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Suppressive Effects of Young Radish Cultivated with Sulfur on Growth and Metastasis of B16-F10 Melanoma Cells

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The oral administration of extracts of young radishes cultivated with sulfur after intravenous tumor cell injection achieved a marked reduction of pulmonary colonization in mice. Treatment of the mice with extracts of young radish cultivated with sulfur did not show any increase in the number of CD8+ or NK T cells in the spleen, indicating no influence on host immunity. Sulforaphane, which could be a candidate for an active compound from young radishes cultivated with sulfur, inhibited cell growth of B16-F10 melanoma cells. In addition, extracts of the young radish cultivated with sulfur-fed group showed enhanced quinine reductase (QR) activities in the liver and lung and a slight increase of glutathione S-transferase (GST) activity in the liver. These results suggested that the administration of extracts of young radishes cultivated with sulfur suppressed pulmonary tumorigenesis, possibly due to increased activity of detoxification enzymes in the liver and lung, and partly due to cell cytotoxicity.

Key words: Quinone reductase, Radish, Sulfur, Isothiocyanate, Melanoma

INTRODUCTION

The consumption of cruciferous vegetables such as broccoli, Brussels sprouts and cabbage has been associated with a decreased risk for many cancers. Epidemiological studies indicate an inverse relationship between the consumption of cruciferous vegetables and the incidence of cancer (Haenszel *et al.*, 1980). Two main groups of anticarcinogens have been discovered, the isothiocyanates and the indoles. Both compounds are metabolites of the glucosinolate and thioglycoside compounds that are contained in cruciferous plants (Zhang and Talay, 1998). Isothiocyanates are released upon chewing or maceration of certain cruciferous vegetables. Myrosinase, which is released from a separate cellular compartment, hydrolyzes the glucosinolate-producing isothiocyanates and other products. Substantial amounts of glucosinolates are known to occur in a wide variety of cruciferous dietary vegetables (Hecht, 2000).

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The remarkable ability of isothiocyanates to prevent cancer treated with carcinogens can be caused by their effects on carcinogen metabolism. Most dietary and environmental carcinogens require enzymic transformation (metabolic activation), to exert their carcinogenic effects *in vivo*. The process of detoxification competes with metabolic activation. The mechanism of this chemo-protective effect is not entirely understood, but has been linked to the induction of detoxification enzymes, including phase I enzymes of the cytochrome P-450 family, and phase II enzymes, such as glucuronosyl transferases, glutathione S-transferase (GST), and quinine reductase (QR) (Wortelboer *et al.*, 1992). Consumption of diets containing cruciferous vegetables has been shown to increase drug detoxification in both the human and in rodents. In rat studies, diets containing Brussel sprouts induced phase-I and II enzymes in liver and small intestine (Wortelboer *et al.*, 1992). Sulforaphane isolated from broccoli also blocked mammary tumor development in rats treated with 9, 10-dimethyl-1,2-benzanthracene (DMBA) (Zhang *et al.*, 1994). Sulforaphane appears to have dual actions by inhibiting *Helicobacter* infections and preventing benzopyrene-induced stomach cancers (Fahey *et al.*, 2002). Sulforaphane also inhibited extracellular, intracellular, and antibiotic-

resistant strains of *Helicobacter pyroli* and prevented benzo[a]pyrene-induced stomach tumors and prevented colonic aberrant cryptic foci (Chung *et al.*, 2000).

In the present study, extracts of young radishes that have been cultivated with sulfur were administered to mice to prevent pulmonary colonization that is induced by B16-F10 melanoma cells. Fourteen days after the intravenous injection of the tumor cells, the spleen, liver and lungs of the mice were evaluated to determine host immunity and the possible induction of detoxification enzymes.

MATERIALS AND METHODS

Materials

Selenium, Trizma base, BSA, FAD, NADH, 2, 6-dichloroindophenol (DCP), Tween 20, 1-chloro-2, 4-dinitrobenzene (CDNB) and dicumarol were obtained from the Sigma Chemical Co. (St. Louis, U.S.A.). FITC labeled anti-mouse CD4 antibody (Ab), anti-mouse CD8 Ab, anti-mouse NK1.1 Ab were obtained from Pharmingen (San-Diego, CA). Sulforaphane and sulforaphene were purchased from the Alexis Corporation (SanDiego, CA). Young radishes were cultivated at the Agricultural Institute of Kyungsang Namdo (Jinjoo, Korea). The young radishes were grown in soil that contained sulfur (1,818 g/m^3) (>95%, Dae-Do Chemical., Jinjoo, Korea).

