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# 17 $\beta$ -Estradiol Modulates Gastroduodenal Preneoplastic Alterations in Rats Exposed to the Carcinogen *N*-Methyl-*N'*-Nitro-Nitrosoguanidine\*

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## ABSTRACT

Gastric cancers are a significant cause of morbidity worldwide. Epidemiological studies and animal models show that males have higher incidences of gastric cancers compared with females, suggesting that sex hormones may modulate gastric cancer risk. An animal model of the initiation phase of gastric cancer was used to determine the effects of systemic estrogen administration on morphological progression of preneoplastic lesions and to define cell populations at which estrogens may act. Preneoplastic progression in antral and duodenal mucosa was examined in male rats that received the chemical carcinogen, *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG), during treatment with implants containing 17 $\beta$ -estradiol or oil vehicle. Histopathological changes in antral and duodenal gland morphology, numbers of proliferating cells and apoptotic bodies, and antral gastrin cell numbers and protein storage levels were determined 4 weeks

later. With MNNG treatment, duodenal villous heights were significantly decreased, and epithelial cells displayed histological features of hyperplasia and dysplasia. Antral glands showed epithelial hyperplasia and dysplasia, increased mucosal height, and decreased mucin levels. Antral gastrin storage protein levels were decreased by MNNG. Systemic treatment with 17 $\beta$ -estradiol significantly reversed MNNG-induced alterations in duodenal gland heights while increasing mucin and gastrin levels in antral glands. Cell proliferation and apoptosis rates were not significantly different between groups. The present results indicate that systemic 17 $\beta$ -estradiol treatment influences antral and duodenal gland differentiation during the initiation phase of chemical gastroduodenal carcinogenesis in male rats. These results explain, in part, a potential pathway through which protective effects of estrogens on chemical carcinogenesis are mediated in the upper gastrointestinal tract. (*Endocrinology* 140: 4886–4894, 1999)

**G**ASTRIC CANCER is one of the most common neoplasms worldwide, and numerous clinical and experimental studies suggest that sex hormones alter gastric cancer risk (1–3). Gastric cancer incidence rates are approximately 2-fold higher in males than in females in nearly every country studied. Differences in sex ratios also depend on gastric site as ratios for proximal cancers are approximately 7-fold higher in males than females (4). Estrogen replacement therapy (ERT) in postmenopausal women significantly reduces the incidence of colorectal adenocarcinomas (reviewed in Ref. 5). Epidemiological studies for effects of ERT on gastric cancer risk have not been performed in the United States, though a trend for risk reduction was found in Italian women (6). Women with late onset of menopause also have a reduced risk of gastric cancer (7). Several groups have clearly shown a higher incidence for experimental gastric tumors in male animals compared with females (rats, mice, hamsters) (8–11).

Environmental factors are important etiological factors in human gastric carcinogenesis with *N*-nitrosamino compounds the most commonly investigated agents. These compounds are found in cured meats, some drinking water, and certain vegetables. They can be converted by certain bacteria such as *Helicobacter pylori* into nitrosamines that have been found to cause gastric cancer in animals. Chronic administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in drinking water to rats produces adenocarcinomas in the distal glandular stomach (antrum) and upper small intestines (duodenum) (11–15). The MNNG model has been used extensively to study mechanisms of human chemical gastric carcinogenesis because the antral adenocarcinomas share gender-related incidences, histological features, and distribution patterns with human upper gastrointestinal (GI) adenocarcinomas (11, 16). MNNG acts directly on upper GI epithelial cells, causing methylation of DNA and proteins. It is rapidly converted to *N*-methyl-*N'*-nitrosoguanidine that is not carcinogenic. Adenocarcinomas develop at the gastric lesser curvature wherein higher cell proliferative rates and thiol concentrations may increase local susceptibility (17, 18).

Large differences have been observed in the sensitivity of different rat strains to MNNG-induced tumors (reviewed in Ref. 19). These differences were observed following identical treatment with MNNG in the drinking water. Male Wistar and ACI rats were very sensitive to MNNG, whereas male Sprague Dawley rats had intermediate sensitivity and Buffalo rats were resistant. Several parameters have been stud-

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ied to account for genetic differences in sensitivity to MNNG that include MNNG metabolism by *N*-denitrosation, tissue glutathione levels, *O*-methylguanine demethylation (DNA repair), and cell proliferation rates (19, 20). Of all these factors, cell proliferation rate (number and range of distribution) was the only factor that predicted strain sensitivity (20). Thus, the proliferative response of the gastric mucosa was proposed to be a key factor in the difference of susceptibility to gastric carcinogens between male rats of various strains.

Within these strains, gender differences in tumor incidence have also been determined (21). Rats were administered MNNG (83  $\mu\text{g}/\text{ml}$ ) in their drinking water for 8 months and were killed at 18 months. The incidence of gastric adenocarcinoma in ACI rats was 80% in males and 47% in females. In contrast, the incidence was 18% in males and 0% in females in Buffalo rats. The F1 progeny of sensitive (ACI) and resistant (Buffalo) strains had a dominant resistant phenotype in males and sensitive phenotype in females (17% male *vs.* 8% female). These incidences indicate complex interactions between MNNG susceptibility and gender. Sprague Dawley rats are reported to have intermediate sensitivity (19, 22), but gender incidences have been not reported. In another study, administration of MNNG for 4 months with tumor incidences determined at 12 months resulted in gastric carcinomas in 80% of Wistar male rats compared with an incidence of < 6% of ovariectomized and intact female rats (8). Castration decreased the incidence to 30%. Another report from the same laboratory showed a slight reduction in tumor incidences in male rats receiving estradiol treatment (88% *vs.* 68% (23). Estradiol was administered as twice weekly injections (400  $\mu\text{g}/\text{kg}$ ) for 4 months starting 2 months after beginning MNNG treatment. This evidence suggests that female hormones suppress, and male hormones promote, gastric cancer risk. Although male rats are known to be more susceptible than females to MNNG-induced gastric tumors, the gender-specific mechanism has not been investigated.

