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Promotion by Bombesin of Gastric Carcinogenesis Induced by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Wistar Rats

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ABSTRACT

The effects of bombesin on the incidence, number, histological type, and depth of involvement of gastric cancers induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were investigated in male Wistar rats. Rats received alternate-day s.c. administration of 20 or 40 μg/kg body weight of bombesin in depot form after p.o. treatment with the carcinogen for 25 weeks. Prolonged administration of bombesin at 40 μg/kg led to a significant increase in the incidence and number per rat of gastric cancers of the glandular stomach at Week 52. In rats that had received alternate-day injections of 20 μg/kg of bombesin, the number of gastric cancers per rat, but not the incidence of cancer, was significantly more than in untreated rats. However, bombesin at both dosages did not affect the histological appearance of the lesions or their depth of involvement. At Weeks 30 and 52, norepinephrine concentrations in the fundic and antral portion of the gastric walls and labeling indices in the antral and fundic mucosae were significantly higher in rats treated with bombesin at both dosages than in untreated rats. These findings indicate that bombesin enhances gastric carcinogenesis after administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine is stopped and that this effect may be related to its effects in increasing tissue norepinephrine concentrations in the stomach wall and increasing cell proliferation in the gastric mucosa.

INTRODUCTION

Bombesin is a tetradecapeptide isolated from amphibian skin (1). In mammals, bombesin-like activity has been reported in the gastrointestinal tract, and bombesin-like peptides are considered to be putative neurotransmitters (2–4). Therefore, bombesin has important roles in the physiology and pathophysiology of the gastrointestinal system (4–9).

A new and intriguing recent development is the discovery that neuropeptides such as bombesin, vasopressin, vasoactive peptide, and neurotensin can affect the growth of various malignant cells and tissues (10, 11). We recently found that prolonged administration of neurotensin in depot form after MNNG[2] treatment significantly increased the incidence of gastric cancer (12). Bombesin-like peptides are potent mitogens for Swiss 3T3 cells (10) and have attracted interest as possible autocrine growth factors for small-cell lung carcinoma (13). Furthermore, Lhoste and Longnecker (14) found that bombesin stimulates the growth of preneoplastic acinar cell lesions of the pancreas. These findings indicate that bombesin may be closely associated with gastric carcinogenesis. However, to our knowledge, there have been no reports regarding such an effect of bombesin. Therefore, in the present work, we examined the effects of prolonged administration of bombesin on the development of gastric cancer in Wistar rats.

MATERIALS AND METHODS

Animals. Ninety young (6-week-old) male Wistar rats were used. Animals were purchased from SLC, Japan (Shizuoka, Japan). The animals 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, Tokyo, Japan).

Experimental Design. The animals were given drinking water containing MNNG (25 μg/ml; Aldrich, Milwaukee, WI) for 25 weeks. Beginning at Week 26, the animals were given normal tap water *ad libitum* and were randomly divided into three groups. They were given s.c. injections every other day, until the end of the experiment at Week 52, as follows: Group 1 (30 rats) received the vehicle (olive oil) only. Groups 2 and 3 (30 rats each) received bombesin in depot form at dosages of 20 and 40 μg/kg body weight/day, respectively.

Bombesin (Sigma Chemical Co., St. Louis, MO) was given as a suspension in olive oil. Injections were given s.c. at various sites every other day in a volume of 1 ml/kg body weight, between 2:00 and 3:00 p.m. each day. The rats in Group 1 were given 1 ml/kg body weight of plain olive oil, administered as for Groups 2 and 3.

Histological Observation. Animals that survived for more than 49 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 at Week 49. All surviving animals were killed at the end of the experiment at Week 52. The stomach was opened along the greater curvature and fixed with Zamboni's (15) solution for histological examination. Serial sections 5 μm thick were stained with hematoxylin and eosin and examined without knowledge of which group they were form.

Definition and Classification of Gastric Cancers. Histologically, we defined adenocarcinomas as lesions in which neoplastic cells had penetrated the muscularis mucosae to involve the submucosa or deeper layers. As previously reported (16), the adenocarcinomas were classified into highly well-differentiated, well-differentiated, and poorly differentiated types.

