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Dietary selenium fails to influence cigarette smoke-induced lung tumorigenesis in A/J mice

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Abstract

The goal of the study was to determine if dietary selenium inhibited the induction of lung tumorigenesis by cigarette smoke in A/J mice. Purified diets containing 0.15, 0.5, or 2.0 mg/kg selenium in the form of sodium selenite were fed to female A/J mice. Half of the mice in each dietary group were exposed to cigarette smoke 6 hours/day, 5 days/week for five months followed by a four month recovery period in ambient air, while the other half was used as controls. After the recovery period, the mice were euthanized, and their lungs were removed for further analysis. Mice exposed to smoke had a higher tumor incidence and a higher tumor multiplicity, whereas dietary Se did not affect either the tumor incidence or tumor multiplicity. An increase in dietary selenium led to increased levels of selenium in the lung as well as GPx protein levels, but dietary Se did not affect lung SOD protein levels. In conclusion, these data confirm the carcinogenic activity of cigarette smoke in mice but show that dietary Se does not affect smoke-induced carcinogenesis in this model.

Keywords

selenium; cigarette smoke; lung; carcinogenesis; antioxidant

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

1. INTRODUCTION

Lung cancer is the leading cause of death from cancer in the USA and in the world [1; 2]. The major cause of lung cancer is cigarette smoking. Both active and passive smoking have been strongly implicated in lung cancer.

An experimental model of lung cancer has been developed by Witschi and colleagues [3; 4], using A/J mice. This strain of mice has an increased spontaneous incidence of lung tumors compared to other mouse strains, and is susceptible to a greatly enhanced incidence and multiplicity of lung tumors when exposed to cigarette smoke or some chemical agents, such as urethane [5; 6]. In this model, exposure of mice to sidestream cigarette smoke (SSCS) for 5 months followed by another 4 month recovery in ambient air significantly increases the incidence or multiplicity of tumors in the exposed group in comparison to controls. So far only a few preventive agents have been tested for their efficacy against tobacco smoke-induced lung tumorigenesis in animal models. Unfortunately, except for the combination of dexamethasone and myoinositol [7], none have been found effective against smoke-induced lung tumors in the A/J mouse model [6; 8]. In contrast, significant protection has been reported against tobacco-derived 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)- and benzo[a]pyrene (B[a]P)-induced mouse lung tumors by several agents, including isothiocyanates, diallyl sulfide, and selenocompounds [9; 10; 11; 12].

One chemopreventive measure that has been successful in the prevention of lung cancer is supplementation with the essential nutrient selenium (Se). The Nutritional Prevention of Cancer (NPC) study, which was originally designed to determine if Se could prevent recurrence of skin cancers, observed that individuals consuming Se yeast supplements (200 mg/day) had a lower risk of developing lung, prostate, and colon carcinomas [13]. The much-larger Selenium and Vitamin E Cancer Prevention Trial (SELECT) was subsequently performed to determine if selenium and vitamin E, alone or in combination, could prevent these cancers in men [14]. None of the supplements, however, was found to inhibit the development of any of these types of cancer. The SELECT study has been criticized for its use of selenomethionine as the form of selenium used and for the use of subjects with better nutritional status for Se compared to the NPC study [15].

In this study, we hypothesized that feeding supplemental levels of selenium above the recommended level would decrease the induction of lung tumors using the A/J mouse model of environmental tobacco smoke carcinogenesis. Mice were fed a diet containing the recommended level of Se and two higher levels. The higher levels of dietary Se, however, did not inhibit lung carcinogenesis by environmental tobacco smoke.

2. MATERIALS AND METHODS

Materials

The University of Kentucky reference research cigarettes 3R4F (Lexington, KY) were used. Materials for purified diets were obtained from Harlan Teklad (Madison, WI) or Dyets (Gardners, PA). Sodium selenite was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals, unless noted, were purchased from Sigma.

Smoke Exposure System

Inhalation exposures to smoke was carried out in a whole-body Hinners type stainless steel/glass chamber as described earlier [16]. Cigarette smoke was generated from 3R4F University of Kentucky research cigarettes. The concentration of smoke particulates in the exposure chamber atmosphere averaged 46 ± 3 mg TPM/m³.