Estrogens regulate cell growth and differentiation in numerous tissues in both females and males and have critical roles in breast and uterine carcinogenesis (24). Estrogens act on cells through intracellular estrogen receptors (ER) that regulate target gene expression although nongenomic effects have also been reported (25). Recently, two ER subtypes, ER $\alpha$  and ER $\beta$ , have been characterized with differences in tissue distribution, ligand binding, transcriptional activity, and dimerization specificity (26–28). Expression of both ER $\alpha$  and ER $\beta$  have been reported in epithelial cells of the upper GI tract with a predominance of ER $\beta$  over ER $\alpha$  expression (29–31). Additional complexity would be expected in those cell types that express both receptors as ER homodimer (ER $\alpha$ /ER $\alpha$ ; ER $\beta$ /ER $\beta$ ) or heterodimer (ER $\alpha$ /ER $\beta$ ) formation can occur (32).

Mechanisms for protective effects of estrogens in gastric carcinogenesis are not known and could involve direct effects on mucosal cells, indirect effects mediated through other agents, or both. As a first step in identifying the roles of estrogens in GI carcinogenesis, a rat model for human gastric cancer was used to examine the influence of systemic estrogen exposure on morphological progression of preneoplastic lesions and to define the cell populations at which

estrogens may act. Systemic and local effects of 17 $\beta$ -estradiol administration were examined in male rats that received sc implants of 17 $\beta$ -estradiol (E2) or oil vehicle before and during the initiation phase of gastroduodenal carcinogenesis induced by MNNG.

## Materials and Methods

### Reagents

Serum testosterone and estradiol levels were determined by RIA at Equitech Labs (Alachua, FL). MNNG was purchased from Aldrich, Milwaukee, WI). All other reagents were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Norcross, GA).

### Animals and experimental protocol

Young adult male Sprague Dawley rats (6 weeks old, weight ~150 g, *n* = 48; Harlan Sprague Dawley, Inc., Indianapolis, IN) were used. All experiments were approved by the Institutional Animal Care and Use Committee using protocols according to the NIH Guide for the Care and Use of Laboratory Animals and by the Environmental Health and Safety office at the University of Florida. Rats were randomly assigned in groups of 2–3/cage and housed on wood chips (14-h light, 10-h dark cycle). Rats were acclimated to a powdered diet (AIN 76A, Tekland, Madison, WI) containing casein protein rather than soy protein, a major source of genistein and other phytochemicals with potential estrogenic activity (33). Following 1 week on the AIN diet, rats were anesthetized and SILASTIC brand silicon tubing (Dow Corning, Midland, MI) containing either 17 $\beta$ -estradiol (E<sub>2</sub>; 300  $\mu\text{g}/\text{ml}$  in sesame oil; Sigma) or oil vehicle were placed sc as previously described (29). All implants were replaced after 2.5 weeks.

One week following the first implant placement, rats were given MNNG (CAS: 70–25–7)(100  $\mu\text{g}/\text{ml}$ , (12)) or plain tap water. Stock solutions of MNNG (1 mg/ml in tap water) were prepared fresh weekly and stored at 4 C. The stock solution was diluted with tap water just before use and placed in a tin-foil covered bottle to prevent photolysis of MNNG. Food and water intakes were recorded and drinking solutions replenished three times a week. Weekly body weights were determined.

After 4 weeks of MNNG treatment and 2 h before they were killed, rats received an injection of the thymidine analog bromo-deoxyuridine (BrdU; 50 mg/kg ip) to label cells in the S phase of the cell cycle. Two hours later, rats were anesthetized with sodium pentobarbital (60 mg/kg) and the abdominal aorta was cannulated for serum collection and *in vivo* perfusion with 100 ml phosphate buffer (PBS, 10 mM KPO<sub>4</sub>, 150 mM NaCl, pH 7.4) followed by 200 ml 2% paraformaldehyde-lysine-periodate (PLP) (34). During perfusion, the stomach was distended with 2 ml PLP. The stomach and duodenum (proximal 5 cm) were removed and opened and ingesta rinsed off with PBS. Tissues were postfixed overnight in PLP at 4 C. Sections of fundus, antrum (midline on lesser curvature) and duodenum were embedded in paraffin for light microscopy. Sections from antrum were embedded in Lowicryl resin for immunogold labeling as previously described (35) or in plastic resin (TAAB 812, Merivac, Halifax, Nova Scotia). Blood samples were collected before perfusion, stored on ice for 30 min, and spun at 3000  $\times$  g for 20 min. Serum was collected and stored at –75 C.

### Histopathology

Alterations in mucosal morphology were determined on sections immersion-fixed in 10% formalin and stained with hematoxylin-eosin for nuclear detail and periodic acid-Schiff (PAS) histochemistry for mucin staining. Histological classification of lesions was made using established criteria from human pathology for grade of dysplasia (0–3 scale, none to severe) (36). Sections were coded so that the observer (GYL) was unaware of the treatment group.

### Image analysis

Antral gland heights and duodenal villus height and crypt depth were measured in regions in which full-length glands were oriented perpendicular to the submucosa. Height of antral and duodenal glands

was measured using a Carl Zeiss Axiophot microscope fitted with a 10× objective and 1.25 optivar setting. Images were captured with a video camera (Hamamatsu C2400, Hamamatsu City, Japan) linked to a personal computer with frame grabber and image analysis software (Vidas 2.1, Carl Zeiss, Thornwood, NY). The image analysis system was calibrated for each objective and optivar combination by tracing a reference distance on a stage micrometer. For each rat, 5–8 antral glands were measured in proximal, middle and distal regions. Duodenal villous and crypt heights were measured in four to eight glands from proximal and distal regions in each rat. Total duodenal gland height was calculated as the sum of villous and crypt height for each gland.

