A High Isoflavone Soy Protein Diet and Intravenous Genistein Delay Rejection of Rat Cardiac Allografts

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ABSTRACT Genistein, a soy isoflavone, has in vitro immunosuppressive properties. We investigated whether genistein or dietary soy protein containing isoflavones could influence the outcome of rat cardiac allografts. Lewis rats were fed a diet with protein from high isoflavone soy protein fraction (HIS), casein (CAS) or casein with isoflavones added (CI) starting 1 wk before heart transplants from Wistar Furth donors, and continuing throughout the study. HIS-fed rats had significantly prolonged time to rejection compared with CAS- and CI-fed recipients (10.8 ± 2.62 vs. 7.18 ± 0.75 and 7.22 ± 0.44 d, P < 0.001). Intravenous genistein [20mg/(kg · d)] for 14 d significantly prolonged heart survival compared with controls and dissolvent-treated recipients (23.2 ± 7.4 vs. 8.4 ± 1.3 and 11.4+/−3.6 d, P < 0.0005), and had an additive effect when given to heart recipients also receiving low dose cyclosporine for 7 d (30.8 ± 2.3 vs. 23.4 ± 2.4 d, P < 0.005). Concanavalin A–stimulated lymphocytes, isolated from Lewis rats given intraperitoneal genistein for 7 d, had decreased production of interferon γ compared with controls or dimethyl sulfoxide–treated groups (22.6 ± 9.9 vs 149 ± 105 and 154 ± 103 μg/L, P < 0.05). In conclusion, a high isoflavone soy diet and intravenous genistein, but not isoflavone extract alone, delay rejection of rat cardiac allografts, with an additive effect in cyclosporine-treated rats. In addition, intraperitoneal genistein has immunosuppressive properties in vivo. J. Nutr. 132: 2283–2287, 2002.

KEY WORDS: • soy • genistein • allograft • rejection • isoflavone • rats

Organ transplantation is a life-extending and life-saving treatment for persons afflicted with many diseases that cause organ failure. It is hampered by the risk of rejection, and thus requires long-term use of expensive medications, typically costing $6000 to $15,000/y (1). Current immunosuppression drug regimens have serious side effects, including hyperlipidemia, hyperglycemia, hypertension and infectious risks (2,3). Dietary and nutritional supplements might have a role in augmenting the effectiveness of these regimens without increasing side effects if they have a favorable effect on rejection risk. Some evidence exists for this approach. L-Arginine, fish oil, canola oil and sunflower oil have improved time to rejection in a rat heart transplant model (4–8). Canola oil and sunflower oil, canola oil and sunflower oil have improved time to rejection compared with controls and dissolvent-treated recipients (23.2 ± 7.4 vs. 8.4 ± 1.3 and 11.4+/−3.6 d, P < 0.0005), and had an additive effect when given to heart recipients also receiving low dose cyclosporine for 7 d (30.8 ± 2.3 vs. 23.4 ± 2.4 d, P < 0.005). Concanavalin A–stimulated lymphocytes, isolated from Lewis rats given intraperitoneal genistein for 7 d, had decreased production of interferon γ compared with controls or dimethyl sulfoxide–treated groups (22.6 ± 9.9 vs 149 ± 105 and 154 ± 103 μg/L, P < 0.05). In conclusion, a high isoflavone soy diet and intravenous genistein, but not isoflavone extract alone, delay rejection of rat cardiac allografts, with an additive effect in cyclosporine-treated rats. In addition, intraperitoneal genistein has immunosuppressive properties in vivo. J. Nutr. 132: 2283–2287, 2002.
and lipoxygenase. Many of these bioactive compounds are found in the isoflavone-enriched fraction of soy protein. The isoflavone-enriched fraction is so named because of its high concentration of estrogenic isoflavones, particularly genistein and daidzein (11–13).

