Published in final edited form as:

Carcinogenesis. 2007 December ; 28(12): 2560–2566.

Decreased 7,12-dimethylbenz[a]anthracene-induced carcinogenesis coincides with the induction of antitumor immunities in adult female B6C3F1 mice pretreated with genistein

Tai L. Guo^{*}, Rui P. Chi, Denise M. Hernandez, Wimolnut Auttachoat, and Jian F. Zheng
Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298-0613, USA

Abstract

The objective of this study was to determine if genistein (GEN) modulation of the immune responses might contribute to the increased host resistances to tumors. A time-course study was performed in adult female B6C3F1 mice that had been exposed to GEN for 1–4 weeks at the dose level of 20 mg/kg by gavage. A significant increase in *ex vivo* cytotoxic T lymphocyte (CTL) activity was observed in the periods of 2 weeks and 4 weeks. Moreover, increased activities of CTLs were associated with a decrease in the percentage of CD4⁺CD25⁺ T cells and an increase in the production of interferon- γ and activation of STAT1 (signal transducer and activator of transcription 1) and STAT4. Additionally, exposure of mice to GEN increased the activities of *in vivo* CTLs. An increased activity of natural killer (NK) cells was also observed. Further study in the B16F10 tumor model suggested that GEN-mediated enhancement in host resistance to B16F10 tumor was partially related to its potentiating effect on NK cells. Finally, 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumor model was employed to determine the chemopreventive effect of oral GEN treatment. Mice pretreated with GEN for 2 weeks by gavage, the time when an enhanced CTL activity had been produced, had a decreased susceptibility toward DMBA-mediated carcinogenesis, while treatment with GEN after tumor induction conferred no protection. In conclusion, pretreatment with GEN by gavage could enhance host resistances to the B16F10 tumor and DMBA-induced carcinogenesis, suggesting that GEN modulation of immune response was, at least partially, responsible for the antitumor effect of this compound.

Introduction

The isoflavone genistein (GEN) (4,7,4'-trihydroxyisoflavone) is a phytoestrogen found in high levels in soy products. Ingestion of GEN-containing soy food in Asian populations has been associated with reductions in the risks of breast and prostate cancers (1,2). The potential beneficial effects of GEN in the human population are supported by similar findings in experimental animals (3,4). Although GEN has been shown to inhibit the growth of a wide variety of tumor cells in culture by inhibiting the activity of some enzymes, e.g. protein tyrosine kinases and topoisomerase II, this effect of GEN has not been associated with an antitumor effect *in vivo* (3,5). In contrast, there is a large amount of evidence to demonstrate that GEN has weak estrogenic effects on the reproductive systems, especially at low concentrations (6, 7). However, it is still not exactly clear how phytoestrogen GEN exerts its chemopreventive effect.

^{*}To whom correspondence should be addressed. Tel: +1 804 828 6732; Fax: +1 804 828 5604; Email: tlguo@vcu.edu

Conflict of Interest Statement: None declared.

One possible mechanism for GEN to inhibit tumor development may involve its effect on the immune system. In addition to estrogen-dependent tumors, GEN has been shown to be effective in preventing the development of tumors that there is no strong evidence to support their requirement for estrogen (8). Furthermore, in athymic mice that lack the development of T cells, no inhibitory effect was observed on the growth of estrogen-dependent or estrogen-independent tumors when the animals were exposed to dietary GEN (9,10). Considering the importance of both innate immunity and acquired immunity in antitumor mechanisms, it was hypothesized that GEN modulation of the immune responses in female B6C3F1 mice might contribute to the increased host resistances to transplantable tumors and chemical-induced [e.g. 7,12-dimethylbenz[*a*]anthracene (DMBA)] tumors. Previously, we demonstrated that oral exposure to GEN by gavage could increase host resistance to the B16F10 tumor, and this enhancement was associated with increases in cytotoxic T-cell and natural killer (NK) cell activities (11). In this study, we have determined the molecular mechanisms associated with the enhanced antitumor immunities after oral GEN exposure by focusing on the CD4⁺CD25⁺ T cells, cytokines and signal transducer and activator of transcriptions (STATs). Furthermore, DMBA-induced tumor model was employed to determine the chemopreventive effects of oral GEN exposure because DMBA has been widely used to induce carcinogenesis in rodents (12). Mice pretreated with GEN for 14 days had decreased susceptibility toward DMBA-mediated carcinogenesis, suggesting a tumor modifier role for GEN. Decreased DMBA-induced carcinogenesis in adult female B6C3F1 mice following oral GEN pretreatment coincided with the induction of antitumor immunities.

Materials and methods

Animals and diets

Female B6C3F1 mice were obtained from Charles River Breeding Laboratories (Portage, MI) or Taconic Farm (Germantown, NY). Mice arrived at 4–6 weeks of age and were quarantined upon arrival. The mice were between 8 and 10 weeks old at the beginning of the studies. Mice were housed four per cage in plastic shoe box cages with hardwood chip bedding and fed Harlan Teklad Laboratory Diets (NIH 07; Madison, WI) and tap water from water bottles *ad libitum*. The diet contained crude protein (22.5%), crude fats (4.5%) and crude fiber (4.5%). The animal room was maintained at 21–24°C and the relative humidity between 40 and 70%. The light/dark cycle was maintained on 12 h intervals. All animal procedures were conducted under an animal protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

For the feeding study, the diet in a powdered form (5K96, purchased from Purina Mills, St Louis, MO) was based on the NIH-31 formula, except that casein replaced the protein contributed by soy and alfalfa, soy oil was replaced by corn oil and the vitamin mix was adjusted for irradiation. The control diet was assayed for GEN and daidzein after hydrolysis of conjugates. The concentrations of both GEN and daidzein of this diet were determined by Liquid chromatography with electrospray mass spectrometry and tandem mass spectrometry to be ~0.5 p.p.m. (13). GEN (Toronto Research Chemicals, North York, Ontario, Canada) with purity >99% was mixed into the standard 5K96 feed every 3 months by the Diet Preparation Staff, Bionetics at the National Center for Toxicological Research (Jefferson, AR). Each dosed batch of feed was analyzed by the Division of Chemistry, and it was stable for at least 6 months when stored refrigerated.

