Induction of cell cycle arrest and apoptosis by grape seed procyanidin extract in human bladder cancer BIU87 cells

J. LIU¹, W.-Y. ZHANG², Z.-H. KONG¹, D.-G. DING¹

¹Department of Urology, Henan Provincial People’s Hospital, Zhengzhou, China
²Department of Obstetrics and Gynecology, The First Affiliated Hospital of Henan University of Traditional Chinese Medicine, Zhengzhou, China

Abstract. – OBJECTIVE: The aim of this study was to evaluate the effects of grape seed procyanidin extract (GSPE) on cell proliferation and apoptosis in human bladder cancer BIU87 cells and to investigate its molecular mechanism in vitro.

MATERIALS AND METHODS: BIU87 cells were treated with different concentrations of GSPE for 24h in vitro while an untreated group was taken as control. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, Hoechst 33258 staining, flow cytometry, RT-PCR and Western blot were used to detect the anti-proliferation and apoptotic induction effects of GSPE on BIU87 cells.

RESULTS: It was found that GSPE inhibited the cell growth through cell cycle arrest at G1 phase and induced cell apoptosis in BIU87 cells in a dose-dependent manner. Semi-quantitated RT-PCR and Western blot analysis indicated that GSPE increased caspase-3 (p<0.01), but decreased the expression of cyclinD1, CDK4 and survivin (p<0.01).

CONCLUSIONS: GSPE inhibits cell proliferation by inducing cell cycle arrest and apoptosis in BIU87 cells, and the effect may be related with its down-regulation of cyclinD1, CDK4 and survivin.

Key Words: Apoptosis, Bladder cancer, Cell cycle, Grape seed procyanidin extract (GSPE).

Introduction

Bladder cancer is the most common malignant tumor in the urinary system, and the sixth most common malignancies in developed countries, where most bladder tumors are transitional cell carcinomas (TCCs). Approximately 75% of uroepithelial TCCs are superficial (Tis, Ta, and T1), most of the superficial cases are treated by transurethral removal of the tumor conservatively, followed by adjuvant intravesical therapy without removing the bladder. As a main therapeutic regimen, adjuvant intravesical therapy is used extensively on patients with superficial urothelial carcinoma of the bladder in the clinic to treat the tumor and preclude the recurrence of bladder cancer, but the toxicity, set effects and drug resistances depress the therapeutic effect of the drugs. Of the superficial tumors, 50-70% recur one or more times, but do not progress into invasive disease, and 10-30% progress to invasive and potentially lethal disease after transurethral resection. Thus, to decrease recurrence and progress of bladder cancer, it is critical for urologists to find a new and safe drug agent for intravesical therapy.

Procyanidins are a class of polyphenolic compounds which distributed in plants extensively, and derived from common dietary foods (fruits, beans and chocolate, etc.) and beverages (fruit juices, wine and tea, etc.). Grape seeds are a particularly rich source of procyanidins, and the procyanidins are typical from the other derivatives. Grape seed procyanidin extract is a mixture of several polyphenols mostly contain dimers, trimers, tetramers and other oligomers of (+)-catechin and (-)-epicatechin, mainly oligomers. Recent cell culture studies have shown that treatment of human breast cancer MCF-7 and MDA-MB468, human lung cancer A-427, human gastric adenocarcinoma CRL-1739, human oral squamous cell cancer CAL27 and SCC25, human prostate cancer DU145 and LNCaP, human colorectal cancer HT29, LoVo and CaCo2 cells with grape seed procyanidin extract results in a inhibition of cell proliferation and/or an induction of cell apoptosis. Loss of the fine balance between cell proliferation and apoptotic death contributes to...
increase in cellular mass and tumor progression. The agent, however, is not be reported in bladder cancer cells. Thus, the aim of this study was to demonstrate the antiproliferative and proapoptotic effect of grape seed procyanidin extract (GSPE) in human bladder cancer cell line BIU87, and delineate the mechanism of those effects.

Materials and Methods

Cell and reagents

Human bladder cancer cell line BIU87 was obtained from the Institute of Urology, Peking University. GSPE (purity ≥95%, Nanjing Qingze Herbal Technology Development Co. Ltd, China) was dissolved in RPMI-1640 (without fetal bovine serum, FBS) as a 10 mg/ml stock solution and diluted by RPMI1640 as desired concentrations, stored at -20°C in light-tight containers until used. FBS (Gibco, Grand Island, NY, USA), RPMI 1640 (Gibco, USA), Trizol (Invitrogen, Carlsbad, CA, USA), Hoechst 33258 apoptosis staining kit (Beyotime Institute of Biotechnology, Jiangsu, China), Annexin-V-FITC/PI apoptosis assay kit (Jingmei Biotech Co. Ltd, China), anti-cyclinD1, anti-CDK4, anti-caspase-3, anti-survivin and anti-β-actin were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA), ECL reagent kit (Pierce, Waltham, MA, USA).