One of the better-characterized bioactive factors found in the soy protein fraction is genistein. Genistein, a principal isoflavone found in soybeans, has several biochemical activities. It has been demonstrated to inhibit protein tyrosine kinases (14), acts as an antioxidant (15), modulates transforming growth factor-β (TGF-β) (16), inhibits DNA topoisomerase II (17) and acts as a weak estrogen (18). Signal transduction after engagement of the TCR, a critical event in initiation of acute cellular rejection, is inhibited by genistein in vitro by inhibition of the tyrosine kinase p56lck (19). Genistein inhibits T-cell activation in vitro (20,21). Intraperitoneal administration of genistein delayed time to rejection of rat small bowel allografts in one study (22), demonstrating the in vitro immunosuppressive ability of this isoflavone.

Accordingly, we examined whether a diet containing high isoflavone soy protein or the isoflavone-enriched fraction of soy protein could prolong the time to rejection of rat cardiac allografts. We also examined the effects of intravenous genistein alone on the outcome of such rat heart transplants. Third, we examined whether the soy isoflavone genistein could inhibit activity of stimulated lymphocytes, a possible factor in its immunosuppressive potential, by measurement of IFN-γ production.

MATERIALS AND METHODS

Animals. All procedures were reviewed and approved by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee. Male Lewis rats (RT1l) (Harlan, Indianapolis, IN) 12 wk of age served as heart recipients, and were fed diets as described. Male Wistar Furth rats (RT1u) (Harlan), 12 wk of age, served as heart donors and received nonpurified rat diet (Lab Rodent Diet 5001, PMI Nutrition International, Richmond, IN). The proximate composition of this diet (per kg) is 230 g protein, 4.5 g fat, 6 g fiber and 12.72 MJ total metabolizable energy. Rats were housed two per cage, except heart recipients, which were housed singly after transplant. Food and water was consumed ad libitum throughout the study.

Study 1

Diet. The experimental diets were prepared in powdered form and placed in cuffed lid bowls in the rat cages beginning 7 d before transplant and continuing throughout the study. Only the protein component of the diet was modified; all other nutrients were constant and were based on the AIN-93 diet (23). Consumption of purified diets was not quantitated. All rats gained weight similarly before transplant, and resumed food intake within 1 d of transplant (data not shown).

Three experimental diets were used in Study 1, varying only in type of protein and the absence or presence of exogenous isoflavone (see Table 1). The protein source was either high isoflavone soy protein (HIS) [5.65 mg isoflavones/g protein, SUPRO SOY brand isolated soy protein (a Solae protein), gift from DuPont Protein Technologies International, St Louis, MO; or casein (CAS), ICN Biomedicals, Irvine, CA]. The third diet contained casein, and had purified isoflavones added (CI) (Novosoy, gift from Archers Daniels Midland, Decatur, IL). Similar amounts of isoflavones were present in both the HIS and CI diets.

Heart transplantation. Heart transplantation was performed according to a modification of the technique of Ono and Lindsay (24). Rats were anesthetized with phenobarbital 50 mg/kg intraperitoneally. The donor’s anterior thoracic wall was incised and elevated superiorly; 5 mL heparin (10,000 U/L) given intravenously, superior and inferior vena cavae ligated, pulmonary artery and aorta transected, pulmonary veins ligated, and the heart removed and plunged into saline slush. The cardiac allograft was anastomosed to the recipient Lewis rat’s infrarenal abdominal aorta and vena cava in end-to-side fashion using 9–0 nylon suture. The hearts started beating within a few minutes of reperfusion and topical rewarming with saline.

Experimental procedure. Three groups of Lewis rats were fed diets as described above, beginning 7 d before transplant. Diets were continued for each recipient for the duration of the study. Hearts were monitored daily by palpation of allograft heartbeat in the rat abdomen. Rejection was defined as the day on which no allograft heartbeat was palpable. Laparotomy was performed to confirm whether or not the heart had ceased beating. Anesthetized rats were killed by transection of vena cava and aorta. Allograft hearts were removed to formalin, then sectioned for histlogic analysis with hematoxylin and eosin stain to confirm rejection.

