Genistein potentiates inhibition of tumor growth by radiation in a prostate cancer orthotopic model

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Abstract

Objective: We have shown previously that pretreatment with genistein potentiated cell killing induced by radiation in human PC-3 prostate carcinoma cell line *in vitro*. We tested this approach *in vivo* using an orthotopic prostate carcinoma model of PC-3 cells in nude mice. **Methods:** Established prostate tumors were pretreated with p.o. genistein at a dose of 5 mg/d for 2 days followed by tumor irradiation with 5 Gy photons. One day after radiation, genistein was resumed and given every other day for 4 weeks. **Results:** Genistein combined with radiation caused a significantly greater inhibition of primary tumor growth (87%) compared with genistein (30%) or radiation (73%) alone. The number of metastatic lymph nodes was also significantly decreased following genistein and radiation. Paradoxically, genistein alone increased the size of lymph nodes associated with heavy tumor infiltration. Genistein-treated prostate tumors were large with necrosis, apoptotic cells, and giant cells and have a lower proliferation index than in control tumors. Following radiation, areas of tumor destruction replaced by fibrotic tissue and inflammatory cells as well as giant cells were observed, which are typical of radiation effect. After radiation and genistein treatment, an increase in giant cells, apoptosis, inflammatory cells, and fibrosis was observed with decreased tumor cell proliferation consistent with increased tumor cell destruction. Long-term therapy with genistein after prostate tumor irradiation significantly increased survival. **Conclusions:** Genistein combined with prostate tumor irradiation led to a greater control of the growth of the primary tumor and metastasis to lymph nodes than genistein or radiation alone, resulting in greater survival. [Mol Cancer Ther 2004;3(10):1271–9]

Introduction

Genistein is an isoflavone, a major metabolite of soy produced by the intestinal bacteria, which is believed to be one of the anticancer agents found in soybeans (1, 2). Epidemiologic studies have shown that Japanese and Chinese men, who consume soy products, have the lowest incidence of prostate carcinoma in the world (3). Genistein has a heterocyclic diphenolic structure similar to estrogen (4) and has shown antitumor and antiangiogenic activities (5, 6). Genistein was found to inhibit tyrosine protein kinases (except p40 protein tyrosine kinase; ref. 7), topoisomerase I and II (8), and protein histidine kinase and 5α-reductase (9). Genistein has been shown to inhibit cell growth of tumor cell lines from various malignancies including breast, lung, melanoma, prostate, head and neck squamous cell carcinoma, leukemia, and lymphoma independent of hormone receptor status (10–17). We have shown previously that genistein inhibited the cell growth of androgen-dependent (LNCaP) and androgen-independent (PC-3) human prostate carcinoma cell lines *in vitro* (10). Genistein affected the cell cycle and induced apoptosis and thus could be used as a cytotoxic agent for prostate cancer.

Prostate carcinoma is the most common malignant tumor in men, with >180,400 newly diagnosed cases annually, resulting in >31,000 deaths each year (18). Localized prostate carcinoma is sensitive to conventional radiotherapy using megavoltage photons (X-rays); however, residual disease often causes clinical relapse (19). To improve the local control of prostate carcinoma, the combination of radiation with additional antitumor agents should be considered. We have tested previously the combination of genistein with radiation *in vitro*. We showed that pretreatment with genistein potentiated cell killing induced by photon or neutron radiation in human PC-3 prostate carcinoma cell line *in vitro* (20). Genistein at 15 μmol/L caused a significant inhibition in DNA synthesis, cell growth, and colony formation in the range of 40% to 60%. Pretreatment of cells for 1 to 2 days with 15 μmol/L genistein potentiated the effect of low-dose photon (2-3 Gy) or neutron (1-1.5 Gy) radiation on DNA synthesis, cell growth, and colony formation causing up to 80% to 95% inhibition compared with 50% with radiation alone (20). Our data indicate that genistein combined with radiation inhibits DNA synthesis, resulting in inhibition of cell division and growth. These results were highly reproducible in subsequent studies and suggest the potential for combining genistein with radiation for the treatment of

prostate carcinoma. The goal of the current study was to test this approach in a preclinical model of prostate carcinoma *in vivo*. We used an orthotopic prostate carcinoma model consisting of implantation of PC-3 cells in the prostate of nude mice, which leads to the formation of a primary prostate tumor and metastasis to para-aortic lymph nodes (21, 22). The experimental conditions for the treatment of prostate tumor-bearing mice, combining genistein with radiation, were designed based on our *in vitro* observations regarding the sequence and exposure of genistein. The effect of radiation and genistein treatment was more significant when PC-3 cells were first treated with genistein for 24 hours prior to irradiation compared with the reverse sequence of irradiation prior to genistein. To get an optimal effect, continuous exposure of the cells to genistein before and after radiation was needed. Mice bearing established PC-3 prostate tumors were pretreated with genistein for 2 to 3 days. Then, selective photon irradiation was given to prostate tumors, and a day later, genistein was resumed for the duration of the experiment. This combined treatment with genistein and radiation led to a greater control of the growth of the primary tumor and metastasis to lymph nodes than genistein or radiation alone, resulting in greater survival.