Preparation of extracts of young radishes cultivated with sulfur

Leaves of the young radishes were washed with distilled water, suspended with 80% methanol, and disrupted by pulsing for 5 min with an ice-chilled glass bead beater (Biospec Products, Bartlesville, OK, U.S.A.). The homogenate was filtered through several layers of cheese clothe to remove debris and the filtrate was then centrifuged at 10,000×g for 40 min in a Model T-324 with an A-8.24 rotor refrigerated centrifuge (Kontron Instruments, Zurich, Switzerland). The supernatant was evaporated to remove methanol, the residue dissolved in distilled water, and concentrated completely by ultrafiltration using an Amicon YM 10 membrane thereby removing large molecular weight products. Fraction of lower molecular mass were dissolved in phosphate-buffered saline, and sterilized for injection of the mice.

Tumor cell lines and animal experiments

B16-F10 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For animal experiments, cells were passed two to five times with medium after thawing. Cells in a log growth phase were detached from tissue culture flasks using a mixture of 0.25% trypsin and 0.03% EDTA. The cells were then washed and suspended in phosphate-based saline (PBS) just before inoculation to mice. On day zero, 2×10^5 cells as a 0.2 mL suspension were injected into the tail vein of seven-week old male C57BL/6 mice (~20 g). Extracts of young radishes cultivated with sulfur (8 mg/kg) or selenium (2 mg/kg) in a solution of PBS was administered to mice by the oral route (gavage) on days -6, -4, -2, 0, +2, and +4. PBS was injected as control to a group of mice. At the indicated days after tumor inoculation, mice were sacrificed and the lung, spleen, and liver organs were removed for study. The numbers of black metastatic colonies in the lungs were counted using a dissecting microscope. For subsequent enzymatic analyses, the lung and liver tissues were dissected and stored for future assay. The spleens were removed for the counting of total cells and the different T cell populations were analyzed by flow cytometry.

Flow cytometry

The isolated cells (5×10^5/ sample) were incubated in incubation buffer (phosphate buffered saline, 10% human serum and 0.1% sodium azide) with Fc blocker for 30 min, then stained with FITC-labeled CD3, and PE-labeled CD4, CD8, NK1.1 for 30 min on ice. Fluorescence-labeled rat IgG was used as an isotype control. The cells were washed with PBS and flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA, U.S.A.).

Cell viability

Cell viability was determined by measuring the exclusion of Trypan blue. Cells (3×10^5) were plated and incubated under the indicated conditions. Viable cells were collected, and counted using a hemacytometer.

Enzyme analysis

Lung and liver tissues were washed and sliced by hand. The sliced cells were homogenized for 5 min using a glass homogenizer and left on ice for 10 min. The homogenates were centrifuged at 13,000×g for 30 min at 4°C. Supernatants were transferred to a new tube and protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.). The activity of GST in the cytosol fractions was determined spectrophotometrically, at 25°C, with 1-chloro-2, 4-dinitrobenzene (CDNB). The reaction mixture contained 100 mM KPO_4, pH 6.5, 1 mM glutathione, and 1 mM CDNB. The reaction was started by the addition of the cytosol extracts. QR was determined by measuring the reduction of 2, 6-dichloroindophenol. The reaction mixture contained 25 mM Tris-HCl, pH 7.4, 60 ug bovine serum albumin, 5 uM FAD, 0.2 mM NADH, 80 uM 2,6-dichloroindophenol (DCP), and 0.01% Tween 20 in 1 mL and the reaction continued for 5 min at room

temperature; termination of the reaction was through the addition of final concentration of 30 uM dicumarol. The absorbance of the reaction mixture was read on a spectrophotometer at 600 nm.

