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Enhancement by monochloramine of the development of gastric cancers in rats: A possible mechanism of *Helicobacter pylori*-associated gastric carcinogenesis

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Abstract: The effects of cytotoxic monochloramine on the development of gastric cancers induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were investigated in Wistar rats. After oral administration of drinking water containing the carcinogen and regular chow pellets for 25 weeks, rats received regular chow pellets or chow pellets containing 20% ammonium acetate, and normal tap water or water containing 30 mM sodium hypochlorite, with or without s.c. injection of taurine, until the end of the experiment in week 52. Treatment with both ammonium acetate and sodium hypochlorite significantly increased the incidence of gastric cancers in week 52, while the concomitant use of taurine with ammonium acetate and sodium hypochlorite significantly attenuated the enhanced gastric carcinogenesis. Spectrophotometric examinations revealed that taurine scavenged monochloramine. These findings suggest that *Helicobacter pylori*-associated gastric carcinogenesis may be mediated by monochloramine.

Key words: *Helicobacter pylori*, monochloramine, gastric carcinogenesis

Introduction

Large cohort studies have shown a higher risk of gastric cancer for persons infected with *Helicobacter pylori*;[1–4] however, the mechanism of the increased risk is still unclear. Recent studies have suggested that ammonia is involved in the development of *H. pylori*-associated gastric cancers,[5,6] and the ammonia-monochloramine system has been shown to play an important role in *H. pylori*-associated gastric mucosal injury.[7] Monochloramine is a reactive oxidant produced by the reaction of neutrophil-derived hypochlorous acid and ammonia[7] and is more toxic to gastric mucosal cells than is sodium hypochlorite or ammonium chloride.[8,9] These findings indicate that monochloramine may be more closely related to the development of gastric cancers than is ammonia. To investigate this possibility, we examined the effects of monochloramine in gastric cavities produced by oral administration of ammonium acetate and sodium hypochlorite on the development of gastric cancers induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in Wistar rats.

Materials and methods

Animals

One hundred and fifty inbred male Wistar rats (6 weeks old) were purchased from SLC (Shizuoka, Japan). The animals were housed in suspended, wire-bottomed metal cages in our animal quarters, at controlled temperature (20°C–22°C) and humidity (30%–50%), with a 12-h/12-h light-dark cycle.

Experimental design

The animals were given drinking water containing MNNG (50 μg/ml; Aldrich, Milwaukee, Wis., USA) and regular chow pellets (Nihon Nosan, Yokohama, Japan) for 25 weeks. The MNNG was dissolved in deionized water at a concentration of 1 mg/ml and kept in a cool (4°C), dark place. Just before use, the stock solution was diluted to 50 μg/ml with tap water. Each rat was given 40 ml of MNNG solution (less than a single rat can consume in 48 h) from a bottle covered with aluminium foil to prevent photolysis of MNNG; the solution was replenished every other day.

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At week 26, the animals were divided randomly into six groups of 25 each. Until the end of the experiment in week 52, each group was given a different combination of chow pellets, drinking water, and alternate-day s.c. injections. Group 1, the control group, was given regular chow pellets, normal tap water, and 0.9% NaCl injections; group 2 was given chow pellets containing 20% ammonium acetate (Sigma, St. Louis, Mo., USA), drinking water containing 30 mM sodium hypochlorite (Sigma), and 0.9% NaCl injections; group 3 was given chow pellets containing 20% ammonium acetate, drinking water containing 30 mM sodium hypochlorite, and taurine (300 mg/kg body weight, Sigma) injections; group 4 was given chow pellets containing 20% ammonium acetate, normal tap water, and 0.9% NaCl injections; group 5 was given regular chow pellets, drinking water containing 30 mM sodium hypochlorite, and 0.9% NaCl injections; and group 6 was given regular chow pellets, normal tap water, and taurine (300 mg/kg body weight) injections. Taurine in 0.9% NaCl solution and 0.9% NaCl solution were injected at various sites, in a volume of 2 ml/kg body weight, between 2 and 3 p.m. every other day.

