Enhancement by Organosulfur Compounds from Garlic and Onions of Diethylnitrosamine-induced Glutathione S-Transferase Positive Foci in the Rat Liver

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INTRODUCTION

Environmental compounds are known to be involved in the generation of many human cancers. Elimination of carcinogetic compounds from our environment would be expected to help prevent human cancer, but this is not a practical proposition. Therefore, it is important to discover naturally occurring or synthetic compounds which can suppress or prevent the process of carcinogenesis (1–12).

We have focused on some OSCs which are contained in garlic and onion. Some OSCs have been proved to be chemopreventive in animal models. For example, diallyl sulfide inhibits development of colon cancer, esophageal cancer, pulmonary adenoma, and forestomach tumors in rodents when administered prior to administration of several carcinogens (13–16). Moreover, DAS inhibited hepatocarcinogenesis when administered after an initiating procedure, but this has not been consistently observed (17, 18).

DAS, diallyl disulfide, diallyl trisulfide, allyl methyl sulfide, allyl methyl trisulfide, and propylene sulfide had enhancing effects on focus formation. In contrast, high doses of methyl propyl disulfide and propylene sulfide significantly decreased the number of glutathione S-transferase placental form-positive foci. In the third experiment, combined treatment with the five chemicals that had enhancing activity were fed at low doses and increased the induction of glutathione S-transferase placental form-positive foci. To investigate the mechanism of the modifying effect on hepatocarcinogenesis, ornithine decarboxylase activity was measured in diallyl sulfide-, allyl methyl sulfide-, and dipropyl sulfide-treated liver tissue without prior initiation with diethylnitrosamine, and its activity was increased compared to controls. Spermidine/spermine N1-acetyltransferase activity was not significantly changed. Formation of 8-hydroxydeoxyguanosine, a DNA adduct generated by activated oxygen species, and lipid peroxidation (2-thiobarbituric acid-reacting substance production) were also not changed. These results suggest that the promoting effect could be caused by increased cell proliferation with increased polyamine biosynthesis. In evaluating relationships between diet and cancer, it is appropriate to consider not only the possible protective role of garlic and onions but also their enhancing effects.

MATERIALS AND METHODS

Chemicals. Organosulfur compounds used are DAS, DDS, DAT, AMS, AMT, DPS, DPD, MPD, PS, and DMD (19, 20). DAT and AMT were obtained from Oxford Chemical Co., Northamptonshire, United Kingdom; MPD was from Aldrich Chemical Co., Milwaukee, WI; and the other OSCs and DEN from Tokyo Chemical Industry Co., Tokyo, Japan.

A total of 150 F344 rats obtained at 5 weeks of age (Charles River Japan Inc., Hino, Japan) were housed in an air-conditioned room at 23 ± 1°C (SD), a relative humidity of 36–62%, with a 12-h light/12-h dark cycle, and were given diet (Oriental MF; Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. The diet is a commercial diet, which is a modified NIH open formula diet (NIH-07). Rats were acclimatized for 1 week before use.

Experiments 1 and 2 were performed to investigate modifying effects of each OSC on hepatocarcinogenesis. In experiment 1, a total of 150 rats were divided into 12 groups. The rats in groups 1 to 5 were given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks on basal diet, they received DAS (100 mg/kg body weight; group 3), DDS (25 mg/kg body weight; group 2), AMS (150 mg/kg body weight; group 3), DPS (150 mg/kg body weight; group 4), and DPD (150 mg/kg body weight; group 5) dissolved in corn oil (1 ml/kg) by i.g. gavage 5 times/week for 6 weeks. Animals were subjected to PH at week 3 to maximize any interaction between proliferation and the effects of the compounds tested. Group 6 was given DEN and PH without administration of any test compounds. Animals in groups 7 to 11 received saline instead of DEN solution but were subjected to administration of test compounds and PH. Group 12 animals were given saline injections and then subjected to administration of corn oil instead of PH. Animals in each group were killed for examination at week 8 (Fig. 1). The livers were examined immunohistochemically for GST-P expression.

