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INHIBITIONS BY 6-HYDROXYDOPAMINE AND NEOSTIGMINE SINGLY OR TOGETHER OF GASTRIC CARCINOGENESIS INDUCED BY N-METHYL-N′-NITRO-N-NITROSOGUANIDINE IN WISTAR RATS

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The effects of chemical sympathectomy induced by 6-hydroxydopamine (6-OHDA) and administration of the acetylcholinesterase inhibitor neostigmine, singly or together, on gastric carcinogenesis induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), and on the tissue catecholamine concentration of the gastric wall and the labeling index of the gastric mucosa, were investigated in inbred Wistar rats. Rats received s.c. injections of neostigmine (0.075 mg/kg), and/or i.p. injections of 6-OHDA (42 mg/kg twice within 24 hr, and then 105 mg/kg every 2 weeks from 1 week later) 25 weeks after oral treatment with MNNG. Prolonged administration of 6-OHDA or neostigmine significantly reduced the incidence of gastric cancers by week 52, and in combination they had a significantly greater inhibitory effect. 6-OHDA and/or neostigmine had no influence on the histology of gastric cancers. Administration of 6-OHDA, but not neostigmine, significantly decreased the norepinephrine concentration in the antral portion of the gastric wall. The labeling index of the antral mucosa was decreased significantly by treatment with 6-OHDA or neostigmine, and decreased even more significantly by 6-OHDA plus neostigmine. Our findings indicate that 6-OHDA and neostigmine have protective effects against gastric carcinogenesis and that in combination their effects are additive. These results imply that the activities of the sympathetic and parasympathetic autonomic systems together influence gastric carcinogenesis.

The possible role of the nervous system in the mechanisms of chemical carcinogenesis has been discussed (Gurkalo and Volfson, 1980, 1982; Tutton and Barkla, 1980). Pharmacological compounds that enhance the sympathetic action of the autonomic nervous system stimulate carcinogenesis, whereas compounds that enhance the cholinergic influence or exert an antiadrenergic action inhibit carcinogenesis (Gurkalo and Volfson, 1982). We previously found that chemical sympathectomy by treatment with 6-OHDA significantly reduced the incidence and number of gastric cancers induced by MNNG (Tatsuta *et al.,* 1989*c*). Recently, we also found that prolonged administration of the monoamine oxidase inhibitor nialamide significantly increased the NE concentration in the gastric wall and the incidence and number of gastric cancers (Tatsuta *et al.,* 1989*b*). Moreover, we showed that long-term administration of neostigmine significantly reduced the incidence of gastric cancers induced by MNNG (Tatsuta *et al.,* 1989*a*). These findings indicate that the sympathetic and parasympathetic nervous systems are involved in the development of gastric cancers. However, the relationship between the sympathetic and parasympathetic nervous systems in gastric carcinogenesis is still not understood. Therefore, in the present work, we examined the effects of prolonged administration of 6-OHDA and/or neostigmine on gastric carcinogenesis in Wistar rats previously treated with MNNG.

MATERIAL AND METHODS

Animals

One hundred and sixty 6-week-old male inbred Wistar rats were purchased from SLC (Shizuoka, Japan). The animals were housed in suspended, wire-bottomed metal cages in animal quarters at controlled temperature (21-22°C), humidity (30-50%), and lighting (12 hr darkness/12 hr light), and given free access to regular chow pellets (Oriental Yeast, Tokyo, Japan).

Experimental design

The animals were given drinking water containing MNNG (75 μg/ml; Aldrich, 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 75 μg/ml with tap water just before use. Rats were given 40 ml of MNNG solution each, supplied from bottles covered with aluminum foil to prevent light-denaturation of MNNG, and the solution was renewed every other day. Safety precautions for the use of MNNG were taken as far as possible. From week 26, the rats were given normal tap water *ad libitum* and were randomly divided into 4 groups of 40 rats each. These groups were treated as follows until the end of the experiment in week 52: Group 1 was given only the vehicles, plain olive oil s.c. and 0.9% NaCl solution i.p. Group 2 was given neostigmine s.c. and 0.9% NaCl solution i.p. Group 3 was given plain olive oil s.c. and 6-OHDA i.p. Group 4 was given neostigmine s.c. and 6-OHDA i.p.

