Research Article

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Cyclosporine-A induces apoptosis in human prostate cancer cells PC3 and DU145 via downregulation of COX-2 and upregulation of TGFβ

Siklosporin-A, İnsan Prostat Kanseri Hücreleri PC3 ve DU145 'te COX-2'yi Azaltarak ve TGFβ'yi Arttırarak Apoptozu İndükler

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Abstract

Background: Potential targets for prostate cancer therapy are urgently needed for curative of patients. Cyclosporine-A (CsA), an immunosuppressive and a selective cyclooxygenase-2 (COX-2) inhibitor, exerts antitumor activity. However, the molecular effects of CsA is not fully understood in prostate cancer. In this research, we sought to determine role and mechanism of CsA in prostate cancer.
Materials and methods: PC3 and DU145 cells were treated with CsA time (12, 24, 48 h) and dose dependent (2.5, 10, 25 μM) and cell survival, migration, colony formation, expression of apoptosis related proteins/genes using MTT assay, scratch assay, Western blotting/qPCR. At the same time, cells treated with CsA to test on the effects of COX-2 promoter activity using luciferase reporter plasmid. Lastly, functional role in the CsA treatment prostate cancer cells were interrogated for relationship of TGFβ, Akt, caspases and COX-2.
Results: These study findings provided direct evidences that the CsA induced apoptosis and downregulated migration.
Conclusions: CsA downregulated Akt as well as COX-2 and upregulated TGFβ, resulting in the suppression of cell migration which was augmented a potential therapeutic of CsA in prostate cancer cells.

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Keywords: Cyclosporine; Prostate cancer; Apoptosis; Migration; Anti-cancer; Androgen.

Öz

Amaç: Prostat kanser tedavisi için hastalara yeni tedavi edici potansiyel ajanlar gerekmektedir. Siklosporin-A (CsA) bir immunsupressör olarak bilinir ve COX-2 inhibitörüdür aynı zamanda anti-tümör özelliği de bildirilir. Fakat CsA'nın prostat kanserinde moleküler etkileri henüz bilinmemektedir. Bu çalışma CsA'nın prostat kanserindeki mekanizması ve rolünü aydınlatmayı amaçlamıştır.
Gereç ve Yöntem: İnsan PC3 ve DU145 hücreleri zamana (12, 24, 48 saat) ve doza bağlı olarak (2.5, 10, 25 μM) inkübe edilerek hücre canlılığı, migrasyon, koloni oluşumu, apoptoz ilişkili protein/genlerin ekspresyon düzeyleri MTT, migrasyon-mesafe ölçümü, Western blot/qPCR kullanılarak yapıldı. Aynı zamanda hücrelerde CsA ile inkübasyonunun COX-2 promoter aktivitesi üzerindeki etkisi lusiferaz aktivitesi ile ölçüldü. Son olarak CsA tedavisinin prostat kanserinde TGFβ, Kaspazlar ve COX-2 ilişkisi sorgulandı.
Bulgular: Bu çalışmanın bulguları CsA'nın prostat kanserinde apoptozu indüklediği ve migrasyonu azalttığı yönündedir.
Sonuç: CsA tedavisi prostat kanserinde Akt ve COX-2'yu azaltıp, TGFβ'yı arttırmıştır. Böylece hücre migrasyonunu baskılaması ile prostat kanseri tedavisinde potansiyel bir terapötiktir.

Anahtar Kelimeler: Siklosporin; Prostat kanseri; Apoptoz; Migrasyon; Androjen.

