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Protective Effect of Oral Cysteamine Against Induction of Gastric Cancer by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Wistar Rats

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Abstract—*The effect of oral administration of cysteamine (2-aminoethanethiol hydrochloride) on the incidence and histology of gastric adenocarcinomas induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was investigated in inbred Wistar rats. Oral administration of 0.4% cysteamine in food after treatment with MNNG for 25 weeks significantly reduced the incidence and number of adenocarcinomas of the glandular stomach in experimental Week 52. Histological examination showed that adenocarcinomas that did develop in rats fed on cysteamine had high mucin-producing activity. Furthermore, oral administration of cysteamine caused a significant increase in serum gastrin level and significant decreases in the antral mucosal pH and the labeling indices of the antral mucosa. These findings indicate that cysteamine inhibits the development of gastric adenocarcinomas when given orally. This effect may be related to its ability to decrease proliferation of antral mucosal cells.*

INTRODUCTION

SOME gastrointestinal peptides, such as secretin [1], vasoactive intestinal peptide [2] and cholecystokinin and its analogs [3], have been found to be closely associated with carcinogenesis in the gastrointestinal tract and the pancreas. We have previously found [4] that long-term administration of tetragastrin in depot form after MNNG treatment resulted in a significant reduction in the incidence of gastric adenocarcinomas in the stomach of Wistar rats. Further, we have recently found [5] that prolonged subcutaneous administration of 2-aminoethanethiol hydrochloride (cysteamine, a potent duodenal ulcerogen) after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) caused significant increases in serum gastrin and acid secretion and a significant decrease in the incidence and number of adenocarcinomas of the glandular stomach. Deveny *et al.* [6] also reported that hypergastrinemia has a protective effect against gastric cancer induced by MNNG in rats, and that this effect is probably mediated by acid secretion. These findings suggest that pharmacological compounds causing hypergastrinemia and hypersecretion of acid should inhibit gastric carcinogenesis. However, to assess their potential value in the prevention of gastric cancers in humans, a suppressive effect on the development of adenocarcinomas of the stomach by oral administration of these compounds should be established. Therefore, in the present work, we examined the effect of oral administration of cysteamine on the incidence, number and histological types of adenocarcinomas induced by MNNG in rats.

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MATERIALS AND METHODS

Young male Wistar rats (n = 105), aged about 6 weeks and weighing 100–130 g, were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Of the 105 rats, 75 were given drinking water containing MNNG (50 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 kept in a cool, dark place. The stock solution was diluted to 50 μg/ml with tap water just before use. Forty milliliters of MNNG solution (which is less than any rat consumes in 48 h) were given to each rat from bottles covered with aluminum foil to prevent de-

naturation of the MNNG by light. The bottles were replenished every other day after confirming that they were empty. When there was residual water in the bottles, its volume was measured and a corresponding volume of MNNG solution was added. From experimental Week 26 onwards, the MNNG-treated rats were given normal tap water. They were divided into two groups, which were treated as follows until the end of the experiment: Group 1 (35 rats) was given *ad libitum* regular laboratory pellets (Oriental Yeast Co., Tokyo, Japan) containing no cysteamine; Group 2 (35 rats) received 0.4% cysteamine orally. Cysteamine (Aldrich Chemical Co.) was added to regular laboratory pellets and given *ad libitum* until the end of the experiment. As a control group, Group 3 (35 rats) received cysteamine orally from Week 26 onwards but did not received MNNG.

The three groups were kept in the same room in different cages with wire mesh bottoms to reduce coprophagia throughout the experiment. The pellets with or without cysteamine were given in isocaloric amounts (60 kcal/day). Body weights were measured every 4 weeks until the end of the experiment. Food consumption was measured in 10 rats from each group for a period of 1 week at the end of Weeks 30 and 45.

Animals that survived for more than 45 experimental weeks were included in effective numbers, because the first tumor of the glandular stomach was found in a rat in Group 1 that died in Week 45. Animals were killed when they became moribund during the experiment or at the end of experimental Week 52. All animals were autopsied and the stomach and other organs were carefully examined. The stomach was opened, pinned flat on a cork mat and fixed with Zamboni's solution [7] for histological examination. Longitudinal strips 3 mm wide were prepared from visible tumors and suspicious lesions. The specimens were then embedded in paraffin and sections 5 μm thick were stained with hematoxylin and eosin. In addition to tumors, flat mucosa from the fixed stomach with no visible tumors was cut into strips 3 mm wide, and serial sections were examined microscopically for cancerous foci. Serial sections were made whenever necessary to ensure that individual cancerous lesions were not counted more than once. Sections were examined without knowledge of which group they were from.

