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Original Research

Sex-Associated Difference in Estrogen Receptor β Expression in N-Methyl-N′-Nitro-N-Nitrosoguanidine-Induced Gastric Cancers in Rats

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Epidemiologic studies indicate that the incidence of gastric cancer is higher in males than in females. Although the mechanisms mediating this difference are unclear, a role for estrogens has been proposed. We used Western blotting to evaluate the role of estrogen receptor (ER) subtypes ERα and ERβ and proliferating cell nuclear antigen (PCNA) in *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in Wistar rats; ERα and ERβ mRNA levels also were analyzed by quantitative real-time RT-PCR analysis. The incidence of gastric cancer was significantly higher in male than female rats. In both sexes, ERα expression was similar in MNNG-treated cancerous and noncancerous tissues and normal gastric tissue. However, ERβ expression in MNNG-treated cancerous and noncancerous tissues was significantly lower in male rats and higher in female rats than that in normal gastric tissue; MNNG-induced cancerous tissue showed the highest ERβ expression. PCNA expression in MNNG-treated cancerous tissues was higher than that in noncancerous tissues, and was higher in male rats than female rats. Western blotting results were consistent with the mRNA changes determined by quantitative real-time RT-PCR. The present study provides evidence of a sex-associated difference in ERβ and PCNA expression in MNNG-induced gastric cancers in Wistar rats.

Abbreviations: MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; ER, estrogen receptor; PCNA, proliferating cell nuclear antigen.

Globally, gastric cancer is the second most common cause of cancer death (after lung cancer), causing more than 700,000 human deaths annually.[7] Although the incidence of gastric cancer shows a male:female ratio of about 2:1, the increased incidence in males cannot be attributed entirely to differences between the sexes in the prevalence of known risk factors.[7] Epidemiologic and experimental studies have provided some support in favor of the hypothesis that the male predominance in the incidence of gastric cancer is due to sex hormones, mainly through a decreased risk in women, who are highly exposed to estrogen.[25] Moreover, studies have demonstrated that treatment with tamoxifen, an antiestrogen, might increase the incidence of gastric cancer in women and that men who have been treated with estrogen have a decreased risk.[8,9] However, investigations of potential mediators for this sex-associated difference have been limited.[7,8,25]

Estrogen receptor (ER) signaling pathways regulate important physiologic processes, such as cell growth and differentiation, and at least 2 ER subtypes, ERα and ERβ, mediate the genomic actions of estrogens.[2,11,15,17,21,31,37] Although the stomach has not been identified as a direct target organ of sex hormones, ER have been reported in the human and rodent gastric mucosa.[3,4,6,11] However, the biologic significance of the ER in gastric cancer remains inconclusive.[7,9,29,40,44,45]

Administration of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in the drinking water induces high incidences of gastric cancer in rats, hamsters, and dogs.[14,28,34] MNNG-induced gastric carcinogenesis occurs more frequently in male than female Wistar rats.[14] Moreover, the incidence of MNNG-induced rat gastric cancer is higher in ovariecotomized female rats, and the administration of female sex hormones to male rats or castrated male rats decreases its incidence.[4,14,15,41] Proliferating cell nuclear antigen (PCNA) has been useful for the identification of cells in the proliferative phase and can be a potential prognostic marker in gastric carcinoma malignancy.[33]In the present study, we investigated protein and mRNA levels of ERα and ERβ and protein levels of PCNA in rat MNNG-induced gastric cancers to better understand the sex-associated difference in gastric carcinogenesis.

Materials and Methods

Animals, chemicals and treatments. Male (n = 36) and female (n = 36) Wistar rats (age, 5 wk; Japan Slc, Hamamatsu, Japan) were housed at 3 rats per 20 × 45 × 25-cm clear polypropylene cage with wire tops on heat-sterilized hardwood-chip bedding in an