RESULTS AND DISCUSSION

Previously, extracts of young radish cultivated with sulfur were shown to contain isothiocyanate-like compounds by HPLC analysis and QR-inducing activities in Hepa 1c1c cells. The structure of isothiocyanate-like compounds found in extracts of young radishes cultivated with sulfur has not been defined (Kim *et al.*, 2004). We have attempted to determine the effect of extracts of young radishes that had been cultivated with sulfur in an experimental B16-F10 melanoma metastasis model. Fourteen days after the intravenous infusion of B16-F10 cells, saline-treated mice had approximately 105±37 colonies per lungs of mice, whereas extracts of young radishes cultivated with sulfur in the absence and presence of selenium had 10±14 and 21±30 colonies, respectively (Fig. 1A and 1B). We also treated the mice with the extracts of young radishes cultivated without sulfur and found a reduction of B16-F10 melanoma-induced colonies, but to a lesser extent (data not shown).

We have measured the total number of lymphocytes (CD4+, CD8+, and NK cells) by FACS analysis to investigate whether host immunity is a factor in the anti-tumor activity of extracts of the young radishes cultivated with sulfur. No significant increase of the CD8+, and NK cells was observed in extracts of the young radishes cultivated with sulfur and only slight decreases of these cells were in that with addition of selenium-treated group (Table I). These results suggest that the anti-tumor activity of extracts of young radish cultivated with sulfur in the absence or presence of selenium-treated groups could be

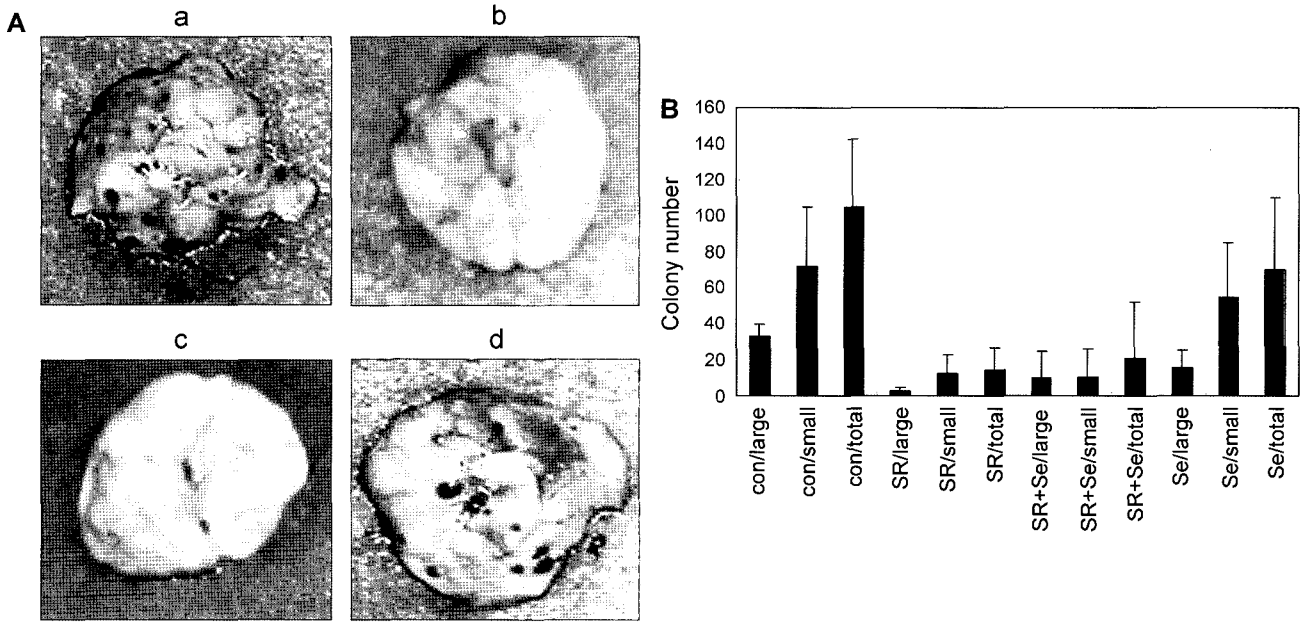

Fig. 1. Effect of extracts of young radishes cultivated with sulfur on pulmonary metastasis by B16-F10 tumor cells. (A) B16-F10 melanoma cells were injected intravenously into saline (a), extracts of young radish cultivated with sulfur in the absence (b) or in the presence of selenium (c), and selenium (d)-fed mice. (B) The number of metastatic colonies in the lungs of these mice was counted 14 days after the injection of the tumor cells. Effectors were administered orally on day -6, -4, -2, 0, 2, and 4. Results are the means ± SD. The total number of colonies in the young radish cultivated with sulfur-fed group was statistically significant versus saline-fed alone ($P<0.05$). Four mice were used for each group; results are representative of three experiments.