Histologic observations

Animals that survived for more than 49 weeks were included in the effective numbers, as the first tumor of the glandular stomach was found in a rat in group 1 that died in week 49. All surviving rats were killed at the end of the experiment in week 52 and autopsied. 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 picric acid-formaldehyde solution for histologic examination. The fixed stomach was cut into longitudinal strips 3 mm wide. Specimens were embedded in paraffin, and serial sections, 5-μm-thick, were stained with hematoxylin and eosin. Sections were examined without knowledge of which group they were from.

Definition and classification of gastric cancers

Histologically, adenocarcinomas were defined as lesions in which neoplastic cells penetrated the muscularis mucosae to invade the submucosa or deeper layers, and they were classified as very well differentiated, well differentiated, or poorly differentiated.[10]

Measurement of labeling index

The labeling index of gastric mucosal cells was determined in weeks 30 and 52, in five rats in each group, with an immunohistochemical analysis kit for bromodeoxyuridine (BrdU) incorporation (Becton-Dickinson Immunocytometry Systems, Mountain View, Calif., USA).[11,12] Briefly, nonfasting rats received s.c. injections of 0.9% NaCl (groups 1, 2, 4, and 5) or 300 mg/kg body weight of taurine (groups 3 and 6) in a volume of 2 ml/kg body weight. One h later, they received an i.p. injection of 20 mg/kg body weight of BrdU and were killed with ether 1 h later. The stomach was removed and fixed in 70% ethanol for 4 h. The fixed stomach was cut into longitudinal strips 3 mm wide. Specimens were embedded in paraffin, and sections (3-μm-thick) were immersed in 2 N HCl for 30 min at room temperature and neutralized in 0.1 N $Na_2B_4O_7$, then immersed in methanol containing 0.3% H_2O_2 for 30 min and treated with 10% horse serum. Next, sections were stained with anti-BrdU monoclonal antibody (diluted 1:20) for 2 h at room temperature, stained with biotin-conjugated horse anti-mouse antibody (diluted 1:200; Vector Laboratories, Burlingame, Calif., USA) for 30 min, and then treated with avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min. The reaction product was localized with 3,3′-diaminobenzidine tetrahydrochloride. The BrdU-labeled cells were identified by the presence of dark pigment throughout their nuclei.

To determine the labeling index of gastric mucosal cells, BrdU-labeled and -unlabeled cells in the zone of proliferating cells[13] were counted without knowledge of which group the preparations were from. The zone of proliferating cells in the fundic mucosa was defined as a 250-μm rectangle between the highest and the lowest labeled cells in a well-oriented section; ten such rectangular areas per rat were examined. In the antral mucosa, all cells below the highest labeled cell in each gland were regarded as being within the zone of proliferating cells. In this case, 100 well oriented glands were examined from each rat. From these measurements, we calculated the labeling index as the number of BrdU-labeled cells per total number of cells within the zone of proliferating cells.

Preparation of chloramines

Monochloramine and taurine chloramine were synthesized by the method of Thomas et al.,[14] with a slight modification: 0.5 ml of sodium hypochlorite (Sigma) was added to 5 ml of 6.8 mM ammonium acetate (Ishizu, Osaka, Japan) in 50 mM sodium phosphate buffer (pH 7.8) at room temperature five times at 1-min intervals. The product was identified and quantified as 3.4 mM monochloramine from its absorbance at the λ_{max} wavelength at 242 nm with an E_m value of 429 M/cm. To synthesize 1.5 mM taurine chloramine, 3.4 mM taurine was used instead of 6.8 mM ammonium acetate in the procedure described above, and taurine chloramine was

identified and quantified with an E_m value of 429 M/cm at 252 nm.

Quantification of monochloramine in mixture of food and drinking water

The amounts of N-Cl derivatives were determined by measuring the amount of 5-thio-2-nitrobenzoic acid (Nbs) that was oxidized to 5,5′-dithiobis-(2-nitrobenzoic acid) after the addition of excess Nbs.[14] Since two kinds of N-Cl derivatives, monochloramine and $NHCl_2$, came to equilibrium in the presence of amines and in the absence of hypochlorite, we expressed the amount of monochloramine as the amount of N-Cl derivatives.