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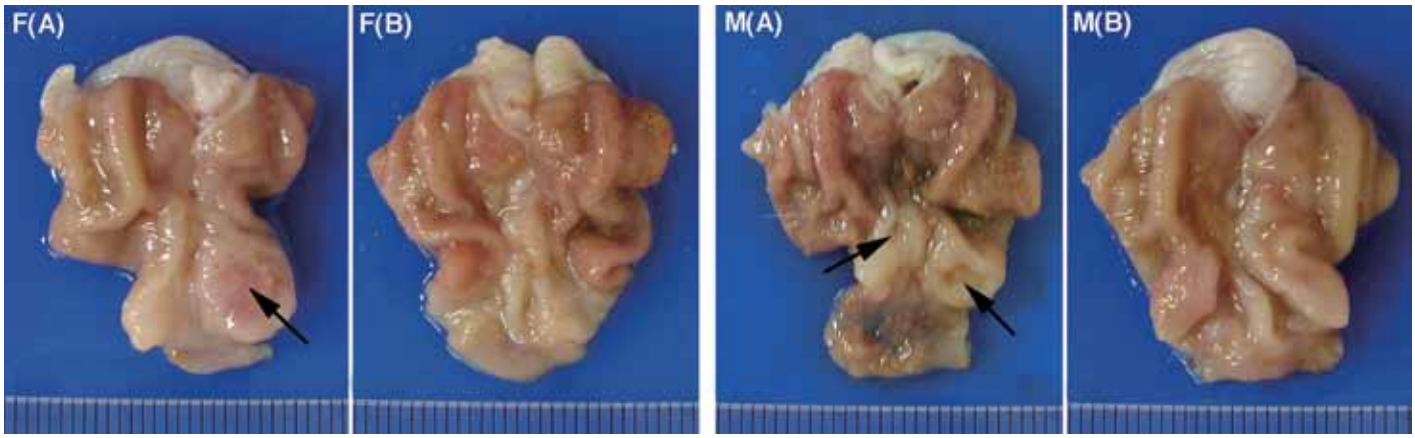

Figure 1. Representative images of fresh rat stomachs opened along the greater curvature without being pinned to a flat board. Gastric carcinoma (arrows) is seen at the pyloric region. Ruler lines indicate 1 mm. F(A), female rat of MNNG-treated group; F(B), female rat of vehicle-treated group; M(A), male rat of MNNG-treated group; M(B), male rat of vehicle-treated group.

Table 1. Gastric tumor variables in male and female rats exposed to MNNG

	Male	Female
No. of rats	24	24
Body weight (g)	379.3 ± 35.3	330.2 ± 25.2
Tumor incidence (%)	18 (75%)[a]	9 (38%)
No. of tumors per rat	2.35 ± 0.63[a]	1.75 ± 0.32
Mean tumor size (mm^2)	12.34 ± 1.76	10.77 ± 1.88

Data are given as mean ± SEM.
For comparison, vehicle-control male rats (n = 12) weighed 355.5 ± 34.8 g whereas female rats (n = 12) weighed 328.8 ± 22.1 g.
[a]Significantly ($P < 0.05$ by Student t test) different from value for female rats.

environment-controlled room on a 12:12-h light:dark cycle at 22 ± 2 °C and 55% ± 5% relative humidity and were fed a conventional diet (MF, Oriental Yeast, Tokyo, Japan). All experimental procedures were conducted following approval of the Animal Care and Use Committee of the Azabu University School of Veterinary Medicine. Guidelines set by NIH and the Public Health Service Policy on the Humane Use and Care of Laboratory Animals were followed at all times.[19] MNNG was obtained from Aldrich Chemical Industry (Tokyo, Japan). Seven-week-old male (n = 24) and female (n = 24) rats were given 100 ppm MNNG in their drinking water for 24 wk and then were supplied with normal tap water for 30 wk. Seven-week-old male (n = 12) and female (n = 12) rats were given normal tap water for 54 wk as a vehicle-treated control group. All rats survived to each endpoint without any abnormal clinical signs. The dose of MNNG was based on those used in previous studies.[14,28,42] After drug treatment, rats were anesthetized and euthanized by CO_2 overdose, the stomach was removed from the rats, opened along the greater curvature, and pinned flat on a corkboard. Serial step sections 2 to 3 mm thick taken from along the lesser curvature were frozen without fixation and stored at –80 °C. In addition, corresponding serial sections were fixed with 10% neutral formalin, dehydrated, embedded in paraffin, and sectioned at 4 μm. The sections were stained with hematoxylin and eosin.

