

Gastric Tumor Induction by 1,2-Dimethylhydrazine in Wistar Rats with Intestinal Metaplasia Caused by X-Irradiation

Hiromitsu Watanabe,^{1,3} Toshihiro Uesaka,¹ Shoichirou Kido,¹ Yoshimasa Ishimura,¹ Kazuhisa Shiraki,¹ Ken Kuramoto,² Shitau Hirata,⁴ Shuneki Shoji,¹ Osamu Katoh¹ and Nariaki Fujimoto³

¹Department of Environment and Mutation, ²Department of Hematology and Oncology, ³Department of Cancer Research, Research Institute for Radiation Biology and Medicine and ⁴Department of Otolaryngology, School of Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553

Five-week-old male Wistar rats were X-irradiated with a total of 20 Gy in 2 equal fractions at a 3-day interval. 1,2-Dimethylhydrazine (DMH) solution was injected i.m. into the back musculature at a dose of 20 mg/kg body weight weekly for 10 weeks, beginning 20 weeks after the final irradiation. Twelve months after the initial carcinogen treatment, tumors in the fundus of the glandular stomach were observed in 5 of 23 animals receiving both X-irradiation and DMH treatment. No tumors of the glandular stomach were observed in the DMH and X-ray alone or nontreatment groups. It is concluded that the presence of intestinal metaplasia may increase sensitivity to the induction of gastric tumors by carcinogens like DMH.

Key words: Stomach tumor — 1,2-Dimethylhydrazine — Wistar rat — Intestinal metaplasia

Based on investigations in humans, intestinal metaplastic changes in the stomach have been considered as precancerous lesions or a predisposing condition for differentiated gastric carcinoma development.^{1–7} However, we have reported an inverse relationship between the numbers of intestinal metaplasias, with or without Paneth cells, and gastric tumor development and found that the presence of intestinal metaplasia does not exert a positive influence on induction of gastric neoplasia by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)⁸ or *N*-methylnitrosourea (MNU)⁹ in rats. The situation is complex because Nakagawa *et al.*¹⁰ have indicated that colorectal mucosa implanted into the glandular stomach, like the intrinsic large intestine, is sensitive to tumorigenesis caused by 1,2-dimethylhydrazine (DMH), in contrast to the normal gastric mucosa. Furthermore, Ando *et al.* reported that induction of intestinal metaplastic mucosa with Paneth cells in the glandular stomach was associated with susceptibility to tumorigenesis induced by DMH.¹¹ We previously reported that intestinal metaplasia (large intestinal or incomplete type) without both Paneth cells and alkaline phosphatase (ALP)-positive foci is induced in Wistar rats by X-irradiation, with most of the goblet cells having sulfomucins, revealed by high iron diamine-alcian blue staining.¹² The present study was designed to examine further whether intestinal metaplasia, without Paneth cells, might also be a target for DMH-induction of malignant tumors in the glandular stomach.

MATERIALS AND METHODS

Male 5-week-old-Crj:Wistar rats were X-irradiated according to the method described previously,^{12, 13} with two X-ray doses of 10 Gy at a three-day interval (total dose, 20 Gy) to the gastric region. Rats were provided with normal diet and tap water *ad libitum*. DMH (Nacalai Tesque Inc., Kyoto) was injected i.m. into the back musculature at a dose of 20 mg/kg body weight once weekly for 10 weeks beginning 20 weeks after the final irradiation (group 1). Group 2 rats received DMH after sham irradiation, group 3 received X-irradiation alone and group 4 was untreated. Animals were killed and autopsied when they became moribund and all remaining rats were killed 12 months after the initial DMH treatment. The stomach, and the small and large intestinal tracts were removed, opened and extended on cardboard for inspection. The location of individual tumors was recorded by measuring the distance from the pyloric ring in the small intestine and from the anus in the large intestine and recorded along with numbers and sizes. All tissues were fixed in 10% neutral formalin. ALP-positive foci in the gastric mucosa were detected by the naphthol-AS-MX-phosphate-fast blue RR staining method¹⁴ and the numbers of ALP-positive foci in the whole gastric mucosa per rat were counted using a dissection microscope and a double-blind protocol. Colon specimens were stained with 0.5% methylene blue for 15–30 min, then placed on glass slides with the luminal side up and viewed under a stereomicroscope at a magnification of ×20–30, to allow assessment of the presence of aberrant crypt foci (ACF). Sections of paraffin-embedded tissue were also routinely stained with hematoxylin and

