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Genistein Induces a Protective Immunomodulatory Effect in a Mouse Model of Cervical Cancer

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ABSTRACT

Background: Genistein (GEN), a naturally occurring flavonoid present in soy bean, has attracted scientific interest for its possible benefits in cancer. **Objective**: The potential immunomodulatory effects of genistein on the immune system and against TC-1 tumor cell line were evaluated in adult female C57BL/6 mice. **Methods**: Mice were treated with GEN 10 days before to 10 days after the tumor induction. Thirty days after the last GEN treatment, lymphocyte proliferation, Lactase Dehydrogenase (LDH) cytolytic activity and cytokine secretion were analyzed in GEN and control groups. **Results**: The results showed that ingestion of genistein significantly increased lymphocyte proliferation and LDH release. Furthermore, the treatment with genistein also caused a significant increment in interferon gamma (IFN-γ). In addition, the treatment achieved significant therapeutic effect in tumor models compared to the control group. These results indicated that the effect of GEN on tumor growth may be attributed to its effect on lymphocyte proliferation, cytolytic activity and IFN-γ production. **Conclusion**: These results demonstrate that GEN exerts an immunomodulatory effect in a mouse model of Human Papillomavirus (HPV) associated-cervical cancer.

Keywords: Cervical Cancer, Genistein, Human Papillomavirus, Imunomodulation, TC-1

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INTRODUCTION

Human papillomavirus (HPV) has been identified as the etiological agent of cervical carcinoma, the second most common malignancy in women worldwide. Moreover, HPVs are suspected to be also involved in the induction of other tumors, and the development of treatment against HPV remains of utmost priority (1). Chemoprevention is an important strategy to control the process of carcinogenesis. The potential of using medicinal herbs as cancer chemopreventive nutraceuticals and functional foods is promising. Thus, there is a need for exploring drugs/agents which act as chemopreventive agents.
Genistein (GEN) (4, 7, 4′-trihydroxyisoflavone) is a phytoestrogen found in high levels in soy bean products (2). Phytostrogens comprise a family of biologically active plant agents called isoflavones, possessing an estrogenic effect (3).
GEN-containing dietary supplements and soy bean food ingestion has been linked through epidemiological studies, with a range of potential health effects. These include prevention of breast, colon, and prostate cancers and cardiovascular disease (4-5). The potential beneficial effects of GEN in the human population are supported by similar findings in experimental animals (6).
Although GEN has been shown to inhibit cancer cell growth by inhibiting the activity of some enzymes in vitro, this effect of GEN has not been associated with an antitumor effect in vivo (7).
One possible mechanism for GEN to prevent tumor development may involve its effect on the immune system and the modulation of the immune response (8). In contrast, it has been shown that GEN has phytosteroid and estrogen-like activity. Therefore, there has been concern that the estrogenic effects of these compounds might have side effects in some situations (9). Due to controversial reports on chemopreventive potential of genistein, the antitumor and immune induced responses of genistein in HPV-associated tumor was investigated in this study.

MATERIALS AND METHODS

Mice and Cells. C57BL/6 mice (6–8 weeks old) were purchased from the Pasteur Institute (Karaj, Iran). Mice were housed for 1 week before the experiment, given free access to food and water, and maintained in a light/dark cycle with lights on from 6: 00 to 18: 00 h. All experiments were done according to the guidelines for the care and use and the guidelines of the laboratory animal ethical commission of shefa neuroscience research center.
TC-1, (Part of the Johns Hopkins Special Collection) was derived from primary epithelial cells of C57BL/6 mice cotransformed with HPV16 E6 and E7 and activated c-Ha-ras oncogene. TC-1 cell line which is HPV-16 E7+ was used as a tumor model in an H-2b murine system.TC-1 cell line was grown in Roswell Park Memorial Institute medium (RPMI 1640) (Gibco-BRL) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin/ streptomycin 50 U/ml, 2 mM glutamine, 1 mM sodium pyruvate, 2mM nonessential amino acids, and G418 0.4 mg/ml at 37°C with 5% CO2.
Tumor Therapy Assay. For in vivo tumor experiments, C57BL/6 mice (ten per group) were challenged by subcutaneous injection in the right flank with 2×10^{5} TC-1 cells

suspended in 100 µl PBS. PBS- treated mice were used as background control group in all experiments.

