Effects of Takrisodokyeum Water Extracts on LNCaP Prostate Cancer Cells

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Androgen receptors (AR) play a crucial role in the development and progression of prostate cancer. Many studies have suggested that prostate cancer cell proliferation is inhibited by AR downregulation, and it has been reported that Takrisodokyeum (TRSDY) induced apoptotic cell death and suppressed tumorigenesis in human leukemia cells. Therefore, this study was conducted to elucidate the mechanism by which TRSDY affects cell growth and AR expression in androgen-dependent prostate cancer cells (LNCaP cells). We investigated the proliferation and apoptosis of LNCaP cells using MTT and DNA fragmentation assays. In addition, we used western blot analysis to assess the effects of TRSDY on the expression of the AR target gene, prostate-specific antigen (PSA). Furthermore, the mechanism of AR downregulation by TRSDY was investigated using EMSA to analyze the binding activity of AR to androgen response elements (ARE). TRSDY significantly suppressed proliferation and induced apoptosis in LNCaP cells. In addition, TRSDY-induced apoptotic cell death was accompanied by activation of caspase-3 and cleavage of its substrate, poly(ADP-ribose) polymerase. TRSDY also inhibited the constitutively expressed or 5α-dihydrotestosterone (DHT)-induced AR/PSA protein levels. However, these effects were mediated by inhibition of the binding of AR to ARE. TRSDY-mediated AR/PSA downregulation contributes to the inhibition of cell proliferation and the induction of apoptosis in LNCaP human prostate cancer cells. Our findings suggest that TRSDY may be used as a chemopreventive or chemotherapeutic agent for the treatment of prostate cancer.

Key words: takrisodokyeum, apoptosis, androgen receptor, prostate specific antigen, prostate cancer

Introduction

Prostate cancer is the most common cancer and the second-leading cause of cancer-related death among men in Western countries. The human prostate is an androgen-sensitive organ that depends on androgens for growth and development. However, potent androgens such as testosterone and 5α-dihydrotestosterone (DHT) and their corresponding nuclear androgen receptors (AR) have been implicated in normal prostate development as well as in the development of prostatic diseases including prostate cancer. Prostate cancer is initially dependent upon androgens for growth and survival, however, regression occurs when androgens are removed.

Therefore, androgen deprivation therapy is still the most commonly used therapy for treatment of androgen-dependent prostate cancer. However, most tumors eventually become refractory to androgen ablation. During the development of a normal prostate and of prostate cancer, cell survival depends primarily on the AR, which is required for tumor initiation and progression. It has been proposed that AR activity is a key factor in the progression of prostate cancer to the resistant phenotype. Among the factors that alter AR activity, increased AR expression is frequently observed in ablation-resistant prostate cancer. It has been also reported that AR mRNA levels were increased by up to 70-fold in metastatic androgen-independent prostate cancer cells when compared to primary prostate cancer cells. Chen et al. also reported that increased AR expression effectively transformed prostate cancer growth from a hormone-sensitive to a hormone-refractory form, thereby causing AR antagonists to function as weak agonists, resulting in AR activation. Therefore, AR down-regulation agents have emerged as an attractive target for the development anti-prostate cancer drugs.

One of the attractive strategies currently being considered for the treatment of prostate cancer is the use of herbal or pharmaceutical manipulation to induce the death of malignant cells through apoptosis. One potentially promising example is PC-SPES, which is comprised of the extracts of eight...
Chinese and American herbs. This treatment is widely used by individuals suffering from advanced prostate cancer15,16.

Another treatment is takrutosideyum (TRSDY), which is an herbal mixture comprised of twelve herbs that has been used for the treatment of cancer in Asia. We previously demonstrated that TRSDY induces apoptosis via the generation of reactive oxygen species in human leukemia cells25.

