Inhibition of Wnt/β-catenin signaling mediates ursolic acid-induced apoptosis in PC-3 prostate cancer cells

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Abstract:
Background: Ursolic acid, a pentacyclic triterpenoid, is known to exert antitumor activity in breast, lung, liver and colon cancers. Nonetheless, the underlying mechanism of ursolic acid in prostate cancer cells still remains unclear. To investigate the antitumor mechanism, the apoptotic mechanism of ursolic acid via Wnt/β-catenin signaling was examined in PC-3 prostate cancer cells.

Methods: Cytotoxicity assay, flow cytometry, immunofluorescence assay and western blotting were performed.

Results: Ursolic acid showed cytotoxicity against PC-3, LNCaP and DU145 prostate cancer cells with IC50 of 35 µM, 47 µM and 80 µM, respectively. Also, ursolic acid significantly increased the number of ethidium homodimer stained cells and apoptotic bodies, and dose-dependently enhanced the sub-G1 apoptotic accumulation in PC-3 cells. Consistently, western blotting revealed that ursolic acid effectively cleaved poly (ADP-ribose) polymerase (PARP), activated caspase-9 and -3, suppressed the expression of survival proteins such as Bcl-XL, Bcl-2 and Mcl-1, and upregulated the expression of Bax in PC-3 cells. Interestingly, ursolic acid suppressed the expression of Wnt5α/β and β-catenin, and enhanced the phosphorylation of glycogen synthase kinase 3 β (GSK3β). Furthermore, the GSK3β inhibitor SB216763 or Wnt3a-conditioned medium (Wnt3a-CM) reversed the cleavages of caspase-3 and PARP induced by ursolic acid in PC-3 cells.

Conclusions: Our findings suggest that ursolic acid induces apoptosis via inhibition of the Wnt5/β-catenin pathway and activation of caspase in PC-3 prostate cancer cells. These results support scientific evidence that medicinal plants containing ursolic acid can be applied to cancer prevention and treatment as a complement and alternative medicine (CAM) agent.

Key words: ursolic acid, apoptosis, caspase, Wnt/GSK3β/β-catenin signaling

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Introduction

Prostate cancer is internationally the second most common cancer diagnosed in men and the sixth most common cause of cancer death among men. According to the NCI report, the incidence rate of prostate cancer is approximately 16.22% in men born today [2, 6]. Recent clinical practice shows that medicinal herbs with antitumor activities have chemopreventive potential with few side effects [4, 35].

Ursolic acid as a pentacyclic triterpene acid is mainly contained in several fruits such as hawthorn, prunes, peppermint, apples, rosemary, lavender, and also medicinal plants including Rosmarinus officinalis, Oldenlandia diffusa, Eriobotrya japonica, and Glechoma hederacea [31]. As a whole, ursolic acid has been suggested as a non-toxic chemopreventive/chemoprotective agent in clinical practice [20], even though there are some evidences that ursolic acid causes the DNA damages in human endothelial cells [18] and reduces the sperm motility [3].

Previous evidence shows that ursolic acid induces apoptosis in several cancers such as colon cancer [1], breast cancer [11], leukemia [15], melanoma [12] and prostate cancer [24]. Several papers studied the effect of ursolic acid in vivo models. Ursolic acid significantly inhibited tumor growth in transgenic mouse prostate model [24] and prostate cancer xenograft in nude mice [23]. Although ursolic acid exerts antitumor activity via inhibition of CXCR4/CXCL12 [23], matrix metalloproteinase 2 (MMP2) [12], P2Y2/Src/p38/COX-2 [13] and the AKT pathway [37] as well as autophagy [25], the antitumor mechanism of ursolic acid still remains unclear in prostate cancers. Thus, in the present study, the role of Wnt/β-catenin signaling was elucidated in ursolic acid-induced apoptosis in PC-3 prostate cancer cells using XTT assay, cell cycle analysis, and western blotting.

Materials and Methods

Cell culture

PC-3, DU145, LNCaP prostate cancer cells, Raw 264.7 (leukemic monocyte macrophage cells) and HEK293 (human embryonic kidney 293) cells were obtained from American Type Culture Collection (ATCC). HEK293, PC-3 and DU145 cells were main-tained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO₂. LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 µmol/l L-glutamine, 10 µmol/l HEPES, 1 µmol/l sodium pyruvate, and 4.5% D-glucose without antibiotics at 37°C in a humidified atmosphere containing 5% CO₂. Wnt3a CM was prepared as previously described [21].

