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The Effects of Sodium Chloride, Miso or Ethanol on Development of Intestinal Metaplasia after X-Irradiation of the Rat Glandular Stomach

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The influence of sodium chloride (NaCl), miso (Japanese soybean paste) and ethanol on development of intestinal metaplasia was examined. Five-week-old male CD(SD): Crj rats were treated with two 10 Gy doses of X-rays to the gastric region at a 3-day interval (total 20 Gy). After irradiation, the rats received supplementation with NaCl (1% or 10% in diet), miso (10% in diet) or ethanol (10% in drinking water) for 12 months. The number of alkaline phosphatase-positive foci of intestinal metaplasia in rats given 1% NaCl diet (Group 3) after X-rays was significantly elevated as compared to that in rats given X-rays alone (Group 1) ($P<0.01$) or X-rays with 10% NaCl (Group 2) ($P<0.01$). In the pyloric gland mucosae, the total numbers of metaplastic foci in rats of Group 3 were much higher than in Group 2, or after miso diet (Group 4) or ethanol supplementation (Group 5) ($P<0.01$), but no difference was found between Group 2, 4 or 5 and Group 1. Atypical hyperplasia only appeared at incidences of less than 6% in Groups 1–3 and no promoting effect on gastric tumorigenesis was evident in Group 2. The present results thus showed that the occurrence of intestinal metaplasia induced by X-irradiation can be significantly increased by administration of 1% NaCl and decreased by 10% NaCl and ethanol, but this is not associated with any influence on gastric neoplasia.

Key words: Intestinal metaplasia — Rat — NaCl — Ethanol — Miso

Intestinal metaplasia in the glandular stomach has been considered to be a precursor lesion for well differentiated gastric adenocarcinoma.[1-6] Imai *et al.* reported that intestinal metaplasia was more frequent in Japanese individuals than in American whites,[7,8] and considered this was chiefly based on differences in food intake between the two countries.[7-9] Intestinal metaplasia can be experimentally induced by chemical carcinogens,[10-14] X-irradiation,[15-19] chemical carcinogens plus X-irradiation[20-22] or application of xenogenic stomach antigens.[23] Nevertheless, its pathogenesis remains unclear. The present paper describes an investigation of the effects of NaCl, miso or ethanol on intestinal metaplasia induced by X-irradiation. The aim of the experiments was to clarify the potential role of cultural differences in consumption of these important materials in generating the observed differences in intestinal metaplasia.

MATERIALS AND METHODS

Animals Five-week-old male Crj:CD(SD) rats (Charles River Japan Inc., Hino) were used in the present study. They were housed three or four per cage in polycarbonate cages and were kept under constant conditions of temperature (24±2°C), and relative humidity (55±10%) with a 12 h light/12 h dark cycle. 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-irradiated according to the method described previously.[15-20] A 0.6-cm-thick lead cover, with a hole 1.8 cm in diameter, was ositioned so that the hole lay over the gastric region of the rats. All animals were given two X-ray doses of 10 Gy each with a three-day interval (total 20 Gy). Exposure factors were as follows: 200 kVp, filter 0.5 Cu + 1.0 Al, half-value layer 1.18 mm Cu, at a dose rate of 90 R/min as measured with a Radicon 555 decimeter. The X-ray air dose (in R) was then converted to the absorbed dose (in cGy) using a factor of 0.95 cGy/R.

Experimental design Two hundred and twenty-eight rats were divided into 10 groups. After the second irradiation, rats were fed normal (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Group 1, total NaCl concentration 0.32%) diet supplemented with 10% NaCl (Group 2, Wako Pure Chemical Ind., Ltd., Osaka; total NaCl concentration 10.32%), 1% NaCl (Group 3; total NaCl concentration 1.32%), or 10% miso (Group 4, Japanese soybean paste, Cyuou Miso Kyoukai, Tokyo; total NaCl concentration 2.52%), or drinking water supplemented with 10% ethanol (Group 5) throughout the experiment. Unirradiated control animals were maintained on the 10% NaCl (Group 6), 1% NaCl diet (Group 7), miso diet (Group 8) and ethanol alone (Group 9) or without any supple-

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ment (Group 10). All had free access to food and water throughout.

Examination of animals All animals were regularly observed and killed at the termination of the experiment after 12 months. The stomachs were cut open along the greater curvature, stretched out and pinned on a board with the mucosal surface facing upward. Each stomach was washed several times with physiological saline before gross examination, and fixed in 10% neutral formalin. Alkaline phosphatase (ALP) activity in the gastric mucosa was determined by the naphthol-AS-MX-phosphate-fast blue RR staining method.[24] The numbers of crypts with ALP activity in both the pylorus and fundus were counted using a dissecting microscope employing a double-blind protocol.

