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Modifying Effects of Capsaicin, Allyl Sulfide, Indole-3-Carbinol and Germanium on the Induction of Pepsinogen 1 Altered Pyloric Glands in Rats Initiated with N-Methyl-N'-Nitro-N-Nitrosoguanidine

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Quantitative analyses were made of pepsinogen 1 (Pg 1) decreased pyloric glands after treating male Wistar rats with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and NaCl and then with various test chemicals. Animals received MNNG in drinking water (100 μg/ml) and 10% NaCl in diet for 8 weeks (group 1), followed by basal diet containing 0.01% capsaicin, 0.5% allyl sulfide, 0.5% indole-3-carbinol and 0.05% germanium until 20 weeks (groups 2-5). Groups 6 to 9 received test chemicals alone without MNNG and NaCl treatment. Group 10 was received basal diet and tap water. All animals sacrificed at week 20. Sections of pyloric mucosa were investigated for Pg 1 immunostaining. Induction of Pg 1 altered pyloric glands (PAPG) was compared with corresponding control. Allyl sulfide significantly decreased the number of PAPG and morphological changes induced by MNNG and NaCl. Other chemicals, such as capsaicin, indole-3-carbinol and germanium, showed no significant modifying effects on PAPG induction.

The results suggest that allyl sulfide has inhibitory effect on MNNG-induced rat gastric carcinogenesis.

INTRODUCTION

The human diet contains, in addition to a great variety of natural carcinogens, many inhibitors of experimental carcinogenesis, including antioxidants, ethoxyquin, retinoids, flavones, indoles and selenium (Miller *et al.*, 1986; Pariza *et al.*, 1986; Ames, 1983). Epidemiological evidence from different

geographical locations and dietary customs suggests that such food constituents play an important modulatory role in human cancer incidences (Doll *et al.*, 1981; Parkin *et al.*, 1980). Some can exhibit promotional as well as inhibitory behavior (Jang *et al.*, 1989a, 1988; Ito *et al.*, 1987), and thus it is of fundamental importance that such opposing activities be fully understood both at the mechanistic level and in terms of their promotional versus inhibitory potencies.

In vivo mid-term screening tests for several organs including liver, lung and urinary bladder carcinogens and promoters based on qualitative putative preneoplastic changes have been devloped (Yun *et al.*, 1988; Ito *et al.*, 1988; Fukushima *et al.*, 1983). However, there is no adequate *in vivo* mid-term screening tests for gastric carcinogens and promoters due to the lack of a comparable marker of putative preneoplastic change in the stomach. This is particularly important since induction of gastric cancer takes a long time. There is therefore a need for a marker which could act in the same way as glutathione S-transferase placental type (GST-P) positive foci in the liver. Recently Tatemastsu *et al.* (1988; 1987a,b) demonstrated that Pg 1 decreased pyloric glands might be a useful marker for rapid detection of gastric carcinogens and promoters.

There are three isozymes of pepsinogens (Pg 1, Pg 3 and Pg 4) in normal rat pyloric mucosa (Furihata *et al.*, 1973). Pg 1 content reduced in normal-appearing pyloric mucosa, and also in adenomatous hyperplasia and adenocarcinoma in glandular stomach of rats treated with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). In an immunohistochemical study of Pg 1, pyloric glands low in Pg 1, termed Pg 1-altered pyloric glands (PAPG), were demonstrated in normal-appearing pyloric mucosa in the early stages of MNNG-induced stomach carcinogenesis in rats. Induction of PAPG was dose-dependent, numbers of PAPG gradually increased with time. PAPG detected immunohistochemically is therefore considered to be a putative preneoplastic change in the glandular stomach of rats (Tatematsu *et al.*, 1988, 1987b).

In the present study, we tested modifying effects of capsaicin, allyl sulfide, indole-3-carbinol which occur commonly in Korean food and germanium using PAPG as a preneoplastic marker of rat gastric carcinogenesis.

