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Journal of Nutritional Biochemistry 17 (2006) 204–210

Journal of Nutritional Biochemistry

Genistein modulates prostate epithelial cell proliferation via estrogen- and extracellular signal-regulated kinase-dependent pathways

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Received 18 May 2005; received in revised form 15 July 2005; accepted 18 July 2005

Abstract

Epidemiological evidence suggests that consumption of soy is associated with a decreased risk for prostate cancer. Genistein, the most abundant isoflavone present in soy, is thought to be responsible, in part, for these anticancer effects. The present study examined the effects of genistein on cellular proliferation, extracellular signal-regulated kinase (ERK1/2) activity and apoptosis in a nontumorigenic human prostate epithelial cell line (RWPE-1). Low concentrations of genistein (0–12.5 μmol/L) significantly increased cell proliferation and ERK1/2 activity ($P<.01$) in RWPE-1 cells, while higher concentrations (50 and 100 μmol/L) of genistein significantly inhibited cell proliferation and ERK1/2 activity ($P<.001$). A similar biphasic effect of genistein on MEK1 activity, an ERK1/2 kinase, was also observed. Pretreatment of cells with a MEK1 inhibitor (PD 098059) significantly blocked genistein-induced proliferation and ERK1/2 activity ($P<.01$). In addition, treatment of cells with ICI 182,780, a pure antiestrogen, inhibited genistein-induced RWPE-1 proliferation and ERK1/2 signaling. Taken together, these results suggest that genistein modulates RWPE-1 cell proliferation and signal transduction via an estrogen-dependent pathway involving ERK1/2 activation.

Keywords: Genistein; Prostate; Extracellular signal-regulated kinase

1. Introduction

Prostate cancer is the second most commonly diagnosed cancer among men in the United States, accounting for over 30% of new cancer cases annually [1]. Epidemiological evidence indicates that the incidence and mortality of prostate cancer are considerably lower in Asian populations than in the American population [2–5], although precancerous lesion rates are approximately the same for both populations [6,7]. Interestingly, an increase in prostate cancer risk was observed in immigrants from Asia to the United States who abandoned their traditional diets [8,9]. These associations suggest that environmental and lifestyle factors such as diet may play an important role in the development of prostate cancer. Increased consumption of soy and soy-based foods among Asians per capita as compared to Americans is thought to be responsible, in part, for this reduced prostate cancer risk. A diet high in soy is associated with a variety of health benefits, which have been largely attributed to the isoflavones present in these foods. Genistein (4′,5,7-trihydroxyisoflavone), the most abundant isoflavone in soy, may be responsible, in part, for these anticancer effects due to its ability to inhibit tyrosine kinases [10], topoisomerases [10], promote cell cycle arrest [11–13], induce apoptosis [14] and modulate signal transduction pathways associated with cancer development.

Androgens are the primary hormones regulating prostate growth and development. However, estrogens are also important hormonal mediators for development of this gland. Both androgens and estrogen have been implicated in prostate carcinogenesis. For example, estrogen in combination with the androgen dihydrotestosterone (DHT) can increase aberrant growth and induce neoplastic transformation in mice [15]. In contrast, estrogenic signaling, specifically through estrogen receptor beta (ERβ), may protect against prostate carcinogenesis [15] by decreasing the occurrence of hyperplasia [16] and increasing protection against oxidative injury via induction of glutathione S-transferase (GST) and quinone reductase (reviewed in Ref. [15]). Interestingly, most phytoestrogens, such as genistein, have been shown to bind primarily ERβ.

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doi:10.1016/j.jnutbio.2005.07.005

This may explain, in part, the anticancer effects of these compounds in the prostate. However, data supporting this hypothesis are limited.