Strips of the stomach were cut perpendicularly to the mucosal surface, two strips being taken through the lesser curvature and four through the greater curvature. The strips were embedded in paraffin and serially sectioned at 3 μm. Sections were routinely stained with hematoxylin and eosin, and for clarification when necessary, the periodic acid Schiff (PAS)-alcian blue and high-iron diamine (HID)-alcian blue (AB)[25] staining procedures were introduced.

Gastric lesions in the glandular stomach were classified into atypical hyperplasia and adenocarcinoma categories.[22] 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.

For quantitative analysis, heights of stomach mucosa in both the pyloric and fundic areas were measured with the aid of an image analyzer (Model CIA 102, Olympus Co., Tokyo) coupled with a color TV monitor.

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

RESULTS

Thirty-one animals died from pneumonia and dehydration. The mean body weights of rats at the 1 year autopsy time point in the 10% NaCl and ethanol groups were significantly decreased as compared to the respective control values (Table I).

ALP-positive metaplastic foci in both the pylorus and fundus were more frequently found in the X-irradiated Groups 1, 3 and 4 than in Groups 2 and 5, and were almost absent in the non-irradiated Groups 6 to 10 (Table I). Most foci developed in the pylorus. In Group 3, ALP foci were larger than those in Group 1, 2 or 4 (Fig. 1). The average numbers of foci of intestinal metaplasia with ALP activity in both pylorus and fundus combined were significantly increased in Group 3 (X-ray+1%NaCl) over those in Group 1 (X-ray alone, $P<0.01$) and Groups 2, 4 and 5 ($P<0.01$; Table II).

Total incidences of intestinal metaplasia on the basis of histological findings were over 88% in the irradiated Groups 1 to 5, and 10 to 23% in Groups 6 to 8 with no metaplastic glands being observed in Groups 9 to 10 (Table I). Average numbers of foci of intestinal metaplasia are summarized in Table II. In the pylorus, total numbers of intestinal metaplasia were decreased in the animals receiving 10% NaCl or ethanol while supple-

Table I. Body Weight and Incidences of Intestinal Metaplasia and Gastric Tumors

Group	Treatment	Animal No.		Body weight (g) (mean±SD)	Intestinal metaplasia (%)				ATP[b]
		Initial	Effective		ALP[a]	Pylorus	Fundus	Total	
1	X-ray	20	16	628±88	(88)	15 (94)	10 (63)	16 (100)	1 (6)
2	X-ray+10%NaCl	20	16	419±73[c,d]	(30)	16 (100)	3 (19)	16 (100)	1 (6)
3	X-ray+1%NaCl	30	26	599±81	(100)	25 (96)	6 (23)	26 (100)	0
4	X-ray+Miso	35	31	609±92	(94)	29 (94)	10 (29)	30 (97)	1 (3)
5	X-ray+Ethanol	20	16	399±91[c]	(14)	13 (81)	4 (25)	14 (88)	0
6	10%NaCl	20	17	522±76[c]	(0)	4 (23)	0	4 (23)	0
7	1%NaCl	20	19	661±53	(11)	4 (21)	0	4 (21)	0
8	Miso	30	29	625±63	(0)	3 (10)	0	3 (10)	0
9	Ethanol	18	15	418±41[d]	(0)	0	0	0	0
10	Control	15	12	611±32	(0)	0	0	0	0

a) Foci detected as ALP-positive.
b) Tubular atypical hyperplasia.
c) Significantly different as compared to Group 1 ($P<0.01$).
d) Significantly different as compared to Group 10 ($P<0.01$).