In experiment 2, a total of 150 rats were divided into 12 groups. Rats in each group were treated by the same medium-term bioassay system as
experiment 1. They received DAT (150 mg/kg body weight; group 1), AMT (100 mg/kg body weight; group 2), MPD (100 mg/kg body weight; group 3), PS (50 mg/kg body weight; group 4), and DMD (50 mg/kg body weight; group 5) dissolved in corn oil by i.g. gavage 5 times/week. All rats were killed at week 8, and livers were examined immunohistochemically for GST-P expression, formation of 8-OHdGuo, and production of TBA-reacting substances.

The dose of DAS and DDS were one-half of the doses (200 and 50 mg/kg body weight) used in a previous experiment by us (18). The doses of the other chemicals tested were the same or nearly equimolar concentration as DAS or DDS. Each dose is less than the maximum tolerated dose.

In experiment 3, the modifying effects with combined treatment of OSCs on rat hepatocarcinogenesis modification were examined. A total of 105 rats were divided into 7 groups. Rats in each group were given DEN and PH with or without combined treatment of 5 test compounds. The test compounds which increased the areas and numbers of GST-P-positive foci from experiments 1 and 2 were DAS, DAT, AMS, AMT, and DDS. The doses of the combined treatment of five OSCs are shown in Table 1. The determination of doses were based on the ratio of their content in garlic (groups 1–3) or using equimolar concentrations (groups 4–6). For these two combinations, three dose levels were used.

In experiment 4, ODC and SAT activity were measured in the liver following simple administration of the OSCs. A total of 20 rats were divided into 4 groups. From the beginning of the experiment, the rats received DAS (100 mg/kg body weight; group 1), AMS (150 mg/kg body weight; group 2), or DDS (150 mg/kg body weight; group 3) in corn oil (1 ml/kg) by i.g. gavage 5 times/week. Group 4 animals received corn oil alone. All rats were killed at week 3.

Tissue Processing. At autopsy, livers were excised and sections 2–3 mm thick were cut 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 experiments 1–3. To measure the formation of 8-OHdGuo and lipid peroxidation of rat liver in experiment 2 and OSC and SAT activity of rat liver in experiment 4, rat livers were frozen by liquid nitrogen.

GST-P Immunohistochemistry. The avidin-biotin-peroxidase complex method was used to demonstrate GST-P-positive liver foci, a putative preneoplastic lesion (5, 25, 26). After deparaffinization, liver sections were treated sequentially with normal goat serum, rabbit anti-GST-P (1:8000) (provided by Dr. K. Sato, Hirosaki University) biotin-labeled goat anti-rabbit IgG (1:400) and avidin-biotin-peroxidase complex. The sites of peroxidase activity were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-GST-P antibody binding, preimmune rabbit serum was used instead of antiserum. 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).

Determination of 8-OHdGuo Formation in DNA. Liver sample portions ~2 g, wet weight, were used. Liver DNA was isolated and digested as described by Takagi et al. (27). The level of 8-OHdGuo formation in resulting samples was determined by high performance liquid chromatography by an adaptation of the methods of Floyd et al. (28, 29) and Kasi et al. (30–32) as described previously (33). The level of 8-OHdGuo formation was expressed as the number of 8-OHdGuo residues formed/10^9 of total deoxyguanosine.

Determination of Lipid Peroxidation (TBA-Reacting Substance Production). Peroxidation of liver lipids was quantitated by measuring accumulation of TBA (Sigma)-reacting substances and expressed as nmol value equivalent (eq) to a standard amount of malondialdehyde (dimethyl acetal: Aldrich) by an adaptation of the method of Yagi (34) as described previously (35).

Measurement of SAT and ODC Activity and Polyamine Biosynthesis. We measured SAT and ODC activity by the method of Matsui et al. (36) and Otani et al. (37), respectively. The frozen piece of rat liver was suspended in 0.5 ml of 50 mm Tris (pH 7.5) containing 0.25 m sucrose and disrupted by a homogenizer for 30 seconds. The homogenized suspensions were centrifuged at 100,000 x g for 30 min, and the supernatant was assayed for ODC and SAT activity by measurement of the amount of radioactive putrescine produced from [5,14C]ornithine and the amount of acetyl moiety transferred from [1,14C]acetyl-CoA to spermidine, respectively.

