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Effect of selenium-enriched broccoli diet on differential gene expression in min mouse liver[1,2]

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Abstract

Multiple intestinal neoplasia (Min) mice are a good model for investigating the effects of dietary alterations in a genetic model for intestinal cancer. Previous studies have shown that selenium-enriched broccoli effectively reduces colon cancer susceptibility. Although colon cancer cells mainly metastasize to the liver, little is known about the effects of selenium-enriched broccoli on gene expression in mouse liver. To better understand the protective role for selenium-enriched broccoli in tumorigenesis, a gene profile of the mouse liver was analyzed. Mice were fed either 0.11 mg selenium/kg control diet or 2.1 mg selenium/kg selenobroccoli diets for 10 weeks. Use of mouse pathway finder-1 GEArrays revealed that selenium-enriched broccoli moderately increased ikB$\alpha\kappa$B, hsp86, gadd45 gene transcripts. In addition, analysis of the binding of liver nuclear proteins to ^{32}P-labeled probes demonstrated that selenium-enriched broccoli enhanced the binding of transcription factor p53, NFκB, AP-1 to their *cis*-acting elements. Collectively, these results suggest for the first time that selenium-enriched broccoli activates certain pro-apoptotic genes linked to p53, NFκB and stress signal pathways in response to "danger signals" such as tumorigenesis. Published by Elsevier Inc. All rights reserved.

Keywords: Selenium; Broccoli; Min mouse; Regulatory element; Gene array; Nuclear protein

1. Introduction

Selenium is an essential trace element for humans and many other forms of life [1]. In addition to its known essentiality, a protective role for selenium has been observed in colon cancer risk [2–5]. Grains and vegetables contain organic forms of the element such as selenomethionine or selenocystine. Broccoli contains primarily Se-methylselenocystine, which is more readily converted to methyl selenol than other organic forms of selenium [6]. It has been hypothesized that the production of methyl selenol is required for cancer prevention [7]. The Min mouse is highly susceptible to spontaneous formation of numerous tumors in both the small and large intestines [8,9]. Recent studies suggest that selenium from selenium-enriched broccoli is more effective than other inorganic forms of selenium against aberrant crypts formation, a preneoplastic lesion for colon cancer [10]. Selenium-enriched broccoli also decreased intestinal tumor formation in the Min mouse [11]. In the case of colon cancer development, the genes associated with initiation and progression are well documented [12]. For example, the mutation of the adenomatous polyposis coli (APC) gene can result in a marked predisposition to colon cancer [12]. In contrast, although liver metastasis is usually responsible for the mortality of colon cancer patients, little is known about its mechanism. The Min mouse presents an opportunity to examine the early molecular events in mouse liver. The current study investigated whether selenium-enriched broccoli would affect the expression of genes linked to apoptosis.

2. Materials and methods

2.1. Chemicals

T4 polynucleotide kinase was obtained from Promega (Madison, WI). Adenosine 5′-triphosphate (γ-^{32}P) and cy-

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tidine 5′-triphosphate (α-^{32}P) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotides were synthesized by Gibco BRL (Rockville, MD).

2.2. Animals and diets

Twenty-eight heterozygotic male Min (C57Bl/6J - $^{APCMin/+Apc}$) mice were obtained at 5 weeks of age from Jackson Laboratories (Bar Harbor, ME). All mice were housed individually in a room with controlled humidity, temperature and light. Mice were provided free access to demineralized water and purified diet. The basal diet was an AIN-93 diet [13] containing either low-selenium broccoli or an equivalent amount of high-selenium broccoli. The broccoli was produced as described [10] and accounted for 2.2 g/kg of the diet. By analysis, the diets contained 0.11 and 2.1 mg selenium/kg diet for the control diet and selenobroccoli diets, respectively. Mice consumed their diets for 10 weeks [11].

This study was approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center, and mice were maintained in accordance with the guidelines for the care and use of laboratory animals.