Study 2

To examine whether the soy isoflavone genistein affects time to rejection, we administered genistein intravenously. Lewis rats were fed a nonpurified rat diet (Laboratory Rodent Diet 5001, PMI) before and after heart transplant from Wistar Furth donors. To examine whether any effect seen would also occur in the presence of a calcineurin inhibitor, some rats were also given low dose cyclosporine (CA). The third diet contained casein, and had purified isoflavones added (CI) (Novosoy, gift from Archers Daniels Midland, Decatur, IL). Similar amounts of isoflavones were present in both the HIS and CI diets.

TABLE 1

<table>
<thead>
<tr>
<th>Diet Composition for Study 1†</th>
<th>Casein (CAS)</th>
<th>Casein + Isoflavones (CI)</th>
<th>High-Isoflavone Soy Protein (HIS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein2</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Soy protein3</td>
<td>—</td>
<td>—</td>
<td>200</td>
</tr>
<tr>
<td>Sucrose</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
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</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Vitamins mix4</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mix4</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>DL-α-Tocopherol</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Soy isoflavone powder5</td>
<td>—</td>
<td>1.2</td>
<td>—</td>
</tr>
</tbody>
</table>

† Total metabolizable energy content = 15.67 MJ/kg diet. Macronutrient distribution (% metabolizable energy): protein (18.6), carbohydrate (69.4), fat (12.0), based on macronutrient energy contents of 16.7 kg/J protein, 37.6 kg/J fat, and 16.7 kg/J available carbohydrate (33).

1 High-isoflavone soy protein [SUPRO SOY brand isolated soy protein (a Solae protein), DuPont Protein Technologies International, St Louis, MO], 87% protein, 4.8% fat, 4.2% ash, 5.65 mg total soy isoflavones/g protein.

4 Vitamin and mineral mixes, AIN-93 (23) (ICN Biomedicals).

5 Soy isoflavone powder (Archer Daniels Midland, Decatur, IL), 833 mg total soy isoflavones/g product. Final diet composition 5 mg isoflavone/g protein in CI diet.
Study 3

Male Lewis rats, 10–12 wk of age, were given genistein (gift from Archers Daniel Midland) at 20 mg/kg·d dissolved in 0.1 mL DMSO, or 0.1 mL DMSO by intraperitoneal injection for 7 d or no treatment. Rats were fed nonpurified rat diet (Lab Rodent Diet 5001, PMI). After 7 d, cervical lymph nodes were removed, minced in RPMI-1640 (BioWhittaker, Walkersville, MD) with 5% fetal bovine serum, passed through a 70-μm nylon screen, and centrifuged at 200 × g for 10 min. Pellets were resuspended and recentrifuged two additional times, and RBC were lysed with ammonium chloride. Cells (1 × 10^6) were added to 96-well plates, 0.1 mL supernatant added and incubated at room temperature for 2 h, wells washed and detection antibody (biotinylated anti-rat INF-γ monoclonal antibody) added for 1 h, wells washed, avidin-horseradish peroxidase added for 30 min, washed and developer (tetramethyl benzidine substrate) added. After 30 min, plates were read at 450 nm. INF-γ (pg) was quantified on the basis of a standard curve generated with each assay. Lymphocyte stimulation IFN-γ assays were performed in quadruplicate for each rat.

Statistics. Groups were analyzed by ANOVA. Duncan’s multiple comparisons test was used to determine group differences. IFN-γ levels were also analyzed by ANOVA using log transformed data due to large variances observed. Differences were considered significant at P < 0.05.

RESULTS

Study 1

The number of days until rejection of each cardiac allograft is shown in Table 2. Acute rejection was confirmed by histologic examination in all cases. Recipients fed the HIS diet had a significantly longer time to rejection than either the CAS or CI groups. Addition of isoflavone extract to the casein diet (CI) did not improve graft survival compared with casein alone.

Study 2

Cardiac allograft survival was significantly longer in recipients receiving intravenous genistein than in rats fed no treatment or dissolvent only (Table 3). Intravenous genistein led to significantly improved graft survival compared with the HIS diet from Study 1 (Table 2).