Cell culture

Medium for BIU87 cell line was supplemented with 10% FBS and 1% penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) solution. Cell cultures were maintained in 25 cm² cell culture flasks or six-well cell culture plates (Iwaki, Japan) at 37°C and 5% CO₂ in humidified chambers. When BIU87 cell was plated to 80%-90% confluency, cells were fed with fresh medium and were treated with either RPMI 1640 alone (control group) or different concentrations of GSPE which were diluted by RPMI1640 (as described above), and cultured for 24 h to get the cells for following tests. Three separate, independent replications of each test were performed.

MTT assay for cellular viability

Cell viability was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA] assay. The cells were plated at 1×10⁴ cells per well 200 μl of complete culture medium in 96 well microtiter plates, and treated with designed concentrations (12.5, 25, 50, 100, 200 μg/ml) of GSPE till 80%-90% confluency. Each concentration of GSPE was repeated in 6 wells. After incubation for specified times at 37°C in a humidified incubator, MTT reagent [20 μl, 5 mg/ml in phosphate buffered saline (PBS) (10 nmol/l, pH 7.45)] was added to each well and incubated for 4 h. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) (150 μl). Absorbance (A) was recorded on a microplate reader at a wavelength of 490 nm. The effect of GSPE on growth inhibition was assessed as the percentage of inhibition in cell growth where untreated cells were taken as 100% viable. Percent viability was calculated as [value of drug-treated group (A)/ control group (A)] ×100.

Apoptosis Assessment by Hoechst 33258 Staining

After treatment with GSPE, BIU87 cells on six-well plates were immersed in 0.5 ml methanol for 10 min, followed by a rinse with PBS for 3 min twice. Then cells were stained with 1 mg/ml Hoechst 33258 compounds in a dark chamber at room temperature for 5 min and rinsed for 3 min twice in PBS again. Cells were analyzed by fluorescence microscopy (Leica, Wetzlar, German). The apoptotic cells are featured as pyknotic and fragmented nuclei emitting intense fluorescence.

Cell Cycle Analysis by Flow Cytometry

The effect of GSPE on BIU87 cell cycle phase distribution was assessed using flow cytometry. Briefly, after treatment with GSPE (0, 50, 100 and 200 μg/ml concentrations) for 24 h, floated cells were discarded by aspiration and the attached cells were trypsinized and thereafter washed twice with cold PBS and centrifuged. The pellet was resuspended in cold ethanol for 12h at 4°C. The cells were centrifuged at 200 g for 5 min, the pellet washed twice with cold PBS, suspended in 500 μl PBS and incubated with 5 μl RNAase (200 μg/ml final concentration) at 37°C for 30 min. The cells were then chilled over ice for 10 min and stained with propidium iodide (PI, 50 μg/ml final concentration) for 30 min for analysis by flow cytometry. Flow cytometry was performed with a FACScan (BD FACS Calibur, San José, CA, USA). A minimum of 10,000 cells/sample were collected, and the DNA histograms were further analyzed using Cellquest software (Becton Dickinson, Franklin Lakes, NJ, USA) for cell cycle analysis.
Apoptosis Analysis by Flow Cytometry

To quantify GSPE induced apoptosis of BIU87 cells, annexin V and PI staining was performed followed by flow cytometry. After treatment of cells with different concentration of GSPE for 24h, both floating and attached cells on six-well plates were collected by digestive juice (0.25% trypsinization plus 0.02% EDTA) and washed with 4°C PBS twice, and subjected to annexin V and PI staining using Apoptosis Assay Kit following the step-by-step protocol provided by the manufacturer. In brief, cells were resuspended in 250 μl of bind buffer and adjusted the density for 1× 10⁶. For each sample, 100 μl cell suspension was aspirated and added 5 μl of annexin V-FITC and 10 μl of PI. After 15 min incubation in the dark at room temperature, each sample was diluted with 400 μl of PBS to obtain a final volume appropriate for flow cytometry.

