Nutrition and Cancer

Dietary Supplementation with High-Selenium Soy Protein Reduces Pulmonary Metastasis of Melanoma Cells in Mice1,2

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ABSTRACT The effect of high-selenium (Se) soy protein on pulmonary metastasis of murine B16BL6 melanoma cells was investigated in male C57BL6 mice. Isolated soy proteins (ISP) from soybeans grown with and without Se foliar application during seed development were compared. Five diets were studied, a basal AIN-93G diet or a basal diet containing 10% low-Se ISP, 5% low-Se + 5% high-Se ISP, 10% high-Se ISP, or 10% low-Se ISP supplemented with Se equivalent to that of the 10% high-Se ISP diet. The Se concentrations of the 5 diets were 0.13, 0.13, 1.9, 3.6, and 3.0 μg/g, respectively. Mice were fed the diet for 2 wk before and 2 wk after an i.v. injection of 5 × 10⁶ viable cells. At necropsy, the number and size of tumors that had developed in the lungs were determined. In the control group, 13/18 mice exhibited ≥50 tumors. The numbers of mice with ≥50 tumors were 8/18, 7/18, 3/18, and 6/17 in the ISP-fed groups, respectively. The differences between the 10% high-Se ISP group, the Se-supplemented 10% low-Se group, and the control were significant (P < 0.05). Dietary supplementation with 10% low-Se ISP significantly decreased the mean number of tumors per group and the tumor size compared with the control. A greater reduction in these variables occurred in mice fed the 10% high-Se ISP diet. The inhibition by the Se-supplemented 10% low-Se ISP diet was similar to that by the 10% high-Se ISP diet. The whole-blood Se concentration was inversely related to the tumor number (R = −0.87, P = 0.052), tumor cross-sectional area (R = −0.91, P < 0.05), and tumor volume (R = −0.93, P < 0.05). These findings suggest that Se is responsible for the greater antimetastatic effect of the high-Se ISP. We conclude that the high-Se soy protein has a greater inhibitory effect than the low-Se soy protein on pulmonary metastasis of melanoma cells in mice. J. Nutr. 134: 1536–1540, 2004.

KEY WORDS: • selenium • soy protein • melanoma • metastasis • mice

Selenium (Se) is an essential trace element whose geographical abundance varies dramatically worldwide. A lower amount of Se in soil results in a deficiency of the element throughout the food system, which in turn leads to Se deficiency in humans and livestock that consume the food. For this reason, Se fertilization has been employed to raise the Se content in plant food in certain parts of the world in which Se is deficient in the soil. Selenium deficiency is related to the occurrence of certain chronic diseases including cancer in humans. By contrast, an adequate Se intake is associated with a reduction in the risk of cancer (1).

The United States produces approximately half of the world’s soybeans, and the soil Se content of the major soy-producing states varies dramatically. For example, South Dakota and Nebraska have sufficient or adequate Se in soil, whereas Indiana and Ohio are marginally or severely Se deficient (2). The Se content of soybeans reflects the soil Se status of the producing states. The selenium concentration in soybeans collected from different states varies from ≤0.07 to 1.5 μg/g (3–5). We found that fresh beans from Gregory County, South Dakota contained 1.0–2.9 μg/g Se (unpublished data). A large amount of soybeans are produced each year in states that are sufficient or adequate in Se. However, the health benefits of this great plant protein resource have not been explored.

Worldwide epidemiologic studies demonstrate that consumption of soyfoods (6–8) or an adequate intake of Se (9,10) is associated with a lower occurrence of certain cancers in humans. Results from animal studies show that dietary supplementation with soy protein or Se inhibits experimentally induced tumorgenesis in various models, including mammary (11–13), prostate (14,15), urinary bladder (16), and gastrointestinal (17,18) tumors in animals. Although many studies have focused on the effects of soy and Se on primary tumor development and growth, their ability to influence metastasis has not been examined in detail.