#### Gastrin immunogold analysis

Gastrin protein levels within storage granules were quantified by immunogold analysis. Ultrathin sections were placed on formvar-coated nickel grids and incubated with 0.1 M NH<sub>4</sub>Cl for 1 h. Grids were washed in Tris buffer (TBS; 10 mM Tris HCl, pH 7.5, 150 mM NaCl) followed by blocking buffer (10 mM Tris HCl, pH 7.5, 500 mM NaCl, 1% BSA). Grids were incubated with rabbit polyclonal antibodies to gastrin (1:500) in blocking buffer overnight at 4°C followed by washing in TBS. Grids were incubated with goat antirabbit antiserum conjugated with 15 nm gold particles (1:200, Amersham Pharmacia Biotech) followed by washing with TBS. Sections were counterstained with uranyl acetate and lead citrate before examination using a Carl Zeiss transmission electron microscope. Gastrin cells were randomly selected and cells containing a nucleus were photographed. Storage granules were selected from photographs (~16,000 final magnification) and defined as symmetrical gray densities 0.3–0.7 mm in diameter. Gold particles within four to eight storage granules were counted and data expressed as average number per storage granule/cell. This average was then used to calculate a mean value for each rat (4–7 gastrin cells/rat). Controls for immunogold staining included replacement of the primary antisera with normal rabbit serum or PBS.

#### Immunohistochemistry for BrdU incorporation and gastrin cell numbers

Cells in the S phase of the cell cycle and gastrin-producing neuroendocrine cells were counted following immunohistochemistry. Following rehydration to PBS, tissue sections were placed in citrate buffer (10 mM, pH 6.0) and heated in a microwave at full power (750 W) for 10 min with buffer replenishment every 2.5 min. Sections were cooled for 20 min at room temperature and washed in PBS for 5 min. Sections were incubated in 10% normal goat serum for 10 min following by the primary antibody overnight at 4°C (mouse monoclonal anti-BrdU peroxidase conjugate, Roche Molecular Biochemicals; rabbit polyclonal anti-gastrin). Sections were washed in PBS and incubated in 3% H<sub>2</sub>O<sub>2</sub>/PBS for 10 min to inactivate endogenous peroxidase. Sections were incubated for 10 min with peroxidase conjugated antirabbit antibodies for gastrin cell detection. Immunoreactivity was localized by incubation with diaminobenzidine tetrahydrochloride substrate. Cell counting was performed in regions with glands oriented perpendicular to the submucosa. Numbers of positive BrdU nuclei and gastrin cells in the antral glands were counted in five to eight rectangular reference frames with the 40× objective and 1.6 optivar setting. Number of positive BrdU nuclei in duodenal crypts were counted as above using the 20× objective and 2.0 optivar setting. The reference frame from the imaging system was overlaid on the section with the shortest side aligned at the muscularis mucosa to encompass four to five glands. The viewer was unaware of treatment conditions.

#### Apoptosis rates in antral glands

Several methods are available for determining rates of apoptosis including morphological assessment on stained sections and *in situ* DNA end labeling methods (37, 38). Cell necrosis following inadequate fixation has been reported to effect apoptosis rates determined by the end labeling method in the rat intestinal tract (38). The accuracy of apoptosis detection was enhanced by using semithin plastic resin sections with toluidine blue staining rather than using H&E sections (39). Cells undergoing apoptosis were estimated by counting numbers of apoptotic cell bodies/500 cells. Apoptotic bodies were identified as small, round

cells with dense nuclear staining surrounded by a distinct clear cytoplasm. Cells in the entire antral gland unit were counted from superficial epithelium to the base of glands in regions where glands were oriented perpendicular to the submucosa.

#### Data analysis

Three independent experimental trials were performed with 2–6 rats/treatment group in each trial. Data are presented as mean ± SEM (N = number of rats/treatment group) and were analyzed by one-way ANOVA with posthoc *t*-tests for significant differences (*P* < 0.05).

## Results

#### Morphological alterations in antrum and duodenum induced by MNNG

Data in Fig. 1 show that MNNG induced striking alterations in antral and duodenal gland morphology. In the antrum, MNNG induced mucosal hyperplasia and surface epithelial dysplasia (Fig. 1B). Nuclear alterations were observed in surface cells following MNNG. Surface epithelial cells in the proximal glandular region (gastric fundus) did not show hyperplasia or dysplasia (not shown). Staining for mucin, determined by PAS histochemistry, was reduced throughout the antral glands particularly in superficial and neck mucous cells. Grade of antral dysplasia was significantly increased compared with control (Table 1) and dysplastic foci or adenocarcinoma were detected in two animals. In contrast, MNNG induced duodenal villus shortening and widening and mucosal hyperplasia and epithelial dysplasia (Fig. 1, D–F). Systemic 17β-estradiol treatment modulated glandular alterations induced by MNNG in both antrum and duodenum. Mucin staining was increased to control levels throughout the antral glands following 17β-estradiol treatment (Fig. 1C). As well, antral dysplastic foci or adenocarcinomas were not observed and grade of dysplasia was improved by 17β-estradiol treatment (Table 1). Duodenal gland heights were increased in 17β-estradiol treated rats (Fig. 1F). These data show that MNNG induced differential patterns of changes in antral and duodenal glands and that these alterations could be modulated by 17β-estradiol administration.

Antral and duodenal gland heights were quantified using a calibrated image analysis system. MNNG treatment resulted in significantly increased antral mucosal gland heights, whereas duodenal gland heights were decreased (Fig. 2). These effects represented an increase of 164% in antral gland height and a decrease of 47% in duodenal villous height compared with control rats. Duodenal crypt height was not changed by MNNG treatment (Fig. 2). 17β-Estradiol administration significantly reversed MNNG-induced alterations in duodenal total gland and villous heights. Increases in antral gland heights induced by MNNG were slightly decreased by 17β-estradiol cotreatment (*P* = 0.068) (Fig. 2). Thus, 17β-estradiol treatment primarily had a protective effect on duodenal gland height in this model.

