Preclinical and Clinical Evaluation of Broccoli Supplements as Inducers of Glutathione S-Transferase Activity

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ABSTRACT

Previous studies suggest that cruciferous vegetables may provide protection against carcinogen exposure by inducing detoxification enzymes. ICR(Ha) mice were gavaged with broccoli tablets (1 g/kg), and colon tissues were collected after treatment. Glutathione S-transferase (GST) activity was assayed and peaked on days 1 and 2 after treatment, respectively (P = 0.03). Elevations in GST activity were attributed to the increased expression of μ and π. These data supported a clinical assessment of broccoli supplements.

Twenty-nine subjects at increased risk for colorectal cancer were randomized to group 1 (no cruciferous vegetables) or group 2 (broccoli supplements, 3 g/day) for 14 days. Blood samples and colon biopsies were obtained pre- and postintervention. No significant difference was observed between the GST activities of the control and broccoli supplementation groups posttreatment. Mean lymphocyte GST activity was 107% of baseline in the broccoli supplementation group (range, 75–158%) and 102% of baseline in the control group (range, 75–158%).

Correlation of the GST activities of blood lymphocytes and colon mucosa taken simultaneously suggested that the GST activity of blood lymphocytes may be used as a biomarker of the responsiveness of colon tissue to chemopreventive regimens. Future clinical studies evaluating cruciferous vegetables should consider using concentrated dietary supplements in subjects with a previous history of colorectal cancer.

INTRODUCTION

Recent analyses indicate that dietary and environmental factors may contribute to 85–90% of all cases of colorectal cancer (1). Although dietary factors may account for as much as 30% of attributable risk, diets high in fiber, calcium, and various micronutrients appear to decrease one’s risk for colorectal cancer. The ability of dietary constituents to regulate detoxification enzyme expression suggests that these enzymes may represent an essential link between diet and the prevention of colorectal cancer as well as a critical target for intervention.

Reviews of the dietary practices of colon cancer patients have repeatedly documented a decreased ingestion of cruciferous vegetables (2–4). After monitoring the diet of cancer patients for several years, it was estimated that individuals who rarely or never ate cabbage were at a 3-fold increased risk for colon cancer (3). Identical results were obtained for Brussels sprouts, broccoli, and turnips. An inverse, dose-dependent relationship between the ingestion of cruciferous vegetables and cancer incidence was also observed in patients with cancer of the rectum (4).

Direct evidence for the chemopreventive activity of cruciferous vegetables has been derived from animal models of chemically induced carcinogenesis. Rats fed cabbage and broccoli daily failed to develop mammary tumors after the administration of 7,12-dimethylbenz(a)anthracene (5). Independent administration of two major components of these vegetables, benzyl isothiocyanate and indole-3-carbinol, indicated that both were able to inhibit mammary tumor formation (6, 7). In a separate study, rats were fed either a purified diet containing aflatoxin B1 or the same diet supplemented with 25% freeze-dried cabbage for 26 weeks (8). The inclusion of cabbage in the diet decreased elevations in plasma α-fetoprotein induced by aflatoxin B1 and significantly inhibited hepatic tumor formation by more than 50% compared with carcinogen-treated controls.

Although the mechanism(s) by which cruciferous vegetables provide protection against carcinogen exposure remains unclear, elevations in detoxification enzyme levels after vegetable supplementation have been observed repeatedly. Among the common enzymatic alterations are inductions in GST and NQO activities. The GSTs (α, μ, π, and σ) are a family of Phase II detoxification enzymes that catalyze the conjugation of electrophilic compounds with glutathione. The resulting conjugate is more water soluble and, in most instances, no longer cytotoxic. NQO is a flavoprotein that catalyzes the reduction of a broad range of quinones, quinone imines, and azo dyes via a two-electron transfer (9). It is speculated that the protective enzyme activity is due to its successful competition with
one-electron reduction reactions and inhibition of free radical intermediates (10).

