Down-regulation of Apurinic/Apyrimidinic Endonuclease 1/Redox Factor-1 Expression by Soy Isoflavones Enhances Prostate Cancer Radiotherapy In vitro and In vivo


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Abstract
We previously showed that genistein, the major bioactive component of soy isoflavones, acts as a radiosensitizer and potentiates prostate tumor cell killing by radiation in vitro and in animal tumor models in vivo. However, when given alone in vivo, pure genistein promoted increased lymph node metastasis, which was not observed with a soy isoflavone mixture consisting of genistein, daidzein, and glycitein. In this study, we show that soy inhibits tumor cell growth and potentiates radiation-induced cell killing in vitro like pure genistein. In an orthotopic model, combining soy isoflavones with tumor irradiation inhibited prostate tumor growth. To determine the molecular mechanisms by which soy isoflavones potentiate radiotherapy, we investigated apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) and nuclear factor-κB (NF-κB), two signaling molecules involved in survival pathways. Soy isoflavones decreased APE1/Ref-1 expression in vitro, whereas radiation up-regulated it. Pretreatment with soy isoflavones followed by radiation inhibited APE1/Ref-1 expression. APE1/Ref-1 decrease correlated with decreased DNA-binding activity of NF-κB mediated by soy isoflavones and radiation, thus promoting cell killing. In vitro treatment of prostate tumors with soy isoflavones and radiation down-regulated APE1/Ref-1 protein expression and NF-κB activity, confirming the molecular alterations observed in vitro. The down-regulation of APE1/Ref-1 and NF-κB by isoflavones, in vitro and in vivo, supports our hypothesis that these markers represent biological targets of isoflavones. Indeed, a 2-fold increase in APE1/Ref-1 expression, obtained by cDNA transfection, resulted in a 2-fold increase in NF-κB DNA-binding activity, and both of which were down-regulated by soy isoflavones, confirming the cross-talk between these molecules and, in turn, causing radiosensitization. [Cancer Res 2007;67(5):2141–9]

Introduction
Prostate cancer is the most commonly diagnosed cancer in American men as well as the second leading cause of male cancer deaths (1). Epidemiologic studies have shown that men who consume diets rich in soy isoflavones have lower incidence of prostate cancer (2, 3). Emerging data suggest that genistein, a major bioactive isoflavone component of soybeans, enhances the efficacy of conventional cancer therapy by modulating key signaling molecules involved in controlling cell proliferation pathways, such as nuclear factor-κB (NF-κB) (4). NF-κB is a major transcription factor involved in the synthesis of critical cell survival proteins in response to cellular stress (5–9). NF-κB is constitutively activated in prostate cancer in vitro and correlates with tumor progression (8, 9). Genistein was found to inhibit the activation of NF-κB leading to cell growth inhibition by affecting the cell cycle and inducing apoptosis in prostate cancer cells in vitro (10–12). Localized prostate cancer is sensitive to conventional radiotherapy using megavoltage photons (X-rays), yet residual disease causes clinical relapse (13). Radiation causes DNA damage, triggering tumor cell apoptosis and death. However, in response to cellular stress, radiation can also cause the activation of DNA repair and/or NF-κB, thus driving the cells towards survival and promoting radioresistance, possibly contributing to clinical relapse. We have previously shown that pure genistein acts as a radiosensitizer and potentiates radiation-induced tumor cell killing of human PC-3 prostate cancer cell line in vitro (14, 15). Translation of these studies in vivo showed that genistein, given with primary prostate tumor irradiation, significantly enhanced inhibition of prostate tumor growth and increased mouse survival, using a metastatic orthotopic PC-3 xenograft tumor model (16). Furthermore, genistein combined with radiation also controlled spontaneous metastasis to para-aortic regional lymph nodes. Paradoxically, we discovered that pure genistein, given as a single treatment modality in this model, promoted increased metastasis to lymph nodes (16). This novel observation was reproduced in a syngeneic orthotopic RM-9 prostate tumor model (17), raising concerns regarding soy-based clinical trials for cancer patients. We recently found that a mixture of soy isoflavones, consisting of genistein, daidzein, and glycitein, was safer than genistein in the in vitro treatment of prostate tumors with soy isoflavones and radiation. In this study, we compared the effect of a soy isoflavone mixture to that of pure genistein, given alone or in conjunction with radiation, on molecular events in vitro and in vivo using human prostate cancer cells and prostate cancer orthotopic tumor model, respectively. Our previous studies showed that genistein causes pleiotropic molecular effects in prostate cancer cells (4), including the...
inactivation of the Akt/NF-κB pathway (10, 14, 19). Our initial studies showed that radiation-induced activation of NF-κB was completely inhibited by the pretreatment of PC-3 cells with genistein (14). Studies of the mechanism of activation of the transcription factor NF-κB indicate that NF-κB DNA binding is redox-regulated by and dependent upon uponicrin/apyrimidinic (AP) endonuclease 1/ redox factor-1 (APE1/Ref-1), a multifunctional protein involved in the maintenance of genomic integrity and in the regulation of gene expression (20–23). Therefore, in the current study, we focused our investigation on APE1/Ref-1 and NF-κB, two major signaling molecules involved in determining tumor cell fate, i.e., death versus survival, in response to treatment.