Materials and Methods

Tumor Model

The human prostate carcinoma PC-3 tumor cell line was purchased from the American Type Culture Collection (Rockville, MD). PC-3 cells were cultured in culture medium consisting of F-12 K nutrient mixture supplemented with 7% heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY), 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1 μmol/L sodium pyruvate (Sigma Chemical Co., St. Louis, MO), 10 mmol/L HEPES buffer, 100 units/mL penicillin/streptomycin, 0.5 μg/mL fungizone, and 50 μg/mL gentamicin. As described previously, PC-3 cells were cultured *in vitro* and then implanted in the prostate of male BALB/c *nu/nu* nude mice (21, 22). New PC-3 prostate tumor cell lines were generated from prostate tumors (PC-3/PI) that were more tumorigenic and induced metastatic prostate tumors with faster kinetics than the original PC-3 cells (21, 22). These new PC-3/PI cell lines were used for prostate implantation. Tumor cells were washed twice in HBSS and a concentration of 5×10^5 cells in 20 μL HBSS was injected into the prostate of 4- to 6-week-old male BALB/c *nu/nu* nude mice (Harlan Sprague-Dawley, Indianapolis, IN). The prostate of anesthetized mice was exposed through a midline laparotomy incision and by retraction of the bladder and male sex accessory glands anteriorly. Injection of cells was done with a 27 gauge needle inserted in the prostatic lobe located at the base of the seminal vesicles as described previously (21, 22). The abdominal wound was sutured using a 4.0 chromic gut suture in a running fashion. Mice were housed and handled under sterile conditions in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. Mice received Lab Diet 5021 (Purina Mills, Inc., Richmond, IN). The animal protocol was approved by Wayne State University Animal Investigation Committee (Detroit, MI).

Prostate Tumor Irradiation

Acrylic jigs were designed to place anesthetized mice in the supine position with their fore and hind limbs restrained by posts to help localize the prostate between the hind legs as described previously (22). Three jigs were positioned on an aluminum frame mounted on the X-ray machine to simultaneously irradiate three mice. A 6.4 mm lead shield was positioned above the jigs with three cut-outs to allow for irradiation of the prostate as confirmed by double-exposure X-ray radiographs (22). The radiation dose to the prostate and the scattered dose to areas of the mouse outside of the radiation field were carefully monitored. Photon irradiation was done with a Stabilipan X-ray set (Siemens Medical Systems, Inc., Malvern, PA) operated at 250 kV, 15 mA with 1 mm copper filtration at a distance of 47.5 cm from the target.

Experimental Protocol

For a short-term experiment of 38 days, mice bearing established PC-3 prostate tumors were treated with p.o. genistein by gavage and tumor irradiation. Following prostate implantation with PC-3/PI cells, established prostate tumors of ~0.4 cm were observed at autopsy by days 14 to 16, whereas the size of normal prostates is ~0.2 cm (22). Before initiating treatment, a few mice were sacrificed to monitor and confirm tumor growth and size in the prostate. On days 13 and 14 following intraprostatic injection of PC-3/PI cells, mice received p.o. genistein at a dose of 5 mg/d per mouse. On day 15, established PC-3 prostate tumors of ~0.4 cm were selectively irradiated with a single dose of 5 Gy photons. One day later, on day 16, genistein treatments were resumed and given every other day for the duration of the experiment. Genistein (LKT Laboratories, Toronto, Ontario, Canada) was dissolved in 0.1 mol/L Na_2CO_3 and mixed with sesame seed oil at 2:1 ratio prior to gavage and delivered p.o. in 0.3 mL at a dose of 5 mg/d per mouse. This is a feasible and reproducible method allowing us to control the dose given. The dose was selected from initial dose titration studies, which were based on previous animal studies using a diet rich in genistein or s.c. administration (23–27). Sesame seed oil was used to facilitate gavage and avoid irritation of the esophagus by Na_2CO_3 and was safe as shown also by others (28). Mice from control groups and radiation-only–treated groups received a mixture of 0.2 mL of 0.1 mol/L Na_2CO_3 and 0.1 mL of sesame oil. Six to seven mice were used per experimental group in this short-term experiment. On day 38 after PC-3 cell injection, mice were sacrificed, autopsied, and examined for gross tumors in the prostate. The tumor-bearing prostates and para-aortic lymph nodes were resected, measured in three dimensions using a caliper, and weighed. A good correlation was observed between tumor volume and tumor weight; therefore, weight data are presented. Prostates and para-aortic lymph nodes were also processed for histologic studies.

For a long-term experiment of 87 days to monitor mouse survival, each experimental group consisted of 10 to 13 mice. Established PC-3 tumors were pretreated on days 8 to 10 after cell injection with p.o. genistein at a dose of 5 mg/d. On day 11, prostate tumors were irradiated with a single dose of 5 Gy photons. One day later, genistein treatment was resumed and given every other day until mice were sacrificed. Mice were monitored daily for survival and sick or moribund animals were sacrificed and autopsied. On day 87, all remaining mice were sacrificed and autopsied to assess primary tumor size and metastases.