Labeling Indices of Gastric Mucosae. Labeling indices for the gastric mucosae were measured at Weeks 30 and 52 with an immunohistochemical analysis kit for assaying BrdUrd incorporation (17, 18) (Becton-Dickinson Immunocytometry System, Mountain View, CA), by the modified method described by Tada *et al.* (19). For this, the rats were fasted for 12 h and then received the following s.c. injections: Group 1, olive oil, 1 ml/kg body weight; Groups 2 and 3, bombesin, 20 and 40 μg/kg body weight, respectively. One h later, the rats received an i.p. injection of BrdUrd, 20 mg/kg body weight, and were killed 1 h later with ether.

To analyze the labeling indices of the gastric mucosae, 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 labeling index was expressed as the number of BrdUrd-labeled cells/total number of cells within the proliferation zone.

Measurement of Norepinephrine and Epinephrine in Gastric Wall Tissue. Norepinephrine and epinephrine concentrations of tissues of the gastric wall were determined by high-performance liquid chromatography as previously reported (21) at Weeks 30 and 52. After a 12-h fast, the rats received the following s.c. injections: Group 1, olive oil, 1 ml/kg; Groups 2 and 3, bombesin, 20 or 40 μg/kg, respectively. One h later, a sample of approximately 50 mg of gastric wall was obtained from each rat from the fundic and antral portions of the stomach.

Serum Gastrin Levels and Gastric Acid Secretion. Serum gastrin levels in the fasting state and after refeeding were determined at experimental Weeks 30 and/or 52. Rats were fasted for 12 h and then received one of the following s.c. injections: 1 ml/kg olive oil (Group 1), or 20 or 40 μg/kg bombesin (Groups 2 and 3, respectively). One h later, one-half of the animals in each group were anesthetized, and blood was obtained by cardiac puncture. The remaining rats in each

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

Table 1 *Incidence and number of gastric cancers in MNNG-treated rats*

Group	Treatment[a]	Body wt (g) Wk 26	Body wt (g) Wk 52	Effective no. of rats	No. of rats with gastric cancers (%)	No. of gastric cancers/rat
1	Olive oil	320 ± 5	379 ± 4	20	3 (15)	0.2 ± 0.1
2	Bombesin, 20 μg/kg	315 ± 3	405 ± 7	20	9 (45)	0.7 ± 0.2[b]
3	Bombesin, 40 μg/kg	319 ± 8	391 ± 6	20	11 (55)[b]	0.7 ± 0.1[b]

[a] Treatment regimens: olive oil: 1 ml/kg of olive oil was given every other day after 25 weeks of oral treatment with MNNG; bombesin, 20 μg/kg: 20 μg/kg of bombesin in depot form was given every other day after 25 weeks of oral treatment with MNNG; bombesin, 40 μg/kg: 40 μg/kg of bombesin in depot form was given every other day after 25 weeks of oral treatment with MNNG.

[b] Significantly different from the value for Group 1 at $P < 0.05$.

group were refed rat chow pellets *ad libitum* for 60 min, after which blood was obtained by cardiac puncture. Gastrin content was assayed with a radioimmunoassay kit from Dainabot Radioisotope Laboratories, Ltd. (Tokyo, Japan) (23).

Gastric acid secretion was measured at Week 52. Gastric acid secretions were collected for 3 h by the method of Shay et al. (24). For this, the rats were starved for 1 h and then received the following s.c. injections: Group 1, 1 ml/kg olive oil; Groups 2 and 3, 20 or 40 μg/kg bombesin, respectively. They were immediately anesthetized with ether, and the stomach pylorus was ligated.

Statistical Analysis. Results were analyzed by the χ^2 test or by one-way analysis of variance with Dunn's multiple comparison (25–27). Data are given as mean ± 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 from each group were killed at Week 30 for measurement of tissue catecholamine concentrations in the gastric wall, labeling index of gastric mucosa, or serum gastrin levels. No rats died before Week 49.