Administration of lyophilized cabbage (20% of the diet) to young mice produced significant elevations in the GST activity and glutathione content of liver, lung, kidney, and jejunal mucosa (11). Stohs et al. (12) reported similar results for the hepatic response of elderly mice to cabbage. The GST activity of small intestinal mucosa and liver was increased (3–150% and 15–180%, respectively) in a dose-dependent manner in the rat by the addition of 2.5–30% Brussels sprouts to a semisynthetic diet. Quantitation of the NQO activity of cultured hepatoma cells after exposure to various vegetable extracts identified members of the Cruciferae family as the most effective enzyme inducers (13). In humans, GST α was significantly elevated in the plasma of subjects ingesting large amounts of Brussels sprouts (14). Elevations in salivary GST, DT-diacerase, and class 3 aldehyde dehydrogenase were observed in an individual after the daily consumption of a similar amount of broccoli (15).

Sulforaphane (\((-\)\)-1-isothiocyanato\((-4R\)-(methylsulfinyl)\)-butane) has been isolated recently from broccoli and identified as both a potent inducer of Phase II detoxification enzymes (16) and an inhibitor of the carcinogen-activating cytochrome P450 2E1 (17). Analysis of purified sulforaphane in a murine hepatoma cell assay indicated that this single component was responsible for 65–80% of the total inducible activity present in the original broccoli extract. Induction of NQO activity independent of either cytochrome P450 1A1 or the aryl hydrocarbon receptor has led to the classification of sulforaphane as a monofunctional enzyme inducer. Oral administration of sulforaphane to mice (15 μg/day) produced elevations in GST and NQO activity in a variety of tissues, including small intestine (16). The effectiveness of sulforaphane in blocking the formation of mammary tumors in rats treated with 9,10 dimethyl-1,2-benz(a)anthracene has been demonstrated (18).

The present study evaluates the ability of broccoli supplements to increase the activity of GST in vivo. Marine studies demonstrating the ability of broccoli to induce GST activity in colon tissue provided the basis for the present clinical investigation. Individuals at increased risk for colorectal cancer have been selected for clinical evaluation based on previous findings from this laboratory, which indicated that these individuals possess significantly lower levels of GST activity than healthy controls at no known risk for cancer (19).

**MATERIALS AND METHODS**

**Preclinical Studies**

**Animal Treatment**

Female ICR(Ha) mice (approximately 6 weeks old) were obtained from the Fox Chase Cancer Center Laboratory Animal Facility’s breeding colony and maintained under standard conditions, receiving food and water *ad libitum*. The broccoli tablets (Broccoli-500 mg Food Concentrate) were generously supplied by Solgar Co., Inc. (Lynbrook, NY) and contained the equivalent of 5 g of fresh broccoli in lyophilized form. All broccoli was grown in the absence of synthetic fertilizers. According to Solgar Co., Inc., the composition of the broccoli tablets is identical to that of fresh broccoli except for vitamin C content (131 mg/100 g). Broccoli tablets were pulverized with a mortar and pestle and suspended in 1% carboxymethylcellulose/25% glycerol. Mice were gavaged with a single dose of the mixture at 1 g/kg, the maximum dose that could be maintained in suspension for gavage, and sacrificed at 1 and 2 days post-treatment. Colon tissues were excised, rinsed in PBS, and immediately frozen on dry ice. All tissues were stored at −70°C until analysis.

**Enzymatic Activity Assays**

GST activity was measured according to the method of Habig et al. (20), using 1-chloro-2,4-dinitrobenzene (Sigma Chemical Co., St. Louis, MO) as substrate. Glutathione conjugates formed in the presence of enzyme were quantified spectrophotometrically. Use of 3,4-dichloronitrobenzene as a substrate allowed specific quantitation of activity associated with the μ class of GST. Results from all assays were normalized to protein content using the Bradford assay (Bio-Rad, Richmond, CA) and expressed as specific activity. Statistical comparisons were performed using the Wilcoxon 2-sample test with normal approximation and a continuity correction of 0.5.

