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Protection by Galanin against Gastric Carcinogenesis Induced by N-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Wistar Rats[1]

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ABSTRACT

The effects of prolonged administration of the neuropeptide galanin on gastric carcinogenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the norepinephrine concentration in the gastric wall, and the labeling index of the gastric mucosa were investigated in Wistar rats. The rats received 2 or 4 μg/kg body weight of galanin s.c. every other day after 25 weeks oral treatment with the carcinogen. Prolonged administration of galanin at 4 μg/kg body weight, but not at 2 μg/kg body weight, significantly decreased the incidence of gastric cancers in experimental week 52. However, it did not influence the histological types of cancers. Galanin at 4 μg/kg body weight also significantly decreased the labeling index of the antral epithelial cells but not the norepinephrine concentration in the gastric wall. These findings indicate that galanin inhibits gastric carcinogenesis and suggest that its effect may be related to the suppression of proliferation of the antral epithelial cells.

INTRODUCTION

The neuropeptide galanin, consisting of 29 amino acids, was originally isolated from porcine intestine by Tatemoto *et al.* (1) and was subsequently found in the brain and peripheral nervous tissue in most parts of the gastrointestinal tract and pancreas (2–4). Galanin exerts various potent biological effects; *in vitro* studies have shown that it inhibits smooth muscle contraction of several animal species, inhibits pancreatic hormone secretion, stimulates insulin and glucagon secretions in the pig, inhibits amylase secretion in the rat, and increases the plasma level of growth hormone (5–9). More recent evidence has suggested that galanin may also be a sympathetic neurotransmitter in the canine pancreas (10, 11). Previously, we found that the sympathetic nervous system is involved in gastric carcinogenesis; stimulation of the sympathetic nervous system enhances carcinogenesis, whereas its suppression inhibits carcinogenesis (12, 13). High concentrations of galanin-like immunoreactivity have been found in human, pig, and rat gastric fundus and antrum (3). Recently, Sethi and Rozengurt (14) reported that galanin stimulated clonal growth of small cell lung cancer cells and that this growth-promoting effect was closely dependent on the galanin concentration. These findings suggest that administration of galanin might enhance gastric carcinogenesis. To test this possibility, in the present work, we examined its effect on gastric carcinogenesis induced by MNNG[3] in Wistar rats.

MATERIALS AND METHODS

Animals. Ninety 6-week-old male inbred Wistar rats were purchased from SLC (Shizuoka, Japan). The rats were housed in suspended, wire-bottomed metal cages in the animal quarters at a controlled temperature (20–22°C) and humidity (30–50%) with a 12-h light-dark cycle and were supplied *ad libitum* with standard laboratory pellets (Oriental Yeast, Tokyo, Japan).

Experimental Design. The animals were given drinking water containing MNNG (50 μg/ml; Aldrich, Milwaukee, WI) for 25 weeks. The MNNG was dissolved in deionized water at a concentration of 2 mg/ml, kept in a cool, dark place and diluted to 50 μg/ml with tap water just before use. The rats were given 40 ml of MNNG solution (less than a single rat can consume in 48 h) from bottles covered with aluminum foil to prevent photolysis of MNNG, and the solution was replenished every other day. From week 26, the rats were given normal tap water *ad libitum* from an autonomic water system and divided randomly into three groups of 30 rats each; they received the following s.c. injections every other day until the end of the experimental week 52: group 1, the control group, was given only the vehicle, olive oil; and groups 2 and 3 were given 2 and 4 μg/kg body weight of galanin, respectively. Galanin (porcine; Sigma, St. Louis, MO) was prepared as a suspension in olive oil. Injections were given s.c. in a volume of 1 ml/kg body weight between 2 and 3 p.m. each day.

Histological Observations. Animals that survived for more than 50 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 50. All surviving rats were killed 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 resected and opened along the greater curvature, pinned on a cork mat, and fixed in Zamboni's solution (15) for histological examination. The fixed stomach was cut into longitudinal strips of 3-mm widths. 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 and were classified as very well-differentiated, well-differentiated, and poorly differentiated, as reported previously (16).

