Suppression of Colorectal Oncogenesis by Selenium-Enriched Milk Proteins: Apoptosis and *K-ras* Mutations

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Abstract

The chemical form and bioavailability of dietary selenium may influence its protectiveness against colorectal cancer. Selenium is readily incorporated into milk proteins by feeding cows with selenized-yeast. This study examined whether a dairy source of organic selenium (as milk proteins) is more effective than a yeast source at inhibiting oncogenesis in carcinogen-treated mice and whether it regulates the homeostatic response to carcinogen-induced DNA damage. Dietary interventions are as follows: selenium-enriched milk protein isolate (Tatura-Bio Se; 0.5 or 1 ppm selenium) or milk protein control and selenized-yeast (Sel-Plex; 1 or 4 ppm selenium) with casein or casein alone as control. After 4 weeks on diet, mice received a single azoxymethane (10 mg/kg) injection to induce mutations and were killed 6 hours later. Measures were as follows: plasma selenium, cell proliferation, and acute apoptotic response to azoxymethane (AARGC). Separate groups of mice on the same diets were given 4 weekly azoxymethane (15 mg/kg) injections to induce oncogenesis. Mice were killed 6 or 30 weeks after the last azoxymethane injection. Measures were as follows: aberrant crypt foci (ACF), cancers, and *K-ras* mutations. Dairy-selenium at 1 ppm significantly suppressed ACF and cancers, whereas yeast-selenium at an equivalent selenium intake had no effect. Dairy-selenium significantly increased plasma selenium levels and AARGC, and reduced cell proliferation and frequency of *K-ras* mutations in ACF relative to an equivalent dose of selenium from yeast. Selenium-enriched milk protein isolate is superior to selenized-yeast in terms of its bioavailability and capacity to suppress oncogenesis. Suppression may be a consequence of enhanced apoptotic deletion of azoxymethane-induced DNA lesions and the subsequent reduction in frequency of *K-ras* mutations. [Cancer Res 2008;68(12):4936–44]

Introduction

Epidemiologic data suggest that people could reduce their cancer risk through dietary change or supplementation with specific micronutrients (1, 2). Colorectal cancer (CRC) is the third most frequent cause of cancer mortality in the world. Chemoprevention represents an important and potentially feasible option for control of this cancer. Selenium, an essential trace element, is required for a number of metabolically important enzymes (3). It was first mentioned as a possible protective agent against human cancer >30 years ago (4). Because then, human epidemiologic studies have indicated a significant inverse relationship between intake of selenium and risk of cancer overall, including CRC (5). Selenium has also been extensively investigated in laboratory studies. Selenium was shown to exhibit inhibitory effects in various tumor cell lines as well as in chemically induced tumors in animals (6).

The selenium concentration in foods varies significantly due to variability in concentration and availability as inorganic salts from soil (7). People living in regions of low selenium intake have relatively higher rates of cancers (8). Improving dietary intake of selenium through selenium-enriched food sources is one strategy for increasing human selenium intake and, perhaps, reducing cancer risk (9). Selenium-enriched plant foods have been shown to significantly protect against CRC in animal models (10, 11). Availability of selenium, however, does depend not only on the dose of selenium, but also the form in which it is administered.

Like most foods, dairy products are not normally considered to be a significant source of selenium. However, feeding organic selenium to cows results in effective and rapid incorporation into milk proteins (12–14). Such a source might be advantageous.

Several potential anticarcinogenic mechanisms have been proposed for selenium, including induction of apoptosis (15), protection from oxidative DNA damage, and improved immune function. In this study, an azoxymethane-induced CRC animal model has been used because this model is characterized by many of the clinical, pathologic, and molecular features of human CRC (16, 17). Azoxymethane causes O^6-methylguanine (O^6-MeG) adduct (18); if O^6-MeG is not repaired by DNA repair enzymes or removed by apoptosis, it may lead to G to A transition, resulting in irreversible mutations in the oncogene *K-ras* (19). Colorectal oncogenesis involves aberrant crypt foci (ACF), which are the earliest identifiable focal preneoplastic lesions of the adenoma-carcinoma sequence, and *K-ras* mutations are early molecular events found in ACF (20, 21). As such, *K-ras* mutations and ACF serve as biomarkers for determining CRC risk. In our previous studies, we showed that administration of azoxymethane to rodents was rapidly followed 6 to 8 hours later by an acute apoptotic response (termed AARGC) in the distal colon. Protection against CRC by some dietary agents and drugs is associated with their ability to up-regulate AARGC for controlling DNA damage (22–27).

In this study, we compared a selenium-enriched milk protein isolate with a selenized-yeast for their relative bioavailabilities at a comparable selenium concentration and effects on azoxymethane-induced oncogenesis. We also compared the two selenium sources for their effects on glutathione peroxidase (GPx) activity, epithelial AARGC and proliferation, and frequency of *K-ras* mutations in resultant ACF.

Materials and Methods

Reagents. Azoxymethane was purchased from Sigma (Australia). Milk protein isolate (control, with no supplemental selenium to cows) and

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selenium-enriched milk protein isolate (Tatura-Bio Se; 85% protein, 5 ppm selenium) were provided by Tatura Milk Industries. The milk protein isolate was prepared from milk, after lipid separation, using ultrafiltration separation with a pore size of 10 kDa. Selenium yeast (Sel-Plex; 1,800 μg selenium per gram dry weight) was provided by Alltech Biotechnology P/L.

Animals. A total of 300 *wild-type* male mice of C57BL/6J strain were obtained from the Animal Resource Centre, Adelaide University, Australia. Animal protocols were approved by the Animal Welfare Committee at Flinders University. Three experiments were performed, 60 mice were used for a short-term experiment (4-wk homeostatic response to DNA damage), 60 mice for a 12-wk ACF experiment, and 180 mice for a long-term (36 wk) tumor study. For each experiment, animals were divided randomly into 5 or 6 equal experimental groups (with comparable initial body weights), housed in cages (four per cage), and maintained in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle at 22 ± 2°C temperature and 80 ± 10% humidity. Animals were given free access to water, weighed weekly, and were monitored closely for clinical signs of ill health throughout the study. Animals appearing sick were euthanased immediately.

