Combined effects of terazosin and genistein on a metastatic, hormone-independent human prostate cancer cell line

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Abstract
Metastatic prostate cancer progresses from androgen-dependent to androgen-independent. Terazosin, a long-acting selective α1-adrenoceptor antagonist, induces apoptosis of prostate cancer cells in an α1-adrenoceptor-independent manner, while genistein, a major soy isoflavone, inhibits the growth of several types of cancer cells. The present study was designed to test the therapeutic potential of a combination of terazosin and genistein using a metastatic, hormone-independent prostatic cancer cell line, DU-145.

Terazosin or genistein treatment inhibited the growth of DU-145 cells in a dose-dependent manner, whereas had no effect on normal prostate epithelial cells. Addition of 1 μg/ml of terazosin, which was inactive alone, augmented the growth inhibitory effect of 5 μg/ml of genistein. Co-treatment with terazosin resulted in the genistein-induced arrest of DU-145 cells in G2/M phase being overridden and an increase in apoptotic cells, as evidenced by procaspase-3 activation and PARP cleavage. The combination also caused a greater decrease in the levels of the apoptosis-regulating protein, Bcl-XL, and of VEGF165 and VEGF121 than genistein alone.

In conclusion, the terazosin/genistein combination was more effective in inhibiting cell growth and VEGF expression as well as inducing apoptosis of the metastatic, androgen-independent prostate cancer cell line, DU-145, than either alone. The doses used in this study are in lower and nontoxic anticancer dosage range, suggesting this combination has potential for therapeutic use.

1. Introduction
Prostate cancer is a leading cause of cancer-related deaths in men [1,2]. Mortality results from metastasis to the bone and lymph nodes and progression from androgen-dependent to androgen-independent prostatic growth [3]. Radiation therapy is curative for localized disease, but there is no treatment for metastatic prostate cancer [1].

Terazosin is a long-acting selective α1-adrenoceptor antagonist that is used clinically to provide acute relief of the obstructive symptoms associated with benign prostatic hypertrophy (BPH) [4–6] and recent studies have shown that it induces apoptosis of prostate epithelial and smooth muscle cells in patients with BPH [7–10]. It also induces apoptosis of prostate cancer cells via an α1-adrenoceptor-independent mechanism [11–17] and has anti-angiogenic effects in the human prostate [18–21]. These findings provide the rationale for the development of an effective therapeutic strategy using terazosin for patients...
with androgen-dependent or androgen-independent prostate cancer.

Epidemiological studies have shown that, in Asia, the decreased occurrence of cancers, including prostate cancer, is associated with consumption of soy [22,23]. Soy isoflavones are natural chemoprotectors against cancer and are not toxic for normal cells [24]; genistein (5,7,4′-trihydroxyisoflavone) is one of the predominant compounds of soy isoflavones [24,25]. Genistein inhibits cell growth both in several types of cancer, including prostate [26,27], breast [28,29], lung [30], bladder [31,32], and liver [33,34] cancers, and in BPH [35] and inhibits angiogenesis in tumors [36].

In an attempt to reduce the therapeutic dosage of terazosin so as to decrease its toxicity in prostate cancer treatment, we tested the effect of combining it with genistein, using a hormone-independent prostate cancer cell line, DU-145. The anti-angiogenesis and apoptosis-related proteins are considered to be the common downstream effectors mediating the effects of terazosin and isoflavones in prostate cancer, those were examined to evaluate therapeutic efficacy.

2. Materials and methods

2.1. Cell culture and viability assay

The DU-145 cell line, an androgen-independent tumor cell type, derived from a human prostate carcinoma, was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in MEM (GibcoBRL, Grand Island, NY) containing 10% fetal bovine serum (GibcoBRL, Grand Island, NY). Normal human prostate epithelial cell (PrEC) was obtained from Clonetics (San Diego, CA) and maintained according to the manufacturer’s instructions using PrEGM medium. PrEC cells were used at passage 3-6.

Cells were seeded in each well of a 24-well culture plate (Corning, New York, USA) and grown at 37 °C in a 5% CO2 incubator. After 24 h incubation, the cells were treated with terazosin (Sigma, St. Louis, MO) and/or genistein (GibcoBRL, Grand Island, NY) for 3 days, and then cell number was counted with crystal violet elution assay for viability, and expressed as a percentage of that of the corresponding control group. Terazosin was dissolved in distilled water. Genistein was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO), the final DMSO concentration being less than 0.5% (v/v); the same concentration of DMSO was added to the controls.