Antibodies for ER. To detect ERα, we used primary 6F11 antiserum (Novocastra Laboratories, Newcastle, UK), a mouse antiER antibody that has no affinity for ERβ.[24] To detect ERβ, we used primary PA1-310 antiserum (Affinity BioReagents, Golden, CO), a rabbit antiER antibody that was generated against amino acids in the C-terminal region of rat ERβ and has only 6% amino acid homology with rat ERα, corresponding to amino acids 467 to 485 of ERβ. These antibodies were affinity-purified by column chromatography, and their immunoreactivities were confirmed previously.[5,6,24,32,42]

Western blot analysis. Rat gastric tissues were homogenized in 50 mM Tris-HCl, 150 mM KCl (pH 7.4), 1% Triton X100, and 0.25 mM PMSF and centrifuged at 8000 × g for 30 min at 4 °C. The supernatant obtained was centrifuged at 100,000 × g for 90 min at 4 °C. The pellet was suspended in 50 mM Tris-HCl (pH 7.4), 1% Triton X100, and 1 mM PMSF, and protein concentrations were determined by using a bicinchonic acid protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Microsomal samples (10 μg each) underwent electrophoresis on a 10% SDS–polyacrylamide gel. The proteins were transferred for 2 h to a nitrocellulose membrane that was blocked by immersing it in 5% nonfat dried milk in PBS with 0.1% (v/v) Tween 20. Western blot analysis was performed by using antiERα antibody (1:1000; Novocastra Laboratories), antiERβ antibody (1:1000; Affinity BioReagents), antiPCNA monoclonal antibody (1:2000; Novocastra Laboratories), and antiβ-actin antibody (1:2000; A5441, Sigma Aldrich, St Louis, MO, dilution 1:2,000) diluted in PBS with 0.1% (v/v) Tween 20 and incubated 1 h at room temperature on an orbital shaker. After being washed 3 times in PBS with 0.1% (v/v) Tween 20, they were incubated with horseradish peroxidase-conjugated antirabbit antibody (1:2500; Amersham Biosciences, Piscataway, NJ) for 1 h on an orbital shaker. After being washed 3 times in PBS with 0.1% (v/v) Tween 20, signals were detected by using chemiluminescence (ECL Plus Western Blotting Detection System, Amersham Biosciences). Protein levels were quantified by densitometric scanning (Image-Pro Plus, Media Cybernetics, San Diego, CA). For better comparison and to compensate for possible unequal loading, ER protein levels in each sample were normalized to that of the housekeeping protein β-actin.

Real-time quantitative RT-PCR analysis. For each RNA sample, 100 ng was used as the template for first-strand cDNA synthe-

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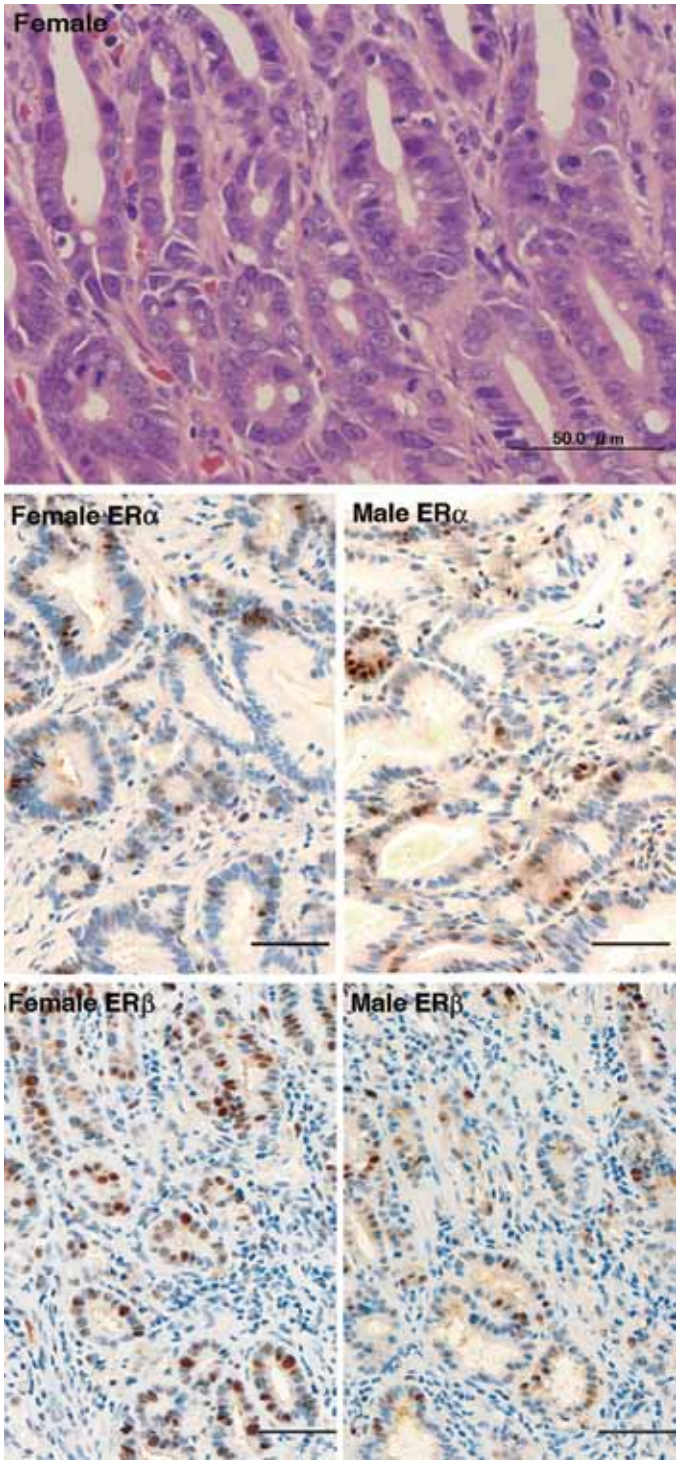