Diets. The experimental diets fed to the animals were based on a modified form of AIN-76A diet for rodents (28). It contained 19% sunflower oil and 20% protein, and L-methionine was not added. Newmark and colleagues (29) have shown this New Western-style Diet to be associated with "spontaneous" tumor development in C57BL/6 mice. Calcium was not included in the mineral mix of milk proteins control (MP-Cont), milk proteins 0.5 ppm selenium diet (MPSe0.5), and milk proteins 1 ppm selenium diet (MPSe1) because the dairy protein sources have relatively high calcium concentrations, but calcium was added as $CaHPO_4$ to balance calcium level (0.46%) in casein control (Cas-Cont), selenium yeast 1 ppm selenium diet (CasYSe1), and selenium yeast 4 ppm selenium diet (CasYSe4). Two control diets were used depending on the protein source, either milk proteins or casein. They both contained relatively low levels of selenium, 0.34 ppm selenium in milk proteins, and 0.29 ppm selenium in casein. Further details of diets are provided in Table 1.

Dietary selenium and homeostatic response to azoxymethane-induced DNA damage. Ten-week-old mice were assigned to each of 5 diet (n = 12 per group). After 4 wk on diet, animals were given a single azoxymethane injection (10 mg/kg s.c.) to induce DNA damage and AARGC. Six hours later, animals were killed by cardiac puncture after ketamine/xylazine anesthesia, this being the time of maximal apoptotic response to azoxymethane in the rodent (24). Two centimeters of distal colon was removed, placed in 10% paraformaldehyde, and embedded in paraffin for histologic and immunohistologic examination. Liver and the remaining colon were fresh frozen immediately in liquid nitrogen and stored at −80°C.

Dietary selenium and azoxymethane-induced ACF formation. Four-week-old mice were assigned to each of 5 diet (n = 12 per group). After 2 wk on diet, animals received azoxymethane injections (15 mg/kg s.c.) weekly for 4 successive wk. All animals remained on the same diet throughout the study until killed 6 wk after the last azoxymethane injection. Colons were removed, opened longitudinally, and fixed flat on hibond C paper in 10% paraformaldehyde. For *K-ras* mutation analysis, ACF were microdissected and trimmed to remove adjacent normal-appearing crypts. A distal segment of colon was taken from animals (n = 6 per group) for assessment of baseline apoptosis and cell proliferation.

Dietary selenium and azoxymethane-induced colon tumor formation. Four-week-old mice were assigned to each of 6 diet (n = 30 per group). After 2 wk on diet, animals received 4 weekly azoxymethane injections (15 mg/kg s.c.). All animals remained on the same diet throughout the study until killed 30 wk after the last azoxymethane injection. Colons were removed, opened, and fixed as described for ACF.

Preparation of blood and analysis of plasma selenium status. Blood samples were obtained by direct heart puncture into heparinized vacutainer tubes. Selenium concentration in the plasma was determined using the fluorometric assay described by McIntosh and colleagues (30).

Assay of GPx activity. GPx activity was measured using a Glutathione Peroxidase Cellular Activity Assay kit (Sigma). Samples were homogenized in a buffer containing 1 mol/L Tris (pH 7.6), 0.5 mol/L EDTA, and centrifuged at 10,000 rpm for 20 min at 4°C. The protein concentration was quantified using the Protein Assay kit (Bio-Rad). GPx activity was determined in duplicate using 2.5 to 5 μL of the supernatant (15–30 μg of proteins), assayed in a 100 μL reaction volume containing 5 mmol/L

Table 1. Composition of experimental diets (grams per 100 g diet)

Ingredient	MP-Cont	MPSe0.5	MPSe1	Cas-Cont	CasYSe1	CasYSe4
	Control-milk protein	Tatura-Bio Se selenium at 0.5 ppm	Tatura-Bio Se selenium at 1 ppm	Control-casein	Sel-Plex selenium at 1 ppm	Sel-Plex selenium at 4 ppm
Casein*	0	0	0	20	20	20
Milk protein*	20	10	0	0	0	0
Tatura-Bio Se*,†	0	10	20	0	0	0
Sucrose	20	20	20	20	20	20
Corn starch	31.3	31.3	31.3	30.88	30.78	30.48
Fiber (α cell)	5	5	5	5	5	5
Sunflower oil	19	19	19	19	19	19
Choline	0.2	0.2	0.2	0.2	0.2	0.2
Mineral mix	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1	1	1
Sel-Plex†	0	0	0	0	0.1	0.4
CaHPO4‡	0	0	0	0.42	0.42	0.42

NOTE: The experimental diets consisted of a modified AIN-76A diet achieved by adding 19% sunflower oil and 20% protein. DL-methionine was not added as explained in Materials and Methods section.

*Milk protein isolate, Tatura-Bio®Se was used as source of protein for diets MP-Cont, MPSe0.5 and MPSe1 and casein used for diets Cas-Cont, CasYSe1 and CasYSe4.

† Tatura-Bio Se and Sel-Plex were used as source of selenium for diets MPSe0.5 and MPSe1, and diets CasYSe1 and CasYSe4.

‡ CaHPO4 was added to Cas-cont, CasYSe1, and CasYSe4 to balance the calcium concentration (0.46%). Calcium was not included in the mineral mix of diets MP-Cont, MPSe0.5, and MPSe1 because the milk proteins have relatively high calcium concentrations.