Cyclosporine 5 mg/(kg·d) intramuscularly for 7 d prolonged graft survival compared with control rats as expected. Addition of intravenous genistein for 14 d significantly prolonged the graft survival compared with cyclosporine alone.

Study 3

INF-γ production measured in lymphocytes from Lewis rats fed standard rat diet and given either no treatment, dissolvent intraperitoneally (DMSO) or genistein intraperitoneally for 7 d is shown in Table 4. There were no significant differences, but there was substantial within-group variability. Therefore, a log (base 10) transformation of data was performed, and the genistein group was found to have significantly lower IFN-γ production than rats treated with DMSO only or untreated rats (P < 0.05).

TABLE 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>Days of graft survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS</td>
<td>15</td>
<td>7, 7, 7, 9, 9, 10, 10, 11, 12, 12, 13, 13, 14, 15</td>
</tr>
<tr>
<td>CAS</td>
<td>11</td>
<td>6, 6, 7, 7, 7, 7, 8, 8, 8, 8</td>
</tr>
<tr>
<td>CI</td>
<td>9</td>
<td>7, 7, 7, 7, 7, 7, 8, 8</td>
</tr>
</tbody>
</table>

1 Values are means ± se. Means without a common letter differ P < 0.001.
2 Diets as defined in Table 1.

TABLE 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Osmotic pump</th>
<th>Genistein</th>
<th>Cyclosporine</th>
<th>n</th>
<th>Days of graft survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>6</td>
<td>14, 15, 24, 24, 31, 31</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7</td>
<td>7, 8, 8, 8, 9, 9, 11</td>
</tr>
<tr>
<td>Dissolvent</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>7</td>
<td>7, 9, 9, 10, 13, 16, 16</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>5</td>
<td>21, 22, 22, 26, 26</td>
</tr>
<tr>
<td>Genistein plus cyclosporine</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>5</td>
<td>28, 29, 31, 33, 33</td>
</tr>
</tbody>
</table>

1 Values are means ± so. Means without a common letter differ, P < 0.005.
TABLE 4

Interferon γ (INF-γ) production by concanavalin A–stimulated lymphocytes in intraperitoneal genistein-treated rats and controls1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>µg/L</th>
<th>log (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>5</td>
<td>149 ± 105</td>
<td>2.07 ± 0.32a</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>8</td>
<td>154 ± 103</td>
<td>1.79 ± 0.65a</td>
</tr>
<tr>
<td>Genistein</td>
<td>11</td>
<td>22.6 ± 9.9</td>
<td>1.31 ± 0.20b</td>
</tr>
</tbody>
</table>

1 Values are means ± so. Log-transformed means without a common letter differ, P < 0.05.

DISCUSSION

These studies demonstrated a significant delay in time to rejection of heart allografts in rats either fed the HIS diet or given intravenous genistein. However, addition of an isoavone supplement to the CAS diet did not improve time to rejection.

In addition, these studies suggest that genistein may have immunosuppressive properties in vivo; it is clearly immunosuppressive in vitro (20,21). Genistein may be responsible for the prolongation of graft survival in rats fed the HIS diet. However, this hypothesis is inconsistent with the lack of an effect from adding the isoavone extract to a casein diet. It is possible that the absorption of isoavones, particularly genistein, is affected by the presence or absence of the soy protein fraction or by the form of isoavone.

As previously mentioned, soybeans contain a plethora of bioactive phytochemicals in addition to isoavones, including saponins, phytic acids, phytosterols, trypsin inhibitors and phenolic acids. Many of these bioactive compounds are found in the isoavone-enriched fraction of soy protein and/or soy protein itself, depending on the processing method used (13). For example, if soy protein is isolated from soybeans via a water isolation process, many bioactive compounds remain in the soy protein isolate. This method was used to prepare the high-isoavone soy protein used in this study. If soy protein is isolated from soybeans via an alcohol wash process, the isoavones, along with many of the other bioactive compounds, are removed from the protein. Thus, we cannot exclude that nonisoavone phytochemicals might have contributed to the improvement in graft survival.