The spread of cancer cells from a primary tumor to a distant organ and the subsequent malignant development is the most devastating aspect of cancer. Advances in surgery and other therapeutic approaches, e.g., radiotherapy or chemotherapy, have greatly improved the treatment of primary neoplastic...
diseases. However, metastasis remains a major cause of death in cancer patients. Practically speaking, any treatment that prevents or slows the spread of malignant cells may reduce the progress of the disease and improve patient prognosis. Results of a previous clinical trial show that combining nutritional intervention (a combination of Se, vitamin C, vitamin E, and β-carotene) with surgical and other therapeutic approaches reduces further metastasis in breast cancer patients with malignant spread to lymph nodes in the axilla (19). Laboratory studies show that dietary supplementation with soy protein reduces the malignant spread of melanoma (20), mammary carcinoma (21), rhabdomyosarcoma, and Lewis lung carcinoma in animals (22,23). Dietary supplementation with Se reduces lung metastasis of melanoma cells (24,25), and Se yeast inhibits the spread of Lewis lung carcinoma cells in mice (26). These findings along with the aforementioned clinical observations suggest that nutritional adjuvants can be useful in reducing metastasis.

The present study was designed to compare the effect of soy proteins from soybeans with and without Se fertilizer application during seed development on pulmonary metastasis of melanoma cells in mice. The aim of this study was to generate data that may lead to future investigations on naturally produced high-Se soybeans as a nutritional adjuvant in cancer prevention, including reducing metastasis.

**MATERIALS AND METHODS**

**Preparation of soy proteins.** The soybean cultivar “Coffax” (27) was planted in Mead, NE, during the 1997 crop year. Sodium selenate (Sigma) was applied during soybean seed development as a foliar spray at stages R1 (14.3 g Se/hectare) and R5 (28.7 g Se/hectare) (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) was planted in Mead, NE, during the 1997 crop year. Sodium selenate (Sigma) was applied during soybean seed development as a foliar spray at stages R1 (14.3 g Se/hectare) and R5 (28.7 g Se/hectare) (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28). After the harvest, the seeds from Se-fertilized (high-Se soybean) and unfertilized (low-Se soybean) plants were dehulled and defatted (28).

**Animals and diets.** The protocol of the present study complied with the NRC guidelines (29) and was approved by the Creighton University Animal Care and Use Committee. Male C57BL/6 mice (3 wk old; Charles River) were housed in groups of 5 in a pathogen-free room on a 12-h light:dark cycle. The temperature in the room was maintained at 25 ± 1°C. Five diets were compared. They were a basal diet containing 20% casein and the basal diet containing 10% low-Se ISP, 5% low-Se + 5% high-Se ISP, 10% high-Se ISP, or 10% low-Se ISP supplemented with Se as selenomethionine (Sigma). The ISP was added to the experimental diets at the expense of casein. The Se content of the Se-supplemented 10% low-Se ISP diet was adjusted to be equivalent to that of the 10% high-Se ISP diet. This group was included to determine whether a greater inhibitory effect by the 10% high-Se ISP diet was due to its Se content. The Se concentrations of these diets were 0.13 ± 0.05, 0.13 ± 0.04, 1.9 ± 0.3, 3.6 ± 0.02, and 3.0 ± 0.5 μg/g (n = 3/group), respectively. Dietary formulations were based on the AIN-93G standard diet (30), except soybean oil was replaced by corn oil. All diets were prepared in our laboratory, and each lot was stored at 4°C for no longer than 3 wk. Diet components, except ISP, were purchased from ICN.

**Cell culture.** Cultures of B16BL6 murine melanoma cells (M.D. Anderson Cancer Center) were maintained in MEM supplemented with 10% heat-inactivated fetal bovine serum as previously described (20). Immediately before injection into the mice, the cells were collected from monolayer cultures by a brief trypsinization (0.05% trypsin and 0.5% mmol/L EDTA), and a single-cell suspension was made in serum-free medium. Cell viability was determined using the trypan blue exclusion assay.

