Modifying responses of allyl sulfide, indole-3-carbinol and germanium in a rat multi-organ carcinogenesis model

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The modifying potential of allyl sulfide (AS), indole-3-carbinol (I3C) and carboxyethylgermanium sesquioxide (GE) on lesion development was examined in a wide-spectrum initiation model. Groups 1-4 were treated sequentially with diethylnitrosamine (DEN) (100 mg/kg, i.p., single dose), *N*-methylnitrosourea (MNU) (20 mg/kg, i.p., four doses at days 2, 5, 8 and 11), and *N,N*-dibutylnitrosamine (DBN) (0.05% in drinking water during weeks 3 and 4). Groups 5-7 received vehicles without carcinogens during the initiation period. Group 8 served as the untreated control. After this initiating procedure, groups 2-7 were administered a diet containing 0.5% AS or I3C and 0.05% GE. All surviving animals were killed 40 weeks after the beginning of the experiment and the target organs were examined. The induction of GST-P$^+$ hepatic foci in rats treated with carcinogens was significantly inhibited by treatment with all three compounds. AS treatment significantly decreased the incidence of hepatic hyperplastic nodules, adenoma of the lung and thyroid, and papillary or nodular hyperplasia of the urinary bladder. Administration of GE also significantly inhibited the development of hepatic nodules and adenoma of the lung and thyroid. However, I3C only inhibited the hyperplastic nodules of the liver. These results demonstrated that this multi-organ initiation model could be useful in confirming organ-specific modification potential and, in addition, the inhibitory effect of AS, I3C and GE on liver, lung, thyroid and urinary bladder carcinogenesis.

Introduction

Recently it has been shown that naturally-occurring chemicals in certain vegetables and fruits can inhibit the process of carcinogenesis in animals. Examples of such compounds are phenols, indoles, aromatic isocyanates, terpenes, organosulphur compounds, retinoids and selenium (1-3). Epidemiological evidence from different geographical locations and dietary customs suggests that such food constituents play an important modulatory role in human cancer incidence (4,5). Some exhibit promotional as well as inhibitory behavior (6-9), and thus it is of fundamental importance that such contradictory activities be fully understood.

Animal and *in vitro* experiments indicate that allyl sulfide (AS) (a component of garlic oil), indole-3-carbinol (I3C) (a component of cruciferous vegetables) and carboxyethylgermanium sesquioxide (GE) (organic trace element) can inhibit several types of tumors and decrease tumor growth and proliferation. Inhibitory effects of AS, I3C and GE on chemical carcinogenesis during, shortly before or shortly after carcinogen exposure had been studied in various experimental systems using a single carcinogen as an initiator (10-17). However, the majority of these studies concerned only one organ system or the effect on the initiation period. In our previous studies it had been found that AS inhibited the induction of GST-P$^+$ hepatic foci and gastric pepsinogen-altered pyloric gland in rat, but I3C and GE did not show any significant modifying potential (8,9).

In general, chemicals show organ specificity in their modifying action and may exert very different effects on different organs. For example, butylated hydroxyanisole not only inhibits but can also strongly enhance carcinogenesis depending on the organ, regardless of whether the carcinogens act directly or require metabolism (18). Similarly, butylated hydroxytoluene promotes urinary bladder carcinogenesis, but inhibits liver carcinogenesis. In addition, it enhances the development of *N*-methylnitrosourea (MNU)-initiated thyroid neoplasia (6). Therefore, the modifying effects of various dietary constituents on carcinogenesis should be tested by a whole-body multi-organ concept of carcinogenesis (19,20), because putative chemopreventive agents can not be allowed to have promoting effects in any organs.

In the present study we investigated the organ-specific modifying potential of AS, I3C and GE in a whole-body carcinogenesis model induced by diethylnitrosamine (DEN), MNU and *N,N*-dibutyl nitrosamine (DBN), especially focused on the effects in the post-initiation period.