Figure 2. The top panel shows a representative image of an MNNG-induced rat gastric tumor, which is histologically a well-differentiated adenocarcinoma. Female Wistar rat. Mayer hematoxylin and eosin stain; bar, 50 µm. The 4 lower panels show representative results of ERα and ERβ immunohistochemical staining in MNNG-induced gastric carcinomas. Similar numbers of nuclei positive for ERα were found in both sexes (Female ERα, Male ERα), whereas female rats (Female ERβ) had more nuclei positive for ERβ than did male rats (Male ERβ). Avidin–biotin complex staining with Mayer hematoxylin counterstaining; bar, 50 µm.

sis by using a *Taq*Man Reverse Transcription kit (PE Applied Biosystems, Foster City, CA), according to the manufacturer's 2-step protocol. Controls included for each reaction were the RNA sample without reverse transcriptase (RNA–RT) and no RNA with reverse transcriptase (no RNA+RT). The final reaction mixes for reverse transcription included 1 × *Taq*Man RT buffer; 5.5 mM $MgCl_2$; 500 µM each dATP, dGTP, and dCTP; 1 mM dTTP; 0.25 µM random hexamers; 1.25 U/µL MuLV reverse transcriptase; and 0.4 U RNase inhibitor (PE Applied Biosystems). Quantitative analyses of target gene (ERα and ERβ) mRNA levels were performed by real-time quantitative PCR (ABI Prism 7700 Sequence Detection System, PE Applied Biosystems) with *Taq*Man chemistry and probes. The *Taq*Man probes and primers for target genes were assay-on-demand gene expression products custom synthesized by PE Applied Biosystems and were oligonucleotides with fluorescent reporter and quencher dyes attached (ERα, ID no. Rn 01430445_mL; ERβ, ID no. Rn 00688791_mL).[1] Optimal primer, probe, and cDNA concentrations were determined in a separate set of experiments to ensure that both target gene and GAPDH fragments were amplified with equal efficiency. PCR reactions were performed with first-strand cDNA (2 µL) from each sample, a Universal PCR Master Mix kit (PE Applied Biosystems), 250 nM *Taq*Man probe, 0.16 U AmpErase UNG (uracil N-glycosylase), and 900 nM forward and reverse primers of the target gene and GAPDH. Three measurements per sample were performed in each of 2 independent experiments. Results were analyzed with the ABI Sequence Detector software version 1.7 (PE Applied Biosystems). For relative quantification of target gene expression, the standard-curve method was applied. The calibrated standard curve of each target gene cDNA and GAPDH amplification plots were examined at 5 different dilutions (containing 100, 50, 25, 10, and 5 ng) of total RNA samples that were obtained from each PCR product by using a TOPO II TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The target gene's normalized value was determined by dividing the average target gene value by the average GAPDH value. The SD of the quotient was calculated from the SD of the target gene and GAPDH by using the following formula:

$$\text{CV} = (\text{SD of quotient}) \,/\, (\text{mean quotient})$$

$$\text{CV}^2 = \text{CV}_1^2 + \text{CV}_2^2$$

$$\text{CV}_1 = (\text{SD of target gene value}) \,/\, (\text{mean target gene value})$$

$$\text{CV}_2 = (\text{SD of GAPDH value}) \,/\, (\text{mean GAPDH})$$

The normalized target gene value is a unitless number that can be used to compare the relative amount of the target genes in different samples. One way to make this comparison is to designate one of the samples as a calibrator. In this study, the gastric tissue of the vehicle-treated control group was designated as the calibrator, and the average target gene value was divided by the average calibrator value according to the manufacturer's instructions for quantification of relative gene expression.[1]

Statistical analysis. For each set, the mean, SD, and SEM were calculated and compared through Student *t*, Scheffé *F*, and χ^2 tests by using Stat View J 5.0 (Abacus Concepts, Cary, NC). A *P* value of less than 0.05 was regarded as statistically significant.