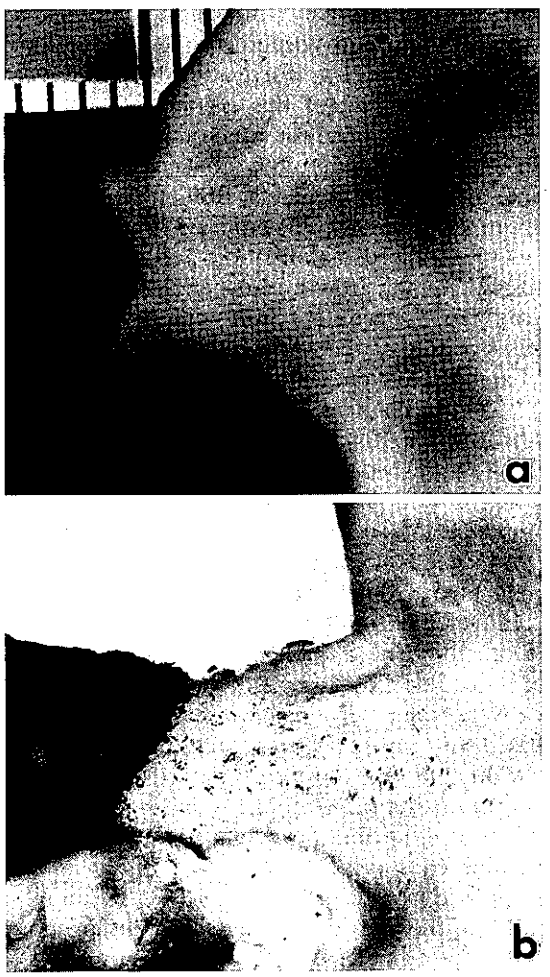

Fig. 1. ALP-positive metaplastic foci in Group 1 (a) were smaller than those in Group 3 (b).

mentation with 1% NaCl was associated with a tendency for increase (Table II). In the fundic gland mucosa, the total numbers of metaplasia were significantly decreased in Group 5 and showed a tendency for reduction with the other treatments. Combination of data for pyloric and fundic mucosae revealed metaplastic foci in Groups 2, 4 and 5 to be significantly fewer than in Group 1 ($P<0.01$).

Pyloric mucosa height was significantly greater in Group 2, 3, 4, or 6 than in Group 10 ($P<0.01$) and lower in Group 9 ($P<0.01$). The height of the fundic glands in Group 1, 3, 4, 6, or 9 was significantly smaller than in Group 10 ($P<0.01$) (Table III). No obvious alteration in cell size was evident in the pyloric or fundic glands of any group. Parietal cell mass in irradiated groups except in Group 2 was decreased.

In Group 2, erosion or ulceration was observed and atypical hyperplasias developed in the glandular stomach at incidences of 6, 6, and 3% in Groups 1, 2 and 4, respectively (Table I). No adenocarcinomas of the glandular stomach were observed in this experiment.

DISCUSSION

In the present investigation, administration of 1% NaCl significantly enhanced the development of intestinal metaplasia after X-irradiation, whereas both 10% NaCl and ethanol were inhibitory. No obvious effect on atypical hyperplasias, considered to be preneoplastic lesions of the glandular stomach, or adenocarcinomas was evident, however.

Many authors consider that stomach cancer and intestinal metaplasia are caused by similar environmental factors.[9, 26, 27] High salt diet has been proposed as a major

Table II. Numbers of Foci of Intestinal Metaplasia (mean ± SD)

Group	Treatment	Pylorus	Fundus	Pylorus + Fundus	
				Total	ALP[a]
1	X-ray	9.8 ± 1.7	3.3 ± 1.3	13.7 ± 2.1	38 ± 8
2	X-ray + 10%NaCl	2.4 ± 0.5[b]	0.7 ± 0.9	2.7 ± 0.5[b]	12 ± 5[d]
3	X-ray + 1%NaCl	15.3 ± 13.7[c]	1.1 ± 2.3	16.9 ± 14.0[c]	93 ± 56[b]
4	X-ray + Miso	7.5 ± 1.3[d]	0.7 ± 1.3	8.4 ± 4.9[b, d]	51 ± 48[d]
5	X-ray + Ethanol	2.2 + 0.6[b]	0.8 ± 0.5[e]	3.3 ± 0.7[b, d]	23 ± 5[d]
6	10%NaCl	0.3 ± 0.1	0	0.3 ± 0.1	1 ± 1
7	1%NaCl	0.3 ± 0.6	0	0.1 ± 0.6	0.3 ± 1.0
8	Miso	0.1 ± 0.4	0	0.1 ± 0.4	0
9	Ethanol	0	0	0	0
10	Control	0.2 ± 0.1	0	0.2 ± 0.1	0

a) Foci detected as ALP-positive.
b) Significantly different from X-ray alone ($P<0.01$).
c) Significantly different from X-ray + 10%NaCl ($P<0.01$).
d) Significantly different from X-ray + 1%NaCl ($P<0.01$).
e) Significantly different from X-ray ($P<0.05$).

