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Progesterone Enhancement of Stomach Tumor Development in SD Rats Treated with *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

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The effects of chronic progesterone treatment on gastric tumorigenesis were examined in 6-week-old male SD rats. The rats were castrated, progesterone or testosterone pellets were implanted, and, starting one week after the operation, 100 mg/liter of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was administered in the drinking water for 16 weeks. Every 2 months the pellets were changed. Group 1 animals received castration plus MNNG while Groups 2 and 3 also received progesterone and testosterone, respectively. In the Group 4 case, progesterone and testosterone were administered alternately for 2-month periods and in Group 5 MNNG was given to intact animals. All survivors were killed one year after the start of MNNG treatment. In Group 1 the incidence of gastric tumors was significantly decreased as compared with the Group 5 value. The Group 2 incidence, in contrast, was similar to that in Group 5, and the size of the observed gastric tumors was massively increased. The area of the pyloric gland mucosa was also greater than in other groups. Testosterone treatment was associated with a less pronounced increase in tumor size and a recovery in incidence. The results indicate that progesterone may exert a promoting influence on gastric tumor development.

Key words: SD rat — MNNG — Gastric tumor — Progesterone — Castration

The factors responsible for human gastric tumors are varied and their respective roles remain to be established. A relationship between gastric tumorigenesis and sex hormones has been suggested, and well differentiated adenocarcinomas are more common in males than in females.[1-3] It has been reported that gastric tumors in young women are linked to toxemia during pregnancy, extrauterine pregnancy or simply pregnancy and delivery.[4-8] Moreover, gastric tumors have receptors for estrogen, testosterone and/or progesterone.[9-11] In animals treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) gastric tumor induction in males is higher than in females, but with castration the incidence is decreased.[12, 13] We earlier showed that prolactin does not promote gastric tumorigenesis.[14] However, only a few reports on the effects of sex hormones on gastric tumorigenesis have appeared in the literature. We therefore conducted the present study of the effects of progesterone and testosterone on stomach tumorigenesis in rats treated with MNNG.

MATERIALS AND METHODS

Animals Six-week-old CD (SD) rats were purchased from Charles River Japan Inc., Hino. They were housed three or four per polycarbonate cage and maintained under constant conditions of room temperature (24±2°C), relative humidity (55±10%) and 12 h light/12 h dark cycle, under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" by Hiroshima University.

MNNG MNNG (Aldrich Chemical Co., Inc., Milwaukee, WI) was dissolved in distilled water at a concentration of 100 mg/liter. Rats were given this solution in light-opaque bottles as their drinking water for 16 weeks. MNNG solution was exchanged at 2- to 3-day intervals.

Steroid hormones Rats were implanted with pellets containing either testosterone or progesterone (Sigma). These were prepared by melting the hormones with cholesterol powder by heating until fusion occurred.[15, 16] Each pellet was individually weighed and cut to an appropriate size for subcutaneous implantation under the back fat pads. Renewal of the 5 mg pellets was performed every 2 months.

Experimental groups One hundred and forty-eight male SD rats were divided at random into 5 groups. One week before the MNNG treatment, rats were castrated and implanted with progesterone (Group 2), testosterone (Group 3), testosterone followed by progesterone alternately every 2 months (Group 4), or no hormone (Group 1). Group 5 rats were given MNNG alone without castration. All animals had free access to food (MF, Oriental Yeast Co., Ltd., Tokyo) throughout the experiment. All animals were observed on a daily basis and killed when moribund or at the termination of the experiment, one year after the initial MNNG treatment.

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Examination of animals The stomach and other major organs were removed, and the liver, heart, kidneys and adrenals were weighed, fixed in 10% neutral formalin and routinely processed for histopathological studies. The stomach was cut open along the greater curvature, stretched and pinned on cardboard with the mucosal surface facing upward, washed with physiological saline for gross examination and fixed in 10% neutral formalin. Alkaline phosphatase (ALP) activity in the gastric mucosa was detected by naphthol-AS-MX-phosphate-fast blue RR staining methods.[17] The numbers of crypts with ALP-positive foci in both the pylorus and fundus were counted by using a dissecting microscope and employing a double-blind protocol.

Strips were cut perpendicularly to the mucosal surface of the stomach, two at the lesser curvature and four at the greater curvature. The strips were embedded in paraffin and serially sectioned at 3 μm. Sections were stained with hematoxylin and eosin, and where necessary for clarification, the periodic acid-Schiff reaction-alcian blue and high iron diamine-alcian blue staining procedures were introduced.

Gastric tumors in the glandular stomach were classified as: 1) atypical hyperplasia of the mucosa if proliferation of the atypical glands was observed or 2) adenocarcinoma if atypical glands proliferated and invaded all the layers of the gastric wall.[18] In the case of animals surviving at the end point, the number and type of intestinal metaplasias were also evaluated. Intestinal metaplasia was classified using the following histological criteria: type A, gastric mucosa with goblet cells; type B, intestinal type crypts without Paneth cells; and type C, intestinal metaplasia with Paneth cells.[19]

For quantitative analysis, areas of stomach mucosa without lesions and sizes of gastric tumor were examined with the aid of a color image analyzer (Model CIA 102, Olympus Co., Tokyo), allowing measurement on a color TV monitor.