Signaling via the ER occurs via two primary mechanisms, those involving modulation of ER-responsive genes (genomic signaling) and those involving the rapid activation of signaling pathways, such as the mitogen-activated protein kinase (MAPK) cascade (nongenomic signaling). Mitogen-activated protein kinases are a family of proteins involved in mediating the effects of extracellular stimuli and are important in the regulation of a host of cellular functions including growth, proliferation, apoptosis, inflammation, differentiation and cell-cycle progression [17]. At present, three MAPK signaling pathways have been characterized in mammalian cells [17,18] and include the extracellular signal-regulated kinase cascade (ERK1/2), which preferentially regulates cell growth and differentiation, and two cellular stress-activated kinase cascades, p38 and c-Jun NH_2-terminal kinase (JNK) [19]. Genistein has been shown to modulate all three of these MAPK signaling cascades in mammary epithelial cells and in prostate cancer cells [20,21]. The ERK1/2 signaling cascade is of particular interest because this pathway has been implicated in cell proliferation and prostate cancer progression [22].

Although the effect of genistein on ERK1/2 signaling in carcinogenic cell lines has been extensively studied, the effects of this isoflavone on cellular proliferation and ERK1/2 signaling in nontumorigenic cells have not. The present study examines the molecular mechanisms by which genistein modulates proliferation of the nontumorigeneic prostate epithelial cell line RWPE-1.

2. Methods and materials

2.1. Materials

Genistein and 3-[4,5 -2-yl]-2-,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). The MEK1 kinase inhibitor, PD 098059, was purchased from Tocris (Ballwin, MO). Genistein, PD 098059 and ICI 182,780 were dissolved in appropriate solvents and diluted into culture medium to achieve indicated concentrations. Final solvent concentrations did not exceed 0.5%. BCA protein assay kit and superSignal chemiluminescent substrate kit were obtained from Pierce (Rockford, IL). ECL Western blotting detection reagent was obtained from Amersham Biosciences (Piscataway, NJ). Monoclonal antibody for estrogen receptor β (PA1-311) was purchased from ABR Affinity Bioreagents (Golden, CO). Polyclonal antibodies for phospho-ERK1/2, total ERK1/2, phospho-MEK1 and total MEK1 were obtained from Cell Signaling-Technology (Beverly, MA). Estrogen receptor α and β antibodies were purchased from Research Diagnostics (Flanders, NJ). Annexin V-FITC apoptosis detection kit was purchased from Oncogene Research Products (Cambridge, MA).

2.2. Cell culture

A human prostate epithelial cell line (RWPE-1) was obtained from the American Type Culture Collection (Rockville, MD). RWPE-1 cells were maintained in keratinocyte serum-free medium (GIBCO Laboratories, Grand Island, NY) supplemented with 50 μg/ml bovine pituitary extract, 5% L-glutamine and 5 ng/ml epidermal growth factor (EGF) in a humidified incubator (5% CO_2, 95% O_2) at 37°C.

2.3. Cell proliferation assay

Cells were plated in 96-well microtiter plates at an initial density of 2×10^4 cells per well. Cells were treated with genistein (0–100 μmol/L) and incubated for an additional 24 h. After incubation, cell proliferation was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was dissolved in PBS at 5 mg/ml. Briefly, 10 μl of MTT solution was added to each well, followed by 4 h of incubation. After incubation, MTT-containing medium was removed, and 100 μl of 0.04 mol/L HCl in isopropanol was added to each well to dissolve formazan crystals. The concentration of formazan was quantified spectrophotometrically at 620 nm. For mechanistic studies, cells were treated with specific signal transduction or receptor inhibitors alone or in combination with genistein for 24 h. Cell proliferation was determined as above.

2.4. Immunoblotting

Cells were grown and treated in 60-mm dishes (75% confluent). After appropriate treatments, growth medium was removed, cells were washed twice with phosphate-buffered saline (PBS), and crude proteins isolated and separated on a 10% SDS-polyacrylamide gel. Separated proteins were transferred onto a nitrocellulose membrane, blocked with 5% nonfat dried milk and incubated with primary antibodies (p-ERK1/2, total ERK1/2, p-MEK1, total MEK1, estrogen receptor α and β antibodies) at recommended dilutions in TTBS (1× TBS with 0.1% Tween-20) overnight at 4°C. After incubation and washing, membranes were incubated with a secondary antibody conjugated with horseradish peroxidase, and signals were detected using enhanced chemiluminescence substrate. Signals were visualized on autoradiography film and quantified by densitometry using Scion imaging software (Frederick, MD).

