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Enhancement by Prolonged Administration of Caerulein of Experimental Carcinogenesis Induced by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Rat Stomach

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ABSTRACT

The effect of caerulein on the incidence and histology of gastric adenocarcinomas induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was investigated in inbred Wistar rats. Prolonged alternate-day administration of caerulein at 10 μg/kg body weight after treatment with the carcinogen for 20 weeks significantly increased the incidence and number of adenocarcinomas of the glandular stomach. Histological examination showed that treatment with caerulein had no influence on the histology of induced adenocarcinomas. Furthermore, administration of caerulein resulted in a significant increase in the bromodeoxyuridine-labeling indices of the antral mucosa but did not influence the bromodeoxyuridine-labeling indices of the fundic mucosa and the carcinomas. These findings indicate that caerulein enhances gastric carcinogenesis and that the effect may be related to the promoting effect of caerulein on cell proliferation in the antral mucosa.

INTRODUCTION

Gastrointestinal peptides have been found to regulate the growth of normal gastrointestinal mucosa and the pancreas (1). Furthermore, several gastrointestinal peptides, such as secretin (2) and vasoactive intestinal peptide (3), have been shown to be closely related to carcinogenesis in various organs. We previously found that prolonged administration of tetragastrin in depot form after MNNG[2] treatment significantly reduced the incidence and number of gastric adenocarcinomas in the glandular stomach of Wistar rats (4). Recently, we concluded that this inhibitory effect of tetragastrin on gastric carcinogenesis may be related to its inhibiting effect on cell proliferation in the antral mucosa (5).

Caerulein, a synthetic peptide sharing all known biological properties of cholecystokinin, is known to have a trophic effect on the pancreas and the gallbladder mucosa (6–8). Recently, caerulein was shown also to exert a trophic action on mucosal cells of the antrum (9–11). Therefore, it seemed likely that prolonged administration of caerulein would enhance gastric carcinogenesis. To test this possibility, we examined the effect of prolonged administration of caerulein in depot form on the incidence and number of gastric carcinomas in Wistar rats that had been treated with MNNG.

MATERIALS AND METHODS

Young male inbred Wistar rats about 8 weeks old, initially weighing 150–170 g, were given drinking water containing MNNG (50 μg/ml; Aldrich Chemical Co., Milwaukee, WI) for 20 weeks. The MNNG was dissolved in deionized water at a concentration of 2 mg/ml and kept in a cool dark place. The stock solution was diluted to 50 μg/ml with tap water just before use and given to rats every other day from bottles covered with aluminum foil to prevent photolysis of MNNG. From Week 21 to the end of the experiment, the rats received normal tap water and were divided into three groups. From 3 days after cessation of MNNG administration, the animals were treated as follows: Group 1 (25 rats) received alternate-day s.c. injections of the vehicle, olive oil, at 1 ml per kg body weight; Groups 2 and 3 (25 rats each) received caerulein in depot form on alternate days at dosages of 2 and 10 μg/kg body weight per day, respectively. Caerulein (Sigma, St. Louis, MO) was given as a suspension in olive oil. Injections were given s.c. in various sites every other day in a volume of 1 ml/kg body weight, between 2 and 3 p.m. each day.

The three groups were kept in different cages under otherwise identical conditions in the same room throughout the experiment and had free access to chow pellets (Oriental Yeast Co., Tokyo, Japan).

Animals that survived for more than 48 experimental weeks were included in the data, because the earliest tumor in the glandular stomach was found in a rat of Group 1 that died at Week 48. Rats were killed when they became moribund, and surviving animals were killed at the end of Week 52. All animals were autopsied and their stomach and other organs were carefully examined. The stomach was opened, pinned flat on a cork mat, and fixed with Zamboni's solution (12) for histological examination. The fixed stomach was cut into longitudinal strips 3 mm wide. The specimens were embedded in paraffin, and serial sections 5 mm wide were stained with hematoxylin and eosin. Sections were examined without any knowledge of which group they were from.

