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Promotion by Nialamide of Gastric Carcinogenesis Induced by N-Methyl-N′-nitro-N-nitrosoguanidine in Wistar Rats

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The effects of nialamide, a monoamine oxidase inhibitor, on the incidence, number, and histology of gastric cancers induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) were investigated in male Wistar rats. Rats were given subcutaneously 50 mg/kg body weight of nialamide in depot form every other day after 25 weeks of oral treatment with MNNG. Prolonged alternate-day administration of nialamide caused a significant increase in the incidence and number of gastric cancers of the glandular stomach in week 52. However, it did not affect the histology of the cancers. Nialamide also caused a significant increase in tissue norepinephrine concentrations in the gastric wall and in the labeling indices of the gastric mucosae. However, nialamide had no influence on serum gastrin levels in the fasting state and after re-feeding. These findings indicate that nialamide promotes gastric carcinogenesis and that this may be related to its effects in increasing norepinephrine in the gastric wall and stimulating proliferation of gastric epithelial cells.

Key words: Nialamide — Monoamine oxidase inhibitor — Norepinephrine — Gastric carcinogenesis

Recently, a possible role for the nervous system in the mechanisms of chemical carcinogenesis has been discussed.[1–3] We have previously found that stimulation[4–6] and blockade[7] of the parasympathetic nervous system may be related to the inhibition and promotion, respectively, of gastric carcinogenesis induced by MNNG[4] in Wistar rats. However, the role of the sympathetic nervous system in gastric carcinogenesis is as yet unclear. Norepinephrine is released by action of the sympathetic nervous system, and MAO normally causes breakdown of norepinephrine in the body.[8] Therefore, in the present work we examined the effect of prolonged administration of nialamide, a nonspecific MAO inhibitor, on gastric carcinogenesis in Wistar rats that had been pretreated with MNNG.

MATERIALS AND METHODS

Animals Sixty young (6-week-old) male Wistar rats were used in this study. Animals were purchased from Shizuoka Laboratory Animal Center (Shizuoka). The rats were housed in suspended wire-bottomed metallic cages in animal quarters with controlled temperature (21–22°C), humidity (30–50%), and light (12-h cycle), and had free access to regular chow pellets (Oriental Yeast Co., Tokyo).

Carcinogen and treatment The animals were given drinking water containing MNNG (25 μg/ml; Aldrich Chemical Co., Inc., Milwaukee, WI) for 25 weeks. The MNNG was dissolved in deionized water at a concentration of 2 mg/ml and the solution was kept in a cool, dark place. Just before use, the stock solution was diluted to 25 μg/ml and was given to the rats every other day from bottles covered with aluminum foil to prevent photolysis of MNNG.

Beginning at week 26, the rats were given normal tap water *ad libitum*, and were randomly divided into two groups. They were injected sc every other day, as follows, until the end of the experiment at week 52. Group 1 (30 rats) was given only the vehicle, plain olive oil. Group 2 (30 rats) was given nialamide in depot form at dosage of 50 mg/kg body weight per day.

Nialamide (Sigma Chemical Co., St. Louis, MO) was prepared as a suspension in olive oil. Injections were given sc at various sites every other day in a volume of 1 ml/kg between 2 and 3 p.m. each day. The rats in Group 1 received 1 ml/kg of plain olive oil, administered as for Group 2.

Tissue sampling Animals that survived for more than 50 weeks were included in the effective numbers because the first tumor of the glandular stomach was found in a rat from Group 1 that died in week 50. Animals were sacrificed at the end of the experiment in week 52. All rats 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 with Zamboni's solution[9] for histological examination. The fixed stomach was cut into longitudinal strips 3 mm wide. Specimens were embedded in paraffin, and serial sections 5 μm thick were cut and stained with

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[4] Abbreviations: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; BrdU, bromodeoxyuridine; MAO, monoamine oxidase.

hematoxylin and eosin. Sections were examined without knowledge of which group they were from.

Histological study Histologically, we defined adenocarcinomas as lesions in which neoplastic glandular tissue has penetrated the muscularis mucosae to involve the submucosa or deeper layers. As previously reported,[10] the adenocarcinomas were classified as highly well-differentiated, well-differentiated, or poorly differentiated.