2.2. Cell cycle analysis

For 48 h with or without terazosin and/or genistein treatment, the distribution of cells in different stages of the cell cycle was estimated by flow cytometric DNA analysis, as described previously [31]. A minimum of 1 × 10⁶ cells per sample was evaluated by a Elite-Esp flow cytometry (Miami FL, US) in each case. The percentage of cells in each cell cycle phase (Sub-G1, G0/G1, S, or G2/M) was calculated using Cell FIT research software (Becton-Dickinson, Mountain View, CA).

2.3. Detection of apoptosis by flow cytometry and fluorescence microscopy

TUNEL staining was performed following the protocol recommended in the commercial kit (Boehringer, Mannheim, Germany). Apoptotic cells were also detected by fluorescence microscopy using Hoechst 33342 dye (Sigma, St. Louis, MO) to label the nuclei and propidium iodide to stain DNA as described previously [37].

2.4. Western blot analysis

Cytosolic extracts were prepared from cells and the protein in the supernatant was quantified using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). A sample (60 μg) was electrophoresed on 12% SDS–polyacrylamide gels then transferred to nitrocellulose membranes. The rabbit polyclonal antibodies against human PARP,
Bcl-X\textsubscript{L}, procaspase-3, or \( \beta \)-actin or mouse monoclonal anti-human Bcl-2 and Bax antibody (Santa Cruz, CA, USA) was used as the first antibody and followed by the appropriate horseradish peroxidase-labeled secondary antibody (PharMingen, San Diego, CA, USA). The bound antibody was quantified by chemiluminescence detection (PerkinElmer Life Sciences, Inc.). \( \beta \)-Actin was used as the internal control. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units for \( \beta \)-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

After total cellular RNA was extracted, the complementary DNA (cDNA) was synthesized. Then, polymerase chain reaction (PCR) was performed using a human VEGF genes set-3 multiplex PCR kit (MBI, So. San Francisco, USA) and cDNA amplification was performed according to the manufacturer’s procedure. The amplified PCR products were separated by gel electrophoresis in 2% agarose, the intensity of each band calculated by densitometry analysis, and the results expressed as a percentage of the density of the corresponding \( \beta \)-actin gene band.

2.6. VEGF secretion assay

The cells (2 \( \times \) 10\textsuperscript{5}) were incubated overnight at 37 °C in 6 cm plastic dishes, then for another 36 h with or without terazosin and/or genistein in MEM containing 10% fetal bovine serum. After incubation, the medium was collected and the concentrations of VEGF were measured using an ELISA kit (R&D, Wiesbaden, Germany) according to the manufacturers’ instructions.

2.7. Statistical analysis

All experiments were repeated for three times. All data are presented as means ± SD. Differences in cell cycle distribution were analyzed using the \( \chi^2 \) test. Other differences were analyzed by analysis of variance, and a Scheffe test was used to identify differences between the individual means. Statistical analyses were performed using SAS (version 6.011; SAS Institute Inc., Cary, NC). A \( p \) value of <0.05 was considered statistically significant.

3. Results

3.1. Effects on cell growth

To gain an initial insight into the effects of terazosin or genistein, alone or in combination, on human prostate cancer cells and normal epithelial cells, DU-145 and PrEC cells were treated for 3 days without or with different doses of terazosin and/or genistein. For 3 days incuba-

**Fig. 2.** Genistein and terazosin induce apoptosis of DU-145 cells. (A) Exponentially growing cells were treated for 48 h and evaluated by TUNEL staining. The gray peak is the vehicle control. *\( p < 0.05 \) compared to the untreated group. #\( p < 0.05 \) compared to the genistein-treated group. (B) Cells were stained using Hoechst 33342 and propidium iodide. C: untreated cells, G5: genistein 5 μg/ml, T1 + G5: terazosin 1 μg/ml and genistein 5 μg/ml.
tion, the untreated DU-145 cell proliferated by 2.3-fold, whereas terazosin or genistein alone inhibited cell growth in a dose-dependent manner, genistein being much more effective than terazosin (Fig. 1A); 5 μg/ml of genistein resulted in more than 50% inhibition and was chosen for use in the combination tests. But there was no effect of terazosin or genistein on PrEC cells (Fig. 1B) under these dosage ranges. Although 1 μg/ml of terazosin had no significant effect on DU-145, the combination of 1 μg/ml of terazosin and 5 μg/ml of genistein was more effective in growth inhibition than 5 μg/ml of genistein alone (Fig. 1C). However, no further increase in cell growth inhibition was seen when the dose of terazosin in the combination was increased up to 20 μg/ml (data not shown). Moreover, the combination of 1 μg/ml of terazosin and 5 μg/ml of genistein had no inhibition of cell growth in the PrEC cells (data not shown). We therefore chose this combination dosage for study.