The incidence and number of gastric cancers per rat in each group are summarized in Table 1. In Group 1 (olive oil only), gastric cancers were found in 3 (15%) of 20 rats examined, and the average number of gastric cancers per rat was 0.2 ± 0.1. In Group 3 (bombesin at 40 μg/kg), the incidence and the number per rat of gastric cancers were significantly higher than in Group 1. In Group 2 (bombesin at 20 μg/kg), the incidence of tumors was elevated, although the difference was not statistically significant. The number of gastric cancers in Group 2 was significantly greater than in Group 1.

Data on the proportion of different histological types and the depth of involvement of gastric cancers in each group are summarized in Table 2. In Group 1, all cancers were histologically highly well-differentiated. In Groups 2 and 3, well-differentiated cancers were slightly increased. However, the difference in the proportion of highly well-differentiated cancers in the three groups was not significant. No poorly differentiated cancers were found in this series. Table 2 also shows that there was no significant difference in the incidence of submucosal cancers in the three groups. All cancers were found in the antral mucosa, and no metastases were seen in any rats.

Tissue Norepinephrine, Labeling Indices, Serum Gastrin, and Gastric Acid Secretion. Table 3 summarizes data on norepinephrine concentrations in the gastric walls and the labeling indices of the gastric mucosae in each group at Weeks 30 and 52. At both times examined, tissue norepinephrine concentrations in the fundic and antral portions and labeling indices of fundic and antral mucosae for Group 2 (bombesin at 20 μg/kg) and Group 3 (bombesin at 40 μg/kg) were significantly higher than in Group 1 (olive oil only). Epinephrine was not detected in any samples obtained from gastric walls at all times examined.

At Weeks 30 and 52, administration of bombesin caused slight increases in serum gastrin levels, in the basal state and after refeeding, and in gastric acid secretion, although there was no significant difference in these parameters among the three groups.

DISCUSSION

In the present work, we found that prolonged alternate-day s.c. injections of bombesin at 20 and 40 μg/kg body weight in depot form after 25 weeks of MNNG treatment significantly increased the number and/or the incidence of gastric cancers in the glandular stomach at Week 52. Although the exact mechanism(s) is still unclear, at least four possible explanations can be considered.

The first is the trophic action of gastrin on the gastric mucosa. Bombesin has been reported to have a powerful stimulatory effect on gastric acid secretion and the release of gastrin from the antral mucosa (8, 9). The trophic effects of gastrin on mucosal cells of fundic mucosa of the stomach are well established (28). However, studies on the effect of gastrin on antral mucosal cells have provided conflicting results. We previously found that prolonged administration of tetragastrin in depot form after MNNG treatment resulted in a significant decrease in the incidence of gastric cancers (29–31) and that tetragastrin significantly decreased the labeling index of the antral mucosa (16). We recently found that prolonged administration of cysteamine after MNNG treatment significantly reduced the incidence and number of gastric cancers and significantly increased the serum gastrin levels (32). These findings indicate that exogenous and endogenous gastrin may be closely linked to inhibition of gastric carcinogenesis. Furthermore, in the present work, we found that prolonged administration of bombesin did not significantly increase the serum gastrin level or gastric acid secretion, although these two parameters were slightly elevated. Although the exact reason for this finding is unknown, Walsh and Reeve (4) reported that prolonged i.v. infusion of bombesin leads to marked diminishing of the gastrin response in plasma to levels near those found during fasting.

The second possible explanation is the effect of other peptides released by bombesin. With larger doses of bombesin, gastric

Table 2 *Histological type and depth of involvement of gastric cancers in MNNG-treated rats*

Group	Treatment[a]	No. of gastric cancers	Histology (%) Highly well-differentiated	Histology (%) Well-differentiated	Depth of involvement (%) Submucosa	Depth of involvement (%) Muscularis propria or deeper
1	Olive oil	3	3 (100)	0 (0)	2 (67)	1 (33)
2	Bombesin, 20 μg/kg	14	9 (64)	5 (36)	12 (86)	2 (14)
3	Bombesin, 40 μg/kg	14	11 (79)	3 (21)	14 (100)	0 (0)

[a] For explanation of treatments, see Table 1.