RNA Isolation and Semiquantitative RT-PCR

Total RNA of cells on 25 cm² culture flasks were extracted by Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the protocols provided by the manufacturer. The quality of each total RNA sample (including its concentration and purity) was checked and controlled by measurement of the optical density of each sample. Up to 10 μl with dimethyl pyrocarbonate (DEPC)-treated water, 1 μg of RNA and 300 pmol of oligo-dT(18) (Takara Bio, Otsu, Shiga, Japan) were added together and were heated at 65 °C for 5 min. 200 units M-MLV reverse transcriptase (Takara Bio, Japan), 1 μl of 10 mM dNTP (Promega, Madison, MI, USA), 20 units RNasin (Promega, USA), and 4 μl of 5×M-MLV RT buffer (Promega, USA) were then added to the mixture and the final volume was brought up to 20 μl with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 1 h and 72°C for 10 min.

PCR amplification was performed in a reaction volume of 25 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 μM, 2.5 μl of 10×RT buffer, 1 μl of 2.5 mM dNTP, and 1 μl of Taq DNA polymerase (TaKaRa, Otsu, Shiga, Japan). Specific primers for human cyclinD1, CDK4, caspase-3, survivin, and β-actin as a loading control: cyclinD1 (size of PCR product: 245 bp): sense 5'-GGGCGTCGTCTCTGTTGGA-3', antisense 5'-GGTGGGCTCCCTCAG- GTTCA -3'[25]; CDK4 (size of PCR product: 498 bp): sense 5'-GCGCAGTGGTGGCCCGACG TTG-3', antisense 5'-GGCAGCCCAATCAACTGTA-3'[25]; caspase-3 (size of PCR product: 422 bp): sense 5'-GACATCTCGGTCTGTTG-3', antisense 5'-CAGTTGCTG TGGAGTA-3'; survivin (size of PCR product: 439 bp): sense 5'-CAGAGGCCTCAATCCATGGC-CA-3' [26]; β-actin (size of PCR product: 580 bp): sense 5'-ATGATATCGCCCGCCTCGTC-3', antisense 5'-CGCTCGGTGAGGATCTTCA-3'[25]. The reaction condition of PCR (caspase-3) was as follows: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45s, annealing at 50°C for 45s, and extension at 72°C for 1 min on a Mastercycler gradient (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). PCR products were electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide. Gene expression was presented by the relative yield of the PCR product from target sequences to that from the β-actin gene.

Western Blot Analysis

BIU87 cells were collected by scraping and washed with ice-cold PBS for two times. The cell pellets were homogenized in extraction buffer (50 mM Tris-HCl, 0.1% SDS, 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium orthovanadate), then incubated at 4°C for 30 min and centrifuged 20 min at 12,000 g/min. The concentration of total protein in the supernatant was quantified by Bradford assay. For immunoblot analyses, 50 μg protein lysates per sample were resolved over 15% SDS-PAGE gels and transferred onto a nitrocellulose membrane. It was followed by blocking with 5% non-fat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. The membranes were incubated with anti-cyclinD1 (1:500), anti-CDK4 (1:500), anti-caspase-3 (1:500), anti-survivin (1:500), and anti-β-actin (1:500) respectively at 4°C overnight. After binding of horseradish peroxidase (HRP)-coupled goat anti-rabbit/mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5000) at room temperature for 2 h, antigens were visualized by enhanced chemiluminescence (ECL-kit, Pierce Biotechnology, Waltham, MA, USA). All results are representative of three independent experiments.
GSPE favors cell cycle arrest and apoptosis in human bladder cancer

Statistical Analysis
SPSS20.0 software (SPSS Chicago, IL, USA) and SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Data were expressed as mean ± SD. All experiments were performed at least three times. The results are presented as the mean ± standard deviation (SD). * represents p<0.01 compared to the control.

Results

Inhibition of BIU87 Cell Growth by GSPE
The effect of GSPE on cell viability was determined employing a MTT assay. BIU87 cells were cultured with GSPE at final concentrations of 12.5, 25, 50, 100 and 200 μg/ml for 24 h, after which MTT assays were then carried out. The cells cultured in GSPE-free media were used as the control. As shown in Figure 1, the viability of the BIU87 cells incubated with GSPE at concentrations of 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml for 24 h was 99.55±0.50% (n=18, p >0.05), 99.15±0.63% (n=18, p >0.05), 86.98±1.48% (n=18, p <0.01), 68.18±2.06% (n=18, p <0.01) and 51.42±1.78% (n=18, p <0.01) of the control value, respectively. A trend of decreasing viability with increasing GSPE concentration was observed at 50, 100 and 200 μg/ml in BIU87 cells. The results of the MTT assay showed that GSPE exerts a dose-dependent cytotoxic effect on the bladder cancer BIU87 cells. For further mechanistic studies, a dose of 50, 100 and 200 μg/ml GSPE was selected.