Experimental Design

Female A/J mice, 7 to 8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME), and allowed to acclimate for one week before the beginning of the study. A total of 180 mice were split into 6 different groups containing 30 mice per group and fed a purified diet similar to the AIN-93M diet formulation, which contained selenium at the concentration of 0.15 mg/kg [17]. The composition of the diet was as follows (% of diet): torula yeast, 30.0; corn starch, 36.0; dextrose, 19.95; cellulose fiber, 5.0; AIN-93M mineral mix, 3.5; AIN-93 vitamin mix, 1.0; choline bitartrate, 0.25; DL-methionine, 0.3; soybean oil, 4.0. Selenium (as sodium selenite) was added to the diet to obtain high selenium diets with selenium concentrations of 0.5 and 2.0 mg selenium/kg diet, respectively. Mice were fed the diets for 3 weeks before beginning smoke exposure, to allow them to adjust to the diets. At this time, one-half of the mice in each dietary group were exposed to cigarette smoke for 5 days per week (6 hours/day) for 5 months followed by a 4 month recovery period in ambient air. Mice were euthanized by overexposure to carbon dioxide gas. After euthanasia, lungs were fixed in Tellyeniczky's solution, and tumors counted under a dissecting microscope. Lungs of some mice were frozen in liquid nitrogen, to be used for selenium and other analyses. Livers were frozen in liquid nitrogen.

Determination of Selenium Status

Lung Se was determined using the method of Spallholz et al. [18]. Four lungs per group were used with two lungs being combined before analysis, for a total of 2 samples per group. Glutathione peroxidase activity was measured in lung homogenates using the method of Lawrence and Burk [19], using hydrogen peroxide as the substrate.

Protein Expression Analyses

Levels of PCNA, Mn-SOD, CuZn-SOD, and GPX proteins in lung homogenates were determined by western analyses. The frozen lungs were homogenized in extraction buffer (Pierce, Rockford, IL) containing protease inhibitors (Sigma, Pittsburgh, PA). Lysed tissue was centrifuged at $8,000 \times g$ for 30 min at 4°C. Protein levels of the supernatants were determined by BCA assay (Pierce, Rockford, IL) and stored at -80°C. Protein samples (30 µg per treatment) were separated using 10% SDS-PAGE and subsequently were transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk buffer and incubated overnight at 4 °C with primary antibodies. After washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase and visualized using ECL detection reagents (Thermo Fisher Scientific Inc., Waltham, MA). Bands were quantified using ImageJ software (NIH, Bethesda, MD) and normalized to β-actin expression. Mn-SOD antibody was purchased from Stressgen Bioreagents (Victoria, BC, Canada). Antibodies for GPX1/2, CuZn-SOD and PCNA were obtained from Santa Cruz

Biotechnology (Santa Cruz, CA). Antibody for β -actin and HRP conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical Analyses

Results were analyzed using an analysis of variance (ANOVA) for a 2×3 factorial arrangement of treatments. There were no significant interactions and therefore only main effects are discussed. Differences between means were determined using Newman-Keuls posthoc test. The results are reported as means $\pm$ standard deviation (SD). For tumor incidence data, results were analyzed using χ^2 analysis. A probability level 0.05 was required for significance.

3. RESULTS

The purpose of this study was to determine if supplemental dietary Se could inhibit the induction of lung cancer by cigarette smoke in A/J mice. Mice were fed the recommended level of selenium or one of two higher levels and were exposed to environmental tobacco smoke for 5 months followed by a 4 month latency period; control mice were fed the three Se diets with no smoke exposure. At the end of the study, the mice exposed to cigarette smoke had higher body weights than unsmoked mice (Table 1). Mice fed the 2.0 mg/kg Se diet had lower body weights than those fed the 0.15 or 0.5 mg/kg diets, but there was no significant difference between the 0.15 and 0.5 mg/kg diets. Mice exposed to environmental tobacco smoke had a higher tumor incidence and a higher tumor multiplicity ($P < 0.05$). Dietary selenium, however, did not significantly affect either the tumor incidence or the tumor multiplicity. All tumors were classified as adenomas.

