J Food Sci Nutr
Vol 12, p 11~15 (2007)

J Food Science and Nutrition

Genistein Inhibits the Proliferation of MDA-MB-231 Cells *In Vivo*

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Abstract

This study was conducted to evaluate the influence of diets containing genistein and soy extract on the growth of MDA-MB-231 cells implanted into female Balb/c mice. Four-week-old female athymic nude mice (Balb/c) were acclimated to an AIN-93G control diet for 1 week and then injected MDA-MB-231 cells (1×10^6/site) and were continued on the on AIN-93G control diet. Five weeks after injecting the MDA-MB-231 cells (1×10^6/site), two experimental groups were assigned to diets containing genistein (750 μg/g AIN-93G diet) or 0.6% soy extract (containing genistein at 750 μg/g AIN-93G diet) until they were sacrificed. Tumor growth was significantly reduced in the groups treated with genistein and soy extract compared to the control group. The results of the proliferating cell nuclear antigen (PCNA) assay also revealed that genistein and soy extract treatment reduced the proliferation of MDA-MB-231 cells *in vivo*. In the present study, dietary isoflavone was provided just before solid tumor formation, and thus the timing of dietary isoflavone administration may be critical to the suppression of tumor growth.

Key words: genistein, soy extract, MDA-MB-231 cell, *in vivo*, tumor growth

INTRODUCTION

Breast cancer is the most frequent cause of cancer deaths in women today. Despite advances in early detection and treatment of breast cancer, the mortality rate from breast cancer has not changed appreciably. Moreover, the incidence of breast cancer increases every year in Asian countries due to changing life style (1). Early epidemiological studies indicated that soy consumption was directly correlated with reduced risk of breast cancer in Asian women. Soy-based Asian diets may be one factor that explains the lower incidence of breast cancer in those countries compared with other diets that include less soy (2,3). The lower breast cancer rates among Asian women appear to be dietary and not genetic since Asian women consuming a traditional diet high in soy have a lower incidence of breast cancer, yet when they immigrate to the USA the second generation loses this protection (4).

The primary component of soybeans associated with chemoprevention is genistein (4, 5, 7-trihydroxyisoflavone), which closely resembles steroid hormones in its structure and possess estrogen-like biological activity (5,6). Several mechanisms have been proposed for genistein's putative anticarcinogenic activity. Genistein has been shown to inhibit the growth of various cancer cells through the modulation of genes that are related to the regulation of cell growth and programmed cell death known as apoptosis (6,7). It has been found that genistein inhibits the activation of the nuclear transcription factor, NF-kappaB and Akt signaling pathway, both of which are known to maintain a balance between cell survival and apoptosis (8,9). Genistein also targets the estrogen and androgen-mediated signaling pathway in the processes of carcinogenesis. Moreover, genistein is also found to be a potent inhibitor of angiogenesis and metastasis (10,11).

However, genistein treatment has led to paradoxical actions when added to cultures of breast cancer cells, both inhibiting and stimulating cell proliferation depending on the concentration to which cells are exposed. Physiological concentrations (1 nM to 10 μM) of genistein stimulate the growth of estrogen dependent human breast cancer (MCF-7) cells *in vitro* and *in vivo* through the estrogen receptor-α (12-16) whereas high concentration of genistein (>10 μM) inhibits the growth and survival of human breast cancer cells via a route independent of estrogen receptors (12,13,16).

Animal studies demonstrated that the timing of exposure to genistein is also important in the development

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of breast cancer (17-22). Exposure to dietary genistein from the neonatal through prepubertal periods protect against chemically induced mammary cancer in rats. The protective effects include increased latency, reduced tumor incidence and multiplicity, and more rapid maturation of undifferentiated end buds to differentiated lobules. In contrast, limiting exposure to dietary genistein to the prenatal or adult periods does not predispose or protect against mammary cancer. In studies using xenograft animal model, treatment of genistein before tumor formation was effective for tumor suppression, however genistein treatment after tumor formation stimulated tumor development.

Timing of genistein exposure appears to be critical in determining its impact on the development of breast cancer. In the present study, we initiated genistein treatment in the period between breast cancer cell injection and tumor formation, which was just prior to solid tumor development, based on a preliminary experiment.

