

Dietary Genistein Reduces Metastasis in a Postsurgical Orthotopic Breast Cancer Model

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Abstract

Metastatic spread, not primary tumor burden, is the leading cause of breast cancer deaths. For patient prognosis to improve, new systemic adjuvant therapies that are capable of effectively inhibiting the outgrowth of seeded tumor cells after surgical treatment of the primary breast tumor are needed. To facilitate the preclinical development of such therapies, relevant animal models of breast cancer metastasis that can mimic the postsurgical adjuvant setting are required. Here we developed a preclinical xenograft model of breast cancer metastasis where the primary tumor was removed by surgical resection before systemic adjuvant treatment. We used this model to assess the antimetastatic effect of postsurgical dietary intervention with the soy isoflavone genistein. The anticancer activity of genistein has been established *in vitro* and *in vivo*, however, few studies have tested the potential of genistein as an antimetastatic therapy. Using our model, we tested the efficacy of adjuvant treatment with genistein to inhibit the outgrowth of metastases postsurgery. To establish primary tumors, human breast carcinoma cells, MDA-MB-435/HAL, were implanted into the mammary fat pad of female nude mice. Primary tumors were left to grow for 5 weeks before being surgically removed. Mice were then randomized into two diet groups: control soy-free diet versus genistein-supplemented diet. Five weeks later, metastatic burden was assessed. Genistein reduced the percent metastatic burden in the lungs by 10-fold. These results indicate that dietary intervention following cancer surgery can affect the outgrowth of seeded tumor cells. The availability of well-characterized, clinically relevant animal models for studying factors that regulate metastatic outgrowth postsurgery will provide an important tool for developing new systemic adjuvant therapies. (Cancer Res 2005; 65(8): 3396-403)

Introduction

Breast cancer remains the most common cancer among women worldwide, with more than 1 million new cases and ~370,000 deaths each year (1). Most breast cancer deaths are due to metastatic disease, not primary tumor burden (2, 3). Metastasis is a multistep process that involves the spread of malignant cells from the primary tumor and the subsequent growth of secondary

tumors in distant tissues and organs of the body (4, 5). For many patients, cancer cells have already been shed from the tumor at the time of initial diagnosis even if metastases are not yet clinically detectable (6, 7). Despite the benefits of improved screening techniques and the advantage of early diagnosis, up to 30% of women treated for stage I breast cancer will relapse with distant metastatic disease within 5 years (8–10). In most cases, surgery and various combinations of radiation therapy, chemotherapy, hormone therapy, and immunotherapy provide effective interventions for controlling the primary tumor burden. Whereas metastatic breast cancer may respond to a variety of these hormonal and chemotherapeutic interventions, it is rare that such therapy is curative (11, 12). If patient survival is to improve, it is imperative that new systemic adjuvant therapies capable of effectively inhibiting the outgrowth of metastases be developed. However, most new antimetastatic therapies are tested in preclinical models for their ability to reduce the growth of primary tumors and not for their ability to affect disseminated tumor cells. Agents that are designed to affect metastasis should be tested in preclinical models of metastasis. Here we present a preclinical xenograft model of breast cancer metastasis where the primary tumor is physically removed by surgical resection before systemic adjuvant therapy, and describe the use of this model to test postsurgical dietary intervention with the soy isoflavone genistein.

Compelling evidence from both clinical and laboratory studies indicates that many dietary constituents, including soy, play a role in cancer prevention (13–16). Epidemiologic studies suggest that the increased consumption of soy products by Asian women contributes to lower breast cancer incidence and mortality rates compared with women from industrialized Western nations (17–19). The protective effects of soy-based diets may be related to the high levels of naturally occurring hormone-like isoflavones found in soybeans (20–22). One of the more active soy isoflavones is genistein (4',5,7-trihydroxyisoflavone), a heterocyclic diphenolic compound structurally similar to 17 β -estradiol with binding affinity for both the α and β isoforms of the estrogen receptor (23–25). Studies have shown that competitive inhibition of the estrogen receptors by genistein at pharmacologic doses results in antiproliferative effects on estrogen receptor–positive cancer cells (26–29). However, genistein has also been reported to inhibit proliferation of estrogen receptor–negative cancer cells (29–31). This effect may be due to the ability of genistein to elicit a wide range of non estrogen receptor–mediated intracellular responses, some of which include potent inhibition of tyrosine kinases, stabilization of topoisomerase II, and transcriptional regulation of transforming growth factor β (32–35). By these mechanisms genistein has been shown to interrupt cell cycle progression, trigger apoptosis, and induce differentiation in a variety of cancer cell types (36–39). Moreover, the antitumor actions of genistein

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are not limited to inhibiting tumor cell proliferation but also include the ability to inhibit both tumor cell invasion and angiogenic activity, suggesting that genistein may have potential as an antimetastatic compound (40–44).

Although genistein has been described as having antimetastatic properties, much of the research has focused on testing the ability of genistein to prevent or shrink primary tumors. Very few studies have examined the efficacy of genistein as treatment for metastatic disease (45–47). In the present study, we used the spontaneously metastasizing MDA-MB-435/HAL human breast cancer xenograft model to examine the effect of genistein treatment in an adjuvant setting. We investigated whether a genistein-supplemented diet, given following surgical resection of primary mammary tumors, could inhibit the outgrowth of metastases.