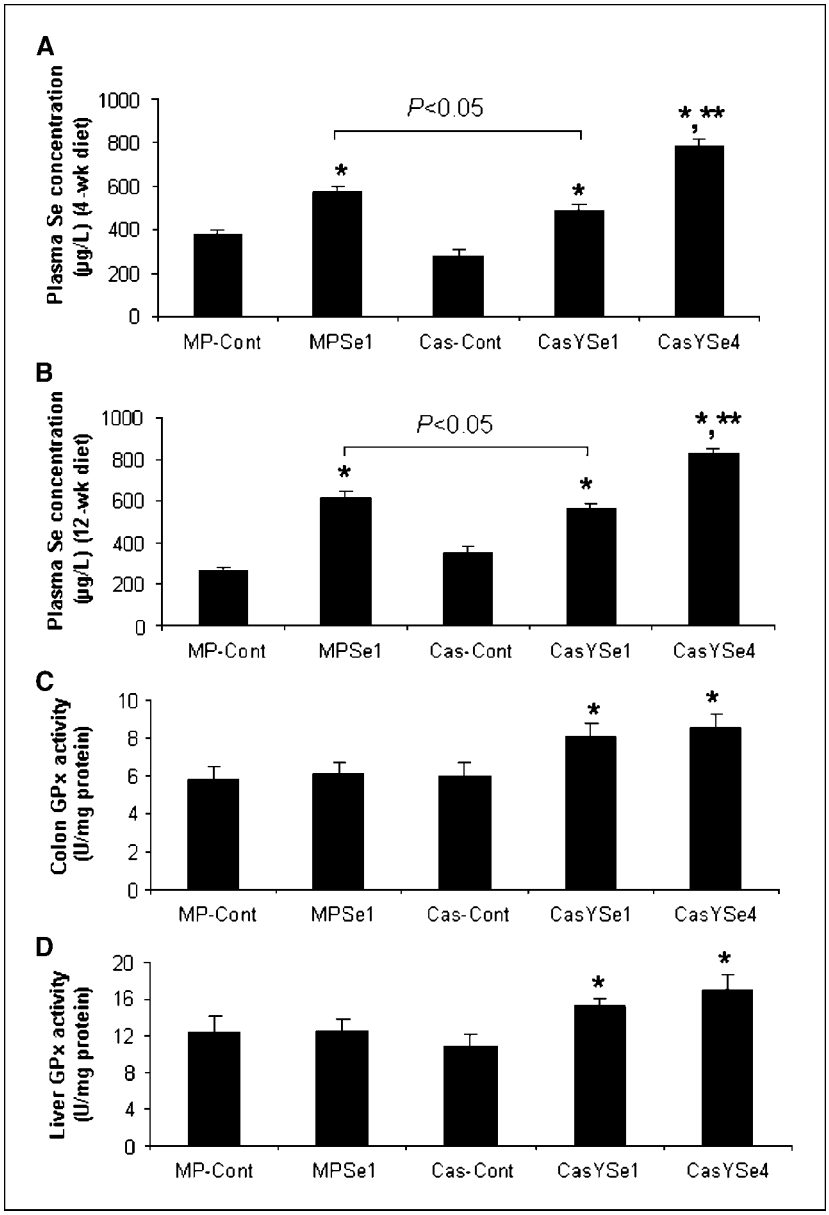

Figure 1. Effects of different selenium-containing diets on plasma selenium concentration for experiment 1 (4 wk on diet; *A*) and for experiment 2 (12 wk on diet; *B*), and on GPx activity for colon (*C*) and liver (*D*) each after 4 wk on diet. Selenium intake as MPSe1, 1 ppm selenium-enriched milk protein isolate, or CasYSe1 or CasYSe4, 1 or 4 ppm selenized-yeast, showed significantly increased plasma selenium concentrations in both experiments. A significant dose-dependent effect was seen with the yeast source. Plasma selenium concentration in mice fed MPSe1 were significantly higher than those fed equivalent selenium in CasYSe1 ($P < 0.05$). Selenium intake as 1 and 4 ppm selenized-yeast significantly increased colon and liver GPx activity, but maximal effect was reached with 1 ppm. Selenium as MPSe1, however, had no significant effect on GPx activity. *Columns,* mean; *bars,* SE. $n = 12$. *, $P < 0.05$ compared with their respective control diets (*MP-Cont* or *Cas-Cont*); **, $P < 0.05$ compared with 1 ppm selenized-yeast.

NADPH, 30 mmol/L H_2O_2, and 42 mmol/L reduced glutathione. The oxidation of NADPH to NADP was monitored at 340 nm on UV/Vis spectrophotometer.

Measurement of apoptosis. The frequency of epithelial cells undergoing apoptosis was determined by hematoxylin staining as previously described in detail by us and validated relative to terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling staining (24). Twenty crypts from distal colonic segments were chosen and assessed by an independent observer unaware of the dietary treatments. The apoptotic index was calculated as the number of apoptotic cells per crypt column divided by the total number of cells in the column and multiplied by 100.

Measurement of epithelial proliferation. Proliferative activity of epithelial cells was measured using immunohistochemical staining with Ki-67 monoclonal antibody (Santa Cruz). In brief, antigen retrieval was carried out by heating sections in 0.1 mol/L citrate buffer for 1 h. Sections were incubated with Ki-67 antibody (1:1,000) overnight after incubation in 3% H_2O_2 for 20 min. Detection was by biotinylated secondary rabbit-anti-mouse antibody (1:200; Dako) for 30 min and avidin/biotinylated peroxidase complex (Signet Laboratories) for 20 min. Slides were visualized by incubating with 3′-diaminobenzamine substrate. The scoring for cell proliferation was the same as the method used to score apoptosis.

Measurement of *K-ras* mutations. DNA was extracted using DNeasy Tissue kit (Qiagen, Inc.). Mutations at codon 12 of *K-ras* gene was analyzed by locked nucleic acid (LNA)-mediated real-time PCR clumping. This is a sensitive new method for detecting point mutations, a modification based on methods described previously in human studies (31). Briefly, a set of primers (forward, 5′-AGGCCTGCTGAAAATGACTG-3′; reverse, 5′-GGTAC-TTCTATCGTAGGGTCGTAC-3′) was chosen to amplify a 121-bp genomic fragment from exon 1; a *wild-type* LNA oligomer (CCTACGCCACCAGCTCC-PH), which covered codons 10 to 14, was used to suppress the amplification of *wild-type* DNA; and two 5′-Cy5–labeled probes were used to enhance the expression of specific mutant sequence, with 12Asp probe (Cy5-TTGCCTACGCCA*T*CAGCTCCAA-PH) for GAT mutation and 12Cys (Cy5-TTGCCTACGCCAC*A*AGCTCCAA-PH) for TGT mutation. Anchor probe was 3′-labeled with fluorescence (CATCCACAAAGTGATTCTGAATTAGCTG-TATCGTCAAGGCGCT-FL).