Materials and methods

Animals, diets and chemicals

A total of 160 6-week-old male F344 rats (Korea Research Institute of Chemical Technology) were used. Four or five animals were kept in each polycarbonate cage in a room at 22 ± 2°C with a 12-h light/dark cycle. They were given diet and tap water *ad libitum*. The composition of powdered control diet which is based on AIN-76A diet is as follows (21): casein, 20%; DL-methionine, 0.3%; corn starch, 15%; sucrose, 50%; cellulose, 5%; AIN-76 mineral mix, 3.5%; AIN-76A vitamin mix, 1%; and choline bitartrate, 0.2%. All diet ingredients were mixed in our laboratory. The incorporation of AS, I3C and GE into the semi-purified diet was done after they were pre-mixed with a small quantity of diet in a food mixer to ensure uniform distribution of these compounds. The dose levels of AS or I3C (0.5%) and GE (0.05%) were determined by the results of our previous experiments (8,9). DEN, DBN, I3C (CAS: 700-06-1) were obtained from Sigma Co., USA; AS (CAS: 592-88-1) was from Fluka Co., Switzerland; MNU was from Nakarai Chemical Co., Japan; GE (CAS: 12758-40-6) was from Takachiho Inc., Japan.

Experimental design

The animals were divided into eight groups. As shown in Figure 1, groups 1 through 4 were pre-treated with three carcinogens. The rats were initially given a single i.p. injection of 100 mg/kg DEN dissolved in 0.9% NaCl solution, MNU was given at 20 mg/kg, i.p., in a citrate-buffered solution adjusted to pH 6.0, four doses on days 2, 5, 8 and 11. DBN was given at 0.05% in drinking water during weeks 3 and 4. Groups 5-7 received vehicles without carcinogens and group 8 served as the untreated control. Subsequently, the animals in groups 2-7 were given a diet containing 0.5% AS or I3C and 0.05% GE until week 40. All surviving animals were killed and their major organs—including thyroid, lungs, esophagus, stomach, intestine, pancreas, liver, kidney and urinary bladder—were removed and fixed in 10% buffered formalin solution. All organs were embedded in paraffin and stained with hematoxylin and eosin for histologic examination.

Abbreviations: AS, allyl sulfide; I3C, indole-3-carbinol; GE, carboxyethylgermanium sesquioxide; MNU, *N*-methylnitrosourea; DEN, diethylnitrosamine; DBN, *N,N*-dibutyl nitrosamine; B[*a*]P, benzo[*a*]pyrene.

* 이 論文은 1991年度 Carcinogenesis Vol. 12 no. 4에 발표된 것임.

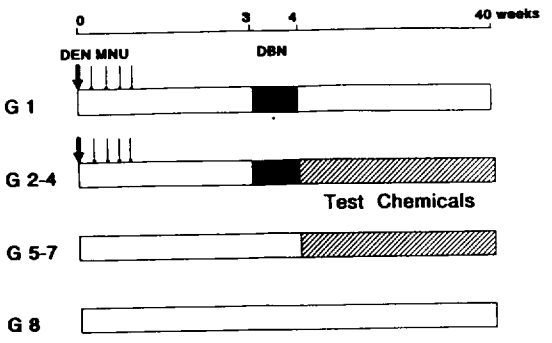

Fig. 1. Experimental design. Animals: 6-week-old male F344 rats. Carcinogens: DEN (100 mg/kg i.p.), MNU (200 mg/kg i.p.), DBN (0.05% in drinking water). Test chemicals: 0.5% AS or I3C, 0.05% GE in diet.

Only the animals which survived until the end of week 40 were included in the effective numbers. For quantitative analysis of GST-P$^+$ hepatic foci, three slices of liver tissue were fixed in ice-cold acetone and processed for embedding in paraffin, and a subsequent immunohistochemical examination was done by the ABC method. The data were measured by a color image processor, model VIDAS (Kontron, FRG).

Data on the incidences of lesions were analyzed for statistical significance with the two-tailed χ^2-test. Other data were analyzed with Student's *t*-test.

