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Enhancing Effects of Calcium-deficient Diet on Gastric Carcinogenesis by N-Methyl-N′-nitro-N-nitrosoguanidine in Wistar Rats

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The effects of *ad libitum* feeding of calcium-deficient diet on the incidence, number and histological types of gastric cancers induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) were investigated in male Wistar rats. Rats were fed standard pellet diet containing 0.5% (normal-calcium diet) or 0.01% calcium (calcium-deficient diet) after oral treatment with MNNG for 25 weeks. Oral administration of the calcium-deficient diet resulted in a significant increase in the incidence, but not the number, of gastric cancers in experimental Week 52. However, it did not affect the histological types of cancer. The calcium-deficient diet also caused a significant increase in tissue norepinephrine concentration of the antral portion of the gastric wall and in the labeling index of the antral epithelial cells. These findings indicate that the calcium-deficient diet enhanced gastric carcinogenesis and suggest that its effect may be related to increase in norepinephrine in the gastric wall and consequent stimulation of proliferation of antral epithelial cells.

Key words: Gastric carcinogenesis — Calcium-deficient diet — N-Methyl-N′-nitro-N-nitrosoguanidine

There is evidence for the involvement of the sympathetic nervous system in chemical carcinogenesis.[1,2] Compounds that enhance the activity of the sympathetic nervous system stimulate carcinogenesis in various organs.[1] Recently, we found that prolonged administration of tyrosine methyl ester, a catecholamine precursor, after oral treatment with MNNG[4] resulted in significant increases in the tissue NE concentration of the gastric wall and the incidence of gastric cancer in Wistar rats.[3]

Dietary intake influences the activity of the sympathetic nervous system in experimental animals. Hagihara *et al.*[4] showed that a calcium-deficient diet (0.01% Ca) increased catecholamine biosynthesis in the adrenal glands. These findings suggest that administration of calcium-deficient diet might enhance gastric carcinogenesis in MNNG-treated rats. To test this possibility, in the present study we investigated the effect of *ad libitum* feeding of calcium-deficient diet on the development of gastric cancers in Wistar rats that had been treated with MNNG.

MATERIALS AND METHODS

Animals Sixty 6-week-old male inbred Wistar rats were purchased from SLC (Shizuoka). The animals were housed in stainless steel suspended wire-mesh cages under controlled environmental conditions of 12 h light and 12 h darkness, 30–50% humidity, and 20–22°C. The rats were fed *ad libitum* on standard laboratory pellets (Oriental Yeast, Tokyo).

Experimental design The animals were given drinking water containing MNNG (25 μg/ml; Aldrich, Milwaukee, WI) for 25 weeks. On each day of its administration, MNNG was dissolved in deionized water at a concentration of 1 mg/ml in a cool, dark place and diluted to 25 μg/ml with tap water just before use. Rats were given 40 ml of MNNG solution each, supplied from bottles covered with aluminum foil to prevent photolysis of MNNG, and the solution was renewed every other day. Appropriate safety precautions were taken in handling MNNG. From Week 26, the rats were given normal tap water *ad libitum* from an automatic watering system and were divided randomly into two groups of 30 rats each. These groups were given standard pellet diet containing 0.5% calcium (normal-calcium diet, Oriental) or 0.01% calcium (calcium-deficient diet, Oriental) until the end of the experiment in Week 52. The pellet diets used contained the constituents listed in Table I. The calcium content of the calcium-deficient diet was based on the results of Hagihara *et al.*[4]

Tissue sampling Animals that survived for more than 48 weeks were included in effective numbers, because the first tumor of the glandular stomach was found in a rat in Group 2 killed in Week 48. All surviving animals were killed at the end of the experiment in Week 52. All rats

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[4] Abbreviations: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; BrdU, bromodeoxyuridine; NE, norepinephrine; ODC, ornithine decarboxylase.

