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Synergism between Sodium Chloride and Sodium Taurocholate and Development of Pepsinogen-altered Pyloric Glands: Relevance to a Medium-term Bioassay System for Gastric Carcinogens and Promoters in Rats

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In an approach to early detection of gastric carcinogens and promoters in an *in vivo* test system, promotion by sodium chloride (NaCl) and the synergistic effects of NaCl and sodium taurocholate (Na-TC) on development of pepsinogen-altered pyloric glands (PAPG) in rat glandular stomach after initiation with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) were investigated. A total of 205 male WKY/NCrj rats were divided into 8 groups. Group 1 was given a single dose of MNNG of 160 mg/kg body weight by gastric intubation, and starting 2 weeks later basal diet containing Na-TC for 18 weeks. In addition, 1 ml doses of saturated NaCl solution were given by gastric intubation at weeks 4, 6, 8 and 10. Similarly, group 2 was treated with MNNG and Na-TC, while group 3 animals received MNNG and NaCl. Group 4 was given MNNG alone. Groups 5–8 served as equivalent controls without MNNG initiation. The results revealed significantly enhanced induction of immunohistochemically defined PAPG in the Na-TC+NaCl ($P<0.001$), Na-TC ($P<0.01$) and NaCl ($P<0.01$) treated animals initiated with MNNG. Sodium chloride demonstrated a clear synergistic effect with Na-TC in promoting the development of PAPG, suggesting possible advantage for its use in medium-term *in vivo* assays for detection of gastric carcinogens and promoters.

Key words: Sodium chloride — Pyloric gland — N-Methyl-N′-nitro-N-nitrosoguanidine — Pepsinogen — Bile acid

Many chemicals have not been tested in long-term carcinogenicity experiments in animals because these experiments are time-consuming and expensive. *In vivo,* short-term carcinogenicity assays based on genetic alterations,[1] or neoplastic cell transformation[2] and various other systems[3,4] have been developed for identification of carcinogenic compounds, but these *in vitro,* short-term tests give high rates of false-positive and false-negative results and are not suitable for detection of promoters and determination of target organs.[4–7] Therefore, an *in vivo,* medium-term bioassay is required which combines the advantages of *in vitro,* short-term assays and *in vivo,* long-term experiments. An *in vivo,* medium-term assay has been developed based on quantitative measurement of enzyme alteration in liver cell foci, which may represent preneoplastic changes.[8–10] In gastric carcinogenesis pyloric glands with an alteration of pepsinogen isozyme 1 (PAPG), which can be detected immunohistochemically, were recently suggested to represent a preneoplastic change,[11–14] because of the three pepsinogen (Pg) isozymes (Pg 1, 3, 4), Pg 1 decreases or disappears preferentially in the pyloric mucosa from an early stage of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis[15] and in gastric tumors.[16] Almost all cells of the pyloric gland cell type in areas of adenomatous hyperplasia and adenocarcinoma have little or no Pg 1 detectable immunohistochemically.[12] The number of PAPG increases with the dose of MNNG[11] and with time after the end of MNNG administration.[11,14] Moreover, a correlation has been found between the susceptibilities of different strains of rats to induction of gastric carcinoma and to induction of PAPG by MNNG.[14]

For establishing an *in vivo,* medium-term bioassay for gastric carcinogens and promoters, we previously carried out sequential, quantitative analyses on PAPG in rats treated first with MNNG and then with N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG) as a second gastric carcinogen or sodium taurocholate (Na-TC) as a gastric promoter.[13] Results showed that induction of PAPG was significantly enhanced by treatment with ENNG from week 8 and with Na-TC from week 16. Partial hepatectomy (PH) during administration of test chemicals was also found to enhance the development of enzyme-altered foci and to shorten the period required for *in vivo,* medium-term screening for liver carcinogens.[17–19] By analogy, as sodium chloride enhances cell proliferation in the gastric mucosa,[20] its administration during treatment with test chemicals in *in vivo,* medium-term screening for gastric carcinogens and promoters should

enhance the induction of PAPG. Therefore, in this work we investigated the effect of sodium chloride on the development of PAPG in the glandular stomach of rats during Na-TC administration to rats initiated by MNNG.

