Soybean Isoflavones Reduce Experimental Metastasis in Mice\textsuperscript{1,2}


Donghua Li, John A. Yee, Michael H. McGuire,\textsuperscript{*}
Patricia A. Murphy\textsuperscript{1} and Lin Yan\textsuperscript{3}

Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, NE 68178, \textit{Department of Surgery}, Creighton University School of Medicine, Omaha, NE 68131 and \textsuperscript{1}Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011

\textbf{ABSTRACT} We investigated the effect of dietary supplementation with isoflavones on pulmonary metastasis of B16BL6 murine melanoma cells in C57BL/6 mice. Mice were fed a basal AIN-93G diet or the basal diet supplemented with the isoflavones genistein and daidzein at 113 \textmu mol/kg, 225 \textmu mol/kg, 450 \textmu mol/kg, or 900 \textmu mol/kg for 2 wk before and after the intravenous injection of 0.5 x 10\textsuperscript{6} melanoma cells. At necropsy, the number and size of tumors that formed in the lungs were determined. The number of mice that had >15 lung tumors was 17 in the control group, and 16, 15, 13, and 10 in the groups fed isoflavones at 113 \textmu mol/kg, 225 \textmu mol/kg, 450 \textmu mol/kg and 900 \textmu mol/kg, respectively. The latter two were significantly different from the control (P \leq 0.05). The median number of tumors in the control group was 67, and those in the isoflavone-supplemented groups were 57, 33, 32, and 17, respectively. The last was significantly different from the control (P \leq 0.05). Dietary supplementation with isoflavones at 225 \textmu mol/kg, 450 \textmu mol/kg, and 900 \textmu mol/kg also significantly decreased tumor size (median cross-sectional area and volume) compared to the control values. We conclude that dietary supplementation with isoflavones reduces experimental metastasis of melanoma cells in mice. \textit{J. Nutr. 129: 1075–1078, 1999.}

\textbf{KEY WORDS:} • mice • genistein • daidzein • melanoma • metastasis

Epidemiologic studies suggest that consumption of foods that are high in soybean-based products is associated with a reduced risk of breast (Lee et al. 1991), prostate (Severson et al. 1989), uterine (Goodman et al. 1997), and gastric cancers (Nagai et al. 1982) in humans. Dietary supplementation with soybean protein isolate (SPI)\textsuperscript{4} (Hawrylewicz et al. 1991) or soybean chips (Barnes et al. 1990) reduces mammary carcinogenesis in female rats. Adding autoclaved raw soybean to the diet of male mice inhibits carcinogenicity of N-nitroso compounds in the liver and urinary bladder (Mokhtar et al. 1988). These protective effects are associated with soy isoflavones, e.g. genistein and daidzein. Long-term intraperitoneal administration of genistein and daidzein to young rats (Constantinou et al. 1996) and subcutaneous injection of genistein to neonatal rats (Lamartiniere et al. 1995) reduce the development of mammary carcinoma after exposure of the animals to carcinogens. Other studies showed that soy-derived products have no effect on tumorigenesis in some animal models (Reddy et al. 1976).

Soybean is a rich source of dietary isoflavones (Murphy 1982). Isoflavones exist in soybean primarily as conjugated glycosides. Following ingestion, they are hydrolyzed to aglycones by glycosidases produced by intestinal bacteria. The conjugates genistin, daidzin, and glycitin and their aglycones, genistein, daidzein, and glycine constitute 90–95% of the total soy isoflavones (Murphy 1982). It appears that the unconjugated aglycones are associated with many biological properties of isoflavones that may be responsible for their anticancer activities. These include antiestrogenic activity (Folman and Pope 1966), inhibition of protein tyrosine kinases (Akiyama et al. 1987), regulation of cell cycle progression and apoptosis (Kerner et al. 1995), and antiangiogenic activity (Fotis et al. 1993). Metastasis, the spread of malignant cells from a primary neoplasm to distant organs that results in the development of secondary tumors, is one of the most devastating aspects of cancer. Advances in surgical techniques and adjuvant therapies have proven useful in the treatment of primary tumors. However, metastasis remains a major cause of poor prognosis and death in cancer patients. We recently reported that dietary supplementation with SPI reduces pulmonary metastasis of murine melanoma cells in mice (Yan et al. 1997). Connolly et al. (1997) reported that soybean chips inhibit metastasis of human mammary carcinoma cells in athymic nude mice. These studies suggest that dietary soybean is useful in preventing the spread of malignant cells.