One ml of 30 mM sodium hypochlorite in 100 mM sodium phosphate buffer (pH 7.6) was added to 23 mg of chow pellets (with and without 20% ammonium acetate), which had been wet with 100 µl of phosphate buffer (pH 7.6); 1 ml of phosphate buffer was added to 23 mg of chow pellets containing ammonium acetate (control) five times at 1-min intervals at room temperature. The mixture was centrifuged at 10000 *g* for 2 min, and the supernatant was aspirated. Five µl of 30% H_2O_2 was added to 1 ml of the 50% diluted supernatant to reduce unreacted hypochlorite, and then 2 µl of catalase (27 mg/ml; Sigma) was added. Immediately after this, 50 µl of 0.68 mM Nbs in phosphate buffer (pH 7.0) was added, and the absorbance at 412 nm (Em = 13600) was measured.

In vitro measurement of scavenging effect of taurine

The scavenging effect of taurine on pro-oxidant monochloramine was investigated spectrophotometrically. After 0.25 ml of 1.9 mM monochloramine was added to 2.25 ml of 1.7 mM taurine in 50 mM sodium phosphate buffer (pH 7.8) at room temperature, the absorption spectrum of the reaction mixture was compared with that of sodium phosphate buffer containing 0.19 mM monochloramine, 1.7 mM taurine, or 0.31 mM taurine chloramine.

Statistical analysis

Results were analyzed by the χ^2 test, Fisher's exact probability test, or one-way analysis of variance with Dunn's multiple comparison.[15] Values are given as means ± SE. Significant indicates a calculated *P* value of less than 0.05.

Results

Incidence, number, histologic type, and depth of involvement of gastric cancers

Two rats in group 3 died before experimental week 49. Because tumors were not found in these animals, they were excluded from the effective numbers. At week 52, the animals that had received chow pellets containing 20% ammonium acetate (groups 2, 3, and 4) weighed significantly less than those in control group 1 (Table 1).

The incidence of gastric cancers in group 2 (ammonium acetate + sodium hypochlorite) (17 of 20; 85%) was significantly higher than that in the controls (group 1) (10 of 20; 50%); the numbers of gastric cancers per tumor-bearing rat were not significantly different in the two groups (Table 1). The incidence of gastric cancers in rats that received ammonium acetate, sodium hypochlorite, and taurine (group 3) was significantly lower than that in group 2. Administration of ammonium acetate alone (group 4) slightly, but not significantly, in-

Table 1. Body weight, and incidence and number of gastric cancers, in MNNG-treated rats

Group number	Treatment[a]	Body weight (g)		Effective number of rats	Number of rats with gastric cancer (%)	Number of gastric cancers per tumor-bearing rat
		Week 26	Week 52			
1	Control	320 ± 6	363 ± 11	20	10 (50)	1.8 ± 0.4
2	NH_4 + SH	317 ± 4	311 ± 9[d]	20	17 (85)[b]	1.7 ± 0.2
3	NH_4 + SH + taurine	315 ± 4	315 ± 6[d]	18	7 (39)[e]	1.6 ± 0.3
4	NH_4	333 ± 4	323 ± 5[c]	20	14 (70)	1.4 ± 0.2
5	SH	320 ± 6	351 ± 11	20	12 (60)	1.4 ± 0.2
6	Taurine	312 ± 8	370 ± 7	20	12 (60)	2.2 ± 0.3

All values are expressed as Means ± SE

[a] Treatment: After treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) for 25 weeks, each group of rats was given chow pellets containing ammonium acetate (NH_4; 20%), drinking water containing sodium hypochlorite (SH; 30 mM), and alternate-day s.c. injections of taurine (300 mg/kg body weight), alone or in combination. Control rats received normal chow pellets, normal tap water, and alternate-day s.c. injections of 0.9% NaCl after treatment with MNNG for 25 weeks

[b–d] Significantly different from the value for group 1: [b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$ (one-way analysis of variance; two-tailed)

[e] Significantly different from the value for group 2 at $P < 0.01$ (one-way analysis of variance; two-tailed)

Table 2. Histologic type (all were adenocarcinomas) and depth of involvement of gastric cancers in MNNG-treated rats

