RESEARCH COMMUNICATION

Genistein Mechanisms and Timing of Prostate Cancer Chemoprevention in Lobund-Wistar Rats

Jun Wang[1]*, Isam-Eldin Eltoum[2,3], Mark Carpenter[4], Coral A Lamartiniere[1,3]

Abstract

The objective of the present study was to determine if a specific window of development (neonatal/ prepubertal only, adult only, or life-time) is effective for genistein chemoprevention of prostate cancer, and the potential mechanisms of genistein chemoprevention *in vivo*. Male Lobund-Wistar (L-W) rats were fed zero or 250 mg genistein/kg AIN-76A diet at designated periods of time and then injected with N-methylnitrosourea (NMU) into the dorsolateral prostate (DLP) on day 70 for cancer initiation. Rats were necropsied at 11 months. The incidence of poorly differentiated (PD) carcinomas was 43.5% in rats fed a phytoestrogen-free AIN-76A diet only, 29.6% in rats provided genistein in the diet from postnatal days 1-35, 28.6% in rats fed genistein from months 3-11, and 20% in rats provided genistein from birth through 11 months. Genistein induces cell apoptosis and inhibits cell proliferation in both prostate cancerous and nontumorigenic DLP. These actions are accompanied with the regulation of PTEN/Akt-AR axis. Our data demonstrate that dietary genistein reduces the incidence of advanced prostate cancer induced by NMU in L-W rats during adult and life-time exposure, the latter being more effective. The regulation of AR/Akt/PTEN axis by genistein may be one of the molecular mechanisms by which it inhibits cell proliferation and induces apoptosis, thus providing evidence of roles of genistein in prostate cancer prevention and treatment.

Key Words: Chemoprevention - prostate cancer - genistein - rat model

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Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men. Epidemiological data indicate that Asian men consuming a traditional diet high in soy products have a lower incidence of clinically manifested prostate cancer compared with American and European men (Adlercreutz, 1995).Yet, Asians who immigrate to the United States and adopt a western diet lose this protection, and the earlier in life of their arrival, the more risk for developing prostate cancer (Shimizu et al., 1991; Cook et al., 1999). These associations indicate the importance of environment, including nutrition and timing of exposure on the development of prostate cancer.

Asian men have traditionally consumed a soy-based diet containing isoflavonic phytochemicals, resulting in higher genistein concentrations in the blood, urine and prostatic fluid compared to American men (Adlercreutz et al., 1991; Morton et al., 1997). Genistein, the most abundant phytoestrogen component of soy, has been implicated in prevention of hormone-sensitive cancers including the breast and prostate (Adlercreutz, 1995). Genistein may reduce the risk of cancer due to its ability to inhibit protein-tyrosine kinase (Akiyama et al., 1987), angiogenesis (Fotsis et al., 1993), its ability to induce G2/M cell cycle arrest (Pagliacci et al., 1994), to enhance apoptosis in breast cancer cells (Li et al., 1999), its ability to regulate Akt signaling pathway (Li et al., 2002), and its antioxidant properties (Wei et al., 1995). Genistein also enhances tumor cell differentiation (Constantinou and Huberman, 1995).

The balance between cell proliferation and cell loss through apoptosis can determine how fast a neoplasm develops. Cancer chemoprevention is generally associated with inhibition of cellular hyperproliferation and induction of apoptosis. Genistein has been reported to inhibit cell proliferation and induce apoptosis in both prostate cancer cells and nontumorigenic prostate epithelial cells (Davis et al., 1998). AR, Akt and PTEN signaling pathways play important roles in prostatic tumorigenesis by modulating cell proliferative and apoptotic effects on homeostasis of prostatic epithelium.