**Experimental design.** Mice were fed the basal diet for 2 d before being randomly assigned to 5 groups of 18 each. The mice in each group were then fed either the basal diet or one of the ISP-supple-mented diets. After 2 wk of consuming the experimental diets, each mouse was injected with 5 × 10⁵ viable cells in 0.2 mL serum-free medium via the lateral tail vein. To limit the possibility that differences in the viability of cells injected into individual mice might affect their metastasis to the lungs, the cells were injected into mice within 30 min after their collection, and the order that the cells were injected into mice from different groups was randomized. After the injection, mice consumed their respective diets for an additional 2 wk. One week before tumor cell injection, 6 mice from each group were transferred to metabolic cages. Their food intake was recorded for 7 d. At the end of the experiment, mice were anesthetized using ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight), and then killed by cervical dislocation. Their lungs were excised and fixed in 10% phosphate-buffered formalin. The number of lung tumors was determined by counting visible black foci using a dissecting microscope (20). Tumor cross-sectional area and volume were measured. The cross-sectional area of tumors in randomly selected fields was measured using a Quantimet 500 image analysis system (Leica). Tumor volume was calculated using the mean of the longest and the shortest diameters measured and the assumption that tumors were spherical (31). Major organs were weighed and grossly examined for metastasis. Blood and liver were collected for Se analysis. Selenium in diets, whole blood, and liver was analyzed fluorometrically (32).

**Statistical analysis.** Fisher’s exact test was used to analyze the frequency distribution of the mice that had ≥50 tumors. Bartlett’s test was used to test the homogeneity of variances. Parametric data were analyzed using 1-way ANOVA and the Student-Newman-Keuls multiple comparison test. Nonparametric data were analyzed using the Kruskal-Wallis and Dunn’s multiple comparison tests. Linear regression was performed to determine the correlations between dietary Se and whole blood and liver Se and those between blood Se level and tumor number, tumor cross-sectional area, and tumor volume after log-transformation of data. The data were analyzed using the statistics program Instat 2.01 for Macintosh (GraphPad). Differences are considered significant at P = 0.05.

**RESULTS**

The overall body weight of mice at the beginning and at the end of the experiment was 11 ± 0.1 and 23 ± 0.2 g, respectively. The mean food intake of all mice was 3.3 ± 0.1 g/d (n = 30). There were no differences in body weight and food intake among the groups throughout the experiment (data not shown). At necropsy, there were no differences in liver, kidney, and heart weights among the groups (data not shown), and no tumors were observed in these organs by gross examination. Therefore, dietary supplementation with 10% high-Se ISP did not affect the growth of the mice during the experiment. One mouse in the Se-supplemented 10% low-Se ISP group was excluded from the study before tumor cell inoculation because of its slow growth rate.

Intravenous injection of B16BL6 melanoma cells into C57BL6 mice results in pulmonary metastasis (33). In the present study, injection of 5 × 10⁴ melanoma cells via the lateral tail vein resulted in lung metastasis in all of the mice. The effect of the high-Se soy protein on lung metastasis of melanoma cells was determined in 2 ways. First, the mice within each group were separated into 2 categories based on the number of tumors per mouse, ≤50 tumors and ≥50 tumors. Of the mice fed the basal diet, 72% (13/18) had ≥50 tumors. The percentage of mice with ≥50 tumors was 44% (8/18), 39% (7/18), 17% (3/18), and 35% (6/17) in the 10% low-Se ISP, the 5% low-Se + 5% high-Se ISP, the 10% high-Se ISP, and the Se-supplemented 10% low-Se ISP groups, respectively. The last-mentioned 2 differed from the control (P < 0.01 and P < 0.05, respectively). Second, the number of lung tumors in the control and the ISP-fed groups...
Effect of dietary supplementation with low-Se and high-Se ISP on pulmonary metastasis of melanoma cells in mice

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumors, n/mouse</th>
<th>Tumors, n/group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71 ± 6</td>
<td>1273</td>
</tr>
<tr>
<td>10% low-Se ISP</td>
<td>53 ± 7a</td>
<td>946</td>
</tr>
<tr>
<td>5% low-Se + 5% high-Se ISP</td>
<td>42 ± 6b</td>
<td>756</td>
</tr>
<tr>
<td>10% high-Se ISP</td>
<td>39 ± 4b</td>
<td>699</td>
</tr>
<tr>
<td>10% low-Se ISP + Se</td>
<td>43 ± 5b</td>
<td>725</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Letters in a column indicate different from the control, aP < 0.05 and bP < 0.01. Data were analyzed using ANOVA and the Student-Newman-Keuls multiple comparison test.