Cytotoxicity assay

To investigate the cytotoxicity, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) bromide colorimetric assay was used. Prostate cancer cells were plated at 10⁴ cells in 96-well plates and incubated overnight. Cells were treated with various concentrations of ursolic acid. After 24-h incubation, 100 ml of MTT (Sigma Chemical Co., St. Louis, MO, USA) was added to each well and incubated for 4 h at 37°C. Formazan crystals were dissolved by addition of 100 ml DMSO solution. The microplate reader at 570 nM was used to determine the absorbance of each well.

Ethidium homodimer assay

To measure cell death, we used the DAPI and ethidium homodimer dye following the manufacturers’ instructions (Molecular Probes). In brief, PC-3 cells were treated with 30 µM ursolic acid for 24 h. After incubation, cells were fixed in 4% methanol-free formaldehyde solution and stained with the 5 µM ethidium homodimer and then incubated at 37°C for 30 min in the dark. Then, the cells were mounted with mounting medium containing DAPI and visualized under an Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc., Weimar, Germany).

Cell cycle analysis

PC3 cells treated with ursolic acid or GSK3β inhibitor SB216763 (10 µM) for 24 h were fixed in 75% ethanol at −20°C, resuspended in PBS containing RNase A (1 mg/ml), and incubated for 1 h at 37°C. The fixed cells were stained with propidium iodide (50 µg/ml) for 30 min at room temperature. The DNA contents were analyzed using CellQuest Software with the FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).
Western blotting

Whole cell lysates from the prostate cancer cells exposed to ursolic acid or SB216763 (10 µM) or Wnt3a-CM for 24 h were prepared using lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaN3 and protease inhibitor cocktail). The protein contents in the supernatants were measured by using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, USA), separated on 4–12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and electro-transferred onto a Hybond ECL transfer membrane (GE Health Are Bio-Science, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat dry milk and immunoblotted with anti-cleaved caspase-3, caspase-3, cleaved caspase-9, caspase-9, PARP, Bax, Bcl-2, Bcl-xL, Mcl-1, Wnt3C/β, p-GSK3β, GSK3β or β-catenin (Cell Signaling, Danvers, MA, USA) antibodies.

Immunofluorescence assay

PC 3 cells in the absence or presence of ursolic acid were fixed with 4% paraformaldehyde and permeabilized in cold methanol for 15 min at −20°C. Fixed cells were then washed twice with 1 × PBS, followed by blocking with 10% normal goat serum blocking solution (Zymed Laboratories, Carlsbad, CA, USA) for 30 min. Cells were incubated with the primary antibodies against β-catenin (Cell Signaling, Danvers, MA, USA) for overnight at 4°C. The cells were washed three times in 1 × PBS and incubated with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Carlsbad CA, USA) for 45 min at room temperature in a humidified chamber. Cells were washed in 1 × PBS, mounted with Vectashield/DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by a Carl Zeiss LSM5 confocal microscope.

Statistical analysis

Statistical analysis of the data was conducted using Sigmaplot version 12 software (Systat Software Inc., San Jose, CA, USA). All data were expressed as the means ± standard deviation (SD). The statistically significant differences between control and ursolic acid-treated cells were calculated by the Student’s t-test.

Results

Cytotoxic effects of ursolic acid on PC-3, DU145 and LNCaP prostate cancer cells

The cytotoxicity of ursolic acid (Fig. 1A) was evaluated against PC-3, DU145 and LNCaP prostate cancer cells using MTT assay. Cells were treated with various concentrations of ursolic acid (0, 5, 10, 20, 40 or 80 µM) for 24 h. As shown in Figure 1B, ursolic acid exerted cytotoxicity in PC-3, DU145 more than in LNCaP cells. In contrast, ursolic acid did not show significant cytotoxicity against Raw 264.7 leukemic monocyte macrophage cells, while only high concentration of ursolic acid (80 µM) affected the viability of the cells.