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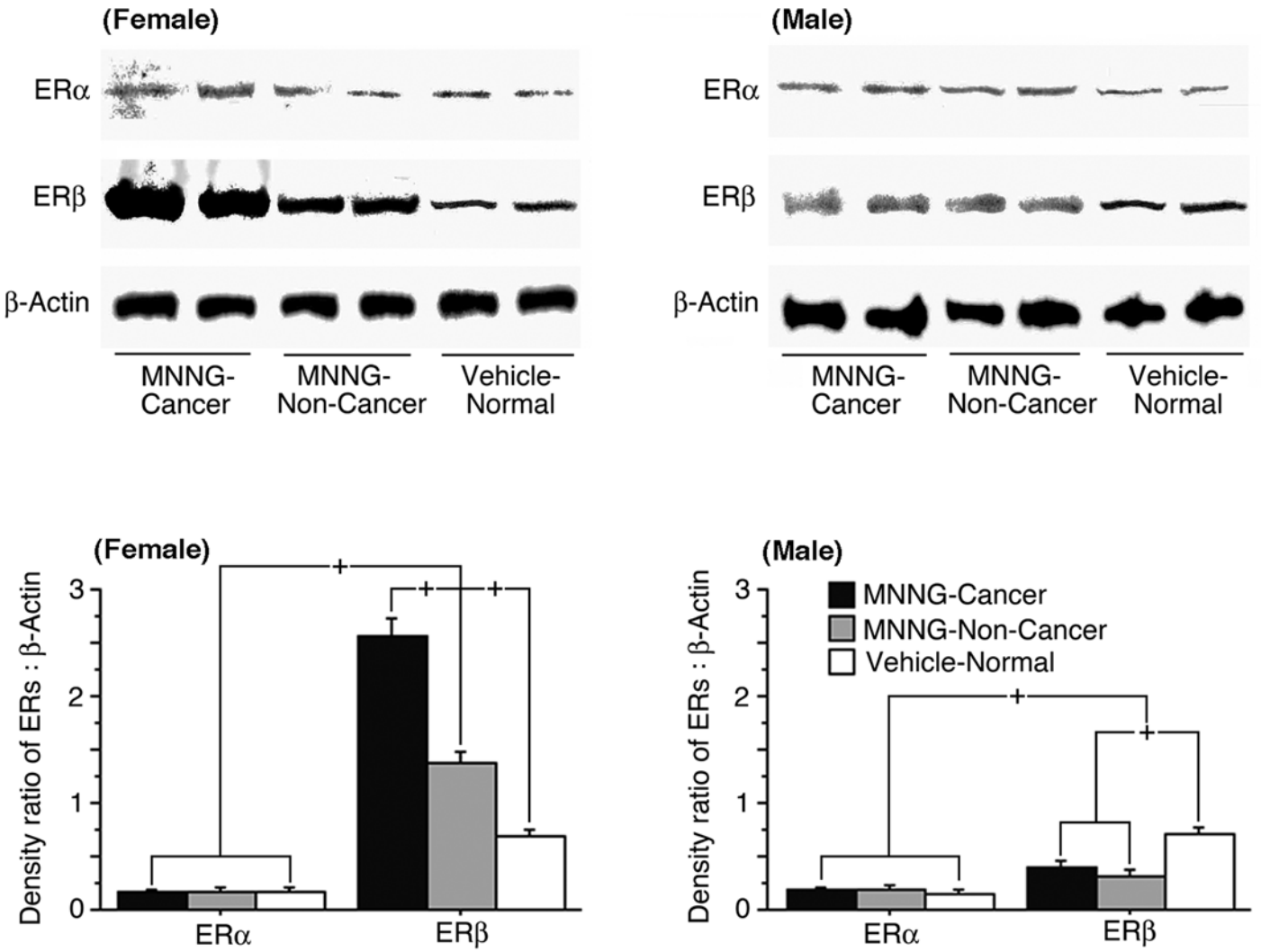