**Western Blot Analyses**

Tissue homogenates were applied to 12% polyacrylamide slab gels and separated according to the method of Laemml (21). Maximal separation of GST isozymes was achieved by running a prestained M, 18,000 molecular weight marker (Diversified Biotech, Newton Center, MA) to the bottom of the slab gel. Proteins were electrophoretically transferred to nitrocellulose in 25 mM Tris and 192 mM glycine (pH 8.3) containing 20% methanol (150 mA overnight; Ref. 22). Membranes were rinsed three times (10 min each) with 50 mM Tris and 400 mM NaCl (pH 7.5) containing 0.05% Tween 20, and were incubated sequentially for 1 h at room temperature with Tris-buffered saline containing 3% BSA and either rabbit anti-rat GST α, rabbit anti-rat GST μ, or rabbit anti-mouse GST γ antibodies diluted 1/1000 (Biotrin International, Dublin, Ireland). These antibodies have been characterized and cross-react only with other members of the same GST class. Membranes were rinsed, incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), and developed using 4-chloro-1-naphthol as substrate (Bio-Rad).

**Clinical Trial**

**Study Population**

Individuals were either recruited from the Outpatient Clinic at the Fox Chase Cancer Center or were members of the community who responded to a trial advertisement in the local newspaper. All eligible subjects were at increased risk for colorectal carcinoma and included men and women over the age of 18 with a family history of colorectal cancer, a personal history of colon polyps, or a personal history of colorectal cancer (>2 years from definitive treatment). Individuals were deemed ineligible if they had: (a) a known untreated primary or metastatic carcinoma; (b) hereditary nonpolyposis colorectal carcinoma; (c) familial adenomatous polyposis syndrome; (d) a history of colitis (inflammatory, radiation-induced, or Crohn’s disease); or (e) significant medical or psychiatric problems that would make
were obtained from the sigmoid colon (-20 cm from the anus), immediately frozen in liquid nitrogen, and stored at -80°C.

Peripheral blood samples were obtained at the time of each endoscopy. Blood samples (~10 ml) were drawn into heparinized tubes by venipuncture for biochemical and molecular analyses. The blood was immediately transported to the laboratory at room temperature. Two ml of whole blood were aliquoted and stored at -70°C for subsequent GST μ phenotyping, and the remainder was layered over Lymphocyte Separation Media (Organon Teknika Corp., Durham, NC) and centrifuged (400 × g) at room temperature for 25 min. The resulting mononuclear cell layer was washed twice with PBS and stored as a dry pellet at -70°C.

**Enzyme Analyses**

**GST μ Phenotyping.** The presence of GST μ (M1) in whole blood (100 μl) was determined using an enzyme-linked immunoadsorbent kit supplied by Biotrin International. Assays were performed according to the manufacturer’s instructions. All samples were assayed in duplicate with corresponding positive and negative controls.

**Enzyme Activity Assays.** At the time of analysis, all samples were thawed on ice and immersed in 1 ml of 10 mM Tris-HCl (pH 7.8). Blood lymphocytes were lysed by pulsed sonication for 30 s (Fisher 550 sonic dismembrator, Pittsburgh, PA) and centrifuged at 10,000 × g for 3 min. Biopsy samples of colon mucosa (one specimen per milliliter) were homogenized (Omni 1000 homogenizer, Waterbury, CT) and centrifuged at 10,000 rpm for 15 min at 4°C. The protein content of the resulting supernatant was determined using the Bradford assay (Bio-Rad).

GST assays were performed as described previously for murine tissues. All results were normalized to protein content and expressed as specific activities.

**RESULTS**

**Preclinical Studies.** Experimentation was conducted in mice to determine the ability of broccoli supplements to induce Phase II detoxification enzyme activity. A single dose of lyophilized broccoli (1 g/kg) produced significant elevations in GST activity (Fig. 2). GST activity peaked in colon tissue on day 1 posttreatment ($P = 0.03$), but was not significantly different from vehicle-treated controls on day 2 posttreatment.