For the histological examination, we defined adenocarcinomas as lesions in which neoplastic glands had penetrated the muscularis mucosae to involve the submucosa or deeper layers. As reported previously (5), the adenocarcinomas were classified as highly well, well, or poorly differentiated. On the basis of their mucin-producing activity, well-differentiated adenocarcinomas were subdivided into common and mucinous types, and poorly differentiated cancers were subdivided into anaplastic and signet-ring cell carcinomas.

The BrdUrd-labeling indices of the gastric mucosa and cancers were examined at Weeks 25 and/or 52 with an immunohistochemical analysis kit for assay of BrdUrd incorporation (13, 14) (Becton Dickinson Immunocytometry System, Mountain View, CA), by the modified method of Tada *et al.* (15). After starvation for 12 h, the rats received one of the following s.c. injections: 1 ml/kg body weight olive oil (Group 1); or 2 or 10 μg/kg body weight caerulein (Groups 2 and 3, respectively). Three h later, they received an i.p. injection of 20 mg/kg body weight BrdUrd, and 1 h later they were killed with ether. Cells containing BrdUrd were identified by the presence of dark pigment over the nuclei. For analysis of the BrdUrd-labeling index of the gastric mucosa, the numbers of BrdUrd-labeled and unlabeled cells in the zone of proliferating cells were counted without knowledge of which treatment group the samples were from (16). The zone of proliferating cells in the fundic mucosa was defined as a rectangular 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 BrdUrd-labeling index (number of BrdUrd-labeled cells/total number of cells within the zone of proliferating cells). For analysis of BrdUrd-labeling index of the gastric cancers, the numbers of BrdUrd-labeled and unlabeled cells were counted in 20 or more neoplastic glands at the peripheral part of the tumors. The

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

BrdUrd-labeling index of the cancers is expressed as number of BrdUrd-labeled cells/total number of cancer cells.

Gastric acid secretion and serum gastrin level were examined at Week 52. Gastric secretions were collected for 3 h by the method of Shay *et al.* (17). After the stomach pylorus was ligated, the rats received the following s.c. injections: 1 ml/kg body weight olive oil (Group 1); or 2 or 10 μg/kg body weight of caerulein (Groups 2 and 3, respectively). Three h later, the fluid in the gastric cavity was collected, and its acid content was determined by titration of a 2-ml portion with 0.1 N NaOH to pH 7.0 using a glass electrode. Then the acid output was calculated.

To measure the serum gastrin level, rats were starved for 24 h and then given the following s.c. injections: 1 ml/kg body weight olive oil (Group 1); or 2 or 10 μg/kg body weight of caerulein (Groups 2 and 3, respectively). One h later, blood was obtained by cardiac puncture. Gastrin content was assayed with a radioimmunoassay kit from Dainabot Radioisotope Laboratories, Ltd. (Tokyo, Japan) (18).

Results were analyzed by the χ^2 test (19) or one-way analysis of variance with Dunn's multiple comparison (20–22). Data are given as means ± SE. "Significant" indicates a calculated P value of <0.05.

RESULTS

Incidences and Numbers of Gastric Cancers. At Week 52, all rats that had received the carcinogen and then caerulein had slightly, but not significantly, higher body weights than the control group.

Five rats in each group were killed at Week 25 and the labeling index of the gastric mucosa was determined. No animals died between Weeks 26 and 48.

The incidences and numbers of gastric cancers per animal in each group are summarized in Table 1. In Group 1 (MNNG and olive oil), gastric cancers were found in 7 (35%) of 20 rats examined, and the average number of gastric cancers per animal was 0.5 ± 0.2. In Group 3 (MNNG and caerulein at 10 μg/kg) the incidence of gastric cancers and number of cancers per rat were significantly higher than in Group 1. In contrast, the incidence and number of gastric cancers in Group 2 (MNNG and caerulein at 2 μg/kg) were similar to those in Group 1.

All cancers were found in the antral mucosa, and no metastases were seen in any rats.

Histological Types and Depths of Involvement of Gastric Cancers. Table 2 shows data on the incidence of different histological types and the depth of involvement of gastric cancer in each group. All the cancers induced in the glandular stomach were identified histologically as adenocarcinomas. There were no significant differences in the histological types of the adenocarcinomas in the three groups. No poorly differentiated adenocarcinomas were found in any group. Submucosal cancers were more frequent, but not significantly so, in Group 3 (MNNG and caerulein at 10 μg/kg).

