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ORNITHINE DECARBOXYLASE INHIBITOR LESSENS THE RAT GASTRIC CARCINOGENESIS ENHANCEMENT CAUSED BY TYROSINE METHYL ESTER

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The effects of combined administration of a catecholamine precursor, tyrosine methyl ester (TME), and an ornithine decarboxylase (ODC) inhibitor, 1,3-diaminopropane (DAP), on the incidence of gastric cancers induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG), the norepinephrine (NE) concentration and ODC activity of the gastric wall, and the labeling index of the gastric mucosa were investigated in inbred Wistar rats. Rats received s.c. injections of TME, 512 mg/kg body weight, every other day and drinking water with or without 2.5 g/l of DAP after 25 weeks of oral administration of MNNG. At week 52, administration of TME resulted in significant increases in the incidence of gastric cancers, in the NE concentration and the ODC activity of the antral portion of the gastric wall, and in the labeling index of antral epithelial cells. Administration of both TME and DAP significantly reduced the enhancements by TME of gastric carcinogenesis, NE concentration and ODC activity of the antral wall, and the labeling index of the antral mucosa. Our results suggest that ODC inhibition lessens enhancement by TME of gastric carcinogenesis and that the enhancement by TME of gastric carcinogenesis is mediated in part by polyamine biosynthesis. ***Int. J. Cancer*** **73:113–116, 1997.**
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The possible role of the autonomic nervous system in the mechanisms of chemical carcinogenesis has been discussed (Gurkalo and Volfson, 1982; Tutton and Barkla, 1980): pharmacological compounds enhancing the sympathetic action of the autonomic nervous system promote carcinogenesis in various organs (Tatsuta *et al.,* 1989*a,b,* 1991*a,* 1992*a,b,c*). Moreover, we also found that administration of a catecholamine precursor, tyrosine methyl ester (TME), caused significant increases in the incidence of gastric cancers induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MMNG) and in tissue norepinephrine (NE) concentrations in the antral portion of the gastric wall in Wistar rats (Tatsuta *et al.,* 1991*b*). These findings also indicate that enhanced activity of the sympathetic nervous system stimulates gastric carcinogenesis, but the exact mechanism by which increased activity of the sympathetic nervous system enhances carcinogenesis is not clearly understood. Kodama *et al.* (1987) examined the effect of NE on the ornithine decarboxylase (ODC) activity of rat lung and found that the induction rate of ODC increased dose-dependently with NE to a level 100-fold higher than control. Therefore, if ODC is necessary for expression of enhancement of gastric carcinogenesis by TME, inhibition of ODC should lessen the response to TME. To investigate this possibility, we examined the effect of TME with and without 1,3-diaminopropane (DAP), a potent inhibitor of ODC, on the development of gastric cancers induced by MNNG in Wistar rats.

MATERIAL AND METHODS

Animals

One hundred inbred 6-week-old male Wistar rats were used in this study. Animals were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in suspended wire-bottom metal cages in animal quarters with controlled temperature (21 to 22°C), humidity (30 to 50%), and light (12-hr cycle). The rats had free access to regular chow pellets (Nihon-Nosan, Yokohama, Japan).

Experimental design

The animals were given drinking water containing MNNG (25 µg/ml; Aldrich, Milwaukee, WI) for 25 weeks. The MNNG was dissolved in deionized water at a concentration of 1 mg/ml. The resulting stock solution was stored in a cool, dark place and diluted to 25 µg/ml with tap water just before use. Forty milliliters of MNNG solution per rat was supplied from bottles that were covered with aluminum foil to prevent photolysis and were refilled every other day.

At week 26, the animals were divided into 4 groups of 25 rats each. Until the end of the experiment in week 52, each group was given a different combination of injections, with or without TME, and tap water, with or without DAP (2.5 g/l; Sigma, St. Louis, MO). Group 1, the control group, was given injections of 0.9% NaCl solution and normal tap water; group 2 was given injections of TME and normal tap water; group 3 was given injections of TME and drinking water containing DAP; and group 4 was given injections of 0.9% NaCl solution and drinking water containing DAP. TME (512 mg/kg body weight; equimolar to 400 mg/kg body weight of tyrosine; Sigma) was dissolved in 0.9% NaCl solution and injected s.c. at various sites in a volume of 2 ml/kg body weight between 2 and 3 P.M., every other day.

Tyrosine itself is not high in water-solubility and its absorption is slow. TME increases the tyrosine concentration both in serum and in the brain. Therefore, TME is a useful tool for studying the effect of increased concentrations of catecholamine (Oishi and Szabo, 1987).