MATERIALS AND METHODS

Chemicals

Chemicals and enzymes were purchased from Sigma, Inc. (St. Louis, MO, USA) except where otherwise indicated. Genistein was obtained from Indofine Chemical Company (Somerville, NJ). Novasoy® was obtained from Archer Daniels Midland (ADM, Decatur, IL), and consisted (w/w) of 40% isoflavones (1.3:1.0:0.3 ratio of the glycosides genistin, daidzin, and glycitin), 40% natural soy components (mostly saponins), 7~12% protein, 1% fat, and 4% ash. All isoflavone forms, such as malonyl and acetyl, were included in the isoflavone determinations. The concentration of aglycones was less than 1%.

Cell culture

MDA-MB-231 cells were routinely cultured in RPMI-1640 medium (MEM, Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (FBS, Atlanta Biologics, Norcross, Georgia), 2 mM L-glutamine and 80 μg/mL penicillin, and grown at 37°C with 5% CO_2. Cells were quantified by hemocytometer counting, and were viable at greater than 90%, as determined by trypan blue exclusion.

Animal studies

Four-week-old female athymic nude mice (Balb/c) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were housed in a climate controlled room (22±2°C, 50±10% relative humidity) with a 12 hour light/dark cycle and provided diet and water *ad libitum*. The mice were acclimated to an AIN-93G control diet for 1 week and then the animals were injected with MDA-MB-231 cells (1×10^6/site) into both the right and left flank on the back of each animal and continued to receive the AIN-93G control diet. Five weeks after injecting the MDA-MB-231 cells (1×10^6/site), the animals were randomly assigned to one of three treatment groups. Each group was provided with control diet (G1), diet containing genistein (750 μg/g AIN-93G diet) (G2) or 0.6% soy extract (containing genistein at 750 μg/g AIN-93G diet) (G3) until sacrifice. During the study, tumor growth and body weight were monitored weekly, and dietary intakes were also measured every week. Animals were sacrificed 13 weeks after cell inoculation and tumors were removed.

Immunohistochemistry

Tumor cell proliferation was measured by immunohistochemical analysis using proliferating cell nuclear antigen (PCNA). Tumors were removed, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Four μm serial sections were cut from paraffin blocks and placed on slides. The slide sections were incubated in xylene to remove paraffin and rehydrated using a graded alcohol series of decreasing concentration. Visual staining was achieved using a mouse monoclonal PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by Streptividine-HRP conjugate and diaminobenzidine (DAB) substrate. Both PCNA-positive proliferating cells and total tumor cells were counted in 3-non-necreotic areas of each section using light microscopy at 400-fold magnification. The proliferation index was calculated as the percentage of PCNA-positive tumor cells to total tumor cells.

Statistical analysis

All data are expressed as the mean±standard error. Statistically significant differences ($p<0.05$) among means were determined by one-way ANOVA using a PC-based version of SPSS (Version SPSS/PC 11.5, Chicago, IL)

RESULTS

Body weight and food intake

The mice fed each diet gained weight at similar rates and no significant difference was observed among the control and experimental groups (Fig. 1). In addition, the body weights of growing animals increased with time, indicating that implanting MDA-MB-231 cells caused no physiological alterations. Food intake was also measured periodically throughout the study. From ten week and on, the food intake of soy extract treated group was somewhat higher than the other groups, however

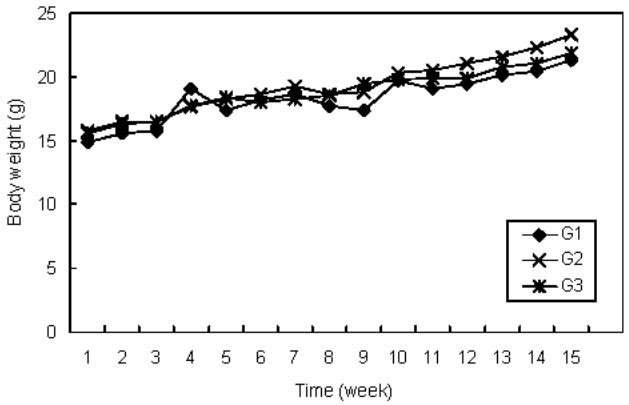

Fig. 1. Body weight change.
Values are mean±SE (n=8).
G1: DMSO+MDA-MB-231 cell (1×10^6/site),
G2: MDA-MB-231 cell (1×10^6/site)+genistein (750 μg/g AIN-93G diet), G3: MDA-MB-231 cell (1×10^6/site)+0.6% Nova soy (750 μg/g AIN-93G diet).