BrdUrd-labeling Index, Serum Gastrin Level, and Gastric Acid Secretion. Table 3 summarizes the data on the BrdUrd-labeling indices of gastric mucosa and carcinoma in each group at Weeks 25 and/or 52. At both weeks the BrdUrd-labeling index of the antral mucosa was significantly higher than the control (Group 1) value in Group 3 (MNNG and caerulein at 10 g/kg), but not in Group 2 (MNNG and caerulein at 2 μg/kg). In contrast, at neither Week 25 nor Week 52 did caerulein at either dosage show an influence on the BrdUrd-labeling indices of the fundic mucosa and gastric carcinomas.

Table 4 also shows that the caerulein dosages in Groups 2 and 3 resulted in a significant increase in the gastric acid secretion but had no influence on serum gastrin level.

Table 1 *Incidences and numbers of gastric cancers in MNNG-treated rats*

Group	Treatment[a]	Body wt (g) Wk 20	Body wt (g) Wk 52	Effective no. of rats	No. of rats with gastric cancer (%)	No. of gastric cancers/rat
1	MNNG + olive oil	358 ± 5	410 ± 13	20	7 (35)	0.5 ± 0.2
2	MNNG + caerulein, 2 μg/kg	355 ± 8	414 ± 7	20	6 (30)	0.5 ± 0.2
3	MNNG + caerulein, 10 μg/kg	360 ± 9	415 ± 10	20	15 (75)[b]	1.1 ± 0.2[b]

[a] Group 1, 1 ml/kg of olive oil was given after MNNG treatment for 20 weeks. Group 2, 2 μg/kg of caerulein were given every other day after MNNG treatment for 20 weeks; Group 3, 10 μg/kg of caerulein were given every other day after MNNG treatment for 20 weeks.

[b] Significantly different from the value in Group 1; $P < 0.05$.

DISCUSSION

In the present work, we found that administration of caerulein in depot form after MNNG treatment for 20 weeks resulted in a significant increase in the incidence and number of gastric carcinomas in the glandular stomach at Week 52.

Hudd *et al.* (23) reported that cholecystokinin inhibited the growth of human cholangiocarcinoma xenografted into nude mice. Moreover, Yasui *et al.* (24) recently examined the effects of four types of COOH-terminal cholecystokinin fragments on the growth of xenotransplantable human gastric cancer (SC-6-JCK) derived from a poorly differentiated adenocarcinoma, and found that treatment with cholecystokinin octapeptide and its glutaryl analogue for 30 days caused a significant decrease in the weight and size of the tumors compared with those of the control. They previously demonstrated that prolonged administration of pentagastrin promotes the growth of the xenotransplantable human carcinoma (SC-6-JCK) in nude mice (25) and concluded that proliferation of gastrin-dependent human gastric cancers may be suppressed by cholecystokinin in competition with gastrin (24). However, Kobori *et al.* (26) examined the effects of many gastrointestinal peptides on the growth of the rat gastric carcinoma cell line BV9 and found that cholecystokinin-pancreozymin and caerulein increased the number of stomach cancer cells from 150% to 310% of the number of control cells cultured in a serum-free, hormone-free medium. Furthermore, unlike the gastric carcinoma cell line they used, we previously reported that prolonged administration of tetragastrin resulted in a significant decrease in the incidence and number of gastric carcinomas induced by MNNG (5). In the present work, we found that caerulein had no influence on the BrdUrd-labeling index of the gastric cancer cells.

Gastrointestinal peptides are well known to regulate growth of normal cells in the gastrointestinal tract and pancreas (1).