Figure 3. Representative Western blots of ERα and ERβ in cancerous (MNNG-Cancer) and noncancerous (MNNG-Non-Cancer) tissues from rats treated with MNNG and normal gastric tissue (Vehicle-Normal) from vehicle-treated rats; results from 2 rats of each group are shown. The protein concentration was determined by using a bicinchonic acid protein assay reagent kit (Pierce) with bovine serum albumin as the standard. Microsome samples (10 µg) were applied for Western blotting analysis, and immunoreactive proteins were detected by using chemiluminescence. The upper panels show representative bands of ERα and ERβ; lower panels show ERα:β-actin and ERβ:β-actin density ratios; results were obtained by screening samples from 6 rats of each group. Lower panels show that ERα expression is similar in both sexes in MNNG-treated cancerous (MNNG-Cancer) and noncancerous (MNNG-Non-Cancer) tissues and normal gastric tissue (Vehicle-Normal). In contrast, ERβ expression in MNNG-treated cancerous (MNNG-Cancer) and noncancerous (MNNG-Non-Cancer) tissues was significantly (+, $P < 0.01$, Scheffé F test) lower in male rats and higher in female rats than that in normal gastric tissue (Vehicle-Normal). MNNG-induced cancerous tissue (MNNG-Cancer) from female rats showed the highest ERβ expression. Data presented as mean ± 1 SD.

Results

Body weight, cancer incidence and morphology. The incidences, numbers, and sizes of the gastric cancers in each group are summarized in Table 1. The mean body weight of rats at autopsy did not differ between the MNNG- and vehicle-treated groups of either sex. The incidence and absolute counts of MNNG-induced gastric cancers were significantly ($P < 0.05$) higher in male than female rats. All tumors were found in the lesser curvature of the pyloric region (Figure 1). No gastric tumors were found in vehicle-treated rats of either sex. At necropsy, no nodules or tumor masses were found in any tissue except stomach. All cancers in all rats were histologically diagnosed as well-differentiated adenocarcinoma (Figure 2).

Western blot analysis. In the vehicle-treated group, the relative protein level of ERα was lower ($P < 0.01$) than that of ERβ, with no significant difference between sexes (Figure 3). In both sexes, relative levels of ERα protein were similar in MNNG-treated cancerous and noncancerous tissues and vehicle-treated normal gastric tissues (Figure 3). In female rats, the relative level of ERβ protein in MNNG-induced cancerous tissues was higher ($P < 0.01$) than that of noncancerous tissues, and both of these were higher ($P < 0.01$) than that of vehicle-treated normal gastric tissues (Figure 3). In male rats, relative levels of ERβ protein in the MNNG-treated group were similar in cancerous and noncancerous tissues but lower ($P < 0.01$) than that of vehicle-treated normal gastric tissues (Figure 3). PCNA expression in MNNG-treated cancerous tissues

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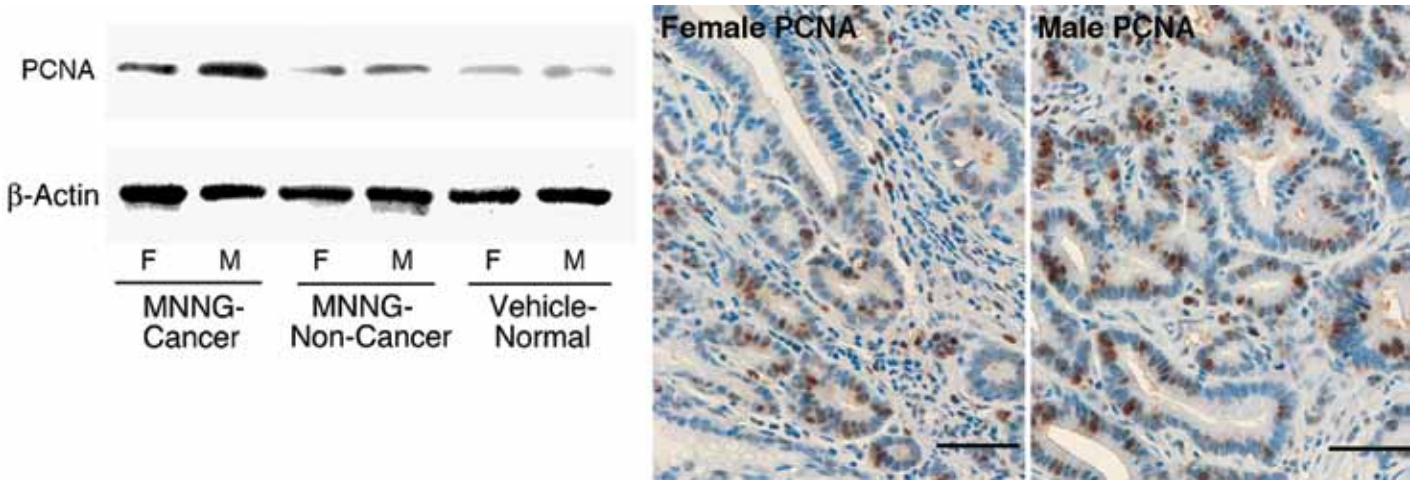