³ To whom correspondence should be addressed.
E-mail: tonko@ipc.hiroshima-u.ac.jp

eosin, and for clarification, when necessary, periodic acid Schiff (PAS)-Alcian-blue (AB) staining of sialomucin (AB-positive) and sulfomucin [high-iron diamine (HID)-positive] was performed. Intestinal metaplasias were categorized using the following histological criteria^{1,13}: 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 metaplastic crypts were counted separately for 2 sections through the lesser curvature (pylorus) and 4 through the greater curvature (fundus) in a double blind fashion for each animal. Tumors in the stomach, small intestine and large intestine were classified into well-differentiated and poorly-differentiated types, the latter including both mucinous and signet ring cell forms.

The immunoglobulin enzyme bridge technique (indirect method) was employed for immunohistochemical studies. Dewaxed tissues were cut and sections were incubated overnight at room temperature with monoclonal anti 8-hydroxydeoxyguanosine (Nihon Yushi Co., Tokyo). Then

the sections were sequentially incubated with a biotin-labeled secondary antibody and an alkaline phosphatase-conjugated streptavidine complex using new fuchsin as the chromogen substrate, all purchased from Dako (Dako Co., Carpinteria, CA; LSAB kit alkaline phosphatase system 40, 40, K0628).

The significance of differences in numerical data was evaluated using the χ^2 and Student's *t* tests.

RESULTS

Mean survival and final body weights did not significantly differ among the groups. Liver relative weights were significantly decreased in the DMH-treated animals as compared to the control groups (Table I) along with kidney absolute and/or relative weights.

Tumors were found in 17 of 23 (74%), 7 of 12 (58%) and 8 out of 12 animals in the X-ray+DMH, X-ray and DMH groups, respectively (Table II). Multiple tumors were significantly more frequent in the X-ray+DMH group (Table III).

Table I. Body and Organ Weights (Relative Weights)

Group	Body (g)	Liver (g)	Spleen (g)	Adrenal (mg)	Kidney (g)	Testis (g)
X-ray+DMH	518±126	13.7±3.7 ^{a)} (25.8±4.8) ^{b,c)}	1.13±0.56 (2.16±1.2)	111±78 (0.23±0.18)	3.25±0.54 ^{a)} (6.29±1.20)	3.54±0.53 (6.90±1.49)
X-ray	537±179	15.6±5.4 (28.9±4.6)	0.93±0.49 (1.67±0.44) ^{c)}	88±19 (0.19±0.08)	4.74±4.16 (8.35±4.47)	3.29±0.94 (6.29±1.65)
DMH	561±153	18.1±6.3 (25.6±4.5) ^{b)}	1.52±0.78 (2.81±1.16) ^{b)}	121±152 (0.20±0.25)	3.39±0.53 ^{a)} (5.65±0.83) ^{b)}	3.75±0.54 (6.60±1.66)
Control	597±165	20.0±5.0 (31.1±5.5)	1.18±0.40 (1.82±0.38)	83±14 (0.14±0.05)	4.21±0.66 (6.67±1.04)	3.58±0.67 (5.86±2.00)

a) Significantly different from the control value (*P*<0.01).

b) Significantly different from the control value (*P*<0.05).

c) Significantly different from the DMH value (*P*<0.05).

Table II. Tumor Incidences

Group	Effective No. of animals	Mean survival	Total tumors	Gastric	Small intestine	Large intestine	Other
X-ray+DMH	23	318±49	17 (74)	5 (22)	3 (13)	9 (39) ^{a,b)}	5 (22) Kidney 3 (13) Pancreas 3 (13) Adrenal 1 (4) Lung sarcoma
X-ray	12	328±47	7 (58)	0	0	0	5 (42) ^{a)} Pancreas 2 (17) Adrenal
DMH	12	308±72	8 (67)	0	2 (17)	5 (42)	1 (8) Hemangioma
Control	9	334±32	0	0	0	0	

a) Significantly different from the control value (*P*<0.05).

b) Significantly different from X-ray value (*P*<0.05).

Gastric tumors in the glandular stomach were observed in 5 of 23 (22%) animals in the X-ray+DMH group but not in any of the other animals. Four of these tumors were well-differentiated, two with goblet cells or PAS-negative mucin (Fig.1, a and b), and there was one signet ring cell carcinoma. They were located in the middle or lower por-

tion of the fundus and there were no tumors in the pylorus. There was no positive geographical relation between gastric tumors and intestinal metaplasia. They did not react with antibody against alkaline phosphatase of intestinal type.