2.3. Preparation of total cellular RNA pools and nuclear protein pools

Mice were deprived of food overnight before killing and mouse liver nuclei were isolated as described [14]. Unless otherwise indicated, all operations were performed at 4°C. Briefly, equal amounts of each mouse liver from the same diet group (14 mice) were excised, washed with PBS and mixed as a “liver pool”. First, total cellular RNA pool was isolated from a “liver pool” by using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, and the integrity of RNA samples was checked by electrophoresis. Second, to prepare a nuclear protein pool, a “liver pool” was homogenized in 4 volumes of STM buffer (20 mM Hepes, pH 7.6, 250 mM sucrose, 5 mM $MgSO_4$, and 1 mM PMSF) using a Wheaton Dounce homogenizer. After filtering through cheesecloth, the homogenate was centrifuged at 750 x g for 10 min. The pellet was mixed with an equal volume of cushion buffer (20 mM Hepes, pH 7.6, 2 M sucrose, 10% glycerol, 15 mM KCl, and 1 mM PMSF). The mixture was loaded onto 15 ml of the cushion buffer and centrifuged at 25,401 x g for 75 min. The nuclear pellet was lysed for 30 min in nuclear extraction buffer (20 mM HEPES pH 7.6, 20% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothteitol, 0.1% Nonidet p-40 containing protease inhibitor cocktail [Sigma, St. Louis, MO]), and then centrifuged at 15,000 x g for 15 min. The supernatant was designated the nuclear fraction and kept at −80°C. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Labs, Hercules, CA) with various dilutions of bovine serum albumin (BSA) as standards. In addition, protein concentrations were confirmed by GelCode Blue protein staining on 10% SDS polyacrylamide gels (Pierce, Rockford, IL) (Fig. 1).

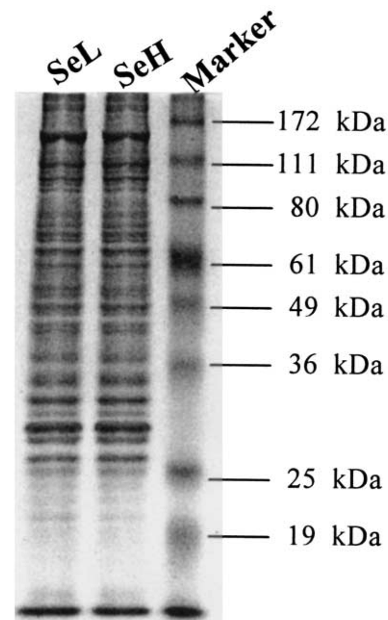

Fig. 1. Confirmation of Bio-Rad protein concentration assay. SeL,16 μg of the liver nuclear protein pool from 14 mice fed a control diet containing broccoli and SeH,16 μg of the liver nuclear protein pool from 14 mice fed selenium-enriched broccoli were loaded on 10% SDS polyacrylamide gels with GelCode Blue protein staining. Marker, protein ladder. The same protein staining-intensity of SeL and SeH was consistently seen in three experiments.

2.4. Gene array analysis

Each cDNA probe was prepared from 5 μg of a total cellular RNA pool, and hybridized to a mouse pathway finder-1 GEArray membrance (Superarray, Bethesda, MD). The membrane contain 23 sequence-verified known marker genes. After hybridization, each gene signal was normalized by 10% of the β-actin signal in the same membrane, and only those gene signals (~10% of the β-actin signal) that were well above background were considered specific gene signals. These data were collected, stored and analyzed with Molecular Dynamics Image-Quant system (Sunnyvale, CA).