2.5. Annexin/propidium iodide staining

Apoptotic cells were quantified by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining according to the manufacturer's instructions. Briefly, RWPE-1 cells were grown in 60-mm dishes until 75% confluent. Cells were treated with 100 μmol/L genistein for 24 h at 37°C. Following treatment, attached and detached cells were collected on ice and centrifuged at 2500×*g* for

5 min at 4°C. The cellular pellet was resuspended with 1 ml 1× cold binding buffer to a concentration of 1×10^6 cells/ml, followed by the addition of annexin V-FITC (0.5 μg/ml) and PI (0.6 μg/ml). Annexin-positive (FITC-stained apoptotic) and PI-positive (PI-stained necrotic) cells were quantified by fluorescence microscopy. A minimum of 300 cells were counted for each treatment ($n=3$).

2.6. Morphology

RWPE-1 cells were seeded in 60-mm dishes at an initial concentration of 1×10^5 cells/ml. Cells were incubated for 24 h in the presence or absence of genistein (100 μmol/L). The morphology of both the control and the treatment was observed by phase-contrast microscopy (100× and 200×).

2.7. Statistical analyses

Densitometric data from Western blots were obtained using Scion Image software (Frederick, MD). Basal levels of protein expression are given as 100%, and twice this level is reported as 200% of the control. Statistical significance between groups was determined by one-way analysis of variance (ANOVA) with Tukey's post hoc comparisons (SigmaStat Software, Chicago, IL). Data are presented as means±S.E.M. Values of $P<.05$ were considered significant.

3. Results

3.1. Genistein and RWPE-1 cell proliferation

Treatment of RWPE-1 cells with 1.5, 3, 6 and 12.5 μmol/L genistein for 24 h increased cell proliferation by 4%, 11%, 32% and 58%, respectively, compared to untreated controls (Fig. 1A). A statistically significant increase in cell proliferation was observed with 6 and 12.5 μmol/L genistein ($P<.01$). Cell proliferation was maximal at 12.5 μmol/L and decreased with increasing concentrations of genistein. Treatment with 50 and 100 μmol/L genistein significantly decreased RWPE-1 cell proliferation by 18% and 60%, respectively, compared to untreated controls ($P<.01$).

3.2. Genistein modulates ERK1/2 and MEK1 activity in RWPE-1 cells

Treatment of RWPE-1 cells with 10 μmol/L genistein (2 h) significantly increased ERK1/2 activity (p-ERK1/2), while 100 μmol/L genistein significantly decreased this activity (Fig. 1B). Total ERK1/2 (t-ERK1/2) levels remained unchanged with treatments. Treatment of cells with 10 μmol/L genistein (2 h) slightly increased MEK1 activity (p-MEK1), while 100 μmol/L genistein significantly decreased MEK1 activity (Fig. 1C). Total MEK1 levels remained unchanged with all treatments.

Low concentrations of genistein increased ERK1/2 activity in RWPE-1 cells (Fig. 2A). A significant increase in ERK1/2 activity was observed upon treatment (2 h) of cells with 5 and 12.5 μmol/L genistein (Fig. 2B; $P<.01$).