Chemicals and reagents

DMBA was purchased from Sigma (St Louis, MO). Phosphate-buffered formalin (10%) was purchased from Fisher Scientific (Fair Lawn, NJ). For gavage, GEN solutions were freshly prepared daily in 25 mmol/l Na₂CO₃ at concentrations of 0.02, 0.2, 0.6 and 2 g/l (2,11). Mice

were administered with GEN solutions or the vehicle (25 mmol/l Na₂CO₃) by gavage (0.1 ml/10 g body wt); and this treatment resulted in dose levels of 0.2, 2, 6 and 20 mg/kg body wt, respectively.

DMBA administration by gavage and pathology

DMBA was dissolved in corn oil to give a 5 mg/ml stock concentration. Mice were gavaged with 0.2 ml (1 mg) DMBA once a week for 5 weeks as described (14). In the first study, mice were gavaged with GEN for 2 weeks before the first dose of DMBA (on day 15 of GEN exposure) and continued until the end of the experiment. In the second study, GEN treatment was not started until the animals received all the five doses of DMBA, which was 1 month after the first DMBA treatment. Gross/clinical examinations of mice were done weekly, for a total of 25–27 weeks after the first DMBA treatment, to monitor body weights, skin papilloma and tumor progression. Mice were sacrificed either at the end of the study or earlier if they displayed significant weight loss, signs of distress (ruffled fur, leg paralysis, etc.) or palpable tumors >2 cm in diameter.

All mice were necropsied. Tissues including tumors were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin and subjected to a blind review by a pathologist. Skin papilloma multiplicity was calculated based on the number of papillomas per mouse. Skin papilloma incidence was calculated based on the numbers of mice showing skin papillomas out of the total number of mice in the group. Total tumor multiplicity was calculated based on the combination of observed tumors and skin papillomas divided by the number of mice per group.

B16F10 melanoma tumor model

Host resistance to the B16F10 melanoma tumor was assayed as described (11,15). Briefly, mice (12 mice per group) were challenged with 1×10^5 B16F10 melanoma cells in 0.2 ml phosphate-buffered saline intravenously. Eighteen days after tumor cell challenge, mice were killed by CO₂ inhalation. The lungs were removed, placed in Bouin's solution (Sigma) and counted for nodules. In some studies, one day prior to killing, the mice were pulsed (intravenously) with ¹²⁵I-UdR. Twenty-four hours after ¹²⁵I-UdR injections, mice were killed and lungs were removed and radioassayed. For NK cell depletion, mice were injected with anti-NK1.1 monoclonal antibody (mAb) (intraperitoneally 250 µg per mouse; obtained from the National Cell Culture Center, Minneapolis, MN) 2 days prior to challenge with B16F10 melanoma cells.

Cytotoxic T lymphocyte activity

The assay for *ex vivo* cytotoxic T lymphocyte (CTL) activity was performed as described (11). The splenocytes from control and treated mice were washed once with earle's balanced salt solutions. Splenocytes were sensitized *in vitro* with mitomycin C-treated P815 mastocytoma cells at a responder:sensitizer ratio of 50:1 for 5 days at 37°C in 5% CO₂. For the preparation of mitomycin C-treated P815 cells, cells were incubated in dark with mitomycin C at a concentration of 50 µg per 2×10^7 cells for 30 min at 37°C. Then the cells were washed four times. Following the sensitization phase, cultured splenocytes were harvested and re-suspended in Eagle's minimal essential medium (Hazelton, Lenexa, KS) for determination of CTL activity. Supernatants were saved for enzyme-linked immunosorbent assay (ELISA) to measure the levels of cytokines. ⁵¹Cr-labeled P815 cells (2×10^4 cells/100 µl) were co-cultured in duplicate with 100 µl of graded numbers of splenic effector cells in U-bottom microtiter culture plates to yield a serial half-dilution of effector:target (E:T) ratios from 25:1 to 0.75:1. For the preparation of ⁵¹Cr-labeled P815 cells, 15×10^6 cells were incubated with 500 µCi of ⁵¹Cr for 60 min at 37°C with finger vortex every 15–20 min followed by four washes. After a 4 h incubation at 37°C and 5% CO₂, the plates were centrifuged for 10 min at 250g. One

hundred microliters of supernatant from each well were removed and counted in a LKB gamma spectrophotometer. Controls for spontaneous and maximum release were generated by culturing labeled target cells in the presence of either Eagle's minimal essential medium or 0.1% Triton X-100, respectively.

For *in vivo* CTL activity determination, mice were injected with 10×10^6 viable P815 tumor cells on day 18 of GEN exposure. On day 10 after P815 injection, mice were euthanized by CO₂ asphyxiation, spleen removed and splenocytes obtained. CTL activity was determined using a 4 h ⁵¹Cr release assay as described for the *ex vivo* CTL assay and is presented as percent cytotoxicity. Briefly, the splenocytes at different dilutions were mixed with the target cells to obtain E:T ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. The spontaneous release was determined by adding 100 µl of medium to 12 replicate cultures containing the targets. The maximum release was determined by adding 100 µl of the P815 cells and 100 µl 0.1% Triton X-100 to each of 12 replicate wells. The lytic unit is defined as the number of splenocytes required to kill 10% of the target cells, which is approximately half of the percent cytotoxicity at the E:T ratio of 200:1.