As a major component of the DNA base excision repair pathway, APE1/Ref-1 is the primary enzyme responsible for the recognition and incision of noncoding and mutagenic AP sites in DNA (24). However, APE1/Ref-1 also functions as a redox activator of multiple cellular transcription factors, including NF-κB (25–28). Levels of APE1/Ref-1 have been shown to be dramatically elevated in various types of cancer, including prostate cancer (29–32). Increased APE1/Ref-1 expression was shown to promote tumor resistance to both ionizing radiation and chemotherapy(32–34). However, a decrease in APE1/Ref-1 expression was shown to render cells more sensitive to a variety of DNA damaging agents, including ionizing radiation (35, 36). Therefore, in the current study, we explored the potential of APE1/Ref-1 as a molecular target for the enhanced prostate cancer radiosensitivity mediated by soy isoflavones.

The effect of soy isoflavones combined with radiation on APE1/Ref-1 modulation was compared with that on NF-κB regulation based on previous studies demonstrating cross-talk between these two molecules (20–23, 26). Our data show that soy isoflavones decreased the expression of APE1/Ref-1 in vitro and in vivo, whereas radiation caused up-regulation. However, pre-treatment with soy isoflavones followed by radiation inhibited the expression of APE1/Ref-1. The decrease in APE1/Ref-1 expression correlated with decreased DNA-binding activity of NF-κB mediated by soy isoflavones and radiation in vitro and in vivo, indicating a possible mechanism of increased cell killing and tumor growth inhibition. To our knowledge, this is the first study demonstrating increased prostate tumor radiosensitivity by decreasing APE1/Ref-1 using soy isoflavones.

Materials and Methods
Prostate cancer cells. PC-3 human prostate cancer cell line (American Type Culture Collection, Rockville, MD) was cultured in F-12K culture medium (CM) with supplements (14, 15).

Treatment with soy isoflavones. The soy isoflavones mixture (formulation 115H7625 COA; Solae, St. Louis, MO) consists of 43% genistein, 21% daidzein, 2% glycitein, 2.5% protein, 11.9% fat, and 1.7% water, with the remainder being carbohydrate. Both soy isoflavones and pure genistein (LKT Laboratories, Toronto, Canada) powders were dissolved in 0.1 mol/L Na2CO3 to make a 10 mmol/L stock solution. Then, soy isoflavones or genistein were serially diluted 1:10 and 1:100 in culture medium and added to cells plated in flasks or plates to obtain final concentrations of 5 to 60 μmol/L (14). Control cells were incubated with equivalent dilutions of Na2CO3 in CM.

For animal experiments, soy isoflavones or genistein were dissolved in 0.1 mol/L Na2CO3 and mixed with sesame seed oil at a 2:1 ratio before treatment to facilitate oral gavage and to avoid irritation of the esophagus by Na2CO3 (16). Mice were treated p.o. by gavage either with 1 mg/day soy or an equivalent dose of 0.43 mg/day pure genistein. Control and radiation-only–treated groups received a mixture of 0.1 mol/L Na2CO3 and sesame seed oil.

Treatment with radiation. Cells in 15-mL tubes, T25 flasks, or T75 flasks were irradiated with photons using a 60Co unit (Atomic Energy of Canada Ltd. Theratron 780, Mississauga, Ontario, Canada) as previously detailed (14, 15).

Quantification of apoptosis by histone/DNA ELISA. PC-3 cells were treated with 0 to 60 μmol/L pure genistein or soy isoflavones for 72 h and then treated with 3 Gy radiation. At 24 h after radiation treatment, cells were processed for detection of apoptosis using the Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) as previously described (37). Spectrophotometric absorbance of the samples was determined using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 405 nm. The enrichment factor was calculated by dividing absorbance of treated cells by absorbance of control untreated cells.

Analysis of cell survival by clonogenic assay. Cells were treated with soy isoflavones for 24 h, then irradiated with 3 Gy photons (14, 15). After treatment, cells were plated in triplicate wells of six-well plates in 2 mL CM as follows: 500 cells per well for control, 1,000 cells per well for soy isoflavones or radiation alone, and 3,000 cells per well for soy isoflavones + radiation treatments, based on cell titrations previously determined (14). Following 10 days incubation, in the presence of soy isoflavones, at 37°C in a 5% CO2/5% O2/90% N2 incubator, colonies counted (clones of at least 50 cells were counted as one colony). The plating efficiency was calculated for each well, and the surviving fraction was normalized to control cells (14).