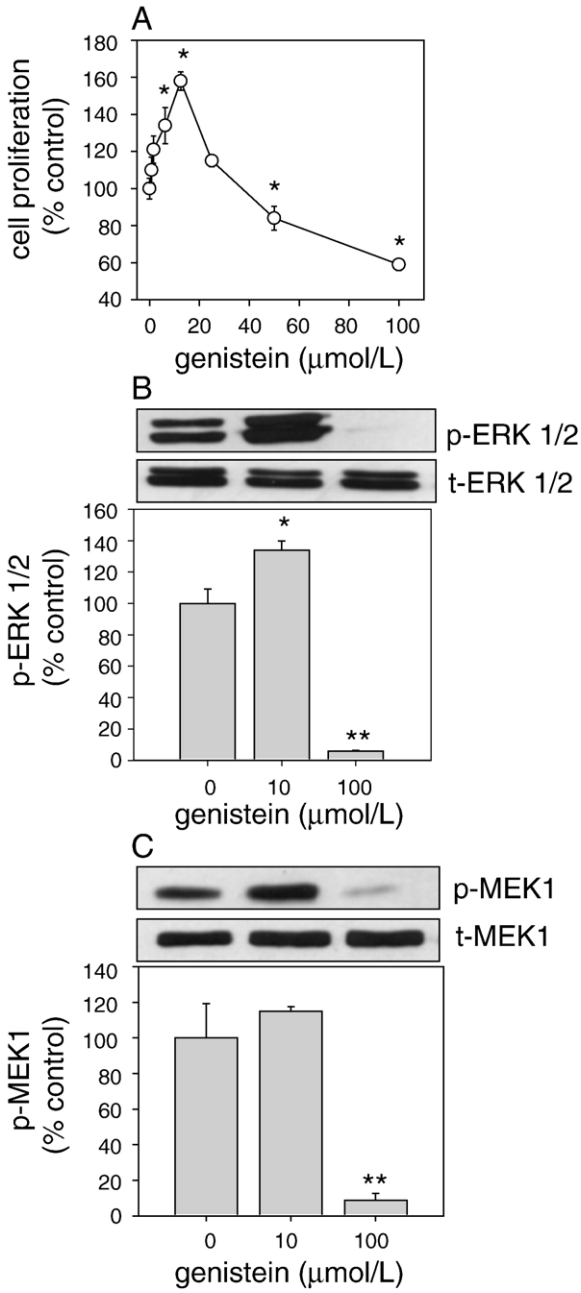

Fig. 1. Genistein modulates RWPE-1 cell proliferation and ERK1/2 signaling. (A) RWPE-1 cell proliferation 24 h following treatment with genistein (0–100 μmol/L) as determined by MTT assay. Data represent mean±S.E.M. ($n=24$) with significant differences from control indicated (*, $P<.01$). (B) Modulation of activated (p-ERK1/2) and total ERK (t-ERK1/2) 2 h following treatment with genistein (0, 10 and 100 μmol/L), given as representative immunoblot and for quantification. (C) Modulation of activated (p-MEK1) and total MEK1 (t-MEK1) 2 h following treatment with genistein (0, 10 and 100 μmol/L), given as representative immunoblot and for quantification. Data represent mean±S.E.M. ($n=3$) with significant differences in ERK1/2 and MEK1 activity from controls indicated by * ($P<.01$) and ** ($P<.001$).

A maximum increase in ERK1/2 activity was achieved at 12.5 μmol/L genistein. A decrease in ERK1/2 activity was observed at concentrations of genistein greater than 12.5 μmol/L.