Statistical Analysis. Statistical analysis of the observed values was performed using Student’s t test or Dunnett’s t test.

RESULTS

In DEN-initiated groups of experiments 1 and 2, final body weights were significantly decreased in rats treated with DPD, DAT, and PS compared to rats treated without test chemicals. Relative liver weights were significantly increased in DEN-initiated rats treated with DDS,
DPS, DAT, AMT, MPD, PS, and DMD and decreased in DPD-treated group (Tables 2 and 3).

Tables 4 and 5 show the 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 groups given DAS, AMS, and DPS were significantly increased over control levels. In particular, DPS was most effective. In experiment 2, in groups given DAT or AMT, the number and area of GST-P-positive foci were significantly increased. In contrast, in groups given MPD or PS, values for the number of foci were significantly decreased. In experiments 1 and 2, GST-P-positive foci were not seen, and the histological findings of livers were normal in groups without DEN.

For experiment 3, final body and relative liver weights are summarized in Table 6. Final body weights were significantly decreased in rats treated concomitantly with high doses of test chemicals (groups 1 and 4). Relative liver weights were significantly increased in all of the treated groups, and a dose dependency could be observed. The proportion of numbers and areas of GST-P-positive foci in the different groups compared to group 7 (control) are summarized in Fig. 2. They were significantly increased by the combined treatments compared to the control group. In each group treated with test chemicals, no significant differences were observed, but there was a tendency toward a dose-dependent response.

Results for determinations of 8-OHdGuo formation in DNA and TBA-reacting substances produced in the livers of rats initiated by DEN in experiment 2 are shown in Table 7. Formation of 8-OHdGuo tended to decrease in groups treated with MPD or PS. Results for TBA-reacting substances displayed significant decreases in rat livers with MPD and PS treatment. In groups treated with DAT or AMT, formation of 8-OHdGuo and TBA-reacting substances hardly increased compared to control group.

For experiment 4, the results of ODC and SAT activity in the liver are shown in Table 8. ODC activity increased in DAS (42.9 ± 12.5 pmol/mg), AMS (137.5 ± 157.6 pmol/mg), and DPD (38.7 ± 34.5 pmol/mg)-treated rats over the control level (13.5 ± 6.6 pmol/mg) and was especially elevated significantly in the group treated with DAS. SAT activity was not increased in any of the groups.

### Table 4
Numbers and areas of GST-P-positive foci in the liver of rats initiated with DEN in experiment 1 followed by treatment with various OSCs

<table>
<thead>
<tr>
<th>Group</th>
<th>Test chemical</th>
<th>No. of rats</th>
<th>No./cm²</th>
<th>Area (mm²/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DAS</td>
<td>14</td>
<td>6.58 ± 2.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>DDS</td>
<td>14</td>
<td>4.39 ± 1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>AMS</td>
<td>14</td>
<td>6.14 ± 1.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>DAS</td>
<td>14</td>
<td>8.45 ± 2.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>DPD</td>
<td>14</td>
<td>5.53 ± 1.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>DPD</td>
<td>14</td>
<td>4.69 ± 1.61 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.38 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD.
<sup>b</sup> Significantly different from group 6 at P < 0.05 (Student's t test).
<sup>c</sup> Significantly different from group 7 at P < 0.01 (Student's t test).

### Table 5
Numbers and areas of GST-P-positive foci in the liver of rats initiated with DEN in experiment 2 followed by treatment with various OSCs

<table>
<thead>
<tr>
<th>Group</th>
<th>Test chemical</th>
<th>No. of rats</th>
<th>No./cm²</th>
<th>Area (mm²/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DAT</td>
<td>15</td>
<td>6.62 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>AMT</td>
<td>14</td>
<td>8.24 ± 2.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>DAS</td>
<td>14</td>
<td>3.28 ± 1.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>DPD</td>
<td>14</td>
<td>3.17 ± 1.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>DPD</td>
<td>15</td>
<td>4.09 ± 1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>DPD</td>
<td>15</td>
<td>4.71 ± 1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD.
<sup>b</sup> Significantly different from group 6 at P < 0.05 (Student's t test).
<sup>c</sup> Significantly different from group 6 at P < 0.01 (Student's t test).

