Chemoprevention of N-Nitrosomethylbenzylamine-induced Esophageal Cancer in Rats by the Naturally Occurring Thioether, Diallyl Sulfide

Michael J. Wargovich, Cynthia Woods, Vincent W. S. Eng, L. Clifton Stephens, and Kenneth Gray

Section of Gastrointestinal Oncology and Digestive Diseases, Department of Medical Oncology [M. J. W., C. W.], and Division of Veterinary Medicine and Surgery [L. C. S., K. G.J, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030, and Ludwig Institute for Cancer Research ¡V. W. S. E], Toronto, Ontario M4Y 1M4, Canada

ABSTRACT

Diallyl sulfide (DAS) is a principal thioether of garlic (Allium sativum) accounting, in part, for the flavor and fragrance of this herb. Previous studies have shown that DAS is a potent inhibitor of experimentally induced colon cancer in mice. Metabolic studies of other garlic-derived substances suggested that DAS could prevent tumorigenicity of other hepatic activated carcinogens. The present study was designed to determine whether DAS could inhibit the DNA-damaging and tumorigenic effects of N-nitrosomethylbenzylamine in rat esophagus. A dose of 200 mg/kg of DAS given p.o. 3 h prior to N-nitrosomethylbenzylamine administration was found to inhibit the carcinogen-induced nuclear toxicity by 64% to 56% at the two doses (3 and 5 mg/kg) of NMBA tested. These results suggested that the compound was potentially anticarcinogenic. In the carcinogenicity experiment it was found that DAS totally inhibited tumor formation in rats treated with a carcinogenic dose of NMBA (100% inhibition of papilloma and squamous cell carcinoma incidence, P < 0.0001). Additionally DAS was found to substantially reduce hepatic microsomal metabolism of the carcinogen. These data demonstrate that DAS is unique in its anticarcinogenic activity. It strongly suppresses the tumorigenic effects of potent, metabolically activated monoalkylating carcinogens in the gastrointestinal tract.

INTRODUCTION

DAS is one of several thioethers identified as flavor and fragrance components of garlic (1). Recent studies have focused on pharmacologically active elements in garlic that could account for the reported antithrombotic and antihypertensive effects of eating the herb (2, 3). Other investigators have examined garlic for its anticancer effects. Indeed, Belman (4) reported that the topical application of garlic or onion oil inhibited tumor promotion in DMBA/TPA-induced skin cancer in mice. His conclusions led us to test DAS for potential activity against intestinal carcinogens. Using the NA assay, we found DAS to be unique among the thioethers in garlic; it strongly inhibited the early nucleotoxic effects of dimethylhydrazine in the colons of mice (5). This result suggested that DAS could have anticarcinogenic properties; the compound was subsequently found to be a potent inhibitor of DMBA-induced colon neoplasia (6). Because DMH undergoes partial metabolic activation in the liver to the proximate form of the carcinogen (7), we theorized that DAS could act by modulation of metabolic conversion of DMH and could affect similarly activated carcinogens.

MATERIALS AND METHODS

Chemicals. NMBA was synthesized according to the methods of White (10), purified under reduced pressure, and a gift provided by Dr. Peter Zucker of the Ontario Cancer Institute, Toronto, Ontario, Canada. DAS was purchased from Aldrich Chemical Co., Milwaukee, WI; its purity was at least 97% by high-pressure liquid chromatography separation.

Animals and Treatments. Male Sprague-Dawley rats (Charles River, Wilmington, MA) were purchased at 100 to 125 g of initial body weight. The first shipment of 18 rats was used for nuclear aberration assay. The animals were housed 3 to a cage and given constant access to laboratory chow and water. The light-dark cycle alternated every 12 h. For the nuclear aberration assay, the 18 rats were subdivided into 6 groups of 3 animals each. Since we observed maximal antitumorigenic activity for DAS at a dose of 200 mg/kg in our previous study, this dose was retained for the current investigation (6). Groups 1, 2, and 3 received 200 mg/kg of DAS (mixed in corn oil) intragastrically (i.g.) 3 h prior to administration of NMBA; Groups 4, 5, and 6 received only corn oil and were given NMBA i.g. NMBA was dissolved in saline and injected s.c. at a dose of either 3 mg/kg (Groups 1 and 4) or 5 mg/kg (Groups 2 and 5). Groups 3 and 6 received only an s.c. injection of saline. Animals were killed 24 h following NMBA injection, and the esophagi were removed and processed for NA analysis.