Effect of dietary supplementation with low-Se and high-Se ISP on tumor size of metastatic tumors in the lungs of mice

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor cross-sectional area, mm²</th>
<th>Tumor volume, mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.154</td>
<td>0.046</td>
</tr>
<tr>
<td>10% low-Se ISP</td>
<td>0.084a</td>
<td>0.020a</td>
</tr>
<tr>
<td>5% low-Se + 5% high-Se ISP</td>
<td>0.093a</td>
<td>0.022a</td>
</tr>
<tr>
<td>10% high-Se ISP</td>
<td>0.055a,b,c</td>
<td>0.010a,b,c</td>
</tr>
<tr>
<td>10% low-Se ISP + Se</td>
<td>0.043a,b,c</td>
<td>0.007a,b,c</td>
</tr>
</tbody>
</table>

1 Letters in a column indicate: different from the control, aP < 0.01; different from the 10% low-Se ISP group, bP < 0.01; different from the 5% low-Se + 5% high-Se ISP group, cP < 0.01.

2 Because of the heterogeneous variances among sample populations, data were analyzed using the Kruskal-Wallis nonparametric and Dunn’s multiple comparison tests.

Effect of dietary supplementation with low-Se and high-Se ISP on pulmonary metastasis of melanoma cells in mice

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Se</th>
<th>Liver Se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.005 ± 0.001</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>10% low-Se ISP</td>
<td>0.005 ± 0.001</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td>5% low-Se + 5% high-Se ISP</td>
<td>0.016 ± 0.004</td>
<td>0.029 ± 0.004</td>
</tr>
<tr>
<td>10% high-Se ISP</td>
<td>0.024 ± 0.001</td>
<td>0.060 ± 0.009</td>
</tr>
<tr>
<td>10% low-Se ISP + Se</td>
<td>0.026 ± 0.002</td>
<td>0.074 ± 0.014</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 4.

To determine Se status of the mice, blood and liver Se concentrations were quantified at the end of the experiment (Table 3). The Se concentration in whole blood (R = 0.98, P < 0.05) and liver (R = 0.92, P < 0.05) was positively correlated with the dietary Se concentration.

DISCUSSION

The present study was undertaken to investigate whether high-Se soy protein is more effective than low-Se soy protein in inhibiting pulmonary metastasis of B16BL6 melanoma cells in mice. Intravenous injection of B16BL6 cells produced tumor nodules in the lungs. Mice fed the diet containing 10% low-Se ISP developed fewer and smaller tumors in the lungs than mice fed the basal diet. These results are consistent with previous findings that dietary supplementation with soy protein reduces lung metastasis of melanoma cells in mice (20). In the present study, mice fed the 10% high-Se ISP diet had even fewer and smaller tumors than those fed the 10% low-Se ISP diet, indicating that the high-Se soy protein had a greater inhibitory effect than the low-Se soy protein on lung metastasis of melanoma cells in mice.

Several observations from the present study suggest that the greater inhibitory effect of the high-Se ISP on pulmonary metastasis is due to its higher Se content. The 10% high-Se ISP diet (containing 3.6 µg/g Se) was more effective in reducing tumor number and tumor size than the 10% low-Se ISP diet (containing 0.13 µg/g Se). Increasing the Se content of the 10% low-Se ISP diet to the level equivalent to that of the 10% high-Se ISP diet, by adding selenomethionine to the diet, was as effective as the 10% high-Se ISP diet. The regression
analyses showed that the Se status of the mice was inversely correlated with tumor number and tumor size. The greater inhibition of metastasis by the high-Se soy protein suggests that an additive or even synergistic effect of Se with soy protein exists. The present study was not designed to determine such an effect. However, findings from this study certainly provide a foundation for future investigations with a factorial design aimed at examining an additive or synergistic effect. Nevertheless, results of the present study clearly show that dietary supplementation with the high-Se soy protein combines beneficial effects of soy protein and Se in reducing pulmonary spread of melanoma cells in mice.