MATERIALS AND METHODS

Animals and Chemicals

MNNG was obtained from Aldrich Chemical Co. (Milwaukee, WI). Male Wistar rats were purchased from Korea Research Institute of Chemical Technology, Toxicology Center and used at 6 weeks of age for this study. Animals were housed (3-4/polycarbonate cage) on hardwood chips in an air-conditioned room with a 12 h light/dark cycle. They were given continuous access to pellet diet made by NIH-open formula and water. Rats of groups 1 to 5 were given drinking water containing 100 μg/ml of MNNG for 8 weeks and 10% NaCl in diet. After then animals were received basal diet or diet containing 0.01% capsaicin (Fluka Co., Switzerland), 0.5% allyl sulfide (Sigma Co., USA), 0.5% indole-3-carbinol (Sigma Co., USA) and 0.05% germanium (GE-132) (Takachihe Inc., Japan), kindly donated by Dr. Duk-

Sam Rhoe until week 20. Groups 6 to 9 were given test chemicals alone without MNNG and 10% NaCl treatment from week 8 till week 20. Control group (group 10) was given normal tap water and basal diet. All rats were sacrificed at week 20 of the experiment. The stomach was removed and fixed in sublimated formaldehyde, cut longitudinally into about 8 strips, embedded in paraffin for histopathology.

Histopathological Analyses

Sections of stomach tissues were stained by H & E. Lesions of pyloric mucosa were categorized as normal-appearing mucosa, changed mucosa (mucosal hyperplasia or atrophy) and adenomatous hyperplasia according to the criteria previously reported (Tatematsu *et al.*, 1987b).

Immunohistochemical and histochemical procedures

Anti-Pg 1 serum was generous gift from Dr. Chie Furihata (Department of Molecular Oncology, Institute of Medical Science, University of Tokyo). The ABC method was used to determine the localization of Pg 1 in the pyloric mucosa. Biotin-labelled goat anti-rabbit immunoglobulin (IgG and avidin-biotin-peroxidase complex; Vectastain ABC Kit, PK4001) was obtained from Vector Laboratories (Burlingame, CA, USA). Before immunostaining sections were treated with 80% iodo-alcohol and 5% sodium thiosulfate for the removal of mercuric precipitates. Endogenous peroxidase activity was blocked by methanolic hydrogen peroxide. The sections were then washed with distilled water transferred to PBS and stained by the ABC method. The sections were treated sequentially with a) diluted normal goat serum, b) rabbit anti-rat Pg 1 IgG (1: 10000), c) biotin-labelled goat anti-rabbit IgG (1:400), and d) ABC. The site of peroxidase binding was determined by the diaminobenzidine. Then section were counterstained with hematoxylin. As a negative control for the specificity of anti-Pg 1 antibody, preimmune rabbit serum was used instead of anti-Pg 1 IgG. To evaluate mucin content, paradoxical concanavalin A (Con A) staining (PA-reduction-Con A-HRP) were used as previously described (Katsuyama *et al.*, 1978). By this method, class III mucins stain brown, as distinct from unstained class II mucins. Class III mucins were found in pyloric gland cells, mucous neck cells and Brunner's gland cells, all of which normally contained Pg 1. In normal-appearing pyloric mucosa, pyloric glands that stained weakly or negative for Pg 1 were defined immunohistochemically as Pg 1-altered pyloric gland (PAPG). Pyloric glands that were cut longitudinally from the neck to the bottom in the central part were examined, and the number of PAPG per 100 pyloric glands were calculated by counting over 500 pyloric glands. Because glands consisting of pyloric gland-type cells in areas of changed mucosa and adenomatous hyperplasia varied in size, areas of cells of the pyloric gland cell type containing little or no Pg 1 were measured by VIDAS Automatic Image Analysis System (Kontron Ltd., West Germany).

RESULTS

Results of mean body weight and daily intake of diet are summarized in Table 1. The mean body weights of MNNG-treated groups were generally lower than MNNG-untreated groups. However, daily consumption of diet revealed no differences between corresponding groups. Results on morphological changes and immunohistochemical staining for Pg 1 were summarized at Table 2. Changed pyloric mucosa, showing atrophic or hyperplastic change, was occasionally observed in MNNG-treated groups. A few cases of adenomatous hyperplasia were also found in groups 1, 2, 4 and 5, however, no adenomat-

