Jpn. J. Cancer Res. 85, 1067–1072, November 1994

Enhancing Effects of Organosulfur Compounds from Garlic and Onions on Hepatocarcinogenesis in Rats: Association with Increased Cell Proliferation and Elevated Ornithine Decarboxylase Activity

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Four organosulfur compounds from garlic and onions were examined for modifying effects on diethylnitrosamine (DEN)-induced neoplasia of the liver in male F344 rats using the medium-term bioassay system based on the two-step model of hepatocarcinogenesis. Carcinogenic potential was scored by comparing the numbers and areas per cm^2 of induced glutathione S-transferase placental form-positive foci. Isothiocyanic acid isobutyl ester (IAIE), dipropyl trisulfide (DPT), and allyl mercapton (AM) exerted enhancing effects on their development, while dimethyl trisulfide also tended to increase them. To investigate possible mechanisms of the modifying influence, sequential changes in ornithine decarboxylase activity (ODC) over 24 h were measured in AM-treated liver tissue without prior DEN initiation. The activity started to increase by 4 h after AM-treatment, and reached maximum at 16 h, compared to controls. Spermidine/spermine N^1-acetyltransferase activity was not significantly changed. An increase in proliferating cell nuclear antigen-positive cells followed the elevation of ODC activity. These results suggest that IAIE, DPT, and AM promote rat hepatocarcinogenesis and their promoting effect might be caused by increased cell proliferation with increased polyamine biosynthesis. In evaluating relationships between diet and cancer, it is thus appropriate to consider not only a possible protective role of garlic and onions , but also enhancing effects.

Key words: Organosulfur compound — Hepatocarcinogenesis — Medium-term bioassay system — Polyamine biosynthesis

Environmental compounds are known to be involved in the development of many human cancers, and their elimination from our environment would be expected to help in the prevention of human cancer. However, this is not a practical proposition, and therefore, it is important to discover naturally occurring or synthetic compounds which can suppress or prevent the process of carcinogenesis.[1–10]

We have focused our attention on organosulfur compounds (OSCs) contained in garlic and onions, which have been proved to be chemopreventive in some animal models. For example, diallyl sulfide (DAS) inhibits development of colon carcinomas, esophageal carcinomas, pulmonary adenomas, and forestomach tumors in rodents when administered prior to carcinogen exposure.[11–14] Moreover, DAS inhibited hepatocarcinogenesis when administered after an initiating procedure, though this has not been consistently observed.[15, 16]

In our previous experiment, simple second-stage administration of five OSCs, DAS, diallyl trisulfide (DAT), allyl methyl sulfide (AMS), allyl methyl trisulfide (AMT), and dipropyl sulfide (DPS), resulted in enhancement of a putative preneoplastic lesion,[5] i.e., glutathione S-transferase placental form (GST-P)-positive focus formation in the liver, with combined treatments at low doses exerting dose-dependent enhancing effects. Moreover, this proved to be associated with increased cell proliferation and elevated polyamine biosynthesis. On the other hand, methyl propyl disulfide (MPD) and propylene sulfide (PS) significantly decreased the GST-P-positive focus formation.[17]

In the present study, the promoting stage-modifying potential of four OSCs contained in garlic and onion were therefore examined in the rat liver medium-term bioassay system defined by Ito *et al.*[5] To investigate possible mechanisms that might be involved, the activities of ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SAT), rate-limiting enzymes of polyamine biosynthesis which are reported to be increased in the promotion stage of skin and bladder carcinogenesis,[18, 19] were also measured.

MATERIALS AND METHODS

Chemicals Dipropyl trisulfide (DPT) and dimethyl trisulfide (DMT) were obtained from Oxford Chemicals Co., Northhamptonshire, UK, and allyl mercapton (AM), isothiocyanic acid isobutyl ester (IAIE) and N-

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diethylnitrosamine (DEN) were from Tokyo Chemical Industry Co., Tokyo.

Animals and treatments Male F344 rats obtained at 5 weeks of age (Charles River Japan, Inc., Hino, Shiga) were housed in an air-conditioned room at a temperature of 23±1°C and a relative humidity of 36±6%, with a 12 h light-12 h dark cycle, and were given pellet diet (Oriental MF; Oriental Yeast Co., Tokyo) and tap water *ad libitum* during the experiment. They were acclimatized for 1 week before use.