Group number	Treatment[a]	Number of gastric cancers	Histology (%)		Depth of involvement (%)	
			Very well differentiated	Well differentiated	Submucose	Muscle layer or deeper
1	Control	18	16 (89)	2 (11)	16 (89)	2 (11)
2	NH_4 + SH	29	20 (69)	9 (31)	22 (76)	7 (24)
3	NH_4 + SH + taurine	10	6 (60)	4 (40)	9 (90)	1 (10)
4	NH_4	19	15 (79)	4 (21)	17 (89)	2 (11)
5	SH	17	14 (82)	3 (18)	15 (88)	2 (12)
6	Taurine	26	23 (88)	3 (12)	24 (92)	2 (8)

[a] For explanation of treatments, see Table 1

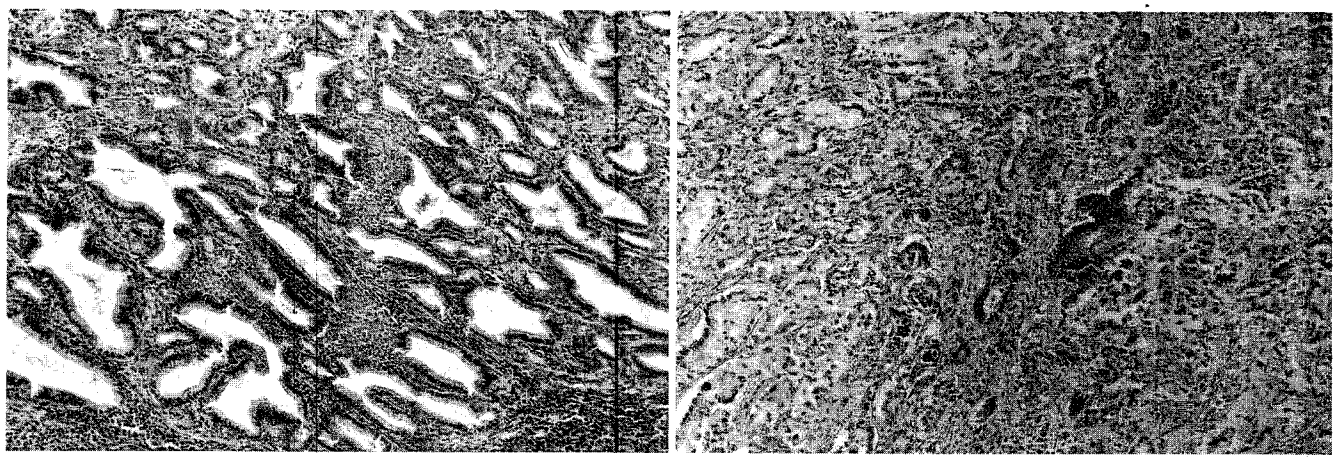

Fig. 1a,b. Histologic classification of gastric adenocarcinomas in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-treated rats. **a** Very well differentiated adenocarcinoma. **b** Well differentiated adenocarcinoma. H&E, × 100

creased the incidence of gastric cancers compared with that in group 1. Administration of sodium hypochlorite alone (group 5) or taurine alone (group 6) had no effect on the incidence of gastric cancers compared with that in group 1. Administration of taurine alone (group 6) slightly, but not significantly, increased the number of gastric cancers per tumor-bearing rat compared with that in group 1.

All gastric cancers were found in the antral mucosa and were histologically adenocarcinomas (Table 2). There were no significant differences in the histologic types of adenocarcinomas in the six groups: most of the adenocarcinomas were very well differentiated and the rest were well differentiated (Fig. 1). No poorly differentiated cancers were found in this series. There were no significant differences in the depth of involvement of gastric cancers in the six groups. Severe erosions with and without intramucosal hemorrhage were found in rats treated with ammonium acetate and sodium hypochlorite (group 2), but erosions were mild in rats simultaneously treated with taurine (group 3).