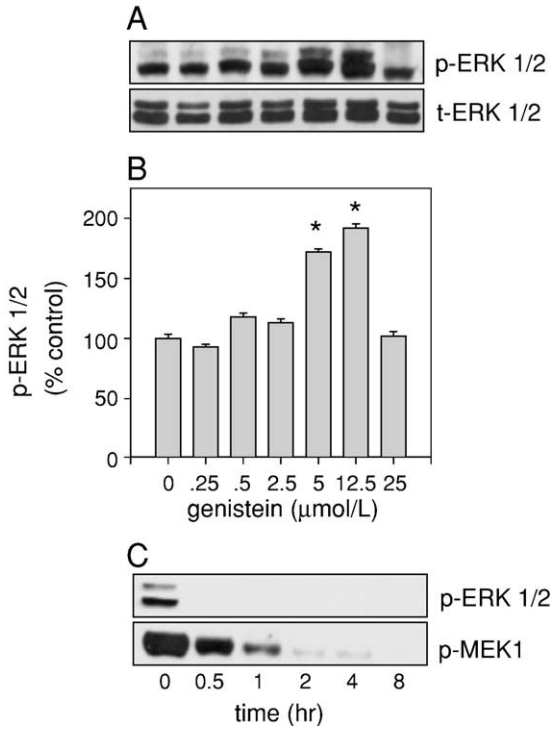

Fig. 2. Concentration and time-dependent modulation of ERK1/2 signaling by genistein in RWPE-1 cells. Active (p-ERK1/2) and total (t-ERK1/2) 2 h following treatment with genistein (0–25 μmol/L) given as representative immunoblot (A) and for quantification (B). Data represent mean±S.E.M. ($n=3$) with significant differences in ERK1/2 activity from controls indicated by * ($P<.01$). (C) Time-dependent inactivation of active ERK1/2 and MEK1, 0–8 h following treatment with 100 μmol/L genistein. Representative Western blot from three independent experiments is given.

Genistein inactivates ERK1/2 and MEK1 in a time-dependent manner (Fig. 2C). ERK1/2 activity was completely inhibited 0.5 h following treatment with genistein (100 μmol/L) and remained inactive for 8 h. MEK1 activity was reduced in a time-dependent manner at 0.5, 1, 2 and 4 h following genistein treatment (100 μmol/L), with complete inactivation observed by 8 h. Total ERK1/2 and MEK1 levels did not change at any time point (data not shown).

3.3. Mechanism of genistein-induced RWPE-1 cell proliferation

To elucidate the mechanism of genistein-induced RWPE-1 proliferation, cells were treated with genistein (10 and 50 μmol/L) alone or in combination with PD 098059 (50 μmol/L) for 24 h (Fig. 3A). As before, an increase and a decrease in RWPE-1 cell proliferation were observed following treatment with 10 and 50 μmol/L genistein, respectively, compared to controls. Treatment with PD098059 alone significantly reduced RWPE-1 cell proliferation by approximately 15%, compared to controls. Treatment with PD098059, in combination with genistein at 10 and 50 μmol/L, reduced RWPE-1 cell proliferation by approximately 20% and 40%, respectively, compared to controls.

The effect of PD098059 on ERK1/2 and MEK1 activity 2 h following treatment with genistein (0–50 μmol/L) is given as immunoblot and for quantification in Fig. 3B. As previously observed (Fig. 1B), treatment with 10 μmol/L genistein significantly increased ERK1/2 activity by approximately 20%, while 50 μmol/L genistein decreased ERK1/2 activity by approximately 40% ($P<.05$) (Fig. 3B). Treatment of cells with PD098059 (50 μmol/L) alone significantly decreased ERK1/2 activity by 30% ($P<.01$). Treatment of cells with PD098059 (50 μmol/L) in combination with genistein (10 μmol/L) significantly reduced ERK1/2 activity by 25% ($P<.01$). In addition, treatment of cells with PD 098059 (50 μmol/L) in combination with genistein (50 μmol/L) further reduced ERK1/2 activity by approximately 80%, compared to controls. No change in