Measurement of Genistein Concentration in Blood

Prior to sacrificing the mice, mice received one last p.o. genistein treatment at 5 mg; 2 hours later, blood was drawn from the heart and the serum was separated to quantitate genistein. Total serum isoflavones were measured using minor modifications of the validated procedure published previously (29). Off-line solid-phase extraction of total plasma isoflavones was done following enzymatic deconjugation (Isolute ENV+, 25 mg, Jones Chromatography, Lakewood, CO) followed by quantification of genistein using isotope dilution liquid chromatography-electrospray-mass spectrometry with d4-genistein internal standards. The method detection limit was ~0.02 μmol/L and the interassay and intraassay precision and accuracy ranged from ~5% to 10%.

Tissue Preparation for Histology and Immunohistochemistry

At completion of experiments, mice were sacrificed and the prostates and para-aortic lymph nodes were resected and processed for histology studies. The tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were stained with H&E. Tumor sections (5 μm thick) were also stained for detection of Ki-67 nuclear antigen with Ki-67 antibody using the biotin-streptavidin immunostaining method in the Benchmark Automated Stainer (Ventana Medical, Tucson, AZ). Slides were deparaffinized, rehydrated, and treated with EDTA for antigen retrieval followed by incubation with Ki-67 antibody (clone MM-1, Ventana Medical) for 32 minutes and completed with the IVIEW DAB detection kit. Slides were counterstained with Mayer's hematoxylin. The Ki-67 index was determined by the average percentage of tumor cells with nuclear staining in three different 400× fields.

Statistical Analysis

Differences in prostate tumor sizes and lymph node sizes among the various treatment groups were analyzed by two-tailed unpaired t test at different time points. Survival curves were constructed using the Kaplan-Meier product limit method. Comparisons of survival curves were carried out using the log-rank test.

Results

Treatment of PC-3 Prostate Tumors with Genistein and Radiation: Effect on Primary Tumor

We have shown previously that pretreatment with genistein potentiated cell killing induced by photon radiation in PC-3 cells *in vitro* (20). This effect was depen-

dent on both the genistein and the radiation dose and was observed at intermediate doses; therefore, high doses giving optimal inhibition by either treatment were not selected. In the PC-3/nude mouse orthotopic model, prostate tumors were found to be radiosensitive (D_0 of 1.5 Gy); thus, an intermediate photon dose of 5 Gy causing 70% inhibition of prostate tumor growth by day 30 was selected for combination with genistein (22). A dose of 5 mg/d per mouse of genistein was selected for p.o. administration as described in Materials and Methods. We have also shown *in vitro* that administration of genistein 1 day prior to radiation treatment followed by continuous exposure of genistein led to optimal PC-3 cell killing (20). Based on these studies, prostate tumor-bearing mice were pretreated with genistein for 2 days followed by prostate irradiation and continued genistein exposure for the duration of the experiment. After 4 weeks of genistein, mice were sacrificed to assess the primary tumor size (day 38 post–PC-3 cell injection). This time point was selected to compare the effect of genistein, radiation, and both combined on the primary tumor because prostate tumors are already very large in control mice. Pathologic observations showed that untreated mice had large vascularized prostate tumors of 0.9 to 1.1 cm in diameter (~400 mm^3) compared with the size of normal prostate of 0.3 cm (~14 mm^3). Following treatment with genistein, large tumors of 0.80 to 1.1 cm (~350 mm^3) were also observed compared with smaller tumors of 0.6 to 0.9 cm (~100-200 mm^3) following irradiation. Small tumors of 0.5 to 0.6 cm (~50 mm^3) were observed following combined treatment of genistein and radiation. Analysis of the mean of prostate tumor weight in the different treatment groups showed that, relative to control tumors, single modalities of genistein or radiation alone caused 30% and 73% tumor growth inhibition, respectively ($P < 0.05$; Fig. 1). The
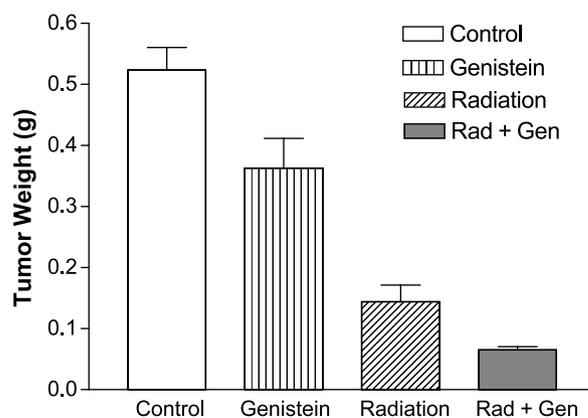

Figure 1. Treatment of PC-3 prostate tumors with genistein and tumor irradiation: response of primary tumors. On days 13 and 14 after PC-3 cell injection in prostate, mice were treated with p.o. genistein at a dose of 5 mg/d. On day 15, established prostate tumors were irradiated with 5 Gy photons. One day later, genistein treatment was resumed and given every other day. On day 38, mice were sacrificed and the prostates, including the tumors, were resected and weighed. *Columns,* mean tumor weights of six to seven mice per treatment group; *bars,* SE.

combined modality of genistein and radiation caused a greater tumor growth inhibition of 87% that is highly significant compared with control (P < 0.0001), genistein (P < 0.0001), or radiation (P < 0.01; Fig. 1).