Experimental Design. Mice were separated in three experimental groups. The mice were gavaged daily with genistein (20 mg/kg) (LC laboratories, USA) dissolved in 4% DMSO (Sigma Aldrich, USA) (Group 1-test group), 4% DMSO (Group 2-Vehicle control). For healthy control group, the mice were mock-infected and PBS-treated (Group 3). All the treatments were done 10 days before to 10 days after the tumor induction.

Subcutaneous tumor volume was estimated according to Carlsson's formula (11); hence, the largest (a) and the smallest (b) superficial diameters of the tumor were measured twice a week and then the volume (V) of the tumor was calculated by the formula:

$$(V=a\times b\times\frac{b}{2})$$

Statistical analysis was performed using Student's t test. All values were expressed as means ± S.D.

Lymphocyte Proliferation Assay (LPA). For assessing lymphocyte proliferation, LPA test was performed. Twenty four hours after the last gavage, spleens from the immunized mice were removed and red blood cells were cleared by incubation in lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA, pH 7.2) for 5 min at 37 °C. Obtained mononuclear cells ($2x10^5$ cells/well) were grown in each 96-well plate (Nunc, Denmark).The preparations were cultured with RPMI-1640 supplemented with 10% fetal calf serum, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% penicillin/streptomycin and induced in the presence of 10 µg/ml genistein. T cell mitogen PHA (phytohemaglutenin, Sigma chemicals) at a concentration of 5 µg/ml was used as a positive control. Unstimulated splenocytes were used as a negative control. After 3 days, MTT (3-(4,5-dimethyl tetrazolyl-2) 2,5 diphenyl) tetrazolyumbromide (Sigma chemicals) in a concentration of 5 µg/ml was added per well and incubated for 5 h at 37°C in 5% CO2. DMSO (dimethyl sulfoxide) (100 µl) was added to dissolve the formazan crystals produced.

Plates were read at 540 nm, and the results were expressed as stimulation index (SI). The SI was determined as follows: OD values of stimulated cells (Cs) minus the relative cell number of the unstimulated cells (Cu) multiplied or divided by relative OD values of unstimulated cells. All tests were performed in triplicate for each mouse.

Cell Cytotoxicity Assay. In order to assess cell cytotoxicity, lactate dehydrogenase (LDH) level was determined in splenocytes. Twenty-four hours after the final administration, mice were sacrificed and their splenocytes were isolated. For each sample obtained from individual mouse, single cell suspension of mononuclear cells (used as the effecter cells) were cocultured in RPMI 1640 medium with washed EL4 target cells at various effector-to-target cell ratios (E/T ratios) for 4h in phenol red-free RPMI 1640 containing 3% FCS. For preparation of the target cells, EL-4 cells were stimulated with genistein antigen (5 µg/ml).

After centrifugation, the supernatants (50 µl/well) were transferred to the 96-well flat-bottom plates, and lysis of target cells were determined by measuring LDH release using Cytotoxicity Detection Kit (LDH) according to the procedures stated by the manufacturer (Takara Company). Several controls were used for the cytotoxicity assay.