Serum gastrin levels Serum gastrin levels in the fasting state and after re-feeding were determined in 5 rats in each group at experimental weeks 30 and/or 52. Rats were fasted for 12 h and then received one of the following sc injections: 1 ml/kg olive oil (Group 1), or 50 mg/kg nialamide (Group 2). One hour later, half of the animals in each group were anesthetized and blood was obtained by cardiac puncture. The remaining rats in each group were re-fed rat chow pellets *ad libitum* for 60 min, after which they were anesthetized and blood was obtained by cardiac puncture. The serum was separated and stored at −20°C for not more than 1 week. Its gastrin content was assayed with a radioimmunoassay kit from Dainabot Radioisotope Laboratories, Ltd. (Tokyo).[11]

Norepinephrine concentration in gastric wall tissue Norepinephrine and epinephrine concentrations in tissues of the gastric wall were determined by high-performance liquid chromatography as reported[12] in weeks 30 and 52. After a 12-h fast, five rats in each group received the following sc injections: Group 1, olive oil, 1 ml/kg; Group 2 , nialamide, 50 mg/kg. One hour later a sample of approximately 50 mg of all layers of gastric wall with no visible tumors was obtained from each rat from the fundic and antral portion of the stomach. The samples were homogenized with 4.0 ml of 0.4 *N* perchloric acid and centrifuged at 2500 rpm for 10 min. The supernatant was mixed with 1.0 ml of 0.2 *M* disodium ethylenediamine tetraacetate (EDTA), and the mixture was adjusted to pH 6.0 with ammonium hydroxide. This mixture was then added to 300 mg of purified alumina (Woelm Neutral Active Grade I) following the method described by Anton and Sayre,[13] and the pH was adjusted to 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 aspirated and discarded. The precipitated aluminum was washed twice with distilled water and then was shaken vigorously with 2.5 ml of 0.4 *N* acetate. The mixture was centrifuged, and the clear supernatant was transferred to a small glass tube and lyophilized for 3 h. The residue was dissolved in 0.5 ml of 0.2 *N* acetic acid, and a 50-μl aliquot of this solution was injected into a liquid-chromatographic column (Hitachi 3011-C gel column, 2.6×250 mm). Materials were 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, consisting of 0.0075% potassium ferricyanide, 0.1% ascorbic acid, and 5 *N* sodium hydroxide. The resulting fluorescent products were examined with a highly sensitive spectrofluorophotometer (Hitachi 650-10, Hitachi Ltd., Tokyo).

Labeling indices of gastric mucosa The labeling index of the gastric mucosa was measured in weeks 30 and 52 using an immunohistochemical analysis kit for assay of BrdU incorporation[14, 15] (Becton-Dickinson Immunocytometry System, Mountain View, CA), by the modified method described by Tada *et al.*[16] Briefly, five rats in each group were fasted for 12 h and then received the following sc injections: Group 1, olive oil, 1 ml/kg; Group 2, 50 mg/kg nialamide. One hour later the rats received an ip injection of BrdU (20 mg/kg) and were killed by ether overdose after one more hour. The stomach was fixed in 70% ethanol for 4 h. Sections 3 μm thick 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, stained with biotin-conjugated horse anti-mouse antibody (at a dilution of 1:200) for 30 min, and stained with avidin-biotin-peroxidase complex for 30 min. The reaction product was localized with 3,3′-diaminobenzidine-tetrahydrochloride. Cells containing BrdU were identified by the presence of dark pigment over their nuclei. For analysis of the BrdU labeling index of the gastric mucosa, the numbers of BrdU-labeled and unlabeled cells in the zone of proliferating cells were counted without knowledge of which treatment group the samples were from.[17] The zone of proliferating cells in the fundic mucosa was defined as a rectangle 250 μm wide between the highest and lowest labeled cells in a well-oriented section. Ten such rectangular areas were selected in each rat. In the antral mucosa, all cells below the highest labeled cell in each pit-gland column were regarded as being within the zone of the proliferating cells. We selected 100 well-oriented columns of pits and glands in each rat. From these measurements we derived the BrdU labeling index (number of BrdU-labeled cells/total number of cells within the zone of proliferation).