Effect of Genistein and Radiation on Spontaneous Metastases

We have shown previously that prostate implantation of PC-3 cells led to the formation of a primary tumor and spontaneous metastases to para-aortic draining lymph nodes (21, 22). Large prostate tumors are always accompanied by enlarged para-aortic lymph nodes from which tumor cells can be isolated (21, 22). The para-aortic lymph nodes are in the lumbar part of the spine along the aorta. We estimate that some are in the field of radiation at ∼3 to 4 mm from the prostate, whereas the main two para-aortic lymph nodes are located higher along the lumbar spine above the field of radiation at ∼7 mm from the prostate (22). Scattered radiation outside of the radiation field was carefully monitored and found to be 1% to 2% of the treatment dose. In contrast to a size of 1 to 1.5 mm in normal mice, enlarged para-aortic lymph nodes of 4 to 5 mm were prominent in control mice bearing large prostate tumors. Following genistein treatment, larger para-aortic lymph nodes of 5 to 8 mm were isolated on day 38, although primary tumors were smaller than in control mice. The mean weight of para-aortic lymph nodes showed a significant 2-fold increase in lymph node weight of genistein-treated mice compared with control (P = 0.017; Fig. 2). In mice treated with radiation alone, about one half of the lymph nodes were enlarged. Following radiation combined with genistein, few lymph nodes were enlarged and the mean weight was significantly lower than in control tumor-bearing mice or genistein-treated mice (P < 0.005; Fig. 2). From all lymph nodes resected in each treatment group, the proportion of enlarged lymph nodes was 13 of 13 in control mice compared with 13 of 14 in genistein-treated mice, 7 of 12 in radiation-treated mice, and 3 of 14 in radiation plus genistein–treated mice (Table 1).
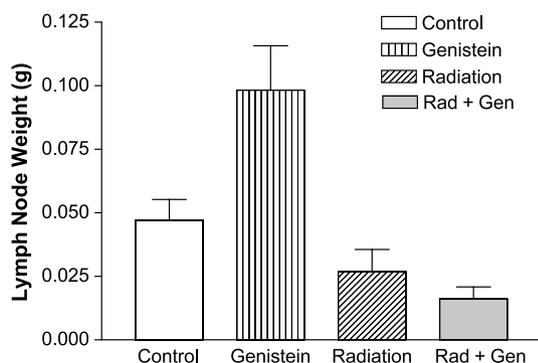

Figure 2. Treatment of PC-3 prostate tumors with genistein and tumor irradiation: response of lymph node metastases. Para-aortic lymph nodes were resected on day 38 from mice of the experiment described in Fig. 1. At least two lymph nodes were obtained from each mouse and were weighed. *Columns,* mean lymph node weights from six to seven mice per treatment group; *bars,* SE.

Genistein Levels in Blood

Based on initial studies, measurements of genistein levels in the serum of mice were done at 2 hours after treatment as we found that genistein level is optimal in the serum at 2 hours after p.o. genistein and then progressively decreases with time. Genistein levels of 31.47 ± 16.79 μmol/L (SD, n = 8) were measured in the serum of mice treated with genistein. These levels were significantly higher compared with 0.213 ± 0.048 μmol/L (SD, n = 8) in mice treated with the vehicle only (Na_2CO_3/sesame oil; P < 0.0012). The low genistein levels observed in control mice are likely the result of the soy content in the animal diet.

Effect of Long-term Treatment with Genistein after Prostate Tumor Irradiation

To assess the long-term effects of continuous genistein treatment after prostate tumor irradiation, PC-3 prostate tumor-bearing mice were treated with genistein alone or radiation or both and were followed for 3 months in a survival experiment as described in Materials and Methods. The median survival of prostate tumor-bearing mice was similar and <45 days in control and genistein-treated mice, but it was increased to 68 days in radiation-treated mice. The median survival was 87 days in mice treated with combined genistein and radiation (Fig. 3). Comparisons between the survival curves between the various treatment groups showed that genistein did not increase mouse survival compared with control mice (P > 0.05; Fig. 3). In these two groups, mice were sacrificed or died by days 45 to 48 and 90% of these mice had large prostate tumors at autopsy and enlarged para-aortic lymph nodes. Genistein-treated mice reproducibly showed ∼2-fold increase in the size of para-aortic lymph nodes compared with lymph nodes from control mice, confirming the data shown in Fig. 2. Treatment with radiation alone or radiation combined with genistein led to a significant increase in mouse survival compared with that of control or genistein-treated mice (P < 0.0001; Fig. 3). Large prostate tumors were observed in 80% of the mice treated with radiation alone at autopsy often associated with enlarged lymph nodes. The combined radiation and genistein treatment resulted in greater mouse survival than radiation alone (P < 0.0025; Fig. 3). By day 87, the remaining 7 of 13 mice treated with radiation plus genistein showed a mixed tumor response at autopsy, with small tumors of 0.5 cm in 4 of 7 mice and larger tumors of 1 cm in 3 of 7 mice.

In both short-term and long-term experiments, following genistein treatment alone or combined with radiation, no signs of toxicity were apparent during treatment. When experiments were terminated, mice showed no toxic effects in the surrounding organs, with no reduction in size, atrophy, or bleeding at autopsy.