Introduction

Prostate cancer is the most common primary tumor of the urological system in man. Prostate cancer constituent of progression and metastasis is urgently required to understanding of molecular mechanisms. Generally

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standard first-line treatment for metastatic prostate cancer is recommending hormone therapy but it is not curative. As well, prostate cancer patients recurrent with first-line starting hormone therapy that an alternative approach is to urgently require novel therapeutics to treatment as suppress progression [1]. Prostate cancer metastasis proceeds through a complex series of molecular events that include consistently cyclooxygenase-2 (COX-2) overexpressed in a large percentage. Furthermore, it was suggested that COX-2 is an attractive target for the treatment or prevention of prostate cancer and it is well established that selective COX-2 inhibitors suppresses prostate cancer tumor dissemination and invasion [2]. COX-2 suppression plays a role in induction of apoptosis, suppression of angiogenesis, reduction of metastasis, involving carcinogenesis in prostate cancer [3, 4]. However, overexpression of COX-2 can induce tumor formation potential mediated suppression of apoptotic proteins and poorer prognosis [5]. Previous studies with independent of androgen prostate cancer cells suggested that a COX-2 expression is connection with chemorefractory and resistant to radiotherapy in patients which is a specific marker for tumor formation [6, 7].

Recent evidence has shown that in prostate cancer patients have a high expression of COX-2 and activation of transforming growth factor beta (TGFβ) and vigorously collaboration with mortality from prostate carcinoma [8]. COX-2 inhibitors have role of anti-proliferative activity and suppression of COX-2 related to growth inhibition in prostate cancer cells [9]. Cyclosporine-A (CsA) is known a selective COX-2 inhibitor and immunosuppressive a drug commonly used for transplantation and immune related disease [10]. On the other hand CsA regulate gene expression via microRNAs on cancer therapy in human glioblastoma cells and contribute to prevention of cancer [11]. In addition, we evaluated the molecular activity of the COX-2 and TGFβ pathway affected by CsA in androgen independent of prostate cancer cells.

Materials and methods

Cell culture and cell survival assay

Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PC3 and DU145 human prostate cancer cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and kept in a humidified atmosphere at 37°C incubator with 5% CO_2 in air. These cells were chosen as they represent major aspects of androgen independent human prostate cancer and their progression. To determine the cell viability, cells were trypsinized and seeded into 96-well plates (1×10^4 cells/well). The cells were treated with different concentrations (0–250 μM) of CsA and incubated for 24 h. After the incubation cells washed with PBS and added to 100 μL RPMI-1640. Ten microliter of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Vybrant, Invitrogen) labeling reagent was added to each well and incubated for 4 h in humidified atmosphere at 37°C incubator with 5% CO_2 in air. After the incubation 100 μL of the SDS buffer was added into each well for solubilization of formazan precipitate. Then absorbance was measured by microplate reader at 570 nm and was carried out in triplicate of each assay.

Cell growing assays

In order to investigate the effects of CsA on cell survival of PC3 and DU145 cells, we incubated with CsA at a final concentration of 10 μM in growth medium (without antibiotics) for 12 h, 24 h and 48 h. After the incubation, methylene blue staining was used for cell survivals. The cells were extracted with 1% SDS in PBS solution and stained with 0.01% methylene blue solutions. The absorbance was measured at 600 nm with microplate reader. Each experiment was repeated three times.

Determination of COX-2 promoter activity

Cells were seeded in 24 well plates and co-transfected with totally 0.5 μg plasmids of pCOX-2-Luc [12] and pRL-SV40 (Promega) using the Lipofectamine transfection reagents (Invitrogen). At 24 h after transfection, cells were treated with CsA for 24 h. End of the incubation, cells were lysed in the Passive Lysis Buffer in order to dual luciferase-renilla assays. Luciferase reporter activity was performed on a Luminometer (Lumate, USA) with dual luciferase activity kit (Promega) following to manufacturing procedure.

Colony formation assay

PC3 and DU145 cells were seeded in six well plate at a concentration of 1000 cells per well and incubated with CsA and maintained at 37°C for 14 days. The medium was changed every 3 days. At the end of incubation, each well in plate was washed with PBS, fixed with cold methanol/acetic acid, stained with % 0.5 crystal violet staining solution for 15 min and washed with ddH_2O, sequentially. The stained cells were examined with microscope and imaging

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system. The number of colonies in each well was counted and analyzed.