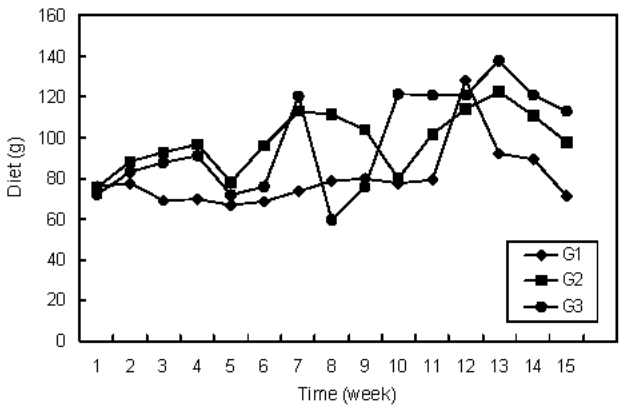

Fig. 2. Dietary intake per week.
Values are mean±SE (n=8).
Groups are the same as in Fig. 1.

no significant difference was observed among the groups (Fig. 2).

Tumor growth

To determine the treatment time, we conducted a preliminary study, which demonstrated that development of palpable tumors was first observed in mice at 7 weeks after injecting MDA-MB-231 cells. Therefore, we decided the treatment time point just before solid tumor formation at week 5 after breast cancer cell injection. After 7 weeks from injecting MDA-MB-231 cells, the tumor volumes were steeply increased in the control group, however, the genistein and soy extract significantly reduced tumor volume up to 50% compared of control (Fig. 3).

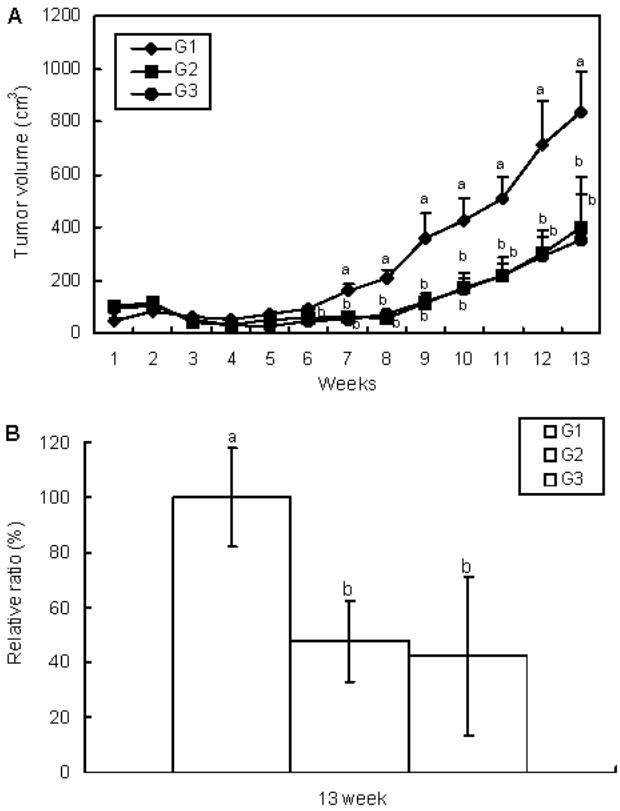

Fig. 3. Tumor growth (A) and relative ratio of tumor mass at 13 week (B) in mice implanted MDA-MB-231 cells.
Values are mean±SE (n=8).
Groups are the same as in Fig. 1.

Tumor cell proliferation

Tumor cell proliferation was measured by immunohistochemical detection of PCNA.

The results of PCNA also revealed that genistein and soy extract treatment reduced cell proliferation of tumors

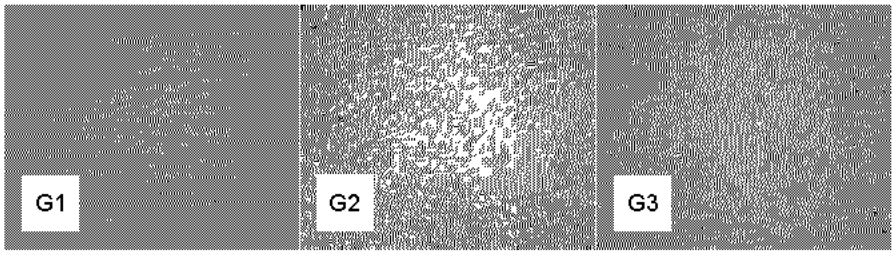

Fig. 4. The proliferation of MDA-MB-231 cells implanted in athymic mice measured by immunohistochemical analysis using proliferating cell nuclear antigen (PCNA). Groups are the same as in Fig. 1.

implanted in athymic nude mice (Fig. 4).