Protein extraction and Western blot analysis. Extraction of nuclear proteins and cytoplasmic proteins was accomplished using Sigma Celllytic NuCLEAR Extraction Kit (Sigma-Aldrich, St. Louis, MO). Western blot analysis was completed as previously described (14) using monoclonal anti-APE1/Ref-1 antibody (Novus Biologicals, Littleton, CO). AlphaEaseFC imaging software (Alphalnotech, San Leandro, CA) was used to quantify resultant bands (28). As an internal control for nuclear protein loading, membranes were reprobed with anti-retinoblastoma antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or for cytoplasmic protein loading with anti-jβ-actin antibody (Abcam, Cambridge, MA; ref. 37).

Fluorescence staining for confocal microscopy. PC-3 cells were cultured onto coverslips in six-well plates and treated with 30 μmol/L soy isoflavones for 48 h, then irradiated with 3 Gy. One hour after radiation, cells were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated overnight at 4°C with monoclonal anti-APE1/Ref-1 antibody (Novus Biologicals). After washing with PBS, cells were exposed for 2 h to FITC-conjugated secondary goat anti-mouse immunoglobulin G (Alexa Fluor 488, Invitrogen, Carlsbad, CA) prepared in 4% polyacrylamide gel using buffer containing Tris-glycine buffer (pH, 8.5) and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1. Equal protein loading was ensured by immunoblotting 10 μg of nuclear protein with antiretinoblastoma antibody (37).

Transfection of PC-3 cells with APE1/Ref-1 cDNA. PC-3 cells were transfected either with empty plasmid pCMV6-XL5 (pCMV6) or with the plasmid containing APEX nuclease 1 (pCMV6.APE1; OriGene, Rockville, MD). PC-3 cells were transfected with the cDNA plasmid vectors (5 μg DNA) using LipofectAMINE 2000 (Invitrogen). After 6 h incubation, the medium was replaced, and the cells were further incubated for 48 h. Then, the cells were treated with soy for 24 h at a concentration of 60 μmol/L, which showed a significant inhibition of APE1/Ref-1 (Fig. 4B) because the exposure to soy was only 24 h. Nuclear extracts were prepared using the Sigma Celllytic NuCLEAR Extraction Kit as described above. APE1/Ref-1 Western blot analysis and EMSA analysis for NF-κB were done using the transfected and nontransfected PC-3 nuclear extracts.

Cancer Res 2007; 67: (5). March 1, 2007 2142 www.aacrjournals.org
Experimental animals. Male nude mice (BALB/c nu/nu, 5–6 weeks old; Harlan, Indianapolis, IN) were housed in sterile facilities, fed sterile water and Lab Diet 5021 (Purina Mills, Inc., Richmond, IN) ad libitum. The mice were used in accordance with Animal Care and User Guidelines of Wayne State University under a protocol approved by the Institutional Animal Care and Use Committee as required by the American Association for the Accreditation of Laboratory Animal Care.

Experimental protocol in orthotopic PC-3 animal model. PC-3/PI prostate tumor cell lines were generated from PC-3 human prostate tumors as previously described (16, 39, 40). PC-3/PI cells (5 × 10^6) in 20 µL HBSS were injected into the mouse prostate as previously detailed (16, 17, 39, 40). Following prostate implantation with PC-3/PI cells, before initiating treatment, a few mice were killed to monitor and confirm tumor growth and size in the prostate. On days 7 to 10 after cell injection, mice had established 0.4-cm prostate tumors compared with 0.2-cm normal prostate.

Ten mice per group were used, and mice were randomized into the following six treatment groups: (a) untreated control; (b) genistein, daily oral gavage; (c) soy isoflavones, daily oral gavage; (d) radiation only; (e) genistein + radiation; (f) soy isoflavones + radiation. On day 8, treatment with soy isoflavones mixture or pure genistein was initiated by oral gavage as described above and continued once a day for 3 days. On day 11, prostate tumors were irradiated with 5 Gy photon radiation as previously detailed (16, 40). Daily isoflavone treatment was resumed on day 12 and continued for the duration of the experiment (16, 17). In separate experiments, mice were killed on day 19 postinjection for Western blot and EMSA analyses of prostate tumor extracts and on day 32 postinjection to resect and weigh tumor-bearing prostates (16, 17).

Tissue preparation for histology. Tissues were fixed in 10% zinc formalin, embedded in paraffin, and sectioned (16, 17). Tumor sections (5 µm) were stained with monoclonal anti-APE1/Ref-1 antibody (Novus Biologicals) by immunohistochemistry as previously described (16).