Table 1. Mean body weight and daily intake of diet

Group	Effective no. of rats	Body weight (g)	Daily intake of diet (g/rat/day)
1. MNNG	24	289.1±143.3*	22.6±1.6
2. MNNG→CAP	18	278.0±107.9	21.3±2.7
3. MNNG→AS	18	278.9±111.8	22.1±2.2
4. MNNG→I3C	17	262.6± 98.1	23.4±2.8
5. MNNG→GER	19	278.3±107.6	24.2±2.6
6. CAP	13	389.5±105.5	22.1±1.0
7. AS	10	388.8±103.7	23.9±0.4
8. I3C	10	392.7± 96.9	25.9±1.5
9. GER	10	395.2± 98.6	24.8±0.7
10. CONTROL	9	317.7± 84.8	24.2±0.4

* : Mean±SD

MNNG : N-methyl-N'-nitro-N-nitrosoguanidine, CAP : Capsaicin, AS : Allyl sulfide, I3C : Indole-3-carbinol, GER : Germanium

ous hyperplasia was observed in group 3. In control rats, no pyloric glands with low Pg 1 contents were found. In groups 1 to 5, all rats showed Pg 1 altered pyloric glands in normal-looking pyloric mucosa. Treatment of allyl sulfide after MNNG and NaCl showed significant inhibition of PAPG induction. However, other chemicals including capsaicin, indole-3-carbinol and germanium did not show any modifying effects on PAPG induction. In changed mucosa, some cells of the pyloric gland type cells with class III mucins had a high Pg 1 content like normal pyloric gland cells, whereas others contained little or no Pg 1. Cells of the pyloric gland cell type with a low Pg 1 content in changed mucosa constituted about 25% of the area of pyloric gland type cells in the groups of 1 to 5. In adenomatous hyperplasia, almost all cells of the pyloric gland cell type had class III mucins and a low Pg 1 content.

DISCUSSION

The present findings clearly show that allyl sulfide possessed inhibitory effects on the induction of PAPG pretreated with MNNG and NaCl. Dietary inhibitors of mutagenesis and carcinogenesis are of particular interest because they may be useful for human cancer prevention. Many natural compounds of

Table 2. Incidences of morphological changes and PAPG in pyloric glands

Group	Effective no. of rats	Normal looking		Changed Mucosa		Adenomatous hyperp.	
		Incidence of PAPG(%)	No. of PAPG[a,b]	Incidence (%)	PCNPG[c,d] area	Incidence (%)	PCNPG area
MNNG	24	100	2.4 ±1.5	40	24.5±9.7	10	95.7±3.2
MNNG→CAP	18	100	2.6 ±1.7	42	28.7±10.6	8	96.7±4.6
MNNG→AS	18	100	1.1 ±0.4*	17	13.8±4.6	0	0
MNNG→I3C	17	100	2.1 ±1.2	35	24.4±11.2	12	98.5±4.7
MNNG→GER	19	100	2.2 ±1.4	38	26.5±12.3	11	97.4±4.9
CAP	13	20	0.8 ±0.3	0	0	0	0
AS	10	10	0.4	0	0	0	0
I3C	10	10	0.6	0	0	0	0
GER	10	0	0	0	0	0	0
Control	9	0	0	0	0	0	0

[a]PAPG = pyloric glands with little or no Pg 1 content
[b]No. of PAPG = No. of PAPG/100 pyloric glands
[c]PCNPG = pyloric gland-type cells with little or no Pg 1
[d]PCNPG area = area (mm^2) of PCNPG/area ($100mm^2$) of pyloric gland-type-cells

Significantly different from MNNG group at *$p<0.001$

food have been designated anticarcinogens because they may inhibit various initiating and promoting mechanisms of chemical carcinogenesis (Pariza *et al.*, 1986). Dietary factors exert the greatest environmental influence on carcinogenesis and Doll and Peto have estimated that diet is responsible for approximately 35% of the total cancer deaths in the USA (Doll *et al.*, 1981).

Recently, attention has been paid to the pharmacologic activity of allium extracts and oils in the inhibition of carcinogenesis. Animal and *in vitro* experiments indicate that compounds in allium vegetables (e.g., allyl sulfides) inhibit several types of tumors and decrease tumor growth and proliferation (Jang *et al.*, 1989b; Hayes *et al.*, 1987; Wargovich *et al.*, 1987). Data on the effects of dietary intake of allium vegetables from study performed in a population at high risk for stomach cancer in Shandong, China also revealed a significant reduction in gastric cancer risk with increasing consumption of allium vegetables (You *et al.*, 1989). A possible mechanism for this protective effect is the selective inhibition of cytochrome P450IIE1, which is involved in the initial hepatic activation of the procarcinogen and suppression of its level in microsomes (Bradly *et al.*, 1988). You *et al.* (1989) suggest that since garlic

has antibacterial properties, it may influence stomach cancer risk by inhibiting bacterial growth in the gastric cavity, thus resulting in less conversion of nitrates to nitrites and a lowered probability of endogenous formation of N-nitroso compounds. We also reported that inhibition of rat GST-P hepatic foci development by allyl sulfide (Jang *et al.*, 1989b). Those findings are in agreement with the data from experiments using other carcinogens suggesting that this modifying potential is independent of initiator.