Table I. Composition of Pellet Diets Used

Ingredient	Content (g/100 g)	
	Normal-calcium diet	Calcium-deficient diet
β-Corn starch	41.5	41.5
Milk casein	25.0	25.0
α-Potato starch	10.0	10.0
Cellulose	8.0	8.0
Linol salad oil	6.0	6.0
Sucrose	5.0	3.51
Mineral mix	3.5[a]	3.5[b]
Vitamin mix[c]	1.0	1.0
H_3PO_4	0.0	1.49

a) Mineral mix contains 50 g of $CaHPO_4$, 7.4 g of NaCl, 22 g of $K_3C_6H_5O_7 \cdot H_2O$, 5.2 g of K_2SO_4, 2.4 g of MgO, 0.35 g of $MnCO_3$, 0.6 g of Fe-citrate (Fe 17%), 0.16 g of $ZnCO_3$, 0.03 g of $CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$, 0.001 g of $Na_2SeO_3 \cdot 5H_2O$, 0.001 g of KIO_3, and 0.055 g of $CrK(SO_4)_2 \cdot 12H_2O$ per 100 g.
b) Mineral mix contains the same components as the mineral mix for normal-calcium diet except for 50 g of $CaHPO_4$.
c) Vitamin mix contains 40,000 IU of vitamin A acetate, 10,000 IU of vitamin D_3, 500 mg of vitamin E acetate, 0.5 g of vitamin K_3, 60 mg of vitamin B_1, 60 mg of vitamin B_2, 70 mg of vitamin B_6, 0.1 mg of vitamin B_{12}, 2 mg of D-biotin, 20 mg of folic acid, 160 mg of Ca-pantothate, 300 mg of nicotinic acid, and 20 mg of choline bitartrate per 100 g.

were autopsied, and the stomach and other organs were carefully examined. The stomach was opened along the greater curvature, pinned flat on a cork mat, and fixed in Zamboni's solution[5] for histological examination. The fixed stomach was cut into longitudinal, 3 mm wide strips. The specimens were embedded in paraffin, and 5 μm thick serial sections were stained with hematoxylin and eosin. Sections were examined without knowledge of which group they were from.

Classification of gastric cancers Histologically, adenocarcinomas were defined as lesions in which neoplastic glands had penetrated the muscularis mucosae to the submucosa or deeper layers. Adenocarcinomas were classified as very well-differentiated, well-differentiated, and poorly differentiated, as reported previously.[6]

Measurement of catecholamines in the gastric wall The NE and epinephrine concentrations in gastric wall tissue were determined in Weeks 30 and 52 by high-performance liquid chromatography as reported previously.[7] For this, five unstarved rats in each group were killed by cervical dislocation between 10:00 and 11:00 A.M. and samples of about 50 mg of the wall of the fundic and antral portions of the stomach were removed from each rat. Each sample was homogenized with 4.0 ml of 0.4 *N* perchloric acid and centrifuged at 1,100*g* for 10 min. The supernatant was mixed with 1.0 ml of 1.2 *M* disodium ethylenediamine tetraacetate (EDTA), adjusted to pH 6.0 with ammonium hydroxide, mixed with 300 mg of purified alumina (Woelm Neutral Active Grade I), and adjusted to pH 8.4–8.8 with ammonium hydroxide. The mixture was stirred for 5 min and centrifuged at 10,000*g* for 10 min, and the supernatant was discarded. The precipitated alumina was washed twice with distilled water, shaken vigorously with 2.5 ml of 0.4 *N* acetate, and centrifuged. The clear supernatant was transferred to a small glass tube and lyophilized, and the residue was dissolved in 0.5 ml of 0.2 *N* acetic acid. A sample of 50 μl of this solution was injected into a liquid chromatographic column (Hitachi 3011-C gel column, 2.6×250 mm), and material was eluted with 0.1 *M* KH_2PO_4 containing 0.05% H_3PO_4 at a constant flow rate of 0.5 ml/min at 45.0±0.2°C. The effluent was mixed with the reagent for the trihydroxyindole reaction (0.0075% potassium ferricyanide, 0.1% ascorbic acid, and 5 *N* sodium hydroxide) and the resulting fluorescent products were examined in a highly sensitive spectrofluorophotometer (Hitachi 650-10, Hitachi, Tokyo).