The primers, probes, and LNA oligomer were purchased from TIB MOLBIOL.

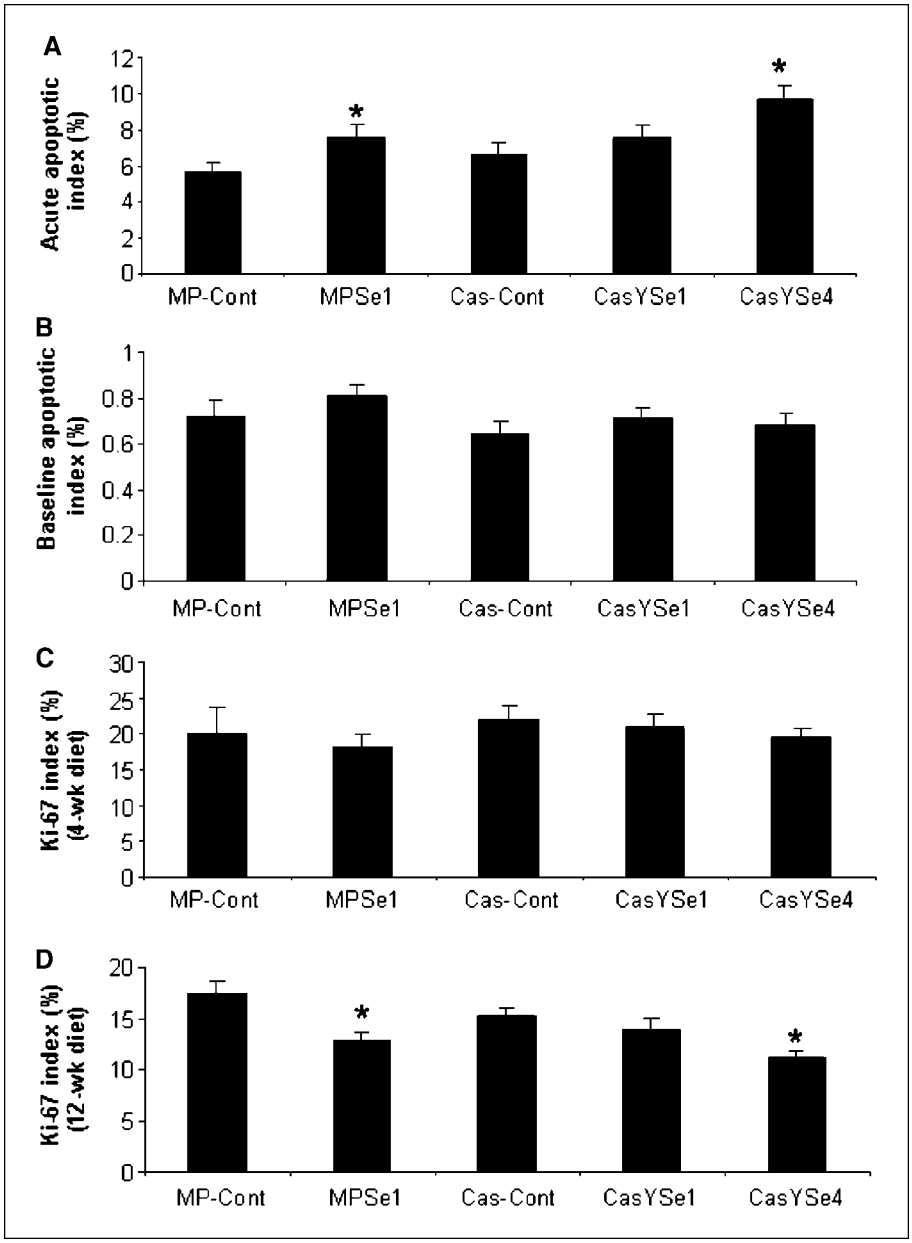

Figure 2. Effects of different selenium-containing diets on colonic epithelial acute apoptotic response to genotoxic carcinogen (AARGC; *A*), baseline epithelial apoptosis (*B*), and cell proliferation for experiment 1 (4 wk on diet; *C*) and experiment 2 (12 wk on diet; *D*). Selenium intake as MPSe1, 1 ppm selenium-enriched milk protein isolate, and CasYSe4, 4 ppm selenized-yeast, significantly increased apoptotic index (AARGC), whereas they did not significantly affect baseline apoptosis in colonic epithelial cells. Cell proliferation (Ki-67 labeling index) was not significantly affected by dietary selenium intake after 4 wk on the diets. However, MPSe1 and CasYSe4 significantly reduced Ki-67 labeling index after 12 wk on diets. *Columns,* mean; *bars,* SE; $n = 12$. *, $P < 0.05$ compared with their respective control diets (*MP-Cont* or *Cas-Cont*).

For PCR, 3 mmol/L $MgCl_2$, 0.2 μmol/L sense and anchor probes, 0.005 μmol/L LNA, and 0.5 μmol/L forward and 0.1 μmol/L reverse primer with 20 ng of genomic DNA were added to make a final volume of 20 μL per capillary. PCR was performed on a Rotor Gene 3000 (Corbert Research) with acquisition at 470 nm/660 hp (FAM/Cy5FRET). The running conditions were as follows: 10 min of initial denaturation at 95°C, 45 cycles of 20 s at 95°C for denaturation, 45 s for primer annealing and probe binding at 58°C, and 20 s at 72°C for extension. Fluorescence was acquired at 58°C. After holding the temperature at 95°C for 20 s and at 40°C for 20 s, melting curve was analyzed by increasing the temperature from 40°C to 85°C with a slope of 0.3°C per second. Mutation was confirmed by DNA sequencing using Big Dye Termination Cycle Sequencing Ready Reaction kit (Applied Biosystems). Twenty-four to thirty ACF (small and large) were randomly selected from each group and analyzed for *K-ras* mutations.