### DISCUSSION

Although most OSCs have been shown to be chemopreventive in previous studies, high doses of DAS, DAT, AMS, AMT, and DPS were shown to promote neoplastic lesion development in the rat liver in the present study. Their combined treatment enhanced GST-P-positive focus development. Their effects indicated a remarkable dose dependency in the two different combined groups. Therefore, their combined treatment may exert promoting activities on hepatocarcinogenesis of rats.

In previous studies, the inhibiting effects of OSCs were mainly examined in the initiation stage (13, 38—42). AMT, AMD, DAT, and AMS administered 96 and 48 h prior to the carcinogen inhibited pulmonary adenoma formation in female A/I mice. AMS and AMD inhibited pulmonary adenoma formation in female A/I mice, but neither DAT nor AMT did (41). In those experiments, the administration of OSCs during the initiation stage inhibited the metabolic activation of the carcinogens (43, 44); but in our experiment, since we administered OSCs during the promotion stage, our results imply the involvement of a different mechanism.

The selection of the bioassay system in this study was based on examining the modifying potential of OSCs on the promoting stage of hepatocarcinogenesis. The data obtained from the present investigations unexpectedly showed enhancing effects of five OSCs, DAS, DAT, AMT, AMS, AMT, and DPD, several of which inhibited carcinogenesis by administration during the initiation stage. This bioassay system has been utilized to detect modifying effects of many chemicals on liver carcinogenesis (5). Moreover Ogiso et al. (45, 46) indicated that the degree of induction of GST-P-positive foci and nodules in this bioassay system for liver carcinogenesis corresponds with the incidence of hepatocellular carcinomas revealed in a long-term in vivo assay. Consequently the present study strongly suggests that these OSCs promote hepatocarcinogenesis.
ORBANOSULFIDE ENHANCEMENT OF HEPATOCARCINOGENESIS

Table 7  Induction of oxidative DNA damage and TBA-reacting substance production in rat livers initiated with DEN followed by treatment with various OSCs

<table>
<thead>
<tr>
<th>Group</th>
<th>Test chemical</th>
<th>8-OHdGuo/10^5 deoxyguanosine</th>
<th>TBA-reacting substance production (μmol malondialdehyde/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DAT</td>
<td>4.5 ± 0.9^a</td>
<td>8.7 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>AMS</td>
<td>4.1 ± 0.8</td>
<td>9.4 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>MPS</td>
<td>3.1 ± 1.4</td>
<td>8.0 ± 2.0^a</td>
</tr>
<tr>
<td>4</td>
<td>PS</td>
<td>3.6 ± 1.5</td>
<td>6.9 ± 1.1^a</td>
</tr>
<tr>
<td>5</td>
<td>DMD</td>
<td>4.7 ± 2.0</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>4.7 ± 2.2</td>
<td>11.2 ± 1.8</td>
</tr>
</tbody>
</table>

^a Mean ± SD.  
^b Significantly different from group 6 at P < 0.05 (Student's t test).

Table 8  ODC and SAT activity in liver of rats treated with OSCs

<table>
<thead>
<tr>
<th>Test chemical</th>
<th>ODC activity (pmol/h/mg protein)</th>
<th>SAT activity (pmol/10 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS</td>
<td>42.9 ± 12.5^a</td>
<td>0.24 ± 1.20</td>
</tr>
<tr>
<td>AMS</td>
<td>137.5 ± 157.6^b</td>
<td>0.82 ± 0.90</td>
</tr>
<tr>
<td>DPS</td>
<td>38.7 ± 34.5</td>
<td>0.45 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>35.6 ± 6.6</td>
<td>0.17 ± 0.42</td>
</tr>
</tbody>
</table>

^a Mean ± SD.  
^b Significantly different from the nontreated group at P < 0.05 (Student's t test).

Most of OSCs used may tend to decrease body weights and increase relative liver weights. In further studies, we must examine the dose response for each OSC which enhanced GST-P positive focus formation in order to evaluate the possibility of a practical threshold. In addition, we must dissociate the effects of increasing focus formation from the toxicity of OSCs.