Results

Data concerning mean body wts and relative liver, lung and kidney wts in all groups are shown in Table I. The I3C-treated groups with or without carcinogens showed decreased mean body wt, but there were no significant differences between the corresponding controls. Relative organ wts also showed no statistical difference. Table II shows the average daily and total intake of diet and test chemicals. The AS-alone group had the lowest daily intake of diet and averge food consumption was

Table I. Mean body and organ wts

Group	Treatment	No. of rats	Mean body wt[a] (g)	Relative organ wt[a] (g/100 g body wt[a] (g)		
				Liver	Lungs	Kidneys
1.	DEN + MNU + DBN	16	302 ± 87	3.0 ± 0.3	0.59 ± 0.32	0.90 ± 0.71
2.	DEN + MNU + DBN → AS	18	290 ± 85	3.2 ± 0.4	0.83 ± 0.67	0.82 ± 0.12
3.	DEN + MNU + DBN → I3C	17	273 ± 79	3.1 ± 0.2	0.52 ± 0.10	0.93 ± 0.15
4.	DEN + MNU + DBN → GE	19	301 ± 89	3.0 ± 0.3	0.54 ± 0.21	0.68 ± 0.13
5.	AS	20	333 ± 90	3.1 ± 0.6	0.44 ± 0.06	0.68 ± 0.08
6.	I3C	20	301 ± 68	3.0 ± 0.3	0.61 ± 0.43	0.71 ± 0.08
7.	GE	20	342 ± 87	3.0 ± 0.2	0.50 ± 0.10	0.72 ± 0.09
8.	Control	20	324 ± 82	2.9 ± 0.3	0.49 ± 0.09	0.66 ± 0.07

[a]Values represent mean ± SD.

Table II. Average daily and total intake of diet and test chemical

Group	Treatment	Average daily intake[a]		Total intake of test chemical (g/kg body wt/36 weeks)
		Diet (g/kg body wt/day)	Test chemical (mg/kg body wt/day)	
1.	DEN + MNU + DBN	48.2 ± 3.2	0	0
2.	DEN + MNU + DBN → AS	48.3 ± 4.2	241.5 ± 17.1	60.9
3.	DEN + MNU + DBN → I3C	44.6 ± 9.8	223.0 ± 24.4	56.2
4.	DEN + MNU + DBN → GE	44.9 ± 0.2	22.5 ± 0.1	5.7
5.	AS	43.0 ± 2.0	215.2 ± 8.1	54.2
6.	I3C	54.2 ± 4.6	271.2 ± 9.6	68.3
7.	GE	46.1 ± 2.3	23.1 ± 0.9	5.8
8.	Control	46.9 ± 3.6	0	0

[a]Values represent mean ± SD.

Table III. Quantitative data of GST-P$^+$ foci

Group	Treatment	No. of rats	GST-P$^+$ foci data[a]	
			No./cm^2	Area (mm^2)/cm^2
1.	DEN + MNU + DBN	16	19.2 ± 9.2	1.15 ± 0.71
2.	DEN + MNU + DBN → AS	18	9.5 ± 6.4*	0.58 ± 0.25*
3.	DEN + MNU + DBN → I3C	17	5.2 ± 2.3***	0.25 ± 0.15**
4.	DEN + MNU + DBN → GE	19	8.2 ± 4.4***	0.43 ± 0.24**
5.	AS	20	0.1 ± 0.4	0.01 ± 0.02
6.	I3C	20	0	0
7.	GE	20	0	0
8.	Control	20	0	0

Significantly different from group 1 at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
[a]Values represent mean ± SD.

Table IV. Incidence of neoplastic and pre-neoplastic lesions

	Treatment							
	CAR (16)[a]	CAR + AS (18)	CAR + I3C (17)	CAR + GE (19)	AS (20)	I3C (20)	GE (20)	Control (20)
Liver								
Hyperplastic nodule	8 (50)[b]	1 (6)**	2 (12)*	2 (11)*	0	0	0	0
Lung								
Hyperplasia	2 (13)	3 (17)	3 (18)	2 (11)	0	1 (5)	0	1 (5)
Adenoma	9 (56)	2 (11)**	5 (29)	1 (5)***	0	0	0	0
Carcinoma	3 (19)	0	1 (6)	0	0	0	0	0
Thyroid								
Hyperplasia	3 (19)	3 (17)	2 (12)	4 (21)	0	0	0	1 (5)
Adenoma	8 (50)	2 (11)*	4 (24)	2 (11)*	0	0	0	0
Carcinoma	2 (13)	0	1 (6)	1 (5)	0	0	0	0
Kidney								
Atypical tubules	4 (25)	3 (17)	3 (18)	2 (11)	0	0	0	0
Stromal cell proliferation	1 (6)	1 (6)	2 (12)	1 (5)	0	0	0	0
Nephroblastoma	1 (6)	0	1 (6)	0	0	0	0	0
Urinary bladder								
Simple hyperplasia	14 (88)	10 (56)	11 (65)	9 (47)	0	1 (5)	0	1 (5)
PN hyperplasia	9 (56)	2 (11)**	4 (24)	5 (26)	0	0	0	0
Papilloma	4 (25)	2 (11)	2 (12)	1 (5)	0	0	0	0
Carcinoma	1 (6)	0	1 (6)	0	0	0	0	0
Others	2 (13)	3 (17)	3 (18)	2 (11)	0	0	0	0