Enzyme-linked immunosorbent assay

ELISAs were performed according to the manufacturer's instructions (Phar-Mingen, San Diego, CA). Briefly, 100 µl of diluted capture antibody was added to each well in a 96-well plate (Maxisorb NUNC, Nalge Nunc International, Rochester, NY) and allowed to adhere overnight at 4°C. The capture antibodies were anti-mouse interleukin-4 (IL-4), interferon-γ (IFN-γ) or IL-2 mAbs. Plates were washed, then blocked in 10% fetal bovine serum–phosphate-buffered saline for 1 h at room temperature. After washing, serial dilutions of the standard and samples were prepared in the plates, then allowed to adhere for 2 h at room temperature. After washing, 100 µl of working detector including detector antibody and avidin–horseradish peroxidase reagent was added to each well, then incubated for 1 h at room temperature. The detector antibodies are biotinylated anti-mouse IL-4, IFN-γ or IL-2 mAbs. After washing, 100 µl of tetramethylbenzidine substrate solution was added to each well. After incubation in the dark for 30 min at room temperature, the absorbance was read at 405 nm.

NK cell activity

The activity of NK cells was assayed using the NK-sensitive target, Na⁵¹CrO₄-labeled YAC-1 cells, as described with modification (11). Briefly, the splenocytes at different dilutions were mixed with the target cells to obtain E:T ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. The spontaneous release was determined by adding 100 µl of medium to 12 replicate cultures containing the targets. The maximum release was determined by adding 100 µl of the YAC-1 cells and 100 µl of 0.1% Triton X-100 to each of 12 replicate wells. NK cell-specific lysis (percentage) of ⁵¹Cr-labeled YAC-1 cells was used as the end point of the assay.

Flow cytometric analysis of lymphocytes

The percentages of lymphocytes in the spleen and CTL culture were measured using flow cytometric analysis (11). For CD4⁺CD25⁺ T cells, single-cell suspensions were dually labeled with fluorescein isothiocyanate-conjugated anti-mouse CD25 mAb (1:80; Becton Dickinson, San Jose, CA) and phycoerythrin or peridin–chlorophyll protein-conjugated anti-CD4 mAb (1:80; Phar-Mingen) for 30 min on ice. The cells were washed and enumeration performed on a Becton Dickinson FACScan Flow Cytometer in which a forward scatter threshold was set high enough to eliminate red blood cells. The data were analyzed using the CellQuest software (Becton Dickinson). Irrelevant, isotype-matched antibodies were used as the control.

Western blot analysis for STAT expression

The cells from CTL culture were lysed directly in a 50 mmol/l Tris buffer (pH 7.6) containing 150 mmol/l NaCl, 1% Triton X-100 and inhibitors for protease and phosphatase. Samples were placed on ice for 20 min with occasional vortexing. Equivalent amount of proteins were loaded onto a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, transferred and blotted with antibodies against phosphorylated STATs. After incubation with goat anti-mouse or goat anti-rabbit IgG–horseradish peroxidase, the binding was visualized using the ECL Western Blot Detection System (Amersham Biosciences, Piscataway, NJ). Then the same membrane was stripped and blotted with antibodies for total STATs and β -actin. The antibodies for STATs were obtained from Cell Signaling Technology (Beverly, MA) for phosphorylated STAT6, from New England Biolabs (Beverly, MA) for phosphorylated STAT1 and STAT5, from Zymed Laboratories (San Francisco, CA) for phosphorylated STAT4 and from Santa Cruz Biotechnology (San Diego, CA) for total STATs.

Statistical analysis

The data were analyzed as follows. The Bartlett's test for homogeneity was used to select the type of analysis to be conducted. Homogeneous data were analyzed using a one-way analysis of variance; when significant, the Dunnett's *t*-test was used to determine differences between the experimental and the control groups. For non-homogeneous data, a non-parametric analysis of variance was used; when significant, differences between the control group and the experimental groups were determined using the Wilcoxon rank test. The data for host resistance to DMBA-induced carcinogenesis were determined using Fisher's exact χ^2 test.

Results

To elucidate the mechanisms underlying GEN-mediated enhancement in *ex vivo* CTL activity when P815 cells were used as the target (11), a time-course study was performed in mice that had been exposed to GEN for 1–4 weeks by gavage at the dose level of 20 mg/kg. An increased CTL activity was observed at all the time points with the periods of 2 and 4 weeks reaching the levels of statistical significance (Figure 1).

There is evidence that CD25⁺CD4⁺ T regulatory cells can suppress the activation and proliferation of CTLs (16,17). When the cells harvested from the CTL cultures after a 5 day incubation were stained with antibodies for CD4 and CD25, there was a significant decrease in the percentage of CD4⁺CD25⁺ T cells (Figure 2A and B) in GEN-treated mice, but not CD4⁺CD25⁻ T cells (data not shown). The percentage of CD4⁺CD25⁺ T cells was decreased from 13.5 to 6.9%, which was a 50% reduction. There were no significant differences in the number of recovered splenocytes from the CTL cultures between the control and GEN-treated group (data not shown). A decrease in the percentage of CD4⁺CD25⁺ T cells was also observed in the splenocytes obtained directly from vehicle- or GEN-treated mice (data not shown).

Cytokines have been shown to be important in generating CTL responses (18). When the 5 day CTL culture supernatants were evaluated for the presence of differential cytokines by ELISA, increased levels of IFN- γ , but not IL-2 and IL-4, were observed (Figure 2C and data not shown). Additionally, the messenger RNA levels of IFN- γ were also increased in overnight concanavalin A-treated splenocytes obtained from GEN-treated mice when compared with the control (data not shown).

The signal transduction pathways, such as Jak-STATs, are involved in generating CTL responses (19). Thus, the activation of STATs was evaluated. Increased activation of STAT1 and STAT4 (Figure 2D), but not STAT5 and STAT6 (data not shown), was observed in the CTLs generated from GEN-treated mice when compared with vehicle controls.