Table I. Effects of the supplementation of young radishes grown with sulfur on spleen cell numbers in C57BL/6 mice

	Total lymphocytes ($\times10^5$)	Number of CD4+ ($\times10^5$)	Number of CD8+ ($\times10^5$)	Number of NK ($\times10^5$)
Control	353±101	121±65	21±11	18± 9
SulfurRadish-treated group	287± 95	110±56	23±12	19± 8
SulfurRadish with Selenium-treated group	220± 81	109±45	15± 8	10± 8
Selenium-treated group	203± 98	151±42	25±10	22±10

Data represent the mean ± S.E. of 4 animals.

due to factors other than host immunity.

We also investigated whether the isothiocyanate compounds, such as sulforaphane, had an effect on the growth of B10-F10 melanoma cells. Increasing concentrations of sulforaphane caused a progressive reduction in the numbers of cells after 24 h of incubation (Fig. 2A). An analog of sulforaphane, sulforaphene, also showed a similar pattern (data not shown). Extracts of young radishes cultivated with sulfur exhibited a definite cytotoxicity to the B10-F10 melanoma (Fig. 2B).

We determined the effect of orally administered extracts of young radish cultivated with sulfur on the QR and GST profiles in mouse liver and lung tissues. As shown in Fig. 3A and 3B, extracts of the young radish cultivated with sulfur had higher levels of QR in the mouse liver and lung compared to the saline treated control group. A slight increase of QR was observed in extracts of young radish cultivated with sulfur with the selenium-treated group, but no difference was found in the selenium-treated group and the non-treated group. Compared to QR, a lesser degree of increase was observed in the level of GST between extracts of young radish cultivated with sulfur-treated and the saline-treated controls (Fig. 3C).

In the present study, extracts of young radishes cultivated with sulfur prevented pulmonary colonization induced by the B16-F10 melanoma cells. The multiple (six-time) oral administration of extracts of young radishes cultivated with sulfur before and after intravenous tumor cell injection

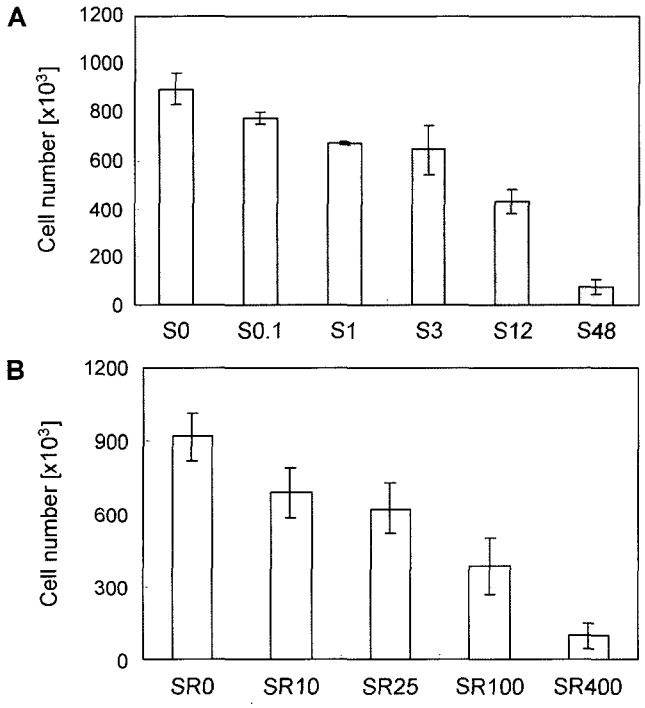

Fig. 2. Inhibition of cell growth by sulforaphane. Numbers of viable B16-F10 melanoma cells cultured in the indicated concentrations of sulforaphane (S) (0, 0.1, 1, 3, 12, and 48 ug/mL) (A) and extracts of young radish cultivated with sulfur (SR) (0, 10, 25, 100, and 400 ug/mL) (B) was counted by trypan blue exclusion. Each sample point was performed in triplicate and the results represented as means ± SD; results are representative of three experiments.