2.5. Electrophoretic gel mobility shift assay

Gel shift experiments were performed as described previously [15]. Briefly, 4 μg of a nuclear protein pool was incubated with 25,000 cpm of ^{32}P-end-labeled doubled strand oligonucleotides individually (1~5 fmol) in a total volume of 20 μl for 20 min on ice and then 15 min at room temperature. Following incubation, the reaction mixture was electrophoresed through a 5% nondenaturing polyacrylamide gel at 4°C. The image signals representing the DNA-liver nuclear protein complex were quantified and analyzed

Table 1
Mouse pathway finder-1 GEArray analysis of the effects of selenium (Se)-enriched broccoli on gene expression in mouse liver

GenBank	Gene name	Se-enriched diet	Low-Se diet	Se-enriched/ Low-Se diet
		Signals 1000 × (intensity units/dot)		
(1) L22472	bax	1.788 ± 0.106	1.828 ± 0.054	0.978
(2) M20157	egr-1	1.306 ± 0.039	1.472 ± 0.219	0.887
(3) U41751	EI24	2.361 ± 0.156	3.295 ± 0.160	0.717
(4) L28177	gadd45	1.147 ± 0.034	0.811 ± 0.350	1.414
(5) X61753	hsf1	1.661 ± 0.261	1.723 ± 0.080	0.964
(6) J04633	hsp86	12.11 ± 0.294	8.230 ± 0.356	1.471
(7) U36277	iκBα	1.089 ± 0.017	0.570 ± 0.183	1.911
(8) M57999	NFκB	2.309 ± 0.089	1.396 ± 0.088	1.654
(9) U24173	$p21^{waf1}$	4.424 ± 0.187	4.138 ± 0.283	1.069
(10) U09968	$p27^{Kip1}$	4.190 ± 0.357	4.297 ± 0.126	0.975
(11) U22399	$p57^{Kip2}$	175.3 ± 0.276	188.0 ± 0.977	0.932

[1]Values are means ± SEM, n = 2.
[2]Signals were normalized with 10% of β-actin signals in the same membrane.
[3]bax, Bc12-associated X protein; egr-1, Mouse Egr-1 (Early growth response 1); EI24, mus musculus p53 responsive (EI24) mRNA; gadd45, DNA-damage inducible transcript 1; hsp86, mouse heat shock protein 86; iκBα, Mus musculus I-kappa β alpha chain; NFκB, nuclear factor of kappa light chain gene enhancer in B-cells; $p21^{waf1}$, cyclin-dependent kinase inhibitor p21Waf1; $p27^{Kip1}$, mouse cyclin-dependent kinase inhibitor p27 Kip1; $p57^{Kip2}$, cyclin-dependent kinase inhibitor p57Kip2.

with Molecular Dynamics Image-Quant system (Sunnyvale, CA). Sequences of the oligonucleotide probes used in above gel shift assays were as follows: AP-1 (activator protein-1), 5′-CGC TTG ATG AGT CAG CCG GAA-3′; NFκB (Nuclear factor of kappa light chain gene enhancer), 5′-AGC TTC AGA GGG GAT TTC CGA GAG-3′; p53 (protein53 tumor suppressor), 5′-GGA CAT GCC CGG GCA TGT CC-3′; SP-1 (nuclear factor of GC-rich DNA), 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; TCF (T-cell factor), 5′-CCC TTT GAT CTT ACC-3′; mutant TCF, 5′-CCC TTT GGC CTT ACC-3′.

Each probe was tested at least three times with the liver nuclear protein pools. Appropriate positive and negative controls were run in parallel to verify specificity of protein-DNA binding for each probe.

2.6. Statistical analysis

Results are given as means ± SEM. Student's *t*-tests for one sample were used to test whether % increase of DNA binding in selenium-enriched broccoli diet was significantly different from that in control diet. Differences with a P-value <0.05 were considered significant.

3. Results

To determine the effect of selenium-enrich broccoli on the expression of genes linked to apoptosis, we applied gene array and DNA mobility shift assay to define the transcriptional response in Min mouse liver. Through a side-by-side sample preparation, we had total cellular RNA pools and nuclear protein pools. Compared with low selenium control diet, selenium-enriched broccoli up-regulated the mRNA levels of ikBα (91%), NFκB (65%), hsp86 (47%) and gadd45 (41%) (Table 1). Furthermore, selenium-enriched broccoli significantly enhanced the binding of liver nuclear proteins to p53 (62%), NFκB (16.4%), and AP-1 (11.3%) DNA probes (Fig. 2). In contrast, there was little effect on SP-1 DNA binding (Fig. 2).