Taken together, we have demonstrated that oral exposure to GEN increased the activities of CTLs in an *ex vivo* system using P815 cells as the stimulator and the target, possibly by increasing the production of IFN- γ and the activation of STAT1 and 4 and decreasing the percentage of CD4⁺CD25⁺ T cells. Nonetheless, it was important to know if GEN affected the generation of tumor-specific CTLs *in vivo*. To this end, an *in vivo* CTL assay was performed to examine the effect of GEN on CTL generation (20). As shown in Figure 3, GEN exposure by gavage for 28 days produced a dose-related increase in the *in vivo* CTL activity against P815 cells in P815 cell-injected adult female B6C3F1 mice (Figure 3A). The increase was not due to an increased percentage of CD8⁺ T cells (data not shown). Additionally, increased NK cell activity was also observed in GEN-treated mice (Figure 3B). More importantly, when the serum IFN- γ levels were measured using ELISA, significant increases were observed at the GEN dose levels of 2 and 20 mg/kg when compared with the vehicle controls (Figure 3C).

Above all, we have demonstrated that oral exposure to GEN by gavage could modulate the immune responses by increasing the activities of CTLs and NK cells at physiologically relevant doses. There is evidence that the B16F10 tumor is sensitive to the activities of T cells and NK cells (21,22). Indeed, treatment with anti-NK1.1 antibody to deplete NK cells significantly decreased host resistance to the B16F10 tumor (Figure 4A). When the effect of GEN on host resistance in the B16F10 tumor model was evaluated in female B6C3F1 mice that had been exposed to feed containing 25, 250 or 1250 p.p.m. GEN for 28 days and continued on the GEN-containing diet after tumor cell injection for another 18 days, significant increases in host resistance to B16F10 tumor were observed at the 250 and 1250 p.p.m. GEN treatment groups (Figure 4B). For a 25 g mouse consuming 2 g chow every day, these GEN levels are approximately equivalent to 2, 20 and 100 mg GEN/kg/day. When the effect of GEN was further examined in NK-depleted mice, an increased host resistance to B16F10 tumor was still observed (Figure 4D); however, the magnitude of protection was decreased when compared with the animals without antibody treatment (Figure 4C), suggesting that other mechanisms such as CTLs and IFN- γ were also involved.

Next, we determined if GEN-mediated enhancements in antitumor immunities could be translated into an increase in chemical-induced carcinogenesis. Because our data suggested that exposure to GEN by gavage for at least 14 days was needed to produce a significantly enhanced CTL response in female B6C3F1 mice, two studies were performed to address the above question. In both studies, the daily GEN treatments were continued until the end of the experiment.

In the first study, we determined the development of DMBA-induced carcinogenesis in female B6C3F1 mice after GEN treatment for 14 days. The body weight increased linearly with time in mice that only received vehicle. Treatment with vehicle plus DMBA slightly reduced the body weight initially when compared with the vehicle-only group; however, the overall body weights of mice that received DMBA were greater than vehicle-only group at day 50 after the last DMBA treatment and thereafter, which was probably due to the tumor induction. There were no significant differences in body weight among the various DMBA-treated groups (data not shown). During the study, the number of unscheduled death or mice had to be sacrificed due to moribundity resulting from tumor progression was recorded. When the number of cumulative death was compared, treatment with GEN at 6 mg/kg significantly reduced the total number of cumulative death at several time points including weeks of 16, 17 and 25 (Figure 5A). All mice were sacrificed and necropsied at week 27 after the last DMBA treatment. Upon pathological analyses, the incidence of mice presenting with any types of tumor was significantly decreased in the 20 mg/kg GEN group when compared with the vehicle group (Figure 5B). Skin papillomas first started to appear in DMBA-treated mice 8 weeks following the last dose of DMBA. Consistent with the report by Nicol *et al.* (23), skin papillomas were observed in areas ranging from ventral midline to dorsal lateral surfaces, and from subocular

to vaginal opening areas, and were not confined to any specific region. By the end of the 27th week after DMBA treatment, the incidence of skin papillomas was significantly reduced by GEN with statistical significances observed at the 6 and 20 mg/kg GEN dose levels, and the multiplicity of skin papillomas was significantly reduced by GEN at all the dose levels (Table I). Upon pathological analyses, the total tumor multiplicity of mice presenting with any types of tumor was also significantly reduced by GEN at all the dose levels (Table I).

In the second study, the GEN treatment was not started until the animals received all the five doses of DMBA, which was 1 month after the first DMBA treatment. In contrast to the protective effect observed in mice that were pretreated with GEN, daily GEN (20 mg/kg) gavage after the DMBA treatment did not reduce the number of cumulative death (Figure 5C). On the other hand, a significant increase in the number of cumulative death by GEN was observed at week 12 when compared with the vehicle group. Lack of protective effect of GEN treatment after tumor initiation by DMBA was further substantiated by the fact that there was no difference in the number of tumor-free mice at the final necropsy between the vehicle and GEN treatment (Figure 5D).

Discussion

Immune system plays an important role in surveillance against tumor growth. CTLs constitute the major antitumor effector population; NK cells are recognized for their involvement in host resistance against tumor growth and dissemination (24). Consistent with the reported chemopreventive effect of GEN, we have demonstrated that administration of GEN by gavage for > 14 days at physiologically relevant doses in adult female B6C3F1 mice significantly increased CTL activity to P815 mastocytoma tumor cells in both *ex vivo* and *in vivo* systems. Increased activation of NK cells was also observed. Furthermore, pretreatment with GEN for >14 days has increased host resistance to the B16F10 tumors that are sensitive to the activities of NK cells and CTLs and DMBA-induced carcinogenesis in adult female B6C3F1 mice.