The serine/threonine protein kinase Akt, also referred to as protein kinase B (PKB), plays a critical role in controlling the balance between cell survival and apoptosis (Franke et al., 1997). Akt may be activated by

[1]Department of Pharmacology and Toxicology, [2]Department of Pathology, [3]Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, [4]Department of Mathematics and Statistics, Auburn University, Auburn, AL USA
**For Correspondence: jun.wang@ccc.uab.edu*

insulin and various growth and survival factors through activation of phosphatidylinositol 3-kinase (PI3K) (Burgering et al., 1995; Franke et al., 1995). Akt functions to promote cell survival by inhibiting apoptosis by its ability to phosphorylate and inactivate several targets including Bad, Forkhead transcription factors and caspase-9, all of which are involved in apoptosis (Datta et al., 1997; Brunet et al., 1999; Cardone et al., 1998).

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) was identified to play a critical role in tumor suppression (Stiles et al., 2004). Loss of or reduction in PTEN expression has been reported in a wide array of human cancers, including those of the prostate and mammary (Li et al., 1997), and is associated with poor clinical outcome (Depowski et al., 2001). Overexpression of PTEN induces apoptosis in mouse mammary epithelium (Dupont et al., 2002), in breast cancer cells (Weng et al., 1999) and in prostate cancer cells (Davies et al., 1999), and PTEN-deficient murine embryonic fibroblasts are resistant to various apoptotic stimuli (Stambolic et al., 1998). The best elucidated function of PTEN as a tumor suppressor relates to its negative regulation of the PI3- kinase/Akt pathway by virtue of its ability to dephosphorylate phosphatidylinositol 3, 4, 5-triphosphate (Stambolic et al., 1998).

Androgens play a major role in the development, maintenance, and regulation of male phenotype and reproductive physiology. These activities are mediated through the AR, which belongs to the steroid/thyroid receptor superfamily, a group of ligand-regulated transcription factors. The expression, activation and up-regulation of AR may play a privotal role in prostate cancer progression (Culig, 2003). AR and PTEN play opposing roles in the prostate. AR induces proliferation and anti-apoptosis (Denmeade et al., 1996), while PTEN induces apoptosis and growth arrest (Yuan and Wang, 2002). PTEN inhibits the transcriptional activity of AR and works as an antagonist of AR activity through inhibition of Akt activation. This effect is not cell line or promoter dependent (Li et al., 2001; Nan et al., 2003). PI3K/Akt has been shown to promote prostate cancer cell survival and growth via enhancing AR-mediated transcription. It has been reported that blocking the PI3K/Akt pathway reduces the expression of an endogenous AR target gene, and the cross-talk between the androgen and PI3K/Akt pathways is mediated through the modulation of glycogen synthase kinase 3 (GSK3), a downstream substrate of PI3K/Akt (Sharma et al., 2002). Genistein has been shown to induce the expression of the PTEN gene and reduce AR gene expression in prostate cancer cells (Cao et al., 2006). Despite the reported actions of genistein in vitro, the in vivo mechanisms of action that account for chemoprevention remain to be elucidated, particularly at concentrations that represent human exposure.

While there is no perfect model for evaluating prostate cancer, numerous animal models including transgenic and chemically-induced rodent models have their merits and liabilities. Among the model systems of prostate cancer in rodents, the Lobund-Wistar (L-W) rat is unique in its inherent susceptibility to spontaneous and induced metastasizing adenocarcinomas which shares characteristics of tumorigenesis with those in aging men (Pollard, 1998). The L-W rat spontaneously develops a relatively high incidence (26% in an average of 26.6 months) of metastatic adenocarcinomas of the prostate complex and seminal vesicle (Pollard, 1973). In order to shorten the latency period and increase the incidence of tumors in rodents, researchers have used chemical carcinogens to induce tumorigenesis (Pollard and Luckert, 1986; Bosland and Prinsen, 1990). In L-W rats, prostate tumor incidence has been increased to 40-45% by 10-12 months via injections with n-methylnitrosourea (NMU) in the dorsolateral prostate followed by a series of s.c. implants of testosterone propionate (Schleicher et al., 1996). An added benefit of carcinogen injection into the dorsolateral prostate is target organ specificity (Schleicher et al., 1996). The dorsal and lateral lobes of the rat are considered homologues of the human prostate (Price, 1963). This results in an acceptable model for carcinogen-induced prostate cancer and chemoprevention studies.