Labeling index of gastric mucosa

At weeks 30 and 52, administration of ammonium acetate and sodium hypochlorite (group 2) significantly increased the labeling indices of both fundic and antral mucosa compared with those in group 1 (Table 3; Fig. 2). Combined administration of taurine with ammonium acetate and sodium hypochlorite (group 3) significantly decreased the labeling indices of both fundic and antral mucosa, which were elevated by administration of ammonium acetate and sodium hypochlorite (group 2) at both times examined. Administration of ammonium acetate alone (group 4), sodium hypochlorite alone (group 5), or taurine alone (group 6) had no significant effect on the labeling indices of either fundic or antral mucosa compared with those in group 1.

Yield of monochloramine

Six nmole of monochloramine was produced from the reaction of 10 mg of chow pellets containing 20% ammonium acetate with 440 µl of 30 mM sodium hypochlorite.

Table 3. Labeling index of gastric mucosa in MNNG-treated rats

Experimental week	Group number	Treatment[a]	Labeling index (%)	
			Fundic mucosa	Antral mucosa
30	1	Control	13.0 ± 0.7	12.2 ± 0.4
	2	NH_4 + SH	22.8 ± 1.1[b]	21.8 ± 1.1[b]
	3	NH_4 + SH + taurine	13.8 ± 0.6[c]	13.8 ± 0.6[d]
	4	NH_4	16.8 ± 1.2	13.8 ± 1.5
	5	SH	14.4 ± 0.8	13.4 ± 0.5
	6	Taurine	12.4 ± 0.8	11.8 ± 1.1
52	1	Control	10.2 ± 0.9	12.4 ± 0.8
	2	NH_4 + SH	20.8 ± 1.2[b]	21.4 ± 1.2[b]
	3	NH_4 + SH + taurine	14.6 ± 0.9[c]	12.8 ± 1.2[d]
	4	NH_4	12.6 ± 0.8	14.6 ± 1.0
	5	SH	11.6 ± 0.7	13.4 ± 0.8
	6	Taurine	9.8 ± 1.2	11.0 ± 1.1

All values are expressed as Means ± SE
[a] For explanation of treatments, see Table 1
[b] Significantly different from the value for group 1 at $P < 0.001$ (one-way analysis of variance; two-tailed)
[c,d] Significantly different from the value for group 2: [c] $P < 0.01$, [d] $P < 0.001$ (one-way analysis of variance; two-tailed)

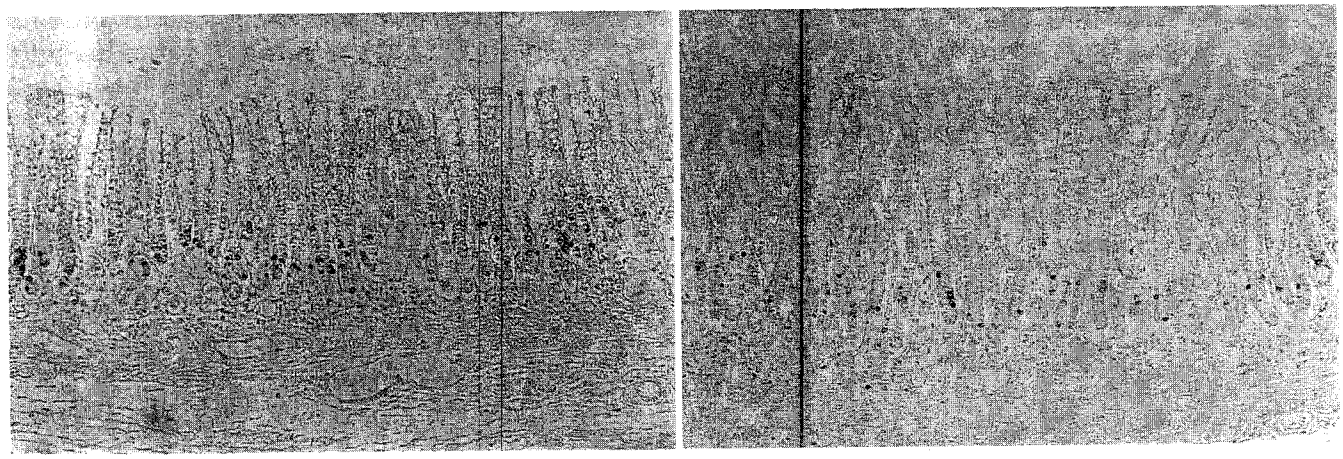

Fig. 2a,b. Immunohistochemical photomicrograph of gastric antral mucosa from **a** a rat treated with and **b** a rat without ammonium acetate plus sodium hypochlorite. Bromodeoxyuridine-labeled cells were frequently found in rats treated with ammonium acetate plus sodium hypochlorite. ×100