In addition, Yang KH showed that TRSDY regressed 3-methylcholanthrene-induced tumor formation and tumorigenesis of 3LL cells, a leukemia cell line36. However, the preventive mechanisms of TRSDY on prostate cancer cells have not yet been clarified. Therefore, this study was conducted to determine whether and how TRSDY regulated cell proliferation and AR expression in LNCaP human prostate cancer cells.

Materials & Methods

1. Culture conditions

LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and propagated in culture dishes at the desired densities in RPMI1640 medium supplemented with 10% fetal calf serum (FCS; HyClone, South Logan, UT) and 1% penicillin/streptomycin under 5% CO2 at 37°C. Next, the cells were cultured in 10% charcoal-stripped FCS (HyClone) for an additional 24h to deplete the endogenous steroid hormones prior to treatment with TRSDY extract at the designated concentrations, with or without 5α-dihydrotestosterone (DHT, Sigma-Aldrich, St, MO) in phenol red free RPMI 1640 medium.

2. Preparation of TRSDY extract

TRSDY was prepared as described previously17. Briefly, TRSDY, which is a mixture of twelve traditional drugs, was obtained from Wonkwang Oriental Medical Hospital and classified and identified by local experts. The amounts of the twelve traditional drugs included in the mixture evaluated in this study are shown in Table 1. One hundred grad by 1 TRSDY were added 900 ml wat in Tabboiled for 2h, filtered and centrifuged at 200 ml. The sterile extract (22.7 g) was then stored at -70°C until needed.

3. MTT assay for cell viability

The viability of cultured cells was determined by assaying the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan18. Briefly, LNCaP cells were seeded overnight in 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson, Franklin Lakes, NJ) at a density of 104 cells per well in 100 μl of medium. The cells were then treated with various concentrations of TRSDY extract for 48 h. Next, the cells were washed twice with PBS, after which MTT (100 μg/100 μl of PBS) was added. The cells were then incubated at 37°C for 1 h, and DMSO (100 μl) was then added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a Spectra MAX PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA).

<table>
<thead>
<tr>
<th>Components</th>
<th>Ratio(%)</th>
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<tbody>
<tr>
<td>1. Flos Lonicerae (flower bud of Lonicera japonica THUNB)</td>
<td>31.67</td>
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<tr>
<td>2. Pergarcium Cni Retzolatae (pepipp of Citrusunshiu MARCOUR)</td>
<td>31.67</td>
</tr>
<tr>
<td>3. Radix Astragali (root of Astragalus membranaceus BUNGE)</td>
<td>21.11</td>
</tr>
<tr>
<td>4. Radix Trichosanthis (root of Trichosanthes kirilowii Maxim)</td>
<td>21.11</td>
</tr>
<tr>
<td>5. Radix Saposhnikoviae (root of Saposhnikovia divaricata SCHSCH-H)</td>
<td>15.5</td>
</tr>
<tr>
<td>6. Radix Angelicae Sinesis (root of Angelica sinesis (DELS)</td>
<td>15.5</td>
</tr>
<tr>
<td>7. Rhizoma Chuanxiong (root and rhizome of Ligusticum chuanxiong HORTR)</td>
<td>15.5</td>
</tr>
<tr>
<td>8. Radix Angelicae Dahuricae (root of Angelica dahurica Fisch)</td>
<td>15.5</td>
</tr>
<tr>
<td>9. Radix Pianwood (root of Paeonodongrandiflorum AUCIT)</td>
<td>15.5</td>
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<tr>
<td>10. Cortex Magnoliae Officinalis (root and pericarp of Magnolia boomentifolia ERCH)</td>
<td>15.5</td>
</tr>
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<td>11. Smarag, Maris (leaves of Manicentadactylusa)</td>
<td>15.5</td>
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<tr>
<td>12. Sperma Geciass (spine of Gedimiaienis AM)</td>
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4. Genomic DNA isolation and DNA-laddering assay

Genomic DNA was isolated from cultured cells using a Wizard Genomic DNA purification kit (Promega). Briefly, the medium was removed and the cells were then lysed with lysis buffer, after which they were incubated for 1h with RNase A. Next, the cell lysates were precipitated for proteins and then centrifuged at 12,000 g for 20 min. The supernatant was then precipitated with isopropanol for the isolation of DNA. After an alcohol wash, the DNA was hydrated and quantified. Equal amounts (10 μg) of DNA were then electrophoresed on a 1.5% agarose gel (with incorporated ethidium bromide). The gel was then photographed under ultraviolet luminescence.