Histology of Prostate Tumors and Lymph Nodes following Radiation and Genistein

To investigate the effect of radiation and genistein on prostate tumors *in situ*, the histology of prostate tumors and para-aortic lymph nodes resected on day 38 from mice treated with radiation, genistein, or both was evaluated.

Table 1. Summary and quantitation of histologic observations

% Viable	% Tumor Cells	Giant Cells	Fibrosis	Necrosis	Apoptosis	Inflammatory Infiltrates	Lymph Node Metastases
Control	80	10	−	+	±	±	++ (100%)
Radiation	50	50	++	±	+	++	+ (58%)
Genistein	60	40	+	++	++	++	++ (93%)
Radiation + genistein	30	70	++	±	++	+++	± (21%)

NOTE: Histologic findings from the experiment presented in Figs. 4 and 5 were quantified including the percentage of viable tumor cells or giant cells in the tumor sections. The extent of fibrosis, necrosis, apoptosis, and inflammatory infiltration were scaled from mild (±) to heavy (+++). The percentage of enlarged metastatic para-aortic lymph nodes from a total of 12 to 14 resected nodes in each treatment group is reported.

Histologically, untreated PC-3 prostate tumors presented as a poorly differentiated epithelial neoplasm consisting of pleomorphic tumor cells with large hyperchromatic nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm (ref. 22; Fig. 4A). These large tumors had a myxoid background and loosely cohesive cystic growth pattern with 80% viable tumor cells and some areas of necrosis with no significant apoptosis, fibrosis, or inflammatory infiltrates (Table 1). Following radiation, tumors were smaller and contained patchy areas of fibrosis infiltrated by inflammatory cells, apoptotic cells, and ∼50% residual viable tumor cells of which 50% looked like giant tumor cells (Fig. 4B; Table 1). Giant tumor cells result from radiation-induced marked cytologic changes such as cytomegaly and nucleomegaly with abnormal chromatin margination or packed chromatin as shown previously (ref. 22; Fig. 4B; Table 1). Following treatment with genistein alone, large tumors exhibited increased myxoid stroma with early fibrosis and geographic areas of necrosis containing apoptotic cells (Fig. 4C; Table 1). However, remaining areas of tumor contained ∼60% viable tumor cells of which 40% looked like giant tumor cells (Fig. 4C; Table 1). The combination of radiation and genistein resulted in much smaller tumors that showed large areas

Figure 3. Survival of PC-3 prostate tumor-bearing mice treated with prostate tumor radiation and genistein. On days 8 to 10 after PC-3 cell injection in prostate, mice were treated with p.o. genistein at a dose of 5 mg/d. On day 11, prostate tumors were irradiated with 5 Gy photons. One day later, genistein treatment was resumed and given every other day. Ten to 13 mice per treatment group were used and followed for survival.

of tumor destruction replaced by fibrous myxoid stroma infiltrated by inflammatory cells (Fig. 4D). About 30% viable remaining tumor cells were observed and 70% of these cells looked like giant cells, some of which undergoing apoptosis with typical condensed nuclei and dense eosinophilic cytoplasm (Fig. 4D; Table 1). Quantitation of histologic findings showed that radiation and genistein caused a greater increase in tumor destruction, apoptosis, inflammatory infiltrates, and cytologic changes (visualized by giant tumor cells) compared with either therapy (Table 1).

The response of metastases to therapy was assessed by histology studies of para-aortic lymph nodes. On day 38, lymph nodes from control mice and genistein-treated mice showed invasion of tumor cells replacing small immune cells with focal remaining lymphoid structures at the periphery in contrast to the typical histology of cortex and medullary cords of normal lymph nodes (Fig. 4E and F). Often, tumor cells replaced completely the medullary regions (Fig. 4F) and invaded most of the peripheral cortical areas as well. These histologic observations confirm that the large nodules isolated along the spine were indeed enlarged lymph nodes heavily infiltrated by tumor metastases. It should be noted that the tumor seen in metastatic lymph node grew in the same loosely cohesive cystic pattern with myxoid background similar to that seen in prostate tumors (Fig. 4E and F). Lymph nodes from mice treated with radiation or radiation plus genistein showed either tumor invasion for enlarged lymph nodes or no tumor invasion for small lymph nodes, confirming the correlation between enlarged lymph nodes and tumor invasion in this model (refs. 21, 22; Table 1).