Figure 4. The left panels show representative Western blots of PCNA in cancerous (MNNG-Cancer) and noncancerous (MNNG-Non-Cancer) tissues from rats treated with MNNG and normal gastric tissue (Vehicle-Normal) from vehicle-treated rats; results from 2 rats of each group are shown. Protein concentration was determined by using a bicinchonic acid protein assay reagent kit (Pierce) with bovine serum albumin as the standard. Microsome samples (10 µg) were applied for Western blotting analysis, and immunoreactive proteins were detected by using chemiluminescence; results were obtained by screening samples from 6 rats of each group. The PCNA levels in MNNG-treated cancerous tissues (MNNG-Cancer) were higher than those in noncancerous tissues (MNNG-Non-Cancer) and were higher in male rats (M) than female rats (F). PCNA expression in vehicle-treated normal gastric tissue (Vehicle-Normal) in both sexes was lower than those in the MNNG-treated group. The right panels show representative results of PCNA immunohistochemical staining in MNNG-induced gastric carcinomas, and male rats (Male PCNA) had more PCNA-positive nuclei than did female rats (Female PCNA). Avidin–biotin complex staining with Mayer hematoxylin counterstaining; bar, 50 µm.

was higher ($P < 0.05$) than that in noncancerous tissues and higher ($P < 0.05$) in male than female rats. PCNA expression in vehicle-treated normal gastric tissue was lower ($P < 0.05$) in both sexes compared with those of the MNNG-treated group (Figure 4).

Real-time quantitative RT-PCR analysis. Quantitative RT-PCR analysis revealed that the mRNA levels of ERα and ERβ (Figure 5) were qualitatively consistent with the patterns for ERα and ERβ proteins observed by Western blotting analysis.

Discussion

The incidence of MNNG-induced gastric cancer was higher in male than female Wistar rats. Although ERα expression was similar in MNNG-treated cancerous, noncancerous, and normal gastric tissues in both sexes, ERβ expression in MNNG-treated cancerous and noncancerous tissues was lower in male rats and higher in female rats than that in normal gastric tissue, and MNNG-induced cancerous tissue showed the highest ERβ expression. PCNA expression in MNNG-treated cancerous tissues was higher than that in noncancerous tissues and higher in male than female rats.

Sex-associated differences in the effects of the 2 types of ER have been reported in several cancers. That is, ERα enhances cellular proliferation in carcinogenesis,[20,23,26,27,30] but ERβ protects against uncontrolled cellular proliferation and malignant transformation.[12,13,22,23] In addition, a general conclusion of previous studies is that higher ERα concomitant with lower ERβ seems to be related to sex-associated differences in cancer risk, such as higher incidences of colon cancer[5,12,13,22] and prostate cancer[30] in males and higher incidences of lung cancer[10,18,20,23,35,44] and breast cancer[27,36,38,44] in females. Meanwhile, although a protective effect for estrogen against gastric cancer has been proposed, statistical associations between the induction of the ER in cancer and clinicopathologic factors have been inconclusive.[7,29,39,43,44,46]

The present study revealed that the incidence of MNNG-induced gastric cancer with high cellular proliferative activity, as revealed by PCNA expression, was significantly higher in male than female Wistar rats. However ERβ expression in cancerous tissue was higher in female rats and lower in male rats than that in normal gastric tissue. Moreover, ERβ also was elevated in the noncancerous tissues of MNNG-treated groups. Furthermore, MNNG induced different ERβ responses in gastric tissues in the 2 sexes, and the ERβ induction with lower PCNA expression that occurred in female gastric cancer might be involved in preventing progression of MNNG-induced rat gastric carcinogenesis.