Materials and Methods

Cell culture. The human breast carcinoma cell line MDA-MB-435/HAL is a GFP-expressing metastasis-derived variant of the MDA-MB-435 cell line (a gift from Dr. David Griggs). This subcloned variant was isolated after multiple *in vivo* passages and was selected for its enhanced tumor growth rate and increased pulmonary metastasis (48). The cells were grown in minimal essential medium with Earle's salts and L-glutamine (MEM, Invitrogen, Burlington, Ontario, Canada), supplemented with 25 mmol/L HEPES buffer (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 1× MEM vitamin solution (Sigma Chemical, St. Louis, MO) and 10% fetal bovine serum (Sigma). Cultures were maintained in a humidified incubator with 5% CO₂ at 37°C and passaged at subconfluency. Cells were harvested by washing briefly with PBS followed by incubation at room temperature with 0.125% trypsin. For *in vivo* injection, cells were harvested at ~70% confluency (log phase of growth), washed twice with sterile PBS, and resuspended at a concentration of 2×10^6 cells/100 μ L PBS.

Animals and diet formulation. Female athymic nude mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Upon arrival animals were maintained under specific pathogen-free conditions in microisolator cages in an isolated colony under controlled light (12-h light and dark cycle), temperature (22–24°C), and humidity (25%). During the course of the experiment, mice were provided with sterilized food and water *ad libitum*. The AIN-76A diet (Harlan Teklad, Madison, WI; ref. 49), a semipurified casein-based diet containing no detectable phytoestrogens (limit of detection, 5 pmol/mL), was selected as the control diet. The genistein diet was based on the control diet, supplemented with 750 μ g/g of genistein and has been used by others in dose response analyses (50–52). This concentration has been shown by others to yield serum genistein levels in mice similar to those observed in humans consuming a diet containing modest amounts of soy products (reviewed in ref. 53). Genistein was chemically synthesized (Toronto Research Chemicals Inc., Toronto, Ontario) and incorporated into the AIN-76A diet at Harlan Teklad. Prepared diets were sterilized by irradiation and stored at –20°C until use. Fresh diet was provided every 5 days and food intake was monitored throughout the studies. Animal care and surgical procedures were conducted in accordance with the standards of the Canadian Council on Animal Care under an approved protocol of the University of Western Ontario Council on Animal Care.

Tumor model and surgical procedures. When mice were 8 weeks of age, tumor cells were injected into the right thoracic mammary fat pad of all mice ($n = 120$) to produce orthotopic primary tumors. A 1.5-cm incision was made on the chest slightly right of the sternum and the skin separated from the chest by gentle blunt dissection. MDA-MB-435/HAL cells (2×10^6 cells/100 μ L PBS) were injected into the animal's right mammary fat pad and the incision closed with wound clips (54). Primary tumor growth was evaluated by measurement with calipers every 5 days. Tumors were measured in two perpendicular dimensions and the volume was estimated using the formula [volume = $0.52 \times (\text{width})^2 \times (\text{length})$] for approximating the volume (mm³) of an ellipsoid.

Tumor resection involved making an elliptic incision around the tumor and dissecting it away from the chest wall. The tumor mass was gently removed with a small margin of the surrounding skin and associated soft tissue (~2 mm) and the incision closed with wound clips. Sham treatment involved making a 2-cm incision lateral to the primary tumor, briefly manipulating the tumor with forceps, leaving the primary mammary fat pad tumor intact and closing the incision with wound clips.

All surgical procedures were done under general anesthesia using a ketamine/xylazine mixture (1.6 mg of ketamine and 0.08 mg of xylazine per 15 g of body mass) administered by i.p. injection.

Study design: protocol of animal experiments. Two studies were conducted to evaluate the effect of genistein on breast tumor growth and metastasis (Fig. 1). The aim of Study 1 was to determine the effect of a genistein-supplemented diet on the growth and metastasis of MDA-MB-435/HAL primary mammary fat pad tumors (unresected). Mice were assigned to either the control diet group ($n = 16$) or the genistein-supplemented diet group ($n = 16$) before tumor cell injection and remained in their diet groups for the duration of the study. Mice were