Effect of Radiation and Genistein on Tumor Cell Proliferation *In situ*

To determine the extent of tumor cell proliferation in prostate tumors and lymph nodes, tumor sections obtained on day 38 were immunostained with antibody specific to Ki-67 proliferation marker. Control prostate tumors showed Ki-67 nuclear-positive staining in 50% of the tumor cells; the staining was uniform across the tumor sections (Fig. 5A). Tumors treated with genistein showed a lower level of staining of 30% of the cells (Fig. 5B). Prostate tumors treated with radiation alone or combined with genistein showed a heterogeneous pattern of Ki-67 staining with areas of low staining and areas of higher staining, correlating with the areas of tumor destruction and areas of viable tumor observed in H&E
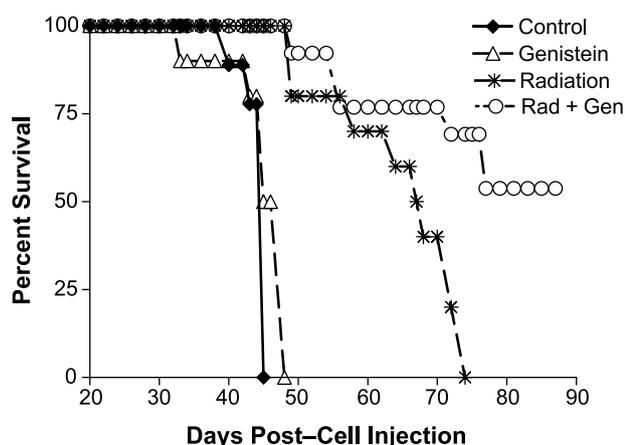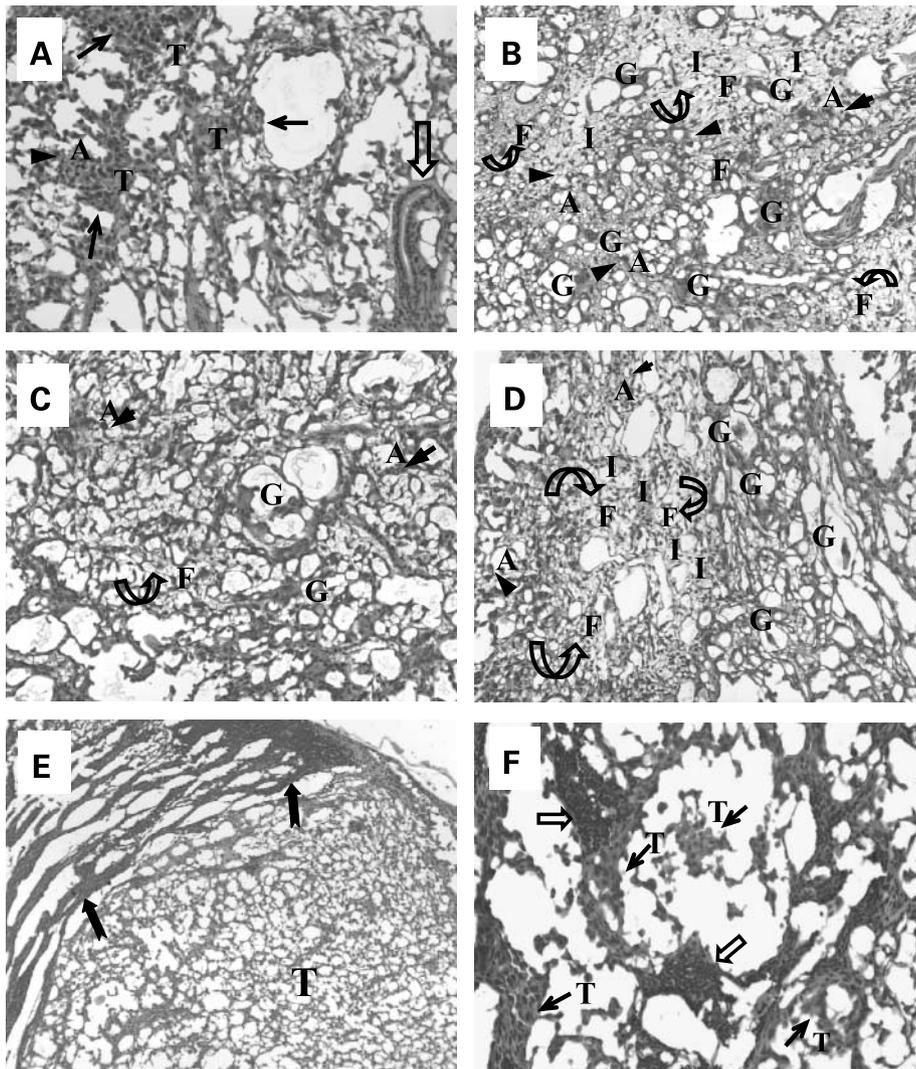
Figure 4. Histology of PC-3 prostate tumors treated with radiation and genistein. Prostate tumors resected from mice of experiment described in Fig. 1 were processed for histology and tumor sections were stained with H&E. The main findings were labeled on the prints with *T* for tumor, *A* for apoptosis, *F* for fibrosis, *G* for giant cells, and *I* for inflammatory cells. **A,** prostate tumor from control mice presenting as loosely cohesive cystic tumor with myxoid background. Tumor cells (*arrows*) are invading the prostate, note remaining prostate gland (*empty arrow*). **B,** irradiated prostate tumor, note areas of tumor destruction showing fibrosis (*curved arrows*) with inflammatory cells and apoptotic cells (*arrowheads*). Some of the remaining tumor cells were giant cells with nuclei margination (*G*). **C,** prostate tumor from mice treated with genistein showing an increase in myxoid stroma with early fibrosis (*curved arrows*), apoptotic cells (*arrowheads*), and multinucleated giant cells (*G*). **D,** prostate tumor from mice treated with radiation and genistein showing large areas of tumor destruction replaced by fibrous myxoid stroma (*curved arrows*) and inflammatory cells. Most of remaining tumor cells looked like giant cells with apoptotic nuclei and eosinophilic cytoplasm (*G*). **E,** lymph nodes from mice treated with genistein; low magnification (×25) shows remaining lymphoid structures at periphery (*large arrows*) and tumor invasion (*arrows*, *T*). **F,** lymph nodes from control mice showing invasion of tumor cells (*arrows*, *T*) replacing normal small immune cells (*empty arrows*). All magnifications ×50 (except **E**).

staining. In lymph nodes, uniform Ki-67 staining of 50% of tumor cells was observed in all metastatic lymph nodes from control mice or mice treated with radiation or genistein and both.