Scratch wound assay

PC3 and DU145 cells seeded in 24 well plates were incubated with 2.5 μM and 10 μM CsA and a scratch was introduced using a sterile 200 μL micropipette tip. Cells were grown for 24 h and the width of the wound was measured with an inverted microscope. The normalized wound area (wound area 24 h/wound area 0 h) was calculated using the Image J software (NIH, USA).

Measurement of caspase-3 activity

PC3 cells were incubated with different concentrations (2.5 μM, 10 μM, 25 μM) of CsA at different times (12 h, 24 h, 48 h) in 12 well plates. After the incubations, cells were harvested with cell lysis buffer. Caspase-3 activity assay was performed with caspase-3 (Cas-3) activity assay kit according to the manufacturer's instructions (Invitrogen).

Western blot analysis

PC3 and DU145 cells were homogenized in cell lysis buffer and protein concentrations were determined by Bradford reagent (Biorad) as previously described [13]. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Santa Cruz). Membrane was blocked with 3% BSA at room temperature and was incubated with primary antibodies. The following primary antibodies at 4°C overnight: anti-Akt, anti-cleaved Cas-3, anti-caspase-8, anti-caspase-3, anti-TGFβ, anti-COX-2 from Santa Cruz. After the incubation of primary antibody, PVDF membrane was washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature. Bands were visualized by the chemiluminescence Western blotting detection reagent in imaging system. β-Actin protein levels were used as a control to verify equal protein loading.

Quantitative real-time PCR analysis

Total RNA was isolated with RNA extraction kit (Invitrogen) according to the manufacturer's instructions, as previously described [14]. A total of 1 μg RNA was reverse transcribed as the template for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosytem) according to the manufacturer's instructions. Quantitative real-time PCR was performed for TGFβ, Cas-3 and GAPDH using primers. The primers were purchased ready-to-order from KiCqstart Sigma Aldrich. One hundred nanograms of cDNA was amplified using Sybr Green PCR Master Mix (Applied Biosytem) on the ABI StepOne Plus detection system, programmed for 95°C for 10 min, then 40 cycles of: 95°C for 15 s, 60°C for 1 min. The amplification results were analyzed using StepOne Software v2.3 (Applied Biosystems, Foster City, CA) and the genes of interest were normalized to the corresponding GAPDH results. Data were expressed as fold induction relative to the control.

Statistical analysis

We used the student t-tests or analyses between groups (ANOVA) for statistical analysis as appropriate. Differences were considered significant p-value of 0.05 or less. All data is represented as mean ± SD, unless otherwise indicated. All experiments were repeated in triplicate. Data from representative experiments are shown.

Results

In order to assess the effects of CsA in cancer therapy, PC3 and DU145 cells were chosen according to the nowadays accepted classification of high metastatic potential prostate cancer based on androgen independent prostate cancer cells.

Biphasic growth of prostate cancer cells in response to increasing concentrations of CsA

First, we analyzed the effects of CsA on human prostate cancer PC3 and DU145 cell lines. In Figure 1A, we show the proliferation of these cells was inhibited by CsA with IC50 values of 8.80 μM in PC3 cells and 12.14 μM in DU145 cells. Cells were treated with CsA (0–250 μM) or control for 36 h. Low concentrations of CsA were able to decrease significant the rate of cell survival in both cell lines after 36 h of exposure, as shown in dose course experiments, as detected by MTT assay (Figure 1B). We next examined whether the cell growing is sustained in different time incubations, which is viewed by 10 μM CsA treatment among PC3 and DU145 prostate cancer cells. The cell growing