3.2. Apoptosis induction

Apoptotic cells were assessed by flow cytometric analysis after TUNEL staining (Fig. 2A). After 48 h treatment with genistein, a significant increase in the percentage of TUNEL-positive apoptotic cells was seen compared to controls. Although terazosin alone did not cause a significant change in the number of apoptotic cells, a stronger effect was observed with combined treatment, the combination resulting in a significant increase in the percentage of apoptotic cells compared to genistein alone (20.13 ± 3.61 vs. 29.54 ± 3.12; p < 0.05). The treatment-induced apoptosis was also apparent from the morphologic changes detected by fluorescence microscopy of Hoechst 33342 dye and propidium iodide-labeled cells (Fig. 2B).

3.3. Cell cycle arrest

To gain an insight into the effects on cell cycle distribution, DU-145 cells were incubated for 48 h with genistein and/or terazosin. As shown in Fig. 3, terazosin had no significant effect on the cell cycle distribution, but genistein caused cell arrest in G2/M phase. Surprisingly, the addition of terazosin to genistein overrode the G2/M phase arrest (percentage of cells in G2/M phase 19.64 ± 2.03% compared to 29.66 ± 2.98%) and increased the percentage of cells in sub-G1 phase from 14.26 ± 2.16% to 22.97 ± 2.48%, but did not significantly change the percentage of cells in G0/G1 or S phase.

3.4. Procaspase-3 activation and PARP cleavage

Caspase-3, a member of the caspase family, is expressed in cells as an inactive 32 kDa proenzyme, procaspase-3. During apoptosis, procaspase-3 is activated by cleavage at specific Asp residues to generate active caspase-3, consisting of 17 and 12 kDa subunits. Caspase-3 then cleaves its substrate, PARP, into 85 and 24 kDa fragments. As shown in the immunoblots in Fig. 4, thirty-six hours treatment of DU-145 cells with genistein alone or terazosin/genistein resulted in a marked decrease in procaspase-3 (Fig. 4A), the terazosin/genistein combination being more effective. In accordance with the activation of procaspase-3, significant generation of the 85 kDa PARP cleavage fragment was seen with all treatments, including terazosin alone (Fig. 4B). These results show that active caspase-3 was generated by either terazosin or genistein and that the combination was more effective than either alone.

3.5. Expression of apoptosis-related proteins

To determine whether the treatment-induced apoptosis was associated with altered expression of apoptosis-regulating proteins, DU-145 cells were treated for 36 h with genistein and/or terazosin, then were subjected to Western blotting. Fig. 5 shows that terazosin had no effect on Bcl-XL levels, whereas genistein caused a marked decrease, and the combination was even more effective. Effects on Bcl-2 could not be tested, as it was undetectable in DU-145 cells, in accordance with a previous report [38]. There was no significant difference in the pro-apoptotic protein

Fig. 3. Effect of genistein and/or terazosin on DU-145 cell cycle progression. Cells were treated for 48 h, and then the distribution of cells in the different phases of the cell cycle was determined by flow cytometry. Sub-G1 represents apoptotic cells. *p < 0.05 compared to the untreated group. #p < 0.05 compared to the genistein-treated group.
Bax expression among all groups (data not shown). Thus, the ratio of anti-apoptotic/pro-apoptotic factor in the combination is the lowest of all groups.

3.6. Expression of angiogenic factors

Since human prostate cancer cells express a variety of angiogenic factors, including VEGF_{165} and VEGF_{121}, which play important roles in new vessel formation, we examined whether the treatments used in this study affected the expression of these angiogenic factors by DU-145 cells. The cells were treated for not more than 36 h with genistein and/or terazosin, then angiogenic factor mRNA levels were measured by RT-PCR. The VEGF mRNA levels were extremely low in control and treated-groups at 6, 12, and 24 h. However, at 36 h treatment, terazosin caused a slight, but not significant, decrease in VEGF_{165} mRNA levels after calibration against \( \beta \)-actin mRNA, whereas both genistein and the combination caused a significant decrease, the effect being greater with the combination. The same

![Fig. 4. Western blotting showing procaspase-3 activation and PARP cleavage in DU-145 cells. After normalized to \( \beta \)-actin, the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. \(^*\) \( P < 0.05 \) compared to the untreated group. \(^\#\) \( P < 0.05 \) compared to the genistein-treated group.](image)

![Fig. 5. Western blotting of Bcl-X\(_{L}\) expression in DU-145 cells. After normalized to \( \beta \)-actin, the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. \(^*\) \( P < 0.05 \) compared to the untreated group. \(^\#\) \( P < 0.05 \) compared to the genistein-treated group.](image)