5. Cellular DNA fragmentation assay

Fragmented DNA was assessed as a specific measurement of apoptotic cell damage. Briefly, the cells were plated on 96-well plates and then allowed to attach for 24 h. Cellular DNA fragmentation was then measured with a commercially available cellular DNA fragmentation ELISA kit (Roche). Proliferating cells in 96-well microtiter plates were then labeled with 10 μM bromo-deoxyuridine (BrdU) overnight, after which they were washed with sterile phosphate-buffered saline (PBS) and treated in the presence or absence of various concentrations of TRSDY for 24 h or 48 h. Next, the cells were washed with PBS and incubated with the DNA fragmentation ELISA kit.
lysis buffer provided in the kit for 30 min at room temperature, after which the soluble BrdU-labeled DNA fragments present in the buffer were quantified using an ELISA kit following the manufacturer's instructions. DNA fragmentation was expressed as a fold increase compared to the control values.

6. Western blot analysis

Cells were homogenized in 100 µl of ice-cold lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin). The homogenates, which contained 20 µg of protein, were then separated by SDS-PAGE with 7.5% (for poly ADP-ribose polymerase (PARP) and AR) or 12% resolving (for caspase-3, prostate specific antigen, and β-actin) and 3% acrylamide stacking gels, after which they were transferred to nitrocellulose sheets (Millipore, Bedford, MA) in a western blot apparatus (Bio-Rad, Hercules, CA). Next, the nitrocellulose paper was blocked with 2% bovine serum albumin and then incubated for 4 h with 1 µg/ml of primary antibody (Santa Cruz Biochemicals,Santa Cruz,Ca). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA) was used as a secondary antibody. The protein expression levels were then determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad).

7. Preparation of the nuclear protein extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from LNCaP cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Binding of AR and androgen receptor responsive element (ARE) was assayed by a gel mobility shift assay using nuclear extracts from control and treated cells. Briefly, an oligonucleotide containing the ARE oligonucleotide (5'-GAAGTCTGGTACAGGGTGTTTCTTTTG-3') was synthesized and used as a probe for the gel retardation assay. The two complementary strands were then annealed and labeled with [γ-32P]dCTP. The labeled oligonucleotides (10,000 cpm) and 10 µg of nuclear extracts and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI-dC), 1 mM dithiothreitol) were then incubated for 30 min at room temperature in a final volume of 20 µl. Next, the reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5-Tris-borate buffer, after which the gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold ARE oligonucleotide.

8. Statistical analysis

Statistical analysis of the data was performed using ANOVA and Duncan’s test. Differences with a p<0.05 were considered statistically significant.

Results

1. TRSDY inhibits androgen-dependent LNCaP cell growth

We tested the effects of TRSDY on cell growth in LNCaP cells using different concentrations of TRSDY extracts. MTT assay of cells that were treated for 24 and 48 h revealed that TRSDY induced a dose-dependent decrease in cell growth (Fig. 1).