We have previously demonstrated that lifetime exposure to physiological concentration of genistein via the diet protects against chemically-induced prostate cancer in L-W rats in a dose dependent manner (Wang et al., 2002). For the present study, we focused on the potential of timing of genistein exposure to protect against prostate cancer, a concept that we have shown to be important in genistein suppressing chemically-induced mammary cancer in rats. In the mammary, neonatal / prepubertal genistein exposure was necessary to program against mammary cancer development (Fritz et al., 1998; Lamartiniere et al., 2002). The goal of this investigation was to determine if a specific window of development (early postnatal life only, adult only or life-time) is responsible for genistein chemoprevention of prostate cancer. Considering that chemically-induced prostate cancer was initiated by injecting NMU orthotopically into the DLP of L-W rats at day 70, we analyzed DLPs of intact rats, as well as in prostate tumors by cell proliferation, apoptosis and AR/Akt/PTEN axis, for mechanisms of genistein chemoprevention.

Materials and Methods

Animals

Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the UAB Animal Care Committee. Seven-week old virgin female L-W rats were purchased from the National Cancer Institute. Animals were kept in a climate-controlled room with a 12:12-h light/dark cycle. These animals were fed AIN-76A diet (Harlan Texlad, Madison WS) for two weeks in our facilities prior to mating. The AIN-76A diet is a semi-purified diet with less than 10 pmol isoflavones/g diet as determined by high-pressure liquid chromatography (HPLC). One female per proven L-W stud was cohabited for two weeks. Day of birth was designated postnatal day zero. The offspring were sexed, and each litter was culled to 10 offspring. Chemically synthesized genistein (98.5% pure by HPLC analysis) was generously provided to us by Roche, Basel,

Switzerland. Genistein was mixed in the diet at 250 mg/kg AIN-76A diet by the laboratory of Dr. Clinton Grubbs (Chemoprevention Center, UAB). Previously, we showed that this dietary genistein concentration resulted in serum genistein concentrations in rats similar to those of humans eating a traditional Asian diet high in soy (Fritz et al., 1998; Adlercreutz et al., 1993), and protected against chemically-induced mammary cancer in rats (Fritz et al., 1998). To determine if a specific window of development (neonatal/prepubertal only, adult only or life-time) is critical for genistein chemoprevention of prostate cancer, the following groups of rats were used:

Group A) Life-time AIN-76A diet (no genistein) as control group.

Group B) 250 mg genistein/kg AIN-76A diet from birth until 35 days of age, then fed AIN-76A diet (neonatal/prepubertal genistein exposure group).

Group C) 250 mg genistein/kg AIN-76A diet starting at 90 days of age, 20 days after cancer initiation, until 11 months of age as adult genistein exposure group.

Group D) 250 mg genistein/kg AIN-76A diet from birth throughout life to demonstrate that postnatal lifetime exposure protects against prostate cancer.

The protocol for the induction of prostate tumors in L-W rats is based on our previous publication (Wang et al., 2002). From days 50–66 postpartum, one male offspring from each litter was gavaged daily with 33 mg Flutamide/kg body weight in sesame oil (Sigma Chemical Co., St Louis, MO) to effect chemical castration. On days 67–69, the rats were injected daily with 25 mg testosterone/kg body weight (Sigma) to stimulate mitosis. On day 70, all rats were anesthetized with ketamine/xylazine, a midline abdominal incision was made, and 42 mg N-methylnitrosourea (NMU)/kg body weight (Sigma) was administered via two injections into the dorsolateral prostate for cancer initiation. One week after NMU administration, rats were given silastic implants (1.8 cm long x 0:078 inch inner diameter x 0:024 inch wall) (SF Medical, Hudson, MA), plugged with medical grade adhesive (Dow Corning, Midland, MI) packed with 25 mg testosterone. The silastic implants were soaked for 2–4 hour and then overnight in phosphate-buffered saline containing 1% BSA. The testosterone capsules were implanted s.c. in the scapular region until 11 months of age (replaced every 12 weeks) to stimulate mitosis and promote tumor growth.