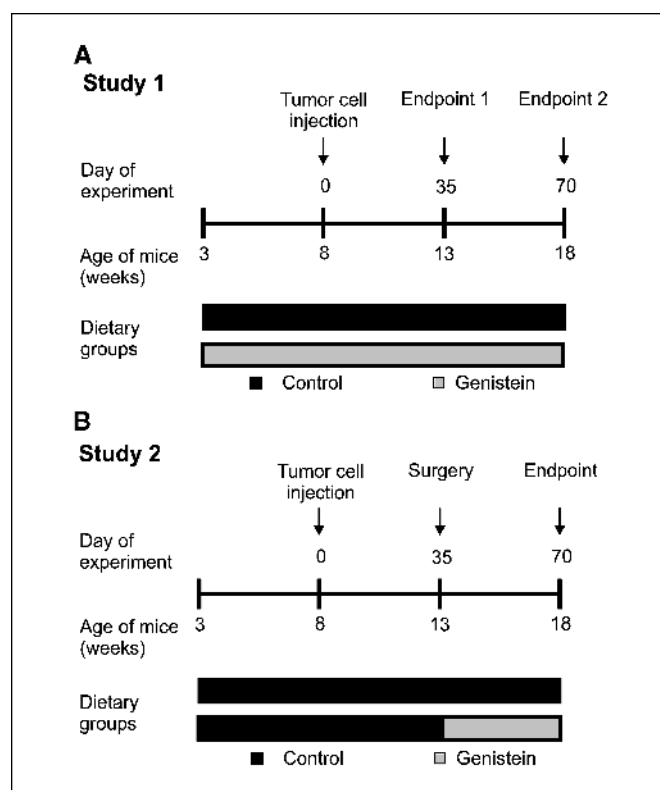


Figure 1. Schematic diagramming the design of the animal experiments. **A**, Study 1 was designed to determine the effect of a genistein-supplemented diet on the growth and metastasis of MDA-MB-435/HAL primary mammary fat pad tumors. Female athymic nude mice were assigned to either the AIN-76A control diet (*Control*, ■; $n = 16$) or AIN-76A supplemented with 750 μ g genistein/g (*Genistein*, ▒; $n = 16$) for the duration of Study 1. At 8 weeks of age (experiment day 0), 2×10^6 MDA-MB-435/HAL cells were injected mammary fat pad and tumor growth was monitored. At endpoints 1 and 2 (experiment days 35 and 70, respectively), mice ($n = 8$) from each diet group were necropsied and examined for metastases. **B**, Study 2 was designed to determine whether a genistein-supplemented diet, given following resection of MDA-MB-435/HAL primary tumors, could inhibit the outgrowth of metastases. All mice were initially fed the AIN-76A control diet ($n = 88$). At 8 weeks of age (experiment day 0), 2×10^6 MDA-MB-435/HAL cells were injected mammary fat pad. On day 35 surgeries were carried out and primary tumors (mean tumor volume ~ 1,750 mm³) were either resected ($n = 44$) or a sham operation was done ($n = 44$). Following surgery, each treatment group (resection and sham) was randomly divided into two diet groups: AIN-76A control diet (*Control*, ■) and AIN-76A supplemented with 750 μ g genistein/g (*Genistein*; ▒). Mice remained on their assigned postsurgery adjuvant diets for 35 days, at which point mice were necropsied (experiment day 70) and examined for metastases.

injected mammary fat pad at 8 weeks of age with MDA-MB-435/HAL cells to initiate tumor growth and the tumors were left to grow for 35 or 70 days, at which times mice were necropsied and assessed for metastases (Fig. 1A). The aim of Study 2 was to determine whether a genistein-supplemented diet, given only following primary mammary tumor resection, could inhibit the outgrowth of metastases. In this study, all mice ($n = 88$) were initially fed the control diet. Mice were injected mammary fat pad at 8 weeks of age with MDA-MB-435/HAL cells and when the mean tumor volume reached $\sim 1,750 \text{ mm}^3$ (~ 35 days after mammary fat pad injection), primary tumors were either resected ($n = 44$) or a sham operation was done ($n = 44$). Immediately following surgery, each treatment group (resection and sham) was randomly divided into two diet groups: control diet and genistein diet. Mice remained on their assigned postsurgery adjuvant diets for 35 days, at which point mice were necropsied (Fig. 1B).

Histologic and immunohistochemical analysis of metastases. At the end points, mice were necropsied and organs (including lungs, lymph nodes, chest wall, brain, liver, spleen, pancreas, kidney, and ovary) were examined for surface metastases using a dissecting microscope ($\times 20$). The lungs and the axillary lymph node from each mouse were fixed in 10% neutral-buffered formalin, embedded in paraffin, and thin sectioned ($4 \mu\text{m}$). Tissue sections were stained with H&E and examined by light microscopy for metastases.

The percent metastatic burden in the lungs was quantified using a stereological point counting method (55, 56). One H&E-stained slide per mouse, with sections of all five lung lobes, was sampled using a light microscope ($\times 200$). Images of each field of view were systematically taken from the whole tissue area and transferred by video camera to a computer screen. A grid was superimposed on each field of view and the number of intersections (9 points per field of view) falling on normal tissue and tumor tissue was counted. Percent metastatic burden for each mouse was calculated as the ratio of tumor points to all points counted $\times 100$ and based on $>1,000$ total points counted.

Proliferative and apoptotic activity within lung metastases was evaluated using $4\text{-}\mu\text{m}$ sections cut adjacent to the H&E-stained lung sections. Immunohistochemical staining with anti-Ki-67 monoclonal antibody, clone MMI1 (1:150; Vector Laboratories, Burlington, Ontario, Canada), was used to analyze nuclear expression of the Ki-67 cell proliferation antigen as described previously (57). To detect apoptotic cells, a standard terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) method was used according to the protocol of the manufacturer (ApopTag Peroxidase *In situ* Apoptosis Detection Kit S7101, Chemicon International, Temecula, CA). The numbers of positively labeled proliferating and apoptosing cells were quantified and expressed as percentages of the total number of tumor cells counted. A minimum of 500 tumor cells, in randomly selected fields, was scored per slide (magnification, $\times 400$). All slides were analyzed and scored in a blind fashion without knowledge of the treatment groups.