Statistical analysis Results were analyzed by the chi-square test or by one-way analysis of variance with Dunn's multiple comparison.[18–20] Data are given as means±SE. "Significant" indicates a calculated *P* value of less than 0.05.

RESULTS

Incidence, number, histological type, and depth of involvement of gastric cancers Ten rats in each group were killed in week 30 for determination of serum gastrin

Table I. Incidence, Number, Histological Type, and Depth of Involvement of Gastric Cancers in MNNG-treated Rats

Group no.	Treatment[a]	Body weight (g)		Effective no.	No. of rats with gastric cancer (%)	No. of gastric cancers	No. of gastric cancers per rat	Histological type		Depth of involvement	
		week 26	week 52					Highly well differentiated (%)	Well differentiated (%)	Submucosal layer (%)	Deeper layers (%)
1	Olive oil	325±5	386±5	20	3(15)	3	0.2±0.1	3(100)	0(0)	2(67)	1(33)
2	Nialamide 50 mg/kg	330±5	365±5[c]	20	11(55)[b]	15	0.8±0.2[b]	14 (93)	1(7)	11(73)	4(27)

a) Treatment regimens: Olive oil; 1 ml/kg of olive oil was given every other day after MNNG treatment for 25 weeks. Nialamide 50 mg/kg; 50 mg/kg of nialamide in depot form was given every other day after MNNG treatment for 25 weeks. Significantly different from the values in Group 1: *b*) $P<0.05$; *c*) $P<0.01$.

Table II. Serum Gastrin Levels, Norepinephrine Concentration in Gastric Wall, and Labeling Indices of Gastric Mucosae

Experimental week	Group no.	Treatment[a]	Serum gastrin level (pg/ml)		Norepinephine (ng/g tissue)		Labeling index	
			In fasting state	After re-feeding	Fundic portion	Antral portion	Fundic mucosa	Antral mucosa
30	1	Olive oil	294±27(5)[b]	—	327.4±11.5(5)	247.6±32.4(5)	0.21±0.01(5)	0.13±0.01(5)
	2	Nialamide 50 mg/kg	334±31(5)	—	468.7±35.3(5)[c]	361.5±14.2(5)[c]	0.29±0.02(5)[d]	0.20±0.01(5)[e]
52	1	Olive oil	397±33(5)	269±28(5)	331.9±13.4(5)	244.4±32.5(5)	0.18±0.01(5)	0.13±0.01(5)
	2	Nialamide 50 mg/kg	359±34(5)	220±13(5)	458.0±32.0(5)[c]	357.9±11.7(5)[d]	0.23±0.01(5)[d]	0.19±0.01(5)[d]

a) For explanation of treatments, see Table I.
b) Numbers in parentheses are numbers of rats examined. Significantly different from the values in Group 1: *c*) $P<0.05$; *d*) $P<0.01$; *e*) $P<0.001$.

levels, tissue norepinephrine concentrations, and the labeling index of the gastric mucosa. No rats died before week 50. Rats that had received prolonged administration of nialamide exhibited significantly lower body weights at the end of the experiment in comparison with untreated animals.

The incidence, number, histological type, and depth of involvement of gastric cancers are summarized in Table I. In Group 1 (olive oil only), gastric cancers were found in three (15%) of 20 rats examined, and the average number of gastric cancers per rat was 0.2±0.1. In Group 2 (nialamide at 50 mg/kg), the incidence and number of gastric cancers per rat were significantly higher than in Group 1.

All tumors induced in the glandular stomach were identified by histology as adenocarcinomas. In Group 1, all cancers were highly well-differentiated. Well-differentiated adenocarcinoma was found in only one rat from Group 2. There were no significant differences in the histological types of adenocarcinoma between the two groups. No poorly differentiated cancers were found in this series. Table I also shows that there were no significant differences in the depth of involvement of gastric cancers between the two groups. All cancers were found in the antral mucosa, and no metastases were seen in any rats.