The objective of the present study was to determine whether isoflavones present in SPI reduce metastasis. To accomplish this, the effect of dietary supplementation with isoflavones genistein and daidzein on pulmonary metastasis of melanoma cells was investigated using an intravenous injection model.

\textbf{MATERIALS AND METHODS}

\textbf{Animals and diets.} The protocol of the present study was reviewed and approved by the Creighton University Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (National Research Council 1985). Three-week-old male C57BL/6 mice were purchased from Charles River (Wilmington, MA). Mice were housed five per box, in wire-topped plastic boxes, in a pathogen-free room on a 12:12-h light-dark cycle. The temperature in the room was maintained at 25 ± 1°C. Mice were given free access to the diet and deionized water and weighed weekly. Five diets were compared: a basal diet and the basal diet supplemented with genistein and daidzein (Lancaster, Windham, NH) at 113, 225, 450, or 900 \textmu mol/kg, which was equivalent to that provided in the diet containing 2.5, 5, 10 or 20\% SPI, respectively (Yan et al. 1997). The concentration of genistein in isoflavone-supplemented diets was 83.3, 166.7, 333.3, and 666.7 \textmu mol/kg, respectively, and the concentration of daidzein was 29.5, 59.1, 118.1, and 236.2 \textmu mol/kg, respectively. Glycitein was omitted from the supplementation because it was not commercially available. Dietary formulations were


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\textsuperscript{3}To whom correspondence should be addressed.

\textsuperscript{4}Abbreviations used: SPI, soybean protein isolate.

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Differences were considered significant at P ≤ 0.05. Because ANOVA can only be used to compare the means of populations with homogeneous variances, the results were analyzed using the Kruskal-Wallis nonparametric and Dunn’s multiple comparison tests (Kruskal and Wallis 1952). The data were analyzed using the statistic program Instat 2.01 for Macintosh. Differences were considered significant at P ≤ 0.05.

RESULTS

To determine the effect of dietary supplementation with isoflavones on growth, mice were weighed weekly, and food intake was recorded. The overall body weight of mice at the beginning and at the end of the experiment was 14 ± 1 g and 24 ± 1 g, respectively. There was no difference in body weight among the groups throughout the experiment (data not shown). The mean food intake of all mice (n = 30) was 2.6 ± 0.6 g/d. There were no differences in food intakes among the groups (data not shown). The daily isoflavone intakes for each group are shown in Table 1. There were no measurable isoflavones in urine from mice fed the basal diet. The urinary excretion of isoflavones (a sum of genistein, daidzein, and daidzein metabolites equol and O-desmethylangolensin) was increased in a dose-dependent manner in mice fed the diets containing isoflavones at 113, 225, 450 or 900 μmol/kg (Table 1). One mouse from the control group and one from the 900 μmol isoflavones/kg group were excluded from the experiment because their growth was significantly less than all other mice. Injection of 0.5 x 105 viable melanoma cells into the lateral tail vein resulted in lung metastasis in all the mice fed the control diet (Table 2). Based on the number of lung tumors per mouse, the mice were placed into one of the two categories: 1) 1–15 tumors and 2) >15 tumors. In the control group, all mice had >15 lung tumors (Table 2). By contrast, 89, 83, 72, and 59% of the mice in groups fed the diets containing isoflavones at 113, 225, 450, and 900 μmol/kg, respectively, had >15 tumors. The latter two were significantly different from the control (P ≤ 0.05). The median number of lung tumors in the control group was 67. The median number of lung tumors reduced in the 900 μmol/kg isoflavone/kg group compared to the control (P ≤ 0.05). The mean number of lung tumors in mice fed the isoflavone-supplemented diets decreased relative to the control in a dose-dependent manner.

