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Lack of Any Positive Effect of Intestinal Metaplasia on Induction of Gastric Tumors in Wistar Rats Treated with *N*-Methyl-*N*-nitrosourea in Their Drinking Water

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The influence of intestinal metaplasia on gastric cancer induction was examined in five-week-old male Wistar:Crj rats. The animals were first treated with two 10 Gy doses of X-rays to the gastric region at a 3-day interval (total 20 Gy) and then, starting two months after the irradiation, received 100 ppm *N*-methyl-*N*-nitrosourea (MNU) in their drinking water for 15 weeks. Thereafter they were maintained for 37 weeks with or without a dietary 1% sodium chloride (NaCl) supplement. The incidences of gastric adenocarcinomas in the MNU or MNU plus NaCl groups were significantly higher than in animals receiving X-rays plus MNU with or without NaCl. Intestinal metaplasias and the numbers of alkaline phosphatase(ALP)-positive foci were significantly increased in the X-ray irradiation groups but the numbers of ALP-positive foci were not increased with or without 1% NaCl. An inverse relationship between incidences of gastric tumors and intestinal metaplasias was apparent. The present experiment thus showed that the presence of intestinal metaplasia does not exert a positive influence on induction of gastric neoplasia by MNU in the rat.

Key words: Intestinal metaplasia — Gastric tumor — MNU — X-ray — Rat

N-Methyl-N-nitrosourea (MNU) is a potent mutagen and direct-acting carcinogen producing tumors in several species in a variety of organs, including the central nervous system, intestine, kidney, stomach, and skin.[1–9] Hirota *et al.*[10] reported that MNU in the drinking water selectively induces glandular stomach carcinomas at a high incidence in F344 rats. This was confirmed by Fujita *et al.*[11] and the MNU-F344 animal model has become established for investigations of gastric carcinogenesis. Various authors consider intestinal metaplasia in the glandular stomach to be a precursor lesion for well differentiated gastric adenocarcinomas.[12–16] The incomplete or large intestinal type of intestinal metaplasia may be particularly associated with gastric tumors[17, 18] and has been thought to provide a region of incipient neoplasia.[19] We earlier reported that intestinal metaplasia without alkaline phosphatase (ALP)-positive foci (incomplete type or large intestinal type) can be induced in Wistar rats by X-irradiation.[20] We further showed that 1% sodium chloride (NaCl) promotes the induction of ALP-positive foci by X-irradiation in SD rats.[21] The present investigation was performed in order to determine whether the presence of intestinal metaplasia and subsequent appearance of ALP-positive foci exert any influence on the induction or development of gastric carcinomas in rats.

MATERIALS AND METHODS

Animals Five-week-old male Crj:Wistar rats (Charles River Japan Co. Ltd., Hino) were used in the present study. They were housed three or four to a polycarbonate cage and kept under constant conditions of temperature (24 ± 2°C), and relative humidity (55 ± 10%) with a 12 h light/12 h dark cycle. The animals were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University.

X-irradiation Rats were anesthetized with Nembutal and X-irradiation was performed according to the method described previously.[20–22] All animals were given two X-ray doses of 10 Gy with a three-day interval (total 20 Gy).

Chemicals MNU was purchased from Sigma Chemical Co., St. Louis and dissolved in distilled water at a concentration of 100 ppm. Rats were given this solution from light-opaque bottles as their drinking water for 15 weeks. The MNU solution was exchanged at 3- to 4-day intervals.

Experimental design One hundred and eighty-six animals were divided into 8 groups. Two months after the second irradiation, at the time when intestinal metaplasias appeared, rats were given MNU, and after that fed normal (Oriental MF, Oriental Yeast Co., Ltd., Tokyo; total NaCl concentration 0.32%) diet or diet supplemented with 1% NaCl (Wako Pure Chemical Ind., Ltd.,

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Osaka; total NaCl concentration 1.32%) for 37 weeks. Group 1 received X-irradiation alone, Group 2 X-irradiation plus NaCl, Group 3 X-irradiation plus MNU and NaCl, Group 4 X-irradiation plus MNU, Group 5 MNU alone, Group 6 MNU plus NaCl, Group 7 NaCl alone and Group 8 served as a nonirradiated control without any supplement (Table I). All animals had free access to food and water throughout.