DISCUSSION

In the present study, the effect of genistein on mammary tumor growth in mice was investigated with dietary supplementation of pure genistein and a standardized commercial soy extract containing genistein. Animal studies on genistein and mammary tumor growth have shown conflicting data. In particular, stimulatory effects of genistein on MCF-7 tumors have been generally reported. Helferich's group (20) observed that dietary treatment with genistein dose-dependently enhanced the growth of MCF-7 tumors *in vivo*. Additionally, dietary genistin, the glycoside form of genistein, stimulated the growth of MCF-7 tumors transplanted in ovariectomized athymic mice (23). Soy protein isolates containing increasing concentrations of isoflavones stimulated dose-dependent growth of MCF-7 tumors transplanted in ovariectomized athymic mice (24). On the other hand, using an estrogen maintained animal model, genistein and soy isoflavones inhibited the growth of MCF-7 tumors (25). In addition, Shao et al showed an inhibitory effect of genistein in MDA-MB-231 cells implanted in athymic mice. They treated the tumor-bearing mice with 500 μg of genistein by oral administration. In contrast, Santell et al. (26) obtained a negative result when they treated the tumor-bearing mice with 750 μg of genistein/g diet. However, in the present study genistein (750 μg/g diet) in pure form and soy extract inhibited tumor growth in mice bearing MDA-MB-231 xenografts, which is similar to the results reported by Hewitt and Singletary (27). In their study, mice were fed diets supplemented with 750 ppm genistein and soy extract containing genistein at 750 ppm for 5 days prior to injection with F3II cells. The results showed that genistein and soy extract exhibited a significant reduction in F3II tumor growth compared to controls, Yuan et al. (28) showed that the treatment of MDA-MB-231 bearing mice with a relative high dose of genistein (about 4 mg per mouse per day) resulted in statistically significant tumor regression. These findings suggest that the inhibitory effect of genistein on tumor growth *in vivo* may be dependent on the dose of genistein administered and ovarian status.

The previous researches demonstrated that the timing of exposure to genistein is important for inhibiting breast cancer development (17-22). Genistein administration during early the period of life protected against chemically induced mammary cancer in rats, however, limiting exposure to dietary genistein in adults does not protect against mammary cancer. Similarly, dietary genistein has been found to both reduce and promote mammary cancer development in different animal model depending on treatment time. Hewitt and Singletary (27) provided 750 ppm genistein in the diet before F3II cell injection and the results exhibited a significant reduction in F3II tumor growth. Zhou et al. (25) also reported that the pretreatment with genistein before breast cancer cell injection significantly reduced MCF-7 tumor growth. Mammary tumor latency was significantly delayed in mammary tumor virus (MMTV)-neu mice fed genistein (29). Once tumors formed, however, genistein did not reduce the number or size of tumors. In contrast, treatment of isoflavone-containing soy extract (soy select) from the beginning of breast cancer cell injection did not stimulate and nor inhibit MCF-7 and MDA-MB-231 cells implanted in athymic mice (30). Of interest in the present study is that the development of tumor was very slow, which is similar to Zhou et al. (25). Therefore, we determined the time of exposure to genistein in the mid of breast cell injection and palpable tumor formation based on the preliminary experiment, notably which is not comparable to other studies. As a result, genistein and soy extract significantly reduced the growth of MDA-MB-231 tumor and no difference between genistein and soy extract was observed. Additionally, the data from the PCNA assay demonstrated that genistein and soy extract treatment inhibited cell proliferation of tumors implanted in athymic mice. Presumably, the timing of dietary isoflavone administration, just before solid tumor formation, was critical to suppressing tumor growth.

ACKNOWLEDGEMENT

This work was supported by the Korea Research Foundation Grant RO5-2003-000-12154-0 (2004) funded by the Korean Government.

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(Received January 24, 2007; Accepted February 28, 2007)