomy exacerbated gastritis in infected female mice comparable with the levels observed in infected males.

Administration of E2 in OVX females attenuated H. pylori gastritis

At 28 WPI, uninfected male mice had developed more severe gastritis than uninfected intact females (Figure 1g and h). Gastric pathology of uninfected OVX females and E2-treated OVX females was comparable with the levels observed in uninfected intact females (Figures 1i, j and 2). Median histological scores in infected males, OVX females and intact females were increased at 28 WPI when compared with 16 WPI (Figures 1k–m and 2). Especially noteworthy was the rapid progression of intestinal metaplasia and dysplasia in infected intact females with advancing age (all $P < 0.01$; Figure 2). Theses results indicate that infected intact females showed a delayed onset of intestinal metaplasia and dysplasia compared with infected males and OVX females. However, there were still significant gender differences of all histological parameters between infected males and intact females at 28 WPI (all $P < 0.01$; Figure 2). The histological score of dysplasia in infected OVX females was significantly higher than those in infected intact females at 28 WPI ($P < 0.01$; Figure 2). Although they did not reach statistical significance, the scores for inflammation, oxyntic atrophy, hyperplasia and intestinal metaplasia in infected OVX females were higher than those in infected intact females (inflammation $P = 0.053$, oxyntic atrophy $P = 0.055$, hyperplasia $P = 0.08$, intestinal metaplasia $P = 0.2$). The scores of all histological parameters in infected E2-treated OVX females were significantly lower than those in infected OVX females ($P < 0.01$ or 0.05; Figures 1n and 2). Infected males and OVX females developed high-grade dysplasia with loss of glandular architecture, marked nuclear atypia and loss of nuclear polarity, which is consistent with high-grade gastrointestinal intraepithelial neoplasia (GIN) (26). High-grade GIN was found in three of seven infected males (42%), 1 of 10 infected OVX females (10%), but was not observed in infected intact females, infected E2-treated OVX females or uninfected mice.

Female hormones modulated gastric epithelial cell proliferation in H. pylori-infected mice

We evaluated epithelial cell proliferation using a Ki-67 LI. Helicobacter pylori infection significantly increased epithelial cell proliferation in all infected groups ($P < 0.05$). Positively stained nuclei were observed from the neck of the glands to the gastric pits in infected
mice. Infected males showed significantly increased epithelial cell proliferation when compared with infected intact females at 16 WPI (\(P < 0.05\); Figure 4a, b and e). Although, at 28 WPI, proliferation index in infected males was higher than that in intact females, this difference was not significant. Ovariectomy alone significantly increased epithelial cell proliferation at 16 and 28 WPI (all \(P < 0.05\); Figure 4c and e). In addition, E2 treatment in infected OVX females significantly reduced gastric epithelial proliferation (\(P < 0.05\); Figure 4d and e).

OVX increased iNOS mRNA level in the gastric tissue of \textit{H. pylori}-infected mice

\textit{Helicobacter pylori} infection significantly increased iNOS mRNA expression in all groups when compared with each uninfected counterpart. iNOS expression in infected males was significantly higher than that in infected intact females at 16 WPI (\(P < 0.01\); Figure 5a). At 28 WPI, iNOS expression in infected males was higher than that in infected intact females; however, this difference was not statistically significant (\(P = 0.07\)). Ovariectomy in infected females significantly increased iNOS expression at 16 WPI and more profoundly at 28 WPI (all \(P < 0.01\)). E2 treatment did not significantly alter iNOS expression.

E2 inhibited pro-inflammatory responses in the gastric tissue of \textit{H. pylori}-infected mice