#### Antral gastrin protein levels

Neuroendocrine gastrin cells were studied to further analyze effects of MNNG on cell differentiation in the antral mucosa. Similar numbers of gastrin cells were found in all groups (Fig. 3). The effect of MNNG on intracellular gastrin protein levels was quantified using immunogold analysis.

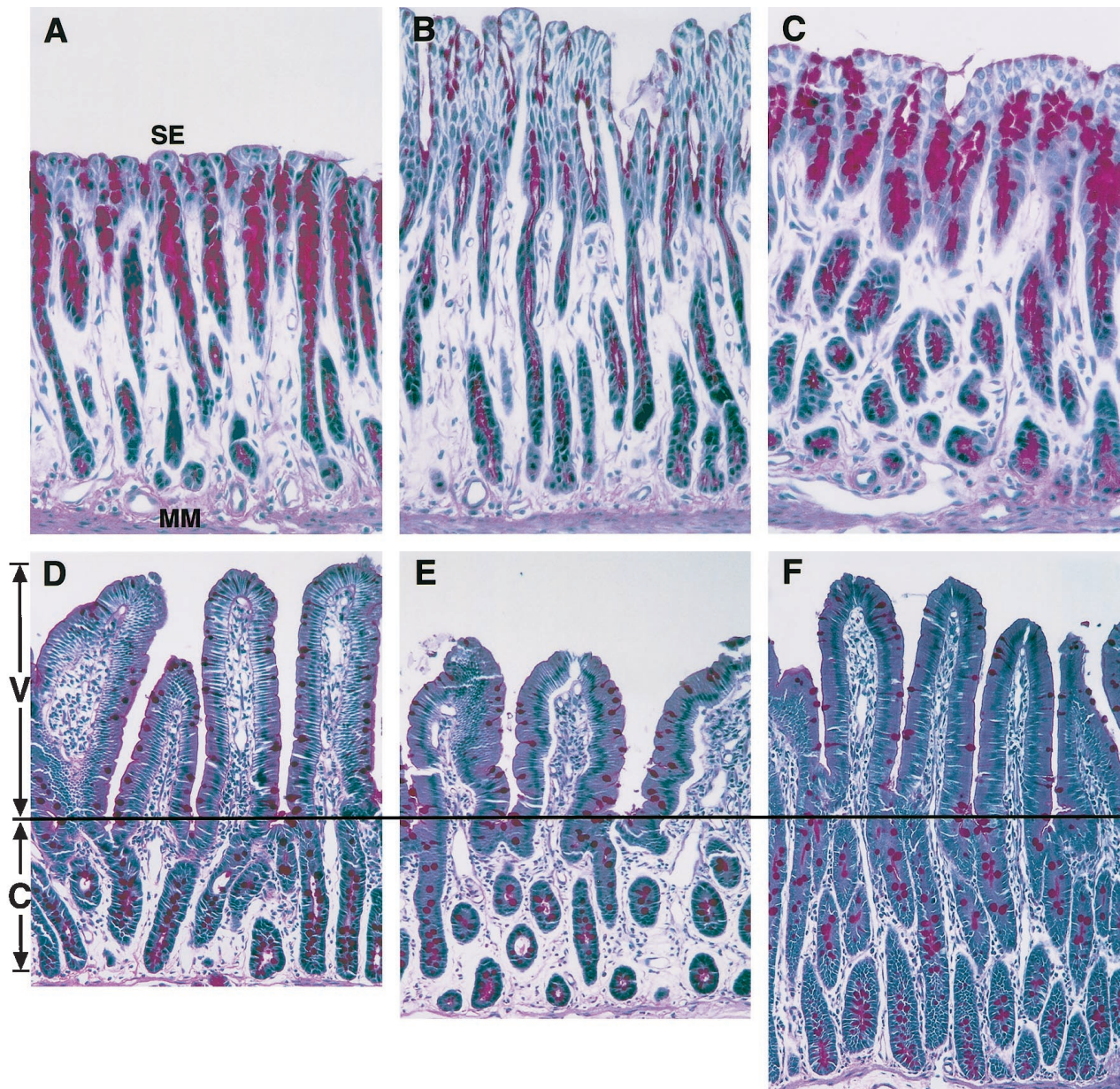


FIG. 1. Alterations in antral (A–C) and duodenal (D–F) gland morphology induced by MNNG. Tissue sections were stained with hematoxylin and periodic acid-Schiff (PAS). Rats were untreated (A, D), MNNG (B, E) or MNNG and  $17\beta$ -estradiol implants (C, F) as described in *Materials and Methods*. For antral sections, sections are aligned at the muscularis mucosa (MM) as shown in a control panel (A). Antral mucosa in MNNG-treated rats (B) is thicker with hyperplasia and dysplasia of the superficial epithelium (SE). MNNG treatment resulted in reduced mucin staining (pink) throughout the glands. MNNG and  $17\beta$ -estradiol treatment (C) reduced superficial dysplasia and increased mucin staining throughout the antral glands. In panels D–F, duodenal glands consist of upper villous (V) and lower crypt (C) regions as shown on the left of a control panel (D). Sections are aligned across villous crypt junctions of representative sections. Villi in the MNNG group (E) are blunted and widened. MNNG and  $17\beta$ -estradiol-treated rats (F) had increased villous heights. Original magnifications: antrum,  $80\times$ ; duodenum,  $62.5\times$ .

Immunoreactivity for gastrin was found in the small, electron dense storage granules, over intracellular membranes and occasionally within the nucleus (Fig. 4).  $17\beta$ -estradiol treatment significantly increased gastrin levels in storage granules compared with MNNG treatment alone (Fig. 4).