Animals were weighed every 2 weeks and observed daily for any signs of illness. Rats were palpated twice a week for prostate tumors starting 7 months after MNU injection, and necropsied at 11 months of age (9 months after MNU injection), or when tumors were approximately 1 cm in diameter, or when animals became moribund. Animals were sacrificed by decapitation and a detailed necropsy was carried out. Particular attention was paid to male genital and pelvic organs. Tumor size, origin and spread were noted. The prostate gland and associated tumors were excised and cut into two equal portions. One portion was snap frozen in liquid nitrogen and stored at -70°C for later measurement of AR, phospho-Akt and PTEN by western blot analyses, and the other portion was fixed in formalin, then paraffin-embedded for histopathology, immunohistochemistry and in situ apoptosis labeling analyses.

Cell proliferation, apoptosis, AR, PTEN and Akt proteins were measured in DLP of intact males receiving AIN-76A diet only and in rats exposed to genistein via the diet from birth until day 70 postpartum. At 70 days of age, the animals were weighed and killed by decapitation. Prostate glands were quickly removed and the DLP microdissected. One half of the DLP was snap frozen in liquid nitrogen and stored in -70°C for subsequent western blot analysis, and the other half fixed in 10% buffed formalin overnight and stored in 70% ethanol for immunohistochemistry of Ki-67 as index of cell proliferation and in situ apoptosis labeling analyses.

Histopathology

Fixed prostate tissue and tumors were processed and embedded in paraffin. Sections (5 μm) were cut from paraffin-embedded tissues and mounted on ProbeOn-Plus slides (Fisher scientific). The sections were coded to prevent reader bias. Histological sections of all tissues were evaluated by a board certified pathologist (Dr. I-E. Eltoum) using a scale that has been established for the TRAMP model (Wechter et al., 2000). Noncancerous lesions were graded as 1: normal tissue, 2: low PIN and 3: high PIN, respectively. Grades 4, 5 and 6 were used to describe well-, moderately- and poorly-differentiated cancerous lesions, respectively.

Cell proliferation

Ki-67 protein was measured in normal DLP tissues and poorly differentiated prostate tumors by immunohistochemistry. Deparaffinized prostate tissue sections were autoclaved for 20 minutes with antigen-unmasking fluid (Vector Laboratories, Burlingame, CA) for antigen retrieval, and sequentially treated with 3% H2O2 for 30 minutes and 2.5% normal horse serum for 30 minutes. The sections were incubated with 1:50 diluted monoclonal mouse anti-rat Ki-67 (MIB-5) antibody (Dako North America, Inc. Carpinteria, CA) for 30 minutes at room temperature and sequentially exposed to ImmPress horse anti-mouse Ig (peroxidase) (Vector Laboratories, Burlingame, CA). Diaminobenzidine substrate kit for peroxidase (Vector Laboratories, Burlingame, CA) was used for visualizing the sites of peroxidase binding. The slides were viewed using a Nikon Labophot-2 microscope (Nikon Corporation, Tokyo, Japan) and digitally recorded using a Nikon 8.0 Mega Pixels CoolPix 8700 Digital Camera (Nikon). The cells were counted using Image J software (Image J, NIH). The cell proliferation indices were generated by counting over 1200 epithelial cells in more than five subsequent microscopic fields showing the highest density of immunostained cells per slide under a microscope at a high magnification of 40x and expressed as numbers of Ki-67-positive cells per 100 prostate epithelial cells. Each group contained six samples.