MATERIALS AND METHODS

Animals and chemicals A total of 205 male WKY/NCrj rats (Charles River Japan, Inc., Kanagawa) were housed in plastic cages with hardwood chips in an air-conditioned room with a 12-h light-12-h dark cycle and were given food (Oriental MF; Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum*. The animals were divided into 8 groups. Group 1 was given a single dose of 160 mg/kg body weight of MNNG (Tokyo Chemical Industry Co., Tokyo) as a solution in dimethylsulfoxide (DMSO) by gastric intubation. From 2 weeks later the rats were given basal diet containing Na-TC (Wako Pure Chemical Industry, Osaka) for 18 weeks. In addition, 1 ml of saturated sodium chloride solution was given by gastric intubation once each in weeks 4, 6, 8 and 10. Group 2 was treated in the same way as group 1 except that saturated sodium chloride solution was not administered. Group 3 was treated in the same way as group 1 but without Na-TC administration. Group 4 was given MNNG alone without saturated sodium chloride solution or Na-TC. Groups 5 to 8 were given 1 ml of DMSO instead of MNNG and then treated in the same way as groups 1, 2, 3 and 4, respectively. Animals in groups 1 to 4 were killed at the end of weeks 12, 16 and 20 of the experiment. Animals in groups 5 to 8 were killed at the end of week 20. The stomach was fixed in sublimed formaldehyde and cut into about 8 strips, which were embedded in paraffin.

Immunohistochemical staining for Pg 1 Anti-Pg 1 serum was prepared as described previously,[21] and anti-Pg 1 IgG was purified by column chromatography on DE-52 (Whatman, Kent, UK). The ABC immunohistochemical method was used to determine the location of Pg 1 in the pyloric mucosa.[22] Affinity-purified, biotin-labeled, goat anti-rabbit immunoglobulin (IgG and avidin-biotin-peroxidase complex; Vectastain ABC kit, PK4001) was obtained from Vector Laboratories (Burlingame, CA). Endogenous peroxidase activity was blocked by treatment with methanolic hydrogen peroxide. Sections were treated sequentially with (1) diluted normal goat serum, (2) rabbit anti-rat Pg 1 IgG (1:6000), (3) biotin-labeled goat anti-rabbit IgG (1:400) and (4) avidin-biotin-peroxidase complex (ABC). The site of peroxidase binding was detected by the diaminobenzidine method of Graham and Karnofsky.[23] Sections were counterstained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-Pg 1 antibody, normal (non-immune) rabbit serum was used in place of anti-Pg 1 IgG. Pyloric glands showing little or no staining for Pg 1, defined immunohistochemically as PAPG, were seen in apparently normal pyloric mucosa and mucosal hyperplasia and atrophy. The number of PAPG per 1 cm of mucosal length was calculated by counting those in all stomach strips (about 8 strips from each animal). The length of pyloric mucosa on histological slides was measured with a general-purpose, color image processor, Olympus Model VIP-21C (Olympus Co., Tokyo).[18]

RESULTS

Altered pyloric mucosa consisting of areas of mucosal hyperplasia and mucosal atrophy was seen from week 12 in groups 1, 2 and 3, and from week 16 in group 4, but was not seen in groups 5, 6, 7 and 8. One adenomatous hyperplasia was found in group 1 in week 20.