In this report, we have extended our previous observation that exposure to GEN (2–20 mg/kg) increased the activity of *ex vivo* splenic CTLs by examining the time-course responses, and demonstrated that a 2 week period was required for a significant increase in CTL activity. Importantly, our data also suggested that GEN treatment after tumor induction by DMBA conferred no protection against DMBA-induced carcinogenesis, which was consistent with the report that GEN did not affect or even enhanced *N*-nitroso-*N*-methylurea-induced mammary tumors in rats when the GEN treatment was started after the *N*-nitroso-*N*-methylurea injection (25). In the study by Cohen *et al.* (26), rats were fed GEN-containing diets 1 week prior to *N*-nitroso-*N*-methylurea administration, and no protective effect was observed either. It seemed that the chemopreventive effect of GEN was not obvious until at least 2 weeks of GEN treatment was completed, which coincided with the fact that 2 weeks of GEN treatment was required for an enhanced antitumor immunity to be produced. The causal relationship between the immunomodulatory and chemo-preventive effects of GEN needs further investigation.

Large efforts have been devoted in the past years to study the direct effects of GEN on tumor cells, and some important observations have been made. One of these findings suggested that breast cancer (BRCA) 1 expression was increased after GEN exposure (27,28). BRCA1 encodes a tumor suppressor gene that is mutated in the germ line of women with a genetic predisposition to breast and ovarian cancers (29). There is evidence that the expression of BRCA1 is required for the up-regulation of STAT1, and BRCA1 is a critical component of IFN- γ -regulated antitumor responses (30). In our studies, both the production of IFN- γ and activation of STAT1 and STAT4 were increased in GEN-treated mice. There is evidence that IFN- γ^+ but not IFN- γ^- T cells from tumor-immunized mice are cytotoxic and mediate tumor rejection (31). IFN- γ response has been suggested to be part of an endogenous tumor

surveillance system (32), and contributes to the rejection of transplantable tumors and the inhibition of chemical-induced carcinogenesis (33). It has been shown that BRCA1 and IFN- γ cooperate to induce expression of target genes such as IRF-7 to stimulate the innate immune response and elicit tumor cell apoptosis (30,34). Simmen *et al.* (35) have shown that sera from soy-treated tumor-bearing rats had higher apoptotic activity toward mammary epithelial MCF-7 cells than casein control. It is interesting to speculate that GEN-mediated increases in the expression of BRCA1 and IFN- γ act together to up-regulate a subset of target genes such as *IRF-7*, *MxA*, *TAP1*, *2',5'-OAS* and *ISG54*. The up-regulation of these genes results in an enhanced immune response through increased antigen presentation, increased chemokine release and ultimately in apoptosis of tumor cells.

The CD25⁺ T cells constitute 5–10% of CD4⁺ T cells and <1% of CD8⁺ T cells in the periphery of naive mice (36). Elimination of CD4⁺CD25⁺ T cells using anti-CD25 mAb *in vivo* induced a potent tumor-specific CTL response and non-specific NK cell response and suppressed the growth of B16 melanoma (16,37). These observations suggested a suppressive function of CD4⁺CD25⁺ T cells. Furthermore, there is evidence that the prevalence of CD4⁺CD25⁺ T cells is increased in patients with various cancers including breast adenocarcinoma (17,38). Recent studies suggested that CD4⁺CD25⁺ T regulatory cells limit the accumulation of IFN- γ -producing T cells in the tumor tissue (39). Although our data suggested that GEN treatment can decrease the percentage of CD4⁺CD25⁺ T cells, further study is needed to determine if this decrease is responsible for the increased production of IFN- γ , and thus, the rejection of transplantable tumors and the inhibition of DMBA-induced carcinogenesis. Although CD4 and CD25 partially identify the T regulatory compartment, foxP3 is currently the most definitive marker of regulatory function (39). Thus, it is also important to determine if the CD4⁺CD25⁺ T cells in our system are indeed regulatory T cells by examining the expression of foxP3 in these cells.

Polycyclic aromatic hydrocarbons, the ubiquitous environmental pollutants found in cigarette smoke, charbroiled meats, smoked foods, air pollution and other environmental sources such as coal tar, have been implicated in causing a variety of cancers including BRCA, and DMBA has been used as a model polycyclic aromatic hydrocarbons to induce carcinogenesis in rodent models (12). DMBA requires bioactivation by the oxidase system to form reactive bay region diolepoxides, which can lead to DMBA–DNA adduct formation. There is evidence that GEN can inhibit DMBA-induced bulky DNA adduct formation (40,41). Although GEN modulation of antitumor immunity is most likely responsible for the chemopreventive effect observed in our studies, we cannot exclude the possibility of inhibitory effect of GEN on DMBA–DNA adduct formation. Additionally, our preliminary data have suggested that the depletion of CD4⁺ cells, but not CD8⁺ cells, produced a significant decrease in CTL activity (data not shown). Thus, further studies are needed to determine if the CD4⁺ T cells are responsible for the GEN-mediated enhanced cytotoxic effect.

In this study, mice were exposed to GEN at dose levels of 0.2–20 mg/kg by gavage and 25–1250 p.p.m. by feeding. These doses are physiologically relevant because soy formula can provide a 4-month-old infant with ~6–9 mg/kg of isoflavones (42). The dose of 20 mg/kg GEN in mice is much lower than a clinical human treatment dose (~100 mg/day) in terms of milligram per square meter of body surface, which usually gives more accurate interspecies extrapolation (43). Additionally, the serum levels of GEN in mice that have been fed 1000 p.p.m. GEN-containing diet (~80 mg/kg) is equivalent to that in men who received 50 mg GEN/day (44,45).