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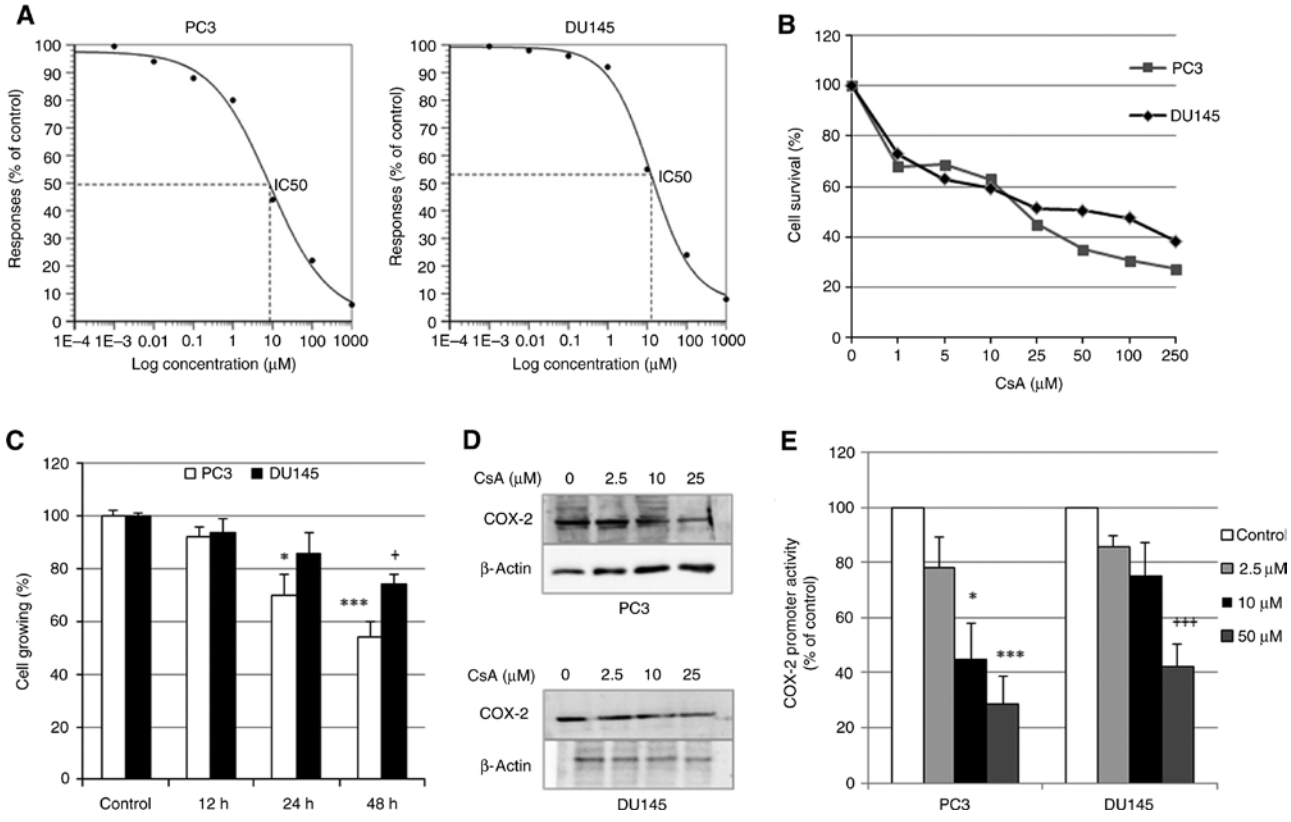


Figure 1: Effects of CsA cell survival in prostate cancer cells.
(A) Proliferation curve generated for PC3 (IC50 = 8.80 μM) and DU145 cells IC50 = 12.14 μM. (B) Cell survival measurement with MTT assay after CsA treatment (0–250 μM) of PC3 and DU145 cell lines for 36 h. (C) Percentage of cell growing in PC3 and DU145 cells with treated of CsA (10 μM) for 12 h, 24 h, 48 h. *$p < 0.05$, ***$p < 0.001$ compare to control in PC3 cells; ++ $p < 0.05$, compare to control in DU145 cells. (D) COX-2 protein expression by Western blot analysis of treated with CsA in PC3 and DU145 cells. (E) COX-2 promoter activity in CsA treated PC3 and DU145 cells (*$p < 0.05$, ***$p < 0.001$ compare to control in PC3 cells; +++$p < 0.001$ compare to control in DU145 cells). Reporter firefly luciferase activities were normalized with internal control Renilla luciferase.

was evaluated 12 h, 24 h and 48 h after adding with CsA (10 μM). As can be seen in Figure 1C, the inhibition of PC3 cells growing at 24 h is more than DU145 cells growing in same time at 10 μM. Furthermore, the cell growing of both cell lines was decreased in response to the treatment of 10 μM CsA for 48 h.