![Fig. 1. Effects of TRSDY on cell viability. LNCaP cells (1x10^6) were treated with various concentrations of TRSDY for 24 or 48 h and its viability was then determined by MTT assay. Each value is the mean ± SEM of three independent experiments. *p<0.05, **p<0.01 vs. untreated control.](image)

2. TRSDY induces apoptosis of LNCaP cells

TRSDY-mediated apoptosis was examined by a DNA fragmentation assay, which is a well-accepted method of determining apoptotic cell death. DNA extracts from LNCaP cells treated with 1.0 mg/ml TRSDY displayed a ladder pattern of discontinuous DNA fragments after 24 h that persisted until 48 h (Fig. 2A). We also examined the BrdU-labeled DNA fragments quantitatively following treatment with various concentrations of TRSDY. As shown in Fig. 2B, TRSDY increased the BrdU-labelled DNA fragments in a dose-dependent manner.

The proteolytic processing of inactive procaspase-3 is an essential component of the apoptotic cell death pathway in many cells. Full-length active PARP is a 116 kDa molecule that is cleaved to fragments of 86 and 30 kDa by caspase-3. As indicated in Fig. 3A, TRSDY caused a significant reduction in procaspase-3 protein expression in a dose-dependent fashion, and this reduction was well correlated with the induction of apoptosis that is shown in Fig. 2B. TRSDY treatment also reduced the levels of active PARP and increased the levels of...
the 85 kDa fragment from 24 h (Fig. 3B). Taken together, these findings suggest that TRSDY induces apoptotic cell death through the activation of caspase-3.

3. TRSDY suppresses AR/PSA expression

Because TRSDY inhibits growth and induces the apoptotic death of LNCaP cells, we evaluated TRSDY to determine if it could affect the expression of PSA, a characteristic biomarker for prostate cancer that is encoded by ARE. PSA was significantly down-regulated in TRSDY-treated LNCaP cells in response to increasing amounts of TRSDY (Fig. 4A). In addition, the expression of PSA and AR was decreased in a time-dependent fashion when cells were treated with 1.0 mg/ml TRSDY (Fig. 4B). To determine if a similar inhibitory effect of TRSDY on endogenous AR and PSA expression in LNCaP cells also occurred in response to treatment with DHT, LNCaP cells were treated with 5.0 nM DHT after being treated with various concentrations of TRSDY for 24 h. When we treated cells with various concentrations of DHT, the AR expression increased in a dose-dependent manner (Fig. 5A). However, these increases were suppressed in cells that were pretreated with TRSDY in a dose dependent manner (Fig. 5B and 5C). These results suggest that TRSDY inhibits the endogenous expression of AR and translocation of AR to the nucleus, which subsequently decreases the expression of PSA.

4. TRSDY inhibits DHT-induced binding of AR/ARE

EMSA was used to explore the effect of TRSDY on the
binding of AR to androgen response element (ARE). Complex formation was observed using the consensus region of ARE and nuclear extracts from LNCaP cells. The treatment of LNCaP cells with various concentrations of DHT enhanced complex formation in a concentration dependent manner (Fig. 6A). However, DHT-stimulated complex formation was gradually inhibited by increasing concentrations of DHT in the presence of TRSDY (Fig. 6B). In competition studies, treatment with a 50-fold molar excess of non-radio-labeled ARE inhibited complex formation (Fig. 6B, lane 7).

Discussion

In recent years, there has been growing interest in applying naturally occurring phytochemical-based compounds with anti-cancer potentials because they are relatively non-toxic, inexpensive and can be administered orally. The compounds are typically prepared by steaming or heating crude plant materials, a method that has been used for centuries in Asia and Europe to manage the symptoms of cancer and even as a conventional treatment of cancer. Although the etiology of prostate cancer is poorly understood, a variety of dietary components including essential and nonessential nutrients are thought to influence the risk of prostate cancer.

Previous studies have shown that TRSDY treatment resulted in apoptotic cell death of HL-60, human leukemia cell line. In the present study, we assessed the anti-cancer effects of TRSDY against prostate cancer. To accomplish this, we employed human prostate cancer cell lines, LNCaP cells. It is known that LNCaP cells are androgen-responsive. In the first experiment, we evaluated TRSDY treatment to determine if it impacts antiproliferative effects in human prostate cancer cells. The results of an MTT assay revealed that treatment of LNCaP cells with TRSDY resulted in a dose-dependent decrease of proliferation. These results indicate that the anti-proliferative effects of TRSDY are dependent on androgen actions.