Control conditions for the immunogold analysis using replacement of the primary antibody with normal rabbit serum or PBS showed occasional nuclear labeling in various cell types (not shown). Gastrin antisera was specific for gas-

trin producing cells as other cell types including neuroendocrine somatostatin and serotonin cells and their respective storage granules were not immunoreactive (not shown).

#### *Cell proliferation and apoptosis rates*

We next used BrdU immunohistochemistry to evaluate the effects of MNNG on antral and duodenal crypt cells undergoing DNA synthesis in the S phase of the cell cycle. In antral

**TABLE 1.** Morphological preneoplastic changes in antral glands in control rats and rats receiving MNNG and oil vehicle (MNNG) or 17 $\beta$ -estradiol implants (MNNG+E<sub>2</sub>)

Treatment	N	Dysplasia Grade	Dysplastic foci (N)	Carcinoma (N)
Control	9	0.2 $\pm$ 0.2	0	0
MNNG	9	1.83 $\pm$ 0.4 <sup>a</sup>	1	1
MNNG+E <sub>2</sub>	8	1.25 $\pm$ 0.3	0	0

Data are mean  $\pm$  SEM.<sup>a</sup>  $P < 0.05$  vs. Control.

glands following MNNG administration, the number of proliferating cells was increased approximately 2.5-fold and their position within the gland shifted from the base upward toward the superficial epithelium (Fig. 5). Proliferating cells were detected in the superficial epithelium of an antral adenocarcinoma that developed in an MNNG-treated rat (Fig. 5). Quantitation of the antral labeling index showed that pretreatment with 17 $\beta$ -estradiol did not decrease antral cell proliferation induced by MNNG (Fig. 5). Similarly in the duodenum, MNNG-induced increases in the labeling index (1.6-fold) were not significantly altered by 17 $\beta$ -estradiol cotreatment (Fig. 5).

Rates of apoptosis were estimated using morphological criteria for apoptotic bodies on plastic sections by light microscopy. Numbers of apoptotic bodies were significantly increased following MNNG treatment (Fig. 6). 17 $\beta$ -estradiol cotreatment did not alter this endpoint.

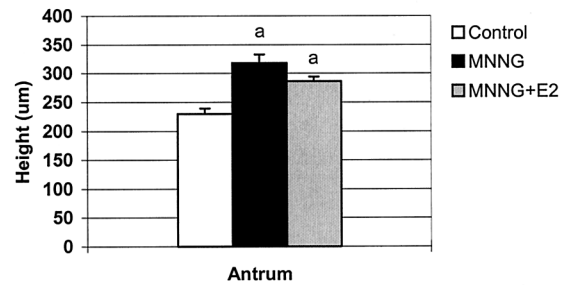
#### General animal health in the MNNG model

Rats given MNNG appeared to be in good health but had decreased weight gain (Table 2). Food intake tended to be lower in the MNNG-treated groups particularly in the rats with 17 $\beta$ -estradiol implants (Table 2). Testicular weights, when normalized to body weight, were not altered by 17 $\beta$ -estradiol treatment and serum testosterone levels were not significantly different between groups (Table 2). Serum estrogen levels measured 18 days following placement of 17 $\beta$ -estradiol implants were similar to those in normal cycling female Sprague Dawley rats from our lab (12.3  $\pm$  3 vs. 12.0  $\pm$  3 pg/ml, respectively,  $n = 6$ /group). These serum levels are similar to other published levels for physiological ranges of estradiol fluctuations during normal estrus cycles (40). The control and MNNG males had serum estrogen concentrations of 7.7  $\pm$  2 and 6.2  $\pm$  1 pg/ml, respectively ( $n = 5$ /group). These values are similar to previously reported serum estrogen levels in male rats (41).

#### Discussion

The majority of experimental studies on the development of human gastric adenocarcinoma have been performed in the rat. Various therapeutic agents enhance or suppress the carcinogenic activity of MNNG (42–46). The present study examined whether systemic estrogens could modify preneoplastic alterations induced by MNNG. The MNNG model was chosen because gender differences, histopathological characteristics and distribution patterns mimic those observed in human upper GI adenocarcinomas (11, 16). An earlier report showed a slight decrease in tumor incidence in

A.



B.

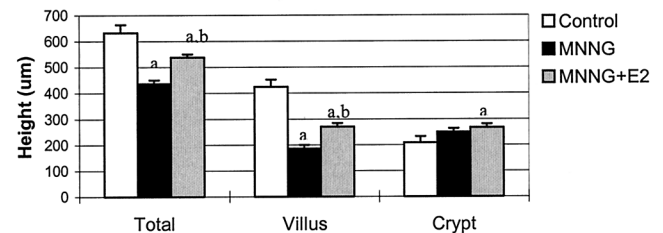


FIG. 2. 17 $\beta$ -estradiol treatment modulates alterations in mucosal gland heights induced by MNNG. Antral (A) gland and duodenal (B) total gland, villous and crypt heights were measured on formalin embedded sections using a calibrated image analysis system. 17 $\beta$ -estradiol significantly increased duodenal total gland and villous heights during MNNG administration (MNNG + E<sub>2</sub>), whereas antral gland heights tended to decrease compared with MNNG. Duodenal crypt heights were significantly increased following MNNG + E<sub>2</sub> compared with controls. Mean  $\pm$  SEM,  $n = 8$ –9, <sup>a</sup>,  $P < 0.05$  from control; <sup>b</sup>,  $P < 0.05$  from MNNG (ANOVA).

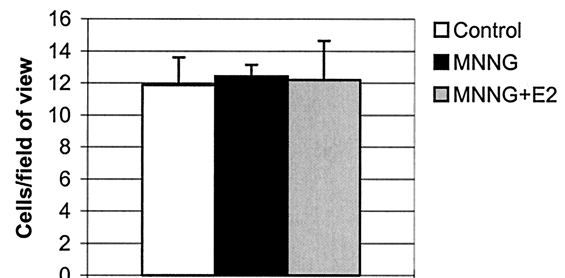


FIG. 3. Gastrin cell numbers were not altered by MNNG treatment. Gastrin cells were stained by immunohistochemistry and numbers of positive cells per field of view were counted as described in *Materials and Methods*. Mean  $\pm$  SEM,  $n = 4$ –6.