Table 3 *Norepinephrine concentration in the stomach wall and labeling index of gastric mucosa in MNNG-treated rats*

Experimental wk	Group	Treatment[a]	Norepinephrine (ng/g tissue)		Labeling index (%)	
			Fundic portion	Antral portion	Fundic mucosa	Antral mucosa
30	1	Olive oil	328 ± 17	232 ± 15	23 ± 1	15 ± 1
	2	Bombesin, 20 μg/kg	425 ± 27[b]	1041 ± 193[c]	39 ± 1[d]	26 ± 2[c]
	3	Bombesin, 40 μg/kg	578 ± 22[d]	1114 ± 137[c]	41 ± 1[d]	31 ± 2[d]
52	1	Olive oil	332 ± 13	244 ± 33	20 ± 1	13 ± 1
	2	Bombesin, 20 μg/kg	469 ± 58[b]	944 ± 165[d]	35 ± 3[b]	21 ± 2[b]
	3	Bombesin, 40 μg/kg	564 ± 57[b]	1085 ± 162[d]	41 ± 4[c]	24 ± 2[c]

[a] For explanation of treatments, see Table 1.
[b] Significantly different from the value for Group 1, $P < 0.05$.
[c] Significantly different from the value for Group 1, $P < 0.01$.
[d] Significantly different from the value for Group 1, $P < 0.001$.

acid secretion declines (33, 34), probably as a result of concomitant release of one or more inhibitory peptides, *e.g.*, somatostatin. In addition to gastrin, bombesin releases many other peptides, *e.g.*, pancreatic polypeptide from the pancreas, somatostatin from the stomach, and cholecystokinin, insulin, glucagon, and neurotensin from the intestine (34). These peptides are known to regulate the growth of the gastrointestinal tract and the pancreas. We recently found that prolonged administration of neurotensin and somatostatin in depot form after 25 weeks of p.o. treatment with MNNG caused a significant increase in the incidence of gastric cancers in the glandular stomach in rats (12).

The third possible explanation is the effect of bombesin on DNA synthesis and cell division. In serum-free medium, bombesin induced DNA synthesis and cell division in the absence of other growth-promoting agents (10). Protein kinase C has received considerable attention because it is a major receptor for the tumor promoters of the phorbol ester family. However, activation of protein kinase C represents one of the pathways through which bombesin can initiate cell proliferation. Bombesin receptor is associated with a thyrosine-specific protein kinase that first phosphorylates the receptor protein itself and then a variety of cytoplasmic proteins (35).

The fourth possible explanation is the effect of bombesin on the sympathetic nervous system (36). Intracisternal administration of bombesin has been found to have dramatic effects on sympathetic activity (37). Brown *et al.* (38) found that bombesin increased sympathoadrenomedullary activity, as assessed by increased plasma norepinephrine and epinephrine. In the present work, we found that prolonged administration of bombesin caused a significant increase in tissue norepinephrine concentrations in the gastric walls. Recently, evidence, both direct and implied, has accumulated to support the concept of neural involvement in controlling cell proliferation in various cell systems. Norepinephrine released by actions of the sympathetic nervous system appears to stimulate crypt cell proliferation in both small and large intestine (39). In the present work, we found that administration of bombesin significantly increased the tissue norepinephrine concentrations in gastric walls. We recently found that the incidence and number per rat of gastric cancers induced by MNNG were significantly greater in spontaneously hypertensive rats than in control normotensive rats (40). We also found that norepinephrine concentrations in the gastric wall and labeling indices in the gastric mucosa were significantly higher in spontaneously hypertensive rats. These findings indicate that increased sympathetic nervous system activity enhances the development of gastric cancers.

In the present work, we found that bombesin promotes gastric carcinogenesis and that its elevation of the labeling indices of gastric mucosae may be related to enhancement of gastric carcinogenesis. Small cell lung cancers were associated with ectopic production of many different hormones, including vasopressin, adrenocorticotropin, and bombesin. In view of the potent mitogenic activity of bombesin in the Swiss 3T3 model system, Woll and Rozengurt (10) suggested that secretion of these neuropeptides could constitute part of an autocrine growth circuit. These findings indicate that endogenous peptide hormones could be tumor promoters and that sustained mitogenesis may be closely related to cancer promotion.

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