4. Discussion

Our previous studies showed that Min mice fed the selenium-enriched broccoli had fewer (p <0.02) small intestinal (46.4 ± 3.7 vs. 65.6 ± 6.1) and large intestinal (0.43 ± 0.17 vs. 1.93 ± 0.27) tumors than those fed the low selenium control diet [11]. Unlike other selenium deficient diet (0.01 mg Se/kg), our low selenium control diet (0.11 mg Se/kg) is closer to "real world" normal diet, and Min mice fed the selenium-enriched broccoli had only small increases in selenium concentration (72 ± 2 vs. 41 ± 3) (nmol/g) and glutathione peroxidase activity (2229 ± 110 vs. 1929 ± 133) (EU/mg protein) in the liver [11]. Therefore, the major stress for these mice is the spontaneous formation of numerous tumors but not the oxidative stress due to different diets. In current study, we examined the effect of selenium-enriched broccoli diet on differential gene expression in the exact same liver tissues of our previous experiments [11].

The changes of mRNA levels in this experiment suggest several important molecular roles of selenium-enriched broccoli in mouse liver. First, ikBα, NFκB are main mediators of the cellular response to a variety of extracellular stress stimuli. For example, NFκB mediates apoptosis

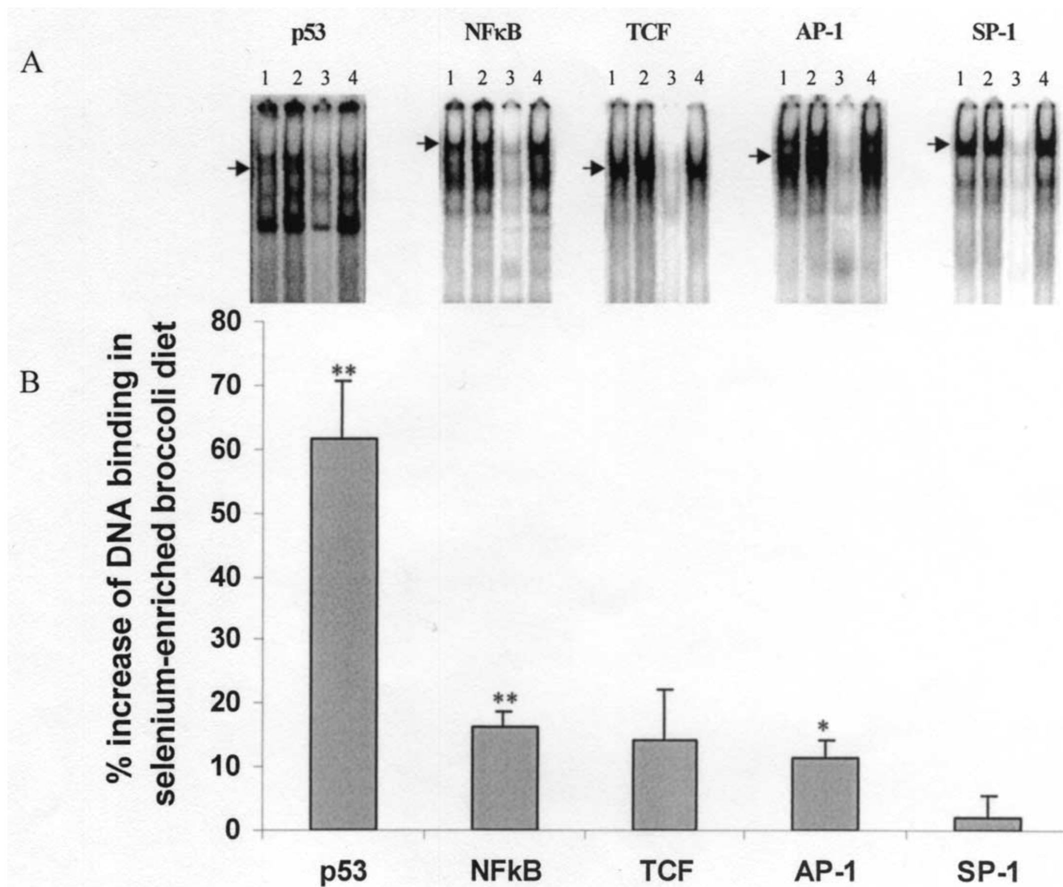

Fig. 2. Effect of the diets on liver nuclear protein DNA binding. *Panel A*: electrophoretic mobility shift assay of liver nuclear protein pool (4 μg) bound to equal amount of respective ^{32}P DNA probe (1~5 fmol). In each binding assay. lane 1 represents the binding activity of control diet; lane 2 represents the binding activity of selenium-enriched broccoli diet; lane 3 and 4 demonstrate the binding specificity. 100 fmol of the respective unlabeled DNA probe or mutant unlabeled TCF DNA probe was used to compete with ^{32}P DNA probe. respectively. The position of respective ^{32}P DNA probe and nuclear protein complex is indicated by the arrow. *Panel B*: The image signals representing the DNA-liver nuclear protein complex were quantified and analyzed with Molecular Dynamics Image-Quant system (Sunnyvale. CA). % increase = 100% x (signals of Se-enriched diet - signals of control diet)/signals of control diet. Values are means ± SEM. n = 3. **P <0.007. *P <0.02.