Wistar male rats receiving 17 $\beta$ -estradiol injections when started 2 months after MNNG treatment (23). In our study, exposure to 17 $\beta$ -estradiol began 1 week before and continued throughout MNNG exposure for 1 month.

The present study confirms earlier reports that preneoplastic proliferative lesions are observed as early as 4 weeks following MNNG treatment and that these early changes correlate with tumor development (14, 45). A greater susceptibility to the carcinogenic effects of MNNG has been reported for the upper small intestine (duodenum) compared with the glandular stomach in rats with preneoplastic alterations consisting of villus blunting and crypt elongation as shown in this study (15, 47). We present new data showing

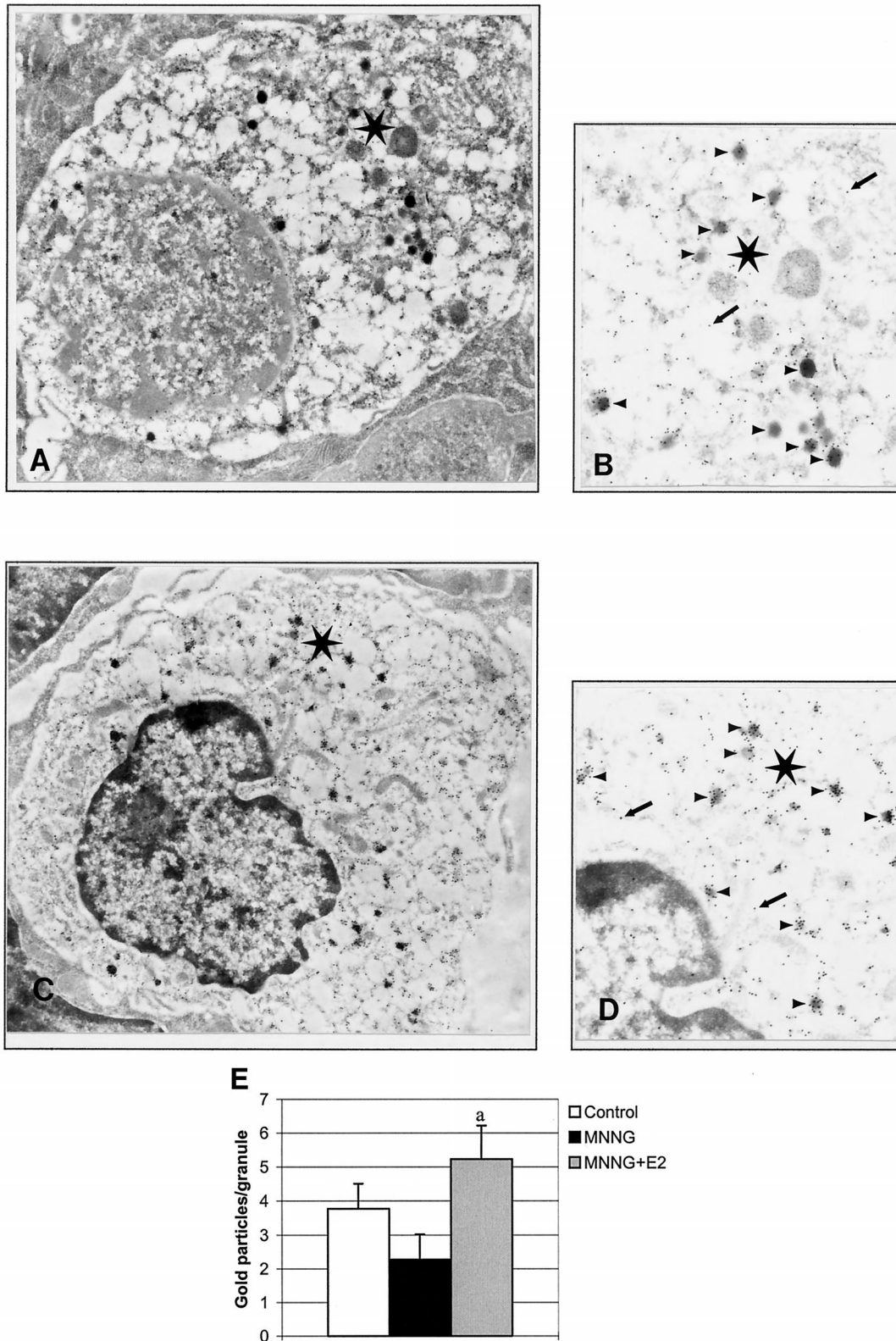


FIG. 4.  $17\beta$ -estradiol treatment increased gastrin immunoreactivity during MNNG treatment. Gastrin protein levels in storage granules were estimated by immunogold analysis as described in *Materials and Methods*. Representative photomicrographs of gastrin cells from MNNG (A, B) and MNNG+E<sub>2</sub> (C, D) treated rats (original magnification  $\sim 16,000\times$ ). Regions (*stars*) within each cell have been enlarged and prints developed with less contrast to emphasize localization of gold particles (*arrows*) within storage granules (*arrowheads*). E, Numbers of gold particles/storage granule were significantly increased by MNNG and  $17\beta$ -estradiol treatment. Mean  $\pm$  SEM, <sup>a</sup>,  $P < 0.05$  from MNNG,  $n = 4-6$ .