Neostigmine (Sigma, St. Louis, MO) was given as a suspension in olive oil at a concentration of 0.075 mg/kg body weight. Injections were given s.c. at various sites every other day in a volume of 1 ml/kg body weight between 2 and 3 p.m. Groups 1 and 3 were given 1 ml/kg of plain olive oil s.c. every other day in the same way as Groups 2 and 4.

According to the method of Fronek (1980), 6-OHDA (Sigma) in 0.9% NaCl solution was injected repeatedly i.p. to produce long-term sympathectomy: 42 mg/kg of 6-OHDA was administered i.p. twice within 24 hr, and then from one week later 105 mg/kg were administered every other week. At the same times Groups 1 and 2 were treated i.p. with 2 ml/kg of 0.9% NaCl solution.

Tissue sampling

Animals that survived for more than 49 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 49. All surviving animals were killed at the end of the experiment in week 52. All rats were autopsied, and the stomach and other organs were carefully examined. The stomach was opened along the greater curvature, pinned flat on a cork mat and fixed in Zamboni's solution (Stefanini *et al.,* 1967) for histological examination. The fixed stomach was cut into longitudinal strips, 3 mm wide. The specimens were embedded in paraffin, and serial sections 5-μm thick were stained with hematoxylin

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Abbreviations: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; BUdR, bromodeoxyuridine; 6-OHDA, 6-hydroxydopamine bromide; neostigmine, neostigmine methyl sulfate; NE, norepinephrine; MAb, monoclonal antibody.

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and eosin. Sections were examined without knowledge of their group of origin.

Classification of gastric cancers

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

Measurement of norepinephrine in the gastric wall

NE concentration in tissues of the gastric wall was determined in weeks 30 and 52 by high-performance liquid chromatography as reported (Tatsuta *et al.*, 1983). For this purpose, 5 rats in each group were used 24 hr or 14 days after treatment with 0.9% NaCl solution (Groups 1 and 2) or 6-OHDA (Groups 3 and 4). Two hours after injections of 1 ml/kg olive oil or 0.075 mg/kg neostigmine (Groups 2 and 4), rats were killed by cervical dislocation. Samples of about 50 mg of the fundic and antral portions of the gastric wall were taken from each rat, homogenized with 4 ml of 0.4 N perchloric acid, and centrifuged at 1,100 *g* for 10 min. The supernatant was mixed with 1 ml of 0.2 M disodium ethylenediaminetetraacetate (EDTA), and the mixture was adjusted to pH 6.0 with ammonium hydroxide. Then the mixture was added to 300 mg of purified aluminum (Woelm Neutral Active Grade I) and the pH was adjusted to 8.4–8.8 with ammonium hydroxide. The mixture was stirred for 5 min and centrifuged at 10,000 *g* for 10 min, then the supernatant was aspirated and discarded. The precipitated aluminum was washed twice with distilled water and then shaken vigorously with 2.5 ml of 0.4 N acetate. The mixture was centrifuged, the clear supernatant was transferred to a small glass tube and lyophilized for 3 hr, and the residue was dissolved in 0.5 ml of 0.2 N acetic acid. A 50-μl aliquot of this solution was injected into a liquid chromatographic column (Hitachi 3011-C gel column, 2.6 × 250 mm). Materials were 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 the reagent for the trihydroxyindol reaction, consisting of 0.0075% potassium ferricyanide, 0.1% ascorbic acid, and 5 N sodium hydroxide. The resulting fluorescent products were examined in a highly sensitive spectrofluorophotometer (Hitachi 650-10, Hitachi, Tokyo, Japan).