CAR: Carcinogens (DEN + MNU + DBN).
Significantly different from CAR group at $*P < 0.02$, $**P < 0.01$, $***P < 0.001$.
[a]Number of rats examined.
[b]Number of tumor-bearing rats/rats examined (%).

slightly less in groups 3 and 4 than in group 1. Several rats died of pneumonia in the initiation stage from three carcinogens. These animals were not included in the effective numbers.

The induction of GST-P$^+$ hepatic foci in rats treated with carcinogens was significantly inhibited by treatment with 0.5% AS or I3C and 0.05% GE (Table III). Two out of 20 rats in the AS-alone group showed a few GST-P$^+$ foci. Table IV shows the incidence of neoplastic and pre-neoplastic lesions induced by three carcinogens. In rats treated with a combination of DEN, MNU and DBN, neoplastic and pre-neoplastic lesions were primarily induced in the liver, lung, thyroid, kidney and urinary bladder. AS significantly decreased the incidences of hepatic nodules, adenoma of the lung and thyroid and papillary or nodular (PN) hyperplasia of the urinary bladder ($P < 0.02$ or $P < 0.01$). Similarly, the incidence of hepatic nodules and adenoma of the lung and thyroid was lower in rats given GE after carcinogen exposure ($P < 0.02$ or $P < 0.001$). However, administration of I3C only inhibited the hyperplastic nodules of the liver. Although some malignant lesions were induced in the thyroid, kidney and other organs by carcinogen treatment, their incidences were not significantly different in the AS-, I3C- or GE-supplemented groups. Without carcinogen pre-treatment, none of the test chemicals induced any neoplastic lesions, and although I3C alone was associated with one case of hyperplasia of lung and urinary bladder, this was not significant. The untreated control rat also showed a case of hyperplastic lesion in the lung, thyroid and urinary bladder. No specific toxic effects of test chemicals in non-initiated animals were observed.

Discussion

The results of the present experiment clearly demonstrated that AS and GE exert an inhibitory potential on the various organs initiated by the combined carcinogen treatments.

Sequential treatment with potent carcinogens having different wide-spectrum initiating activities was used as the initiation step in this study. DEN is known to be a strong initiator of hepato-carcinogenesis, whereas MNU initiates the thyroid, urinary bladder and hematopoietic systems. DBN is carcinogenic to the esophagus, forestomach, liver and urinary bladder of rats. Ito *et al.* (18–20) already reported that the wide-spectrum initiation model could be used for the confirmation of site-specific modification potential. The results of the present experiment also demonstrated that these three carcinogens can induce pre-neoplastic or neoplastic lesions of the various organs. Although more experiments are necessary to optimize the observation period and dose of the initiating carcinogens, they do suggest that this model is very useful in detecting the modifying effects of the test chemicals on multiple organs.

Recently, attention has been paid to the pharmacologic activity of allium extracts and oils in the inhibition of carcinogenesis. Animal experiments suggest that allium compounds can inhibit both the initiation and promotion stages of carcinogenesis. AS, administered 96 and 48 h prior to the carcinogen, was found to inhibit benzo[*a*]pyrene (B[*a*]P)-induced neoplasia of the forestomach in female A/J mice (10,11). AS, administered by gavage 3 h prior to the carcinogen, inhibited 1,2-dimethyl-hydrazine-induced neoplasia of the large bowel in mice and