GSPE Treatment Caused G1 Phase Arrest in BIU87 Cells
To investigate whether GSPE treatment has effects on the cell cycle regulation, its effect on cell cycle distribution by flow cytometry after staining with PI was determined. As shown in Figure 2, concomitant with growth inhibitory effects, GSPE treatment induced a strong G1 phase arrest in a dose-dependent manner. In BIU87 cells G1 phase distribution was 48.46, 55.73, 69.81 and 79.18% at 0, 50, 100 and 200 μg/ml concentrations of GSPE, respectively.

Effect of GSPE on the expression of CyclinD1 and CDK4 in BIU87 cells
Since GSPE was observed to cause an arrest of cells in G1 phase, we next assessed its effect on the expression of cyclinD1 and CDK4 which were intimate with G1 cell cycle regulation. As shown in Figure 3 and Figure 4, GSPE treatment of cells caused a dose-dependent decrease

![Figure 1. Effect of GSPE on the viability of BIU87 cells. Cells were treated with specified concentrations of GSPE for 24h, and cell viability was determined by MTT assay. The experiments were repeated at least three times. The results are presented as the mean± standard deviation (SD). * represents p<0.01 compared to the control.](image1)

![Figure 2. GSPE induces G1 phase arrest in the cell cycle of BIU87 cells. The cells treated with GSPE at 24 h were collected and stained with PI followed by flow cytometry. Data are representative of three independent experiments.](image2)
Figure 3. Expression of cyclinD1 and CDK4 mRNA in response to GSPE treatment in BIU87 cells. Cells were treated with GSPE for 24 h, harvested and total RNA was isolated and analyzed by RT-PCR for cyclinD1 and CDK4 mRNA expression. The housekeeping gene β-actin was used as a control. Results are representative of data obtained from three separated experiments. (*represents $p < 0.01$ compared to the control. Δ represents $p < 0.01$ compared to 50 μg/ml group. # represents $p < 0.01$ compared to 100 μg/ml group).

Figure 4. Expression of cyclinD1 and CDK4 protein in response to GSPE treatment in BIU87 cells. Cells were treated with specified concentrations of GSPE for 24 h, harvested and total cell lysates were prepared. The expression of cyclinD1 and CDK4 were determined by Western blot analysis. A typical immunoblot from three separate experiments with similar results is shown here. (*represents $p < 0.01$ compared to the control. Δ represents $p < 0.01$ compared to 50 μg/ml group. # represents $p < 0.01$ compared to 100 μg/ml group).
in the levels of CyclinD1 and CDK4. Semi-quantitative RT-PCR analysis indicated that the expression level of CyclinD1 and CDK4 mRNA significantly decreased following GSPE treatment in BIU87 cells ($p < 0.01$). Similar results of the expression of cyclinD1 and CDK4 protein could be drawn by semiquantitative Western blot analysis ($p < 0.01$).

**Induction of Apoptosis by GSPE in BIU87 Cells**

Apoptosis was confirmed by analyzing the nuclear morphology of GSPE-treated BIU87 cells. Nuclear morphology was evaluated with membrane-permeable blue Hoechst 33258. Figure 5 showed representative Hoechst 33258 fluorescence photo-micrographs of cultured BIU87 cells treated with or without GSPE, respectively. In control cultures, nuclei of BIU87 cells appeared with regular contours and were round and large in size, none of the BIU87 cells with smaller nuclei and condensed chromatin were seen. In contrast, most of the GSPE-treated BIU87 cells appeared hyper-condensed (brightly stained) and fragmented nuclei. The numbers of apoptotic nuclei containing condensed chromatin increased significantly with the rise of incubation concentration.

For the quantification of apoptotic BIU87 cells, annexin V and PI were used for staining. Annexin V$^+$ and PI$^-$ cells were designated as apoptotic. As shown in Figure 6, compared with the RPMI 1640-treated vehicle control cells, which almost no apoptotic cells were detected, 24 h of GSPE treatment at 50, 100, and 200 μg/ml doses resulted in 8.7, 28.2, and 40.6% apoptotic cells, respectively. When the results were analyzed in terms of annexin-V plus PI staining of the cells, a dose-dependent effect of GSPE was evident ($p < 0.01$).