Histologically, adenocarcinomas were defined as lesions in which neoplastic glands had penetrated the muscularis mucosae to involve the submucosa or deeper layers. As previously reported [8], the adenocarcinomas were classified into highly, well, and poorly differentiated adenocarcinomas. On the basis of their mucin-producing activity, well-differentiated adenocarcinomas were subdivided into a common type and a mucinous type, while poorly differentiated cancers were subdivided into anaplastic and signet-ring-cell carcinomas.

A lesion in which the constituent cells of the gland stained hyperchromatically, the course and size of the glands were irregular and the nuclei were pleomorphic was defined as atypical glandular hyperplasia (AGH) when it was confined to the mucosa and did not penetrate the muscularis mucosae. AGH was classified into three grades: mild, moderate and severe [9].

Serum gastrin level, antral pH and the labeling index of gastric mucosa were examined in Weeks 30 and 52. All examinations were done within 1 week.

Five non-moribund animals from each group were used in both weeks for measurement of basal serum gastrin level and basal antral mucosal pH. Rats were fasted for 24 h and then anesthetized. Blood was obtained from a femoral vein, and the pH of the antral mucosa was measured with a fine pH electrode after opening the stomach along the greater curvature. A different set of five non-moribund rats from each group was used in both weeks for measurement of the serum gastrin response to refeeding and antral pH after refeeding. For this, the rats in Group 1 and in Groups 2 and 3 were fasted for 24 h and then re-fed *ad libitum* on rat pellets, with or without cysteamine, for 60 min, after which the animals were anesthetized and blood was obtained from a femoral vein. The pH of the antral mucosa was also measured after removal of gastric contents. The serum was separated and stored at −20°C, and within 1 week its gastrin content was assayed with a radioimmunoassay kit from Dainabott Radioisotope Laboratories (Tokyo, Japan) [9].

The labeling index of gastric mucosa was determined in different rats from those used for the gastrin or pH measurements. The labeling index of gastric mucosa was measured in five non-moribund rats from each group in both weeks with an immunohistochemical analysis kit for assay of bromodeoxyuridine (BrdU) incorporation [10, 11] from Becton Dickinson Immunocytometry System (Mountain View, CA), by the modified method described by Tada *et al.* [12]. Briefly, the rats in Group 1 and in Groups 2 and 3 were fasted for 24 h and then re-fed *ad libitum* on rat pellets with or without cysteamine for 2 h. One hour later they received an intraperitoneal injection of 20 mg/kg body wt of BrdU and were killed 1 h later with ether. 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 immersed in methanol containing 3% H_2O_2 for 30 min and treated with 10% porcine serum. They were next stained with anti-BrdU monoclonal antibody (dilution 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 that contained BrdU were identified by the presence of a dark pigment over their nuclei. To measure the labeling index, only sections that were cut perpendicular to the surface of the gastric mucosa and contained the entire length of the gland from its bottom to its mouth were used in all groups. For this, the numbers of BrdU-labeled cells were counted in 25 glands or among 1000 cells of gastric mucosa on each slide, in ignorance of the treatment group, and the labeling index was expressed as the number of labeled nuclei per gland or the percentage of labeled cells among the total cells counted.

Results were analyzed by the chi-square test [13] or by one-way analysis of variance with Dunn's multiple comparison [14–16]. Data are given as means ± S.E. 'Significance' indicates a calculated P value of less than 0.05.

RESULTS

Body weight and carcinogen and food consumption

Body weights of three groups were comparable throughout the experimental period. Until the end of Week 25, Group 1 and 2 rats consumed similar amounts of water containing MNNG. Food consumption measured at the end of Weeks 30 and 45 indicated that there was no difference in food intake among the three groups. The average amounts of ingested food per day in Groups 1, 2 and 3 were 23, 25 and 25 g, respectively, in Week 30, and 15, 15 and 17 g, respectively, in Week 45.