Fig. 1. Cytotoxic effect of ursolic acid in prostate cancer cells. (A) Chemical structure of ursolic acid (MW = 456). (B) Human prostate cancer cell lines PC-3, DU145, LNCaP or leukemic monocyte macrophage cell line Raw 264.7 were treated with various concentrations of ursolic acid (0, 5, 10, 20, 40 or 80 µM) for 24 h. Cell viability was determined by XTT assay. The data represent the means ± SD. (C) Morphological feature of PC-3 cells were observed in inverted microscope (100×). Cells were treated with ursolic acid (0, 7.5, 15 or 30 µM) for 24 h. (D) PC-3 cells were treated with 30 µM of ursolic acid and then stained with etidium bromide homodimer dye. Signals were visualized under an Axios vision 4.0 fluorescence microscope.
Apoptotic bodies were also observed in ursolic acid-treated PC-3 cells under inverted microscope, indicating apoptotic feature of ursolic acid, while intact morphology was shown in untreated control (Fig. 1C). Similarly, cell death was confirmed in ursolic acid treated PC-3 cells by ethidium homodimer assay. As shown in Figure 1D, ethidium homodimer assay revealed apoptotic features in ursolic acid treated PC-3 cells compared to untreated control.

Ursolic acid increased the sub-G1 apoptotic portion in PC-3 cells

To confirm whether the cytotoxicity of ursolic acid against PC-3 cells was due to apoptosis induction, cell cycle analysis was carried out. As shown in Figure 2, ursolic acid increased sub-G1 apoptotic portion by \(1.30 \pm 0.02\), \(5.95 \pm 1.43\), \(24.33 \pm 0.16\), and \(49.93 \pm 4.09\)% at the concentrations of 7.5, 15, 30 and 60 µM, respectively, compared to untreated control (1.45 ± 0.23%).
Ursolic acid activated caspase cascades and regulated the Bcl-2 family proteins in PC-3 cells

Generally, apoptosis is induced through two distinctive pathways such as cell death extrinsic pathway and mitochondrial dependent intrinsic pathway [8]. Western blotting showed that ursolic acid activated caspase-9 and -3 and cleaved PARP in PC-3 cells as shown in Figure 3A, implying that ursolic acid induces apoptosis via mitochondrial dependent pathway in PC-3 cells.

Anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL are frequently overexpressed in cancers [22]. In the present study, western blotting revealed that ursolic acid suppressed the expression of Bcl-X<sub>L</sub>, Bcl 2, and Mcl-1<sub>L</sub> as anti-apoptotic genes and also up-regulated the expression of Bax in PC-3 cells as shown in Figure 3B.

To further compare the apoptotic activity of ursolic acid in other prostate cancer cells, the effect of ursolic acid on apoptosis related proteins such as PARP, caspase 3, Bcl-xL, Bcl-2 in DU145 and LNCaP cells was examined by western blotting. Here, ursolic acid activated PARP and Bax as well as suppressed the expression of Bcl-<sub>XL</sub> and Bcl-2 in DU145 cells, while ursolic acid cleaved PARP and suppressed the expression of Bcl-<sub>XL</sub> in LNCaP cells (Figs. 3C and D).

Glycogen synthase kinase 3β (GSK3β), a regulator of glycogen metabolism is involved in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility and apoptosis [9]. GSK3β plays a critical role in Wnt/β-catenin signaling pathway [34]. Here, ursolic acid suppressed the expression of Wnt5<sub>α</sub>/β and β-catenin, and enhanced the phosphorylation of GSK3β at Ser 9 in PC-3 cells (Fig. 4A). GSK3β selective inhibitor SB216763 blocked the decreased expression of Wnt5<sub>α</sub>/β and β-catenin, and the activated phosphorylation of GSK3β at Ser 9 induced by ursolic acid (Fig. 4B). Likewise, GSK3 inhibitor SB216763 prevented the cleavages of caspase-3 and PARP induced by ursolic acid in PC-3 cells (Fig. 4C), implying that ursolic acid-induced apoptosis is controlled through the Wnt5/GSK3β/β-catenin signaling. Wnt (Wnt1, Wnt3a, and Wnt8) signalings promote the dissociation of β-catenin from Axin and inhibit β-catenin phosphorylation and subsequently β-catenin degradation [14, 16]. As shown in Figure 4D, ursolic treatment induced the degradation of β-catenin (Fig. 4D lower panel), while β-catenin exhibited sub-
cellular localization in PC-3 cells (Fig. 4D upper panel). HEK 293 cells treated with 20 µM of ursolic acid in the presence of Wnt3a-conditioned medium blocked the apoptotic signaling such as PARP and caspase 3 (Fig. 4E). Also, ursolic acid attenuated the expression of Wnt5a and β-catenin in DU145 and LNCaP cells (Figs. 4 F and G).