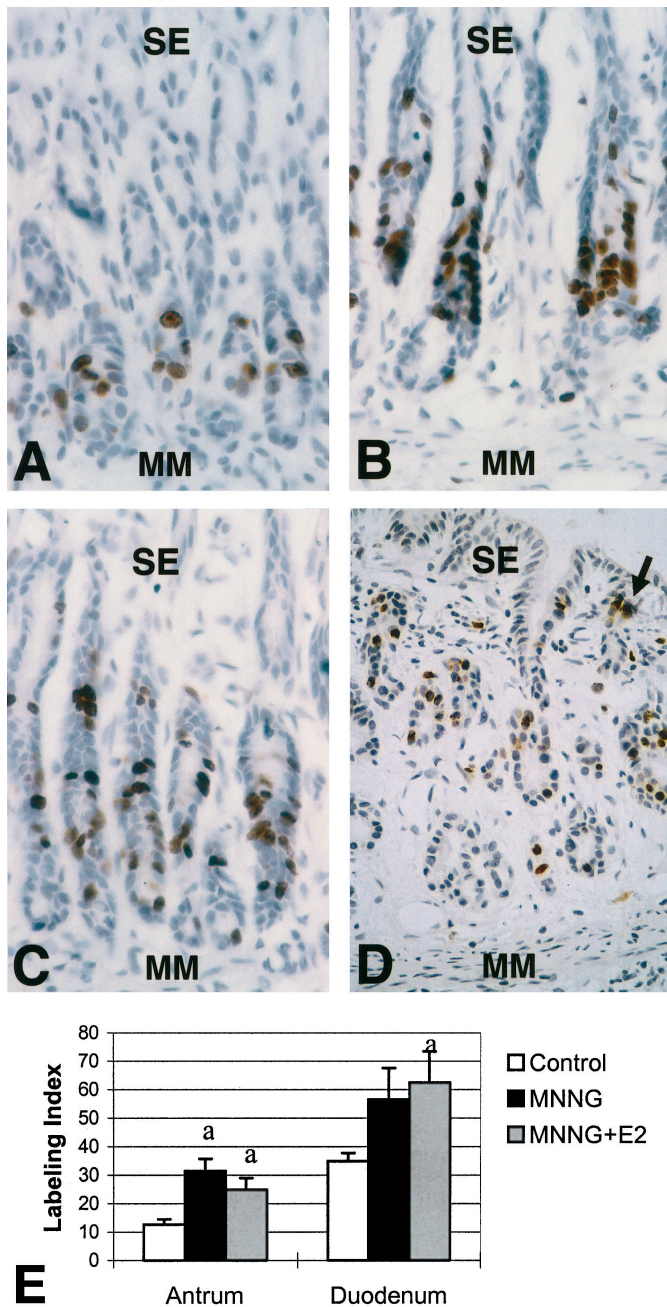


FIG. 5. Distribution and numbers of S phase cells in antral and duodenal glands following MNNG. Antral stem cells in S phase of the cell cycle were detected by incorporation of bromodeoxyuridine followed by immunohistochemistry. Sections are aligned at the muscularis mucosa (MM). In control rats (A), few positive nuclei were detected in the base of antral glands. MNNG treatment (B) increased numbers of proliferating cells with an upward shift of the proliferating zone toward the superficial epithelium (SE). 17 $\beta$ -estradiol co-treatment with MNNG (C) did not significantly alter position of proliferating cells. D, Section of an antral adenocarcinoma from a male rat treated with MNNG demonstrating neoplastic glands and extension of proliferating cells to the surface epithelium (arrow). Original magnifications: A–C, 80 $\times$ ; D, 40 $\times$ . E, BrdU-positive nuclei were counted in antral glands and duodenal crypts as described in *Materials and Methods*. MNNG and MNNG + E<sub>2</sub> treatments significantly increased numbers of proliferating cells. Data are expressed as number/field of view (labeling index) (mean  $\pm$  SEM for n = 4–6; <sup>a</sup> P < 0.05 from control).

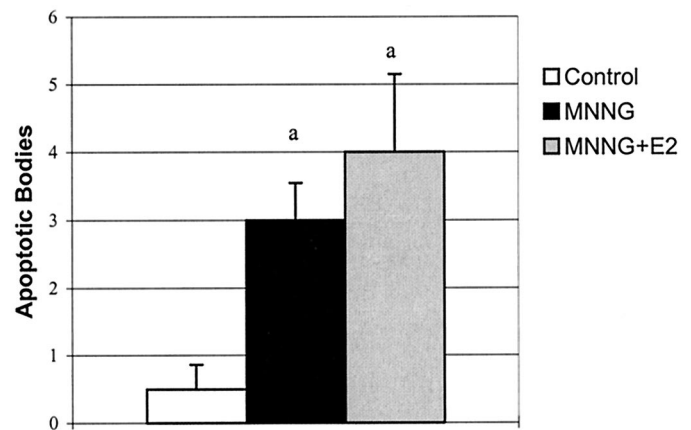


FIG. 6. Numbers of apoptotic bodies in antral glands following MNNG treatment. Apoptotic cell bodies were counted in antral glands from resin embedded sections as described in *Materials and Methods*. Rates of apoptosis were increased by MNNG and MNNG+E<sub>2</sub> treatments. Data are expressed as number/500 cells (mean  $\pm$  SEM, n = 3–5, <sup>a</sup> P < 0.05 from control).

that the upper intestine is a particularly sensitive site for estrogenic protection. These data are compelling in light of the probable protective effect of estrogen replacement therapy on reducing large intestinal, *i.e.* colon, cancer risk in postmenopausal women (reviewed in Ref. 5). Altered mucus secretion is also a premalignant alteration observed following MNNG treatment (48, 49). This study shows that mucin levels were similar to control conditions in rats receiving MNNG and 17 $\beta$ -estradiol treatments.