ERα and ERβ are transcription factors that mediate the estrogen-signaling pathway.[16] If the ER pathways are relevant to carcinogenesis, downstream target genes likely will be affected by the different ER. Heterodimerization of ERβ with ERα reportedly inhibits ERα-mediated transcription in human breast carcinoma,[16,39] and an increase in ERβ blocks ERα transcriptional activity in lung cancer.[20] Moreover, previous studies on colon, prostate, lung, and breast cancers indicate that the progression of carcinogenesis is proportional to the ERα induction,[5,9,10,13,23,31,35,36,40] but this result was not observed in the present study of MNNG-induced gastric carcinogenesis in Wistar rats. The ERα and ERβ signaling pathways in gastric cancer and other organ cancers in rats may be different or the gastric carcinogenic process might be different in animal species.

Evidence is not yet available for a sex-dependent phenotype beyond ERα and ERβ in gastric carcinogenesis. Further detailed study concerning genomic differences in the sexual dimorphism of the expression of ER in gastric cancer is required.

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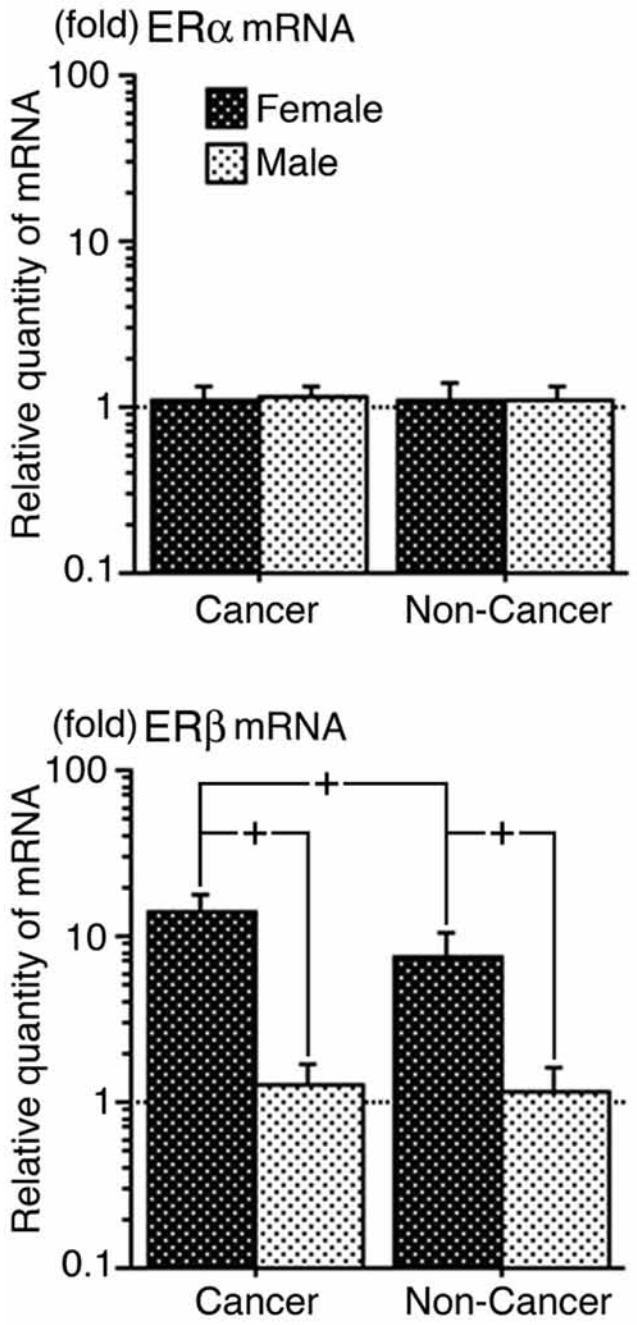

Figure 5. ERα and ERβ mRNA levels in MNNG-induced rat gastric carcinogenesis. The indicated mRNA levels were determined by real-time quantitative RT-PCR, with analysis by using the standard curve method; 1-fold of the relative quantity of mRNA of the vehicle-treated normal gastric tissues was designated as the calibrator value. Each ER mRNA value was normalized to that of the endogenous housekeeping gene GAPDH in each tissue. The results were obtained by screening samples from 9 rats of each group. ERα mRNA levels in MNNG-treated cancerous (Cancer) and noncancerous (Non-Cancer) were similar between sexes. The amount of ERβ mRNA in MNNG-treated cancerous tissue (Cancer) was significantly (+, $P < 0.01$, Scheffé F test) greater than that of noncancerous (Non-Cancer) tissues; that of female rats was greater in MNNG-treated cancerous (Cancer) than noncancerous (Non-Cancer) tissues; and that of male rats was similar between MNNG-treated cancerous (Cancer) and noncancerous (Non-Cancer) tissues. Data are presented as mean ± 1 SD.

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