Measurement of labeling index of gastric mucosa

The labeling index of gastric mucosa was examined in weeks 30 and 52 with an immunohistochemical analysis kit for assay of BUdR incorporation (Becton-Dickinson, Mountain View, CA) (Gratzner, 1982; Morstyn *et al.*, 1983). For this purpose, 5 rats from each group were used 24 hr or 14 days after i.p. treatment with 0.9% NaCl solution (Groups 1 and 2) or 6-OHDA (Groups 3 and 4). Rats received 1 ml/kg of olive oil s.c. (Groups 1 and 3) or 0.075 mg/kg of neostigmine (Groups 2 and 4) 2 hr before killing, and 20 mg/kg of BUdR i.p. 1 hr before killing. The stomach was removed and fixed in 70% ethanol for 4 hr. Sections 3 μm in 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-BUdR MAb (diluted 1:100) for 2 hr at room temperature, washed, treated with biotin-conjugated horse anti-mouse antibody (at 1:200 dilution) for 30 min, and stained with avidine-biotin-peroxidase complex for 30 min. The reaction product was localized with 3,3′-diaminobenzidine tetrahydrochloride. Cells containing BUdR were identified by the presence of dark pigment over their nucleus.

The labeling index of the gastric mucosa was determined by counting BUdR-labeled and -unlabeled cells in the proliferating zone (Eastwood and Quimby, 1983) without knowledge of the group of origin of the sample. The zone of proliferating cells in the fundic mucosa was defined as a 250-μm rectangular area between the highest and lowest labeled cells in well-oriented sections. Ten such rectangular areas in each rat were examined. In the antral mucosa, all cells below the highest labeled cells in each pit-gland column were regarded as being within the zone of proliferating cells. In each rat, 100 well-oriented columns of pits and glands were examined, and the labeling index was calculated as the number of BUdR-labeled cells/total number of cells within the proliferating zone.

Measurement of antral pH

The antral pH was examined in weeks 30 and 52. For this purpose, 5 rats from each group were used 24 hr or 14 days after i.p. treatment with 0.9% NaCl solution (Groups 1 and 3) or 6-OHDA (Groups 3 and 4). The rats received an i.p. injection of 1 ml/kg olive oil (Groups 1 and 3) or 0.075 mg/kg neostigmine (Groups 2 and 4) 2 hr before being killed. The stomach was opened and pinned flat on a cork mat, and the antral pH was measured with a fine electrode.

Statistical analysis

Results were analyzed by the Chi-square test or Fisher's exact probability test (Siegel, 1956), or by one-way analysis of variance with Dunn's multiple comparison (Snedecor and Cochran, 1967; Miller, 1966). Data are shown as means ± SE. "Significant" indicates a calculated p-value of less than 0.05.

RESULTS

Incidence, number and histological types of gastric cancers

Ten rats in each group were killed in week 30 for determination of catecholamine of the gastric wall and the labeling index of the gastric mucosa. Two rats in Group 2 and 3 rats in Group 4 died before week 49. No tumors were found in these animals, and they were excluded from the effective numbers. In week 52, the animals that had received 6-OHDA with and without neostigmine had significantly lower body weights than the untreated rats.

The incidence, number and histological types of gastric cancers in each group are summarized in Table I. In Group 1 (NaCl + olive oil), gastric cancers were found in 16 (80%) of 20 rats examined. In Group 2 (NaCl + neostigmine) and Group 3 (6-OHDA + olive oil), the incidence of gastric cancer

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

Group number	Treatment[1]		Body weight (g)		Effective number of rats	Number of rats with gastric cancer (%)	Number of gastric cancers		Histology (%)	
	i.p.	s.c.	26W	52W			Total	Number/rat	Very well differentiated	Well differentiated
1	NaCl	Olive oil	325 ± 7	409 ± 7	20	16 (80)	21	1.1 ± 0.2	16 (76)	5 (24)
2	NaCl	Neostigmine	331 ± 6	398 ± 5	18	7 (39)[2]	8	0.4 ± 0.1[2]	8 (100)	0 (0)
3	6-OHDA	Olive oil	334 ± 5	348 ± 10[3]	20	9 (45)[2]	11	0.6 ± 0.2	9 (82)	2 (18)
4	6-OHDA	Neostigmine	331 ± 5	351 ± 7[3]	17	1 (6)[4,5]	1	0.1 ± 0.1	1 (100)	0 (0)