MNNG treatment resulted in antral cell hyperplasia with displacement of stem cells toward the superficial epithelium. These changes are considered obligatory preneoplastic lesions that increase the potential for direct stem cell exposure to MNNG (49). One potential mechanism of action of estrogens on mucosal cells could involve modulation of stem cells leading to reductions in cell proliferation. The present study shows a significant increase in number of cells undergoing DNA synthesis in antral glands and duodenal crypts following MNNG treatment. These alterations in labeling indices paralleled changes in antral gland and duodenal crypt heights. 17 $\beta$ -estradiol administration did not alter proliferation rates under these conditions. A compensatory mechanism for excessive cell proliferation is an increased rate of programmed cell death or apoptosis (50). Apoptosis rates were increased following MNNG treatment but were not altered by 17 $\beta$ -estradiol treatment. More sensitive detection methods, such as ultrastructural analysis by electron microscopy, may be required to detect significant differences between treatment groups, if they exist, as basal rates of apoptosis in the antrum are very low (37, 50). Apoptosis rates in human gastric carcinomas are closely associated with cell proliferation rates and apparently do not have biological significance as a prognostic factor (51).

Gastrin is a well-characterized hormonal regulator of proximal gastric and duodenal cell growth and mucosal barrier production (52, 53). Gastrin is synthesized as a prohormone, and following processing in the Golgi apparatus, is concentrated in small electron dense storage granules that contain several processing enzymes (54). This study reports



**TABLE 2.** Physical parameters (body weight, testicular weight, food intake) and serum testosterone levels in control rats and rats receiving MNNG and oil vehicle (MNNG) or 17 $\beta$ -estradiol implants (MNNG+E<sub>2</sub>)

Treatment	Body weight (g)	Testicular weight (g)	Testicular weight (% bw)	Total food intake (g)	Testosterone (pg/ml)
Control	354 $\pm$ 7	3.58 $\pm$ 0.08	1.01 $\pm$ 0.03	788 $\pm$ 14	1041 $\pm$ 295
MNNG	330 $\pm$ 9 <sup>a</sup>	3.73 $\pm$ 0.08	1.14 $\pm$ 0.03 <sup>a,c</sup>	751 $\pm$ 20	1591 $\pm$ 145
MNNG+E <sub>2</sub>	294 $\pm$ 9 <sup>a,b</sup>	3.11 $\pm$ 0.07 <sup>a,b</sup>	1.06 $\pm$ 0.02	728 $\pm$ 19	1122 $\pm$ 284

Data are mean  $\pm$  SEM (N = 9–12 except for serum levels, N = 4–5). Food intake was determined each week for cages containing three rats with an average food intake/rat estimated from three independent experiments. Total food intake was the sum of the six weekly averages.

<sup>a</sup> P < 0.05 vs. Control; <sup>b</sup> P < 0.05 vs. MNNG; <sup>c</sup> P < 0.05 vs. MNNG+E<sub>2</sub>.

for the first time that gastrin protein levels in storage granules are significantly increased following MNNG and 17 $\beta$ -estradiol treatments. The differences in intragranular gastrin protein levels are not expected to yield significant alterations in serum gastrin levels as gastrin cell numbers and messenger RNA steady-state levels (data not shown) are not different between groups. The effects of sex steroid hormones on serum gastrin levels have been studied. In Sprague Dawley rats and guinea pigs, males have higher levels of serum gastrin levels than females and serum levels correlate with antral gastrin concentrations or gastrin cell numbers (55–57). However, 17 $\beta$ -estradiol treatment in either castrated or ovariectomized rats does not significantly lower serum gastrin levels implying that estrogens alone may not have a role in gender differences (55, 56). In contrast, 17 $\beta$ -estradiol administration in ovariectomized mice results in significant increases in serum gastrin levels (58).

The effects of systemic estrogens on differentiation of mucous and gastrin cells could result from improved mucosal barrier function as both cell types have apical membranes raising the potential for direct exposure to luminal MNNG. This proposal is supported by studies showing protective roles of estrogens in acute rat gastric injury models (59). Interestingly, estrogens were prescribed at one time for duodenal ulcers in humans (60). Mechanisms for improved mucosal barrier function could result from alterations in MNNG metabolism or luminal pH. Gender differences in glutathione levels and antioxidant enzymes are known for different regions of the rat GI tract (61). Glutathione levels are significantly higher in gastric and duodenal mucosa of female Sprague Dawley rats compared with males (61). However, higher glutathione levels would probably increase MNNG susceptibility (18, 19, 62). Glutathione peroxidase activity is also significantly higher in gastric mucosa of females compared with males (61). 17 $\beta$ -estradiol treatment decreases gastric acid secretion in ovariectomized rats however MNNG is more efficiently absorbed at higher pH levels (63, 64). Taken together, these data suggest a mechanism distinct from known effects of estrogens in the upper GI tract for mediating protective effects during MNNG carcinogenesis.

Our data confirm decreased body weight gain following MNNG treatment (14) and further show that decreased body weight gain was associated with small reductions in food intake. Reduction of appetite by estrogens has been previously reported (65). As expected, rats treated with both 17 $\beta$ -estradiol and MNNG had decreased weight gain and food intake, with testicular weights reduced in parallel with body

weights. These changes occurred with serum testosterone levels remaining similar between treatment groups.

Despite the severity of lesions induced by MNNG, systemic estrogen treatment in male rats resulted in significant protective effects. Direct effects in mucosal cells, secondary effects mediated by other agents, or both, could mediate protective effects. The potential for a direct effect is suggested by studies showing that ER, particularly the recently identified ER $\beta$  subtype, are expressed in rat antral and duodenal epithelium (29, 66). In another study using male rats, expression of antral ER $\alpha$ , determined by RIA, remained constant while androgen and epidermal growth factor receptors declined throughout the course of MNNG treatment (67). ER expression in GI epithelial cells suggests that direct hormonal manipulation of cell function is possible. Further studies in conjunction with *in vitro* cell culture models and ER gene knock-out animal models (68), particularly those with deletions for ER $\beta$ , are needed to further test this proposal. Such studies would have relevance for understanding potential mechanisms by which female hormones reduce GI cancer risk.

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