Discussion

Radiotherapy of prostate carcinoma is a well-established treatment. However, recurrence is observed in a significant number of patients. To improve the local control of prostate carcinoma, we have tested the combination of radiation with genistein. We and others have clearly documented the cytotoxic biological effects of isoflavones on tumor cells from several malignancies, including human prostate carcinoma cells, resulting in cell cycle arrest and cell death (10–17). We also showed *in vitro* that pretreatment of PC-3 cells with genistein potentiates the radiation-induced cell killing (20). These studies showed that the sequence of genistein first fol-

lowed by radiation and continuous exposure of genistein led to optimal PC-3 cell killing. Based on these findings, the combination of genistein and radiation was investigated in a preclinical PC-3/nude mouse orthotopic prostate carcinoma model (21, 22). Established prostate tumors were pretreated with genistein for 2 to 3 days followed by prostate tumor irradiation and then with continued genistein treatment.

In a short-term study, we found that genistein combined with radiation caused a significant 87% inhibition of tumor growth in the prostate compared with 30% by genistein treatment alone and 73% by radiation alone. These data in the preclinical orthotopic model corroborate our *in vitro* findings of genistein potentiation of radiation-induced cell killing (20). Histologic findings showed that irradiated tumors contained areas of tumor destruction replaced by fibrotic tissue, inflammatory cells, and apoptotic cells. A large number of remaining tumor cells (50%) presented as multinucleated giant cells with abnormal chromatin

patterns compared with 10% in control tumors as shown in our previous studies (30). We showed that giant cells are multinuclear, undergo abnormal mitosis, and ultimately die. Radiation might cause alterations in cell division at the level of cytokinesis, leading to giant cell formation, a potential mechanism of late cell death induced by radiation in addition to apoptosis and necrosis (31, 32). Interestingly, genistein also caused a 40% increase of multinucleated giant cells. Marked geographic necrosis was observed in these large tumors still containing ~60% of tumor cells, which looked viable, but Ki-67 staining indicated a lower level of proliferation compared with control tumors. After combined radiation and genistein treatment, tumors were much smaller and showed large areas of tumor destruction replaced by fibrous myxoid stroma infiltrated by inflammatory cells. The majority of the remaining tumor cells (70%) were giant cells, some of which undergoing apoptosis. These findings are reflective
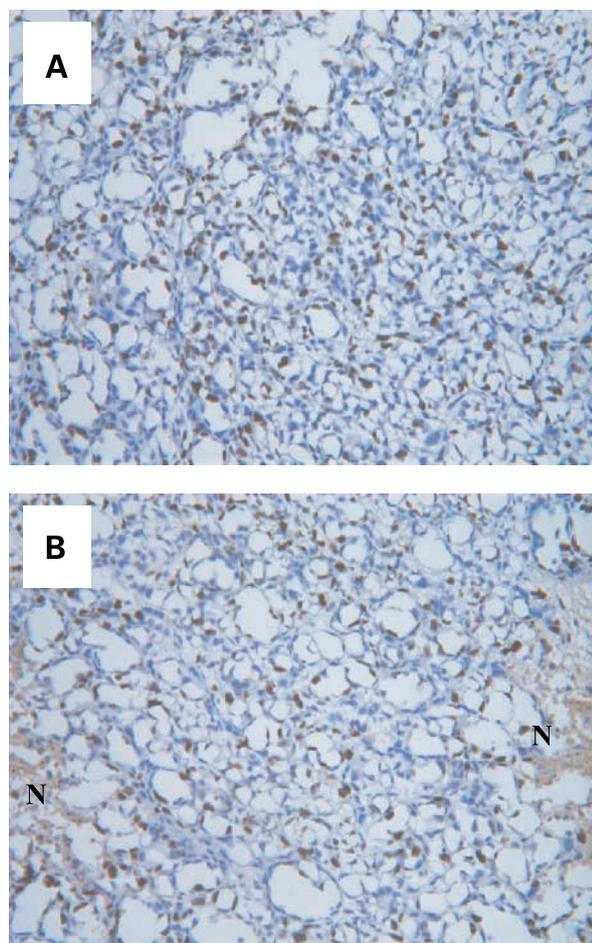

Figure 5. Ki-67 proliferation marker staining of prostate tumors and lymph nodes from mice treated with radiation and genistein. Sections of para-aortic lymph nodes obtained on day 38 from the experiment described in Figs. 4 and 5 were immunostained for Ki-67. **A,** control prostate tumor showing 50% uniform staining. **B,** genistein-treated prostate tumor with staining of ~30% of the cells showing necrotic areas (*N*). Magnifications ×25.

of increased cell destruction and alterations effected by genistein and radiation. Taken together, these data indicate a better control of tumor growth following radiation and genistein compared with each modality alone.