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the major pungent ingredient of hot peppers of the plant genus *Capsicum*. Chili extract and capsaicin are reported to be mutagenic as determined by the Ames test and the micronucleus test (Nagabhushan *et al.*, 1985). There are also some reports on the induction of neoplastic changes by capsaicin (Jang *et al.*, 1988; Toch *et al.*, 1984). However, some investigators could not detect definite carcinogenic activity of capsaicin at rather low dose level (Agrawal *et al.*, 1987). Our recent study revealed inhibitory effect of capsaicin on mouse lung tumor development (Jang *et al.*, 1989a). However, the present results disclose no significant modifying effects on rat gastric PAPG induction.

Indole-3-carbinol (I3C) occurs naturally in edible cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, and cauliflower. It exerts several important effects in experimental carcinogenesis models, exhibiting both inhibitory and promotional activity (Bailey *et al.*, 1987; Nixon *et al.*, 1984). I3C did not show any modifying effect on the present rat gastric PAPG induction system.

Germanium is present in all living plant and animal matter in micro-trace quantities. Ge-132 exhibited significant antitumor activity against a wide spectrum of tumor cell lines, including Walker 256, Ehrlich Ascites, BC47, Lewis lung, IMC carcinoma, AH 66, AH 43, MH 134 (Brutkiewicz *et al.*, 1987). In another study of the effect of Sanumgerman on tumor genesis in mice, it significantly lowered the incidence of tumors. However, our present studies showed no inhibitory effect on the induction of PAPG.

Some compounds, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can exert either enhancement or inhibition depending upon the organ (Ito *et al.*, 1987). BHA significantly enhanced tumor development in the forestomach and urinary bladder, but inhibited the development of liver lesions. Similarly, BHT promoted urinary bladder carcinogenesis, but inhibited liver carcinogenesis, and in addition it enhanced the development of MNU-initiated thyroid neoplasia. Therefore, the modifying effects of allyl sulfide on carcinogenesis in other organs should be tested, preferably by introducing a whole body carcinogenesis concept.

Further research on allium vegetables and their constituents may provide insights into the primary prevention of stomach cancer, still the most common cause of death among cancer patients of Korean people.

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Capsaicin, Allyl sulfide, Indole-3-Carbinol 및 Germanium의 MNNG 유발 랫트 폡시노젠 1 변이 위 유문선 발현 수식효과

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한국인의 상용식품중 대표적인 고추, 마늘, 배추의 주성분으로 알려진 Capsaicin, Allyl sulfide, Indole-3-carbinol 및 Germanium의 랫트 위암발생 수식효과를 검토하기 위하여 MNNG 및 10% NaCl로 Initiation 시킨후 각종 검색물질을 사료에 섞어 투여하였다. 발암수식 효과는 전암병변 Marker로 알려진 폡시노젠 1변이 유문선 (PAPG) 발현양상을 면역조직화학적 방법으로 검색하여 상응하는 대조군과 비교하였고 그 결과는 다음과 같다.

1. MNNG 및 10% NaCl투여군은 비투여군에 비해 체중이 현저히 감소하였으나 검색물질 투여군 상호간에는 큰 차이를 보이지 않았다. 일일 평균 사료 섭취량은 각군간에 차이가 없었다.
2. MNNG와 NaCl 치치후 Allyl sulfide를 투여한 군에서 MNNG 단독 투여군에 비해 PAPG발현이 유의하게 감소하였고, 유문 점막의 증식성 변화도 감소하는 경향을 보였다. 따라서 Allyl sulfide 가 MNNG 유발 랫트 위암발생을 억제함을 강력히 시사하고 있다.
3. Capsaicin, Indole-3-carbinol 및 Germanium은 PAPG 발현에 유의한 수식효과를 나타내지 않았다.