We examined mRNA levels of pro-inflammatory cytokines (IFN-\(\gamma\), TNF-\(\alpha\) and IL-1\(\beta\)) and the anti-inflammatory cytokine IL-10 (Figure 5b–e). \textit{Helicobacter pylori} infection significantly increased both IFN-\(\gamma\) and TNF-\(\alpha\) mRNA expression in all groups at 16 and 28 WPI when compared with each uninfected counterpart (all \(P < 0.01\); Figure 5b and c). Although a gender difference for the mRNA levels of IFN-\(\gamma\) and TNF-\(\alpha\) in infected mice was not observed at 16 WPI, a significant gender difference was detected in TNF-\(\alpha\) mRNA level at 28 WPI (\(P < 0.01\)). Likewise, the effects of ovariectomy on the mRNA levels of IFN-\(\gamma\) and TNF-\(\alpha\) were not detected at 16 WPI in infected mice;
however, both IFNγ and TNF-α mRNA levels in infected OVX females were significantly higher than those in infected intact females at 28 WPI (all P < 0.01). E2 treatment tended to decrease the mRNA level of TNF-α (P < 0.07), but not IFN-γ. Helicobacter pylori infection did not alter the mRNA level of IL-1β in any of the groups at 16 WPI. However, H. pylori infection significantly increased the mRNA level of IL-1β in males and OVX females at 28 WPI when compared with each uninfected counterpart (all P < 0.01) but not in intact females and E2-treated OVX females. It is noteworthy that ovariectomy significantly increased the mRNA level of IL-1β in infected females (P < 0.01). Helicobacter pylori infection significantly increased the mRNA level of IL-10 in intact females at both time points when compared with untreated counterparts (all P < 0.01). E2 treatment in infected OVX females significantly increased the IL-10 mRNA level (P < 0.01; Figure 5d), although ovariectomy did not alter it. No differences in cytokine expressions were observed in the uninfected groups associated with gender or ovariectomy.

**Discussion**

In the present study, we report for the first time the in vivo effect of female hormones on H. pylori-induced gastric carcinogenesis in a rodent model. We confirmed that severe dysplasia and cancer is male predominant in H. pylori-infected INS-GAS mice, whereas infected females showed a relatively delayed onset of dysplasia when compared with males (19,20). Our results are consistent with epidemiological observations by Sipponen et al. (2), who demonstrated that the annual rise in incidence of intestinal-type gastric cancer in women was delayed 10–15 years when compared with males. In addition, we demonstrated that ovariectomy in infected females exacerbated mucosal dysplasia, whereas administration of E2 in OVX females attenuated dysplastic progression. This agrees with the findings that medication with hormone replacement therapy decreased the risk of gastric cancer in post-menopausal women (9).

Unlike INS-GAS female mice, it is not known why outbred female gerbils mount a more aggressive Th1-mediated immune response than their male counterpart which results in more severe gastritis in females (27). Studies directed at studying the differences in gender susceptibility to H. pylori infection in INS/GAS mice and gerbils are warranted.

At 16 WPI, both infected males and OVX females developed significantly more severe gastritis than sexually intact infected females. Similarly, iNOS mRNA level and Ki-67 LI in infected males and OVX females were significantly higher than values in intact females at 16 WPI. iNOS is an important promotinal regulator of mucosal inflammation and cell cycling (14) and is induced by lipopolysaccaride
and pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ (28). E2 is also an important regulator of iNOS expression. In vitro studies showed that E2 either increased or suppressed iNOS expression depending on the cell type (29,30). In our study, infected OVX females showed significantly greater gastric iNOS expression compared with intact females at 16 WPI. This up-regulation occurred in the absence of any significant changes in the mRNA levels of TNF-α, IL-1β and IFN-γ. At 28 WPI, however, infected OVX females showed significantly increased mRNA levels of both iNOS and pro-inflammatory cytokines compared with infected intact females. The intense up-regulation of iNOS mRNA level at 28 WPI may have been mediated by both increased pro-inflammatory cytokines and decreased levels of female hormones. Nitric oxide-mediated endogenous nitrosamine formation has been implicated in gastric carcinogenesis (31). Hence, the up-regulation of iNOS associated with decreased female hormones may play a direct promoting role in gastric carcinogenesis. The finding that E2 treatment did not alter the mRNA level of iNOS has several possible explanations. For example, any suppression of iNOS transcription by E2 may have been offset by pro-inflammatory cytokines. Also, another female hormone, progesterone, could modulate iNOS expression. Recently, a putative progesterone response element was identified upstream of the iNOS promoter region in murine astrocytes (32). Whether a similar mechanism exists within cells of the gastric compartment will require further studies.