To determine the effect of isoflavones on the growth of metastatic tumors, tumor cross-sectional area and volume were determined. The median cross-sectional area was 0.55 mm2, and median volume was 0.21 mm3 in mice fed the basal diet (Table 3). Dietary supplementation with isoflavones decreased both variables in a dose-dependent manner. The difference in tumor cross-sectional area and volume between the control and the groups supplemented with isoflavones at 225, 450, or 900 μmol/kg was significant (P ≤ 0.01).

DISCUSSION

We reported that dietary supplementation with SPI reduces experimental metastasis (Yan et al. 1997). Results of the present study demonstrate that dietary supplementation with
Isoflavones

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Control 17 139 0.55 0.65

Group Mice, n Tumors, n effective in reducing metastasis. Thus, they were unable to determine whether or not daidzein was massive lung tumors in control and daidzein-treated animals. melanoma cells into mice led to the development of uncountable study described by Menon et al. (1998), injection of1x1 0 6
difficult to conclude from the currently available data. In the attributable to genistein. Whether daidzein is without an effect is of dietary isoflavones on experimental metastasis may be largely the concentration of genistein in the experimental diets was with either genistein or daidzein alone was not tested. However, however, the concentration of genistein in the experimental diets was threefold greater than that of daidzein. Thus, the protective effect of dietary isoflavones on experimental metastasis may be largely attributed to genistein. Whether daidzein is without an effect is difficult to conclude from the currently available data. In the study described by Menon et al. (1998), injection of 1 x 10^6 melanoma cells into mice led to the development of uncountable massive lung tumors in control and daidzein-treated animals. Thus, they were unable to determine whether or not daidzein was effective in reducing metastasis.

At present, the mechanism whereby dietary soybean or isoflavones at concentrations equivalent to that provided in the SPI diets decreased the number of lung tumors and the tumor cross-sectional area and volume compared to the controls. The dietary isoflavone content was positively correlated with both the urinary isoflavone concentration and the magnitude of the inhibitory effect on metastasis. Dietary supplementation with isoflavones up to 900 μmol/kg had no adverse effect on the growth of mice during the experimental period. These results indicate that isoflavones effectively reduced pulmonary metastasis of melanoma cells and also retarded the growth of those tumors that developed in the lungs.

The observations from this study provide the first evidence that dietary supplementation with isoflavones reduces experimental metastasis. This is supported by the findings that oral administration of genistein inhibits lung metastasis of melanoma cells intravenously injected into mice (Menon et al. 1998). Interestingly, they found that daidzein was ineffective in reducing metastasis. In the present study, the effect of dietary supplementation with either genistein or daidzein alone was not tested. However, the concentration of genistein in the experimental diets was threefold greater than that of daidzein. Thus, the protective effect of dietary isoflavones on experimental metastasis may be largely attributed to genistein. Whether daidzein is without an effect is difficult to conclude from the currently available data. In the study described by Menon et al. (1998), injection of 1 x 10^6 melanoma cells into mice led to the development of uncountable massive lung tumors in control and daidzein-treated animals. Thus, they were unable to determine whether or not daidzein was effective in reducing metastasis.

TABLE 2
Effect of dietary supplementation of isoflavones on pulmonary metastasis of melanoma cells in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>1–15 Tumors</th>
<th>&gt;15 Tumors^1</th>
<th>Median^2</th>
<th>Mean ± SEM^3</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>0</td>
<td>17</td>
<td>67</td>
<td>67 ± 8</td>
<td>19–110</td>
</tr>
<tr>
<td>Isoflavones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>113 μmol/kg</td>
<td>18</td>
<td>2</td>
<td>16</td>
<td>57</td>
<td>71 ± 11</td>
<td>4–167</td>
</tr>
<tr>
<td>225 μmol/kg</td>
<td>18</td>
<td>3</td>
<td>15</td>
<td>33</td>
<td>50 ± 12</td>
<td>5–201</td>
</tr>
<tr>
<td>450 μmol/kg</td>
<td>18</td>
<td>5</td>
<td>13*</td>
<td>32</td>
<td>45 ± 11</td>
<td>3–157</td>
</tr>
<tr>
<td>900 μmol/kg</td>
<td>17</td>
<td>7</td>
<td>10**</td>
<td>17*</td>
<td>29 ± 6</td>
<td>4–81</td>
</tr>
</tbody>
</table>