"High control" was the total LDH released from the target cells, and all EL4 cells were lysed by a medium containing 1% Triton X-100. "Low control" was the natural release of LDH from the target cells which was obtained by adding only EL4 cells in the assay medium. "T-cell control" was used to measure the natural release of LDH from T cells, obtained by only adding the different ratios of T cells in the assay medium. The assay for all samples, including the controls, was performed in triplicate (10).
The LDH-mediated conversion of the tetrazolium salt into red formazan product was measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolysis was determined by the following formula:

$$\%\ \text{Cytotoxicity} = \left[(\text{experimental value} - \text{effector cell control}) - \frac{\text{low control}}{\text{high control} - \text{low control}}\right] \times 100$$

Cytokine Assay. Twenty- four hours after the last oral administration, mice were sacrificed and their splenocytes were isolated.
Mononuclear cells from spleens of immunized mice at a concentration of 2×10^6 cells/well were incubated in 24-well plates (Nunc, Denmark) for 2 days in a total volume of 1.5 ml of RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, 1% HEPES, 0.1% 2ME, 0.1% penicillin/streptomycin and pulsed with 10 µg/ml genistein. The cell supernatants were collected and assayed for the presence of cytokines using commercially available sandwich-based ELISA kits (eBioscience, USA) following manufacturer's instruction. All tests were performed in triplicate for each mouse.
Data Analysis. To compare results among different groups, one way analysis of variance (ANOVA) with scheffe post hoc comparison was performed. SPSS statistical software version 11.0 was utilized for statistical analyses. Differences were considered statistically significant when p values were <0.05.

RESULTS

Lymphocyte Proliferation Response. In order to evaluate the effect of GEN treatment on the proliferation of T lymphocytes, three mice per group were used for this experiment. The mice were gavaged daily with GEN or DMSO. The animals were sacrificed and the spleen cells were harvested. The lymphocytes were activated in vitro by GEN and evaluated by MTT methods. A significant increase ($p<0.01$) in the lymphocyte proliferation was evident in GEN treated groups over those of negative control ones, following stimulation of the spleen cells with antigen, as shown in Figure 1.
In order to assess the effect of GEN on the lactate dehydrogenase release, 3 mice/group were used. E/T ratio of 50:1 demonstrated the most significant results among different groups and was selected for further analysis. As shown in Figure 2, lymphocytes in immunized mice with GEN (29.4 ± 1.2%) increased specific cytolytic activity at an E/T ratio of 50:1 significantly as compared to that of DMSO (vehicle control) group (18.5 ± 1.9%) or the healthy control group (13.5 ± 2.9) ($p<0.01$).
However, no statistically significant difference in cytolytic activity was found between the DMSO (vehicle control) group and the healthy control group ($p>0.05$).

The most significant difference among all these groups was at an E/T ratio of 50:1 (data not shown). Based on the results, the CTL activities in immunized mice with GEN group was stronger than those in the control ones.

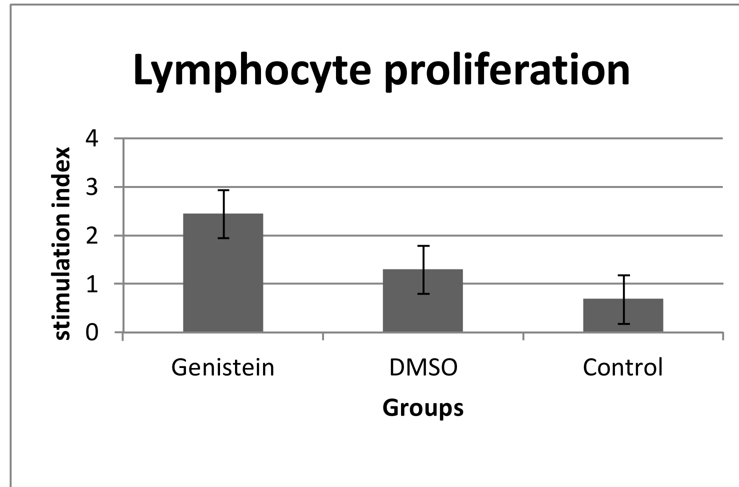

Figure 1. Splenocyte proliferation levels after *in vitro* stimulation with GEN antigen. C57BL/6 mice were challenged by subcutaneous injection of TC-1 cells. Twenty-four hours after final immunization, spleens of mice (three per group) were removed and lymphocyte proliferation was evaluated with MTT method. Formazan crystal formation after incubation of MTT was determined by solving the crystals in DMSO and optical density (OD) was read at 540 nm. Values are mean ± standard error of the mean for the experiments. Lymphocyte proliferation of GEN group was significantly higher than those in control ones ($p \leq 0.01$).