Measurement of labeling index of gastric mucosa The labeling index of gastric mucosa was measured in Weeks 30 and 52 with an immunohistochemical analysis kit (Becton Dickinson, Mountain View, CA) for assaying BrdU incorporation.[8,9] Briefly, five unstarved rats in each group received an i.p. injection of BrdU (20 mg/kg body weight) between 9:00 and 10:00 A.M., and were killed 1 h later with ether. The stomach was fixed in 70% ethanol for 4 h. Sections of 3 μm thickness were immersed in 2 *N* HCl solution for 30 min at room temperature, and then in 0.1 *M* $Na_2B_4O_7$ to neutralize the acid. The sections were then stained with anti-BrdU monoclonal antibody (diluted 1:100) for 2 h at room temperature, washed, treated with biotin-conjugated horse anti-mouse antibody (at 1:200 dilution) for 30 min, and stained with avidin-biotin-peroxidase complex for 30 min. The reaction product was located with 3,3′-diaminobenzidine-tetrahydrochloride. Cells containing BrdU were identified by the presence of dark pigment over their nuclei.

The labeling index of the gastric mucosa was determined by counting BrdU-labeled and -unlabeled cells in the proliferating zone[10] without knowledge of which group the sample was from. The zone of proliferating cells in the fundic mucosa was defined as a 250-μm rectangular area between the highest and lowest labeled cells in well-oriented sections. Ten such rectangular areas in each rat were examined. In the antral mucosa, all cells below the highest labeled cells in each pit-gland column were regarded as being within the zone of proliferating cells. In each rat, 100 well-oriented columns of pits and glands were examined, and the labeling index was calculated as the number of BrdU-labeled cells/total number of cells within the proliferating zone.

Statistical analyses Results were analyzed by the use of Student's t test[11] or Fisher's exact probability test.[12] Data are shown as means±SE. Calculated P values of less than 0.05 were regarded as significant.

RESULTS

Incidences, numbers and depths of involvement of gastric cancers Five rats from each group were killed in Week 30 for measurement of the catecholamine concentration in the gastric wall and the labeling index of the gastric mucosa. One rat in Group 1 and one in Group 2 were killed in Weeks 49 and 48, respectively, because they became moribund. Tumors were found in these animals, which were included in the effective numbers.

The incidences, numbers and depths of involvement of gastric cancers, and the mean body weights in the groups are shown in Table II. The body weights of rats given the calcium-deficient diet were slightly, but not significantly, lower than those of control rats. In Group 1 (normal-calcium diet), gastric cancers were found in only 3 (15%) of the 20 rats examined. In Group 2 (calcium-deficient diet), the incidence of gastric cancers was significantly higher than that in Group 1. The average number of gastric cancers per tumor-bearing rat was 1.0±0.0 in Group 1, and slightly, but not significantly higher in Group 2.

All tumors induced in the glandular stomach were identified histologically as adenocarcinomas. In Groups 1 and 2, all cancers were very well-differentiated: no poorly differentiated cancers were found. Table II also shows that submucosal tumors were slightly, but not significantly, more frequent in Group 2 than in Group 1. All cancers were in the antral mucosa, and no metastasis was found in any rat.

Tissue norepinephrine, and labeling index of gastric mucosa Table III summarizes data on the NE concentrations in the gastric wall, and labeling indices of the gastric mucosa. At both times examined, the tissue NE concentration in the antral, but not the fundic portion of the stomach wall, and the labeling index of the antral, but not the fundic mucosa were significantly higher in Group 2 (calcium-deficient diet) than in Group 1 (normal-calcium diet). Epinephrine was not detected in any gastric wall sample at any time.