Examination of animals All animals were regularly observed and killed upon observation of paralysis of one or two legs or at the termination of the experiment 1 year after the start of MNU treatment. At the time of necropsy, the body, liver and other major organs were weighed and prepared for histopathologic studies. Particular attention was paid to the stomach. Each was cut open along the greater curvature, stretched out, pinned on a board with the mucosal surface facing upward and washed several times with physiological saline before gross examination and fixation in 10% neutral formalin. ALP activity in the gastric mucosa was visualized by the naphthol-AS-MX-phosphate-fast blue RR staining method.[23] The numbers of crypts with ALP activity in the pylorus and in the fundus were counted using a dissecting microscope, with a double-blind protocol.

Strips of stomach were cut perpendicularly to the mucosal surface, two strips being taken at the lesser curvature and four at the greater curvature. The strips were embedded in paraffin and serially sectioned at 3 μm. Sections were also routinely stained with hematoxylin and eosin (HE), and with alcian-blue-periodic acid-Schiff (AB-PAS). Sections were also stained for mucin with high-iron diamine(HID)-AB.

Tumors were classified as: 1) atypical hyperplasia of mucosa if proliferation of atypical glands was observed, or 2) adenocarcinoma if atypical glands proliferated and invaded all the layers of the gastric wall.

Intestinal metaplasias were categorized using the following histological criteria[21, 22]: type A, gastric mucosa with goblet cells which were positive for AB-PAS and HID; type B, intestinal-type crypts without Paneth cells; type C, intestinal metaplasia with Paneth cells (ALP-positive foci). Using these criteria, the numbers of intestinal metaplastic crypts on the same slide were counted separately for both the pyloric glands and the fundic glands in a double-blind fashion. Metaplastic crypts within 5 crypts from the pyloric ring were not scored.

Table I. Initial and Effective Numbers of Animals

Group	Treatment			Initial No. of animals	Effective No. of animals
	X-ray	MNU	NaCl		
1	+	−	−	20	18
2	+	−	+	20	19
3	+	+	+	32	30
4	+	+	−	24	24
5	−	+	−	20	19
6	−	+	+	24	23
7	−	−	+	20	20
8	−	−	−	26	26

Table II. Tumor Incidence Data

Group	Effective No. of animals	No. (%) of animals with tumors							
		Total	Gastric lesions			Sarcoma	Kidney	Adrenal	Other[a]
			Atypical hyperplasia	Adeno-carcinoma	Total				
1	18	9 (50)	0	0	0	2 (11)	1 (6)	5 (38)	2[e]
2	19	6 (32)	0	0	0	3 (16)	0	3 (16)	1[f]
3	30	18 (60)	1 (3)	6 (20)	7 (23)[b, c]	4 (13)	8 (27)	1 (3)	3[g]
4	24	16 (67)	1 (4)	6 (25)	7 (29)[c, d]	4 (17)	4 (17)	4 (17)	4[h]
5	19	14 (74)	0	13 (68)	13 (68)	2 (11)	2 (11)	0	3[i]
6	23	19 (82)	3 (13)	13 (57)	16 (70)	1 (4)	3 (13)	1 (4)	4[j]
7	20	2 (10)	0	0	0	1 (5)	0	0	0
8	26	2 (8)	0	0	0	2 (8)	0	0	0

a) Some animals had more than one kind of tumor.
b) Significantly different from Group 5 ($P<0.01$).
c) Significantly different from Group 6 ($P<0.01$).
d) Significantly different from Group 5 ($P<0.05$).
e) Thymic lymphoma 1, pancreatic tumor 1.
f) Pituitary tumor 1.
g) One animal (pancreatic tumor 1, hemangioma 1), fibroadenoma 1, colon adenoma 1, brain tumor 1.
h) Testicular tumor 1, pancreatic tumor 2, hemangioma 1.
i) Papilloma 1, brain tumor 2.
j) Duodenal adenoma 1, testicular tumor 1, skin papilloma 1, one animal (lung tumor 1, spinal cord tumor 1).

Statistical analysis The significance of differences in numerical data was evaluated by the chi-squared test, Student's t test and by fitting to a linear equation.