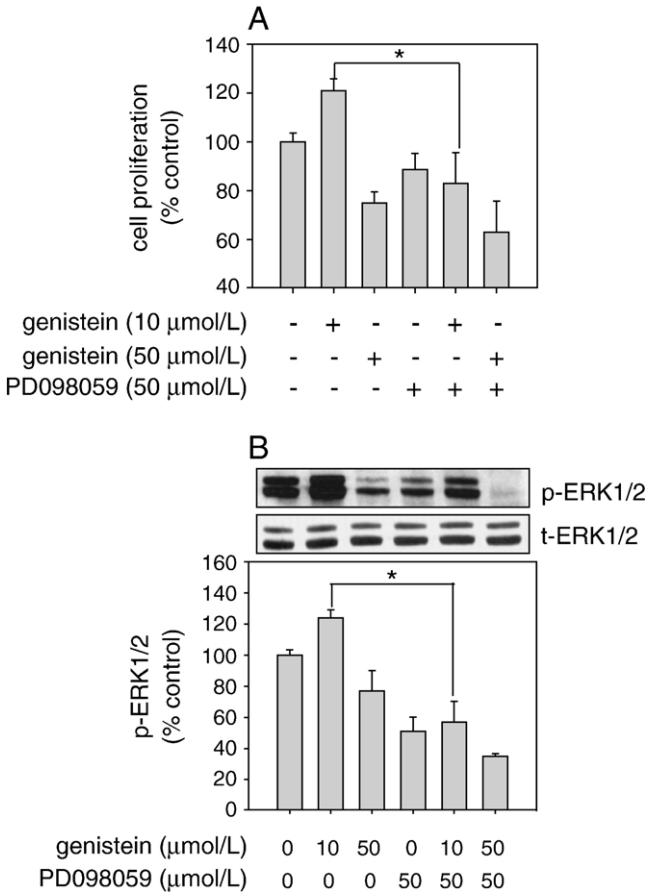

Fig. 3. MEK1 inhibition modulates genistein-induced RWPE-1 cell proliferation and ERK1/2 signaling. (A) RWPE-1 cell proliferation 24 h following treatment with either PD098059 (50 μmol/L) or genistein (10 and 50 μmol/L) alone and in combination, as determined by MTT assay. Data represent mean±S.E.M. ($n=24$). Significant reduction in genistein-induced cell proliferation with PD098059 treatment is indicated (*, $P<.01$). (B) Modulation of activated (p-ERK1/2) and total ERK (t-ERK1/2) 2 h following treatment with either genistein (0, 10 and 50 μmol/L) or PD098059 (50 μmol/L) alone and in combination given as representative immunoblot and for quantification. Data represent mean±S.E.M. ($n=3$). Significant reduction in genistein-induced ERK1/2 activation with PD098059 treatment is indicated (*, $P<.01$).

total ERK1/2 protein was observed with any treatment. Similar results were obtained with PD098059 treatment on genistein-induced MEK1 activity (data not shown).

3.4. Estrogenic effects of genistein in RWPE-1 cell proliferation and ERK1/2 signaling

To determine the possible estrogenic effects of genistein on RWPE-1 cell proliferation and ERK1/2 signaling, cells were treated with the antiestrogen ICI 182,780 (10 μmol/L), alone or in combination with genistein (10 μmol/L) (Fig. 4). Genistein (10 μmol/L) significantly increased RWPE-1 cell proliferation by approximately 22%, compared to controls ($P<.05$). Treatment with ICI 182,780 (24 h) alone did not alter RWPE-1 cell proliferation; however, ICI 182,780 significantly reduced genistein-induced cell proliferation ($P<.05$).

The effect of ICI 182,780 on genistein-induced ERK1/2 activity is given as immunoblot and for quantification in Fig. 4B. As previously observed (Fig. 1B), treatment of cells with genistein significantly increased ERK1/2 activity by approximately 22%, compared to controls, while treatment with ICI 182,780 alone did not significantly alter ERK1/2 activity. Treatment of cells with ICI 182,780, in combination with genistein, significantly reduced ERK1/2 activation, compared to those cells receiving genistein alone ($P<.05$).