N-nitrosomethylbenzylamine-induced esophageal carcinogenesis in the rat (12,13). Previously we observed that AS inhibited the induction of GST-P$^+$ hepatic foci and pepsinogen-altered pyloric gland in rat stomach (8,9). Nishino *et al.* also reported that garlic extracts inhibited skin tumor promotion in mice (22). Data on the dietary intake of allium vegetables from the study performed on a population at high risk for stomach cancer in Shandong, China, also revealed a significant reduction in gastric cancer risk that correlated with an increased consumption of allium vegetables (23). A possible mechanism for these anticarcinogenic effects of AS is the selective inhibition of cytochrome P450IIE1, which is involved in the initial hepatic activation of the pro-carcinogen and suppression of its level in microsomes (24). The present findings, which are in agreement with the data from experiments using other carcinogens, suggest that the inhibitory potential of AS in carcinogenesis is independent of the initiator. A few GST-P$^+$ foci which were observed in the AS-alone group were considered as spontaneously-induced lesions, because the incidence and numbers are negligible. It may be due to individual differences in susceptibility or to some unknown factors.

In this experiment, GE showed an inhibitory effect on the development of neoplastic or pre-neoplastic lesions of the liver, lung and thyroid. Germanium is present in all living plant and animal matter in micro-trace quantities. The average daily human intake of GE ranges from 0.9−3.2 mg (25). *In vitro* cytotoxicity studies of Spirogermanium with HeLa cells and Chinese hamster cells demonstrated the highly significant inhibition of DNA, RNA and protein synthesis at micromolar concentrations (26), and Hill *et al.* (27) documented the cytotoxic activity of Spirogermanium on NIL 8 hamster cells and a wide range of human cancer cell lines. Ge-132 exhibited moderate anti-tumor activity against transplantable Sarcoma 180, Melanoma B16 and Lewis lung carcinoma in mice (28). In another study of the effect of Sanumgerman on tumorigenesis in mice, it was shown to significantly lower the incidence of tumors. These results were identically reproduced by Lekim and Kehlbeck (14), strongly indicating the protective influence of GE against fibrosarcoma. The anti-tumor activity of GE appears to be expressed by activation of the immune mechanisms, including macrophages and/or T lymphocytes (27). Our previous studies showed equivocal modifying effects on rat liver and stomach (8,9).

The present results also showed that I3C possesses an inhibitory effect on hepatic nodules and GST-P$^+$ foci induction after sequential treatment with DEN, MNU and DBN. I3C, a good inducer of aryl hydrocarbon hydroxylase and glutathione-S-transferase, has been found to inhibit B[*a*]P-induced neoplasia in ICR/Ha forestomach, DMBA-induced mammary neoplasia in Sprague−Dawley rats and aflatoxin B_1-induced hepatic carcinogenesis in rainbow trout when administered by gavage or in the diet (15,16). However, dietary I3C enhanced colon tumorigenicity induced by dimethylhydrazine in rats when administered in the diet for the duration of the experiment, while enhancing aflatoxin B_1 tumorigenesis in trout liver when given after initiation (30,31). Investigation of the mechanism responsible for the anti-carcinogenic effects of I3C in trout revealed substantial changes in the *in vivo* uptake, distribution and metabolism of aflatoxin B_1 such that carcinogen−DNA binding in the target organ was significantly attenuated (17). In the present study, I3C did not exert any significant modifying activity for multi-organ carcinogenesis, except for inhibition of hepatic lesion. We have no explanation for this result, but speculate that differences in carcinogens, the dose and the duration of the I3C treatment, as well as differences in the animal species used, could account for the near absence of an inhibitory effect of I3C in the present study.

The results of the present study provide evidence for the diversity of naturally occurring compounds having the capacity to inhibit wide-spectrum initiation carcinogenesis. Humans are repeatedly exposed to complex and various mixtures that contain both initiators and promoters. Therefore, the impact of such inhibitory effects on these multiple carcinogens could be important, but further research would be required to understand the underlying mechanisms for such inhibitory effects.

Acknowledgements

The authors wish to thank Professor Kiyomi Sato of Hirosaki University, Hirosaki, for his generous gift of GST-P antibody, along with Mr Won Suk Jang, Miss Sun Joo Lee and Miss Mi Jung Eui for their excellent technical assistance. This study was supported by a National Project Grant from the Ministry of Sciences and Technology.

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Received on October 11, 1990; revised on January 3, 1991; accepted on January 14, 1991