Histologic observations

Animals that survived for more than 50 weeks were included in the effective numbers because the first tumor of the glandular stomach was found in a rat from group 2 that died in week 49. Animals were killed at the end of the experiment in week 52. All rats, including their stomach and other organs, were carefully examined at autopsy. The stomach was opened along the greater curvature, pinned flat on a cork mat, and fixed with a buffered picric-acid-formaldehyde solution for histologic examination. The stomach was cut into longitudinal strips of 3-mm width. Specimens were embedded in paraffin, and serial 5-µm-thick sections were stained with hematoxylin and eosin. Sections were examined without knowledge of which group they belonged to.

Histologic type of gastric cancers

Histologically, adenocarcinomas were defined as lesions in which neoplastic cells had penetrated the muscularis mucosae to involve the submucosa or deeper layers. As previously reported (Tatsuta *et al.,* 1988), the adenocarcinomas were classified as very well-differentiated, well-differentiated, or poorly differentiated.

Measurement of tissue NE

The NE concentration of the gastric wall was determined in weeks 30 and 52 with high-performance liquid chromatography, as

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reported previously (Tatsuta *et al.*, 1983). For this purpose, 5 rats from each group were kept without food for 12 hr and then treated with a usual injection. Two hours later, rats were killed by cervical dislocation and 50-mg samples 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 1,100*g* for 10 min. The supernatant was mixed with 1.0 ml of 1.2 M disodium EDTA and adjusted to pH 6.0 with ammonium hydrochloride. It was then mixed with 300 mg of purified alumina (Woelm Neutral Active Grade I) and adjusted to pH 8.4 to 8.8 with ammonium hydroxide. The mixture was stirred for 5 min and centrifuged at 10,000*g* for 10 min, after which the supernatant was discarded. The precipitated alumina was washed twice with distilled water, shaken vigorously with 2.5 ml of 0.4 N acetate to elute the NE from the alumina, and centrifuged. The clear supernatant was transferred to a small glass tube and lyophilized, and the residue was dissolved in 0.5 ml of 0.2 N acetic acid. A 50-µl sample of this solution was injected into a liquid chromatography column (2.6 × 250 mm, Hitachi 3011-C gel column, Hitachi, Tokyo, Japan), and this material was 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 a reagent initiating the trihydroxyindole reaction (0.0075% potassium ferricyanide, 0.1% ascorbic acid, and 5 N sodium hydroxide) and the resulting fluorescent products were examined in a highly sensitive spectrophotofluorometer (Hitachi 650-10).

ODC assay

The ODC activity of the gastric wall was assayed at weeks 30 and 52 in 5 rats of each group. For this purpose, rats were kept without food for 12 hr and then treated with a usual injection. Two hours later, the rats were killed by cervical dislocation, and the stomach was opened and pinned flat. Small samples (about 100 mg) were obtained from the antral and fundic portions of the stomach having no visible tumors. Enzyme activity was assayed with DL[1-^{14}C]ornithine as a substrate by the method of Russell and Snyder (1968). Fifty-microliter samples of subcellular fraction were preincubated with 140 µl of phosphate buffer (pH 7.2) and then placed on ice. Then 0.5 µCi of DL[1-^{14}C]ornithine was added by syringe, and the mixtures were incubated at 37°C for 1 hr with slight stirring. The reaction was stopped by injecting 500 µl of 40% citric acid into the outer tube through the test tube cap; stirring at room temperature was continued for another 30 min to trap all the $^{14}CO_2$ released in the basic solution in the central well. The solution in the central well was then transferred to a Picofluor (Packard, Meriden, CT) scintillator, and its radioactivity was assayed with a Beckman (Fullerton, CA) LS-7000 liquid scintillation counter. The protein content of the enzyme extract was measured with the method of Lowry *et al.* (1951) with BSA as a standard. Results are expressed as picomoles of $^{14}CO_2$ released in 30 min per milligram of protein.

Measurement of labeling index

The labeling index of gastric mucosa was measured at weeks 30 and 52 in 5 rats of each group with an immunohistochemical analysis kit for assaying bromodeoxyuridine (BrdU) incorporation (Gratzner, 1982) (Becton-Dickinson, Mountain View, CA). The rats were kept without food for 12 hr and then treated with a usual injection. One hour later, the animals were given an intraperitoneal injection of BrdU (20 mg/kg body weight) and were killed after another hour. The stomach was fixed in 70% ethanol for 4 hr. Sections of 3-µm thickness 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 stained with anti-BrdU monoclonal antibody (diluted 1:100) for 2 hr at room temperature, washed, stained with biotin-conjugated horse anti-mouse antibody (at a dilution of 1:200) for 30 min, and stained with the avidin-biotin-peroxidase complex method for 30 min. The reaction product was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Cells containing BrdU were identified by the presence of dark pigment over the nuclei. For analysis of the BrdU-labeling index of the gastric mucosa, BrdU-labeled and unlabeled cells in the zone of proliferating cells were counted without knowledge of which treatment group the samples belonged to. The zone of proliferating cells in the fundic mucosa was defined as a rectangle of 250-µm width between the highest and lowest 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 gland column were regarded as being within the zone of proliferating cells. We selected 100 well-oriented columns of glands in each rat. From these measurements we derived the labeling index as the number of BrdU-labeled cells per total number of cells within the zone of proliferating cells.