[1]For explanation of treatments, see "Material and Methods".–[2,3]Significantly different from the value for Group 1: [2]$p < 0.05$, [3]$p < 0.001$.–[4]Significantly different from the value for Group 2 at $p < 0.05$.–[5]Significantly different from the value for Group 3 at $p < 0.02$.

was significantly lower than that in Group 1. Moreover, concomitant administration of 6-OHDA and neostigmine (Group 4) resulted in a significantly lower incidence of gastric cancer than in Groups 2 and 3. The number of gastric cancers per rat in Group 1 was 1.1 ± 0.2, and was significantly less in Group 2, but not in Group 3. Combined administration of 6-OHDA and neostigmine did not result in fewer gastric cancers than in Groups 2 and 3.

Table I also shows data on the distribution of different histological types of gastric cancers in the 4 groups. All the tumors induced in the glandular stomach were histologically identified as adenocarcinomas. Almost all were very well differentiated, and no poorly differentiated cancers were found. There were no significant differences in the histological types of adenocarcinomas between the 4 groups. All cancers were found in the antral mucosa, and no metastases were found.

Labeling index of gastric mucosa and antral pH

Table II summarizes data on the labeling indices of the gastric mucosa and antral pH in weeks 30 and 52. At both times, Group 2 (NaCl + neostigmine) and Group 3 (6-OHDA + olive oil) had significantly decreased labeling indices in the antral mucosa and a significantly lower antral pH than Group 1 (NaCl + olive oil) 24 hr and 14 days after i.p. injection of 0.9% NaCl solution (Groups 1 and 2) or 6-OHDA (Groups 3 and 4). The combination of 6-OHDA and neostigmine (Group 4) significantly decreased the labeling index in the antral mucosa and antral pH relative to those in Groups 2 and 3. At both times, administration of 6-OHDA, but not of neostigmine alone, caused a significant decrease in the labeling index in the fundic mucosa.

Tissue catecholamine concentrations in gastric wall

Table III summarizes data on the NE concentrations in the gastric wall in each group at weeks 30 and 52. At both times, administration of 6-OHDA to Group 3 (6-OHDA + olive oil) and Group 4 (6-OHDA + neostigmine) caused significant decreases in NE concentration in the fundic and antral portions of the stomach wall relative to those in untreated Groups 1 and 2. However, there was no significant difference between the values in Groups 1 and 2, and in Groups 3 and 4.

DISCUSSION

In the present work, we confirm our previous observations that prolonged administration of 6-OHDA or neostigmine after 25 weeks of oral treatment with MNNG significantly reduced the incidence of gastric cancers at week 52 (Tatsuta *et al.*, 1989*a,b*). Moreover, we found that administration of 6-OHDA plus neostigmine reduced the incidence of gastric cancers significantly more than treatment with 6-OHDA or neostigmine alone. Thus 6-OHDA and neostigmine, administered together, had additive inhibitory effects on gastric carcinogenesis.

The reason for this additive effect of a combination of 6-OHDA and neostigmine is not known, but at least 2 possible mechanisms may be considered. One is an increase in gastric secretion. Increased secretion of gastric acid may be closely related with inhibition of the development of gastric cancers. We found previously that prolonged administration of tetragastrin in depot form resulted in a significant increase in gastric acid secretion and a significant reduction in the incidence of gastric cancers (Tatsuta *et al.*, 1977). This effect may be due to the digestion of cancerous lesions as a result of increased gastric secretion. Mallory (1940) and Palmer and Humphreys