The availability of class-specific GST antibodies allowed an investigation of the class of isozymes responsible for the observed increases in GST activity. Western blot analyses demonstrated that only GST μ was detectable in the colon of vehicle-treated animals (Fig. 3). Elevations in GST μ expression were observed in the colon of broccoli-treated animals on day 1 and were accompanied by the induction of GST π to detectable levels. Both GST μ and π levels were decreased close to baseline by day 2 posttreatment. The ability of broccoli concentrate to induce GST μ expression was confirmed by enzyme activity assays, which used a substrate specific for the μ isozyme. Enzymatic activity increased on day 1 to 3.5-fold that of vehicle-treated controls. Expression of GST α was nonde-
Broccoli as a GST Inducer

Fig. 2 Activity of GST in the colons of mice 2 days after vehicle treatment (V) or 1 and 2 days after the administration of broccoli supplement (1 g/kg). Bars, SE.

tectable by Western blot in colon tissue from both vehicle- and broccoli-treated mice.

Clinical Trial. Of the 31 patients who consented to trial participation, 29 completed all study requirements. All 29 subjects were compliant as determined by self-report and confirmed by pill counts. The mean age of compliant subjects was 57, with ages ranging from 31–75. Fifty-five percent of all subjects were male, and 94% were Caucasian. The majority of the subjects (66%) were identified as high risk due to a family history of colon cancer (one or more first-degree relatives with colon cancer). Twenty-eight percent possessed a personal history of colon cancer, and 17% possessed a personal history of colon polyps. Seven percent of all subjects possessed two of the above risk factors. Data extracted from food frequency questionnaires indicated that 45 percent of all subjects routinely ate broccoli 2–4 times per week.

The total GST activity of blood lymphocytes and "normal" colon mucosa from high-risk individuals before treatment was 68.0 ± 4 and 75.7 ± 15 nmol/min/mg (mean ± SE), respectively. No relationship was observed between either caloric intake, dietary fat, dietary fiber, or body mass index and baseline GST activities.

Fig. 4 summarizes the response of subjects to the 14-day intervention as measured by changes in the GST activity of blood lymphocytes. Responses were defined as elevations in posttreatment GST activities that exceeded the endogenous intracellular variability in baseline GST activity over time (10%), as established in an independent study (19). After the intervention, the mean GST activity of groups 1 and 2 did not differ significantly (102 and 107% baseline, respectively). Elevations in posttreatment blood lymphocyte GST activity measurements indicative of a response were observed in 36% (4 of 11) of the subjects in the broccoli supplementation group (group 2; range, 111–158%; mean 131%) and in 29% (5 of 17) of the individuals in the control group (group 1; range, 115–158%; mean 127%). Posttreatment GST activity was decreased compared with baseline (>10%) in 29% of individuals in group 1 and in 27% of individuals in group 2. The GST M1 null phenotype was present in 39% of all subjects and was unrelated to treatment outcome.

Examination of each individual’s response based on their corresponding risk factors indicated that 50% (2 of 4) of the individuals in group 2 with a personal history of colorectal cancer responded to supplementation. GST activities were either maintained or decreased during the 14-day intervention period in all individuals with a personal history of colorectal cancer (n = 4) who were randomized to the control group.

Correlation of the GST activities of blood lymphocytes and colon mucosa taken simultaneously from the same individual yielded a Spearman’s rank correlation of 0.71 (Fig. 5). This correlation includes the analysis of both pre- and postintervention activity measurements from groups 1 and 2.

DISCUSSION

Results from the preclinical study demonstrate the ability of commercial broccoli supplements to induce the expression of GST within murine colon. The reported GST response is similar to that observed in rats after the administration of Brussels sprouts (24). The induction of GST μ (M1) by both broccoli and Brussels sprouts is of particular interest due to the polymorphic expression of this enzyme in humans and its potential link with cancer susceptibility (25–27). Approximately 50% of the general population is unable to express GST M1 due to a gene deletion (25). The contribution of the null genotype to colorectal cancer remains equivocal (19, 28, 29).