Experiment 1 was performed to investigate the modifying effects of each OSC on hepatocarcinogenesis (Fig. 1). A total of 125 rats were divided into 10 groups. The rats in groups 1 to 4 were given a single i.p. injection of DEN (200 mg/kg body weight (b.w.)) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, they received DPT (150 mg/kg b.w., group 1), DMT (100 mg/kg b.w., group 2), AM (50 mg/kg b.w., group 3), and IAIE (100 mg/kg b.w., group 4) dissolved in corn oil (1 ml/kg) by i.g. gavage 5 times per week for 6 weeks. Animals were subjected to two-thirds partial hepatectomy (PH) at week 3 to maximize any interaction between proliferation and the effects of the compounds tested. Group 5 was given DEN and PH without administration of any test compound. Animals of groups 6 to 9 received saline instead of DEN solution, and were then given test compounds and PH. Group 10 animals were injected with saline and then administered corn oil, instead of test compounds, and they also underwent PH. Living rats in each group were all killed at the end of week 8, and the livers were excised for immunohistochemical examination of GST-P expression.

The doses of the chemicals tested were the same as or approximately equimolar to those used in previous experiments.[17] Each dose was less than the maximum tolerated dose.

In experiment 2, sequential changes of ODC and SAT activities and levels of proliferating cell nuclear antigen (PCNA) were measured in livers following a single OSC administration. At the beginning of the experiment, the rats received AM (50 mg/kg b.w., i.g.) in corn oil (1 ml/kg) or corn oil as a control, and then sub-groups were killed at 0, 4, 8, 12, 16, 20, 24 h. The ODC and SAT activities and levels of PCNA-positive cells were assessed.

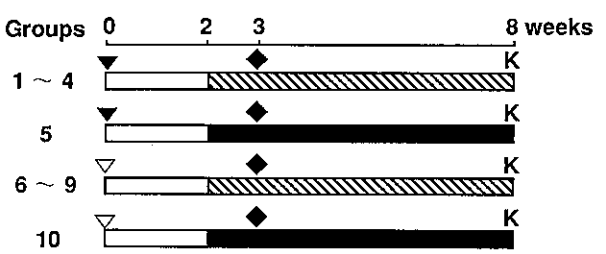

Fig. 1. Liver medium-term bioassay protocol. ▼, DEN 200 mg/kg b.w. (i.p.); ▽, saline 5 ml/kg b.w. (i.p.); ◆, partial hepatectomy; K, kill; ▨, test chemicals (i.g.) 5 times per week; ■, corn oil, 1 ml/kg b.w. (i.g.).

Tissue processing For experiment 1, at autopsy, the excised livers were cut into 2–3 mm slices with a razor blade. Three slices, one each from the right posterior, anterior, and caudate lobes, were fixed in ice-cold acetone for immunohistochemical examination of GST-P. In experiment 2, rat liver samples were frozen in liquid nitrogen for biochemical measurements, and separate slices were fixed in 10% buffered formalin for immunohistochemical staining of PCNA.

Immunohistochemical staining for GST-P and PCNA The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*[20] was used. After deparaffinization, liver sections were treated sequentially with normal goat or horse serum, anti-GST-P[21] (1:8000) (provided by Dr. K. Sato, Hirosaki University) or anti-PCNA antibody (DAKO Japan Co., Ltd.), biotin-labeled goat anti-rabbit or horse anti-mouse IgG (1:400) and ABC. The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. The numbers and the areas of GST-P-positive foci >0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (VIP-21C). PCNA-positive cells with stained nuclei were counted under the microscope and expressed as the proportion of 1000 hepatocytes.

Measurement of SAT and ODC activities We measured SAT and ODC activities by the method of Matsui *et al.*[22] and Otani *et al.*,[23] respectively. Frozen rat liver samples were suspended in 0.5 ml of 50 m*M* Tris (pH 7.5) containing 0.25 *M* sucrose and disrupted with a homogenizer for 30 s. The homogenized suspensions were centrifuged at 100,000*g* for 30 min, and the supernatant was assayed for ODC and SAT activity by measurement of the amount of radioactive putrescine produced from [5-^{14}C]ornithine and the amount of acetyl moiety transferred from [1-^{14}C]acetyl coenzyme A to spermidine, respectively.