In situ apoptosis labeling

For apoptosis assessment, TUNEL assay was performed with ApopTag Plus Peroxidase In Situ

Apoptosis Detection kit (Chemicon International, Temecula, CA) following the manufacturer's instructions. For quantitation of apoptotic cells, two parameters were used to identify apoptotic cells. First, we used positive immunolabeling as detected by ApopTag staining. Second, cells that were positive by immunolabeling were then confirmed by morphological characteristics typical of cells undergoing programmed cell death, i.e., including chromatin aggregation, nuclear, and cytoplasmic condensation as well as fragmentation of the dying cell into a cluster of membrane bound segments. If a cell was judged to be apoptotic by both criteria, it was judged positive. For apoptosis measurement in tumor tissues, the cell apoptotic indices were generated by counting over 1000 cells in more than four subsequent microscopic fields showing the highest density of immunostained cells per slide under a microscope at a high magnification of 400x and expressed as numbers of positive cells per 100 prostate cells. For measurement in normal DLP tissues, the total number of gland acini (over 200 counted) in whole section of each sample was counted under a Nikon microscope at a low magnification (10x) view. All identified apoptotic cells in the gland acini in a section were recorded under high-power (40x), and the apoptotic index was defined as the total number of apoptotic cells per 100 gland acini in each prostate tissues (Hu et al., 1998). Each treatment group contained six animals. All data were expressed as the mean ± SEM. Control and treatment groups were compared using a two-tailed paired t-test, and the differences was considered significant at the level of $p < 0.05$.

Western blot analysis

Protein concentration of each homogenized sample in lysis buffer was determined using the Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules,CA). 40 μg of protein from each sample was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules,CA). Membranes were blocked and incubated with the appropriate antibodies. Nitrocellulose membranes were incubated with an appropriate secondary antibody conjugated to HRP (Cell Signaling Technology), and followed with a chemiluminescent substrate (Pierce). Membranes were then exposed to autoradiography film, and bands quantitated by densitometry analysis using Versa Doc Imaging System and QuantityOne 1-D Analysis Software (Bio-Rad). Precision plus protein prestained standards (Bio-Rad, # 161-0375) was loaded as molecular weight ladders. The following antibodies and positive controls were used for detection of AR, p-Akt and PTEN by Western blots. A polyclonal antibody for AR (N-20, Santa Cruz, CA, sc-816) recognizes AR protein mapping at the N-terminus of AR of human origin and cross-reacts with rats at molecular weight of 110 kDa. LNCap cell lysate (Santa Cruz, sc-2231) was used as AR positive control. The p-Akt antibody (Santa Cruz, CA, sc-7985R) is recommended for detection of Akt1 phosphorylated Ser-473 and correspondingly phosphorylated Akt2 and Akt3 of rat and human origin by western blotting at molecular weight of 60 kDa. A whole cell lysate of A-431-EGF (Santa Cruz, sc-2202) was used as a positive control for p-Akt. PTEN antibody (Cell Signaling Technology, Danvers, MA, #9552) detects endogenous levels of total PTEN protein at 54 kDa molecular weight by western blotting. NIN/3T3 whole cell lysate (Santa Cruz, sc-2210) was used as PTEN positive control.

Statistical analysis

Statistical analysis of histological specimens used exact Cochran-Armitage Trend Test to determine significance ($p < 0.05$). Mean values for cell proliferation index, apoptosis index and band densitometry of western blots from animals exposed to genistein were compared to controls using two-sample student t-test assuming unequal variances, with subsequent multiple comparisons. We considered $p < 0.05$ to be significant.

Results

Genistein in the diet reduced chemically-induced prostate cancer incidence in L-W rats

By 11 months of age, 56% of the control rats had prostate cancer lesions (Grades 4-6) with most of these (43%) being poorly differentiated prostate lesions (Table 1). Twenty-two percent of the Controls (no genistein) had normal tissue at 11 months, 4% and 17% had low- and high-grade PIN, respectively. In regards to genistein treatment, a total of 108 L-W male rats were stratified among four different groups. From a 4 x 6 contingency table analysis based on the exact likelihood ratio chi-square test, there was significant association between treatments: Controls (no genistein), genistein for days 1-35, genistein for adult, and genistein for life-time and tumor grade (1-6), p-value = 0.022. However, when comparing each of the genistein treatment groups to the controls, only genistein treatment for 1-11 months was significantly different from controls ($p = 0.023$). In addition, each genistein treatment was compared to the