Scavenging effect of taurine

As shown in Fig. 3, the taurine solution did not show a detectable absorbance peak in the range of the ultraviolet wavelength, but the mixed solution of monochloramine and taurine showed an absorbance peak (λ_{max} at 252 nm) that was consistent with that of taurine chloramine and distinct from that of monochloramine (λ_{max} at 242 nm).

Discussion

Ammonia produced by *H. pylori* has been considered a factor in the development of *H. pylori*-associated gastric mucosal injury.[5] The ammonia concentration in gastric juice in *H. pylori*-infected patients is higher than in noninfected patients.[16] Kawano et al.[17] found that oral administration of 0.01% and 0.1% solutions of ammonia for 2–4 weeks decreased mucosal thickness, parietal cell number, and fundic gland number in rats, with the decrease in mucosal thickness being more severe in the antral mucosa. Mégraud et al.[18] reported that the degree of vacuolation and viability of Hep2 cells was dependent on ammonia concentration. Tsujii et al.[19] found that oral administration of 0.01% ammonia accelerated mucosal cell migration, especially in the antrum, and led to mucosal atrophy of the stomach in rats.

Ammonia is also believed to be involved in the development of *H. pylori*-associated gastric cancers. Tsujii et

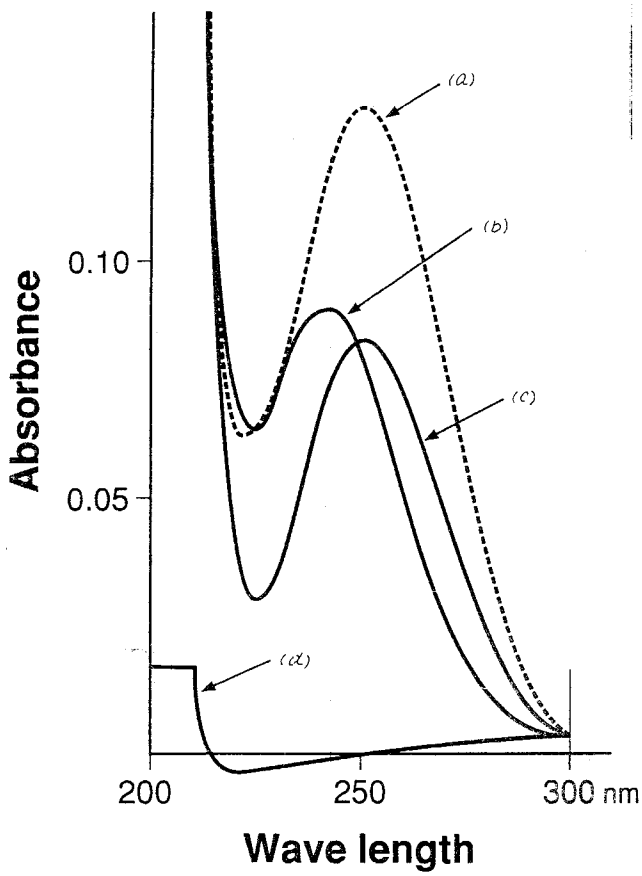

Fig. 3. Scavenging effect of taurine on monochloramine. (*a*) 0.31 mM taurine-chloramine, (*b*) 0.19 mM monochloramine, (*c*) mixture of 1.7 mM taurine and 0.19 mM monochloramine, (*d*) 1.7 mM taurine. All measurements were made in sodium phosphate buffer (pH 7.8)

al.[5,6] found that oral administration of ammonia significantly increased the incidence of gastric cancers induced by MNNG in rats. In the present study, we found that oral treatment with ammonium acetate and sodium hypochlorite (group 2) significantly increased the incidence of gastric cancers induced by MNNG in rats. Administration of ammonium acetate alone (group 4) slightly, but not significantly, increased the incidence of gastric cancers, and the difference between groups 2 and 4 did not reach significance. Since sodium hypochlorite reacts with ammonium acetate to yield cytotoxic monochloramine, a potent oxidizing agent that rapidly reacts with target cell components,[20] these findings suggest that *H. pylori*-associated gastric carcinogenesis may be mediated by the ammonium-monochloramine system.