Statistical analysis. Comparisons among the various treatment groups of the apoptosis, Western blot, and EMSA assays were analyzed by two-tailed unpaired Student’s t test. For the clonogenic assay, ordinary least-squares regression was used to analyze the relationship between soy dose and radiation on cell survival. For in vivo data analysis, the shape of the outcome was assessed to determine whether the assumptions of normal theory tests were tenable. For tumor weight, no useful transformation could be found; therefore, Kruskal-Wallis rank tests were used to evaluate the statistical significance of differences between groups. Multiple comparisons between treatment conditions for each end point were made using Holm’s procedure to protect against inflated type I errors.

Results

Soy isoflavones potentiate apoptosis and inhibition of cell growth by radiation in vitro. We have previously shown that pure genistein enhances apoptosis and inhibition of cell growth caused by radiation (14, 15). To determine whether the soy isoflavone mixture is effective at potentiating of radiation-induced cell killing, we have compared pure genistein to soy isoflavones. Initially, the effect of various doses of soy isoflavones on the induction of apoptotic cell death was tested compared with identical doses of genistein. PC-3 cells were treated with 0, 15, 30, and 60 µmol/L genistein or soy isoflavones for 72 h and then tested for apoptosis using the histone/DNA ELISA detection kit. A dose-dependent increase in apoptotic cell death was observed with soy isoflavones at levels comparable to those obtained with pure genistein (Fig. 1A). An intermediate dose of 30 µmol/L genistein or soy isoflavones was selected for the combined treatment with radiation. PC-3 cells were pretreated with 30 µmol/L genistein or soy isoflavones for 72 h then irradiated with 3 Gy photons and, 24 h later, were tested for apoptosis (Fig. 1B). The level of apoptosis caused by genistein or soy isoflavones combined with radiation (11- to 13-fold increase) was significantly greater than that caused by isoflavones (4-fold increase, P < 0.001) or radiation alone (5.7-fold increase, P < 0.005).

To evaluate the long-term effect of soy isoflavones and radiation on cell growth, treated cells were assayed using a clonogenic assay. Dose titration clonogenic assays using pure genistein alone or combined with radiation in vitro showed that pretreatment of PC-3 cells with genistein at 15 µmol/L enhanced the cell killing caused by low-dose 3 Gy photon radiation (14, 15). These studies were repeated using soy isoflavones instead of pure genistein. PC-3 cells were pretreated with 5, 10, and 15 µmol/L soy isoflavones or 15 µmol/L pure genistein (G) for 24 h then irradiated with 3 Gy photons (R). Cells were plated in a clonogenic assay after soy isoflavones and/or radiation treatment. Column, mean inhibition of survival fraction of triplicates for each single and combined treatment; bars, SE. *, value statistically significant from control at P < 0.05.

Figure 1. Increased apoptosis and inhibition of cell survival by soy isoflavones or genistein combined with radiation in PC-3 cells. PC-3 cells treated with isoflavones alone or combined with radiation were assayed using histone/DNA ELISA for detection of apoptosis. A, dose response of genistein or soy isoflavones–induced apoptosis. PC-3 cells were treated with 0, 15, 30, and 60 µmol/L genistein or soy isoflavones for 72 h. B, apoptosis induced by soy isoflavones and radiation. PC-3 cells were pretreated with 30 µmol/L genistein or soy isoflavones for 72 h then irradiated with 3 Gy photons and 24 h later tested using histone/DNA ELISA. C, cell survival measured in clonogenic assay. PC-3 cells were treated with 5, 10, and 15 µmol/L soy isoflavones or 15 µmol/L pure genistein (G) for 24 h then irradiated with 3 Gy photons (R). Cells were plated in a clonogenic assay after soy isoflavones and/or radiation treatment. Columns, mean inhibition of survival fraction of triplicates for each single and combined treatment; bars, SE. *, value statistically significant from control at P < 0.05.
not shown). A significant inhibition in cell survival fraction was obtained with increasing doses of 10 and 15 μM/L soy isoflavones (P < 0.001; Fig. 1C). These doses caused a further and significant cell survival inhibition up to 95% when combined with radiation compared with radiation alone or soy alone (P < 0.001; Fig. 1C). The effect of soy alone at 15 μM/L or combined with radiation was more potent than that of genistein at 15 μM/L alone or with radiation (P < 0.001; Fig. 1C). The combined treatment caused an inhibition greater than additive relative to each single treatment alone. These data were consistently reproduced in independent experiments.