Incidence and number of gastric cancers

Fifteen rats in each group were sacrificed in Week 30 for examination of serum gastrin level, antral pH and the labeling index of gastric mucosa. No rats died between Weeks 30 and 45. One rat died in Groups 1 and 2 in Weeks 45 and 47, respectively. One gastric cancer was found in these two animals, which were included in the effective numbers.

The incidence and number of gastric cancers in each group are summarized in Table 1. In Group 1 (MNNG alone), gastric cancers were found in 16 (80%) of the 20 rats. The incidence of gastric cancers in Group 2 (MNNG and cysteamine) was significantly less than in Group 1. The number of gastric cancers was 1.6 ± 0.3 per rat in Group 1, but significantly less in Group 2 given oral cysteamine. No cancers were found in Group 3 (cysteamine alone). All cancers found were in the antral mucosa, and no metastases were seen in any rats.

Even in Week 52, the incidence and number per rat of gastric cancers were significantly less in Group 2 than in Group 1.

Histological type and depth of involvement of gastric cancer

Table 2 shows data on the incidence of different types of gastric cancers and the depth of involvement of the cancers in MNNG-treated groups. All tumors induced in the glandular stomach were adenocarcinomas. In Group 1 (MNNG alone), 31 (97%) of the 32 cancers were highly differentiated. In Group 2 (MNNG and cysteamine), the incidence of highly differentiated adenocarcinomas was significantly less than that in Group 1. However, no poorly differentiated cancers were found in any of the groups. The depth of involvement of the gastric cancers did not differ in the two groups.

Incidence and number of AGH

Table 3 summarizes the incidence, number and grade of AGH in the MNNG-treated groups. There was no difference in the incidence of AGH between the two groups, but the average number of AGH per rat was significantly less in Group 2 (MNNG and cysteamine) than in Group 1 (MNNG alone). There was no significant difference between the grades of AGH in the different groups. AGH was usually found in the antral mucosa and only rarely in the fundic mucosa.

Table 4 shows the incidence of histological lesions in the duodenum in the three groups. No histological abnormalities were found in any rats in Group 1. The incidence of duodenal lesions in Groups 2 and 3 was slightly greater than in Group 1. However, no chronic active duodenal ulcers were found in rats fed on cysteamine.

Serum gastrin level, antral mucosal pH and labeling index of gastric mucosa

Table 5 summarizes data on the serum gastrin level, antral mucosal pH and labelling index of the gastric mucosa in each group in Weeks 30 and 52. On both occasions, serum gastrin levels in response to refeeding were significantly elevated, and the antral pH after refeeding was more significantly decreased in Groups 2 (MNNG and cysteamine) and 3 (cysteamine alone) than in Group 1 (MNNG alone). However, the serum gastrin levels in the basal state did not differ in the three groups on either occasion, whereas in Week 52, antral pH in the basal state was significantly decreased in Groups 2 and 3 as compared with Group 1.

On both occasions, the two methods of counting BrdU-labeled nuclei show that oral administration of cysteamine to Groups 2 and 3 resulted in a significant decrease in the labeling index of the antral mucosa but not of the fundic mucosa, as compared with those in Group 1 rats treated only with the carcinogen.

DISCUSSION

The trophic effects of gastrin on mucosal cells of the stomach are established [17]. However, studies

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

Group No.	Treatment*	Body weight (g) Week 26	Body weight (g) Week 52	Effective No. of rats	No. of rats with gastric cancer (%)	No. of gastric cancers per rat
1	MNNG alone	290 ± 5 (35)†	354 ± 32 (19)	20	16 (80)	1.6 ± 0.3
2	MNNG + cysteamine	285 ± 10 (35)	360 ± 25 (19)	20	9 (45)‡	0.6 ± 0.2§
3	Cysteamine alone	292 ± 5 (35)	350 ± 30 (20)	20	0 (0)	0.0 ± 0.0

*MNNG alone; 50 μg MNNG/ml was given in the drinking water for 25 weeks, followed by regular pellets. MNNG + cysteamine; 0.4% cysteamine was given orally in pellets after MNNG treatment for 25 weeks. Cysteamine alone: 0.4% cysteamine was given orally in pellets from Week 26, with no prior MNNG.
†Numbers in parentheses are numbers of rats examined.
Significance of difference from the value for Group 1; ‡$P < 0.05$, §$P < 0.001$.