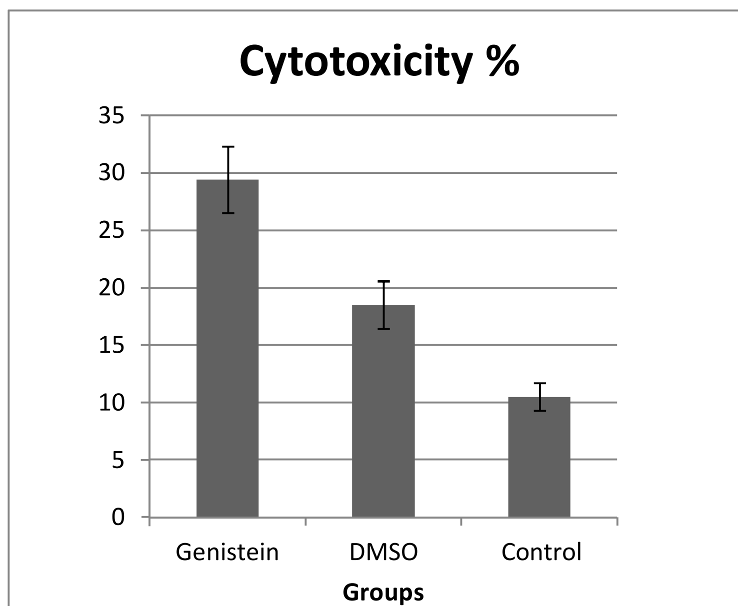

Figure 2. CTL assays to demonstrate lymphocytes in mice vaccinated with GEN using quantitative measurement of LDH release assay. After mice were immunized with GEN for 20 days, spleens were harvested as described in Materials and Methods. Data were collected from LDH results at various E/T ratios (E/T=25:1, 50:1, 100:1) and expressed as cytotoxicity percentage ± SD. Data shown here are from three independent experiments performed in triplicate ($p < 0.01$).

Cytokine Assay. To determine whether treatment with GEN could modulate cytokine secretion and increase specific immune responses, we measured IL-4 and IFN-γ levels in the supernatants of mononuclear cells from treated mice re-stimulated in vitro with GEN antigen. ELISA results obtained from culture supernatants harvested at 72 h showed that, in response to GEN, spleen cells from the GEN group produced higher level of T helper cell type 1 (Th1)-associated cytokine IFN-γ and lower level of the T helper cell type 2 (Th2)-associated cytokine IL-4 ($p<0.001$), compared to the control groups, as shown in Figure 3. The results indicate that GEN regimen predominantly displays a Th1 cytokine profile.