**Soy isoflavones down-regulate expression of APE1/Ref-1 protein.** To elucidate the role of survival pathway molecules in the inhibition of cell growth by genistein/soy isoflavones, we analyzed the expression of APE1/Ref-1, a molecule involved in DNA repair that also functions as a redox activator of multiple cellular transcription factors. PC-3 cells were treated with 15, 30, and 60 μM/L pure genistein or soy isoflavones for 72 h, reproducing the conditions selected for the detection of apoptosis in histone/DNA ELISA assay (Fig. 1A). Expression of APE1/Ref-1 was determined in nuclear extracts from treated cells by Western blot analysis. PC-3 cells strongly express APE1/Ref-1 protein (Fig. 2A). A dose-dependent decrease in nuclear expression of APE1/Ref-1 protein was observed with increasing doses of either pure genistein or soy isoflavones. Based on these data, a dose of 30 μM/L pure genistein or soy isoflavones that caused a marked decrease in APE1/Ref-1 protein expression was selected to determine the kinetics of APE1/Ref-1 down-regulation. PC-3 cells were treated with 30 μM/L pure genistein or soy isoflavones for 24, 48, and 72 h, and nuclear APE1/Ref-1 protein expression was determined by Western blot analysis. A time-dependent decrease in the expression of APE1/Ref-1 protein was shown, resulting in an almost complete down-regulation of APE1/Ref-1 after 72 h treatment with 30 μM/L pure genistein or soy isoflavones (Fig. 2C). A strong basal level of APE1/Ref-1 protein was observed in the cytosol of PC-3 cells by Western blot analysis, which was decreased by treatment with either with 30 μM/L of pure genistein or soy isoflavones (Fig. 2C).

**Inhibition of radiation-induced APE1/Ref-1 expression in PC-3 cells pretreated with soy isoflavones.** We have shown that genistein/soy isoflavones down-regulate APE1/Ref-1 protein expression in a dose- and time-dependent manner (Fig. 2A and B). To investigate the role of APE1/Ref-1 down-regulation in potentiation of radiation-induced cell killing by genistein/soy isoflavones, we analyzed the expression of APE1/Ref-1 following combined treatment. PC-3 cells were pretreated with 30 μM/L genistein or 30 μM/L of soy isoflavones for 72 h followed by 3 Gy photon radiation. Cells were harvested at 1 h postradiation, and expression of APE1/Ref-1 protein was determined in the nuclear fraction by Western blot analysis. Both genistein and soy isoflavones caused a decrease in nuclear expression of APE/Ref-1 compared with control cells (Fig. 2D). The expression of APE1/Ref-1 was increased by 3 Gy of radiation (Fig. 2D), representing an early event in the cell response to radiation because of its role in DNA repair. This up-regulation of APE1/Ref-1 expression by radiation was inhibited by pretreatment with genistein or soy isoflavones (Fig. 2D).

Confocal microscopy analysis of APE1/Ref-1 cellular localization revealed that APE1/Ref-1 was strongly expressed in the cell nucleus and cytoplasm (Fig. 3A). Following radiation, an increased nuclear staining was observed (Fig. 3B), whereas soy isoflavones alone (Fig. 3C) or combined with radiation (Fig. 3D) caused decreased levels of APE1/Ref-1 in the nucleus. These data corroborate our findings using Western blot analysis of APE1/Ref-1 protein expression (Fig. 2D).

**Soy isoflavones inhibit NF-κB DNA-binding activity.** We have previously shown that genistein caused a decrease in NF-κB DNA-binding activity, a well-known transcription factor critically involved in the survival of cells (4, 14). In contrast, we found that radiation increased NF-κB activity, possibly promoting radioresistance. However, radiation-induced activation of NF-κB activity was completely inhibited by the pretreatment of cells with genistein (14). To determine whether the soy isoflavone mixture
also down-regulates NF-κB DNA-binding activity and inhibits radiation-induced activation, these experiments were repeated with soy isoflavones. PC-3 cells were treated with 15, 30, and 60 μmol/L pure genistein or soy isoflavones for 72 h, reproducing the conditions selected for previous experiments. A dose-dependent decrease in NF-κB DNA-binding activity was observed with increasing doses of either pure genistein or soy isoflavones (Fig. 4A). Based on these data, a dose of 30 μmol/L pure genistein or soy isoflavones for 24, 48, and 72 h, and NF-κB DNA-binding activity was determined by EMSA. A time-dependent decrease in NF-κB DNA-binding activity was shown, resulting in almost complete inhibition of NF-κB DNA binding after 72 h treatment with 30 μmol/L pure genistein or soy isoflavones (Fig. 4B).