The present study showed that dietary supplementation with 5% high-Se ISP, 10% high-Se ISP, and Se-supplemented 10% low-Se ISP tended to cause 20, 26, and 25% reductions, respectively, in tumor number compared with the 10% low-Se ISP group. The intravenous injection model used in the present study may help explain the lack of significant differences. The tumor diet. Melanoma cells inoculated into the peritoneum of mice at the time of tumor cell injection, which is a restricting factor on the exposure time of dietary intervention before tumor cell inoculation. A model that allows adequate preincubation dietary exposure may result in more meaningful results. Furthermore, an increase in sample size may increase the power of data analysis. We shall take these into consideration when designing future investigations.

The metastasis of melanoma is a multistep process. Tumor cells must acquire the capacity to separate from the primary tumor and invade the underlying basal lamina to gain access to the cardiovascular or lymphatic circulation. Circulating tumor cells must then exit the cardiovascular system and proliferate within the stroma of the lung to produce a secondary tumor. In the current study, we used an intravenous injection model to study this process. This model does not duplicate all of the steps required for metastasis from a primary tumor. However, it has a principle advantage of standardizing the onset of the invasion by injecting tumor cells into the blood circulation, and measures the ability of malignant cells to extravasate and form tumors in the lungs. The present study demonstrated that the high-Se soy protein reduced the number of mice that had ≥50 tumors and the number of tumors per group compared with the low-Se soy protein–fed group, and the reduction in tumor number was correlated with the Se status of the mice. A greater reduction in the number of tumors is likely due to a greater inhibition of the malignant extravasation from the cardiovascular system into the pulmonary interstitial space, or the survival of melanoma cells while they are in the blood stream, or both.

We found that the tumor size of mice fed the 10% high-Se ISP diet was significantly smaller than that of mice fed the 10% low-Se ISP diet. Melanoma cells are cleared from the blood circulation by ~24 h after the i.v. injection (34). This suggests that the window of opportunity for malignant cells to reach the interstitium of the lungs is relatively brief, and the initiation of cell proliferation and tumor development after extravasation is approximately the same in all mice. Thus, the increased reduction in tumor size is likely due to a greater inhibition on tumor growth.

In the present study, we found that dietary supplementation with high-Se soy protein increased blood and liver Se, and the 10% high-Se ISP and the Se-supplemented 10% low-Se ISP groups did not differ. We chose selenomethionine as the Se supplement because Se takes the place of sulfur during amino acid synthesis in seed development and presents mainly as selenomethionine in soy (35). We did not determine the forms of Se in the high-Se soybeans. However, our results demon-

strated that Se from the high-Se ISP was absorbable and bioavailable to the mice.

The mechanisms by which the high-Se ISP reduces metastasis remain to be elucidated. Reduced cell proliferation, inhibition of angiogenesis, and increased apoptosis are factors related to the inhibition of tumor development and growth. Dietary supplementation with soy isoflavone extract inhibits tumor cell proliferation and angiogenesis and increases apoptosis of tumor cells in mice inoculated with prostate (36) or urinary bladder tumor cells (16). Dietary supplementation with isoflavones reduces pulmonary metastasis of melanoma cells in mice (37). These findings suggest that soy isoflavones contributed at least in part to the inhibitory effect of soy protein on melanoma metastasis in the present study. In vitro studies reveal that Se inhibits the ability of malignant cells to adhere to and invade the extracellular matrix (38,39). These findings suggest that Se can affect the invasion of the extracellular matrix during the spread of malignant cells.

In summary, the present study demonstrated that dietary supplementation with the high-Se ISP had a greater inhibitory effect than supplementation with the low-Se ISP on lung metastasis of melanoma cells in mice. It suggests the potential use of high-Se soy protein products as a nutritional adjuvant in reducing malignant spread in cancer patients. Although the present study was conducted using the protein from Se-fertilized high-Se soybeans, an investigation of naturally produced high-Se soybeans is certainly warranted.

ACKNOWLEDGMENTS

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