CsA suppress COX-2 protein expression and COX-2 promoter activity in PC3 and DU145 cells

In order to find out how CsA influenced COX-2 protein expression, we selected PC3 and DU145 prostate cancer cells for the following research. We firstly observed the COX-2 protein expression levels of these cells after treatment with CsA (between 2.5 μM and 25 μM concentration) for 36 h using Western blotting. There was remarkable downregulate COX-2 protein expression change of PC3 and DU145 prostate cancer cells treated with the CsA at the high dose. (Figure 1D, representative bands more than two fold). Next, we investigated COX-2 promoter activity after treatment of CsA, and tested whether any differences PC3 and DU145 cells. COX-2 promoter activities with treatment of CsA in PC3 were 78.00, 44.67 and 28.53%, respectively. Similarly, COX-2 promoter activities with treatment of CsA in DU145 were 85.90, 75.07, 42.17%, respectively (Figure 1E).

Colony forming ability of PC3 and DU145 cells with CsA treatment

Firstly, we examined that the dose-response effects of CsA treatment on the migration of PC3 and DU145 cells using qualitative wound-healing assay for metastatic potential. As shown Figure 2A, when we exposed PC3 and DU145 cells to different concentrations 2.5 μM and 10 μM

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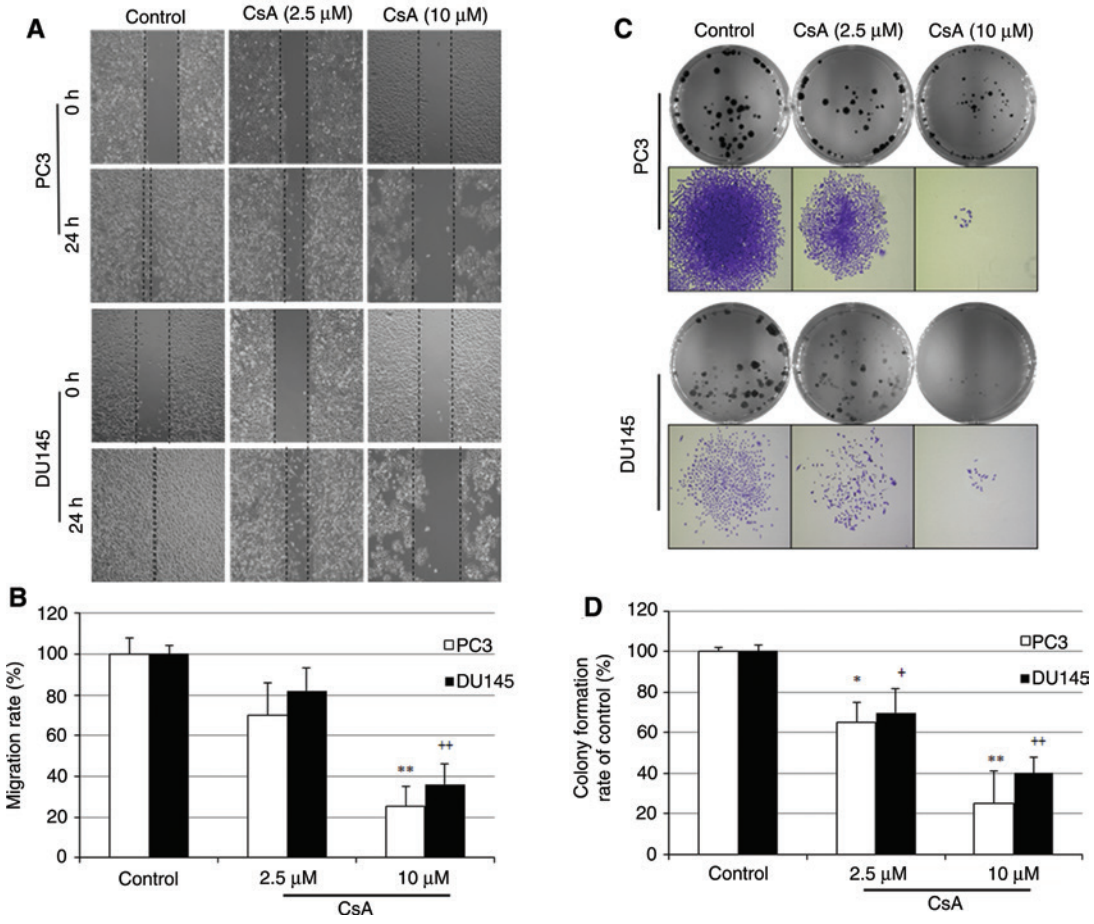