The present clinical study demonstrates the feasibility of administering a cruciferous vegetable supplement to individuals at increased risk for cancer on a chronic basis. Use of these supplements for clinical investigation was advantageous to previous attempts by others (14, 15) to increase the daily consumption of broccoli in the diet for several reasons: (a) the dosage of broccoli could be standardized; (b) the potential for intersubject variability in processing the vegetable for consumption could be circumvented; (c) compliance could be readily assessed using pill counts; and (d) broccoli could be administered in a dosage exceeding that normally consumed in the diet. Although the participants understood that they would receive no apparent benefit from the short-term use of this supplement, 94% re-
The intraindividual variability in (Group 1) to a control diet phocytes from high-risk subjects baseline pretreatment measurement for each individual (n = 25). The intraindividual variability in the GST activity of blood lymphocytes over time is approximately 10%.

Fig. 4 Response of blood lymphocytes from high-risk subjects to a control diet (Group 1) or broccoli supplementation (Group 2). The points represent the difference between the GST activity after the 14-day intervention and the baseline pretreatment measurement for each individual (n = 25). The identification of sulforaphane as the predominant detoxification enzyme in-

The mean GST activity of blood lymphocytes from study participants at baseline was similar to that reported previously by us for individuals at increased risk for colorectal cancer (62.7 ± 3, n = 60; Ref. 19). The correlation between the baseline GST activity of blood lymphocytes and colon mucosa from individuals in this study also was comparable to that established by us previously (19). Maintenance of the correlation between the GST activity of blood lymphocytes and colon mucosa upon the inclusion of posttreatment data from subjects demonstrates that the detoxification enzymes in these two tissues are coordinately regulated. Although other investigators have hypothesized that enzymatic elevations in plasma (14) and saliva (15) after cruciferous vegetable consumption reflect similar changes in other organs, tissue samples were not examined in these previous studies. Our findings support the use of the enzyme activity of blood lymphocytes as a biomarker of the responsiveness of colon tissue to other chemopreventive regimens. The detoxification enzyme expression of blood lymphocytes has been used successfully to monitor the response of colon mucosa to oltipraz (a dithiolthione structurally similar to those found in cruciferous vegetables) in individuals at increased risk for colorectal cancer (30).

Although the absolute dosage of broccoli required to significantly elevate the detoxification enzyme activity of human colon mucosa remains unknown, the findings from the present trial and others suggest that a daily serving of broccoli may be insufficient to alter GST expression. Administration of broccoli to a volunteer for 12 days at 300 g/day produced elevations in both the GST and DT-diaphorase activities of saliva (3- and 4-fold, respectively), which resolved to baseline upon termination of the intervention (15). Unfortunately, the relationship between salivary and lymphocytic levels of GST was not examined in this study. Consumption of an equivalent amount of Brussels sprouts daily for 3 weeks produced a significant elevation (1.4-fold) in the level of GST α in plasma (14). The dosage of broccoli and Brussels sprouts used in the above studies is 10-fold more than that used in the present study and equivalent to approximately 10 servings/day. The dosage used in this study was based on the manufacturer’s recommended daily allowance of the broccoli supplement. Any increase in dosage beyond this level would have required additional approval by the Food and Drug Administration.

The requirement to administer large dosages of cruciferous vegetables over an extended period of time in future chemoprevention trials dictates the need to formulate these extracts in a more concentrated or purified form. Until recently, the breakdown products of glucosinolates were thought to be primarily responsible for the chemopreventive activity of cruciferous vegetables (31). The identification of sulforaphane as the predominant detoxification enzyme in-

fig. 5 Correlation of the GST activity of colon mucosa and blood lymphocytes from individuals at increased risk for colorectal cancer. Colon mucosa and blood lymphocytes were collected simultaneously from each individual and assayed for enzyme activity. Comparison of the activities of these tissues (both pre- and posttreatment samples, n = 51) yielded a Spearman’s rank correlation of 0.71. This correlation was reported initially using baseline data from 13 of these individuals (19).
ducers in broccoli (16) and subsequent demonstration of its ability to inhibit mammary carcinogenesis (18) supports its investigation as a clinical chemopreventive agent. Nutritional supplements of sulforaphane have become available very recently (Solgar Co., Inc.).

In summary, the results from the present study support the future evaluation of concentrated broccoli supplements in patients at increased risk for colorectal cancer. These data suggest that subjects with a previous history of colorectal cancer may benefit most from this chemopreventive regimen.

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