**Soy isoflavones inhibit APE1/Ref-1–induced NF-κB activity in APE1/Ref-1 overexpressing PC-3 cells.** We have shown that soy isoflavones down-regulate APE1/Ref-1 expression and inhibit the DNA-binding activity of NF-κB (Figs. 2 and 4A and B). To elucidate whether a molecular cross-talk occurs between APE1/Ref-1 and NF-κB that is involved in soy-induced killing of PC-3 cells, the effect of overexpressing APE1/Ref-1 on NF-κB DNA-binding activity was investigated using APE1/Ref-1 cDNA plasmid transfection of PC-3 cells. PC-3 cells were transfected with pCMV6.APE1 for 48 h, then treated with soy isoflavones for 24 h, and tested for APE1/Ref-1 expression and the DNA-binding activity of NF-κB. Transfection of PC-3 cells with empty pCMV6 plasmid did not affect APE1/Ref-1 expression and did not alter the APE1/Ref-1 decrease mediated by soy treatment (Fig. 4C). Compared with control, nontransfected cells, and pCMV6-transfected cells, PC-3 cells transfected with pCMV6.APE1 showed a 2-fold increase in nuclear expression of APE1/Ref-1, confirming its overexpression induced by gene transfection (Fig. 4C). Treatment of APE1/Ref-1 overexpressing PC-3 cells with soy isoflavones caused about a 50% decrease in APE1/Ref-1 expression (Fig. 4C). The effect of 2-fold overexpression of APE1/Ref-1 was striking and also resulted in a 2-fold increase in NF-κB DNA-binding activity compared with control and pCMV6-transfected PC-3 cells (Fig. 4C). This 2-fold increase in NF-κB DNA-binding activity of pCMV6.APE1-transfected cells was decreased by almost 50% by soy treatment (Fig. 4C). Transfection of PC-3 cells with empty plasmid did not affect NF-κB DNA-binding activity or its inhibition by soy treatment (Fig. 4C).

**Pretreatment with soy isoflavones inhibits radiation-induced activation of NF-κB.** We have shown that genistein/soy isoflavones down-regulate NF-κB DNA-binding activity in a dose- and time-dependent manner (Fig. 4A and B). To investigate whether the down-regulation of NF-κB DNA-binding activity could potentiate radiation-induced cell killing by genistein/soy isoflavones, we analyzed NF-κB DNA-binding activity following combined treatment. PC-3 cells were pretreated with 30 μmol/L...
genistein or 30 μmol/L of soy isoflavones for 72 h followed by 3 Gy photon radiation. Cells were harvested at 1 h postradiation, and NF-κB DNA-binding activity was determined by EMSA. Both genistein and soy isoflavones caused a decrease in NF-κB DNA-binding activity compared with control cells (Fig. 4D). NF-κB DNA binding was increased in response to 3 Gy radiation (Fig. 4D), representing an early response to cell stress induced by radiation. This up-regulation of NF-κB DNA-binding activity by radiation was inhibited by pretreatment with genistein or soy isoflavones (Fig. 4D). It is important to note that soy isoflavones are as potent as pure genistein at inhibiting NF-κB DNA-binding activity.

Soy isoflavones potentiate the inhibition of tumor growth by radiation and down-regulate APE1/Ref-1 and NF-κB in vivo.

We have shown in vitro that soy isoflavones potentiate tumor cell killing by radiation and cause alterations in the expression and activity of APE1/Ref-1 and NF-κB, two major signaling molecules involved in cell death or survival pathways. To investigate whether these findings occur in vivo, we have treated established PC-3 prostate tumors with a combination of soy isoflavones and tumor irradiation. Mice bearing established PC-3 prostate tumors (0.4 cm in size) were pretreated with p.o. soy isoflavones mixture, given by gavage at a dose of 1 mg/day for 3 days, or an equivalent dose of pure genistein (0.43 mg/day) followed by prostate tumor irradiation at a dose of 5 Gy photons as previously described (16). One day after radiation, soy isoflavones or genistein treatments were resumed and given everyday for 3 continuous weeks (Fig. 5A). Serum levels of about 3 μmol/L genistein and 0.5 μmol/L daidzein were measured in mice treated with genistein, whereas equal levels of genistein (1.7 μmol/L) and daidzein (1.6 μmol/L) were measured in mice treated with soy isoflavones compared with baseline levels in control mice (0.3 μmol/L; ref. 18). The effect of genistein alone was not significant; however, genistein + radiation significantly inhibited tumor growth by 85% compared with each modality alone (P < 0.05; Fig. 5B), confirming our previous studies (16). Soy isoflavones combined with radiation caused a significant inhibition of primary prostate tumor growth (86%; P < 0.001) compared with soy isoflavones alone (40%, P < 0.001) or radiation alone (70%, P < 0.05; Fig. 5B). Therefore, these findings show that the combination of soy isoflavones with radiation led to a greater control of primary tumor growth than soy isoflavones or radiation alone, similar to genistein and radiation treatment (16).