Detection of ACF. The colons were stained with 0.1% methylene blue and counted at ×40 magnification using a dissecting microscope. ACF were identified by increased size, elevated appearance from the surrounding mucosa, and the slit-like shape of the luminal opening and scored from the distal to proximal end without knowledge of the dietary treatment. Crypt multiplicity was defined as the number of crypts in each focus, categorized as small (1–3 crypts per focus) and large ACF (4 or more crypts per focus).

Detection of colon tumors. The colons were scored for macroscopically visible tumors. Tumors were dissected, embedded in paraffin, and stained by H&E for histopathologic evaluation to classify them as adenoma and adenocarcinoma (23). Endpoints were colon tumor incidence (i.e., proportion of mice affected by a tumor), tumor multiplicity, tumor size, and number of mice with adenocarcinomas (cancers). Tumor size was calculated by the formula: $\log10\ [\Sigma(\pi(\text{diameter 1} + \text{diameter 2}))^2/2]$ (32).

Statistical analyses. Statistical analyses were performed using SPSS for Windows, version 14.0 (SPSS, Inc.) and Stata version 10.0 (StataCorp). Data were expressed as mean ±SE. Between-group comparisons of apoptotic index, Ki-67 index, plasma selenium concentration, and GPx activity were assessed using one-way ANOVA with correction for multiple comparisons by Tukey's *post hoc* test. Between-group comparisons of *K-ras,* ACF counts and tumor measures were assessed using logistic and Poisson regression, respectively, with simple linear contrasts. Comparison of selenium at

equivalent concentrations (1 ppm) on tumor end points was further assessed by two-way factorial Poisson models with an interaction between selenium sources and protein type. Differences between groups were considered significant when P value was <0.05.

Results

General observation. Dietary selenium supplementation either as selenium-enriched milk protein isolate or selenized-yeast had no significant effect on the animals during 4, 12, or 36 weeks of feeding, at concentrations of 0.5, 1, or 4 ppm throughout the studies. The body weights of animals fed selenium-containing diets were comparable with those of the control diets (data not shown). Twelve sick mice were killed before the termination of tumor study; tumor end points from these mice were not included.

Effects of dietary selenium intake on plasma selenium status. Plasma selenium concentration reflected dietary selenium supplementation; it was lower in animals fed the control diets than in animals fed the selenium-containing diets. Four weeks of feeding of MPSe1 as 1 ppm selenium-enriched milk protein isolate, or CasYSe1 or CasYSe4 as 1 or 4 ppm selenized-yeast significantly increased plasma selenium concentrations compared with their respective control diets (MP-Cont or Cas-Cont), with selenized-yeast showing a significant dose-dependent effect (Fig. 1*A*). Importantly, plasma selenium concentration in animals fed MPSe1 was significantly higher than those fed a selenium-equivalent CasYSe1. Twelve weeks of selenium feeding had a similar effect to that of short-term selenium feeding (4-week) on plasma selenium concentrations (Fig. 1*B*).

Effects of dietary selenium intake on GPx activity. GPx activities in the colon and liver were significantly increased in animals fed yeast-selenium diets, but the level in those fed CasYSe4 was not significant higher than in those fed CasYSe1 (Fig. 1*C* and *D*). Our observations suggest GPx activity is maximized when feeding 1 ppm selenized-yeast. In contrast, GPx activity were not different in animals fed MPSe1 compared with animals fed MP-Cont.

Effects of dietary selenium intake on apoptosis. AARGC was significantly increased in the distal colon of animals fed MPSe1, compared with those of MP-Cont (Fig. 2*A*). AARGC was also significantly increased in animals fed CasYSe4 relative to those of Cas-Cont. However, although animals that were fed CasYSe1 showed a trend to increased AARGC, it was not significant compared with animals fed Cas-Cont.

Although dietary selenium intake as MPSe1 or CasYSe4 significantly increased AARGC in the short-term study, it did not affect the baseline apoptosis rate in normal epithelium in experiment 2 (Fig. 2*B*).

Effects of dietary selenium intake on colonic epithelial proliferation. In the short-term study, Ki-67 index in colonic crypts was not significantly affected (Fig. 2*C*). However, significant inhibition of proliferation was seen after 12 weeks of feeding MPSe1 or CasYSe 4 (Fig. 2*D*), compared with their respective control diets (MP-Cont or Cas-Cont).

Effect of dietary selenium intake on ACF formation. ACF were observed predominantly in the distal colon. Results are shown in Table 2 for ACF number, crypt multiplicity, and the occasional macroscopic tumor. Animals fed MPSe1 had significantly lower numbers of small, large, and total ACF, compared with animals fed MP-Cont. There was no significant difference in ACF formation between the animals fed CasYSe1 and those fed Cas-Cont. However, CasYSe4 significantly suppressed the number of large ACF but not that of small ACF or total ACF regardless of size. Importantly, MPSe1 significantly reduced all measures of ACF compared with CasYSe1.

Although ACF was the main end point in this study, macroscopic tumors were also observed in several animals fed control diets (MP-Cont or Cas-Cont) and yeast-selenium diets (CasYSe1 or CasYSe4), but no macroscopic tumors were observed in animals fed dairy-selenium diet (MPSe1). Statistical analysis was not undertaken for tumor data alone due to the small numbers; however, statistical analysis was performed after pooling large ACF

Table 2. Effect of dietary selenium intake on ACF, macroscopic tumor formation, and *K-ras* mutations in azoxymethane-treated C57BL/6J mice studied 6 wk after the last injection of azoxymethane

Diet	Selenium in diet (ppm)	Mice (n)	Total ACF per mouse* (mean ± SE)	Small ACF per mouse† (mean ± SE)	Large ACF/per mouse‡ (mean ± SE)	Total No of tumors§ (macroscopic lesions)	Total no of large ACF and tumors	No of ACF selected	Frequency of *K-ras* mutations
MP-Cont	0.07	10	11.6 ± 1.29	7.9 ± 0.91	3.7 ± 0.68	3	36	30	11/30 (37%)
MPSe1	1	12	5.3 ± 0.79‖	3.8 ± 0.78‖	1.5 ± 0.26‖	0	17‖	29	4/29 (14%)‖
Cas-Cont	0.06	12	8.9 ± 1.27	6.2 ± 1.12	2.7 ± 0.43	2	32	26	8/26 (31%)
CasYSe1	1	12	8.7 ± 1.1¶	6.3 ± 0.98¶	2.4 ± 0.47¶	1	32¶	24	7/24 (29%)
CasYSe4	4	12	8.3 ± 0.75	7.5 ± 0.65	0.8 ± 0.27**	1	11**	27	5/27 (19%)

NOTE: ACF study was undertaken by feeding animals with 5 experimental diets (12 mice per group), containing either control diets or selenium from dairy or yeast sources. Animals received 4 weekly azoxymethane (15 mg/kg) injections to induce ACF and were killed 6 wk later. All statistically significant differences are identified.