Table 1. Effect of Genistein on Chemically-Induced Prostate Cancer Incidence in Lobund-Wistar Rats

			Incidence with Pathological Score*					
Treatment	Time	Group Size	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)
A) No Genistein	(Controls)	23	5 (22)	1 (4)	4 (17)	1 (4)	2 (9)	10 (43)
B) Genistein	(1-35 Days)	27	10 (37)	1 (4)	4 (15)	2 (7)	2 (7)	8 (30)
C) Genistein	(3-11 Months)	28	9 (32)	7 (25)	4 (14)	0 (0)	0 (0)	8 (29)[a]
D) Genistein	(1-11 Months)	30	13 (43)	7 (23)	0 (0)	1 (3)	3 (10)	6 (20)[a,b]

*Key to Pathology report: 1-Normal tissue; 2- Low-grade PIN; 3-High-grade PIN; 4-Well-differentiated lesion; 5-Moderately differentiated lesion; 6-Poorly differentiated lesion; [a]< 0.05 compared to controls based on the exact Cochran-Armitage trend test; [b]< 0.05 compared to Controls only using the chi-square test

controls using the exact Cochran-Armitage trend test. Based on this test, the genistein treatment for months 1-11 and genistein for months 3-11 groups had statistically significant downward trends as compared to the control group for poorly differentiatied tumors (grade 6), p = 0.016 and 0.041, respectively. There was no significant trend for genistein 1-35 days as compared to controls (p = 0.134). The greatest effect was seen in L-W rats exposed to genistein throughout life (1 – 11 months) with a 54% decrease in poorly-differentiated cancerous lesions (Grade 6) compared to controls (43.5% to 20%). Consistent with this was a 50% increase in normal prostate tissues (22% to 43%) for control compared to lifetime genistein treatment. These data strongly suggest that lifetime, and adult only, genistein exposure(s) can suppress the development of chemically-induced prostate cancer in L-W rats.

Since the life-time genistein treatment resulted in the most significant suppression of prostate cancer, we used genistein treatment from birth until day 70 (time of NMU injection) for determining the effect of genistein on cell proliferation and apoptosis and protein levels of AR, phospho-AKT and PTEN in DLP of intact rats.

Genistein decreased cell proliferation and increased apoptosis in normal DLP tissues and prostate tumors

Using Ki-67 labeling as indicator of cell proliferation and the TUNEL assay for apoptosis, we found that genistein significantly decreased cell proliferation by 52% and increased apoptosis by 45% in the DLP tissues of normal rats as compared to the controls. In poorly differentiated prostate tumors, we determined that genistein decreased cell proliferation by 44% and increased apoptosis by 55%. Importantly, cell proliferation to apoptotic ratios were significantly lower by 78% in normal DLP and 74% in the prostate tumors, respectively, as compared to the controls.

AR and phospho-Akt, but not PTEN, were regulated by dietary genistein in the DLP of normal 70 day old rats

We investigated whether genistein in the diet regulated the protein expressions of AR, phospho-Akt, and the tumor suppressor, PTEN in the DLP of intact rats. AR and phospho-Akt protein levels were determined to be significantly down-regulated by 62% and 32%, respectively. PTEN protein expression was not significantly altered in rats exposed from day 1-70 to genistein (Figure 1).

Figure 1. AR, p-Akt and PTEN Protein Expression. The proteins were analyzed by western blots. Above, lanes 1, 3, 5, 7, 9 and 11, animals on control diet; lanes 2, 4, 6, 8, 10 and 12, animals on genistein containing diet from birth until day 70 postpartum.