We next evaluated the inhibition of LNCaP cells to determine if it was mediated via apoptosis. As shown in Fig. 2, treatment of LNCaP cells with TRSDY resulted in the formation of a DNA ladder, which is a hallmark of apoptosis. These results were quantitatively verified by a BrdU-labelled DNA fragments assay using ELISA kits. Apoptotic cell death of LNCaP cells by TRSDY is caused by activation of caspase-3 and subsequent PARP cleavage. Apoptosis is a physiologic process that involves the elimination of cells with damaged DNA. In addition, apoptosis is a distinct form of cell death that differs from necrotic cell death and occurs as a result of a series of precisely regulated events that are frequently altered in tumor cells. Therefore, agents that can modulate apoptosis may be useful for cancer management and therapy.

PSA is a tissue-specific glycoprotein with kallikrein-like serine protease activity that is elevated in benign prostate hyperplasia and primary/metastatic prostate cancer tissues. Therefore, most studies have emphasized its use as a serum marker for diagnosis of patients with prostate carcinoma, as well as for monitoring their responses to different forms of therapy. It has also been reported that PSA stimulates cell proliferation and DNA synthesis in LNCaP cells and that such growth regulations are partially neutralized by incubation with PSA-specific antibody in a concentration-dependent manner. In this study, TRSDY was found to downregulate PSA protein levels in a dose- and time-dependent manner. These results suggest that the anti-proliferative effects of TRSDY on LNCaP cells might be mediated by inhibition of PSA expression.

AR is known to be a critical factor in the growth of prostate cancers and in the development of ablation-resistant prostate cancer development. When using whole cell lysates, AR expression levels were decreased after LNCaP cells were incubated with 1.0 mg/ml TRSDY (Fig. 4B), which indicates that TRSDY decreases AR expression at the post-translational level. TRSDY appears to destabilize AR protein or activate its degradation. The addition of novel and potent drug candidates to the existing therapy would certainly improve the outcome of prostate cancer therapy and AR down-regulating agents have emerged as an attractive target for the development anti-prostate cancer drugs. To date, most of the agents found to be AR-down-regulating agents have been natural compounds, which is consistent with the finding that TRSDY contains bioactive compounds capable of regulating AR.

The 5' upstream promoter and enhancer region of the PSA gene contains several androgen response elements (ARE) to which ligand-activated AR binds and induces expression of PSA. Therefore, TRSDY was evaluated to determine if it could inhibit the ability of ligand-activated AR to act as a transcription factor. In LNCaP cells, DHT stimulated AR expression in nuclear fractions in a dose-dependent fashion (Fig. 5A). Further studies showed that DHT increased the binding of DHT-activated AR to ARE, as shown in Fig. 6A. However, pretreatment with TRSDY decreased DHT-induced expression of AR and PSA levels as indicated by Western blot (Fig. 5B & 5C). TRSDY also inhibited formation of the AR/ARE complex induced by DHT. Therefore, the ability of TRSDY to diminish the expression of PSA is probably dependent on decreasing the down-regulation of ARE.
transcriptional activity.

In conclusion, the results of this study demonstrate that TRSDY suppressed androgen-dependent prostate cancer cell proliferation and induced apoptosis, which occurred via caspase-3 activation and PARP cleavage. Those effects of TRSDY might be mediated by suppression of constitutive or DHT-induced AR expression.

Recently, two complex herbal formulations, denoted as PC-SPES® and EquiguardTM, were investigated as prostate cancer therapeutic agents. These results suggest that TRSDY may also be a candidate for developing anti-tumor drugs with increased potency for the prevention and treatment of prostate cancer.

Acknowledgments

This work was supported by Wonkwang University(2007).

References