DISCUSSION

In the present study, we found that the calcium-deficient diet enhanced gastric carcinogenesis in Wistar

Table II. Incidence and Number of Gastric Cancers in MNNG-treated Rats

Group no.	Dietary calcium[a]	Body weight (g)		Effective no. of rats	No. of rats with gastric cancer (%)	No. of gastric cancer per tumor-bearing rat	No. of gastric cancers	Depth of involvement (%)	
		Week 26	Week 52					Submucosal layer	Muscle layer or deeper
1	0.5%	313±4	431±6	20	3 (15)	1.0±0.0	3	3 (100)	0 (0)
2	0.01%	309±5	416±12	20	12 (60)[b]	1.4±0.2	17	14 (82)	3 (18)

a) Rats were given rat chow pellets containing 0.5% calcium (normal-calcium diet) or 0.01% calcium (calcium-deficient diet) after oral treatment with MNNG for 25 weeks.
b) Significantly different from the value for Group 1 at $P<0.01$.

Table III. Norepinephrine Concentration in the Stomach Wall, Labeling Index of Gastric Mucosa and Serum Calcium Concentration in MNNG-treated Rats

Experimental week	Group no.	Dietary calcium[a]	Norepinephrine (ng/g tissue)		Labeling index (%)		Serum calcium concentration (mEq/liter)
			Fundic portion	Antral portion	Fundic mucosa	Antral mucosa	
30	1	0.5%	394±14	235±8	14.2±1.3	15.2±1.4	5.9±0.1
	2	0.01%	398±8	303±12[b]	16.6±2.4	24.4±2.1[b]	5.4±0.1[b]
52	1	0.5%	389±19	246±5	15.8±1.4	15.4±2.2	6.2±0.1
	2	0.01%	404±12	316±9[c]	17.4±1.6	27.4±2.7[b]	5.4±0.1[b]

a) For explanation of treatments, see Table I.
b, c) Significantly different from the value for Group 1: *b*) $P<0.01$, *c*) $P<0.001$.

rats. After oral treatment with MNNG for 25 weeks, *ad libitum* feeding of the calcium-deficient diet containing 0.01% calcium resulted in a significantly higher incidence of gastric cancers in Week 52 than that in rats fed normal-calcium diet containing 0.5% calcium.

Lipkin and Newmark[13] observed that oral treatment of a group of high-risk patients with calcium resulted in the return of hyperproliferative colon mucosa to a near-normal state. A plausible explanation for the antiproliferative effect of calcium is its binding to bile acids.[14, 15] However, calcium has been demonstrated to cause direct inhibition of proliferation of various epithelial cells, including mammary,[16] esophageal,[17] bronchial[18] and urothelial[19] cells, in the absence of bile acid. Guo *et al.*[20] examined the direct effect of calcium on the growth of human gastric cancer cells, finding that these cells were relatively insensitive to loss of extracellular calcium. However, Zwiller *et al.*[21] found that DNA synthesis in calcium-deprived rat liver cells was stimulated by an inositol phosphate-activated protein phosphatase. Reshef *et al.*[22] examined the effect of a calcium-enriched diet on hyperproliferation of colonic epithelium induced by MNNG in rats on a low calcium (0.05% calcium ion) and low fat diet. They found that the hyperproliferation during induction of colon cancers by the carcinogen in rats on the low calcium diet was reduced by calcium enrichment of the diet even when fat intake was low. Furihata *et al.*[23] observed that calcium chloride inhibited stimulation of replicative DNA synthesis induced in the antral mucosa of male Fischer 344 rats by sodium chloride, which is a tumor promoter in the glandular stomach. In the present study, we also found that the calcium-deficient diet increased the labeling index of the antral epithelial cells. These findings suggest that enhancement of gastric carcinogenesis by the calcium-deficient diet is related to stimulation of proliferation of antral epithelial cells.