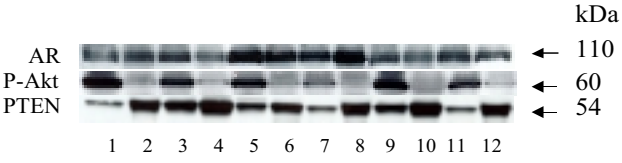

Figure 2. AR, p-Akt and PTEN Protein Expression in PD Prostate Tumors. The proteins were analyzed by western blots. Above, lanes 1, 3, 5, 7, 9 and 11, animals on control diet; lanes 2, 4, 6, 8, 10 and 12, animals on genistein diet.

Phospho-Akt and PTEN, but not AR, were regulated by dietary genistein in the prostate tumors of 11 month old L-W rats

To compare with normal tissue, we investigated these proteins in prostate tumors. There was a significant decrease (56%) in the phosphorylated Akt protein in prostate tumors (Grade 6) of L-W rats treated with genistein compared to control animals (Figure 2). On the other hand, PTEN protein expression in the prostate tumors was significantly up-regulated by 41% in genistein-treated animals. We did not find any significant change in AR protein expression in the prostate tumors of L-W rats treated with genistein.

Discussion

Prostate cancer is a chronic disease, therefore chemoprevention is an attractive strategy for disease control. In recent years, considerable progress has been made in this direction which has led to the identification of novel cancer chemopreventive agents and their mode of action. Ideally, the efficacy of such chemopreventive agents should be verified in animal models before recommending their use in humans. In this study we have administered genistein via the diet, focusing on timing of exposure, to protect against prostate cancer, a concept that we have shown to be important in genistein suppressing chemically-induced mammary cancer in rats (Fritz et al., 1998; Lamartiniere et al., 2002). In the latter, neonatal/prepubertal genistein exposure was necessary to program against mammary cancer development. A similar manifestation was recently noted for women exposed to soy/genistein during adolescence (Shu et al., 2001). Using the Shanghai Cancer Registry, a case control study reported an inverse relationship (50%) between adolescent (13-15 years) soy food intake and breast cancer incidence later in life (Shu et al., 2001).

For prostate cancer development, we found that life-time genistein exposure resulted in the most effective suppression of prostate cancer lesions. It resulted in a 54% decrease in poorly-differentiated prostate tumors. Consistent with this was a 50% increase in normal prostate tissue. Furthermore, genistein treatment for months 1-11 and genistein for months 3-11 groups had statistically significant downward trends as compared to the control group for poorly differentiatied tumors (grade 6), p = 0.016 and 0.041, respectively. With both of these genistein treatments, the chemoprevention was associated with suppressing the rate of cancer development as evidenced by increased percentage of PIN, (44% to 71% and 44% to 67%, respectively). On the other hand, genistein given only early in postnatal life was not able to exert a permanent protective effect against prostate cancer. Hence,

the direct presence of genistein has to be there in order for genistein to suppress prostate cancer development. This is consistent with the concept of lifetime healthy diet and avoidance of toxic chemicals to marginalize our susceptibility for biochemical insult.

The mechanism of action by which genistein suppresses prostate cancer has been speculated to be via its estrogen action because estrogens are generally considered anti-androgens. However as previously shown by us, genistein in the diet at the concentration used in these chemoprevention studies does not alter serum testosterone and DHT levels (Dalu et al., 1998; Fritz et al., 2002). On the other hand, AR was observed to be down-regulated in L-W rats provided genistein in the diet, confirming earlier work in Sprague Dawley rats (Fritz et al., 2002). This is interesting in that AR expression is believed to be under positive androgen control as demonstrated by the drastic reduction in prostate AR immunostaining following castration (Prins and Birch, 1993). AR is restored to normal levels following androgen administration (Prins and Birch, 1993). In our studies, down-regulation of the AR following exposure to genistein in the diet does not appear to be a result of lower testosterone, suggesting that some other mechanism must be responsible for the regulation of AR and of chemoprevention. Furthermore, this is supportive of dietary genistein having little or no adverse effect on the reproductive tract or fertility. In a previous study, we reported that this concentration of genistein in the diet to L-W rats resulted in genistein concentrations of 861±104 pmol / ml serum in the blood and 775±246 pmol / g tissue in the prostate (Wang et al., 2002).