Statistical analysis. Data are expressed as the mean \pm SE. Statistical analyses were done using Sigma Stat version 2.03 for Windows (Access Softek Inc., San Rafael, CA). A two-sided z test was used to compare proportions. Differences between means were determined using the Student's t test when groups passed both a normality test and an equal variance test. When this was not the case, the Mann-Whitney rank sum test was used. A level of $P \leq 0.05$ was regarded as statistically significant.

Results

Study 1. During Study 1 the body weight and food consumption of mice in both the control diet group and the genistein diet group were monitored. Supplementation with genistein was not associated with food avoidance or toxic side effects and no significant difference in food intake or body weight was recorded between the two diet groups either before or during the experimental period (Table 1).

Table 1. Body weight and food intake of nude mice fed control diet or genistein diet

	Body weight, g			Food intake,* g/cage/d
	3 weeks of age [†] ($n = 16$)	8 weeks of age [‡] ($n = 16$)	13 weeks of age [§] ($n = 16$)	
Control diet	12.7 \pm 0.3	22.9 \pm 0.5	25.3 \pm 0.5	12.5 \pm 0.24
Genistein diet	12.5 \pm 0.4	22.6 \pm 0.4	25.1 \pm 0.6	12.6 \pm 0.18

NOTE: Values are means \pm SE; n , number of mice.

*Food intake recorded daily for 7 days following mammary fat pad injection (4 mice/cage).

[†]Initial body weight.

[‡]Body weight at mammary fat pad injection.

[§]Body weight 35 days post mammary fat pad injection.

The effect of genistein on the growth of orthotopic MDA-MB-435/HAL primary tumors is shown in Fig. 2. Animals were started on either the control diet or the genistein diet on arrival and remained on their assigned diet for the duration of Study 1 (Fig. 1A). An inoculum of 2×10^6 MDA-MB-435/HAL human breast carcinoma cells was injected into the mammary fat pad of mice at 8 weeks of age. The tumors first became palpable 5 days after injection and tumor take was 100% in both diet groups. Tumor volume was slightly but significantly lower in the genistein group from day 10 to day 30 ($n = 16$; $P \leq 0.05$; Fig. 1). By day 35, the mean tumor volume in both diet groups reached $\sim 1,750 \text{ mm}^3$; thereafter, no statistically significant difference in mammary tumor volume between the two groups was observed.

The incidence of lung and lymph node metastases in mice bearing primary mammary fat pad tumors fed the control diet versus the genistein diet is summarized in Table 2. After 35 days of primary tumor growth, metastases were histologically evident in some mice in both groups. The incidence of lung metastases was not statistically different between the control and genistein groups and the percent metastatic burden was calculated to be $0.15 \pm 0.049\%$ and $0.079 \pm 0.044\%$ (SE), respectively ($P = 0.327$; Fig. 3A). By 70 days, however, mice on the genistein-supplemented diet had a significantly lower metastatic burden in the lungs compared with the control-fed mice. Whereas only 38% of mice in the genistein group (3 of 8) had surface metastases, 100% of the mice in the control group (8 of 8) had prominent surface lesions ($P = 0.007$; Table 2). Along with inhibiting the incidence of lung metastases, genistein also significantly limited the metastatic burden to $16.25 \pm 8.98\%$ (SE) of the total lung volume as compared with $46.89 \pm 3.25\%$ of the total lung volume in the control group ($P = 0.023$; Fig. 3B). Pleural metastases were also grossly identified; as well, there was histologic evidence of axillary lymph node involvement, although the incidence was not significantly different between the control and genistein groups (Table 2). Superficial metastases were not seen in other organs in either group.

To investigate whether the reduction in metastatic burden observed with genistein supplementation was due to increased apoptosis and/or decreased proliferation within the metastases, we identified apoptotic tumor cells by TUNEL staining and proliferating tumor cells by Ki-67 immunohistochemistry. Compared with mice fed the control diet, mice treated with dietary