A modifying response of DAS in the promoting stage was examined in a rat multiorgan carcinogenesis model by Jang et al. (17) and Takahashi et al. (18), with different results. Jang et al. reported that DAS administered in the diet significantly decreased the incidence of hepatic hyperplastic nodules in the liver. However, Takahashi et al. and in our study, rats received DAS by i.g. intubation, which was quite different from the method of Jang et al. Moreover, Takahashi et al. reconfirmed their results in the DEN-initiated liver medium-term bioassay concerning promoting effects of DAS. Our present findings are in agreement with the latter. The reason for this discrepancy with Jang et al. is unclear.

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 effects of tumor promoters or nongenotoxic carcinogens (47, 48). Polyamines are involved in epithelial cell proliferation (49). ODC, which is a marker of cell proliferation, increased with epithelial cell proliferation of skin or bladder when a promoting agent was administered (23, 24). ODC activity is high in carcinomas induced experimentally by chemical carcinogens (50, 51) and in carcinomas obtained from patients (52, 53). SAT is a newly established rate-limiting enzyme of biodegradation of polyamines (36) and it also was found to be a biochemical marker of cell proliferation (49). In DAS-, AMS-, and DPS-treated livers without prior initiation, ODC and SAT activities were measured in this study. ODC activity was increased in DAS-, AMS-, and DPS-treated groups, although SAT activity was not changed significantly. This result supports the correlation of increased ODC activity with increased induction of GST-P-positive foci. OSCs which enhance cell proliferation in the rat liver would be expected to promote hepatocarcinogenesis. Further investigation is required to analyze the mechanism of increased ODC activity by OSCs.

Kasai et al. (31) reported increased formation of 8-OHdGuo in cellular DNA by agents producing oxygen radicals and its repair. Recently, Nakae et al. (54) demonstrated that the production of 8-OHdGuo correlates with development of putative preneoplastic γ-glutamyltransferase-positive focal lesions using a rat hepatocarcinogenesis model by a choline-deficient L-amino acid-defined diet. Takahashi et al. (55) reported that lipid peroxidation was increased in gastric mucosa by NaCl, a stomach cancer promoter. That study clearly demonstrated that the gastric tumor promoter NaCl dose-dependently increased TBA-reacting substances production levels in both gastric mucosa and urine, supporting a conclusion that there is enhanced lipid peroxidation in the target tissue. Although increased formation of 8-OHdGuo in DNA and lipid peroxidation were expected to participate in the promoting effects of OSCs, they were not significantly involved as determined in the present study.

In this study, the number but not the area of GST-P-positive foci was significantly decreased in rats given PS or MPD. This suggests that some compounds might have inhibitory effects on hepatocarcinogenesis. In addition, the reason why induction of 8-OHdGuo and lipid peroxidation were decreased in the liver should be clarified with further experiments.

The test chemicals are sulfur-containing, volatile compounds, and especially the monosulfides, except PS, and the trisulfides have a tendency to enhance GST-P-positive focus formation. The number of sulfur atoms may relate to the mechanism of promotion of hepatocarcinogenesis by OSCs (56).

Humans are exposed to numerous environmental chemicals during their life span and the chemicals may act in combination, positively or antagonistically, to affect cancer production. Moreover synergistic effects of chemicals on development of tumors have been repeatedly observed in many organs (57, 58). We observed a dose-dependent tendency in the promoting effects by combined treatment with five OSCs (DAS, DAT, AMS, AMT, and DPS) in two different combination groups. Since we also found two inhibitors, we need additional studies to examine the effects of combined treatment with enhancers and inhibitors. It is possible that low doses of carcinogenic substances in combination are of critical importance in determining effects. This may contribute to the correlation between hepatocarcinogenesis and intake of garlic and onion.

In conclusion, many OSCs which exerted promoting effects in hepatocarcinogenesis exist in garlic and onion. ODC activity increased in the treated groups. Moreover, these data suggest that cell proliferation in liver tissue is important for the promoting effects of OSCs.

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