The incidences of large intestinal tumors were 39% and 42% in the X-ray+DMH and DMH alone groups, respectively. The numbers were also similar. The well-differentiated type was slightly more prominent in the X-ray+DMH group (58%) as compared to the DMH alone case (29%), while mucinous tumors were found in 8% and 43%, respectively, and the incidences of signet ring cell carcinomas were essentially the same. ACF numbered 8.0 per animal in the X-ray+DMH group and 4.5 in the DMH group. Small intestinal tumors developed in 13% of rats receiving X-rays+DMH and 17% of those given DMH. Five kidney, 3 pancreas, and 3 adrenal tumors were also observed with

Table III. Numbers of Tumors per Animal

Group	0	1	2	3	4
X-ray+DMH	6 (26)	8 (35)	8 (35) ^{a)}	0	1 (4)
X-ray	5 (42)	7 (58)	0	0	0
DMH	4 (33)	8 (67)	0	0	0
Control	0	0	0	0	0

a) Significantly different from the other groups.

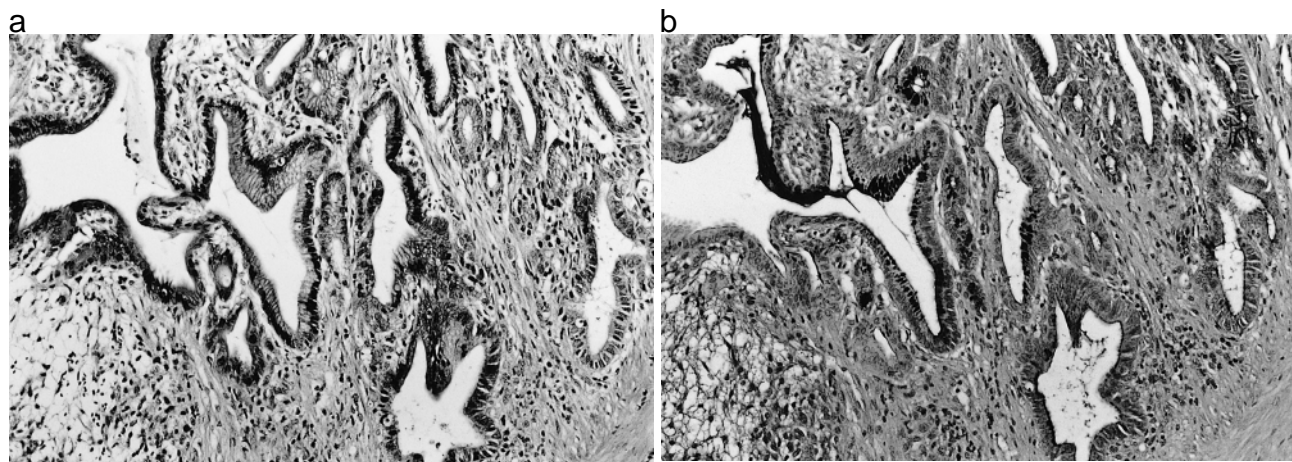


Fig. 1. a: Tumor in the glandular stomach treated with X-rays and DMH, HE staining, $\times 100$. b: PAS-negative mucin with PAS-AI staining.

Table IV. Incidences and Numbers of Intestinal Metaplasias

Group	Pylorus				Fundus				P+F total	ALP
	A	B	C	Total	A	B	C	Total		
X-ray+DMH	23	95	18	100	5	9	0	14	100	36
	0.4 ± 0.8	2.9 ± 3.0	0.3 ± 0.9	3.6 ± 3.7	0.1 ± 0.2	0.6 ± 2.0	0.1 ± 0.6	0.7 ± 2.6	4.3 ± 4.3	4.3 ± 8.8
X-ray	45	100	64 ^{a)}	100	0	27	0	27	100	88
	0.8 ± 1.2	13.1 ± 13.9	2.5 ± 4.1 ^{b)}	16.4 ± 17.5 ^{b)}	0	0.4 ± 0.7	0	0.4 ± 0.7	16.2 ± 17.4 ^{b)}	34.3 ± 51.8 ^{a)}
DMH	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0

A, gastric mucosa with goblet cells positive for AB-PAS and HID; B, intestinal-type crypts without Paneth cells; C, intestinal metaplasia with Paneth cells and alkaline phosphatase (ALP) positive foci.