^1 Significantly different from control, *P ≤ 0.05 and **P ≤ 0.01. Data were analyzed using Fisher’s exact test.
^2 Significantly different from the control, *P ≤ 0.05. Data were analyzed using the Kruskal-Wallis nonparametric and Dunn’s multiple comparison tests.
^3 Because of the heterogenous variances among sample populations, the mean values were not compared by ANOVA.

TABLE 3
Effect of dietary supplementation of isoflavones on tumor cross-sectional area and volume of metastatic tumors that developed in the lungs of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice, n</th>
<th>Tumors, n</th>
<th>Tumor cross-sectional area, mm^2</th>
<th>Tumor volume, mm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>139</td>
<td>0.55 ± 0.03</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Isoflavones</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>113 μmol/kg</td>
<td>18</td>
<td>139</td>
<td>0.52 ± 0.03</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>225 μmol/kg</td>
<td>18</td>
<td>139</td>
<td>0.36** ± 0.04</td>
<td>0.10** ± 0.02</td>
</tr>
<tr>
<td>450 μmol/kg</td>
<td>18</td>
<td>139</td>
<td>0.32** ± 0.04</td>
<td>0.09** ± 0.15</td>
</tr>
<tr>
<td>900 μmol/kg</td>
<td>17</td>
<td>139</td>
<td>0.16** ± 0.02</td>
<td>0.03** ± 0.08</td>
</tr>
</tbody>
</table>

^1 Significantly different from the control, **P ≤ 0.01. Data were analyzed using the Kruskal-Wallis nonparametric and Dunn’s multiple comparison tests.
^2 Because of the heterogenous variances among sample populations, the mean values were not compared by ANOVA.
isoflavone-supplemented diets. Therefore, caution should be taken when data from in vitro experiments are used to explain a dietary effect of soybean or isoflavones in animal studies.

The results of the present study demonstrate that tumor cross-sectional area and volume of mice fed the isoflavone diets were significantly reduced compared to those of mice fed the basal diet. A decrease in tumor size could be due to prolonged retention of tumor cells in the circulatory system or an inhibition of malignant cell proliferation after they take up residence in the lungs. Most circulating B16 melanoma cells rapidly die following their intravenous injection (Fidler 1970). Approximately 1% of the cells survive for 24 h, and one tenth of them form tumor colonies in the lungs. Therefore, it is unlikely that retention in the circulation explains the difference in tumor size between the control and the isoflavone-supplemented groups. Rather, it is more likely that this difference is due to the inhibition of mitosis of malignant cells in the lungs. Investigations in our laboratory designed to determine the effect of dietary isoflavones on cell proliferation and angiogenesis during the formation of metastatic tumors will clarify this possibility.

Comparing results of the present study with our previous report on dietary SPI and experimental metastasis (Yan et al. 1997), it appears that SPI is more effective in reducing the number of lung tumors than the isoflavone-equivalent diets. Soybean contains several potential anticancer components other than isoflavones, e.g., protease inhibitors (Kenedy 1993) and saponins (Koratkar and Rao 1997). Although these agents are largely eliminated during the preparation of the SPI, trace amounts may exist. The SPI also contains phytate that was present in the SPI employed in our previous study (Yan et al. 1997), was not supplemented in the diet in the present study. Thus, these variations could contribute, at least in part, to the differences observed in these two studies.

In summary, results of the present study demonstrate that dietary supplementation with isoflavones reduced experimental metastasis of melanoma cells in mice and also inhibited the growth of metastatic tumors that developed in the lungs. We conclude that isoflavones are responsible, at least in part, for the protective effect of dietary soybean on experimental metastasis of melanoma cells in mice.

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