Measurement of NE in the Gastric Wall. The NE concentration in gastric wall tissue was determined in weeks 30 and 52 by high-performance liquid chromatography as reported previously (17). For this purpose, five starved rats in each group received 1 ml/kg body weight of olive oil (group 1) or 2 or 4 μg/kg body weight of galanin (groups 2 and 3). Two h later, they were killed by cervical dislocation, 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 1100 × *g* for 10 min. The supernatant was mixed with 1.0 ml of 0.2 M EDTA, adjusted to pH 6.0 with ammonium hydroxide, mixed with 300 mg of purified aluminum (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; the supernatant was discarded. The precipitated aluminum 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 disolved 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 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 trihydroxyindol reaction (0.0075% potassium ferricyanide, 0.1% ascorbic acid, and 5 N sodium hydroxide), and the resulting fluorescent products were examined in a high sensitivity spectrofluorophoto-meter (Hitachi 650–10, Hitachi, Tokyo, Japan).

Measurement of the Labeling Index of the Gastric Mucosa. The labeling index of the gastric mucosa was measured in weeks 30 and 52 with an immunohistochemical analysis kit (Becton Dickinson, Mountain View, CA) for assaying BrdUrd incorporation (18, 19). Briefly, another five starved rats in each group were treated s.c. with 1 ml/kg of olive oil (group 1) or 2 or

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[3] The abbreviations used are: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; BrdUrd, bromodeoxyuridine; NE, norepinephrine.

Table 1 *Incidence, number, histological type, and depth of involvement of gastric cancers in MNNG-treated rats*

Group no.	Treatment[a]	Effective no. of rats	No. of rats with gastric cancer (%)	No. of gastric cancers	No. of gastric cancers per tumor-bearing rat	Histology (%)		Depth of involvement (%)	
						Very well differentiated	Well differentiated	Submucosa	Muscle layer or deeper
1	Olive oil	20	10 (50)	13	1.3 ± 0.2	10 (77)	3 (23)	12 (92)	1 (8)
2	Galanin 2 μg/kg	20	10 (50)	15	1.5 ± 0.2	12 (80)	3 (20)	15 (100)	0 (0)
3	Galanin 4 μg/kg	20	3 (15)[b]	3	1.0 ± 0.0	3 (100)	0 (0)	3 (100)	0 (0)

[a] Olive oil, 1 ml/kg of plain olive oil given s.c. every other day after MNNG treatment for 25 weeks; Galanin, 2 or 4 μg/kg of galanin given s.c. every other day after MNNG treatment for 25 weeks.
[b] Significantly different from the value for group 1 at $P < 0.05$.

4 μg/kg of galanin (groups 2 and 3). One h later, BrdUrd (20 mg/kg body weight) was injected i.p., and after another h, the animals were killed with ether. The stomach was removed and fixed in 70% ethanol for 4 h and then embedded in paraffin. Thin sections of 3-μm thickness were immersed in 2 N HCl solution for 30 min and then in 0.1 M $Na_2B_4O_7$. Slides were also immersed in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity and then treated with 10% horse serum. Specimens were incubated with anti-BrdUrd monoclonal antibody (diluted 1:20) for 2 h, washed, and stained first with biotin-conjugated horse anti-mouse antibody (Vector, Burlingame, CA; dilution, 1:200) for 30 min and then with avidin-biotin-peroxidase complex (Vector) for 30 min. The reaction product was detected with 3,3′-diaminobenzidine tetrahydrochloride. Cells that contained BrdUrd were identified by the presence of a dark pigment over their nuclei. For determination of the labeling index of the gastric mucosa, the numbers of BrdUrd-labeled and unlabeled cells in the zone of proliferating cells were counted (20) without knowledge of which treatment group the samples were from. The zone of proliferating cells in the fundic mucosa was defined as a 250-μm rectangular area between the highest and lowest cells in a well-oriented section. Ten such rectangular areas of each rat were examined. In the antral mucosa, all cells below the highest labeled cell in each pit-gland column were regarded as being within the zone of proliferating cells, and 100 well-oriented pit-gland columns in each rat were examined. On the basis of these measurements, the labeling index was calculated as the number of BrdUrd-labeled cells/total number of cells within the zone of proliferating cells.