For the carcinogenicity experiment, 90 rats were assigned to one of 4 groups. Group I (30 rats) and Group II (30 rats) were given injections of NMBA (3.5 mg/kg, s.c.) once per wk for 5 wk; Groups III (15 rats) and IV (15 rats) received injections of saline, s.c., in lieu of NMBA. Three h prior to each weekly injection of NMBA, Groups I and III were given DAS i.g. at a dose of 200 mg/kg in corn oil, and Groups II and IV were gavaged with corn oil only. Animals were fed standard chow and water ad libitum. DAS or vehicle was given to the rats 3 h prior to NMBA based on our previous findings in a DMH-induced colon tumorigenesis experiment (6). Subsequent time course studies with DMH indicate maximal inhibition of DMH during this time. The light-dark cycle was identical to that of the first experiment. After 15 wk, all animals were killed by CO2 asphyxiation, and necropsies were performed.

Nuclear Aberration Assay. Tissue preparation, staining techniques, and descriptive histology for the NA assay have been previously reported (11). For our experiments, the frequency of NA per 500 basal esophageal epithelial cells was scored. Previously published criteria to ensure nonbias were observed (12). The results are reported as the percentage of nuclear aberrations.

NMBA Metabolism Experiment. Ten additional rats maintained on

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2 To whom requests for reprints should be addressed, at Section of Gastrointestinal Oncology and Digestive Diseases, Box 78, University of Texas-M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

3 The abbreviations used are: DAS, diallyl sulfide; NMBA, N-nitrosomethylbenzylamine; DMBA, dimethylbenz(a)anthracene; TPA, tetradecanoylphorbolacetate; HPLC, high-pressure liquid chromatography.

4 A. Baer, unpublished observation.
an AIN-76 A semipurified diet were divided into 2 groups. Five received 200 mg/kg of DAS in corn oil by gavage; 5 received corn oil alone. Three h later all rats were killed. Liver and esophageal microsomes were prepared by the method of Labuc and Archer (13). To detect the effect of DAS on NMBA metabolism the HPLC method of Barch et al. (14) was followed. In our experiment metabolism of NMBA was measured in vitro using liver or esophageal microsomes prepared from the two groups of rats and was assessed by quantifying the peak area of benzaldehyde produced. Detection was determined using a Waters HPLC with isocratic elution in 17% acetonitrile/3% acetic acid, pH 2.45, at a flow rate of 2 ml/min. Detection was conducted by UV absorbance at 260 nm. Results were reported as nmol/min/mg of benzaldehyde produced. The microsomes from 5 rats esophagi were pooled for this assay, but hepatic metabolism was determined from liver microsomes of individual rats.

Carcinogenicity Experiment. Body weights for all animals in groups were measured weekly. At wk 15, Group II rats (NMBA alone) had lower body weights compared to the vehicle control groups (P < 0.05; II = 417 ± 39 g versus IV = 447 ± 44 g). Animals treated with DAS only had a mean body weight of 438 ± 35 g, and animals treated with DAS and NMBA averaged 442 ± 25 g in body weight. At this time several animals showed evidence of rapidly deteriorating pulmonary function, and the experiment was therefore terminated. One animal died of pulmonary complications prior to wk 15 and was not included in the final analysis. All animals were fully necropsied. The esophagus, liver, lung, small and large bowel, and kidneys were preserved for histological analysis. Tissues were fixed in 10% buffered formalin. The esophagi were divided into upper and lower segments. Three step sections per segment were evaluated. Histological evaluation of the tissues was conducted by one of us (L. C. S.). Representative histological sections of esophagi were microphotographed.

Statistics. Statistical analysis of body weights was conducted using the Student t test; tumor incidence data were analyzed by χ² analysis.

RESULTS

Nuclear Aberration Study. The esophagi of rats treated with DAS showed less evidence of the DNA-damaging effects of NMBA as expressed by a reduced frequency of nuclear aberrations (Table 1). At the doses of NMBA tested, DAS pretreatment inhibited the nucleotoxic effect of the esophageal carcinogen by ~56% to 64%. These data suggested that, as short as 3 h prior to NMBA administration, DAS was active in preventing genotoxic events at the target organ. The data also suggested that NMBA could be a potential inhibitor of esophageal tumorigenesis if tested in a carcinogenesis protocol.

NMBA Metabolism Studies: Effect of DAS. Both hepatic and esophageal microsomes from the treated rats converted NMBA to benzaldehyde as shown in Table 2. DAS treatment resulted in a significant reduction in the microsomal conversion of NMBA. However, only hepatic microsomes were affected; metabolism of NMBA using esophageal microsomes was unaffected by pretreatment with DAS. Taken together these results suggest that modification of carcinogen metabolism may only, in part, account for the anticarcinogenic potency of DAS.

Outcome of Esophageal Carcinogenicity Study. Esophageal tumor incidence and frequency of lesions graded according to histological criteria are illustrated in Table 3. Only rats that received NMBA alone developed esophageal tumors. No histological evidence of premalignant or malignant neoplasms was found in the control or in DAS-treated groups. Rats receiving DAS and NMBA routinely demonstrated normal histology markedly similar to that of control animals.

Fig. 1 illustrates the histological types of esophageal lesions encountered in this study. Evidence of metastases was not found in any rat in the study, although NMBA-treated rats (Group I) often showed focal infiltrations of lymphocytes and macrophages in interlobular connective tissue and portal areas of the liver.

DISCUSSION

Members of the Allium family are excellent sources of organic sulfides (15). A long anecdotal history of the curative properties of herbs in this family has suggested that some pharmacological compounds could be present (16). Investigation of organic sulfides for anticarcinogenic or chemopreventative properties was first attempted by Belman (4) and more recently by Sparnins et al. (17, 18). In our previous study we found DAS to strongly inhibit DMH-induced colon cancer in mice (6). In tracer studies, labeled diallyl disulfide, a close analogue of DAS found in garlic, was shown by Pushpendran to be rapidly taken up by the liver (19). Carcinogens such as NMBA and DMH require hepatic activation, and it is possible that some interaction between DAS and the carcinogen may occur.

The results of this study provide some evidence that DAS can modify NMBA metabolism, partially accounting for the observed significant reduction in tumorigenicity in rats pretreated with the thiosulfate. The extent of the inhibition, that is, the complete inhibition observed from the tumorigenicity study, can not be fully explained by the results of metabolic studies. It is possible that the metabolic conversion of NMBA involves more than one P-450 isozyme, and our results can be taken only as indirect evidence for the mechanism of action. Moreover, DAS did not fully inhibit the induction of nuclear aber-

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Table 1: Suppression of NMBA toxicity by DAS in rat esophagus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of nuclear aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>NMBA</td>
<td>3.6</td>
</tr>
<tr>
<td>NMBA + DAS</td>
<td>1.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2: Effect of DAS on microsomal metabolism of NMBA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Esophagus</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>19.6</td>
<td>33.0 ± 12.1f</td>
</tr>
<tr>
<td>DAS (200 mg/kg)</td>
<td>22.5</td>
<td>7.7 ± 0.8f</td>
</tr>
</tbody>
</table>

* Corn oil or DAS was administered by gavage at ~3 h. Rats were killed at 0 h and microsomes prepared.
* Pooled esophageal microsomes from 5 rats; mean.
* Mean ± SEM.
* P = 0.5.

Table 3: Effect of DAS on NMBA-induced esophageal tumorigenesis in male Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor incidence (%)</th>
<th>Histological frequency of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>Hyp: 0</td>
</tr>
<tr>
<td>DAS (15)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NMBA (29)</td>
<td>79d</td>
<td>22</td>
</tr>
<tr>
<td>NMBA/DAS (30)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Rats received NMBA (200 mg/kg, i.g., in corn oil) 3 h prior to NMBA treatment. NMBA was injected s.c. (3.5 mg/kg) once per wk for 5 wk. Vehicle controls received corn oil i.g. and saline, s.c. DAS group received only DAS in corn oil and DMS vehicle. The experiment had a duration of 15 wk.
* Hyp: hyperplasia; Ps Hyp: pseudoneoplastic hyperplasia; Pap: papilloma; Ca Situ: carcinoma in situ; Sq Ca: squamous cell carcinoma.
* Numbers in parentheses, number of rats.
* Significantly different from NMBA/DAS group by χ² analysis (P < 0.001).
INHIBITION OF ESOPHAGEAL CANCER BY Diallyl Sulfide

Figure 1: A series of photomicrographs illustrating the spectrum of proliferative lesions in rat esophagus. H&E, x 250. (A) Normal esophageal epithelium from untreated rats. (B) Normal esophageal epithelium from 750 ppm Diallyl Sulfide treated rats. (C) Normal esophageal epithelium with increased vascularity and hyperplasia of the mucosa. (D) Early carcinoma in situ. (E) Dysplastic changes extend through the full thickness of the mucosa. (F) Squamous cell carcinoma. The basement membrane is invaded by anaplastic squamous epithelial cells.
rations in the esophageal mucosa in the initial pilot study. Since nuclear aberrations reflect exposure of proliferating cells to genotoxic injury (12), they are an acute biological phenomenon. Other factors may affect the process of esophageal tumorigenesis long after the initiation phase of carcinogenesis and were unknown variables affecting this experiment. Factors not investigated that could account for our results include stimulation of pathways of carcinogen detoxification, such as induction of the glutathione-mediated conjugations. Sparnins et al. (17) have recently shown that allyl methyltrisulfide, another organic sulfide in garlic, maximally stimulated glutathione-S-transferase activity in the A/J mice in which they reported 70% inhibition of benzo(a)pyrene forestomach carcinogenesis (17). In another study these investigators found DAS as well to be an effective inhibitor of lung cancer in mice (18). Although chronic stimulation of the biochemical enzymes involving detoxification of NMBA remains an untested possibility in this study, our data suggest that changes in the initial metabolic conversion of NMBA could play some part in the chemopreventive action of DAS. That hepatic metabolism of NMBA was affected by DAS but not esophageal metabolism could reflect the rapid turnover of NMBA in the liver compared to the esophagus as reported by Labuc and Archer (13) and Kawanishi et al. (20) and the possibility that diallyl thioethers are rapidly transported to the liver where they could affect metabolism.

The results of this study confirm our previous finding that DAS is a potent chemopreventive agent of monoalkylating gastrointestinal carcinogens, namely, NMBA and, as we found previously, DMH. Whether DAS is equally potent in protecting the gastrointestinal epithelium from the effect of direct acting carcinogens, such as N-methyl-N'-nitro-N'-nitrosoguanidine and methylnitrosourea is being presently investigated. In order to learn more about the efficacy of the anticarcinogenic activity of DAS we are examining the effect of administration of DAS at graded doses to establish dose-response relationships. These experiments are designed to test DAS at levels more in accord with levels found in the human diet rather than the supraphysiological dose chosen in the current study.

Carcinoma of the esophagus continues to be a common malignancy in Asia and the Middle East (21) and is usually associated with poor prognosis due to inadequate therapeutic regimens (22). The etiology of the disease in parts of China is suggestive of a relationship between inadequate dietary micronutrients and contamination of certain foods with NMBA (23). In summary, DAS, a natural organosulfur product from garlic, can inhibit the carcinogenic effects of NMBA experimentally in animals. Once more is known about the way in which DAS acts to prevent cancer, formulations containing the sulfide could be made available for pharmacological investigations in humans.

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