Se concentrations and GPx activity and protein levels in the lung were quantified to determine if tissue Se levels and Se-dependent proteins responded to dietary Se. Mice fed the 2.0 mg/kg diet had higher lung Se concentrations than those fed the 0.15 mg/kg diet (Figure 1). Mice fed the 0.5 mg/kg diet did not have significantly different lung Se concentrations than mice fed the 0.15 or 2.0 mg/kg diets. Cigarette smoke did not affect lung Se concentrations. Se-dependent GPx activity, however, was increased in smoke-exposed mice (Figure 2), and GPx protein levels, were increased in smoke-exposed mice fed the 0.15 or 0.5 mg/kg diets (Figure 3a). Control mice fed the 2.0 mg/kg diet had higher GPx protein levels than those fed the 0.15 or 0.5 mg/kg diets, but dietary Se did not affect GPx levels in smoke-exposed mice. Dietary Se did not significantly affect Se-dependent GPx activity ($P = 0.14$ in ANOVA).

We had previously observed that dietary Se decreased cell proliferation in lung epithelial cells of both control and smoke-exposed mice [20]. In the present study, we quantified the protein levels of PCNA in whole lung to estimate cell proliferation (Figure 3b). In control mice, PCNA levels were slightly lower in mice fed the high Se diet compared to those fed the low Se diet, in agreement with our previous study, but this was not statistically significant ($P = 0.15$). However, in smoke-exposed mice, no effect was observed. Smoke exposure increased cell proliferation in mice fed the 2.0 mg/kg Se diet, but not in the mice fed the 0.15 or 0.5 mg/kg Se diet.

SOD protein levels were quantified to provide a further measure of cellular antioxidant enzymes. Mn-SOD was not affected by either smoke exposure or dietary Se (Figure 3c). CuZn-SOD was not affected by dietary Se, but was decreased by smoke exposure, but only in mice fed the high Se diet (Figure 3d).

4. DISCUSSION

Studies by Professor Ramesh Gupta and coworkers have reported variable success in inhibiting cigarette smoke-induced DNA damage in various tissues of rodents [21; 22; 23]. In the present study, we examined if dietary Se could inhibit lung carcinogenesis using the Witschi smoke exposure model. Mice exposed to environmental tobacco smoke had an increase in tumor incidence and tumor multiplicity, as has been observed previously [3; 4]. We observed that dietary Se did not affect the tumor incidence or multiplicity, even though increasing dietary selenium produced higher levels of Se in the lung. Thus the results of this study correlate better with the results of the SELECT study rather than the NPC study [13; 14]. It is possible that it is not possible to detect effects of Se using this model; other lung cancer models, such as the model in which mutant ras is overexpressed in the lungs of C57BL/6 mice [24], may be more sensitive to dietary effects.

Previous studies on Se and lung carcinogenesis have produced variable results, depending on the form of Se and carcinogenic agent used. Other studies that have used sodium selenite found that it did not inhibit lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in mice [25; 26; 27]. *In vitro* studies in rat trachea, however, have observed an inhibitory effect of selenite [28; 29]. Other carcinogenesis models have shown sodium selenite to be effective [30; 31; 32; 33; 34; 35; 36; 37; 38]. Se-enriched yeast, selenomethionine, Se-methyl-L-selenocysteine, selenazolidine-4(R)-carboxylic acid (SCA), and 2-methyl-SCA also were found not to affect mouse lung tumor induction by NNK [27; 39; 40]. Two organic forms of Se, 1,4-phenylenebis(methylene)selenocyanate (p-XSC) and 2-oxo-SCA, were both found to inhibit mouse lung tumors induced by NNK [26; 27; 39; 40], and p-XSC was found to inhibit preneoplastic lesions induced by cigarette smoke in guinea pigs [41].

Both lung Se concentrations and the levels of the selenoprotein GPx were increased by higher dietary Se levels, although the effect on GPx was only observed in mice not exposed to cigarette smoke. Lung GPx activity, however, was not significantly affected by dietary Se, although it was increased by smoke exposure. The observation that GPx activities and protein expression did not strictly correlate is not unique. Probucol (a lipid lowering drug) increased GPx activity without the increase of GPx protein [42], and the peroxisome proliferator Wy-14,643 decreased GPx activity but did not affect GPx expression [43].

Another component of the antioxidant defense system is SOD, which is found in the mitochondria, as Mn-SOD, and in the cytosol, as CuZn-SOD [44]. Mn-SOD was not affected by any of the treatments, but CuZn-SOD was decreased by cigarette smoke, but only in mice fed the 2.0 mg/kg Se diet. One of the mechanisms by which smoke exerts its carcinogenic effect is hypothesized to be increased oxidative stress [45; 46]. Therefore, a possible reason for the lack of an effect by the high Se diet is that the antioxidant defense

system was compromised, since smoke decreased both CuZn-SOD and GPx in the high Se diet group.