through transcriptional activation of Fas (CD95) in adenoviral hepatitis [16]. Similarly, the expression of hsp86 and gadd45 genes correlated with induction of apoptosis [17,18]. The up-regulation of ikBα, NFκB, hsp86, gadd45 suggests that selenium-enriched broccoli diet can activate pro-apoptotic gene expression in response to tumorigenic stress. Second, the change with differential gene expression <2.0 is moderate, which is consistent with the observation that compared with mice fed the low selenium control diet, selenium-enriched broccoli diet reduced the number of tumors only by 30% [11]. Although the gene array is a valuable tool, the signals of some critical tumor suppressor genes such as p53 gene were too weak to be specifically detected in the array. Because DNA mobility shift assay is another approach to study gene expression, we then evaluated the binding of liver nuclear proteins to ^{32}P-labeled probes corresponding to several different transcription factors. The transcription factor p53, TCF, AP-1 and NFκB were chosen because their DNA recognition sites are present in the regulatory regions of many inducible genes. The transcription factor p53 is a central molecule in the control of apoptosis induced by loss of cellular integrity [19]. The up-regulation of TCF and AP-1 gene activities has been proposed to mediate the apoptotic response to cellular stress [20,21]. In contrast, SP-1 transcription factor bound to GC-rich elements is ubiquitously expressed in mammalian tissue and not a highly inducible transcription factor which could serve as internal control in the assay [22]. In the current study, the transcription factor p53, AP-1 and NFκB displayed an increase in DNA binding in those mice fed the selenium-enriched broccoli diet. Because these are pro-apoptopic proteins, the increase in DNA binding may alter the expression of a wide spectrum of their target genes. Although the increase in each gene is moderate, they are likely to interact with each other and have a "group effect" on multiple signal pathways associated with apoptosis. Our findings are consistent with the observation that apoptosis triggered by selenium has been implicated as an important mechanism for anticancer effects [23].

Taken as a whole, our results suggest that selenium-

enriched diet not only can increase mRNA levels of pro-apoptotic genes (ikBα,NFκB, hsp86, gadd45) but also can enhance the DNA binding of pro-apoptotic transcription factors (p53, AP-1 and NFκB). This response may due to the stress of spontaneous formation of numerous tumors, and our data demonstrated for the first time that mice fed the selenium-enriched broccoli had stronger apoptotic ability in response to "danger signals" such as tumorigenesis.

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