Statistical analysis

Results were analyzed with the Chi-square test or one-way analysis of variance with Dunn's multiple comparison. Data are shown as means ± SE. A calculated *p* value of less than 0.05 was regarded as significant.

RESULTS

Incidence, number, histologic type and depth of gastric cancers

Five rats from each group were killed in week 30 to measure the NE concentration and ODC activity in the gastric wall and the labeling index of the gastric mucosa. One rat in group 1 and 2 rats in each of groups 2, 3, and 4 died before week 49. No tumors were found in the glandular stomach of any of these animals, which were excluded from the effective numbers of rats. At week 52, the animals that had received TME with and without DAP weighed significantly less than those in the control group 1 (Table I).

In group 1 (control), gastric cancers were found in 7 (37%) of 19 rats examined; the average number of gastric cancers per tumor-

TABLE I – INCIDENCE AND NUMBER OF GASTRIC CANCERS IN MNNG-TREATED RATS

Group number	Treatment[1]	Body weight at weeks: 26	Body weight at weeks: 52	Effective number of rats	Number of rats with gastric cancer (%)	Number of gastric cancers per tumor-bearing rat
1	Control	342 ± 5	363 ± 7	19	7 (37)	1.3 ± 0.2
2	TME	343 ± 4	321 ± 6**	18	15 (83)*	1.3 ± 0.1
3	TME + DAP	353 ± 7	309 ± 5**	18	8 (44)***	1.4 ± 0.2
4	DAP	341 ± 5	352 ± 5	18	7 (39)	1.3 ± 0.2

[1]Treatment (group numbers): Rats were given s.c. injections of 0.9% NaCl solution plus normal tap water (group 1), s.c. injections of 512 mg/kg body weight of TME plus normal tap water (group 2), s.c. injections of 512 mg/kg body weight of TME plus drinking water containing 2.5 g/l of DAP (group 3), or s.c. injections of 0.9% NaCl solution plus drinking water containing 2.5 g/l of DAP (group 4) after 25 weeks of MNNG treatment.–*,**Significantly different from the value for group 1: *$p < 0.02$, **$p < 0.001$.–***Significantly different from the value for group 2 at $p < 0.05$.

bearing rat was 1.3 ± 0.2. In group 2 (TME), the incidence of gastric cancers, but not the number per tumor-bearing rat, was significantly higher than in group 1 (Table I). Administration of both TME and DAP (group 3) resulted in a significantly lower incidence of gastric cancers than that in group 2. DAP alone (group 4) had no effect on the incidence and number of gastric cancers, as compared with those in group 1.

All tumors induced in the glandular stomach were identified histologically as adenocarcinomas. There were no significant differences in the histologic types and depth of involvement of adenocarcinomas among the 4 groups (Table II). No poorly differentiated cancers were found in this series. All cancers were found in the antral mucosa, and no metastases were seen in any rats.

NE concentration, ODC activity and labeling index

At both weeks 30 and 52, treatment with TME (group 2) significantly increased the NE concentration and ODC activity in the antral portion of the gastric wall and the labeling index of the antral mucosa compared with those in group 1 (control; Table III). Administration of both TME and DAP (group 3) significantly decreased the NE concentration and ODC activity of the antral portion of the gastric wall and the labeling index of the antral mucosa, all of which were elevated by TME alone (group 2). Administration of DAP alone (group 4) had no significant effect on the NE content, ODC activity, or labeling index. Moreover, administration of TME or DAP or both had no significant effect on the NE concentration or the ODC activity of the fundic portion of the gastric wall or on the labeling index of the fundic mucosa.

DISCUSSION

Our present study has shown that administration of the ODC inhibitor DAP significantly reduces the enhancement by TME of gastric carcinogenesis.

The mechanism by which DAP inhibits gastric carcinogenesis is not clearly understood, but at least 2 possibilities may be considered. One possibility is that DAP had an influence on c-*myc* oncogene expression. Polyamines are excellent stabilizers of triplex DNA. Thomas *et al.* (1995) have observed a remarkable structural specificity of polyamines in the induction and stabilization of triplex DNA. In their study, MCF-7 cells were treated with a 37mer oligonucleotide to form triplex DNA in the upstream regulatory region of the c-*myc* oncogene in the presence of DAP. Formation of a complex of the oligonucleotide and DAP reduced the c-myc mRNA signal by 65%.