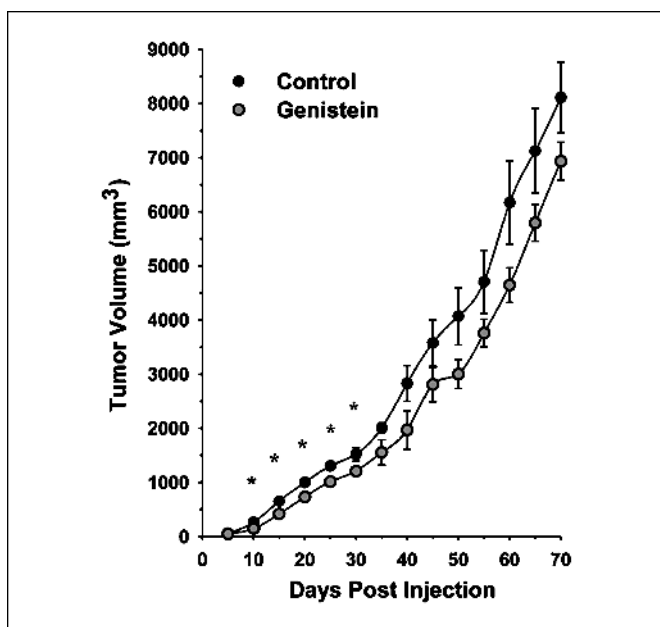


Figure 2. Effect of genistein on the growth of MDA-MB-435/HAL mammary fat pad primary tumors (Study 1). Nude mice were fed either the AIN-76A control diet ($n = 16$) or AIN-76A supplemented with 750 μg genistein/g ($n = 16$) before tumor cell injection through end point. At injection, 2×10^6 MDA-MB-435/HAL cells were implanted into mammary fat pad in 8-week-old mice. Points, mean tumor volume; bars, SE. Tumor growth was found to be significantly reduced in the genistein group versus the control group for days 10 to 30 (*, $P < 0.05$).

genistein showed significantly decreased percentages of proliferating tumor cells ($P = 0.041$; Fig. 3C) within lung metastases as well as increased percentages of tumor cells undergoing apoptosis ($P = 0.02$; Fig. 3D).

Study 2. The effect of adjuvant administration of a genistein-supplemented diet on the incidence of local-regional tumor regrowth and distant metastasis is summarized in Table 3. From Study 1 we determined our surgery time point to be 35 days post mammary fat pad injection, as $>50\%$ of the mice had histologic evidence of metastatic disease at this time. In Study 2 all animals were started on the control diet and underwent tumor resection or

sham operation following 35 days of primary tumor growth (Fig. 1B). After surgery, animals were divided into two diet groups (control diet and genistein diet) and remained on their postsurgery diets for an additional 35 days. In the resection groups primary tumor regrowth occurred in 6 of 22 (27%) of genistein-fed mice and in 10 of 22 (46%) control-fed mice ($P = 0.191$; Table 3); these animals were excluded from any analysis of metastatic burden. At necropsy the incidence of surface lung metastases was not statistically different between the diet groups ($P = 0.161$; Table 3). Similarly, there was no difference in incidence between the diet groups when histologic sections were examined ($P = 0.315$; Table 3). However, when the metastatic burden was quantified, it was revealed that genistein had significantly limited the metastatic burden to $0.47 \pm 0.27\%$ (SE) of the total lung volume as compared with $4.85 \pm 2.19\%$ of the total lung volume in the control group ($P = 0.047$; Fig. 4A). Post-resection adjuvant treatment with genistein thus reduced the percent metastatic burden in the lungs by 10-fold compared with the post-resection control group. Moreover, when compared with the no-surgery control group in Study 1 (Fig. 3B), the combination of tumor resection and adjuvant treatment with genistein reduced the percent metastatic burden 100-fold.

Histologic examination of axillary lymph nodes revealed that post-resection treatment with genistein completely inhibited lymph node metastases (0 of 16 mice) as compared with the control-fed group where 25% (3 of 12) of the mice were lymph node positive ($P = 0.034$; Table 3). Pleural metastases were also grossly identified, although the incidence was not significantly different between the control and genistein groups (5/12 versus 3/16; $P = 0.184$; Table 3).

Ki-67 immunohistochemistry and TUNEL staining again showed that, compared with control diet group, post-resection treatment with dietary genistein significantly decreased the percentage of proliferating tumor cells ($P = 0.01$; Fig. 4B) and significantly increased the percentage of tumor cells undergoing apoptosis ($P = 0.036$; Fig. 4C) within lung metastases.

In the sham groups, ulceration of the primary tumor occurred only in the genistein group (4 of 22 mice) and not in the control group (not significant; $P = 0.119$). These animals were euthanized before the end point and were therefore excluded from any analysis

Table 2. Effect of genistein on the incidence of spontaneous metastases from MDA-MB-435/HAL primary mammary fat pad tumors

Dietary group	Axillary lymph node metastasis	Pulmonary metastasis		Pleural metastasis
		Superficial evidence	Histologic evidence	
Control (35 days p.i.)*	0/8	0/8	5/8	0/8
Genistein (35 days p.i.)	0/8	0/8	3/8	0/8
Control (70 days p.i.)	4/8	8/8	8/8	5/8
Genistein (70 days p.i.)	3/8	3/8†	4/8†	2/8

NOTE: Number of mice with metastases/total number of tumor-bearing mice. Nude mice were fed either control or genistein diets before mammary fat pad injection with MDA-MB-435/HAL cells. Post mammary fat pad injection, mice were maintained on their respective diets for 35 or 70 days whereas primary tumors grew (Study 1). At necropsy, the axillary lymph nodes adjacent the primary tumor were dissected free and lungs were examined for superficial metastases by dissecting microscope. H&E-stained thin sections of lymph nodes and lungs were examined for the presence of metastases.

*p.i., postinjection.

†Significantly different from the control diet group at 70 days p.i.; z test, $P < 0.05$.

