Genistein Enhances Antigen-Specific Cytokine Production in Female DO11.10 Transgenic Mice

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Summary Genistein is a phytoestrogen contained at high levels in soy products and has been shown to regulate immunoresponse. In this study, we evaluated the effects of genistein on the production of cytokines from antigen (Ag)-specific T cells using DO11.10 transgenic mice because the direct effect of genistein on Ag-specific cytokine production has not been elucidated. The oral administration of 20 mg/kg genistein increased IFN-γ and IL-4 production from DO11.10+ T cells in response to ovalbumin (OVA)123–139 peptide in female DO11.10 mice. Analysis of intracellular cytokine synthesis revealed that the percentages of cytokine-producing cells in the control and genistein-treated groups were not different, indicating that increased cytokine production occurred at the single-cell level. In contrast to the female mice, genistein did not increase cytokine production in male mice, suggesting that the effect of genistein on cytokine production is gender-dependent.

Key Words soy isoflavon, genistein, cytokine, T cells, DO11.10 mice

Genistein is a phytoestrogen contained at high levels in soy products. Epidemiological studies, animal studies and in vitro experiments have indicated that genistein exerts beneficial effects for a multitude of disorders, including cancer (1, 2), cardiovascular diseases (3) and osteoporosis (4).

The effects of genistein on immune functions have also been examined but have not been clarified. Genistein has been shown to have an inhibitory effect on the activity of protein tyrosine kinase (5). This inhibitory effect leads to the suppression of lymphocyte functions in vitro (6). In antigen (Ag)-immunized and genistein-treated mice, the magnitudes of delayed-type hypersensitivity (DTH) reaction and antibody (Ab) response were smaller than those in mice not treated with genistein (7, 8). In contrast to the immunosuppressive effect, the administration of genistein significantly increases host resistance to a B16F10 tumor as reflected by a decrease in the number of lung tumor nodules after tumor cell injection. It has shown that the enhancement of cytotoxic T-cell and natural killer (NK)-cell functions is responsible for this resistance (9). Enhancement of the NK-cell function has also been reported (10, 11).

Helper T (Th) cells play an important role in cell-mediated immunity. Ag-specific Th cells are generated by recognition of an antigenic peptide presented on MHC class II molecules of antigen-presenting cells (APCs) through the T-cell receptor (TCR) (12). When Ag-specific Th cells encounter a foreign Ag again, these cells are rapidly activated and expanded, and they produce cytokines to evoke a strong immune response. Th1 cells produce IFN-γ and IL-2, which are prominent mediators of cell-mediated immunity and are involved in organ-specific autoimmune diseases (13). In contrast, Th2 cells selectively secrete IL-4, IL-5, IL-10 and IL-13, stimulate humoral immunity and are responsible for allergic responses (14). In this study, we used DO11.10 transgenic mice, which express TCR specific for chicken ovalbumin (OVA)123–139 peptide (15), and examined the effects of genistein on cytokine production because the direct effect of genistein on Ag-specific cytokine production in vivo has not been elucidated.

MATERIALS AND METHODS

Mice and diet. Female and male DO11.10 mice on a BALB/c background were purchased from Jackson Lab (Bar Harbor, ME, USA) and maintained at the Animal Center of Tokushima University under specific pathogen-free conditions. The room was maintained on a 12-h light:dark cycle at 24°C and 40–50% relative humidity.

Food and water were provided ad libitum. The composition of food used in this study was 200 g casein, 447 g α-starch, 223 g sucrose, 50 g corn oil, 20 g cellulose, 50 g mineral mixture and 10 g vitamin mixture/kg diet. Corn oil was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and other diets were purchased from Oriental Yeast Co., Ltd. (Chiba, Japan).

All experimental procedures were approved by the Animal Research Committee of the University of Tokushima.

Genistein treatment. Genistein (LC Lab., MA, USA) solutions were freshly prepared daily in 25 mM Na2CO3. The mice were administered genistein solutions of the vehicle (25 mM Na2CO3) at 4 or 20 mg/kg body for 28 d
by gavage.