The purified soy isoavone extract used in study 1 (CI) has both glycosylated and free isoavones. This extract was provided in amounts similar to those found in HIS. It is not known whether the effect of isoavone supplementation would have been different if a supplement high in nonglycosylated isoavones had been used. Absorption of glycosylated isoavones such as genistein requires an initial hydrolysis step and may occur more distally in the gut (25,26). Fructo-oligosaccharides have been shown to improve the bioavailability of ingested genistein (27); it remains to be determined whether indigestible oligosaccharides contained in the soy protein fraction enhance absorption and enterhepatic recirculation of genistein and its conjugates. It is also not known whether a higher dose of glycosylated isoavones would have shown a beneficial effect.

The mechanism by which intravenous genistein prolongs graft survival is unclear. It had an additive, but not synergistic effect in combination with cyclosporine. Cyclosporine, a calcineurin inhibitor, blocks TCR signal transduction. Several tyrosine protein kinases that can be inhibited by genistein in vitro exist in the pathway of TCR signal transduction. If inhibition of these tyrosine protein kinases was the principal reason for the genistein effect, a synergistic prolongation of graft survival would be expected if genistein is given with cyclosporine. This study found an additive, rather than synergistic effect of intravenous genistein plus cyclosporine. Other physiologic properties of genistein, such as antioxidant or TGF-β modulation, might also confer beneficial effects on graft survival.

Measurement of INF-γ in Con A–stimulated lymphocytes showed very high variability in the controls. This may be due in part to the heterogeneity of isolated lymph node cells. Statistical analysis of the raw data did detect differences, but because variability within untreated and DMSO groups was high, log-transformed data were analyzed, and the genistein treated rats did have significantly lower INF-γ concentrations than control or DMSO-treated rats. Thus, genistein does have immunosuppressive properties when given intraperitoneally, albeit in amounts greater than could likely be achieved by dietary means alone. We did not test lower doses of intraperitoneal genistein. This depression of INF-γ may be relevant to graft prolongation because IFN-γ production by T helper cells augments the rejection response. Further study is required to determine whether genistein administration in vivo affects other aspects of lymphocyte function.

The roles of soy protein and isoavones have not been studied previously in transplantation. A soy protein diet can decrease serum cholesterol and the risk of heart disease in some patient groups (28). Other potential health benefits of soy include prevention of postmenopausal osteoporosis and possible anticancer effects. Interestingly, kidney transplant recipients of Asian ethnicity have significantly better outcomes in the United States than transplant patients of Caucasian or African-American ethnicity (29). Asians traditionally use more soy-based foodstuffs in their diet. For example, Japanese have a 30- to 60-fold higher intake of isoavones than Caucasians (30). The difference in consumption of soybean products between Pacific Rim countries and Western countries has been postulated to contribute to the decreased risk of coronary artery disease (31). Hence, an intriguing possibility is that a soy-rich diet may contribute to the superior transplant outcomes in Asian-Americans.

A high isoavone soy diet could be an attractive treatment for transplant patients for several reasons. Pharmacologic immunosuppression would still be a requisite, but such a diet might provide a salutary effect on risk of rejection. The cholesterol-lowering effects would be welcome because several immunosuppressant agents cause dyslipidemia, and because of the high prevalence of cardiac disease in transplant recipients. Corticosteroids and calcineurin inhibitors can cause osteoporosis in organ transplant recipients (32). Soy protein has been advocated as beneficial in preventing postmenopausal decreases in bone density, but it is not known whether it could ameliorate this drug-induced bone loss.

In conclusion, these studies support a promising role of a high isoavone soy protein diet in the treatment of transplant patients. We have also reported data that implicate a role of genistein in the antirejection effects of soy. The mechanisms underlying these effects are not known. Further research will help elucidate these mechanisms and also address the specific soybean phytochemicals and doses required to prolong survival of organ transplants.
HIGH ISOFLAVONE SOY AND GENISTEIN DELAY REJECTION

ACKNOWLEDGMENT

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LITERATURE CITED