Figure 2: Effects of CsA on cell migration in prostate cancer cells.
(A) Cell migration measurement with scratch wound assay in CsA treated PC3 and DU145 cells. (B) The rate of wound closure was calculated differences of cells filling the scratched area (**$p < 0.01$ compare to control in PC3 cells; ++ $p < 0.01$ compare to control in DU145 cells). (C) Colony formation assay in PC3 and DU145 cells. Cells were treated with 2.5 μM and 10 μM CsA in PC3 and DU145 cells at six well plates and were cultured for 14 days and stained with crystal violet. Colonies shown as overview images (up) and detailed images individual colony (down). (D) Colony formation ratio was quantitated compared to control groups (*$p < 0.05$, **$p < 0.01$ compare to control in PC3 cells; +$p < 0.05$, ++$p < 0.01$ compare to control in DU145 cells).

of CsA markedly reduced in cell motility in the scratch area compared to control after 24 h of scratch formation. In Figure 2B, CsA showed 70–80%, and 25–33% inhibition of tumor cell motility through wound healing in PC3 and DU145 cells. At the same time, The ability of PC3 and DU145 cells to generate clones and self-renew was evaluated in a serum-starved culture with treated of CsA. Similar to migration assay, treatment of cells with CsA (2.5 μM and 10 μM) resulted in a significant reduction in the number of colonies (65%, 25% in PC3 cells and 70%, 40% in DU145 cells), respectively in Figure 2C–D. Moreover, we observed that the colony formation in PC3 was more effective than DU145 cells in sphere-forming, and the most effective dose was 10 μM CsA. These data suggest that CsA diminish the metastasis effect of prostate cancer cells and highly effective in inhibiting migration.

TGFβ, Cas-3 protein and mRNA expressions are upregulated with CsA in prostate cancer cell lines

To detect whether CsA induced apoptosis of PC3 and DU145 cells, we performed qRT-PCR experiment for gene expressions level of TGFβ and Cas-3. The data suggested that CsA could notably increase the gene expression of TGFβ and Cas-3 in PC3 and DU145 prostate cancer cells (Figure 3A, B). We further detected the protein expression level of TGFβ and Cas-3 for researching the effect of CsA (2.5 μM- 25 μM) in PC3 and DU145 prostate cancer cells. In agreement with the Western blot bands (Figure 3C Supplementary Figure S1), 10 and 25 μM CsA treatment was high protein expression for TGFβ in PC3 and DU145 cells compared to control. As a major member of caspase family,