RESULTS

One hundred and seventy-eight animals which survived beyond 208 days after the start of MNU treatment were counted as effective, those dying before this time point being without tumors (Table I). Mean body weight was significantly decreased in Groups 3 and 5 as compared to that in Group 1, and in Group 5 as compared to that in Group 7 or 8. Liver, kidney, testis, and adrenal weights did not significantly vary among the 8 groups. Spleen weight in Group 2, 3 or 4 was significantly decreased as compared to that in Groups 6 to 8. Mean survival time in Group 3 or 5 was significantly decreased as compared to that in Group 1, 2, 6, 7 or 8.

Total tumor incidences were in the range of 8 to 82%, gastric and kidney (mesenchymal) tumors appearing to predominate in the MNU-treated groups. Sarcomas developed in all groups and adrenal tumors appeared in X-irradiated groups and in Group 6. Tumors of the brain, spinal cord, pancreas, pituitary, colon, testis, duodenum and lung, hemangiomas in the liver, thymic lymphomas and fibroadenomas were sporadic findings (Table II). Greater tumor multiplicity was observed for the MNU groups.

The first gastric tumor was found 208 days after the start of MNU treatment in Group 5 (Fig. 1). All gastric tumors were well differentiated types without intestinal features. A small number of atypical hyperplasias was also apparent. The combined incidences of atypical hyperplasias and gastric adenocarcinomas were significantly higher in Groups 5 and 6 as compared to that in Group 3 or 4. Some gastric adenocarcinomas demonstrated cellular infiltration, fibrosis, myoblast-like cells, and calcification or cartilage in the stromas (Fig. 2), but no intestinal features were found in gastric tumors.

ALP-positive foci were more frequently found in the X-irradiated Groups 1 to 4 than in Groups 5 to 8 (Table III), most developing in the pylorus. The average numbers of ALP-positive foci in Group 1 or 2 were higher than in Group 3 or 4 and administration of 1% NaCl did not exert any significant enhancing effect on the development of ALP-positive foci (Table III).

Total intestinal metaplasia incidences on the basis of histological findings were over 86% in the irradiated Groups 1 to 4, and 19 to 65% in Groups 5 to 8 without irradiation. Type B predominated and type C metaplasia in the pylorus and intestinal metaplasia in the fundus appeared in the X-irradiated groups (Table IV).

Data on average numbers of foci of intestinal metaplasia are summarized in Table V. Total numbers of intestinal metaplasia were smaller in the nonirradiated groups as compared to the irradiated group values.

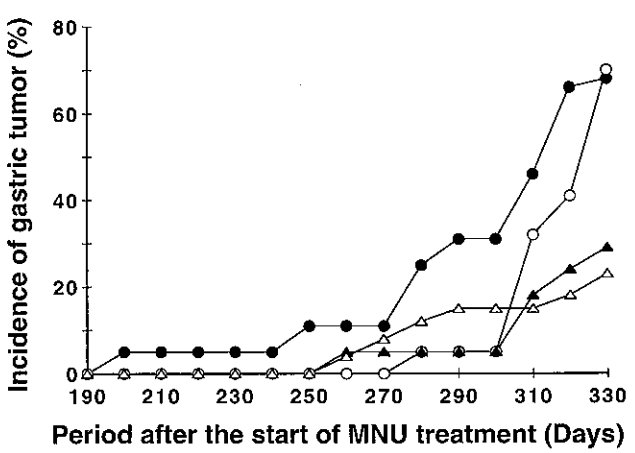

Fig. 1. Cumulative incidences of gastric tumors. △, Group 3; ▲, Group 4; ●, Group 5; ○, Group 6.

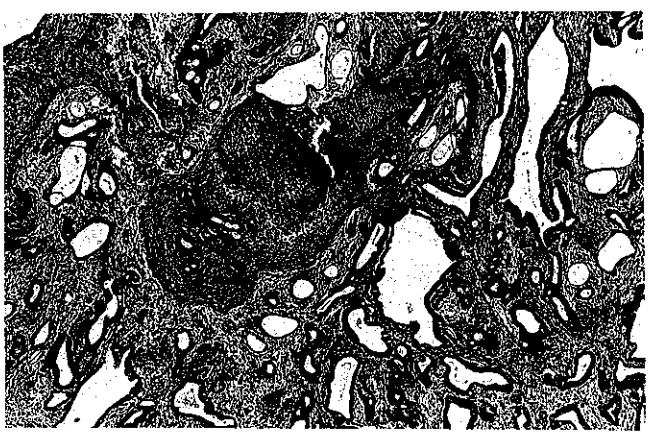

Fig. 2. Gastric adenocarcinoma with stromal cartilage, ×100, HE staining.