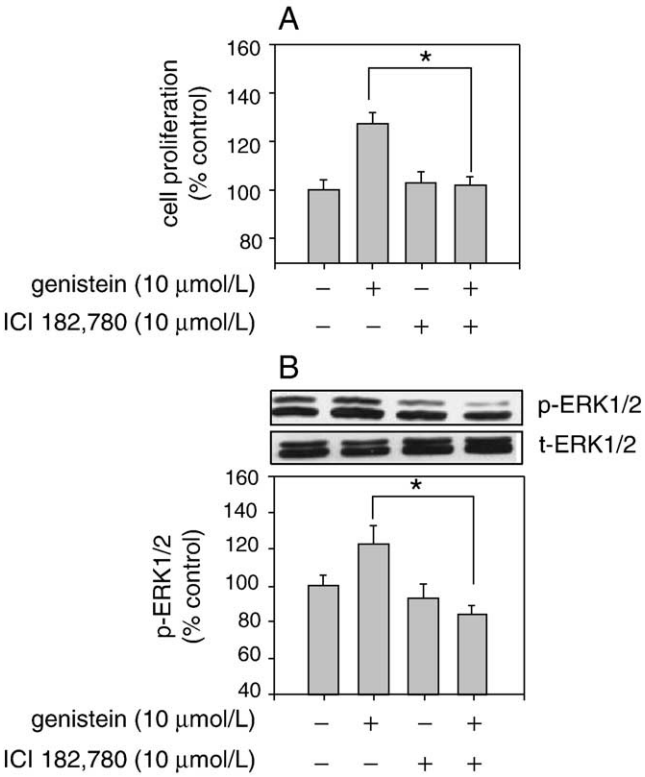

Fig. 4. Estrogen inhibition modulates genistein-induced RWPE-1 cell proliferation and ERK1/2 signaling. (A) RWPE-1 cell proliferation 24 h following treatment with either ICI 182,780 (10 μmol/L) or genistein (10 μmol/L) alone or in combination, as determined by MTT assay. Data represent mean±S.E.M. ($n=48$). Significant reduction in genistein-induced cell proliferation with ICI 182,780 treatment is indicated (*, $P<.01$). (B) Modulation of activated (p-ERK1/2) and total ERK (t-ERK1/2) 2 h following treatment with either genistein (10 μmol/L) or ICI 182,780 (10 μmol/L) alone and in combination given as representative immunoblot and for quantification. Data represent mean±S.E.M. ($n=3$). Significant reduction in genistein-induced ERK1/2 activation with ICI 182,780 treatment is indicated by *($P<.01$).

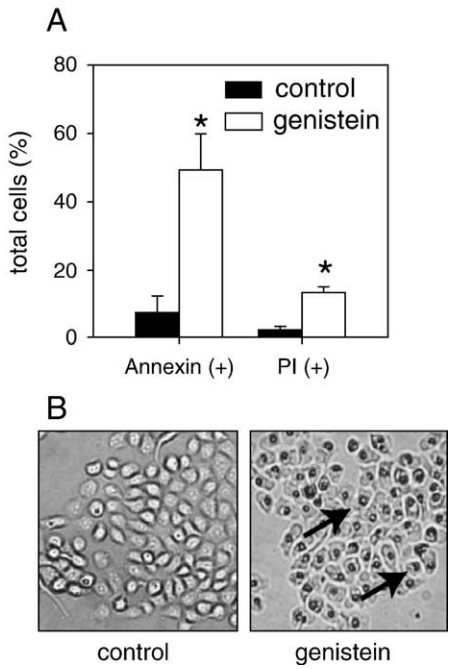

Fig. 5. Genistein-induced apoptosis in RWPE-1 cells. (A) Percentage of RWPE-1 cells staining positive for annexin and PI 24 h following treatment with 100 μmol/L genistein, as determined by fluorescence microscopy. A minimum of 300 cells were counted per treatment ($n=3$). Data are expressed as mean±S.E.M. Statistically significant increases in staining vs. untreated controls are indicated (*, $P<.05$). (B) Morphological analysis of RWPE-1 cells 24 h following treatment with and without genistein (100 μmol/L). Phase-contrast magnification=100×. Arrows indicate nuclear condensation.

3.5. Genistein-induced apoptosis in RWPE-1 cells

Annexin V and PI staining methods were utilized to determine the mechanism by which high concentrations of genistein induce RWPE-1 cell death (Fig. 5). Treatment of cells with 100 μmol/L of genistein for 24 h significantly increased the number of annexin and PI-positive cells compared to untreated controls ($P<.001$) (Fig. 5A). Morphological analysis of RWPE-1 cells shows an increase in nuclear condensation, 24 h following treatment with 100 μmol/L genistein (Fig. 5B).