![Fig. 6. Expression of VEGF\(_{165}\) and VEGF\(_{121}\) in DU-145 cells. Treatment for 36 h, the expressions of VEGF isoforms were analyzed by RT-PCR. The VEGF mRNA expression was normalized to \( \beta \)-actin mRNA, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. \(^*\) \( P < 0.05 \) compared to the untreated group. \(^\#\) \( P < 0.05 \) compared to the genistein-treated group.](image)

![Fig. 7. Effects of genistein and/or terazosin on VEGF secretion in DU-145 cells. The cells were treated for 36 h, and then VEGF secretion to medium was determined by ELISA method. \(^*\) \( P < 0.05 \) compared to the untreated group. \(^\#\) \( P < 0.05 \) compared to the genistein-treated group.](image)
trend was seen with VEGF121 mRNA expression, but genistein alone had a greater effect on VEGF165 levels than on VEGF121 levels, whereas the combination did not (Fig. 6). Moreover, the protein levels of secreted VEGF in medium (Fig. 7) were in accordance with mRNA results.

4. Discussion

Prostate cancer is intrinsically heterogeneous and consists of a simultaneous mixture of androgen-responsive and androgen-unresponsive cells [39,40]. Soy products containing isoflavones are widely available in food and humans consuming soy have micromolar concentrations of isoflavones in the blood [22,24,25]. We recently showed that soy isoflavones inhibit the growth of human bladder and hepatoma cancers both in vitro and in vivo [31–33]. In addition, soy genistein has been shown to inhibit the growth of both benign and malignant prostate tissue [35]. On the other hand, terazosin has been proved to be useful in the treatment of prostate cancer [12–14,17]. In terms of induction of apoptosis or inhibition of angiogenic factor expression, our results demonstrated that the combination of genistein and terazosin was more effective than either alone on hormone-independent human prostate cancer cells. Although, the anticancer effects of terazosin is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. But the reported IC50 of terazosin on prostate cancer cells is higher than is gradually approved by many studies. 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Many anticancer agents and DNA-damaging agents arrest the cell cycle at G1, S, or G2/M phase and induce apoptotic cell death [44–49]. The cell cycle check-points function to ensure that cells have time for DNA repair [3,46,47], whereas apoptotic cell death functions to eliminate irreparable or unrepaired damaged cells [50]. This study showed that, as in other tumor cells [30,33,34], genistein arrested DU-145 cells in G2 phase. Of interest, this G2 phase arrest by genistein was followed by increased apoptotic death after co-treatment with terazosin. The DU-145 cell line carries a mutation in p53 and the bax gene [38]. Studies have shown that alterations in the p53 gene in tumor cells result in defective checkpoint function and sensitize tumor cells to chemotherapy [38,50]. One explanation for the increased sensitivity of p53-mutated tumor cells is that the cells with DNA lesions prematurely enter mitosis because they are unable to regulate cyclin B1/Cdc2 activity and cannot undergo G2 checkpoint arrest [50]. This study suggests that terazosin preferentially over-rides the genistein-induced G2 checkpoint arrest in DU-145 cells with defective p53 function. Moreover, our unpublished data showed the increase in the other checkpoint arrest gene p21 by genistein was reduced after co-treatment with terazosin. On the other hand, the combined effect of terazosin and genistein in reducing Bcl-X, levels would explain, in part, why terazosin pushes the arrested DU-145 cells towards apoptosis.

Human VEGF mRNA is transcribed from eight exons of a single gene and is alternatively spliced into at least six mRNAs, which give rise to mature proteins of 121, 145, 165, 183, 189, and 206 amino acids. VEGF-121 and VEGF-165 are the best characterized and are the most abundant in normal tissues, including blood vessels. Prostate tumors, like most tumors, overexpress VEGF, thereby promoting the development of tumor neovascularization [51,52], and this overexpression correlates with increasing grade, vascularity, and tumorigenicity. Our results showed that VEGF was highly expressed in DU-145 cells, as shown in other studies [52,53], and that genistein decreased VEGF expression and that the combination was even more effective.

Results of the present study clearly demonstrate that a nontoxic dose of terazosin significantly enhances the antitumor activity of genistein on DU-145 human prostate cancer cells suggesting that this combination could potentially be useful in prostate cancer therapy. However, this study is limited because it investigated only one prostate cancer cell line, and the basis of the interaction of these two compounds is not fully clear. It is not known whether other in vivo experiments would have results comparable to those determined in this culture study. More in vivo studies are required to clarify whether the combined treatment is an effective antitumor strategy.

Conflicts of interest statement

None declared.

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