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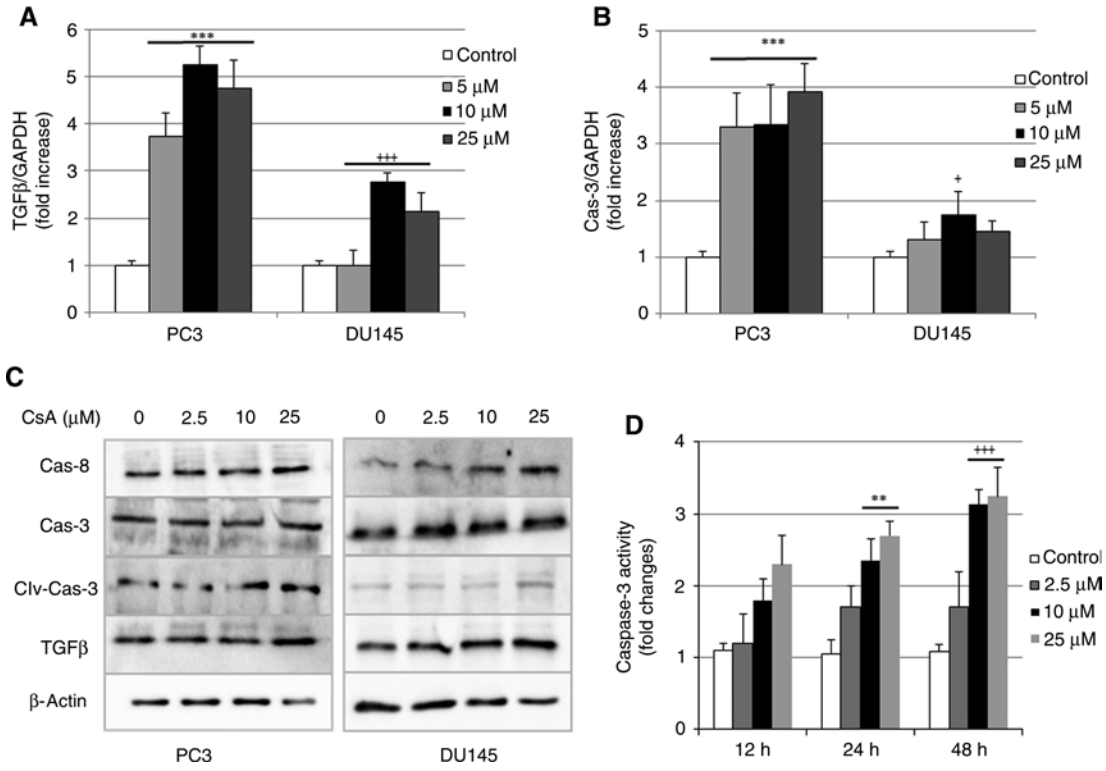


Figure 3: Effects of CsA on apoptotic proteins in prostate cancer cells.
(A–B) Effects of dose dependent CsA on TGFβ and Cas-3 gene expression in PC3 and DU145 cells (***$p < 0.001$ compare to control in PC3 cells; +$p < 0.05$, +++$p < 0.001$ compare to control in DU145 cells). (C) Western blot analysis of expression levels of Cas-3, cleaved Cas-3, caspase-8 (Cas-8) and TGFβ proteins in dose dependent CsA (0, 2.5, 10, 25 μM) treatment at 36 h in PC3 and DU145 cells. Each protein band was normalized to the intensity of β-actin used. (D) Dose and time dependent effects of CsA on the Cas-3 activity in PC3 and DU145 cells. (**$p < 0.01$ compare to control in PC3 cells; +++$p < 0.001$ compare to control in DU145 cells).

Cas-3 activity plays a critical role in apoptosis pathway. Moreover, upregulation of cleaved-Cas-3 is one of the main characteristics of cell death. The results showed that the proetin expression level of Cas-3, and clv-Cas-3 upregulated after treatment with CsA in PC3 and DU145 cells. To verify this hypothesis, we performed Cas-3 activity of time (12–48 h) and dose (2.5 μM–25 μM) dependent with CsA treatment in PC3 and DU145 cells. It was found that dose dependent CsA treatment was increased Cas-3 activity more than two fold after 24 h and 48 h (Figure 3D). On the other hand, we investigated if CsA could induce extrinsic cell death in PC3 and DU145 cells by performing Western blot analysis for Cas-8. The results demonstrated that CsA induced protein expression of Cas-8 in PC3 and DU145 prostate cancer cells (Figure 3C and Supplementary Figure S1). The data demonstrated that CsA treatment contributed to the activation of extrinsic cell death. At the same time, the level of Akt is elevated, whereas that of Akt is increased; significantly resulted activation of Akt to its phosphorylated form concentration-dependent CsA treatment (Supplementary Figure S2).