*Total number of ACF was calculated as the sum of the small and large ACF.

† Small ACF was classified by the number of aberrant crypts per focus (1–3).

‡ Large ACF was classified by the number of aberrant crypts per focus (≥4).

§ No statistical analysis undertaken for macroscopic tumors alone due to the small numbers.

‖ $P < 0.05$, compared with MP-Cont.

¶ $P < 0.05$, compared with MPSe1.

** $P < 0.05$, compared with Cas-Cont.

and tumors (Table 2), and statistically significant differences remained as observed for large ACF alone.

Effect of dietary selenium intake on *K-ras* mutation. The effect of selenium intake on *K-ras* mutation in ACF is summarized in Table 2. Real-time PCR with LNA and specific fluorescent-labeled probes enabled identification of the *K-ras* mutations from the *wild-type* genome through different melting peaks. The melting temperature was 67°C ± 0.29°C for *wild-type* DNA, 72.5°C ± 0.42°C for TGT mutation, and 73°C ± 0.35°C for TGT mutation (Fig. 3). In this study, both G to A transition and G to T transversion mutations were detected in ACF, which differs from reports that only G-A transition mutation are detected in mouse (33–35). *K-ras* mutations were significantly reduced in animals fed MPSe1 but not in animals fed CasYSe1 and CasYSe4.

Effect of dietary selenium intake on tumor and cancer formation. The effect of selenium on macroscopic tumors (adenomas and adenocarcinomas) in the longest-term study was similar to that on ACF. Results summarized in Table 3 show animals fed MPSe1 had significantly lower colon tumor incidence, tumor multiplicity, and tumor size, compared with animals fed MP-Cont or CasYSe1. The percentage inhibition of tumor incidence, multiplicity, and size was 65.5%, 77.6%, and 73.2% versus MP-Cont, and 67.3%, 82.4%, and 77.3% versus CasYSe1. Those fed dairy-selenium diet at selenium 0.5 ppm (MPSe0.5) also showed a trend to protection relative to MP-Cont. Despite protection against large ACF formation, CasYSe4 did not significantly affect tumor incidence, multiplicity, or size, compared with animals fed Cas-Cont, nor did CasYSe1.

Comparison of MPSe1 and CasSe1 on tumor end points using two-way factorial Poisson models with an interaction between selenium sources, and protein type showed that the dairy-selenium significantly reduced tumor incidence (odds ratio, 0.291; $P = 0.048$) and tumor multiplicity (odds ratio, 0.267; $P = 0.021$) with a trend to reduced tumor size ($P = 0.065$). Protein type was not significant in this analysis, although there was a significant interaction between selenium and milk proteins with tumor multiplicity as the end point.

Analysis of the effect of selenium-containing diets on cancer formation specifically was performed after histopathologic examination of tumors (Table 3). Results trended in the same direction as for all macroscopic tumors regardless of whether or not invasion was present; for instance, cancer incidence fell from 19.2% or 22.2% in animals fed MP-Cont or CasYSe1 to 3.3% in those fed MPSe1, but numbers were small and the results did not reach significance.

Discussion

The results of this study provide evidence that a dairy source of selenium (Tatura-Bio Se) is effective in enhancing AARGC, and in reducing azoxymethane-induced ACF, cancers and frequency of *K-ras* mutations. Our results are consistent with previously