Mean number of intestinal metaplasia \pm SD.

a) Significantly different from the X-ray+DMH value ($P < 0.01$).

b) Significantly different from the X-ray+DMH value ($P < 0.05$).

the combined treatment group, and 5 pancreas and 2 adrenal tumors after X-rays alone (Table II).

Intestinal metaplasia was apparent in the X-ray-irradiated, but not the non-irradiated groups. ALP-positive intestinal metaplasias were found in 36% and 88% of animals given X-rays+DMH and X-rays alone, respectively. Numbers of ALP-positive intestinal metaplasias and total numbers of intestinal metaplasias were also significantly higher with X-rays alone than with X-rays+DMH (Table IV). Some intestinal metaplasias and duodenal crypts exhibited cystic structures with pyknotic nuclei. Foci positive for anti 8-hydroxydeoxyguanosine binding were also observed in X-ray+DMH-treated animals.

DISCUSSION

In the present experiment, induction of intestinal metaplastic mucosa in the glandular stomach by X-rays was associated with a tendency for tumorigenesis in response to DMH, in contrast to the non-susceptible normal gastric mucosa. On the other hand, intestinal metaplasia itself was significantly decreased by the carcinogen exposure. We earlier reported that the numbers of intestinal metaplasias with ALP-positive foci induced by X-rays in Donryu rats was similarly decreased by treatment with azoxymethane, but that aberrant crypt-like foci appeared within some affected areas, with the appearance of cystic structures with pyknotic nuclei that exhibited binding of anti 8-hydroxyguanosine.¹⁵⁾ Thus, it would appear that areas of intestinal metaplasia with or without Paneth cells induced by X-irradiation might be susceptible to carcinogen damage, leading either to their deletion or to initiation, giving rise to tumors. Intestinal metaplasias in man are considered by some authors to represent regions of incipient well differentiated gastric adenocarcinoma.⁵⁻⁷⁾ This would be in line with the alternative possibility that the effects of irradiation and DMH on glandular stomach epithelial cells might additively or synergistically cause carcinogenesis. However, there was no direct evidence of a link between different types of intestinal metaplasia and gastric cancer in the present experiment. Further investigations are required to identify sequential changes in the gastric and intestinal metaplastic mucosa following X-irradiation with DMH treatment.

Azoxymethane and methylazoxymethanol, metabolites of DMH, are carcinogenic in both the large intestine and

the small intestine, especially in the duodenum in the latter.^{16, 17)} Campbell *et al.* reported that these carcinogens can reach the target tissues by routes other than the fecal stream¹⁸⁾ and Zedeck *et al.* found that the proximate carcinogen form of the metabolized chemical arrives at the mucosa mainly through the vascular system.¹⁹⁾ Matsubara *et al.* also indicated that carcinogens probably act on intestinal mucosa via the vascular system as well as through biliary transport.²⁰⁾ Thus, intestinal mucosal stem cell(s) would be expected to be susceptible to DMH carcinogenesis, independently of the administration route or their location. We earlier reported that adenocarcinomas develop in successful implants of large intestinal mucosa in the glandular stomach of animals treated with DMH,¹¹⁾ but not MNNG.²¹⁾ With regard to our present finding of gastric carcinomas in animals receiving X-rays+DMH, the report by Ando *et al.* that induction of intestinal metaplasia with Paneth cells in the glandular stomach is associated with a susceptibility to tumorigenesis¹¹⁾ is of clear interest. Thus, the intestinal mucosal phenotype appears to be the important determinant of response to DMH, rather than the intestinal macro-environment itself. The results are compatible with the conclusion that intestinal metaplasias are targets of DMH, in contrast to the counterpart normal gastric mucosa.

In summary, the presence of intestinal metaplasia, with or without Paneth cells, may increase sensitivity to the induction of tumors by carcinogens of the DMH type, but not the MNNG or MNU type. This route, however, is relatively minor compared to the main route of gastric carcinogenesis by carcinogens of the MNNG or MNU type acting on normal glandular mucosa in the stomach. The protocol used in the present experiment may provide a new approach to distinguish between developmental events associated with intestinal metaplasia and gastric tumors. Further investigations are required to explain the variations in the number, size and type of intestinal tumors in the X-irradiated and DMH-treated groups.

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