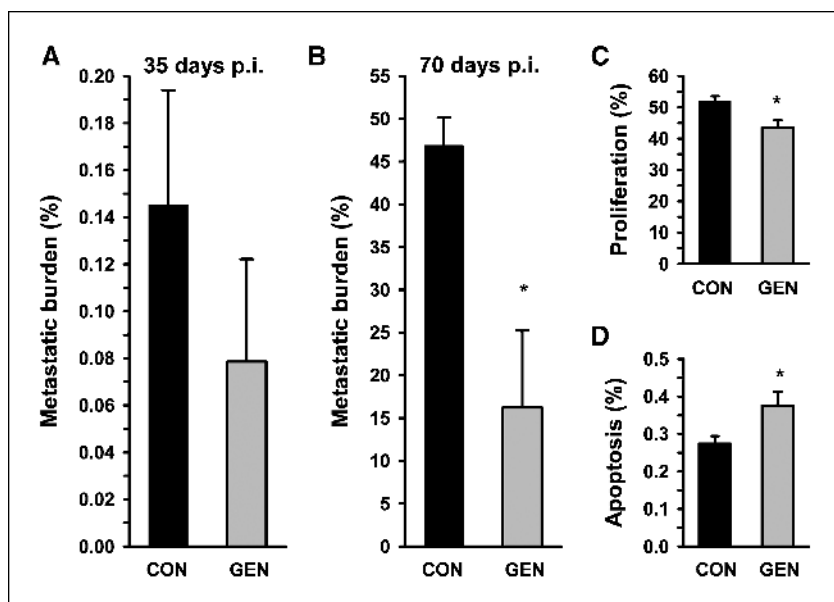


Figure 3. Effect of genistein on the metastatic burden in the lungs of nude mice with intact MDA-MB-435/HAL primary tumors (Study 1). The percent metastatic burden in the lungs was quantified using a stereological point counting method. **A**, lungs of control-fed ($n = 8$) and genistein-fed ($n = 8$) mice were assessed 35 days post mammary fat pad injection. There was no difference in metastatic burden between control-fed and genistein-fed mice. **B**, lungs of control-fed ($n = 8$) and genistein-fed mice ($n = 8$) were assessed 70 days post mammary fat pad injection. Genistein-fed mice had significantly reduced metastatic burden in the lungs. **C**, Ki-67 immunohistochemistry revealed significantly lower levels of proliferating cells within the metastases of genistein-fed mice ($n = 7$) versus control-fed mice ($n = 8$). **D**, TUNEL staining revealed significantly higher levels of apoptosing tumor cells within metastases of genistein-fed mice ($n = 7$) versus control-fed mice ($n = 8$). Columns, mean percentage; bars, SE. Statistical significance was calculated by comparing means of the control group (CON, ■) with means of the genistein group (GEN, □); *, $P < 0.05$.

of metastatic burden. At necropsy the incidence of surface lung metastases was not statistically different between the diet groups ($P = 0.131$; Table 3). Histologic examination of the lung tissue also revealed no significant difference in incidence ($P = 0.871$; Table 3). However, when the metastatic burden was quantified, genistein was seen to have had significantly limited the metastatic burden to $5.9 \pm 1.83\%$ (SE) of the total lung volume as compared with $14.71 \pm 3.54\%$ of the total lung volume in the control group ($P = 0.046$; Fig. 4D). Adjuvant treatment with genistein following a sham operation thus reduced the percent metastatic burden in the lungs by more than 2-fold compared with the post-sham control group. Interestingly, when compared with the no-surgery control group in

Study 1 (Fig. 3B), sham surgery alone reduced the percent metastatic burden 3-fold.

Histologic examination of axillary lymph nodes revealed that treatment with genistein following sham operation significantly inhibited lymph node metastases to 6% (1 of 18) compared with the control fed group where 45% (10 of 22) of the mice were lymph node positive ($P = 0.005$; Table 3). The incidence of pleural metastasis was also dramatically reduced in post-sham, genistein-treated animals where only 22% (4 of 18) had gross metastases as compared with 64% (14 of 22) of control-fed animals ($P = 0.009$; Table 3).

Ki-67 immunohistochemistry revealed significantly lower levels of proliferating cells within the lung metastases of post-sham,

Table 3. Effect of genistein on the incidence of metastases 35 days following surgical resection or sham treatment of MDA-MB-435/HAL primary mammary fat pad tumors

Treatment group	Local recurrence	Axillary lymph node metastasis	Pulmonary metastasis		Pleural metastasis
			Superficial evidence	Histologic evidence	
Control diet postsurgery*	10/22	3/12	3/12	6/12	5/12
Genistein diet postsurgery [†]	6/22	0/16 [‡]	1/16	5/16	3/16
Control diet post-sham	n.a.	10/22	5/22	14/22	14/22
Genistein diet post-sham [§]	n.a.	1/18	1/18	11/18	4/18

NOTE: Number of mice with metastases/total number of mice included in group. Nude mice were fed the control diet before mammary fat pad injection with MDA-MB-435/HAL cells. Post mammary fat pad injection, mice were maintained on the control diet until the mean tumor volume measured $1,750 \text{ mm}^3$ (~35 days post injection; Study 2). Primary tumors were either surgically resected or a sham operation was done. Mice were then randomly divided into two groups: control diet fed or genistein diet fed. At necropsy (35 days postsurgery), the axillary lymph nodes adjacent the primary tumor were dissected free and lungs were examined for superficial metastases by dissecting microscope. H&E-stained thin sections of lymph nodes and lungs were examined for the presence of metastases.