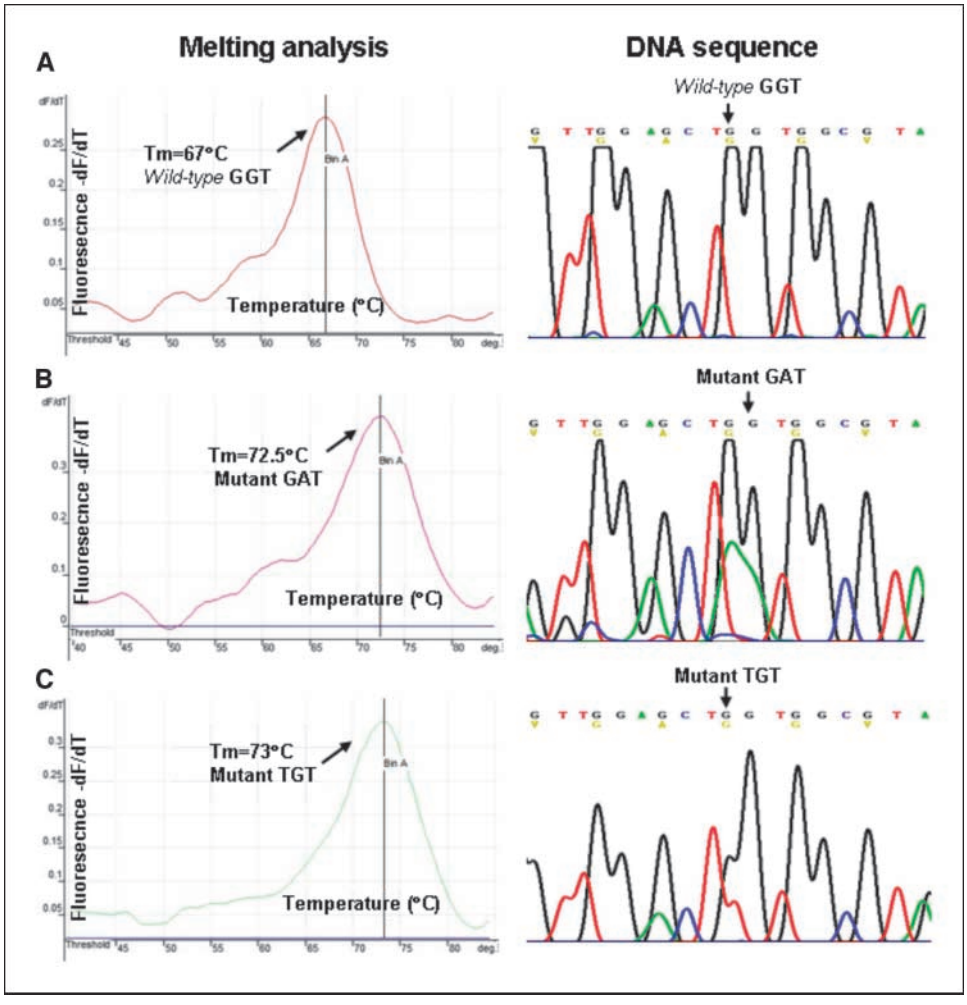

Figure 3. Detection of *K-ras* mutations in codon 12 by LNA-mediated real-time PCR clamping. In melting curve analysis, *wild-type* and mutant *K-ras* can be discriminated through different melting peaks at 67°C ± 0.29°C (*A, wild-type*), 72.5°C ± 0.42°C (*B,* GAT mutation), and 73°C ± 0.35°C (*C,* TGT mutation). Sequence analysis of w*ild-type* DNA (*A*), and mutant DNA; GAT (*B*) and TGT (*C*) confirms the nature of the mutations.

Table 3. Effect of dietary selenium intake on tumor formation (i.e., adenoma plus cancer) and on cancer formation alone in azoxymethane-treated C57BL/6J mice studied 30 wk after the last injection of azoxymethane

Diet	Selenium in diet (ppm)	Incidence (no of mice with tumors)	Incidence (no of mice with cancers)	Multiplicity (no of tumors per mouse; mean ± SE)	Tumor size index (mean ± SE)
MP-Cont	0.07	10/26 (38.5%)	5/26 (19.2%)	0.58 ± 0.17	0.67 ± 0.17
MPSe0.5	0.5	7/30 (23.3%)	4/30 (13.3%)	0.33 ± 0.12	0.41 ± 0.14
MPSe1	1	4/30 (13.3%)*	1/30 (3.3%)	0.13 ± 0.06*	0.18 ± 0.09*
Cas-Cont	0.06	12/25 (48%)	8/25 (32%)	0.72 ± 0.20	0.92 ± 0.21
CasYSe1	1	11/27 (40.7%) †	6/27 (22.2%)	0.74 ± 0.23 †	0.79 ± 0.21 †
CasYSe4	4	11/30 (36.7%)	7/30 (23.3%)	0.47 ± 0.12	0.61 ± 0.15

NOTE: Tumor study was undertaken by feeding animals with 6 different diets (30 mice per group), containing either control diets or selenium from dairy or yeast sources. Animals received 4 weekly azoxymethane (15 mg/kg) injections to induce tumor formation and killed 30 wk later. All statistically significant differences are identified.

*$P < 0.05$, compared with MP-Cont.

†$P < 0.05$, compared with MPSe1.

reported evidence that higher selenium intakes provide greater protection against colon tumor formation, but they also show that efficacy depends on the dietary source of selenium. Furthermore, the effect on each of these end points is significantly greater than with an equivalent selenium dose when fed as selenized-yeast (Sel-Plex). As the dairy-selenium at an equivalent dose, 1 ppm, achieved a significantly higher plasma selenium concentration than the yeast-selenium, the greater anticarcinogenic effect is likely to be due, at least in part, to higher selenium bioavailability but also perhaps to differences between the sources in the nature of their selenium compounds.

Selenium-enriched milk protein isolate was provided at 1 ppm in the diet so as to maintain a constant dietary protein content of 20%. We found that MPSe1 not only inhibited development of all sizes and total numbers of ACF but also significantly suppressed all measures (incidence, multiplicity, and size) of tumor formation including adenocarcinomas (cancers); selenized-yeast at an equivalent amount (CasYSe1) did not. A higher dose of CasYSe4 significantly inhibited formation of large ACF but not other measures of ACF or any measure of tumor formation including cancer. Given dairy-selenium at 1 ppm significantly suppressed ACF, we also used it at 0.5 ppm selenium in the tumor study. A trend to protection was seen at 0.5 ppm indicating a proportionate reduction in tumors. Significant reductions in tumors and cancers were seen at 1 ppm, which indicates the highly effective effect of this dose (10-fold above control) and form. A recent selenium/colon cancer study in rats observed a 45% reduction in tumor burden when they were fed 8 ppm selenium as yeast, but at that level of selenium intake, side effects were observed, including an 11% weight loss (30). Earlier studies in rodent models of CRC showed that selenium in inorganic form (selenite) was effective at suppressing oncogenesis in concentrations ranging from 1 to 4 ppm (36). Selenium-enriched broccoli or wheat at 2 ppm selenium were also effective at reducing ACF (37). Our study indicates that selenium provided as selenium-enriched milk protein isolate is highly effective at the lowest dose in this range. As such, it seems likely to be a highly effective source of selenium for cancer prevention.

Possible chemopreventive mechanisms of selenium include effects on some key cellular events of oncogenesis, such as apoptosis and/or cell proliferation (15). It has been shown that there is a correlation between the effectiveness of selenium as a chemopreventive agent and its ability to inhibit cell growth (38, 39). In this study, we hypothesized that selenium intake exerts its anticarcinogenic effect by enhancing the acute apoptotic response to carcinogen-induced DNA damage (AARGC). This is an important mechanism for removing cells harboring oncogenic DNA adducts and is a regulatory target for a variety of dietary agents and drugs (22, 23, 25–27, 40). We found that AARGC in the colon was significantly enhanced in animals fed MPSe1 and CasYSe4 but not in animals fed CasYSe1. This suggests that selenium may exert a significant proapoptotic effect early in oncogenesis, which protects against persistence of mutations and progression to ACF and eventually cancer. This concept is supported by the observation that specific mutations in *K-ras* were significantly reduced in mice fed MPSe1.