In vitro and *in vivo* studies with genistein yield conflicting results in regards to cell proliferation. Some in vitro studies show that low concentrations of genistein (pmole/ml) stimulate cell proliferation and high doses (nmol/ml) inhibit cell proliferation (Barnes and Lamartiniere, 2003). In animal studies, low pmole/ml blood concentrations are usually associated with no effect and high pmole/ml blood genistein concentrations are usually associated with a biological action, including prostate and mammary cancer chemoprevention (Lamartiniere et al., 2002; Barnes and Lamartiniere, 2003). This dichotomy probably lies with the nature of cell culture studies not being regulated by the complex interplay of interactive cell types, hormones, the immuno-system, etc.

Imbalance between cell proliferation and apoptosis has been considered a key factor in carcinogenesis. Here, we demonstrate that dietary genistein reduced cell proliferation in DLP of intact rats and in chemically-induced L-W rat prostate tumors with 52% and 44% reduction, respectively. The apoptotic labeling indices for epithelial cells in DLP and prostate tumors in the genistein-treated animals were increased 55% and 45% respectively, compared to the control rats. Importantly, genistein in the diet reduced the ratio of cell proliferation to apoptosis by 78% in the DLP, and 74% in prostate tumors. The ratio of cell proliferation to apoptosis is very important because it takes into account the balance between cells replicating and cells dying due to programmed cell death. For instance, a low proliferative index may be offset by a low apoptotic index and actually be biologically irrelevant. In our study, the significant decrease in ratio of cell proliferation and apoptosis most likely plays a major role in prostate chemoprevention with genistein.

Akt is considered a cell survival protein. Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate down-stream targets. Akt may target multiple components of the apoptotic cascade such as Bad, Forkhead transcription factors and caspase-9 (Datta et al., 1997; Cardone et al., 1998; Brunet et al., 1999). Finding significantly down-regulated Akt in DLP tissue and prostate tumors supports the fact that apoptosis was increased in DLP tissue and prostate tumors of genistein-fed animals, hence providing a protective effect against defective and highly proliferative cells.

The tumor suppressor PTEN inhibits cell growth through multiple mechanisms. Proper PTEN function leads to decreased phospho-Akt levels and apoptosis occurs. In contrast, absent or dysfunctional PTEN leads to high levels of phospho-Akt, which is pro-proliferative. PTEN is inactivated frequently in prostate cancer, leading to apoptosis suppression. At present, PTEN and Akt expressions are thought to play key roles in the prevention and therapy of prostate cancer (Simpson and Parsons, 2001). We found that genistein increased PTEN and decreased phosphorylated-Akt protein levels in prostate tumors of rats. Our data provides evidence that the inhibition of Akt function and induction of PTEN activity may be the mechanisms by which genistein inhibits cell growth and induces apoptosis in the prostate to provide chemoprevention.

Another point to be made is that normal cells differ from cancer cells in their ability to undergo apoptosis when cellular DNA damage is initiated (Reed, 1999). We found that genistein regulates the AR in normal prostate, but not in rat prostate tumors induced by NMU. On the other hand, genistein up-regulates PTEN protein expression in prostate tumors, but not in normal prostate tissue. These data suggested that, although genistein could regulate cell proliferation and apoptosis with the same end result in both prostate cancer and non-tumorigenic prostate of rats, the unique mechanism of action regulated by genistein was slightly different.

In summary, we have presented evidence of genistein chemoprevention in a chemically-induced prostate cancer model. Life-time dietary genistein exposure to rats was the most effective in inhibition of prostate cancer progression, but even adult only genistein ingestion appears to provide a suppressive effect against this cancer. By modulating PTEN/Akt and AR signaling pathways in both normal and prostate cancer tissues, genistein induces apoptosis and inhibits cell proliferation, events that could account for it's chemopreventive activity against prostate cancer.

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