To investigate the molecular alterations induced in situ by soy isoflavones and radiation, the in vivo experiment described above was repeated, with the exception that mice were killed earlier to detect molecular changes in prostate tumors. Tumor extracts were obtained on day 19 (Fig. 5A), 1 week after combined treatment with radiation and soy isoflavones to determine the expression and DNA-binding activity of APE1/Ref-1 and NF-κB, respectively. Interestingly, treatment of prostate tumors with either
pure genistein or soy isoflavones down-regulated the expression of APE1/Ref-1 protein, similar to our findings in vitro (Fig. 5C). The expression of APE1/Ref-1 was still relatively high in mice treated with radiation, but was down-regulated in mice treated with radiation combined with genistein or soy isoflavones (Fig. 5C). The DNA-binding activity of NF-κB measured in prostate tumors also followed a similar pattern to that observed in vitro. Treatment with either pure genistein or soy isoflavones alone decreased NF-κB DNA-binding activity, whereas this activity remained high in response to radiation treatment (Fig. 5D). However, treatment with pure genistein or soy isoflavones significantly inhibited the NF-κB DNA-binding activity observed in response to radiation in prostate tumors (Fig. 5D).

Expression of APE1/Ref-1 in prostate tumors treated with soy isoflavones and radiation. To further determine whether soy isoflavones alone or combined with radiation caused decreased in situ alterations in the expression of APE1/Ref-1 in prostate tumors, sections of PC-3 primary prostate tumors were immunostained for APE1/Ref-1 expression. Control tumors showed intensive nuclear staining in >95% of tumor cells, indicating a high level of expression of APE1/Ref-1 in prostate tumors (Fig. 6A) confirming previous observations (29). Following radiation treatment, tumor cells also showed a high level of APE1/Ref-1 staining. Areas of fibrosis as a result of tumor destruction were also observed (Fig. 6B) as previously described (16). Treatment with either genistein or soy isoflavones caused a decrease in the intensity of APE1/Ref-1 staining in the nuclei of tumor cells (Fig. 6C). Combined treatment of radiation with either genistein or soy isoflavones showed comparable findings; these prostate tumors consisted of numerous abnormal giant atypical cells that showed marked cytologic changes such as cytomegaly or nucleomegaly as previously described (16). Interestingly, these giant cells also showed the absence or decreased intensity of nuclear APE1/Ref-1 staining (Fig. 6D) compared with control tumors (Fig. 6A). These observations were consistent with those obtained by Western blot analysis of tumor extracts (Fig. 5C).

Discussion

We previously showed that pure genistein potentiates prostate cancer tumor cell killing by radiation in vitro (14, 15). The current study shows that the mixture of soy isoflavones is equally effective in causing apoptotic cell death, inhibiting prostate cancer cell growth, and potentiates radiation-induced cell killing in vitro as observed with pure genistein (14, 15).

To address the molecular events driving soy isoflavones potentiation of radiation, we studied APE1/Ref-1 expression and NF-κB activity, two signaling molecules involved in survival pathways (5–7, 19, 24–27). These molecules were strongly expressed by PC-3 cells in vitro. Soy isoflavones or pure genistein inhibited APE1/Ref-1 expression in the cell nuclear fraction in a time- and dose-dependent manner. Cytosolic expression of APE1/Ref-1 was also decreased. The nuclear expression of APE1/Ref-1 was increased by radiation, probably representing an early event in the cell response to radiation because of its role in DNA base excision repair. Up-regulation of APE1/Ref-1 expression by radiation was inhibited by pretreatment with soy isoflavones. These findings by Western blot analysis were confirmed by confocal microscopy analysis and suggest that APE1/Ref-1 protein expression is down-regulated by soy isoflavones and up-regulated by radiation, and pretreatment with soy isoflavones inhibits radiation-induced APE1/Ref-1 expression. Microarray analysis also showed that the level of APE1/Ref-1 mRNA is decreased by 5-fold with genistein treatment (data not shown), corroborating our protein expression data.

The down-regulation of APE1/Ref-1 expression by soy isoflavones alone or combined with radiation correlated with the

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Figure 5. Increased prostate tumor growth inhibition and alteration in APE1/Ref-1 protein expression and NF-κB DNA-binding activity in response to genistein or soy isoflavone treatment in vivo. A, flowchart representation of in vivo experimental design and treatment schedule. B, response of PC-3 primary prostate tumors to radiation and soy isoflavones. Columns, means of tumor weights for 7 to 10 mice per group measured on day 32; bars, SE. C, Western blot assay for APE1/Ref-1 protein expression was done on randomly selected tumor tissue nuclear extracts obtained from each treatment group. D, gel shift assay for NF-κB done on nuclear extracts from randomly selected tumor tissues obtained from each treatment group. Western blot analysis of retinoblastoma protein in the nuclear extract was done as an internal loading control. Columns, mean integrated density value of Western blot and EMSA data of the band per microgram of protein loaded; bars, SE. *, value statistically significant from control at P < 0.05.
inhibition of NF-κB DNA-binding activity. We found that soy isoflavones inhibit NF-κB DNA-binding activity in a time- and dose-dependent manner with comparable kinetics to the decrease in APE1/Ref-1.