*Ten mice were excluded from analysis due to local recurrence of the primary tumor.

[†]Six mice were excluded from analysis due to local recurrence of the primary tumor.

[‡]Significantly different from the postsurgery control diet group; z test, $P < 0.05$.

[§]Four mice were excluded from the study due to ulcerating tumors (euthanized before end point).

^{||}Significantly different from the post-sham control diet group; z test, $P < 0.05$.

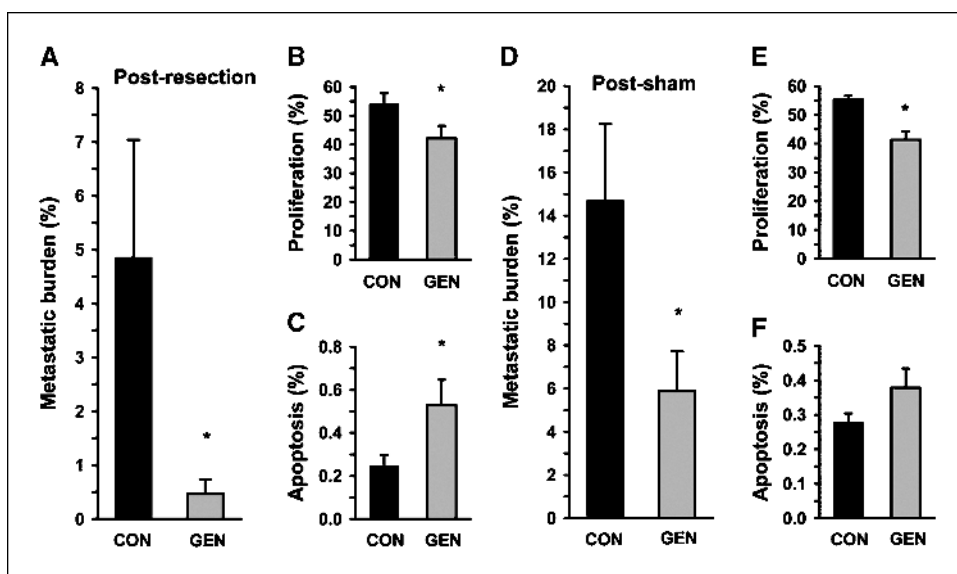


Figure 4. Metastatic burden in the lungs of nude mice treated with genistein for 35 days following tumor resection (A-C) or a sham operation (D-F; Study 2). The percent metastatic burden in the lungs was quantified using a stereological point counting method. A, post-resection, mice fed genistein ($n = 16$) had significantly reduced metastatic burden in the lungs compared with control-fed mice ($n = 12$). B, Ki-67 immunohistochemistry revealed significantly lower levels of proliferating cells within the metastases of genistein-fed mice ($n = 6$) versus control-fed mice ($n = 9$). C, TUNEL staining revealed significantly higher levels of apoptosing tumor cells within metastases of genistein-fed mice ($n = 6$) versus control-fed mice ($n = 9$). D, post-sham (i.e., tumor intact) mice fed genistein ($n = 18$) had significantly reduced metastatic burden in the lungs compared with control-fed mice ($n = 22$). E, Ki-67 immunohistochemistry revealed significantly lower levels of proliferating cells within the metastases of genistein-fed mice ($n = 12$) versus control-fed mice ($n = 19$). F, TUNEL staining revealed a trend towards increased (but not statistically significant) levels of apoptosing tumor cells within metastases of genistein-fed mice ($n = 12$) versus control-fed mice ($n = 19$). Any mice with evidence of local tumor regrowth (resection group) or ulcerating tumors (sham group) were excluded from analysis. Columns, mean percentage; bars, SE. Statistical significance was calculated by comparing means of the control group (■) with means of the genistein group (▒); *, $P < 0.05$.

genistein-fed mice versus post sham, control-fed mice ($P < 0.001$; Fig. 4E). A trend towards increased (but not statistically significant; $P = 0.24$) levels of apoptosing tumor cells within metastases of genistein-fed mice versus control-fed mice was also found by TUNEL staining (Fig. 4F).

Discussion

Whereas surgery often results in the successful removal of the primary tumor, if breast cancer cells have already seeded other organs before surgery, the outgrowth of these metastases commonly leads to treatment failure and even death in breast cancer patients. Treatment with conventional cytotoxic agents or other therapies given after surgical resection has improved the outcome for women with operable breast cancer by reducing locoregional relapses (58). Unfortunately, for breast cancer patients who develop distant metastatic disease, response to these adjuvant treatments is only transient and the majority of patients have evidence of progressive disease within 12 to 24 months of initiating treatment (59). Thus, there is an urgent need for new adjuvant therapies and novel agents that are more effective and less toxic than those currently available, particularly for those women who are at high risk for recurrent breast cancer.