Statistical analysis Statistical analysis of the observed values was performed by using Student's *t* test.

RESULTS

In the DEN-initiated groups of experiment 1, final body weights were significantly decreased in rats treated with AM and IAIE as compared to control rats. Relative liver weights were significantly increased in rats treated with DMT, AM, and IAIE after the DEN initiation (Table I).

Table I. Final Body and Liver Weights of Rats Initiated with DEN in Experiment 1 Followed by Treatment with Various OSCs

Group No.	Test chemical	Effective No. of rats	Final weight (g)	Relative liver weight (g/100 g body wt.)
1	DPT	14	231±21[a)]	2.75±0.43
2	DMT	14	246±14	2.83±0.19[b)]
3	AM	15	228±22[b)]	2.95±0.37[b)]
4	IAIE	15	230±13[b)]	3.09±0.21[b)]
5	–	15	246±13	2.64±0.11

a) Mean±SD.
b) $P<0.05$ (versus group 5, Student's *t* test).

Table II. Numbers and Areas of GST-P-positive Foci in the Livers of Rats Initiated with DEN in Experiment 1 Followed by Treatment with Various OSCs

Group No.	Test chemical	Number of rats	Number (/cm²)	Area (mm²/cm²)
1	DPT	14	7.41±1.98[a, b)]	0.83±0.36
2	DMT	14	7.91±1.99	0.79±0.17
3	AM	15	7.23±1.90[b)]	0.74±0.32
4	IAIE	15	8.26±1.95[b)]	0.76±0.16[b)]
5	–	15	4.41±0.98	0.55±0.14

a) Mean±SD.
b) $P<0.05$ (versus group 5, Student's *t* test).

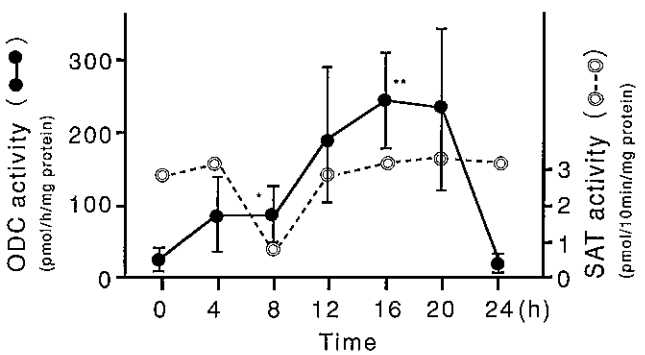

Fig. 2. Sequential changes in ODC and SAT activity in the livers of rats treated with AM (50 mg/kg b.w., i.g.) at the beginning of the experiment (* $P<0.05$, ** $P<0.01$, versus control group, Student's *t* test).

Table II summarizes data for numbers and areas of GST-P-positive foci per unit area of liver sections after DEN initiation in experiment 1. Values for both parameters in the group given IAIE, and for numbers in the groups given DPT and AM, were significantly increased over control levels. DMT also demonstrated a tendency to increase values for the two parameters. In the groups without DEN exposure, GST-P-positive foci were not seen and livers were histologically normal.

The results for sequential changes of liver ODC and SAT activities in experiment 2 are shown in Fig. 2. ODC activity started to increase gradually at 4 h after AM-treatment, and was sharply elevated after 12 h, reaching a maximum level (252.3±52.5 pmol/h/mg protein) at 16 h, as compared to the control value (13.5±6.6 pmol/h/mg protein). SAT activity was not elevated at any time. Data for PCNA-positive cells per 1000 hepatocytes are shown in Fig. 3; their number increased gradually from 4 h with a peak at 20 h.

DISCUSSION

The present study clearly demonstrated that DPT, AM, and IAIE enhance GST-P-positive focus development in the rat liver medium-term bioassay system developed by Ito *et al.* The choice of this system was based its established application for detecting modifying effects of many chemicals on liver carcinogenesis.[5)] Ogiso *et al.*[24, 25)] have proved that the degree of induction of GST-P-positive foci and nodules in this bioassay system for liver carcinogens directly corresponds with the incidence of hepatocellular carcinomas revealed in long-term *in vivo* assays. Consequently the present study results strongly suggest that the OSCs tested would promote liver tumor development.