We observed that ovariectomy resulted in an increased gastric epithelial cell proliferation LI in H. pylori-infected mice, and that this effect was reversed by supplementation of E2. E2 may modulate epithelial cell proliferation in gastric mucosa directly or indirectly. A possible indirect mechanism could be through modulation of gastric iNOS and cytokine expression. A direct effect is suggested by the report that E2 had an anti-proliferative effect on a gastric cancer cell line in vitro (33). The distribution and predominant expression of estrogen receptor α and estrogen receptor β (ERβ) varies by tissue (34). Literature concerning the relative expression of ERs in gastric mucosa is conflicting; however, it appears that the ERβ predominates in the gastric and colonic mucosa (35–37). Paech et al. (38) reported opposing effects of estrogen receptor α and ERβ on modulating gene expression. This observation was supported further by the report that E2 activates cyclin D1 gene expression through estrogen receptor α, but inhibits cyclin D1 expression through ERβ in HeLa cells (39). ERβ gene-deleted mice showed hyperproliferation and decreased apoptosis in colonic epithelium (40).
The mRNA levels of IFN-γ, TNF-α and IL-1β in OVX females were significantly higher than cytokine values in infected intact females at 28, but not 16 WPI. Although the overall expression levels were modest, E2 treatment in infected OVX females significantly decreased IL-1β and increased IL-10 mRNA levels compared with untreated OVX females, suggesting that E2 supplementation (or endogenous E2 in intact females) suppresses mucosal pro-inflammatory responses that promote cancer in the stomach. Enhanced production of IL-1β is associated with an increased risk of gastric cancer (41). E2 inhibits IL-1β expression in monocytes (42) and stimulates IL-10 production in murine dendritic cells (43). Attenuation of IL-1β mRNA level by E2 may be one mechanism by which E2 reduced gastric carcinogenesis in the INS/GAS model. A direct molecular mechanism linking IL-1β and IL-10 expression with E2 has not been reported. However, it is known that estrogen suppress IL-6 gene transcription by inhibiting NF-κB activation (44). Because IL-1β transcription is also regulated by NF-κB (45), the effect of E2 may operate in part through this pathway.

Recently, a study using female gerbils demonstrated that E2 exacerbated H. pylori-induced gastritis, whereas progesterone attenuated inflammation in the early phase of the infection (46). Our study differs from this report in three important respects. First, gender dimorphism in susceptibility to H. pylori infection is different between INS-GAS mice and gerbils. Crabtree et al. (27) reported that female gerbils developed more severe gastritis than did males, and that mRNA expression of IFN-γ and IL-12 p40 was also higher in females than in males. Helicobacter pylori infection in INS-GAS mice induces gastric cancer at a rapid rate in males but not females (19,20), and is more similar to humans in this respect. Second, E2 may have a different biological action depending on whether E2 treatment is initiated in the acute or chronic phase of H. pylori infection. In the gerbil study, E2 treatment commenced at 1 WPI, whereas in our study, E2 supplementation was initiated at 16 WPI. Third, E2 was administered to OVX female mice, whereas in gerbils E2 was given to intact female gerbils.

We observed GIN in only infected males and OVX females but not sexually intact or E2-treated females at 28 WPI; however, the incidence of GIN and median grade of dysplasia were higher in males than in OVX females. This suggests that gender differences beyond sex hormones influence the risk of gastric cancer.

In summary, we have shown that ovarian-dependent female hormones, in particular E2, provide a protective role against gastric carcinogenesis in H. pylori-infected INS-GAS mice, and that tumorigenic potential is positively associated with inflammation as indicated by increased iNOS levels, epithelial cell proliferation and pro-inflammatory cytokine expression in the gastric tissue. Exogenous E2 administration had a beneficial effect against development of gastric cancer that was associated with decreased epithelial cell proliferation, decreased mRNA levels of IL-1β and TNF-α. Although at present the use of hormone replacement therapy would not be an ideal intervention given its likely promotion of breast cancer (47), further characterization of molecular mechanisms by which E2 suppresses gastric carcinogenesis may reveal new therapeutic targets for prevention and treatment of gastric cancer.

Funding

Provided by NIH grants R01AI37750; P01CA26T31; P30ES02109 to J.G.F.; R01CA93405 to T.C.W. and J.G.F.

Acknowledgements

Conflict of Interest Statement: None declared.

References