Table III. Incidences and Numbers of ALP-positive Foci

Group	Effective No. of animals	No. (%) of animals with ALP-positive foci	Average No. per rat (mean±SD)
1	16	15 (94)	31.3±45.7
2	18	15 (83)	33.1±48.7
3	27	13 (48)	12.9±21.4
4	23	16 (70)	19.0±23.4
5	15	0	0
6	20	3 (15)	0.2±0.4
7	19	2 (11)	1.8±4.3
8	25	6 (24)	1.6±4.2

Table IV. Incidences of Intestinal Metaplasia

Group	Type of intestinal metaplasia[a]					
	Pylorus			Fundus		
	A	B	C	A	B	C
1	2 (13)[b]	13 (87)	7 (47)	1 (7)	1 (7)	0
2	0	17 (100)	7 (41)	0	1 (6)	0
3	2 (9)	21 (91)	9 (39)	0	1 (4)	0
4	1 (5)	18 (86)	4 (19)	1 (5)	3 (14)	0
5	1 (7)	5 (36)	0	0	0	0
6	1 (5)	12 (60)	0	0	2 (10)	0
7	0	6 (33)	0	0	0	0
8	0	5 (19)	0	0	0	0

a) A, gastric mucosa with goblet cells; B, intestinal-type crypt without Paneth cells; C, intestinal-type crypt with Paneth cells.
b) Number of animals (%).

Table V. Numbers of Intestinal Metaplasias per Rat (mean ± SD)

Group	Type of intestinal metaplasia[a] (pylorus + fundus)			
	A	B	C	Total
1	0.80±2.57	7.60±12.11	1.67±3.50	10.07±17.70
2	0	7.71±7.86	0.76±1.20	8.47±8.30
3	0.09±0.29	5.22±6.56	0.57±0.95	6.20±7.14
4	0.24±0.77	4.90±5.47	0.38±0.86	5.52±6.21
5	0.07±0.27	1.07±1.69	0	1.14±1.88
6	0.05±0.22	1.15±1.18	0	1.20±1.28
7	0	0.44±0.78	0	0.44±0.78
8	0	0.19±0.41	0	0.19±0.41

a) A, B, C as shown in Table IV.

DISCUSSION

In the present investigation, MNU administration in the drinking water induced gastric tumors in Wistar rats, in line with previous findings for the F344 rat strain.[10, 11] However, pathological findings were different between the F344 and Wistar cases. In F344 animals, the induced gastric tumors differentiate into gastric mucosal and/or intestinal components.[24] In Wistar rats, however, the equivalent gastric lesions were found to be characterized by various mesenchymal components, inflammatory cell infiltration and no differentiation into gastric mucosal or intestinal components by staining with AB-PAS and HID-AB. Thus, the present investigation has provided a new model for gastric tumorigenesis.

The incidences of gastric tumors in the X-irradiated MNU groups were significantly decreased as compared with those in the nonirradiated counterparts. Inverse relationships between incidences of gastric tumors among Groups 1–6 and ALP-positive foci ($y=-4.5x+30.3$, $r=-0.97$, $P<0.01$), numbers of type B metaplasias ($y=-0.11x+8.7$, $r=-0.999$, $P<0.01$) or total numbers of metaplasias ($y=-0.12x+9$, $r=-0.99$, $P<0.01$) were evident in the present experiment. These results are in good agreement with those reported previously[22] and provide further evidence that intestinal metaplasia is not directly associated with development of gastric neoplasms.

In contrast to our earlier results with SD rats[21] administration of 1% NaCl to Wistar rats in the present case did not significantly enhance the development of intestinal metaplasia after X-irradiation. In addition to numbers, the sizes of ALP-positive foci were also smaller than in SD rats, and thus we concluded that 1% NaCl may not be a general promoter of intestinal metaplasia. Clearly, from the present results, there are strain differences which must be taken into account in assessing effects of exogenous agents on different subtypes of metaplasia.

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