Although the biological effect of GEN is complex, the enhancing effect of GEN on host resistances in the B16F10 tumor model and DMBA-induced carcinogenesis is probably due to its estrogenic properties as discussed before (11). There is evidence that dietary GEN affects

DMBA-induced mammary adenocarcinoma in wild-type, but not ER α KO, mice (46). In ovariectomized rats, GEN also had minimal effect on tumor promotion/progression (47). On the other hand, the slight enhancing effect of GEN treatment on DMBA-induced tumors after tumor induction might also be related to the estrogenic effect of GEN because DMBA-induced tumor is estrogen dependent (46). Additionally, daidzein, another phytoestrogen in soy products, can also inhibit DMBA-induced tumor (48). Manjanatha *et al.* (49) have shown that administration of GEN in combination with daidzein commencing 2 weeks prior to carcinogen treatment reduced DMBA-mediated carcinogenicity. Gallo *et al.* (50) also reported that dietary soy treatment for 4 weeks before carcinogen treatment reduced DMBA-mediated carcinogenicity. Therefore, in future evaluation of the effects of soy and soy extract dietary supplements, it will be necessary to consider possible actions of other components of soy on immune function.

In summary, we observed in this study that oral exposure to GEN at physiologically relevant doses significantly increased host resistances in the B16F10 tumor model and DMBA-induced carcinogenesis. Importantly, enhanced host resistance coincided with increased immune responses, e.g. enhanced CTL activity, NK cell activity, IFN- γ production, STAT1 and STAT4 activation and decreased percentage of CD4⁺CD25⁺ T cells. GEN modulation of immune responses may confer a possible explanation for the report that exposure to GEN had no effect on estrogen-dependent and -independent tumors in athymic mice (9,10). Further study on the molecular mechanisms underlying the potentiating effect of GEN on the immune responses will allow us to understand more about its chemopreventive function and the safety issue associated with ingestion of this compound.

Acknowledgements

The authors would like to thank K.L.White and D.L.Musgrove for their discussion and R.D.Brown for her technical help and Connie Weis (National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079) for providing GEN feed in the conducted studies.

Funding

ES012286; National Institute of Environmental Health Sciences contract (NO1-ES-05454).

Abbreviations

BRCA, breast cancer
 CTL, cytotoxic T lymphocyte
 DMBA, 7,12-dimethylbenz[*a*]anthracene
 E:T effector:target
 ELISA, enzyme-linked immunosorbent assay
 GEN, genistein
 IFN- γ , interferon- γ
 IL-4, interleukin-4
 mAb, monoclonal antibody
 NK, natural killer
 STAT, signal transducer and activator of transcription

References

1. Messina MJ, et al. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. *Nutr. Cancer* 1994;21:113–131. [PubMed: 8058523]
2. King RA, et al. Absorption and excretion of the soy isoflavone genistein in rats. *J. Nutr* 1996;126:176–182. [PubMed: 8558299]

3. Constantinou AI, et al. Inhibition of N-methyl-N-nitrosourea-induced mammary tumors in rats by the soybean isoflavones. *Anticancer Res* 1996;16:3293–3298. [PubMed: 9042303]
4. Lamartiniere CA, et al. Genistein alters the ontogeny of mammary gland development and protects against chemically-induced mammary cancer in rats. *Proc. Soc. Exp. Biol. Med* 1998;217:358–364. [PubMed: 9492348]
5. Peterson G. Evaluation of the biochemical targets of genistein in tumor cells. *J. Nutr* 1995;125:784S–789S. [PubMed: 7884565]
6. Cheng EW, et al. Oestrogenic activity of some naturally occurring isoflavones. *Ann. N. Y. Acad. Sci* 1955;61:637–736.
7. Levy JR, et al. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc. Soc. Exp. Biol. Med* 1995;208:60–66. [PubMed: 7892297]
8. Goldwyn S, et al. Promotion of health by soy isoflavones: efficacy, benefit and safety concerns. *Drug Metabol. Drug Interact* 2000;17:261–289. [PubMed: 11201299]
9. Hsieh CY, et al. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. *Cancer Res* 1998;58:3833–3838. [PubMed: 9731492]
10. Santell RC, et al. Genistein inhibits growth of estrogen-independent human breast cancer cells in culture but not in athymic mice. *J. Nutr* 2000;130:1665–1669. [PubMed: 10867033]
11. Guo TL, et al. Genistein modulates immune responses and increases host resistance to B16F10 tumor in adult female B6C3F1 mice. *J. Nutr* 2001;131:3251–3258. [PubMed: 11739876]
12. Prince M, et al. Naturally occurring coumarins inhibit 7,12-dimethylbenz[a]anthracene DNA adduct formation in mouse mammary gland. *Carcinogenesis* 2006;27:1204–1213. [PubMed: 16387742]
13. Doerge DR, et al. On-line sample preparation using restricted-access media in the analysis of the soy isoflavones, genistein and daidzein, in rat serum using liquid chromatography electrospray mass spectrometry. *Rapid Commun. Mass Spectrom* 2000;14:673–678. [PubMed: 10786906]
14. Fischer MF, et al. The effect of dietary fat on the rapid development of mammary tumors induced by 7,12-dimethylbenz[a]anthracene in Sencar mice. *Cancer Res* 1992;52:662–666. [PubMed: 1732055]
15. Murray, MJ., et al. Models for the evaluation of tumor resistance following chemical or drug exposure. In: Dean, JH.; Luster, MI.; Munson, AE.; Amos, H., editors. *Immunotoxicology and Immunopharmacology*. Raven Press; New York: 1985. p. 113-122.
16. Shimizu J, et al. Induction of tumor immunity by removing CD25⁺CD4⁺ T cells: a common basis between tumor immunity and auto-immunity. *J. Immunol* 1999;163:5211–5218. [PubMed: 10553041]
17. Woo EY, et al. Cutting edge: regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J. Immunol* 2002;168:4272–4276. [PubMed: 11970966]
18. Fallarino F, et al. Cutting edge: differentiation of antitumor CTL *in vivo* requires host expression of Stat1. *J. Immunol* 1999;163:4109–4113. [PubMed: 10510345]
19. Thierfelder WE, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996;382:171–174. [PubMed: 8700208]
20. Kerkvliet NI, et al. Inhibition of TC-1 cytokine production, effector cytotoxic T lymphocyte development and alloantibody production by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Immunol* 1996;157:2310–2319. [PubMed: 8805628]
21. Niederkorn JY. T cell subsets involved in the rejection of metastases arising from intraocular melanomas in mice. *Invest. Ophthalmol. Vis. Sci* 1987;28:1397–1403. [PubMed: 3497133]
22. Dobrzanski MJ, et al. Role of effector cell-derived IL-4, IL-5, and perforin in early and late stages of type 2 CD8 effector cell-mediated tumor rejection. *J. Immunol* 2001;167:424–434. [PubMed: 11418679]
23. Nicol CJ, et al. PPARgamma influences susceptibility to DMBA-induced mammary, ovarian and skin carcinogenesis. *Carcinogenesis* 2004;25:1747–1755. [PubMed: 15073042]
24. Whiteside TL, et al. The role of natural killer cells in immune surveillance of cancer. *Curr. Opin. Immunol* 1995;7:704–710. [PubMed: 8573315]
25. Kijkuokool P, et al. Genistein enhances N-nitrosomethylurea-induced rat mammary tumorigenesis. *Cancer Lett* 2006;242:53–59. [PubMed: 16338062]