Several studies reported that Wnt3a activates β-catenin dependent canonical Wnt signaling and inhibits the proliferation [19, 26]. Thus, we investigated whether or not Wnt3a-CM affected the apoptosis induced by ursolic acid. HEK 293 cells were treated with ursolic acid in the absence and presence of Wnt3a-CM. As shown in Figure 4E, Wnt3-CM treatment reversed the decreased β-catenin and increased cleavages of PARP and caspase 3 induced by ursolic acid in HEK 293 cells, implying that Wnt signaling mediates the apoptosis induced by ursolic acid in HEK 293 cells.
GSK3β inhibitor blocked the sub G-1 population in ursolic acid treated PC-3 cells

To determine whether ursolic acid induces apoptosis via GSK3β signaling, we used a GSK3β inhibitor. The cells treated by ursolic acid were cultured in the absence or presence of GSK3β inhibitor and then flow cytometry analysis was performed. As shown in Figure 5, GSK3β inhibitor significantly blocked the sub G-1 population to 11.18 ± 0.61% compared to ursolic acid treated cells (23.31 ± 0.13%), indicating that UA induced the apoptosis through GSK3-β activation.

Discussion

Apoptosis, also known as programmed cell death, is one of cell death type distinct from necrosis. More recently, apoptosis induction has been regarded as the major therapeutic target for cancer chemotherapy [5, 32]. In the current study, ursolic acid showed more significant cytotoxic effects in PC-3 cells than DU145 or LNCaP prostate cancer cells. Thus, we performed subsequent experiments with PC-3 cells. Here we found that the cytotoxic effect of ursolic acid was induced by apoptosis, not necrosis, by observing apoptotic bodies in ursolic acid-treated PC-3 cells. Consistently, ursolic acid significantly increased cell death biomarker ethidium homodimer stained cells and the sub-G1 apoptotic portion in PC-3 cells, implying the apoptotic activity of ursolic acid. Western blotting showed that ursolic acid effectively induced PARP cleavages and also activated caspase-9 and -3 in PC-3 cells. Furthermore, ursolic acid suppressed the expression of survival genes such as Bcl-XL, Bcl-2 and Mcl-1, in PC-3 cells, indicating that the regulation of anti-survival genes mediates ursolic acid-induced apoptosis in PC-3 cells.
The Wnt/β-catenin pathway is involved in regulating cell proliferation and cell differentiation of neural stem/progenitor cells [30]. Also, there are accumulating evidences that GSK3β is closely associated with β-catenin signaling [27, 36]. The axin/GSK3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signaling molecule [10]. GSK3β is involved in a wide range of cellular processes including differentiation, growth, motility and apoptosis. Thus, the aberrant regulation of GSK3β has been implicated in various diseases including Alzheimer’s disease, non-insulin-dependent diabetes mellitus and cancer [7, 17]. Since the balance of inhibitory phospho-serine9 (pSer9; inactive form) and stimulatory phospho-tyrosine216 (pY216; active form) of GSK3β is considered a hallmark of its transient kinase regulation in normal CD34+ cells and several human cell types, previous studies demonstrated that GSK3β phosphorylation at serine enhances apoptosis in breast cancer [29], medulloblastoma [33] and SK-OV-3 cells [28]. Here, ursolic acid suppressed the expression of Wnt5α/β and β-catenin, and induced the phosphorylation of GSK3β at Ser 9, which is an indicator of GSK3β deactivation in PC-3 cells. These data imply that ursolic acid-induced apoptosis can be mediated by Wnt5α/β suppression, β-catenin degradation and GSK3β inactivation in PC-3 cells. Consistently, GSK3β inhibitor SB216763 or Wnt3a-CM blocked the cleavages of caspase-3 and PARP in ursolic acid-treated PC-3 cells, further confirming the involvement of GSK3β phosphorylation in ursolic acid-induced apoptosis in PC-3 cells. These results support scientific evidence that medicinal plants such as Oldenlandia diffusa, Eriobotrya japonica, Rosmarinus officinalis and Glechoma hederacea containing ursolic acid as a leading compound can be applied to cancer prevention and treatment as a complementary and alternative medicine (CAM) agent.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgment: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2012-0005755).

References:


