Chemopreventive effect of S-allylcysteine and its relationship to the detoxification enzyme glutathione S-transferase

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Sulfur-containing substances derived from garlic and onion have been shown to prevent experimental carcinogenesis. One of the hypotheses explaining the mechanisms of the chemopreventive activity of these substances is that they activate detoxification systems such as glutathione S-transferase (GST). In this study the effects of S-allylcysteine (SAC), a water-soluble organosulfur compound derived from garlic, on GST activities in the liver, small intestine and colon were investigated. Additionally, we examined SAC for chemopreventive effects on aberrant crypt foci, which are the most likely precursors of colon cancers. In the rat colonic aberrant crypt assay administration of SAC during the initiation period decreased the number of aberrant crypt foci by 33 and 54% in groups given 40 or 80% maximum tolerated dose (MTD) of SAC respectively. The number of aberrant crypt foci, however, was not changed when SAC was given during the promotion period. GST activity in the liver was increased significantly by 41% 12 h after a single oral administration of 3.5 mmol/kg SAC and this elevated GST level was maintained over a 72 h period. GST levels were increased significantly by the administration of SAC (1.8 mmol/kg/day for 3 days) not only in the liver but also in the proximal and middle small bowel. Isozyme levels of GST after administration of SAC were also determined using Western blotting. Hepatic GST-α and GST-μ were significantly increased by 35 and 42% respectively after oral administration of SAC. GST-π levels were lower than the detection limit (130 ng/mg/protein) in both the control and SAC-treated groups. These results strongly support the previous working hypothesis that SAC exhibits chemopreventive activity by exerting specific effects on carcinogen detoxification systems.

Introduction

The medical properties of garlic (Allium sativum) have been the subject of folklore from ancient times to the present (1,2). A number of oil-soluble organosulfur compounds, such as diallyl sulfide (DAS*), have been reported as chemopreventive agents (3—8). In addition, Sumiyoshi and Wargovich (9) reported that orally administered S-allylcysteine (SAC), a naturally occurring water-soluble organosulfur compound derived from garlic, was active in preventing dimethylhydrazine (DMH)-induced aberrations in the mouse colon. Furthermore, pretreatment with SAC significantly inhibited the development of DMH-induced tumors in long-term tumorigenesis tests in the mouse. They also found that SAC increased glutathione S-transferase (GST) activity significantly in the liver and colon and speculated that the increased GST activity may be responsible for expression of the chemopreventive activities of SAC.

GSTs are a widely distributed family of enzymes that catalyze the conjugation reaction of electrophilic hydrophobic compounds with reduced glutathione (10—12). This event is thought to aid in preventing the tumorigenic process by eliminating electrophilic endogenous and exogenous compounds from the body (13). Many carcinogens and their metabolites have been reported to be detoxified by these enzymes (11,15). Furthermore, as several studies have shown, the cytotoxicity of antineoplastic agents is potentiated by treatment with inhibitors of GST in vitro (15,16) and higher levels of GST activity are associated with clinical resistance to chemotherapy in vivo (17). The contribution of GST activity to antineoplastic drug resistance has been a fertile area of investigation (15).

Multiple isoforms of GST are essential in order for a vast variety of toxic compounds to be detoxified. GST can be classified into three groups, α, μ, π, according to their structural, immunological and catalytic properties, regardless of species and distribution (18,19). These isoenzymes have substrate specificity, although they partly overlap. Thus although many studies on biochemical properties of GST and its isoenzymes have been done, the role of these isoenzymes in cancer chemoprevention is not clearly understood. SAC is relatively non-toxic to animals when compared with other garlic volatiles, such as DAS, and is likely to be more tolerable if used in human prevention studies (9). We therefore chose this agent for subsequent investigation. In this study the chemopreventive effects of SAC and GST activity in the liver, intestine and colon were investigated in rats. The changes in GST isoenzyme levels in the liver were determined using the Western blotting technique to understand a part of their contribution to chemoprevention.

Materials and methods

Animals

Male Fischer-344 rats, 6 weeks old, initially weighing 116—135 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). AIN-76A semipurified diet (Dyets, Bethlehem, PA) was given ad libitum throughout the experimental period. The rats were acclimated to the diet and environment for a period of 1 week after arrival. The rats were housed two or three per cage and had free access to water. The temperature (20—22°C), humidity (45—55%) and lighting (12 h day/night cycle) were constantly controlled.

Experimental schedule

SAC (CH3—CH2—S—CH2—CH(NH2)—COOH) was kindly provided by Wakunaga Pharmaceutical Co. Ltd. (Hiroshima, Japan) and the purity was confirmed to be >98%. In all experiments it was dissolved in distilled water and given orally at a dose of 5 ml/kg. Control rats were intubated with distilled water.

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Aberrant crypt assay

Distribution of GST activity. Rats (n = 8) were given SAC at a dose of 0.25 g/kg diet, which correspond to 40 and 80% maximum tolerated dose (MTD) respectively, were used. DMH (Aldrich, Milwaukee, WI) was dissolved in 0.1% EDTA, pH 6.8, and injected i.p. at a dose of 25 mg/kg once a week for 2 weeks.

Stage of chemopreventive activity. Dietary levels of SAC of 0.125 and 0.25 g/kg diet, which correspond to 40 and 80% maximum tolerated dose (MTD) respectively, were used. DMH (Aldrich, Milwaukee, WI) was dissolved in 0.1% EDTA, pH 6.8, and injected i.p. at a dose of 25 mg/kg once a week for 2 weeks.

Chemopreventive effect on initiation. Rats (n = 10) were fed the diet containing SAC 1 week prior to DMH injection and throughout the experiment. One week after the test diet was initiated the rats received DMH as described above. The rats were killed 2 weeks after the last DMH injection.

Chemopreventive effect on promotion. Rats (n = 10) were given DMH once a week for 2 weeks, then the rats were randomized into three groups. Two weeks after the last injection of DMH the rat experimental groups were placed on a SAC-containing diet for 4 weeks (n = 10). Control rats (n = 10) were fed only the AIN-76A semipurified diet. All rats were killed 6 weeks after the last injection of DMH.

Aberrant crypt assay

The experimental schedule used is shown in Figure 1. DMH dissolved in 0.1% EDTA, pH 6.8, was given i.p. once a week for 2 weeks at 25 mg/kg body wt as a base. Rats were killed by CO2 asphyxiation. Colons were removed immediately, slit longitudinally, flattened and fixed in 70% ethanol for a period of 24 h. The samples were then stained with 0.3% methylene blue (20) and the multiplicity of aberrant crypt foci along the entire length of the colon was determined using a dissecting microscope.

Tissue preparation for GST assay

For this assay rats were killed by cervical dislocation and the livers, small intestines and colons were excised immediately. The small intestine was dissected into three segments; proximal, middle and distal. Each segment was slit longitudinally and washed with phosphate-buffered saline, pH 7.4, and the mucosa was collected by scraping the mucosal surface using a microscope slide. The liver was perfused with saline to remove blood and minced into small pieces. Aliquots of mucosal scrapings and minced liver were homogenized in buffer (0.25 M saccharose, 20 mM Tris, 1 mM dithiothreitol, pH 7.4) using a Tissue-Tearor (Biospec Products, Bartlesville, OK) for 15 s with cooling over ice. The homogenates were centrifuged at 9000 g for 20 min and the resultant supernatants were centrifuged at 150,000 g for 1 h to produce a cytosolic fraction. All procedures were performed on ice and samples were stored at -80C.

Protein assay

Protein concentration was determined in duplicate with a protein assay kit (BioRad Laboratories, Richmond, CA) using bovine serum albumin as the standard. GST activity was determined according to Habig et al. (13) using 1-chloro-2,4-dinitrobenzenzene as substrate.

Western blotting

Aliquots of liver cytosolic samples were applied to a 14% pre-cast polyacrylamide gel (Schleicher & Schuell, Keene, NH) and subjected to electrophoresis. The resulting Western blots were treated with antibodies against rat GST Ya, GST Ybl and GST Yp (Biotrin International, Dublin, Ireland). Specific binding of these antibodies was detected with an electrochemiluminescence kit (Amersham, Little Chalfont, UK) after incubation with peroxidase-linked anti-rabbit Ig (Amersham). Visualized spots were quantitated using a GEL PROSCAN Model 1312 (ISCO, Lincoln, NE). Aliquots of authentic GST antigens GST Ya, GST Ybl and GST Yp were analyzed in the same manner as the standards.

Statistics

Data were presented as the mean ± SE. An unpaired two-tailed Student’s t-test was used to detect statistically significant differences. When the variance among groups was non-uniform the Mann–Whitney U test was used instead of a t-test. Differences with a P value <0.05 were considered significant.

Results

The effects of dietary administration of SAC on initiation and promotion of colon carcinogenesis by DMH are shown in Figure 2. A mean of 85 aberrant crypt foci were found in the colons of DMH-treated rats. The number of aberrant crypt foci was significantly decreased in a dose-dependent manner by 33 and 55% in the groups given 40 and 80% MTD of SAC respectively. However, the number of aberrant crypt foci was not changed in the groups when SAC was given during the promotion period.

Fig. 1. Experimental schedule for aberrant crypt assay.

Week 0 1 2 3 4
DMH SAC SAC SAC SAC DMH
SAC: Orally administered (1mmol/kg/d)
DMH: Administered i.p. (25 mg/kg), 3h after last administration of the test compound

Fig. 2. Comparison of administration periods of SAC. SAC was administered orally at two different times (see text). *P < 0.05.

Fig. 3. Effect of SAC on GST activity in the liver. Rats were administered SAC at 3.5 mmol/kg and killed at the times described in Materials and methods. *P < 0.05, **P < 0.01.
GST activity in the liver was not increased immediately after a single oral administration of SAC, however, it was increased (19% over control) significantly 12 h after SAC. The activity was increased substantially 24 h after SAC (41% over control) administration and this significantly elevated GST activity continued over a 72 h period (Figure 3).

GST activities in the liver, small intestine and colon were compared after administration of SAC. The GST activity was increased by 48, 66, 31 and 68% in the liver, proximal and middle small intestine and colon respectively (Figure 4).

GST isozymes in the liver were determined using Western blotting (Figure 5). GST-α and GST-μ were determined to be 22 300 ± 1300 ng/mg protein and 36 700 ± 4000 ng/mg protein respectively in the control liver cytosolic fraction. These isozymes were significantly increased after administration of SAC by 35.0 and 41.7% respectively. The content of GST-π in the liver was lower than the detection limit, 130 ng/mg protein.

Discussion

The chemopreventive activity of organosulfur compounds derived from garlic has been widely reported (3–8). Furthermore, epidemiological studies have shown that gastric cancer incidence was significantly lower in districts in China and Italy in which people consumed more dietary garlic (21,22). Further, garlic in the diet was found to significantly protect women from colon cancer in the Iowa Women's Health Study (24). We have begun to examine the potential mechanisms of action for certain garlic compounds. SAC is a water-soluble non-toxic compound derived from garlic. In this study we have shown that orally administered SAC decreased carcinogen-induced colonic aberrant crypt foci significantly in rats treated with DMH as the carcinogen. Interestingly, administration of SAC incorporated into the experimental diet significantly decreased the number of aberrant crypt foci when given during the initiation period, but had no effect when given during promotion. The result suggests that SAC affects the initiation phase of carcinogenesis, but not growth or differentiation. We therefore hypothesized that SAC could mediate carcinogenesis through induction of GST. In this study enhancement of GST activity was observed not only in the liver, but also in the small intestine and colon. Other studies have indicated that chemoprevention can be achieved through modulation of the GST system. For instance, biochemical investigations have revealed that several compounds, such as butylated hydroxyanisole and ethoxyquin, induce several GST isozymes and that this induction correlates both with reduced formation of presumably carcinogenic aflatoxin B1 (AFB1)-DNA conjugates and with an increase in GST-catalyzed AFB1-glutathione conjugation (5). Furthermore, administration of a competitive substrate of GST, styrene oxide, increased formation of toxic AFB1-DNA adducts (5). Thus it is suggested that the concentration of reactive metabolites of drugs, plant toxins and environmental pollutants will decrease more rapidly when

![Fig. 5. Western blots of liver cytosolic fraction. (Standard) Lanes 1–4, corresponding authentic GST isozymes (α, 500 ng protein/lane; μ, 800 ng protein/lane; π, 500 ng protein/lane) were diluted serially (×2) and applied to the gel. Resulting staining on the immunoblots was quantified and served as standards for the calculation of absolute amounts of isozymes in the samples. (Sample) Lanes 1–4, aliquots of liver cytosolic samples were diluted serially (×2) and run in parallel with authentic isozymes. One of the visualized spots within the range of the standard curve was used for the calculation. Calculated amounts of isozymes were corrected using corresponding protein contents.](image-url)
GST levels are high. Considering that we are constantly exposed to xenobiotics in our food and environment (3), the significance of maintaining an adequate detoxification system cannot be understated. The mucosa in the gastrointestinal tract is the first line of contact with xenobiotics and a capacity of mucosal cells to detoxify them is essential for protection against carcinogenesis. The protective mechanisms of SAC against colonic carcinogenesis have been investigated extensively in our laboratory, however, little is known about the effect of GST activities at the level of the target organ, the colon. In this study we have shown that oral administration of SAC increased GST activity in the colonic mucosa, where carcinomas are ultimately induced by injection of DMH. We observed a concomitant reduction in aberrant crypt foci during the initiation phase of carcinogenesis, a time when SAC also increased GST enzyme activity. We speculate that specific isozymes of GST may be involved in detoxification of this carcinogen. In this study we also found that hepatic GST-α and GST-μ, but not GST-π, were increased significantly after oral administration of SAC. An increase in one or both of the two isozymes after administration of SAC may be closely related to chemopreventive activity. Further investigations focusing on the role of detoxification in the process of carcinogenesis are currently under way.

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