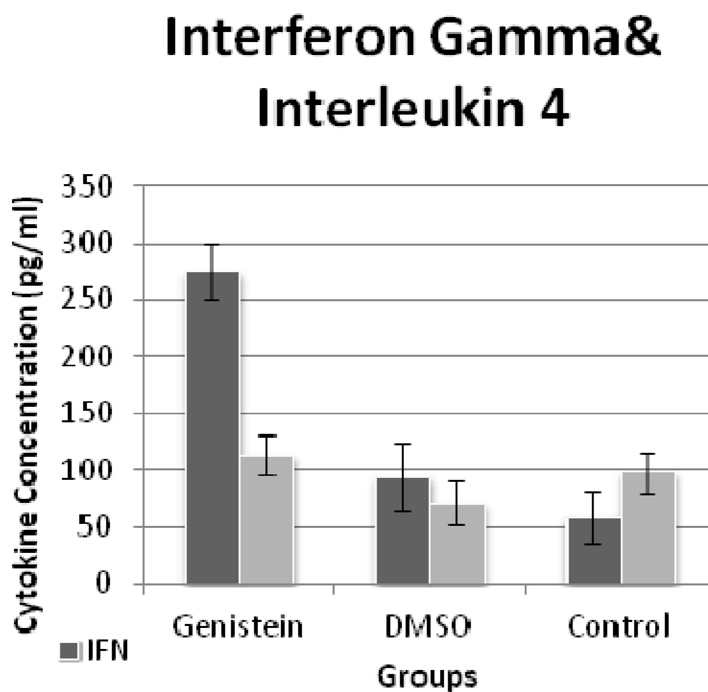

Figure 3. Determination of the production of IFN-γ and IL-4 cytokines. Collected supernatants were screened for the presence of IFN-γ and IL-4 to determine the type (Th1 versus Th2) of the immune response. Concentration of cytokines was determined by comparison to a standard curve of serially diluted positive control samples. The data is represented as mean ± S.D. Each sample was examined in triplicate and results are representative of two experiments ($p< 00.05$).

Tumor Protection. To determine whether the observed enhancement in the number of GEN-specific cellular immune responses after treatment with GEN could be correlated to a GEN-specific anti-tumor effect, we performed an in vivo tumor treatment experiment using a previously characterized E7-expressing tumor model, TC-1 (11).
Mice were injected with 2×10^5 TC-1 cells/mouse subcutaneously on the right flank, and then gavaged daily with GEN from 10 days before to 10 days after the tumor induction. Tumors were measured twice a week once they became palpable. Controls consisted of vehicle control group mice challenged with tumors. The tumor volume was monitored up to 30 days after the last GEN treatment (10 days after tumor induction).

As shown in figure 4, mice treated with GEN significantly reduced both tumor growth (as measured by volume) and tumor weights when compared to the control mice ($p<0.05$). These results indicate that vaccination with GEN could induce therapeutic anti-tumor effects.

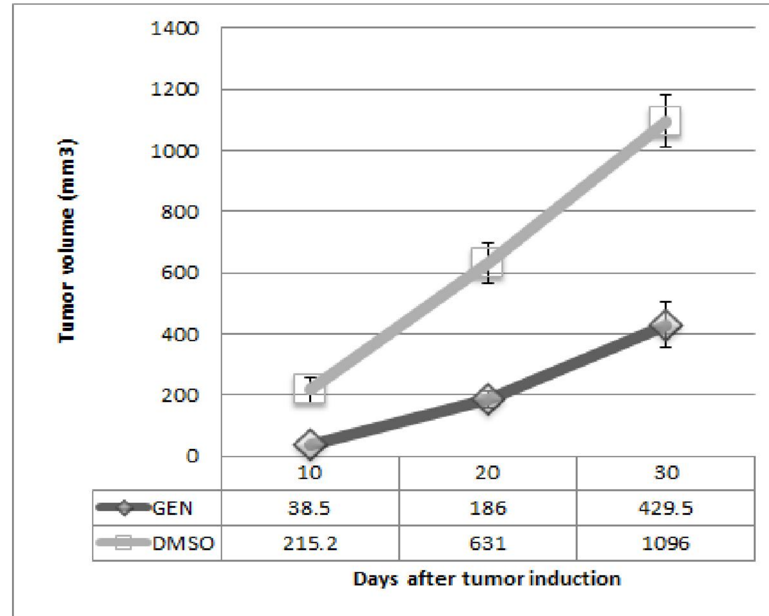

Figure 4. Therapeutic vaccination against TC-1-induced tumors. Mice were inoculated with 2×10^5 TC-1 tumor into the right flank and were monitored for the evidence of tumor growth by palpation and inspection twice a week up to 30 days after the last GEN treatment. For the determination of tumor volume, each individual tumor size was measured. Line and scatter plot graphs depicting the tumor volume (mm^3) are presented. The data presented is a representative of two independent experiments.