Table I. Body and Organ Weights

Group	Treatment	No. of animals	Body weight (g)	Liver (g)	Liver/body weight
1	Cas+MNNG	22	572±88[a,b,c]	11.8±1.8[c,e]	19.9±5.8
2	Cas+MNNG+P	29	620±86	14.3±3.0	22.8±5.2
3	Cas+MNNG+A	29	671±90	15.1±3.7	22.8±6.5
4	Cas+MNNG+A+P	32	634±79	13.6±2.4	21.4±1.9
5	MNNG	36	659±69	18.8±2.4[c,d,e,f]	28.5+2.5[c,d,e,f]

a) Significantly different from Group 5 ($P<0.01$).
b) Significantly different from Group 3 ($P<0.05$).
c) Significantly different from Group 4 ($P<0.01$).
d) Significantly different from Group 1 ($P<0.01$).
e) Significantly different from Group 2 ($P<0.01$).
f) Significantly different from Group 3 ($P<0.01$).
P, progesterone; A, testosterone.

Table II. Tumor Incidence (%)

Group	Treatment	Effective No. of animals	No. of tumor-bearing animals[a]	Gastric tumors			Other tumors (type and number)
				ATP[b]	Adeno-carcinoma	Total	
1	Cas+MNNG	22	9 (41)	3 (10)[c]	2 (9)	5 (22)[c]	6 (duodenum 3, sarcoma 1, squamous cell carcinoma 2)
2	Cas+MNNG+P	29	16 (55)	9 (31)	3 (10)	12 (41)	5 (duodenum 5)
3	Cas+MNNG+A	29	11 (38)	6 (21)[c]	2 (7)	8 (28)	3 (sarcoma 3)
4	Cas+MNNG+A+P	32	11 (40)[d]	3 (9)[e]	5 (16)	8 (25)[c]	2 (lymphoma 1, papilloma 1)
5	MNNG	36	22 (58)	14 (39)	4 (11)	18 (50)	8 (duodenum 4, squamous cell carcinoma 1, sarcoma 2, papilloma 1)

a) Some animals had tumors in two or three different organs.
b) Atypical hyperplasia.
c) Significantly different from Group 5 ($P<0.05$).
d) Significantly different from Group 2 ($P<0.05$).
e) Significantly different from Group 5 ($P<0.01$).

Statistical evaluation The significance of differences in numerical data was evaluated using the chi-square test and Student's t test.

RESULTS

The mean body weights of the rats at the 1 year autopsy time point in Group 1 were significantly decreased as compared to those in Groups 3 to 5. Liver weights in Group 1 were significantly decreased as compared to those in Group 2 or 4, and those in Group 5 were significantly higher than in all other groups (Table I).

Atypical hyperplasias and adenocarcinomas were diagnosed in the pyloric gland region of the stomach in rats receiving MNNG. All gastric tumors were well differentiated. No tumors appeared in the fundic gland mucosa. No metastatic lesions were apparent in the surrounding lymph nodes or in other organs. The incidence of atypical hyperplasias in Group 1 was significantly decreased as compared to Group 5 and that in Group 2 was significantly increased over the Group 3 or 4 value. Incidences of adenocarcinomas were similar among the groups. Total incidences of gastric tumors in Groups 1 and 4 were significantly decreased as compared to that in Group 5 (Table II). Mean size data for gastric tumors are shown in Table III. Gastric tumors in Group 2 were very much larger than in the other groups. Testosterone treatment also appeared to increase tumor size. Non-gastric tumors were slightly more frequent in Group 5 as compared to Group 1 or 3. There was no significant difference in total numbers of different kinds of tumor per tumor-bearing animal (Table IV).

Incidences of ALP-positive intestinal metaplasia within the pyloric gland mucosa, assessed in final survivors, were found to be 9, 28, 21, 9 and 31% in Groups 1 to 5, respectively (Table V). It should be noted that the glands demonstrating intestinal metaplasia were not always located within or adjacent to neoplastic glands. The incidence of type B metaplasia was 10 to 22% and no type C lesions were found. Total numbers of metaplastic glands were few in all groups (maximum 2 crypts per stomach).

The pyloric mucosal area was significantly decreased in Group 1, and higher in Group 2 than in Group 1, 3 or 5 (Table VI). The number of crypts per screen was the same for all experimental groups (data not shown).