PAPGs (Fig. 1) were found immunohistochemically in normal-looking pyloric mucosa and altered pyloric mucosa. Almost all cells of pyloric gland cell type in the adenomatous hyperplasia had low Pg 1 contents. The sequential changes in the numbers of immunohistochemically defined PAPG in each group are summarized in Table I. Induction of PAPG initiated with MNNG was significantly enhanced by Na-TC+NaCl (group 1) and Na-TC (group 2) from week 12 and by NaCl (group 3) from week 16 in comparison with that in group 4. The

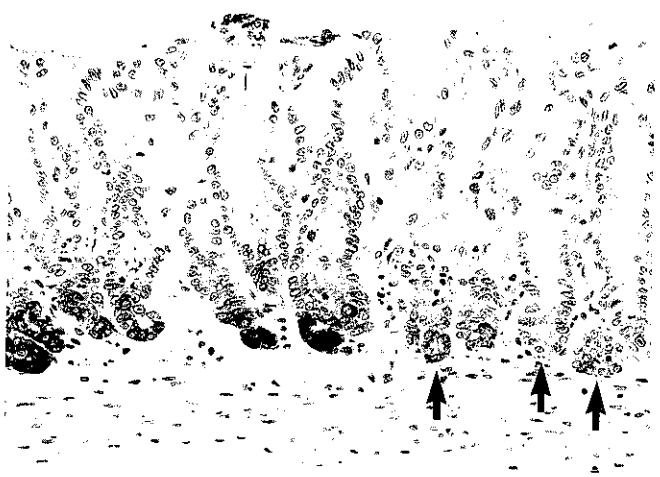

Fig. 1. Immunohistochemical demonstration of Pg 1 altered pyloric glands (arrows) in normal-looking pyloric mucosa in a rat in group 1 in week 16. ×100.

Table I. Sequential Changes in Numbers of Pg 1-altered Pyloric Glands (PAPG) in Pyloric Mucosa

Group	Treatment	Weeks after MNNG administration					
		12		16		20	
		No. of rats	No. of PAPG[a]	No. of rats	No. of PAPG	No. of rats	No. of PAPG
1	MNNG→Na-TC+NaCl	14	6.00±2.07[b, c, d]	14	7.02±2.30[c, d, e]	13	7.30±2.84[b, c, d]
2	MNNG→Na-TC	15	3.65±1.80[f, g]	13	4.61±1.60[f, g]	11	4.30±1.16[f, g]
3	MNNG→NaCl	15	2.26±0.73	15	3.19±1.23[h]	15	3.26±1.34[g]
4	MNNG alone	15	1.88±0.76	15	2.23±0.81	17	1.94±0.69[i]
5	DMSO→Na-TC+NaCl					10	0.53±0.42
6	DMSO→Na-TC					5	0.32±0.30
7	DMSO→NaCl					5	0.12±0.14
8	DMSO alone					5	0.00

Number of PAPG per 1 cm pyloric mucosa; data are means±SD.
a) PAPG, pepsinogen-altered pyloric gland.
b) Significantly different from group 2 at $P<0.01$.
c) Significantly different from group 3 at $P<0.001$.
d) Significantly different from group 4 at $P<0.001$.
e) Significantly different from group 2 at $P<0.05$.
f) Significantly different from group 3 at $P<0.05$
g) Significantly different from group 4 at $P<0.01$.
h) Significantly different from group 4 at $P<0.05$.
i) Significantly different from groups 5, 6 and 7 at $P<0.001$.

number of PAPG in week 12 was significantly greater on treatment with Na-TC+NaCl (group 1) than on treatment with Na-TC (group 2) or NaCl (group 3). Only a few PAPG were found in groups 5, 6 and 7 and no PAPG was found in group 8 (Fig. 2).