DISCUSSION

Immune system plays an important role in protection against tumor growth. Cytotoxic T lymphocytes constitute the major antitumor effector population against tumor growth and dissemination (10,12). Consistent with the reported chemopreventive effect of GEN, the present study demonstrated that administration of GEN by gavage for 20 days at physiologically relevant doses in adult female C57BL/6 mice significantly increased antitumor and immune modulating effects to TC-1 tumor cells in vivo models.
For evaluating the GEN antitumor responses, we examined its effects on different aspects of immune response using three immunoassay experiments, including LDH cytotoxicity test, interferon-γ and lymphocyte proliferation assay. Cytotoxicity and lymphocyte proliferation assays were carried out to test the specific cellular immune response generated by genistein treatment. The capacity to elicit an effective T cell immunity can be shown by the stimulation of lymphocyte proliferation response. Interferon-γ assay was carried out to examine whether the immune response to TC-1 tumor was in a cell-mediated pathway or not. Interferon-γ is a cytokine involved in cell mediated immune response, especially for Cytotoxic T cells and TH1 cells (13). Primed T cells usually respond quickly and strongly when they come in contact with antigens

that they have previously encountered. In the present paper, we demonstrated that GEN administration stimulates Cytotoxic T lymphocytes, T cell immunity and interferon gamma responses compared to controls.
Consistent with the tumor inhibitory effect of GEN reported in humans, increased resistance against cervical cancer was observed after exposure of mice to GEN by gavage for 20 d. This is also in agreement with previous studies that oral administration of GEN inhibited B16F10 lung-tumor nodule formation (14) and decreased B16 tumor volume (15). In another study, Gue et al. showed that at dosages of 6 and 20 mg/kg for 28 d by gavage, GEN significantly decreased B16F10 lung tumor nodule formation (16). In the present study, we used 20 mg/kg GEN for 20 days by gavage based on previous studies which have shown that this dose induces maximum immunomodulation in the mice (16).
There are several possible mechanisms that may be responsible for the inhibitory effect of GEN on cervical cancer tumor growth. Some in vitro studies have demonstrated that GEN can directly inhibit the proliferation of tumor cells (17). However, these mechanisms may not play an important role in our in vivo tumor model.
In general agreement with previous in vivo studies, our results confirm that the chemo-preventive effects of GEN may be related to its immunomodulatory effects. Immunomodulatory effects of GEN have been shown in previous studies. Gue et al. demonstrated significantly increased activities of cytotoxic T cells (CTLs) and natural killer cells by GEN in C57BL/6 mice. The authors conclude that the effect of GEN on CD4+CD25+ regulatory T cells might be responsible in part for its stimulatory effect on T cells (18). In line with these findings, our results show increased CTL and lymphocyte proliferation responses.
Another study showed that the oral administration of 20 mg/kg genistein increased IFN-γ from T cells in response to ovalbumin in female DO11.10 mice. 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 (19). Accordingly, our present findings verify that GEN treatment induces the increased secretion of IFN-γ from the T cells of GEN- treated female mice.
One of the important mechanisms by which GEN exerts its immune response effect is to interact with Estrogen Receptors (ERs) and compete with estrogen for binding (20). ERα is required for 17 β-estradiol-induced increases in IFN-γ expression and Th1 responses. It is possible that binding of GEN to ERβ would leave more free 17 β-estradiol to interact with ERα, thus enhancing T cell activity (21).The main constituent responsible for the immunomodulatory effect of genistein is an isoflavone (4, 7, 4-trihydroxyisoflavone) (22).
These results suggest that orally administrated GEN exerts its immunomodulatory effects in female mouse model of cervical cancer in part by increasing proliferation of splenic lymphocytes, elevating cytolytic activity and IFN-γ production, thereby inducing protective anti-tumor immunity. In conclusion, GEN simulates and modulates the immune response to a desired level against HPV-16 tumor-bearing mice.

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