26. Cohen LA, et al. Effect of intact and isoflavone-depleted soy protein on NMU-induced rat mammary tumorigenesis. *Carcinogenesis* 2000;21:929–935. [PubMed: 10783314]
27. Cabanes A, et al. Prepubertal estradiol and genistein exposures up-regulate BRCA1 mRNA and reduce mammary tumorigenesis. *Carcinogenesis* 2004;25:741–748. [PubMed: 14729590]
28. Fan S, et al. BRCA1 and BRCA2 as molecular targets for phyto-chemicals indole-3-carbinol and genistein in breast and prostate cancer cells. *Br. J. Cancer* 2006;94:407–426. [PubMed: 16434996]
29. Miki Y, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66–71. [PubMed: 7545954]
30. Mullan PB, et al. The 2,5 oligoadenylate synthetase/RNaseL pathway is a novel effector of BRCA1- and interferon-gamma-mediated apoptosis. *Oncogene* 2005;24:5492–5501. [PubMed: 15940267]
31. Becker C, et al. Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. *Nat. Med* 2001;7:1159–1162. [PubMed: 11590442]
32. Ouchi T, et al. Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes. *Proc. Natl Acad. Sci. USA* 2000;97:5208–5213. [PubMed: 10792030]
33. Blankenstein T, et al. The role of IFN-gamma in tumor transplantation immunity and inhibition of chemical carcinogenesis. *Curr. Opin. Immunol* 2003;15:148–154. [PubMed: 12633663]
34. Buckley NE, et al. BRCA1 regulates IFN-gamma signaling through a mechanism involving the type I IFNs. *Mol. Cancer Res* 2007;5:261–270. [PubMed: 17374731]
35. Simmen RC, et al. Inhibition of NMU-induced mammary tumorigenesis by dietary soy. *Cancer Lett* 2005;224:45–52. [PubMed: 15911100]
36. Kuniyasu Y, et al. Naturally anergic and suppressive CD25⁺CD4⁺ T cells as a functionally and phenotypically distinct immunoregulatory T cell subpopulation. *Int. Immunol* 2000;12:1145–1155. [PubMed: 10917889]
37. Steitz J, et al. Depletion of CD25⁺CD4⁺ T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8⁺ T-cell-dependent immune defense of B16 melanoma. *Cancer Res* 2001;61:8643–8646. [PubMed: 11751377]
38. Liyanage UK, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J. Immunol* 2002;169:2756–2761. [PubMed: 12193750]
39. Rudge G, et al. Infiltration of a mesothelioma by IFN-gamma-producing cells and tumor rejection after depletion of regulatory T cells. *J. Immunol* 2007;178:4089–4096. [PubMed: 17371963]
40. Wei H, et al. Isoflavone genistein inhibits the initiation and promotion of two-stage skin carcinogenesis in mice. *Carcinogenesis* 1998;19:1509–1514. [PubMed: 9744550]
41. Chan HY, et al. A potential protective mechanism of soya isoflavones against 7,12-dimethylbenz[*a*]anthracene tumour initiation. *Br. J. Nutr* 2003;90:457–465. [PubMed: 12908908]
42. Irvine CH, et al. Phytoestrogens in soy-based infant foods: concentrations, daily intake, and possible biological effects. *Proc. Soc. Exp. Biol. Med* 1998;217:247–253. [PubMed: 9492332]
43. Hodgson, E. Toxicology testing and risk assessment. In: Hodgson, E.; Levi, PE., editors. *A Textbook of Modern Toxicology*. 2nd edn.. Appleton & Lange; Stamford, CT: 1997. p. 336
44. Yellayi S, et al. The phytoestrogen genistein induces thymic and immune changes: a human health concern? *Proc. Natl Acad. Sci. USA* 2002;99:7616–7621. [PubMed: 12032332]
45. Djuric Z, et al. Effect of soy isoflavone supplementation on markers of oxidative stress in men and women. *Cancer Lett* 2001;172:1–6. [PubMed: 11595123]
46. Day JK, et al. Dietary genistein increased DMBA-induced mammary adenocarcinoma in wild-type, but not ER alpha KO, mice. *Nutr. Cancer* 2001;39:226–232. [PubMed: 11759285]
47. Ueda M, et al. Lack of significant effects of genistein on the progression of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in ovariectomized Sprague-Dawley rats. *Nutr. Cancer* 2003;47:141–147. [PubMed: 15087266]
48. Constantinou AI, et al. Chemopreventive effects of soy protein and purified soy isoflavones on DMBA-induced mammary tumors in female Sprague-Dawley rats. *Nutr. Cancer* 2001;41:75–81. [PubMed: 12094632]

49. Manjanatha MG, et al. Dietary effects of soy isoflavones daidzein and genistein on 7,12-dimethylbenz [*a*]anthracene-induced mammary mutagenesis and carcinogenesis in ovariectomized Big Blue transgenic rats. *Carcinogenesis* 2006;27:2555–2564. [PubMed: 17127718]
50. Gallo D, et al. Chemoprevention of DMBA-induced mammary cancer in rats by dietary soy. *Breast Cancer Res. Treat* 2001;69:153–164. [PubMed: 11759821]

Fig. 1.