Statistical Analysis. Results were analyzed by the χ^2 test or Fisher's exact probability test or by one-way analysis of variance with Dunn's multiple comparison (21–23). Data are shown as means ± SE. $P < 0.05$ was considered significant.

RESULTS

Incidence, Number, Histological Type, and Depth of Involvement of Gastric Cancers. Ten rats in each group were killed in week 30 for measurements of the labeling index of the gastric mucosa and the NE concentration in the gastric wall. One rat in group 2 was killed in week 50 because it became moribund. This rat had a tumor in the glandular stomach and therefore was included in the effective number.

The incidence, number, histological type, and depth of involvement of gastric cancers are summarized in Table 1. In group 1 (olive oil only), gastric cancers were found in 10 (50%) of 20 rats examined. The incidence of gastric cancers in group 3 (galanin at 4 μg/kg) was significantly less than that in group 1. However, administration of galanin at 2 μg/kg had no significant influence on the incidence of gastric cancers.

In group 1, the average number of gastric cancers per tumor-bearing rat was 1.3 ± 0.2. In group 3 (galanin at 4 μg/kg), the number of gastric cancers was slightly, but not significantly, less than that in group 1. Galanin at 2 μg/kg had no influence on the number of gastric cancers.

All tumors induced in the glandular stomach were identified histologically as adenocarcinomas. In group 1, 10 (77%) of 13 cancers were very well-differentiated. In Group 3, all cancers were very well-differentiated, but the difference was not statistically significant. No poorly differentiated cancers were found in this series. Table 1 also shows that there was no significant difference in the depths of involvement of gastric cancers in the three groups. All cancers were found in the antral portion, and no metastases were seen in any rats.

Tissue NE and Labeling Index of the Gastric Mucosa. Table 2 summarizes data on the NE concentrations in the gastric wall, and the labeling indices of gastric mucosa in each group in weeks 30 and 52. At both times, the labeling index of the antral, but not the fundic, mucosa was significantly lower in group 3 (galanin at 4 μg/kg) than in group 1 (olive oil). Treatment with galanin at 2 μg/kg (group 2) had no significant influence on the labeling index of the gastric mucosa.

Table 2 also shows that galanin had no significant influence on the tissue NE concentration in either portion of the mucosa at either time.

DISCUSSION

Our present study showed that galanin after oral treatment with MNNG inhibited gastric carcinogenesis. The dosages of galanin used in this experiment were based on the results of Soldani *et al.* (24). They observed that i.v. infusion of galanin at 4 μg/kg, but not at 2 μg/kg, significantly inhibited the bombesin- and 2-deoxy-D-glucose-stimulated gastric acid output in dogs. Therefore, we suggested

Table 2 *Norepinephrine concentration in gastric wall and labeling index of gastric mucosa*

Experimental week	Group no.	Treatment[a]	Norepinephrine (ng/g tissue)		Labeling index (%)	
			Fundic portion	Antral portion	Fundic mucosa	Antral mucosa
30	1	Olive oil	218 ± 10	235 ± 30	18.0 ± 1.6	27.2 ± 1.9
	2	Galanin 2 μg/kg	226 ± 10	224 ± 28	12.6 ± 1.4	25.6 ± 1.5
	3	Galanin 4 μg/kg	223 ± 26	237 ± 23	17.2 ± 2.2	13.6 ± 1.0[b, c]
52	1	Olive oil	222 ± 11	228 ± 20	16.6 ± 1.1	23.8 ± 1.9
	2	Galanin 2 μg/kg	234 ± 15	232 ± 26	16.8 ± 2.0	20.4 ± 1.2
	3	Galanin 4 μg/kg	224 ± 10	223 ± 22	17.2 ± 1.8	13.6 ± 0.7[b, d]