The mechanisms of cell killing induced by radiation and genistein are currently under investigation. We had shown previously that genistein caused the PC-3 cells to arrest at the G_2-M phase of the cell cycle (10). We found that radiation of PC-3 cells *in vitro* also causes accumulation of cells in the G_2-M phase and a greater accumulation of cells in G_2-M phase was observed with the combination of genistein with radiation.[5] The formation of giant cells observed after genistein and radiation treatment of PC-3 cells both *in vitro* (20) and *in vivo* in the current study may be related to G_2-M arrest and alterations in cytokinesis.

The antitumor activity mediated by genistein on PC-3 prostate tumors correlates with the levels of genistein measured in the mice blood. Mice treated with genistein reached serum levels of ~30 μmol/L compared with 0.2 μmol/L in control mice without apparent toxicity. These levels are comparable with the 15 μmol/L range measured in human volunteers consuming 50 mg isoflavone consisting of 40 mg genistein and daidzein and in other studies (25, 33, 34).

Evaluation of genistein and radiation effect on spontaneous metastases to para-aortic lymph nodes showed that 93% to 100% of lymph nodes resected from control or genistein-treated mice were enlarged compared with 58% following radiation. Histologically, these enlarged lymph nodes showed invasion of tumor cells as shown previously (21, 22). However, following radiation combined with genistein, most of the lymph nodes were smaller with no tumor infiltration and only 21% of the lymph nodes were enlarged with tumor infiltration. Decreased metastasis might correlate with smaller prostate tumors in this treatment group, suggesting that genistein combined with radiation control the tumor growth in the primary site and consequently the incidence of spontaneous metastasis. Compared with lymph nodes from control mice, genistein treatment caused a 2-fold increase in the size of para-aortic lymph nodes due to tumor infiltration. The proliferation pattern of tumor cells in the lymph nodes from genistein-treated mice was comparable with that seen in lymph nodes from control mice. These intriguing data indicate that although genistein could cause some inhibition in the primary tumor it could stimulate metastasis. Genistein has been shown to have antiangiogenic effects (5, 6, 35). Genistein-treated tumors showed greater necrosis, which may be the result from genistein-induced antiangiogenic effects, leading to poor oxygenation and hypoxia. A hypoxic environment in solid tumors causes alterations in gene expression and selection of tumor variants that could contribute to metastatic progression of tumor cells (36).

[5] Hillman et al., in preparation.

Nevertheless, this increased metastasis to lymph nodes was not observed when genistein was combined with tumor irradiation, an observation relevant for clinical application of this therapeutic approach.

Previous animal studies have emphasized the role of genistein in the prevention of prostate cancer and mammary tumors (27, 37–39). A reduced incidence of advanced prostate lesions was observed when genistein was added to the diet of TRAMP mice at an early age prior to prostate carcinoma development (27, 39). Pretreatment with genistein or genistein plus daidzein prior or during chemical treatment to induce prostate cancer in rats decreased the incidence of prostate carcinoma (39–41). Feeding mice with soy phytochemical concentrate (containing genistein, daidzein, and glycitein) reduced tumor growth of s.c. implanted human LNCaP cells in severe combined immunodeficiency mice (35). All these prostate carcinoma animal models show a certain level of chemopreventive effects mediated by genistein. In contrast, other studies showed increase in tumor growth by genistein (42, 43). In a recent prevention study of Zhou et al. (44) using an orthotopic model of LNCaP cells in severe combined immunodeficiency mice similar to our model, soy phytochemical concentrate was more effective than genistein or soy protein. Our current study does not address chemoprevention with genistein but rather addresses inhibition of tumor progression because genistein treatment was initiated on established prostate tumors. Although we showed a 30% inhibition in primary tumor growth, an increased metastasis was observed following genistein treatment. Whether additional compounds such as daidzein and glycitein from the soybean, like in the soy phytochemical concentrate formulation, will protect against increased metastasis mediated by purified genistein remains to be elucidated.

In a survival experiment in which genistein treatment was continued for up to 3 months after prostate tumor irradiation, genistein combined with tumor irradiation led to a significant increase in mouse survival compared with each modality alone. These studies in the orthotopic preclinical model show that combining radiation and genistein for established prostate tumors is an effective approach to enhance the efficacy of radiotherapy resulting in a greater control of the primary tumor and metastasis. Using soy isoflavones in the treatment of prostate carcinoma in conjunction with radiation is a *promising* approach that may also potentially reduce the adverse effects of radiation on normal surrounding tissues because of their antioxidant effects. Furthermore, administration of soy isoflavones has proven to be safe in humans. In an attempt to elucidate the molecular mechanisms of interaction between genistein and radiation, we found that genistein decreases nuclear factor-κB activity whereas photon radiation induces activation of nuclear factor-κB. Radiation-induced activation of nuclear factor-κB activity is completely inhibited by pretreatment of cells with genistein. These findings support our hypothesis that treatment of the cells with genistein will block radiation-induced nuclear factor-κB activation leading to a cascade of molecular events driving the cells to an apoptotic pathway and thus increasing cell killing. Future studies will address the molecular mechanisms involved in genistein/radiation interaction, *in vivo*.

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