GEN increased *ex vivo* splenic CTL activity (lytic units per spleen) in a time-related manner in adult female B6C3F1 mice. Mice were exposed to vehicle (VH) or GEN (20 mg/kg) for 1–4 weeks by gavage as described. Spontaneous release over the 4 h incubation period was 7.5% of maximal release. The lytic units are defined as the number of splenocytes required to kill 10% of the target cells (P815 cells), which is approximately half of the percent cytotoxicity at the E:T ratio of 25:1. The values represent the mean $\pm$ SE, $n = 8$. * $P \leq 0.05$.

Fig. 2.

GEN decreased the percentage of CD4⁺CD25⁺ T cells [A, vehicle (VH) and B, GEN], increased IFN- γ levels in the CTL culture supernatants (C) and increased STAT1 and STAT4 activation in CTL culture cells (D). Adult female B6C3F1 mice were exposed to vehicle or GEN by gavage for 14 days, and the CTL cell culture set-up as described. At the end of the 5 day incubation, the cells were harvested, labeled corresponding antibodies and analyzed using flow cytometry, and 1 ml of supernatant was removed for the determination of IFN- γ levels by ELISA. Western blot analysis was performed using antibodies against total STATs, phosphorylated STATs and β -actin (as loading control). * $P \leq 0.05$, $n = 6-8$.

Fig. 3. GEN increased splenic CTL activity (A), NK cell activity (B) and IFN- γ production (C) in P815 tumor cell-injected adult female B6C3F1 mice *in vivo*. Mice were exposed to vehicle (VH) or GEN (0.2–20 mg/kg) by gavage for 28 days as described. The CTL activity was measured using the P815 cells as the target. Note: the splenocytes from mice without the injection of P815 cells could not kill P815 cells; the splenocytes from P815 cell-injected mice could kill P815 cells but not B16F10 cells. The NK activity was measured using the YAC-1 cells as the target, and the NK cell activity in mice without the injection of P815 cells was identical to P815 cell-injected mice. The values represent the mean $\pm$ SE derived from 6 to 8 mice.

Fig. 4.

GEN increased host resistance to B16F10 tumor in adult female B6C3F1 mice. **(A)** NK cell depletion increased lung tumor burden. **(B)** Decreased lung tumor burden in mice fed with GEN-containing food. Mice were exposed to the vehicle (VH) or GEN-containing feed for 28 days, then challenged with B16F10 melanoma cells as described and continued on GEN-containing feed to the end of the experiment. **(C)** Decreased lung tumor burden in mice treated with GEN by gavage. Mice were exposed to the VH (25 μ M Na_2CO_3) or GEN (20 mg/kg) for 28 days by gavage. Host resistance to the B16F10 melanoma tumor was assayed as described. **(D)** Depletion of NK cells attenuated the decrease in lung tumor burden in mice treated with GEN by gavage. Values represent the mean $\pm$ SE derived from eight animals. * $P \leq 0.05$.

Fig. 5.

Pretreatment with GEN for 14 days decreased DMBA-induced carcinogenesis, while treatment with GEN after tumor induction by DMBA conferred no protection. **(A)** The number of cumulative death during the experiment due to tumor progression in GEN-pretreated mice. **(B)** The number of tumor-free mice at the end of experiment in GEN-pretreated mice. Mice were treated with vehicle (VH) or GEN at 2, 6 and 20 mg/kg for 14 days, and then with DMBA as described. **(C)** The number of cumulative death during the experiment due to tumor progression in mice treated with GEN after tumor induction. **(D)** The number of tumor-free mice at the end of experiment in mice treated with GEN after tumor induction. One month after the first dose of DMBA, daily administration of GEN by gavage was started, and the GEN treatments were continued until the end of the experiment (25 weeks after the first DMBA dose). The daily GEN treatments were continued until the end of the experiment. $n = 16$; $*P \leq 0.05$.

Table I

Effect of GEN pretreatment on DMBA-induced tumors in female B6C3F1 mice

Tissues with tumors	Vehicle	GEN pretreatment (mg/kg)		
		2	6	20
Incidence of skin papillomas	10/16	6/16	4/16 [*]	2/16**
Skin papilloma multiplicity	1.00 ± 0.29	0.38 ± 0.13 [*]	0.25 ± 0.11 [*]	0.13 ± 0.09 [*]
Spleen	1/16	0/16	0/16	0/16
Lung	0/16	0/16	0/16	1/16
Thymus	2/16	0/16	0/16	0/16
Stomach	0/16	0/16	1/16	0/16
Colon	0/16	1/16	2/16	0/16
Vascular	1/16	2/16	2/16	3/16
Total tumor multiplicity	1.25 ± 0.27	0.56 ± 0.16 [*]	0.56 ± 0.13 [*]	0.38 ± 0.13 [*]

Mice were treated as described in the Materials and Methods section. Tumor profiles were sorted by tissue. Statistical comparisons to respective vehicle controls indicated by

* $P \leq 0.05$.