It is also possible that selenium intake has a continuing effect throughout the process of oncogenesis because it also suppressed cell proliferation. Progression to and beyond the ACF stage might be regulated in part by epithelial proliferation. This study provided evidence of selenium affecting cell proliferation because Ki-67 labeling index in colonic crypts was significantly reduced in animals fed MPSe1 and CasYSe4 after feeding for 12 weeks. Given that selenium-enriched milk protein isolate was effective at reducing all sizes of ACF, it is possible that it acts at both initiation and postinitiation stages of azoxymethane-induced carcinogenesis and by a number of different mechanisms that may operate simultaneously or consecutively. Recent cDNA microarray technology has identified the potential selenium-responsive genes that might associate with selenium-induced cell cycle arrest and apoptosis (41), such as *Apo-3, c-jun*, and *cdk5/cyclin D1*.

A study in our laboratory has shown that the time course of induction of AARGC in the colon by azoxymethane (0–72 hours) parallels O^6-MeG adduct formation.[3] One likely important function of enhanced AARGC is to remove cells harboring oncogenic DNA

[3] L.S. Nyskohus, Y. Hu, R.K. Le Leu, G.P. Young, unpublished data.

adducts and so inhibit accumulation of consequent *K-ras* mutations. Therefore, we examined the effect of dietary selenium on *K-ras* mutations because these will reflect the overall burden of unrepaired adducts and so provide further insights into the mechanisms of cancer prevention. We found azoxymethane-treated mice developed not only G to A transition mutation as previously reported (33–35) but also G to T transversion mutation. To our knowledge, this is the first study to show that G to T mutation occurs in the mouse azoxymethane model. In addition, we found a much higher *K-ras* mutation rate than others (33–35). It is not clear why there is a striking difference in terms of mouse *K-ras* mutation spectrum and frequency between our study and others. The highly sensitive PCR technology used (LNA-mediated real-time PCR clumping) may improve the detection of G to T mutation. In addition, the mutation spectrum may differ across species. For example, azoxymethane in rats induces G to A mutation exclusively (42). Furthermore, G to T base substitution is also a preferential target for other methylating carcinogens such as tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (43). By measuring the frequency of both types of *K-ras* mutations, we found that animals fed MPSe1 had a significantly lower frequency than those fed MP-Cont or CasYSe1. This confirms that *K-ras* mutations may be a useful biomarker for chemoprevention.

There has been considerable interest in whether the cancer chemopreventive effect of selenium is mediated through selenoproteins, such as GPx isoforms. GPx acts as an antioxidant to eliminate tumor-promoting reactive oxygen species and seems responsive to dietary selenium intake (44). But GPx induction by selenium has been challenged as the prime mechanism of chemoprevention because GPx activity is saturated at a lower dietary selenium intake than that which shows maximum chemopreventive effect of selenium (45). As cGPx is the most active and abundant isoform, we measured it in this study. Our study showed a disconnect between GPx activity and protection. Although significantly higher GPx activity was found in animals fed yeast-selenium at 1 ppm, increasing intake to 4 ppm did not increase GPx activity further but did lead to suppression of large ACF. Furthermore, dairy-selenium at 1 ppm showed stronger protection against ACF formation and cancer without significantly affecting GPx activity. These findings confirm that an effect of selenium on GPx activity is not crucial for cancer protection and, as such, effects on GPx do not serve as a reliable biomarker. Ganther (46) and others have previously thrown doubt on the GPx hypothesis and have examined a possible role for alternative selenium-containing proteins. It is possible that proteins, such as gastrointestinal GPx (GI-GPx) and selenoprotein-P (SelP) or thioredoxin reductases (TrxR), or non-selenoprotein selenium metabolites, are responsible for the cancer preventive action of selenium (46–48). Future studies are needed to understand the differential effect of the various dietary sources of selenium on GI-GPx, SelP, and TrxR.

When comparing the two selenium sources at equivalent dose (1 ppm), dairy-selenium induced a significantly higher plasma selenium concentration, suggesting that greater bioavailability of selenium is the explanation if bioavailability is accurately reflected in plasma levels. However, yeast-selenium at 4 ppm produced even higher plasma levels than did dairy-selenium at 1 ppm but failed to achieve protection. It is well-known that selenium compounds may differ in type between different food sources, and these could alter efficacy as chemopreventives (49). The milk proteins will not contain low molecular weight (MW) selenium compounds due to the preparative procedure, and any selenocysteine or selenomethionine present will be incorporated into proteins. Further research is warranted to identify the organic forms present within the milk proteins, how they differ from yeast, and their relative chemopreventive actions. It is interesting that the milk protein isolate was protective at 0.5 (trend) or 1 ppm (significant), when compared with the results published on several low MW selenium compounds in the mammary cancer model (50). That review reported 50% reduction in mammary tumors at 2 ppm or greater for all the compounds examined except the garlic compound, selenium-allylselenocysteine, which was effective at 1 ppm. Our data suggest that the dairy protein product is at least as protective as other selenium compounds tested. This warrants further study, including determination of what selenium compounds are released in the gut during digestion and absorption of selenoproteins.

In conclusion, this study has shown a significant difference between dietary sources of selenium in their capacity to inhibit events in colorectal oncogenesis at equivalent selenium concentration. A selenium-enriched milk protein isolate is more effective than a selenized-yeast. The dairy source of selenium had higher bioavailability than the yeast source, although it seems likely that differences in the molecular forms of selenium also contributed to the differences in protection. Inhibition of oncogenesis by dairy-selenium was likely to have been a consequence of reduction in the effect of azoxymethane-induced DNA lesions resulting from enhanced AARGC, reduction in frequency of *K-ras* mutations, and inhibition of cell proliferation. Supplementation of selenium via dairy products might be a practical and effective approach to increasing human selenium intake and preventing cancers such as CRC.

Disclosure of Potential Conflicts of Interest

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