Soy isoflavones also block radiation-induced NF-κB activation, confirming that they are potent inhibitors of NF-κB like pure genistein (14).

In vivo, using the orthotopic PC-3 model, we showed that the combination of soy with radiation led to a greater control of primary tumor growth than soy or radiation alone, as observed with genistein (16). The in vitro molecular alterations of APE1/Ref-1 and NF-κB induced by soy isoflavones and radiation were reproduced in situ in prostate tumors. These molecules were strongly expressed in untreated PC-3 prostate tumors. APE1/Ref-1 protein expression and NF-κB DNA-binding activity were inhibited by treatment with soy isoflavones alone or combined with radiation. Interestingly, APE1/Ref-1 immunostaining was decreased in prostate tumor sections from mice treated with soy isoflavones alone or combined with radiation, confirming in vivo protein expression by Western blot analysis.

Our results clearly show that the down-regulation of APE1/Ref-1 and NF-κB by isoflavones in our in vitro studies could be recapitulated in vivo, supporting our hypothesis that these markers truly represent biological targets of isoflavones. Their down-regulation by isoflavones could potentially radiosensitize prostate cancer tumor cells. This is in direct agreement with previous studies demonstrating that increased APE1/Ref-1 expression promoted tumor resistance to ionizing radiation (32, 33), and a decrease in APE1/Ref-1 expression increased radiosensitivity (35, 36). APE1/Ref-1 and NF-κB DNA are essential for transcription of downstream genes involved in cellular proliferation, tumor growth, invasion, and metastasis (4–7, 27). Their inhibition by soy isoflavones could drive the cells toward apoptotic pathways and block activation of radiation-induced survival pathways, leading to increased cell killing observed in vitro and tumor growth inhibition in vivo. We recently showed that key molecules regulated by NF-κB and involved in cell cycle progression and apoptosis are altered by genistein and radiation (14, 18). These downstream events included the up-regulation of p21WAF1/Cip1 and down-regulation of cyclin B1, the up-regulation of the proapoptotic molecule Bax, and the suppression of the antiapoptotic Bcl-xL and survivin molecules, leading to increased G2-M cell cycle arrest and an increase in apoptosis (14, 18).

The down-regulation of APE1/Ref-1 and NF-κB by soy isoflavones raised the question of the cross-talk between these molecules. In the current study, we have now shown that overexpression of APE1/Ref-1, obtained by cDNA transfection of PC-3 cells, caused a concomitant increase in NF-κB DNA-binding activity. Moreover, soy isoflavones treatment of APE1/Ref-1 over-expressing PC-3 cells significantly inhibited APE1/Ref-1 expression with a corresponding decrease in the NF-κB DNA-binding activity. These findings confirm a molecular cross-talk between APE1/Ref-1 and NF-κB, which is likely to be the molecular mechanism responsible for soy-induced killing of PC-3 cells and radiosensitization. In addition to DNA base excision repair, APE1/Ref-1 functions as a redox activator of multiple cellular transcription factors (25–28). NF-κB activity was found to be redox regulated by and dependent on APE1/Ref-1 (20). APE1/Ref-1–mediated reduction of the p50 subunit in the nucleus was shown to be a necessary step for NF-κB activation (21). Deletion of the redox-sensitive domain of APE1/Ref-1 inhibited tumor necrosis factor-induced NF-κB activation (22). Loss of APE1/Ref-1 resulted in decreased NF-κB DNA binding and transcriptional activation and increased susceptibility to tumor necrosis factor-induced apoptosis (23). Together, these findings establish APE1/Ref-1 as an essential upstream signaling molecule regulating NF-κB.
The dual nature of APE1/Ref-1, redox and repair, could lead to tumor cell survival by ensuring DNA integrity of promoter sequences while simultaneously reducing oxidized transcription factors such as NF-κB (41). Selective targeting of APE1/Ref-1 could enhance conventional cancer treatment such as radiotherapy (42, 43). Soy isoflavones and other dietary agents could downregulate APE1/Ref-1 expression, thereby altering its function as a redox activator of NF-κB, ultimately affecting the balance between cell survival and cell death. Given the safety and efficacy of soy isoflavones, our findings support the clinical use of soy isoflavones combined with tumor irradiation for the treatment of prostate cancer.

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References

Down-regulation of Apurinic/Apyrimidinic Endonuclease 1/Redox Factor-1 Expression by Soy Isoflavones Enhances Prostate Cancer Radiotherapy *In vitro* and *In vivo*


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