In previous studies, inhibiting effects of OSCs were mainly assessed on the initiation stage.[11, 26–30)] Thus AMT, allyl methyl disulfide (AMD), DAT, and DAS administered 96 and 48 h prior to the carcinogen, inhibited benzopyrene-induction of tumors of the forestomach in female A/J mice. DAS and AMD, but not DAT or AMT, inhibited pulmonary adenoma formation in female A/J mice.[29)] In the reported experiments, the administration of OSCs during the initiation stage would have influenced the metabolic activation of the carcinogens,[31, 32)] but in our experiment, since we administered OSCs

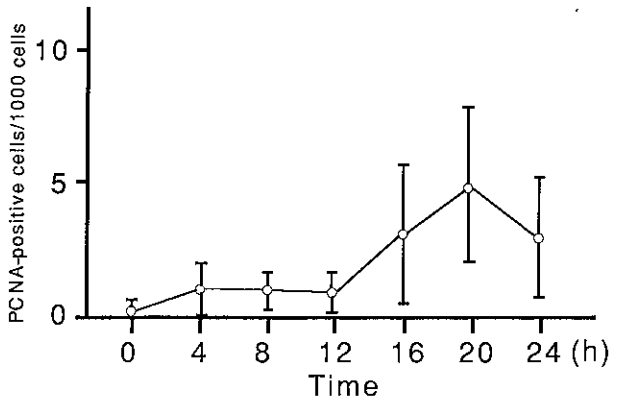

Fig. 3. Sequential changes in PCNA-positive cells in the livers of rats treated with AM (50 mg/kg b.w., i.g.) at the beginning of the experiment.

during the promotion stage, this would not have been the case, and presumably different factors are involved.

The OSCs used in the present experiment exist in trace amounts in garlic or onions. In contrast, DAS, a major sulfur-containing volatile compound, is contained at concentrations of 30–100 μg/g in garlic. The establishment of doses was not based on ratios of contents in garlic, but on equimolar concentrations according to the previous study.[17] Most of the OSCs used tended to decrease body weights and increase relative liver weights, and further dose-response studies are required to evaluate the influence of toxicity as well as to establish whether or not there are practical thresholds.

Cell proliferation has long been suspected of enhancing the frequency of tumor initiation in chemical carcinogenesis. In addition, cell proliferation appears to play an important role in the action of tumor promoters or non-genotoxic carcinogens.[33, 34] Polyamines are involved in epithelial cell diversion[35] and ODC, the key enzyme for their production, increases with epithelial cell proliferation of skin or bladder when promoting agents are administered.[18, 19] ODC activity is high in carcinoma tissues induced experimentally by chemical carcinogens[36, 37] and also in tumors obtained from patients.[38, 39] SAT, a newly established rate-limiting enzyme of biodegradation of polyamines,[22] has similarly been found to be a biochemiclal marker for cell proliferation.[35] But in the AM-treated livers without prior initiation in the present study, only ODC was increased, and the SAT level was not significantly changed. An increase of PCNA-positive cells was also evident following the elevation of ODC activity. These results support a link between increased ODC activity and enhanced induction of GST-P-positive foci, and OSCs which cause cell proliferation in the rat liver might therefore be expected to promote hepatocarcinogenesis. Further investigation is necessary to analyze the reason for the increased ODC activity after OSC application.

Humans are exposed to a wide variety of environmental chemicals during their long life-span and these may act in combination, positively or antagonistically, to affect cancer development. Moreover, synergistic effects of chemicals on tumorigenesis in experimental animals have been observed in many organs.[40, 41] In previous studies, we found that DAS, DAT, AMS, AMT, and DPS enhanced GST-P-positive focus formation in the promoting stage, and that MPD and PS rather exerted inhibition. Additional studies are now needed to examine the effects of combined treatment with enhancers and inhibitors. It is very probable that low doses of carcinogenic or modulating substances in combination are of critical importance in determining effects. Intake of garlic and onion may thus contribute to hepatocarcinogenesis.

ACKNOWLEDGMENTS

This research was supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare and the Ministry of Education, Science and Culture. We would like to express our gratitude to the late Prof. Kiyomi Sato of the Second Department of Biochemistry, Hirosaki University, and Sumitomo Chemical Co. Ltd., Osaka, respectively for the GST-P immunohistochemical staining and analyses.

(Received May 30, 1994/Accepted August 9, 1994)

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