Another possible mechanism by which DAP inhibits gastric carcinogenesis is via its lessening effects on catecholamine and ODC activity. Astancolle *et al.* (1991) found that induction of ODC in the rat liver by dexamethasone or laparotomy was markedly impaired by catecholamine depletion. Cremades *et al.* (1992) found that the existing levels of renal ODC in male mice and the increase induced by testosterone in male, female, castrated male, and hypophysectomized mice were markedly decreased by catecholamine depletion produced by treatment with either alpha-methyl-p-tyrosine or reserpine. Tovar *et al.* (1995) also reported that both peripheral sympathetic nervous factors as well as centrally related factors can modulate the effect of androgens on renal ODC activity. In the present work, we found that administration of both TME and DAP significantly reduced the enhancement by TME of NE concentration and ODC activity of the antral wall. These findings suggest that catecholamines induce ODC activity in various organs.

Using a highly specific, polyclonal antiserum to ODC, Johnson *et al.* (1988) determined that the enzyme is present in the fundic mucosa confined to a narrow band of cells at the base of the gastric pits. In antral mucosa, ODC is present throughout the lower 20% of the mucosa, which consists of the necks of pyloric glands. These findings show that the location of ODC in the gastric mucosa coincides with that of the mucous neck cells, known to be the proliferative cells of the stomach.

In the present work, we found that administration of both TME and DAP significantly lessens the enhanced gastric carcinogenesis by TME and that DAP also significantly lessens TME-enhanced ODC activity. Although we did not examine the effects of any other ODC inhibitors (*e.g.,* α-difluoromethylornithine) on TME enhancement of gastric carcinogenesis induced by MNNG, these findings suggest that enhancement of gastric carcinogenesis by TME may be mediated in part through polyamine biosynthesis.

TABLE II – HISTOLOGIC TYPE AND DEPTH OF INVOLVEMENT OF GASTRIC CANCERS IN MNNG-TREATED RATS

Group number	Treatment[1]	Number of gastric cancers	Histology (%)		Depth of involvement (%)	
			Very well differentiated	Well differentiated	Submucosa	Muscle layer or deeper
1	Control	9	7 (78)	2 (22)	9 (100)	0 (0)
2	TME	20	17 (85)	3 (15)	19 (95)	1 (5)
3	TME + DAP	11	9 (82)	2 (18)	11 (100)	0 (0)
4	DAP	10	8 (80)	2 (20)	9 (90)	1 (10)

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

TABLE III – NOREPINEPHRINE CONCENTRATION AND ODC ACTIVITY OF THE STOMACH WALL, AND LABELING INDEX OF GASTRIC MUCOSA IN MNNG-TREATED RATS

Experimental week	Group number	Treatment[1]	Norepinephrine (ng/g of tissue)		ODC activity (pmol CO_2/30 min/mg protein)		Labeling index (%)	
			Fundic portion	Antral portion	Fundic portion	Antral portion	Fundic mucosa	Antral mucosa
30	1	Control	322 ± 27	284 ± 7	0.58 ± 0.04	3.02 ± 0.14	10.4 ± 0.5	13.8 ± 0.6
	2	TME	377 ± 18	370 ± 14*	0.72 ± 0.04	4.58 ± 0.27*	12.2 ± 0.4	19.8 ± 1.1*
	3	TME + DAP	334 ± 23	356 ± 10**	0.60 ± 0.04	3.12 ± 0.11**	10.4 ± 0.9	13.6 ± 0.7**
	4	DAP	304 ± 27	282 ± 7	0.54 ± 0.05	2.98 ± 0.15	9.4 ± 0.7	12.6 ± 0.4
52	1	Control	390 ± 17	284 ± 11	0.72 ± 0.06	2.90 ± 0.10	9.6 ± 0.5	12.4 ± 0.9
	2	TME	395 ± 20	372 ± 9*	0.84 ± 0.07	4.04 ± 0.12*	10.6 ± 0.9	19.8 ± 0.7*
	3	TME + DAP	384 ± 17	367 ± 8**	0.74 ± 0.05	3.00 ± 0.03**	9.0 ± 0.7	12.6 ± 0.8**
	4	DAP	378 ± 7	278 ± 10	0.60 ± 0.06	2.80 ± 0.05	8.0 ± 0.7	11.2 ± 0.9

[1]For explanation of treatments, see Table I.–*Significantly different from the value for group 1 at $p < 0.001$.–**Significantly different from the value for group 2 at $p < 0.001$.

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