[a] For explanation of treatments, see Table 1.
[b] Significantly different from the value for group 1 at $P < 0.001$.
[c] Significantly different from the value for group 2 at $P < 0.05$.
[d] Significantly different from the value for group 2 at $P < 0.001$.

that prolonged administration of 2 and 4 μg/kg of galanin may have different effects on gastric carcinogenesis. Our present work revealed that long-term s.c. injections of galanin at 4 μg/kg, but not at 2 μg/kg, significantly decreased the incidence of gastric cancers.

Despite the wide distribution of the neuropeptide galanin, its physiological significance is not clear, but the following recent observations indicate that it may be a sympathetic neurotransmitter in the pancreas (10, 11, 25): (*a*) galanin-like immunoreactivity is present in sympathetic nerves innervating pancreatic islets (26); (*b*) administration of galanin changes pancreatic islet hormone secretion observed during electrical sympathetic nerve stimulation (27); and (*c*) the amount of galanin-like immunorectivity released during intense electrical stimulation of sympathetic nerves appears sufficient to mediate the observed impairment of insulin secretion (28).

There is much evidence supporting the concept of neural involvement in control of cell proliferation in various cell systems including rat colonic crypts (29). Moreover, a possible role for the autonomic nervous system in chemical carcinogenesis has been discussed; Gurkalo and Volfson (30) suggested that pharmacological compounds that activate the sympathetic nervous system stimulate carcinogenesis. We also found that the incidence and number of gastric cancers induced by MNNG were significantly greater in spontaneously hypertensive rats than in normotensive rats (12). It is well established that the development of hypertension in these hypertensive rats is dependent on enhanced sympathetic neural activity (31). In the present work, we found that prolonged administration of galanin at 4 μg/kg significantly reduced the incidence of gastric cancers in week 52 and that it did not increase the NE concentration in the gastric wall. These findings suggest that the effect of galanin on gastric carcinogenesis might not be related to its action as a sympathetic neurotransmitter in this experiment.

In addition to the above-mentioned effect, galanin also inhibits the postprandial releases of various neurohumoral substances including insulin, neurotensin, enteroglucagon, somatostatin, pancreatic polypeptide, and vasoactive intestinal peptide (6, 32, 33). Of these neurohumoral substances, neurotensin, somatostatin, and vasoactive intestinal peptide are closely related to the development of gastric cancers. We previously observed that prolonged administration of neurotensin (34), somatostatin (35), or vasoactive intestinal peptide (36) enhanced gastric carcinogenesis induced by MNNG in Wistar rats. In the present work, we found that prolonged administration of galanin at 4 μg/kg significantly reduced the incidence of gastric cancers induced by MNNG and also significantly decreased the labeling index of the antral epithelial cells. These findings suggest that its inhibitory effect on carcinogenesis might be closely related to its inhibition of release of cancer-promoting hormones such as neurotensin, somatostatin, and vasoactive intestinal peptide.

Evidence was rapidly accumulating that neuropeptides acting through distinct receptors and signal transduction pathways can control the proliferation of a variety of cell types (37–39). Sethi and Rozengurt (14) demonstrated that galanin stimulated a rapid mobilization of Ca^{2+} from intracellular stores and induced an increase in the production of inositol phosphates in small cell lung cancer cell lines. Their report showed for the first time that galanin receptors were coupled to inositol phosphate and $[Ca^{2+}]_i$ responses in small cell lung cancer cells and, in particular, that this neuropeptide can act as a direct factor for these human cancer cells.

The present results show that administration of galanin after oral treatment with MNNG for 25 weeks significantly reduced the incidence of gastric cancers and also significantly decreased the labeling index of antral epithelial cells. Our findings suggest that the mechanism(s) of its effect in inhibiting gastric carcinogenesis may be related to its inhibitory effect on the antral cell proliferation.

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