Tissue norepinephrine, labeling index of gastric mucosa, and serum gastrin levels Table II summarizes data on serum gastrin levels, norepinephrine concentrations in the gastric walls, and labeling indices of the gastric mucosae in MNNG-treated rats in weeks 30 and/or 52.

At both times examined, tissue norepinephrine concentrations in both fundic and antral portions of the stomach, and the labeling indices of the antral and fundic mucosae were significantly higher for Group 2 (nialamide at 50 mg/kg) than for Group 1 (olive oil only). Epinephrine was not detected in any gastric wall sample at any time examined. Table II also shows that in

nialamide-treated Group 2, serum gastrin levels in the fasting state and after re-feeding were not significantly different from the values for Group 1.

DISCUSSION

The experiment reported here demonstrates that prolonged alternate-day sc injection of nialamide in depot form after 25 weeks of oral treatment with MNNG resulted in a significantly greater incidence and number of gastric cancers induced in the glandular stomach. These findings indicate that nialamide promotes gastric carcinogenesis.

The exact mechanism by which nialamide promotes gastric carcinogenesis is unclear. However, at least two possible explanations may be considered. The first is the effect of nialamide on cell proliferation in the gastric mucosa. Nialamide is a potent and long-acting MAO inhibitor. Ganrot *et al.*[21] examined MAO activities in human brain and liver in patients who had received MAO inhibitor 1–3 weeks before death with 75–125 mg daily on the indication of metastatic pain, and found that brain and liver activities were less than 5% of the mean control value. Amines in the body, such as norepinephrine, are known to be normally destroyed by MAO.[8] Therefore, nialamide might be expected to increase the norepinephrine concentration in the gastric wall. Nialamide at 50 mg/kg did, in fact, cause a significant increase in tissue norepinephrine concentrations in both fundic and antral portions of the gastric wall. Evidence had previously been obtained to support the concept of neural involvement in control of cell proliferation.[22] Norepinephrine released by action of the sympathetic nervous system appears to stimulate crypt cell proliferation in both small and large intestine.[23, 24] Tutton and Barkla[25] found that inhibition of MAO by nialamide accelerated cell division in colon tumors but did not significantly influence cell proliferation in nonmalignant tissues.

To our knowledge, there have been no reports on the effect of norepinephrine on cell proliferation of normal epithelial and malignant cells of the stomach. However, we recently found that spontaneously hypertensive rats had significantly increased norepinephrine concentrations in both fundic and antral portions of the stomach and significantly elevated labeling indices in the antral and fundic mucosae. We also observed a significantly increased incidence and number of gastric cancers induced by MNNG in comparison with control Wistar Kyoto rats.[26] Moreover, we found that prolonged administration of 6-hydroxydopamine led to a significant reduction in the norepinephrine concentration in the antral portion of the gastric wall and in the labeling index of the antral mucosa, and caused a significant reduction in the incidence and number of gastric cancers.[27] These findings indicate that promotion of gastric carcinogenesis by nialamide may be related to its effect in increasing norepinephrine in the gastric wall and in stimulating proliferation of gastric epithelial cells.

A second possible explanation is the effect of gastrin on gastric carcinogenesis. We have previously found that prolonged alternate-day administration of tetragastrin in depot form after 25 weeks of oral treatment with MNNG caused a significant reduction in the incidence of gastric cancers in Wistar rats.[4–6] Dial *et al.*[28] reported that rats pretreated with nialamide at 200 mg/kg body weight showed a greater rise in feeding-induced serum gastrin than did untreated controls, and concluded that MAO may play an important role in regulation of gastrin release from G-cells by partially controlling the level of amines within these cells. However, in the present work we found that administration of nialamide at 50 mg/kg did not elevate serum gastrin levels, with or without re-feeding. These findings suggest that endogenous serum gastrin may not be related to the promotion by nialamide of gastric carcinogenesis.

In the present work, we found that administration of 50 mg/kg body weight of nialamide in depot form had a stimulatory effect on gastric carcinogenesis. Although further investigations are required, these findings indicate that increased sympathetic nervous system activity may be related to promotion of gastric carcinogenesis.

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