Table 2 *Histological types and depths of involvement of gastric cancers in MNNG-treated rats*

Group	Treatment[a]	No. of gastric cancers	Highly well differentiated (%)	Well differentiated (%) Common type	Well differentiated (%) Mucinous type	Depth of involvement (%) Submucosa	Depth of involvement (%) Muscle layer or deeper
1	MNNG + olive oil	10	6 (60)	4 (40)	0 (0)	8 (80)	2 (20)
2	MNNG + caerulein, 2 μg/kg	9	8 (89)	1 (11)	0 (0)	6 (67)	3 (33)
3	MNNG + caerulein, 10 μg/kg	22	14 (64)	8 (36)	0 (0)	21 (95)	1 (5)

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

Table 3 *BrdUrd-labeling indices of gastric mucosa and carcinoma in MNNG-treated rats*

Experimental Wk	Group	Treatment[a]	BrdUrd-labeling index[b] (mean ± SE)		
			Fundic mucosa	Antral mucosa	Carcinoma
25	1	MNNG + olive oil	0.20 ± 0.02	0.13 ± 0.01	
	2	MNNG + caerulein, 2 μg/kg	0.21 ± 0.02	0.15 ± 0.01	
	3	MNNG + caerulein, 10 μg/kg	0.20 ± 0.02	0.31 ± 0.02[c]	
52	1	MNNG + olive oil	0.20 ± 0.02	0.15 ± 0.01	0.38 ± 0.06
	2	MNNG + caerulein, 2 μg/kg	0.18 ± 0.01	0.20 ± 0.01	
	3	MNNG + caerulein, 10 μg/kg	0.20 ± 0.02	0.39 ± 0.03[c]	0.37 ± 0.07

[a] For explanation of treatments, see Table 1.
[b] BrdUrd-labeling index was expressed as number of BrdUrd-labeled nuclei/total number of cells examined.
[c] Significantly different from the values in Groups 1 and 2; for both $P < 0.001$.

Table 4 *Serum gastrin level and gastric acid secretion in MNNG-treated rats in Wk 52*

Group	Treatment[a]	Mean ± SE	
		Serum gastrin (pg/ml)	Gastric acid secretion (meq/h)
1	MNNG + olive oil	452 ± 63	0.041 ± 0.004
2	MNNG + caerulein, 2 μg/kg	486 ± 88	0.107 ± 0.015[b]
3	MNNG + caerulein 10 μg/kg	435 ± 41	0.092 ± 0.011[c]

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

The first report on the growth-promoting activity of a gastrointestinal peptide, pentagastrin, in the stomach was published by Crean *et al.* (27) in 1969. However, hormonal factors that control cell proliferation in the antrum are still unknown (11). Cholecystokinin and its derivatives such as caerulein are another type of gastrointestinal peptide able to stimulate cell growth in certain areas of the intestine, namely the exocrine pancreas, the duodenum, and the gallbladder mucosa (6–8). Normal growth of gastric fundus mucosa is not stimulated by cholecystokinin. Recently, Caes and Willems (9–11) found that administration of caerulein to normal rats provoked significant increases in both labeling and mitotic indices and a significant acceleration of upward cell migration in the glandular tube of the antral mucosa. In the present work, we found that administration of caerulein at 10 μg/kg body weight to rats resulted in a significant increase in the BrdUrd-labeling index of the antral mucosa. In contrast, Balas *et al.* (28) found that cholecystokinin did not influence labeling index or mucosal weight of the antrum in mice. These discrepancies may be explained by differences in the experimental animals used. However, the direct nature of the trophic effect of caerulein on the antral mucosa could not be established. Caes and Willems (11) pointed out that increased pancreaticobiliary reflux into the stomach, increased gastric acid secretion, and delayed gastric emptying are frequently invoked to explain the observed effect of cholecystokinin on the antral mucosa. In the present work, we found that caerulein stimulates gastric acid secretion, but no data on the stimulation of antral mitotic activity by acid have been reported.

In the present work, we found that administration of caerulein after MNNG treatment enhances gastric carcinogenesis. However, caerulein was administered shortly after cessation of MNNG administration. Under these conditions unrepaired DNA adducts are likely to be present such that stimulation of cell proliferation could enhance neoplastic conversion of cells ultimately resulting in increased cancer formation (29). Therefore, both cocarcinogenic and promoting effects may contribute to caerulein enhancement of gastric carcinogenesis. The exact mechanism is unknown and needs further investigation, but the increased cell proliferation of the antral mucosa induced by caerulein after MNNG treatment may be related to increased development of gastric cancers.

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