Developing experimental animal models that are more akin to the clinical situation is crucial to the development of new types of postsurgical adjuvant treatments. The prevailing model used in preclinical animal studies is the primary tumor xenograft model where agents are tested for their ability to reduce the growth of often ectopic (e.g., subcutaneous) but sometimes orthotopic (mammary fat pad) primary tumors. Preclinical testing of therapies designed to be used as adjuvant treatment to prevent metastatic disease would be more valuable if studied in models of

metastasis. Clinically, systemic adjuvant therapy is given to women with breast cancer following surgical removal of the primary tumor (or in the case of neo-adjuvant therapy, just before surgery), with the aim of preventing outgrowth of cancer cells seeded before surgery. We propose that a more appropriate, clinically relevant preclinical model would assess therapies for their ability to prevent outgrowth of cancer cells known to have seeded from a primary tumor following surgical resection of the tumor. The requirements for such a model include implantation of an orthotopic mammary fat pad tumor in mice; surgical removal of the primary tumor, at a time when tumor cells are known to have been seeded to secondary organs; and treatment with agents following surgery in an adjuvant setting, with the intent to prevent early or late metastatic outgrowth. Here we present a preclinical xenograft model of breast cancer metastasis where the primary tumor is physically removed by surgical resection before systemic adjuvant treatment.

First we characterized the metastatic kinetics of MDA-MB-435/ HAL cells in nude mice (Study 1) and then we evaluated the antimetastatic effects of genistein treatment in a postsurgical adjuvant setting (Study 2). Genistein is a soy-derived isoflavone that elicits pleiotropic molecular effects on tumor cells (32–44). As a potential antimetastatic agent, genistein offers several therapeutic advantages compared with most conventional chemical anticancer agents, as it is a natural compound and has been shown to inhibit the growth of various cancer cells *in vitro* and *in vivo* without toxicity to normal cells (particularly desirable when long-term therapy is necessary; refs. 60–63). The anticancer activity of genistein has in part been attributed to the fact that it acts in a cytostatic fashion by facilitating cell cycle arrest and as a cytotoxic agent through the induction of cellular apoptosis (26, 31, 37–39).

Thus, we hypothesized that genistein could affect the growth of metastatic disease in our model.

In Study 1, where genistein was given both before tumor cell injection and during tumor growth, we clearly showed that dietary genistein decreased spontaneous metastasis of hormone-independent MDA-MB-435/HAL human breast cancer cells from primary mammary fat pad tumors. Furthermore, this decrease in metastatic burden cannot be attributed to a decrease in the rate of primary tumor growth as no significant effect on primary tumor growth was observed. In Study 2, where genistein was given solely as an adjuvant treatment following surgical resection, lymph node metastasis was inhibited and a 10-fold reduction in the percent metastatic burden in the lungs was observed. Interestingly, in the case of the sham-operated group in Study 2, the results suggest that the surgery alone (i.e., making an incision at the primary site) reduced the tumor burden at the metastatic site; however, the mechanisms for this effect are unclear and beyond the scope of this study. The combined results of Study 1 and Study 2 show that genistein is capable of restricting the outgrowth of tumor cells at secondary sites.

We investigated possible underlying mechanisms for the antimetastatic effect of genistein by quantifying the levels of proliferation and apoptosis within metastases. Our analysis revealed that treatment with genistein resulted in both a decrease in tumor cell proliferation and an increase in tumor cell death. The ratio between proliferation and apoptosis regulates metastatic tumor growth (64) and by shifting this ratio, genistein may thus be affecting the expansion of metastases. Tumor metastasis is, however, complex and involves multiple interactions between tumor cells and the local tissue environment. Genistein is known to elicit a wide range of effects in tumor cells but may also be capable of causing host tissue-specific responses that may indirectly affect the growth of tumor cells. In Study 1, we saw that genistein effectively inhibited the growth of tumor cells in the lung and yet had no effect on the growth of tumor cells in the mammary fat pad. The higher affinity of genistein for estrogen receptor β over estrogen receptor α suggests that genistein may affect normal tissues where estrogen receptor β is the more predominant estrogen

receptor isoform, such as the lung, bladder, brain, prostate, ovary, and testis (24, 25). The action of genistein as an estrogen agonist or antagonist will therefore depend on the target tissue, its estrogen receptor status, and the level of endogenous estrogen (14, 53). Other tissue effects include the ability of genistein to impair extracellular matrix signaling (32, 34, 35). By suppressing adhesion-induced protein tyrosine phosphorylation, genistein can restrict cell migration and invasion (39, 40). Genistein has also been shown to limit new vessel formation by directly inhibiting endothelial cell proliferation as well as by blocking matrix-degrading enzymes such as urokinase-type plasminogen activator, matrix metalloproteinase 2, and matrix metalloproteinase 9 (40, 42–44). The actions of genistein at the tissue level may thus influence the ability of the tissue to support metastatic growth. Collectively, genistein may inhibit growth of metastases either through direct effects on the tumor cells or by indirect effects on host tissues.

In summary, these results indicate that dietary intervention following cancer surgery can affect the growth ability of previously seeded, potentially metastatic cells. The antimetastatic action shown here supports the potential of genistein as a postsurgical adjuvant therapy for estrogen receptor-negative breast cancer. However, the use of genistein by breast cancer survivors still remains controversial and should be used with caution as genistein may interfere with the activity of tamoxifen and stimulate estrogen receptor-positive breast cancer in postmenopausal women (47, 53, 65).

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Dietary Genistein Reduces Metastasis in a Postsurgical Orthotopic Breast Cancer Model

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