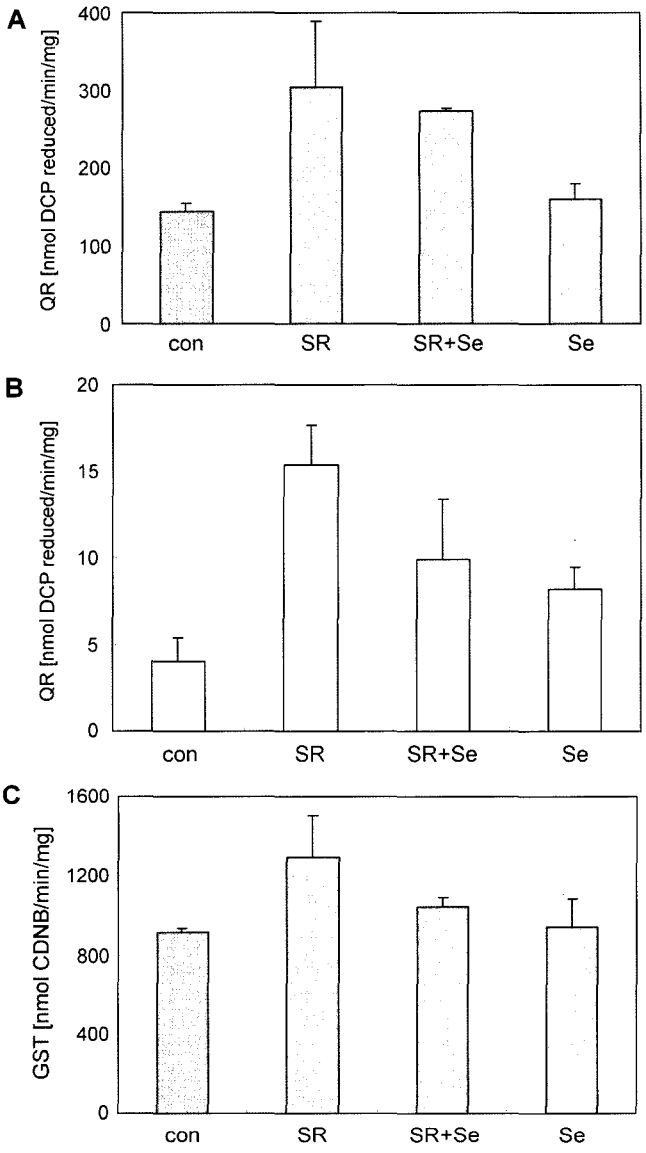

Fig. 3. Induction of Phase II enzymes in mice fed with young radish extract cultivated with sulfur. Induction of QR was determined in mouse liver (A) and Lung (B). Each lung and liver extracts were prepared as described in Material and Methods, and protein concentration was determined by the Bradford assay. QR was determined by measuring the reduction of 2, 6-dichloroindophenol at 600 nm. Both QR activities of liver and lung in young radish cultivated with sulfur-fed group were statistically significant versus saline-fed alone ($P<0.001$). Induction of GST was determined in mouse liver (C). The activity of GST in the cytosol was determined by the formation of CDNB conjugates at 340 nm. The reaction was started by the addition of the cytosol preparations. GST activities in the liver of the young radishes cultivated with sulfur-fed group were statistically significant versus saline-fed alone ($P<0.05$).