Table III. Mean Gastric Tumor Size

Group	Treatment	Gastric tumor (mm^2) (mean±SD)
1	Cas+MNNG	1.6±2.1
2	Cas+MNNG+P	54.5±167.3
3	Cas+MNNG+A	6.7±11.7
4	Cas+MNNG+A+P	3.6±6.6
5	MNNG	1.6±2.2

Table IV. Number of Tumors per Tumor-bearing Rat

Group	Treatment	No. of tumors		
		0	1	2 or more
1	Cas+MNNG	13 (59)	9 (41)	0
2	Cas+MNNG+P	13 (45)	15 (52)	1 (3)
3	Cas+MNNG+A	18 (62)	10 (34)	1 (3)
4	Cas+MNNG+A+P	21 (66)	9 (28)	2 (6)
5	MNNG	15 (42)	18 (50)	3 (8)

DISCUSSION

The present experiment demonstrated a decrease in body weight and the appearance of gastric tumors in rats undergoing castration, with recovery of incidence, and markedly increased size of tumors associated with expansion of the pyloric gland area in rats treated with castration plus progesterone. Pregnancy and/or delivery are thought to accelerate the progress of gastric cancer in humans.[4-8] Yamagata and Uzuka[4] found that artificial abortion or abortion at a late stage was linked with rapid advance of gastric cancer development. Young adults (aged 30 years or younger) with stomach cancer have a

Table V. Incidence of Intestinal Metaplasias

Group	Treatment	No. of animals	ALP-positive foci		Intestinal metaplasia (A+B+C type)	
			Incidence	No. per animal	Incidence	No. per animal
1	Cas+MNNG	22	2 (9)	0.68±2.11	3 (14)	0.15±0.37
2	Cas+MNNG+P	29	8 (28)	0.66±1.37	3 (10)	0.10±0.31
3	Cas+MNNG+A	29	6 (21)	0.85±2.32	4 (14)	0.15±0.36
4	Cas+MNNG+A+P	32	3 (9)	0.24±0.95	5 (16)	0.23±0.43
5	MNNG	36	11 (31)[a]	1.97±5.13	8 (22)	0.16±0.38

a) Significantly different from Group 4 ($P<0.05$).

Table VI. Pyloric Area

Group	Treatment	Pyloric area (mm^2/screen)
1	Cas+MNNG	0.246±0.039
2	Cas+MNNG+P	0.329±0.119[a,b,c]
3	Cas+MNNG+A	0.295±0.060[b]
4	Cas+MNNG+A+P	0.257±0.059[d]
5	MNNG	0.272±0.061

a) Significantly different from Group 5 ($P<0.05$).
b) Significantly different from Group 1 ($P<0.01$).
c) Significantly different from Group 4 ($P<0.01$).
d) Significantly different from Group 3 ($P<0.05$).

low 5-year survival rate because of the advanced stage of the tumors at the time of surgical operation.[5] Furukawa *et al.*[6] reported that female patients aged 34 years or younger who had experienced pregnancy and delivery less than 2 years previously had more advanced stomach cancers than other female or male patients. Recently they also reported gastric cancers diagnosed within 2 years after pregnancy and delivery to be more progressive than their counterparts in other young female patients with or without children.[7] Furukawa *et al.* also documented an increased incidence of gastric cancer with pregnancy and delivery in Wistar rats.[13] They suggested that this was due to accelerated growth of stomach cancer. Gladys[20] also reported that pregnancy had a stimulative effect on the growth of extragenital carcinomas because the increased metabolism during pregnancy accelerated the growth and spread of malignant lesions.

The lutein cells are the principal sites of production of progesterone,[21,22] secretion from the corpus luteum being essential for maintenance of pregnancy during the first 2 months. After this time, the production of progesterone by the placenta is adequate for the continued maintenance of pregnancy. Regulation of egg movement through the fallopian tube, preparation of the uterus to receive the blastocyst, alteration of electrical activity in the brain, control of uterine contraction at parturition, and generation of the secretory system (mammary alveolar system) of the breasts during pregnancy are all dependent on progesterone.[21,22] Some authors have reported that both the incidence of gastric tumors and the height of the pyloric gland mucosa, which are remarkably increased by treatment with gastric tumor promoters,[23-26] were not influenced by prolactin treatment.[14] The present experiment showed that the development of gastric tumors is increased by treatment with progesterone. The correlation between incidence of gastric tumor and area of pyloric glands ($y=185x-20$, $r=0.68$) was not significant but that between tumor size and the pyloric gland area was highly so ($y=0.001x+0.6$, $r=0.87$, $0.5>P>0.05$). Recently we found that gastric tumors appear earlier when growth hormone is added during the promotion phase.[27] As body size is increased during pregnancy (Watanabe *et al.*, unpublished data), cells in some organs may proliferate due to the direct or indirect action of progesterone. In conclusion, the present results provide evidence that progesterone may be a gastric tumor promoter. It might be appropriate for gastric cancer patients with progesterone receptor to receive anti-progesterone therapy. Further study is needed on progesterone doses, serum progesterone and androgen levels, the mechanism of the promoting action during gastric tumorigenesis, and the influence of hormonal treatment on MNNG metabolism.

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