**Effects of GSPE on the Expression of Caspase-3 and Survivin in BIU87 Cells**

To evaluate the possible mechanisms of GSPE induced apoptosis in BIU87 cells, RT-PCR and Western blot assays were established for caspase-3 and survivin. As shown in Figure 7, the mRNA expression level of caspase-3 was increased and survivin was decreased in a dose-dependent manner by GSPE treatment in BIU87 cells for 24h. Semiquantitative RT-PCR indicates that the difference between the groups of different concentrations was significant ($p < 0.01$).
We also detected the protein expression of Caspase-3 and Survivin in BIU87 cells by Western blot analysis after being treated with different concentrations of GSPE for 24h. Similar to caspase-3 and survivin mRNA expression, Western blot analysis indicated that the protein expression levels of Caspase-3 was increased and survivin was decreased in a dose-dependent manner by GSPE treatment. The levels between different groups were significant by semiquantitative Western blot analysis (*represents \( p<0.01 \) compared to the control. A represents \( p<0.01 \) compared to 50 \( \mu \)g/ml group. # represents \( p<0.01 \) compared to 100 \( \mu \)g/ml group).

**Discussion**

Almost 90% of the bladder cancer is superficial transitional cell carcinomas, which always followed an adjuvant intravesical therapy conventionally after transurethral removal of the tumor to preclude the recurrence and invasion. Most of the chemotherapeutic drugs used in the clinic had a toxicity and set effects in some extent. It is critical to find a new and safe drug agent for intravesical therapy.

Several studies have shown that some microchemicals present in the diet and several fruits and vegetables are the most desirable class of agents for the prevention and/or intervention of various cancers\(^{27-29}\). Among these chemicals, GSPE has received increasing attention in recent years for its strong effects of antioxidant and antitumor. Recent investigations\(^{13,16,24,30,31}\) in vitro and in vivo had shown great antitumor effects on most human cancers (such as breast cancer, lung cancer and gastric adenocarcinoma, etc.) by the treatment of GSPE. However, the agent enhances the growth and viability of normal human gastric mucosal cells\(^{16}\) and indicates a lack of toxicity\(^{32}\).

The normal operation of the cell cycle was determined by the regulatory system composed of cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinases inhibitor (CDKI). The cyclinD1-CDK4-pRb (retinoblastoma protein) pathway plays a central role in the transition of G\(_1\)-S phase in the cell cycle and its deregulation is correlated with many types of cancers\(^{33-35}\). When activated by cyclin D1, CDK4 is able to phosphorylate pRb, leading to the release of associated proteins like E\(_2\)F that have the capability to activate genes necessary for cell progression through the G\(_1\) phase\(^{16}\). CyclinD1 and CDK4 play an important role in carcinogenesis, and may be a prognostic factor and therapeutic target for human cancers\(^{37-40}\).

In this study, we found that GSPE treated resulted in G\(_1\) phase arrest in a dose-dependent manner in BIU87 cells. We further investigated the effect...
of GSPE on the expression of G1 regulatory genes. GSPE treatment was found to result in a remarkable decrease in the mRNA and protein levels of cyclinD1 and CDK4 in a dose-dependent manner. It indicated that reduced expression of cyclinD1 and CDK4 may be involved in GSPE induced G1 phase arrest, leading to cell growth inhibition.

Apoptosis is a process of programmed cell death, is critical for the development and maintenance of healthy tissues. Dysregulation of cell death pathways occurs in many types of human cancer. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, plays an important role in inhibition of apoptosis, which binds and inhibits specifically to the terminal effector cell death proteases, caspase-3 and -7 to attend the procedure of apoptosis in tumor cells41-43. It is present during embryonic and fetal development, but undetectable in normal differentiated adult tissue in vivo44. In various human cancers including bladder cancer, survivin is strongly overexpressed45-47 and has been established as a diagnostic, prognostic and therapeutic marker48-53.

In the present work, after treatment of GSPE for 24h, morphological characterization (hyper-condensed and fragmented nuclei) of apoptotic BIU87 cells could be seen by Hoechst 33258 staining, and the number of apoptotic BIU87 cells increased with the concentrations of GSPE by flow cytometry. The mRNA and protein expression of caspase-3 increased and survivin decreased in a dose-dependent manner of the treatment of GSPE. Those indicated that down-regulates the expression of survivin which correlated with apoptosis may be involved in GSPE induced apoptosis in BIU87 cells, weaken the inhibition of caspase-3.

Conclusions

We provided the first evidence that GSPE inhibited cell proliferation by cell cycle arrest and apoptosis in human bladder cancer BIU87 cells which was associated with down-regulation of the expression of cyclinD1, CDK4 and survivin. Because many anticancer drugs are known to achieve their antitumor function by arresting the cell cycle and inducing apoptosis, these abilities of GSPE to arrest the cell cycle and induce apoptosis implying its potential to be a chemotherapeutic agent. Although the precise molecular mechanism of these effects induced by GSPE remains unclear, it might be a potent useful antitumor agent for adjuvant intravesical therapy against human bladder cancer.
Conflict of Interests:
The Authors declare that they have no conflict of interests.

References
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