Discussion

To date, COX-2 plays a major role in enhancing cell migration and metastasis of several cancers through inhibition of apoptosis and multiple signaling pathway proteins. For instance, the association of direct and/or indirect interactions of TGFβ and COX-2 are more complicate and interact with more cellular proteins. It has been supported that TGFβ induces COX-2 expression in mammary epithelial cells [15]. However it was demonstrated that TGFβ reduces overexpression of COX-2 via production of prostaglandins as PGE2 in human adenocarcinoma cells [16]. Therefore, we were selected specific COX-2 inhibitor as CsA and interested to know the cellular response to elevated COX-2 expression. This observation is consistent with our study which indicated that dose dependent manager of CsA, such a specific COX-2 inhibitor, inhibits COX-2 expression and activates TGFβ in prostate cancer cells. We found that COX-2 and TGFβ are opposite interaction molecules, when one is reduced other is enhanced. Meanwhile, TGF-β1 can regulate diversity of cellular process with the inclusion of

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suppressing cell growth, invasion and migration, differentiation and apoptosis. TGFβ induce to apoptotic cell death with different modulator as mitogen activated kinases, Akt (serine-threonine kinase B), NF-κB and death receptors [17]. Our experiments on prostate cancer cells with CsA treatment activated TGFβ gene and protein expression. However, we found that CsA accumulated apoptotic cell death via cas-3 and cas-8. Conversely, TGFβ might inhibit Cas-3 activation for protection of neurons against apoptosis [18]. But, it is well known that TGFβ induce caspase dependent cell death or poly ADP ribose polymerase cleavage for DNA fragmentation [19, 20]. Previous studies have demonstrated that CsA increase TGFβ production in MDCK kidney cells [21], lymphocytes [22], and mesangial cells [23]. Recently, TGFβ is a therapeutics target of prostate cancer: decreasing immune surveillance, suppressing the migration and invasion, leading to obstruction of cell proliferation, progression of cell cycle, induction of apoptotic proteins [24]. In this study, we firstly found that treatment with CsA resulted in reduced migration of human prostate cancer cells via activation of TGFβ and Cas-3. Interestingly, in our results obtained by Western blotting showed that CsA enhanced the upregulation of Akt and activation of Akt phosphorylation in prostate cancer cells. A similar result demonstrate that CsA treatment may play an important role of Akt activation on keratinocytes for preventing and treating skin cancer [25]. Akt protein expression were chosen based on a previous study, in which 20 μM and 12 h incubation with CsA induced significant Akt and phosphorylated Akt in PC3 prostate cancer cells. It has been showed that, CsA concurrently activates the EGFR/PI3K/Akt, Akt inhibitor increased the growth suppressive activity of CsA [26]. In addition, Akt is a known protein kinase B, associated upstream of cell death via caspase dependent [27] which is critical target for molecular therapeutics of cancer [28, 29]. Moreover Akt is most popular target for anticancer drug discovery that modulate tumor formation with cancer cell processes such as apoptotic targeted theraphy and dominate to tumor migration [30, 31]. These data argued that CsA-stimulated Akt activation induce cleave of caspase through which Akt autophosphorylation regulated cell survival and suppresses cell migration and colony formation in prostate cancer cells.

Collectively, these findings here indicate that effects of CsA on the cell death are associated with accumulation of Cas-3, Cas-8, TGFβ, Akt and suppression of COX-2 in prostate cancer cells.

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Conflict of Interest

Authors have no conflict of interest regarding this study.

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