**Cytokine production and enzyme-linked immunosorbent assay (ELISA).** Spleen cells (5×10^6 cells/mL) were incubated with 1 μg/mL OVA_{323-339} peptide for 48 h in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 μM 2-mercaptoethanol, 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C in a humidified atmosphere flushed with 5% CO₂ in air. After culturing, supernatants were collected and stored at −40°C until used. For determination of IFN-γ or IL-4, 96-well plates were coated overnight with primary anti-cytokine capture Ab. Plates were washed with PBS containing 0.05% Tween 20 and blocked with PBS containing 10% FBS, and then dilutions of supernatants or standards were added. Dilutions of the culture supernatant were incubated overnight at 4°C, and after washing, the wells were incubated with biotin-conjugated anti-cytokine-detecting mAb. After a 2-h incubation, the plates were incubated with biotin-conjugated anti-cytokine-detecting mAb. After a 2-h incubation, the plates were washed and streptavidin-conjugated alkaline phosphatase was added. After developing, the OD was determined at 405 nm. The amount of cytokine in each supernatant was extrapolated from the standard curve. The Ab pairs used were as follows (capture antibody/biotinylated detection Ab): IFN-γ, R4-6A2/XMG1.2; IL-4, 11B11/BVD6–24G2. IL-2 content was determined using a mouse IL-2 ELISA system (eBioscience, CA, USA) according to the manufacturer’s instructions.

**Flow cytometric analysis of intracellular cytokine synthesis.** Cells were suspended at 2×10⁶/mL and stimulated with OVA_{323-339} peptide at 1 μg/mL for 48 h. Monencycin was added to the cell culture for the last 4 h. Cells were harvested, washed and stained with FITC-conjugated anti-DO11.10 monoclonal antibody (mAb) (KJ-1-26) for 30 min on ice. After washing the cells, the cells were resuspended in PBS and an equal volume of 4% paraformaldehyde was added for fixation. After fixing for 10 min at room temperature, cells were washed twice with PBS. For intracellular staining, all reagents and washes contained 1% BSA and 0.5% saponin (Sigma, St. Louis, MO, USA), and all incubations were performed at room temperature. After washing and a 10-min incubation with PBS/BSA/saponin, cells were stained with PE-conjugated anti-mouse IFN-γ, IL-2, or IL-4 mAb for 30 min. After staining, cells were washed twice with PBS/BSA/saponin and then with PBS/BSA without saponin to allow membrane closure. Samples were analyzed on a FACScalibur with CellQuest software (Becton Dickinson, Mountain View, CA, USA) after gating out dead cells using forward and side light-scattering.

**Flow cytometric analysis of cell surface antigen.** Thymocytes were stained with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-mouse CD8 mAb for 30 min at 4°C. Spleen cells were stained with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-mouse CD8 mAb or with PE-conjugated anti-mouse CD3 mAb and FITC-conjugated anti-mouse B220 mAb for 30 min at 4°C. Stained cells were analyzed on a FACScalibur with CellQuest software (Becton Dickinson).

**RESULTS**

**Effects of genistein on lymphoid organs and lymphocyte subsets**

Female DO11.10 mice were treated with 4 or 20 mg/kg of genistein for 28 d, and thymus and spleen weights were measured. The weights of the thymi in the group of mice that received 20 mg/kg genistein were greater than those of the control mice. However, a significant difference was not found in the spleen weights.
Effect of Genistein on Cytokine Production

Fig. 2. Cytokine production from female DO11.10 mouse splenocytes treated with genistein. Splenocytes from genistein-treated DO11.10 mice were stimulated with OVA\textsubscript{323–339} for 48 h. IFN-γ (A), IL-2 (B), and IL-4 (C) production levels were determined by ELISA as described in “Materials and Methods.” Production levels of IFN-γ, IL-2, and IL-4 when cultured with medium alone were <1 ng/mL, <500 pg/mL, and <5 pg/mL, respectively. Values are means±SD of seven mice. *p<0.05; **p<0.01.

between the three groups (Fig. 1). To determine whether genistein affects a specific thymocyte population, we investigated thymocyte subsets in genistein-treated mice by flow cytometric analysis. Flow cytometric analysis showed that the expression patterns of CD4 and CD8 in the thymocytes from genistein-treated mice were the same as those in thymocytes from the control mice (Table 1). We also investigated the expression of CD4, CD8, CD3 and B220 molecules in the spleen, but the expression patterns of these molecules in the three groups were the same (data not shown).

Increased cytokine production in genistein-treated female DO11.10 mice

As shown in Fig. 2, levels of IFN-γ and IL-4 production, but not the level of IL-2 production, were significantly increased in 20 mg/kg genistein-treated female DO11.10 mice compared to the levels in control mice. To determine the mechanism, we analyzed intracellular cytokine synthesis at single-cell levels using flow cytometry. Figure 3 shows a typical profile of IFN-γ-producing cells in DO11.10-positive cells upon stimulation with OVA\textsubscript{323–339} peptide. Using this method, we quantified the percentage of cytokine-producing cells. The percentage of cells producing IFN-γ and IL-2 was marginally increased, but the difference was not significant. Genistein had no effect on the percentage of IL-4-producing cells (Fig. 4).