Our present results showed that concomitant treatment with taurine attenuated the gastric carcinogenesis enhanced by ammonium acetate and sodium hypochlorite. Although the incidence of gastric cancers in group 3 (ammonium acetate + sodium hypochlorite + taurine) was slightly, but not significantly, lower than that in group 6 (taurine alone), the reason is not known.

The precise mechanism by which taurine attenuates enhanced gastric carcinogenesis is not clear, but at least three possible explanations may be considered. One possible explanation is an effect of taurine on ornithine decarboxylase (ODC) activity. Okamoto et al.[21] examined the effect of taurine on hepatocarcinogenesis induced by diethylnitrosamine and phenobarbital in male F344 rats and found that taurine inhibited the development of enzyme-altered hepatic lesions and decreased ODC activity in nonneoplastic liver tissue. ODC activity is thought to be involved in the regulation of cell proliferation.[22] More recently, we[23] examined the effect of combined administration of NaCl and the ODC inhibitor 1,3-diaminopropane on the development of gastric cancers induced by MNNG and on ODC activity in the gastric wall in Wistar rats. We found that combined administration of 1,3-diaminopropane significantly reduced both the enhancement by NaCl of gastric carcinogenesis and the ODC activity in the antral wall.

The second possible effect of taurine is on lipid peroxidation. Oral administration of taurine has been reported to protect against lipid peroxidation induced by taumustine and isoprenaline;[24] lipid peroxidation has been investigated as a possible mediator of various pathologic and physiologic processes.[25] Nishikawa et al.[26] found that calcium significantly inhibited the development of gastric preneoplastic hyperplasia in rats treated with MNNG and NaCl. They also found that calcium intake significantly reduced levels of malondialdehyde, a marker of lipid peroxidation, in the gastric mucosa and urine.

The third possible effect of taurine is on oxidant compounds. It has been proposed that taurine acts as a general detoxifier of oxidative species, reacting with and removing chemically active free-radical species.[27,28] Gaull et al.[29] demonstrated that taurine increased the viability of lymphoblastoid cell lines in culture and that these cell lines produced N-chlorotaurine, a condensation product of taurine and hypochlorous acid. They speculated that this condensation system protected the cells; conjugation with taurine may remove oxidative species capable of reacting with and damaging various intracellular compounds, such as nucleic acids, proteins, and carbohydrates.[27] In the present study, we found that taurine scavenged monochloramine to produce taurine chloramine in vitro. These findings also suggest that monochloramine is involved in the development of gastric cancers.

Our present results showed the BrdU labeling indices to be significantly increased, in both fundic and antral mucosa in rats treated with ammonium acetate and sodium hypochlorite (group 2), but all cancers were found in the antrum, not in the fundus. However, administra-

tion of ammonium acetate and sodium hypochlorite markedly increased the incidence of atypical glandular hyperplasias in both fundic and antral mucosa. Atypical glandular hyperplasias were defined as lesions in which the constituent cells of the glands stained hyperchromatically and their nuclei were pleomorphic; the form and size of the glands were irregular, and the lesions were confined to the mucosa and did not penetrate the muscularis mucosae.[10] Atypical glandular hyperplasias are considered to be precancerous lesions.[10] In the present study, adenocarcinomas were defined as lesions in which neoplastic cells penetrated the muscularis mucosae to invade the submucosa or deeper layers.

In conclusion, we found that gastric carcinogenesis induced by MNNG was enhanced by combined treatment with ammonium acetate plus sodium hypochlorite and suppressed by supplementation with taurine, a scavenger of monochloramine. Our findings suggest that monochloramine may be involved in the pathogenesis of *H. pylori*-associated gastric cancers.

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