Table 2. Histological types and depth of involvement of gastric cancers in MNNG-treated rats

Group No.	Treatment*	Effective No. of rats	No. of gastric cancers	Highly differentiated (%)	Well-differentiated (%) Common type	Well-differentiated (%) Mucinous type	Depth of involvement (%) Submucosa	Depth of involvement (%) Muscularis propria or deeper
1	MNNG alone	20	32	31 (97)	1 (3)	0 (0)	19 (59)	13 (41)
2	MNNG + cysteamine	20	12	5 (42)†	7 (58)	0 (0)	7 (58)	5 (42)

*For explanation of treatments, see Table 1.
Significance of difference from the value for Group 1; †$P < 0.001$.

Table 3. Incidence, number, and grade of AGH in glandular stomach of MNNG-treated rats

Group No.	Treatment*	Effective No. of rats	No. of rats with AGH (%)	No. of AGH	No. of AGH per rat	Grade (%) Mild	Grade (%) Moderate	Grade (%) Severe
1	MNNG alone	20	20 (100)	85	4.3 ± 0.5	70 (82)	13 (15)	2 (3)
2	MNNG + cysteamine	20	17 (85)	35	1.8 ± 0.3†	30 (86)	5 (14)	0 (0)

*For explanation of treatments, see Table 1.
Significance of difference from the value for Group 1; $P < 0.001$.

Table 4. Incidence of duodenal lesions in MNNG-treated rats

Group No.	Treatment*	Effective No. of rats	No. of rats with duodenal lesions (%)	No. of duodenal lesions	Duodenal mucosal erosions (%)	Duodenal ulcer (%)		Duodenal cancer (%)
						Healed	Active	
1	MNNG alone	20	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)
2	MNNG + cysteamine	20	3 (15)	4	1 (25)	2 (25)	0 (0)	2 (50)
3	Cysteamine alone	20	5 (25)	7	3 (43)	4 (57)	0 (0)	0 (0)

*For explanation of treatments, see Table 1.

Table 5. Serum gastrin level, antral mucosal pH, and labeling index of gastric mucosa in MNNG-treated rats

Experimental week	Group No.	Treatment*	Serum gastrin level (pg/ml)		Antral mucosal pH		Labeling index (No. of BrdU-labeled nuclei/gland)		Labeling index (% of BrdU-labeled nuclei)	
			Fasting	Refeeding	Fasting	Refeeding	Fundic mucosa	Antral mucosa	Fundic mucosa	Antral mucosa
30	1	MNNG alone	241 ± 14 (5)†	646 ± 99 (5)	3.0 ± 0.0 (5)	4.9 ± 0.3 (5)	1.0 ± 0.1 (5)	1.9 ± 0.1 (5)	1.3 ± 0.1 (5)	8.4 ± 0.5 (5)
	2	MNNG + cysteamine	273 ± 16 (5)	1384 ± 104‡ (5)	3.3 ± 0.2 (5)	3.8 ± 0.2‖ (5)	1.1 ± 0.1 (5)	1.1 ± 0.1* (5)	1.3 ± 0.1 (5)	2.5 ± 0.2‡ (5)
	3	Cysteamine alone	273 ± 13 (5)	1291 ± 130§ (5)	3.3 ± 0.2 (5)	3.0 ± 0.2‡ (5)	1.0 ± 0.1 (5)	1.1 ± 0.1* (5)	1.1 ± 0.1 (5)	2.4 ± 0.2‡ (5)
52	1	MNNG alone	274 ± 30 (5)	469 ± 31 (5)	4.6 ± 0.3 (5)	4.4 ± 0.2 (5)	1.4 ± 0.1 (5)	2.7 ± 0.1 (5)	1.9 ± 0.2 (5)	10.0 ± 1.1 (5)
	2	MNNG + cysteamine	231 ± 9 (5)	954 ± 44‡ (5)	3.4 ± 0.2§ (5)	3.3 ± 0.1§¶ (5)	1.4 ± 0.1 (5)	1.4 ± 0.1* (5)	1.7 ± 0.2 (5)	3.0 ± 0.2‡ (5)
	3	Cysteamine alone	257 ± 26 (5)	839 ± 41‡ (5)	3.6 ± 0.2§ (5)	2.5 ± 0.2‡ (5)	1.2 ± 0.1 (5)	1.2 ± 0.1* (5)	1.3 ± 0.1 (5)	3.3 ± 0.4‡ (5)