Genistein does not increase cytokine production in male DO11.10 mice

Finally, we examined the effects of genistein on cytokine production in male mice because genistein is structurally similar to estrogen. In experiments using female DO11.10 mice, IFN-γ production in 4 mg/kg genistein-treated mice tended to increase but was not significantly different from that in control mice. In contrast to female mice, IL-4 production in genistein-treated male mice was somewhat lower than that in control mice but was not significantly different from that in control mice (Fig. 5).

DISCUSSION

In this study, we found that the oral administration of genistein enhances the production of cytokines from T cells following engagement of the TCR with antigenic peptides presented by MHC class II molecules on APCs in female mice. Genistein displays structural similarity with estrogen, and it has long been known for its estrogenic properties (16). Estrogen inhibits T and B-cell proliferation responses in vitro as does genistein (17). Estrogen also suppresses IL-2/IL-2 receptor induction in Jurkat T cells, and this suppressive effect is associated with the decreased nuclear binding of two important IL-2 promoter transcription factors, NFκB and AP1 (18). However, the effect of estrogen on the immune system
in vivo is not clear. Treatment with estrogen suppressed the DTH responses to keyhole limpet hemocyanin (19), collagen (20) and *Listeria monocytogenes* soluble Ag (21) in mice. With regard to cytokine production from T cells, estrogen is known to up-regulate the production of specific cytokines. Maret et al. examined the effect of estrogen on CD4^+^ T-cell activation and differentiation after immunization with OVA Ag and showed that selective development of INF-γ-producing cells is induced (22). Similarly, increased production of INF-γ and IL-2, but not IL-4, in Con A-activated spleen cells was also reported (23). One of the mechanisms of enhanced INF-γ production has been speculated to be the binding of estrogen/receptor complex to the estrogen-responsive element in the INF-γ promoter region (24). In our study, treatment with genistein enhanced INF-γ and IL-4 production upon stimulation with OVA_{123-139} peptide in female DO11.10 mice (Fig. 2). This might be caused by enhanced cytokine synthesis at the single-cell level but not an increased percentage of cytokine-producing cells (Fig. 4). It has been shown that the effects of genistein on immune cell fuctions are mediated by estrogenic and anti-estrogenic actions (7, 8, 25) and the inhibition of protein tyrosine kinase (5). In experiments using male DO11.10 mice, cytokine production in genistein-treated mice was not significantly different from that in control mice (Fig. 5). These results show that the effect of genistein on cytokine production is gender-dependent. We could not address the mechanism of enhancement of cytokine production by genistein. Considering observations using male and female DO11.10 mice, the estrogenic action of genistein might be responsible for the enhancement of cytokine production in female DO11.10 mice. The study showing that female mice produce more Ag-specific INF-γ than male mice (23) partially supports our theory.

It has been shown that the injection of genistein...
causes dose-responsive decreases in thymic weight. An analysis of thymocyte subsets and apoptotic cells in these mice indicated that CD4\(^+\)8\(^-\) thymocytes undergo apoptosis and that CD4\(^+\)8\(^-\) and CD4\(^+\)8\(^+\) thymocytes are significantly decreased, partially through estrogen receptor-mediated mechanisms (25). However, in our study, the weights of thymi in genistein-treated mice were greater than those in control mice (Fig. 1). Furthermore, genistein did not change thymocyte subsets based on CD4/CD8 expression (Table 1). This discrepancy is thought to be due to endogenous estrogen. The DO11.10 mice used in this study did not undergo ovarioectomy, in contrast to the mice in the study of Yellayi et al. (25). In addition, the different effects of genistein on thymus weight might result from the use of TCR transgenic mice because predominant CD4\(^+\)8\(^-\) cells are differentiated in the DO11.10 mouse thymus (15) in contrast to the intact mice.

In this study, we showed that the oral administration of 20 mg/kg genistein increased IFN-\(\gamma\) and IL-4 production in female DO11.10 mice (Fig. 3). A dose of 20 mg/kg genistein is considered to be relatively high compared to the dose used in human studies (26). However, with regard to the study investigating immune functions, a dose of 20 mg/kg genistein has been shown to affect various immune cell functions (7–11, 27).

In conclusion, this is the first study to show genistein enhances Ag-specific cytokine production in vivo.

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