Table III. Heights of Glandular Stomach Epithelium

Group	Treatment	Height (μm, mean$\pm$SD)	
		Pylorus	Fundus
1	X-ray	201$\pm$53[a]	677$\pm$107[a]
2	X-ray+10%NaCl	287$\pm$42[a]	858$\pm$111
3	X-ray+1%NaCl	220$\pm$59[a]	731$\pm$107[a]
4	X-ray+Miso	210$\pm$52[a]	783$\pm$116[a]
5	X-ray+Ethanol	180$\pm$41	812$\pm$130
6	10%NaCl	291$\pm$55[a]	735$\pm$90[a]
7	1%NaCl	213$\pm$43[a]	908$\pm$117
8	Miso	195$\pm$47[b]	838$\pm$110
9	Ethanol	126$\pm$25[b]	740$\pm$118[a]
10	Control	171$\pm$34	872$\pm$106

a) Significantly different as compared to Group 10 ($P<0.01$).
b) Significantly different as compared to Group 10 ($P<0.05$).

cause of the development of both,[28-30] whereas alcohol consumption appears to be without influence.[31, 32] In rat experiments, gastric tumorigenesis was found to be promoted by 10% NaCl[33, 34] but not 10% ethanol,[34, 35] 1% NaCl or miso diet.[36] It is well known that 10% NaCl causes cell proliferation in the pyloric glands[34] and a number of authors have indicated that enhancing effects on gastric carcinogenesis may be directly related to stimulation of cell proliferation and elongation of the mucosa.[33, 35, 37] If intestinal metaplasia, which is widely considered to have precancerous status for gastric tumors in man,[1-5] is also histogenetically involved in development of glandular stomach tumors in the rat, we should expect it to be increased by 10% NaCl diet. In the present experiment, however, while the pyloric mucosa height was increased by administration of 10% NaCl diet, a decrease in the number of intestinal metaplasias and no increase of gastric tumors were observed. It seems that no intestinal metaplasia was precancerous in nature in this experiment. Recently, extensive study has elucidated many genetic alterations which occur relatively frequently in gastric cancer, e.g., amplification of the c-*erb*B-2 gene, amplification of K-*sam*, point mutation of c-K-*ras*,[38] mutation of *p53*,[39] *TPR-MET* rearrangement,[40] loss of heterozygosity at the *DCC* locus[41] and mutation of the *APC* gene,[42] and in intestinal metaplasia e.g., *TPR-MET* oncogenic rearrangement.[40] Further study at the molecular level will be required to determine the precancerous status of intestinal metaplasia.

In general, daily intake of NaCl in food has been calculated to be about 12.2 g for the Japanese (2.8%)[43] and 5 g/day (recommended) in the USA.[44] The control level of NaCl exposure in the present experiment was therefore similar to that of Japanese. While the higher incidence of intestinal metaplasia in Japanese might be because of the 2.8% NaCl intake as compared to less than half this figure in Western people, the present data do not suggest any clear correlation between consumption and development of this lesion. Thus, the highest level of intestinal metaplasia was found with a 1% NaCl supplement and not the 10% concentration known to promote gastric tumorigenesis. Both 10% salt and ethanol treatments caused a significant decrease in body weight. Generalized malnutrition was recognized as an inhibitor of tumor growth in experimental animals over 70 years ago.[45] Feeding animals calorie-restricted diets decreases mammary-tumor incidence in a dose-dependent manner[46] and decreases colon cancer incidence and size.[47] The mechanisms underlying the observed decrease with both 10% NaCl and alcohol administration were not established, however.

An inverse correlation was observed between the average number of foci of intestinal metaplasia with ALP activity (Table III) and the height of the fundic gland in the irradiated groups ($y=-0.3x+276$, $r=-0.69$). A similar inverse correlation between fundic gland height and total numbers of metaplastic lesions was also observed ($y=-0.08x+71$, $r=-0.91$). In this study, pH values were not ascertained but parietal cell mass was decreased and no increase in parietal cell size was evident. Watanabe *et al.*[25] earlier reported that an inverse relationship exists between the number of parietal cells and pH during normal development, with a decrease in cell number causing an increase in pH value. We therefore proposed as a working hypothesis that an elevation of the pH of the gastric juice due to the disappearance of parietal cells in the fundic gland mucosa may be one of the principal factors responsible for the development of intestinal metaplasia.[16, 17] The results of the present experiment support this working hypothesis.

The fact that erosion and ulceration were observed in the X-ray plus 10% NaCl group, while intestinal metaplasia was decreased, is of interest. In one case intestinal metaplasia appeared in the regenerating epithelium of an ulcer but not in the ulcer itself. Moszkowicz[48] considered that metaplastic glands might be formed in the process of regeneration of ulcer or erosion and Konjetzny[49] pointed out an association between intestinal epithelium and the severity of inflammatory changes. Mangus[50] further suggested that the presence of intestinal epithelium in the stomach is the result of faulty regeneration of surface epithelium in a mucosa repeatedly damaged by gastritis and is an example of metaplasia resulting from chronic irritation. The question of how our data fit with these hypotheses clearly warrants further study, as does the question of whether intact gastric mucosa or regenerating epithelium is prerequisite for induction of intestinal metaplasia. Our model would appear to be of advantage for answering these points.

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