TABLE II – LABELING INDICES OF GASTRIC MUCOSA AND ANTRAL pHs IN MNNG-TREATED RATS

Experimental week	Group number	Treatment[1]		Labeling index (%)				Antral pH	
				Fundic mucosa		Antral mucosa			
		i.p.	s.c.	Day 1[2]	Day 14	Day 1	Day 14	Day 1	Day 14
30	1	NaCl	Olive oil	29.8 ± 1.6	30.4 ± 2.0	28.2 ± 1.4	28.2 ± 2.0	3.9 ± 0.1	4.8 ± 0.1
	2	NaCl	Neostigmine	30.0 ± 1.6	31.6 ± 1.2	18.6 ± 1.0[4]	17.4 ± 1.7[4]	2.6 ± 0.2[3]	3.3 ± 0.1[3]
	3	6-OHDA	Olive oil	14.8 ± 1.6[4]	14.6 ± 1.6[4]	16.2 ± 1.2[4]	16.6 ± 1.4[4]	2.6 ± 0.2[3]	3.2 ± 0.1[3]
	4	6-OHDA	Neostigmine	17.0 ± 1.6[4,7]	16.2 ± 1.0[4,7]	8.4 ± 1.0[4,7,9]	8.0 ± 0.8[4,6,9]	1.7 ± 0.1[4,5,8]	1.7 ± 0.1[3,7,9]
52	1	NaCl	Olive oil	30.0 ± 1.4	29.0 ± 2.2	28.4 ± 1.5	29.2 ± 1.8	4.1 ± 0.2	4.9 ± 0.1
	2	NaCl	Neostigmine	32.4 ± 2.3	31.0 ± 1.9	17.4 ± 1.4[4]	17.6 ± 1.1[4]	2.6 ± 0.2[4]	3.7 ± 0.1[4]
	3	6-OHDA	Olive oil	17.2 ± 1.4[4]	14.6 ± 1.6[4]	16.2 ± 1.2[4]	16.0 ± 1.7[4]	2.8 ± 0.2[3]	3.2 ± 0.1[4]
	4	6-OHDA	Neostigmine	19.0 ± 1.9[3,7]	18.0 ± 2.0[3,6]	7.0 ± 0.7[4,7,9]	5.8 ± 0.4[4,7,9]	1.6 ± 0.1[4,5,9]	1.6 ± 0.1[4,7,9]

[1]For explanation of treatments, see "Material and Methods".–[2]Days after i.p. injection of NaCl solution (Groups 1 and 2) or 6-OHDA (Groups 3 and 4).–[3,4]Significantly different from the value for group 1; [3]$p < 0.001$, [4]$p < 0.01$.–[5,6,7]Significantly different from the value for Group 2; [5]$p < 0.05$, [6]$p < 0.01$, [7]$p < 0.001$.–[8,9,10]Significantly different from the value for Group 3; [8]$p < 0.05$, [9]$p < 0.01$, [10]$p < 0.001$.

TABLE III – NOREPINEPHRINE CONCENTRATIONS IN THE STOMACH WALL IN MNNG-TREATED RATS

Experimental week	Group number	Treatment[1]		Norepinephrine concentration (ng/g tissue)			
				Fundic mucosa		Antral mucosa	
		i.p.	s.c.	Day 1[2]	Day 14	Day 1	Day 14
30	1	NaCl	Olive oil	333 ± 24	356 ± 24	273 ± 19	242 ± 22
	2	NaCl	Neostigmine	288 ± 32	312 ± 55	240 ± 13	249 ± 31
	3	6-OHDA	Olive oil	36 ± 5[3]	31 ± 5[3]	34 ± 4[3]	35 ± 5[3]
	4	6-OHDA	Neostigmine	36 ± 5[3,4]	30 ± 5[3,4]	26 ± 5[3,4]	38 ± 8[3,4]
52	1	NaCl	Olive oil	379 ± 51	346 ± 42	255 ± 32	246 ± 23
	2	NaCl	Neostigmine	321 ± 22	329 ± 36	255 ± 31	257 ± 39
	3	6-OHDA	Olive oil	22 ± 3[3]	32 ± 4[3]	23 ± 5[3]	28 ± 4[3]
	4	6-OHDA	Neostigmine	20 ± 4[3,4]	33 ± 6[3,4]	14 ± 2[3,4]	28 ± 4[3,4]

[1]For explanation of treatments, see "Material and Methods".–[2]Days after i.p. injection of NaCl solution (Groups 1 and 2) or 6-OHDA (Groups 3 and 4).–[3]Significantly different from the value for Group 1 at $p < 0.001$.–[4]Significantly different from the value for Group 2 at $p < 0.001$.