*For explanation of treatments, see Table 1.
†Numbers in parentheses are numbers of rats examined. Fasting serum gastrin/antral pH, serum gastrin/antral pH after refeeding, and labeling index of gastric mucosa were determined in different rats.
Significance of difference from the value for Group 1: $^{\ddagger}P < 0.001$, $\S P < 0.01$, $\|P < 0.05$.
Significance of difference from the value for Group 3: $\P P < 0.05$.

on the effect of gastrin on chemically induced stomach cancer have provided conflicting results. We previously found that prolonged administration of tetragastrin after MNNG treatment for 25 weeks resulted in a significant reduction in the incidence and number of MNNG-induced gastric cancers [4]. In contrast, Tahara *et al.* reported that subcutaneous injections of pentagastrin increased the incidence of gastric cancers induced by MNNG in rats [18]. Furthermore, the effect of hypergastrinemia on gastric carcinogenesis is still unclear. Deveny *et al.* [6] reported that the endogenous hypergastrinemic state exerts a protective effect against gastric carcinogenesis induced by MNNG in rat only when gastric acid secretion is high, and that hypergastrinemia associated with hyposecretion of acid or achlorhydria has no inhibitory effect on the development of gastric cancer. Therefore, they suggested that the inhibitory effect of hypergastrinemia on the development of gastric cancer is probably mediated by acid secretion. We previously found that prolonged subcutaneous administration of cysteamine after MNNG treatment resulted in a significant increase in serum gastrin level and acid secretion, and therefore in a significant reduction in the incidence and number of gastric cancers [5]. Furthermore, in the present work we found that treatment with 0.4% cysteamine also significantly inhibited the development of gastric cancers.

Recently, Jang *et al.* [19] found that intragastric administration of cysteamine exerted an enhancing effect on the development of gastric cancers induced by MNNG when administered intermittently during carcinogen treatment. They gave intragastric cysteamine in two doses of 200 mg/kg body wt at an interval of 4 h every 4 weeks starting 2 weeks after commencement of MNNG treatment for 16 weeks. They suggested that this enhancement may be related to the effect of cysteamine in increasing induction of gastric mucosal erosions. Takahashi *et al.* [20] reported similar results. They studied the effect of ulcers or erosion induced by iodoacetamide on the development of gastric tumors induced by MNNG in rats, and found that ulceration and regeneration of the gastric mucosa after iodoacetamide treatment are important factors in gastric carcinogenesis. Our present results were different from those of Jang *et al.* [19], although the doses of cysteamine they used were similar to those of our present work. These differences in findings may be partly attributable to differences in the methods used to induce hypergastrinemia, the time of induction of hypergastrinemia during gastric carcinogenesis or the experimental system. One of the most important differences may be between cysteamine administration during or after MNNG treatment.

The trophic effect of gastrin on the fundic mucosa of the stomach is well established [17]. However, the hormonal factors that control cell proliferation in the antrum are still unknown. In rats, pentagastrin has been shown to inhibit normal cell proliferation in the antral mucosa [21]. Recently, we have found that the inhibitory effect of tetragastrin in gastric carcinogenesis may be related to its action in decreasing proliferation in the antral mucosa [8]. In the present work, we found that oral administration of cysteamine caused a significant increase in serum gastrin level and a significant decrease in the labeling index of the antral mucosa, but not a significant increase in the labeling index of the fundic mucosa. This apparent failure of the known trophic effect of gastrin on the fundic mucosa might be related to the effect of cysteamine on alterations in nucleic acid and protein synthesis and cell proliferation. There is some evidence that the effects of cystamine may be mediated by activation of adenylcyclase and increased formation of intracellular cyclic AMP [22]. Takagi and Shikata [23] reported that mitotic cells disappeared almost completely within 60 min after addition of cysteamine to HeLa cell cultures.

The present results show that feeding rats 0.4% cysteamine reduced the incidence and number of gastric cancers. However, it is not clear whether cysteamine administration can delay the development or maturation of any foci that may arise: this point requires further investigation. Although the exact mechanism of the effect of cysteamine is still unclear, this is the first report of inhibition of gastric cancer development by oral administration of compounds that induce hypergastrinemia and hypersecretion of acid. This may lead to the eventual development of a method for suppression of gastric carcinogenesis in humans based on dietary alteration or supplementation.

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