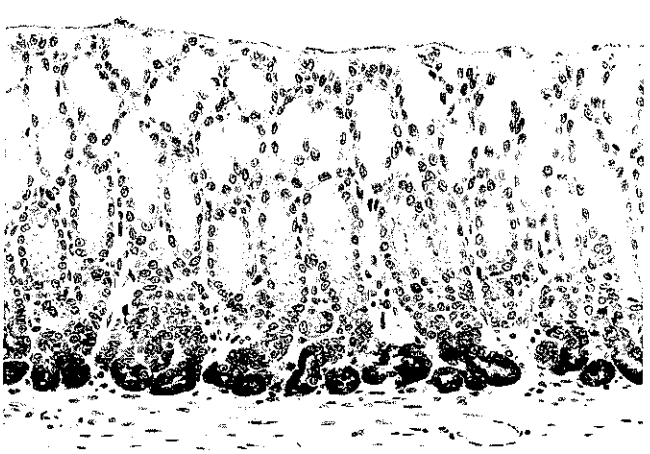

Fig. 2. Immunohistochemical demonstration of Pg 1 in normal-looking pyloric mucosa in a rat in group 8 in week 20. ×100.

DISCUSSION

Previously we reported that Na-TC induced ornithine decarboxylase activity and DNA synthesis in the glandular stomach mucosa of rats[24] and also promoted the development of PAPG in rats initiated with MNNG.[13] The present study also demonstrated promoting activity for Na-TC on the development of PAPG after initiation of MNNG and showed that Na-TC itself can induce significant numbers of PAPG.

Sodium chloride, which clearly promoted the induction of PAPG after MNNG initiation in the present study has been shown both epidemiologically[25, 26] and experimentally[27–31] to increase the risk of gastric cancer. It was found to have promoting[30, 31] and co-initiating[28, 29, 31] effects on two-step gastric carcinogenesis, and a co-carcinogenic effect[27] in MNNG-induced gastric carcinogenesis. These effects of sodium chloride may be related to both disturbance of the mucous barrier,[32, 33] thereby enhancing the penetration of MNNG into the mucosa, and also to repeated injury to the gastric mucosa, resulting in increased cell proliferation.[20, 34]

Administration of sodium chloride during Na-TC feeding resulted in particularly marked promotion of PAPG development which was significant from weeks 12 to 20. Synergistic effects of carcinogens on tumor induction have been well documented in several organs and similar synergism between promoters was also observed

for N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats.[35] The present documented effect during Na-TC feeding may also be considered as synergistic because Na-TC and NaCl are both considered to be gastric promoters.[30, 31, 36, 37]

For use of PAPG as a marker in medium-term *in vivo* screening for gastric carcinogens and promoters, conditions must be found in which PAPG develop rapidly. Rapid production of PAPG after MNNG initiation has been observed using strong gastric carcinogens,[13] but a relatively long period is required for the effects of promoters on the development of PAPG to become significant.[13] Previously we found in a system consisting of initiation by MNNG and administration of test chemicals that 20 weeks was a suitable period for detecting weak gastric carcinogens and promoters.[13] In the present study where we tried to shorten this experimental period by use of sodium chloride, administration of sodium chloride during Na-TC treatment promoted the development of PAPG significantly from week 12 ($P<0.01$–0.001). In the design of an *in vivo* medium-term bioassay protocol, choice of a suitable experimental period is very important, because with too short a period weak gastric carcinogens and gastric promoters may not be detected. Na-TC is considered to be a weak gastric promoter,[36–38] because continuous feeding of taurocholic acid for 28 weeks after MNNG treatment for 12 weeks promoted the development of dysplasias but not adenocarcinomas,[36] and continuous feeding of Na-TC for 52 weeks after MNNG treatment for 13 weeks only resulted in the appearance of adenocarcinomas of small size and at low incidence.[38]

From the results of the present study, we propose that initiation with MNNG, administration of test chemicals for 14 weeks and treatment with sodium chloride during administration of test chemicals might be a suitable protocol for *in vivo*, medium-term screening of chemicals. However, further studies on the effects of many gastric carcinogens, carcinogens without carcinogenic activity in the stomach, gastric promoters and noncarcinogens are in progress to confirm whether this 16-week, *in vivo*, medium-term protocol is indeed applicable for detection of gastric promoters and carcinogens.

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