(1944) reported that cancerous lesions are easily ulcerated by gastric acid. Chemical sympathectomy with 6-OHDA causes complete sympathectomy of the gastric mucosa (Thoenen and Tranzer, 1968). As sympathetic innervation of the stomach inhibits gastric-acid secretion, chemical sympathectomy resulted in a highly significant increase in gastric-acid secretion (Grabner *et al.,* 1984). Larson *et al.* (1984) reported that chemical sympathectomy increased acid secretion in response to submaximal doses of pentagastrin. In the present work, we found that rats treated with 6-OHDA or neostigmine had a significantly lower antral pH than control rats, and that a combination of 6-OHDA and neostigmine caused a significantly greater reduction in antral pH than treatment with either compound alone.

Another possible mechanism is an effect on cell proliferation of gastric epithelial cells. Direct and indirect evidence indicates neural involvement in control of cell proliferation (Kennedy *et al.,* 1983). NE released by the action of the sympathetic nervous system appears to stimulate crypt cell proliferation in both the small and large intestine (Tutton and Helme, 1974; Tutton and Barkla, 1977). In the present and previous studies (Tatsuta *et al.,* 1989*c*), we found that 6-OHDA-treatment significantly reduced the labeling indices of both the antral and the fundic epithelial cells. A trophic influence of the vagus has been suggested (Pearl *et al.,* 1966), but not confirmed (Crean *et al.,* 1969). The demonstration of increased labeling indices after truncal vagotomy does not support the view that the vagus has a trophic influence on human gastric epithelial progenitor cells. As pointed out by Helander (1976), loss of vagal trophic effects could theoretically be compensated for by increased trophic influences of gastrin after vagotomy. However, Håkanson *et al.* (1984) demonstrated a trophic effect of the vagus on the stomach after unilateral vagal sectioning. We found previously that anterior or posterior vagotomy resulted in a significant increase in the labeling indices of both the fundic and the antral epithelial cells on the denervated side (Tatsuta *et al.,* 1988*a*). In the present work, we found that prolonged administration of parasympathomimetic neostigmine significantly reduced the labeling index of the antral epithelial cells. However, there have been no reports on the effect of a combination of 6-OHDA and neostigmine on cell proliferation in gastric mucosa. In the present work, we found that combined administration of these 2 compounds significantly reduced the labeling index of the antral epithelial cells as compared to those of rats treated with 6-OHDA or neostigmine alone.

In the present work, we found that the rats treated with 6-OHDA grew more slowly than the control rats. The lower incidence of gastric cancer in rats treated with 6-OHDA could be explained by caloric restriction. Albanes (1987) found that total caloric intake is an important determinant of tumorigenesis in mice, and that body weight may be a sensitive indicator of this effect. Klurfeld *et al.* (1987) found that rats treated with 7,12-dimethylbenz(a)anthracene and subjected to caloric restriction weighed 40% less and had a significantly lower incidence of mammary and colon tumors than rats fed *ad libitum.* In the present work, we also found that neostigmine affected the labeling index in the antral mucosa, but did not affect the labeling index in the fundic mucosa. We recently found that administration of bombesin resulted in a significant increase in the labeling index in the antral mucosa, but not the fundic mucosa (Iishi *et al.,* 1992). However, it is not known why neostigmine affected the labeling index only in the antral mucosa.

The present results show that 6-OHDA and neostigmine, administered together, had additive inhibitory effects on gastric carcinogenesis and proliferation of antral epithelial cells. These findings indicate that the sympathetic and the parasympathetic nervous system together play important roles in the development of gastric cancers, and suggest that these 2 systems have opposite effects on gastric carcinogenesis.

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