4. Discussion

The estrogenic activity of genistein and its subsequent modulation of cellular processes in nontumorigenic prostate cells are not well characterized. In addition, the majority of studies examining the bioactivity of genistein in cell culture

utilize supraphysiological concentrations not reflective of the plasma and prostatic fluid concentrations found in Asian populations [12,23,24]. The present study demonstrates that physiologically relevant concentrations (1–10 μmol/L) of genistein [23] increased RWPE-1 proliferation and ERK1/2 activity, while supraphysiological concentrations (>25 μmol/L) decreased proliferation and ERK1/2 activity (Fig. 1A,B). Similar biphasic effects of genistein have been observed, in hormone-sensitive [25–28] and intestinal cell lines [11]. In addition, human colon cancer cells treated with the isoflavone daidzein have been shown to exhibit similar biphasic effects on proliferation [29]. The present study is the first to report the biphasic effects of genistein using immortalized nontumorigenic prostate epithelial cells. Several studies have previously examined the effect of genistein on prostate cell proliferation; however, these studies report only decreased cell proliferation following treatment with this isoflavone [30–32]. These discrepancies may be attributed to isoflavone concentration, duration of exposure and/or prostate cell line utilized. Hempstock et al. [32] reported that immortalized nontumorigenic prostate cells treated with genistein (>20 μmol/L) decreased proliferation; however, physiological concentrations of isoflavone were not examined. Wang et al. [31] observed only decreased prostate cell proliferation following treatment with both physiological and supraphysiological concentrations of genistein. However, these observations were made in a tumorigenic prostate cell line following 72 h of treatment with genistein [31].

Activation of the ERK1/2 signaling cascade is essential for the proliferation of numerous cell lines. Genistein-induced proliferation of RWPE-1 cells was blocked using a chemical inhibitor of the ERK1/2 signaling cascade. Therefore, ERK1/2 activity appears to be responsible, in part, in mediating the biphasic effects of genistein. Down-regulation of ERK1/2 activity is of particular interest in regard to carcinogenesis [33] since increased activity of this signaling cascade has been observed and correlated with increased proliferation in hormone-dependent human prostate cancer [34]. Genistein mimics estradiol-induced cell proliferation at low concentrations via ER-dependent ERK1/2 activation in breast cancer epithelium [35]. We hypothesized that genistein-induced ERK1/2 activation and cell proliferation in RWPE-1 cells may also be mediated through ER-dependent signaling pathways. The ability of the antiestrogen ICI 182,780 to suppress genistein-induced ERK1/2 activation and proliferation supports this hypothesis. Similar to normal adult prostate epithelial tissue, RWPE-1 cells express predominantly ERβ (data not shown). Ligand-bound ERβ activation has been shown to protect against prostate carcinogenesis [15] by decreasing the occurrence of hyperplasia [16] and increasing protection against oxidative injury (reviewed in Ref. [15]). In contrast, signaling through ERα is associated with increased hyperplasia and squamous cell metaplasia of the prostate [36]. Therefore, ligands which primarily mediate estrogenic effects via ERβ may offer a protective role against carcinogenesis. The specific involvement of ERβ in genistein-induced RWPE-1 proliferation and ERK1/2 activation and possible protection against prostate carcinogenesis is currently under investigation.

In contrast to the observed effects of physiological concentrations of genistein, supraphysiological concentrations decreased RWPE-1 ERK1/2 activation and proliferation. Initial morphological and biochemical observations strongly suggest that genistein induces apoptosis in RWPE-1 cells. Genistein-induced apoptosis has been observed in a variety of hormone-dependent and -independent prostate cell lines [14,31,37]. These effects are attributed, in part, to the tyrosine kinase inhibitory activity of genistein [10]. Receptor tyrosine kinases are important mediators of ERK1/2 activity, and their inhibition can lead to decreased ERK1/2 activity and apoptosis [31]. Therefore, the tyrosine kinase inhibitory effects of genistein at supraphysiological concentrations appear to antagonize the estrogenic effects observed at physiological concentrations.

Plasma levels of genistein in soy-consuming populations can reach concentrations of 1 to 2.4 μmol/L, and higher levels can be achieved via dietary supplementation [12,23,24]. Schuurmans et al. [38] suggest that levels of genistein may be higher in prostatic fluid than in plasma, due to accumulation in this gland [12]. The present study demonstrates that physiological concentrations of genistein can activate the ERK1/2 signaling cascade via an estrogenic-dependent mechanism, resulting in increased proliferation of a nontumorigenic prostate epithelial cell line. However, the consequence of these genistein-mediated effects on prostate carcinogenesis and chemoprevention is currently unknown.

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