The results of this study are also surprising, considering that dietary Se inhibits cell proliferation in lung epithelial cells after 5 days exposure to cigarette smoke [20]. In the present study, lung PCNA protein levels were slightly decreased by dietary Se, but only in mice not exposed to cigarette smoke. The different results can be attributed to the much longer study period, the study of whole lung rather than epithelial cells, and the use of PCNA as an endpoint rather than BrdU labeling of epithelial cell nuclei. The lack of an effect by dietary Se on PCNA levels in smoke-exposed mice does correlate with the effect observed on tumor incidence and multiplicity. Although smoke exposure significantly increased PCNA levels in the 2.0 Se group, this PCNA level in the smoke-exposed 2.0 Se group was similar to that in both the smoke-exposed and non-exposed 0.15 and 0.5 Se groups.

We observed that mice exposed to cigarette smoke had a higher final body weight compared to unexposed mice. This contrasts with our previous study, in which smoke-exposed mice had lower body weights [20]. In our previous study, mice were exposed to cigarette smoke for 5 days, whereas in the present study mice were exposed to cigarette smoke for 5 months followed by a 4 month recovery period. Therefore the two smoke exposures are not really comparable: mice exposed in the present study had much more time to adjust to smoke exposure and then had 4 additional months with no smoke exposure. This is consistent with other studies that show no weight gain in smoke-exposed animals in comparison to controls during early smoke exposure periods but full weight recovery following completion of 5 + 4 month exposure protocol [3; 4; 47]. Additionally, there is no correlation between final body weights and tumor multiplicity in mice exposed to cigarette smoke [3].

In summary, dietary selenium did not protect A/J mice from the carcinogenic effects of environmental tobacco smoke, using the Witschi model. Selenium concentrations in the lung, however, were increased in mice fed the high-selenium diets. Mice exposed to smoke had a higher tumor incidence.

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Figure 1.

Effect of Dietary Selenium and Environmental Tobacco Smoke on Lung Se Concentrations. Four lungs per group were used with 2 lungs being combined before analysis, for a total of 2 samples per group. Results represent the mean $\pm$ SD. Selenium concentrations were higher in the 2.0 ppm group compared to the 0.15 group (*) ($P < 0.05$).

Figure 2. Effect of Dietary Selenium and Environmental Tobacco Smoke on Se-Dependent GPx Activity. Results represent the mean $\pm$ SD, with 5-6 mice per group. GPx activity was higher in smoke-exposed mice (*) ($P < 0.05$).

Figure 3. Protein Expression of GPX1/2 (A), PCNA (B), Mn-SOD (C) and CuZn-SOD (D) in Mouse Lungs Was Determined by Protein Expression Analyses. Results represent the mean $\pm$ SD with 4 mice per group. Densitometry results were normalized to β -actin. The Western blots shown here are representative images. (*) denotes statistical significance, $p < 0.05$.

Table I

Effect of Cigarette Smoke and Dietary Selenium on Body Weight, Tumor Incidence, and Tumor Multiplicity

Dietary Selenium (mg/kg diet)	Treatment	Final Body Weight (gm)	Tumor Incidence (%)	Tumor Multiplicity (Tumors/mouse)
0.15	Control	26.71 ± 3.23	4/30 (13.3%)	0.133 ± 0.346
	Smoke	27.26 ± 2.78 [*]	14/31 (45.1%) [*]	0.516 ± 0.623 [*]
0.5	Control	26.94 ± 3.21	9/31 (29.0%)	0.290 ± 0.454
	Smoke	28.14 ± 3.06 [*]	19/34 (55.8%) [*]	0.794 ± 0.832 [*]
2.0	Control	24.88 ± 3.52 [‡]	6/27 (22.2%)	0.222 ± 0.416
	Smoke	26.42 ± 2.42 ^{*‡}	15/34 (44.1%) [*]	0.676 ± 0.898 [*]

Data are means ± SD.

^{*} Significant effect of cigarette smoke (P < 0.05)[‡] Significant difference between 2.0 mg/kg diet and both the 0.15 and 0.5 mg/kg diets.