achieved a significant reduction of pulmonary colonization in the C57BL6 mice. Cell cytotoxicity was observed after treatment of sulforaphane in B16-F10 melanoma cells. In addition, specific activities of QR increased in liver and lung, and GST also was enhanced but to a lesser degree in the extracts from the young radish cultivated with sulfur-fed group., No significant changes in CD8+ and natural killer (NK) T cell populations, however, have been observed. These results indicated that suppression of tumor growth by the extracts of the young radish cultivated with sulfur-group could be caused partly by the induction of detoxification enzymes, and the cytotoxicity could possibly be due to the sulforaphane from the extracts of the young radishes cultivated with sulfur rather than an increased host immune response. Assuming that the effective molecule in these extracts is an analog of sulforaphane, we could consider the effect of cell cytotoxicity on B16-F10 melanoma as an anti-tumor activity characteristic of extracts of the young radishes when cultivated in the presence of sulfur. This outcome can be taken to indicate that the extracts of the young radishes cultivated with sulfur might also have a chemo-protective effect in mice when orally administered orally. In our previous reports we showed that extracts of young radish cultivated with sulfur contained isothiocyanate-like compounds (by HPLC analysis and quinine reductase-inducing activity in Hepa 1c1c cells), although we have not elucidated the structure of the isothiocyanate-like compound (Kim *et al.*, 2004). Large scales purification to enable identification of the structure of the active compound(s) is under active investigation.

Total cancer incidence and mortality are significantly reduced by selenium supplementation (Finley *et al.*, 2000). Since selenium treatment has been demonstrated to increase the activity of most seleno-proteins involved in redox reactions (Zhang *et al.*, 2003), we studies selenium with the young radishes cultivated with sulfur. This study did not, however, decrease tumorigenesis by the B16-F10 melanoma cells nor did it indicate an increase in phase II enzymes when administered alone or in combination with young radishes cultivated with sulfur in the melanoma model.

Fruits, vegetables, and several herbs have been shown to be rich sources of cancer chemo-preventive agents (Watenberg, 1992). The targets of these agents could be a blockade of one or more of the following initiation, promotion, or progressive stages of the multi-step processes of carcinogenesis. These agents are also capable to of enhancing the activities of carcinogen-metabolizing enzymes and to bind with toxic substances thus reducing their effective critical concentrations. Chemo-preventive agents may also act as antioxidants and counteract the increased amount of oxidizing agents that are generated by toxic substances. Because oxidative stress is believed to be an important contributing factor in carcinogenesis, it is possible that phase 2 enzymes exert their protective functions not only by inactivation of carcinogenic electrophiles but also through their antioxidant activities (Gao *et al.*, 2001). Sulforaphane cannot react directly with free radicals or ROS and its antioxidant function is secondary to an ability to induce phase II enzymes. Sulforaphane has been shown to be a potent mono-functional inducer of phase II enzymes in cultured cells and mouse tissues *in vivo* (Zhang *et al.*, 1992; Posner *et al.*, 1994). Mono-functional induction has been proposed as a predictor of the chemo-protective activity and several structurally unrelated organic isothiocyanates have been shown to block chemical carcinogenesis and to induce phase II enzymes (Zhang and Talay, 1994). It is reasonable to assume that increased activities of GST and QR in liver and lung of mice fed with young radish extract cultivated with sulfur play a critical role in relation to the observed cancer chemo-preventive effects in mice fed with young radish extract cultivated with sulfur.

The results of these studies indicate that the liver and lungs have a capacity for increasing the activities of phase II detoxification enzymes, particularly QR, when exposed to biologically active phytochemicals. Although the lung possesses lower specific activities of QR than does the liver, the enhancement of absolute QR activity was higher in the lung than in the liver. The activities of both QR and GST were increased in tandem, although the increase of GST was lower than that of QR, implying that a similar regulatory arrangement exists in the liver. This increase in the activity of phase II detoxification enzymes theoretically should protect against carcinogenic compounds. It can be hypothesized that the mixture of compounds present in the young radish extracts cultivated with sulfur could account for the decreased tumorigenesis with B16-F10 melanoma exposure. The present finding indicates chemo-protective effects of extracts of young radishes cultivated with sulfur in an *in vivo* model and also strengthens the validity of the prediction that chemical compounds that induce phase II enzymes are promising candidates for chemo protection. These selective anti-tumor effects may suppress the growth of pre-clinical tumors and thereby contribute to the well-established decreased cancer incidence associated with a diet rich in vegetables.

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