The mechanism of this effect of the calcium-deficient diet is not known, but at least three possible mechanisms may be considered. One is an effect on mucosal ODC activity. Von Leeuwen and Herrmann-Erlee[24] studied the effect of intracellular messenger calcium on ODC activity in fetal rat osteoblasts. They found that calcium is involved in basal ODC activity, and that ODC activity is stimulated via (1) a 3′,5′-cyclic AMP-independent calcium pathway, and (2) a calcium-dependent, cyclic AMP pathway. Arlow *et al.*[25] examined the effect of calcium on azoxymethane-induced colonic mucosal ODC in rats. They reported that maximal stimulation of ODC activity by azoxymethane occurred 5 days after its injection, and that this stimulation was significantly suppressed by rat chow containing 130 mg/day calcium. They also found that 4-h exposure of colon mucosal explants in organ culture to methylazoxymethanol, an active metabolite of the carcinogen, resulted in a significant increase in ODC activity over that of controls, and that addition of calcium chloride significantly suppressed this carcinogen-induced increase in activity of ODC. Similarly Lipkin *et al.*[26] observed a decrease of ODC activity in human and rodent tissue on exposure to an increased level of calcium.

Another possible effect of calcium is on oncogenes. Several types of genetic alterations have been observed in human colon cancers,[27] one potentially important genetic alteration being *ras* gene mutation(s). Point mutations in *ras* protooncogenes have been demonstrated in 40 to 65% of malignant colorectal tumors examined.[27, 28] Jacoby *et al.*[29] reported a high incidence of guanine-to-adenine mutations in the K-*ras* gene in colon cancers induced by 1,2-dimethylhydrazine. Recently, Llor *et al.*[30] examined K-*ras* oncogene mutations in 1,2-dimethylhydrazine-induced colon tumors in rats fed calcium-supplemented diet, and found that one-third of the colon cancers induced in the control group had K-*ras* G-to-A mutations, but that these mutations were not detectable in the cancers of the calcium-supplemented group. Calcium might enhance DNA repair of O^6-methylguanine residues, thereby preventing these mutations. These findings suggest that alteration of K-*ras* mutations may be one mechanism by which calcium influences colon carcinogenesis.

A third possibility is an increase in activity of the sympathetic nervous system. Diet influences the activity of the sympathetic nervous system in experimental animals. Hagihara *et al.*[4] found that a calcium-deficient diet (0.01% Ca) induced an increase in plasma catecholamine in rats. They also reported that hyperparathyroidism caused by a calcium-deficient diet results in increased catecholamine biosynthesis in the adrenal glands. In the present work, we found that the NE concentration in the antral, but not the fundic portion of the gastric wall was increased in rats on calcium-deficient diet. Sympathetic activity can be assayed by measuring the NE concentrations in various tissues. Therefore, these findings indicate that the calcium-deficient diet increased the activity of the sympathetic nervous system in the antral portion of the stomach.

There is evidence of neural involvement in the control of cell proliferation.[31] NE appears to stimulate crypt cell proliferation in both the small and large intestine.[32, 33] We have observed significant increases in the incidence and number of gastric cancers induced by MNNG in spontaneously hypertensive rats over those in control rats. In these rats, the NE concentration in the gastric wall and the labeling index of gastric epithelial cells were both significantly increased.[34] In the present study, we found that prolonged feeding of a calcium-deficient diet caused significant increases in the NE concentration in

the antral portion of the gastric wall and in the labeling index of the antral epithelial cells. These findings indicate that enhancement of gastric carcinogenesis in animals fed the calcium-deficient diet (0.01% Ca) may be related to its effect in increasing the activity of the sympathetic nervous system.

In the present work, we found that a calcium-deficient diet (0.01% Ca) enhanced gastric carcinogenesis. However, the enhancing effect of a calcium-deficient diet may be related to not only the effect of calcium on gastric carcinogenesis but also the dose of calcium used. Guo *et al.*[20] found that in the presence of an inhibitor of intracellular Ca^{2+} release, the growth of colon cancer cell lines was inhibited in a dose-dependent manner, indicating that a minimum basal level of intracellular Ca^{2+} was required for continued proliferation of colon cancer cells. Our findings suggest that this enhancement may be caused in part by increased activity of the sympathetic nervous system.

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