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Ursolic Acid Overcomes Bcl-2-Mediated Resistance to Apoptosis in Prostate Cancer Cells Involving Activation of JNK-Induced Bcl-2 Phosphorylation and Degradation

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ABSTRACT

Androgen-independent prostate cancers express high levels of Bcl-2, and this over-expression of Bcl-2 protects prostate cancer cells from undergoing apoptosis. Ursolic acid (UA) has demonstrated an anti-proliferative effect in various tumor types. The aim of this study is to evaluate the difference between UA-induced apoptosis in androgen-dependent prostate cancer cell line LNCaP cells and androgen-independent prostate cancer cell line LNCaP-AI cells and to reveal the molecular mechanisms underlying the apoptosis. We found that UA treatment in vitro can effectively induce apoptosis in LNCaP and LNCaP-AI cells. UA can overcome Bcl-2-mediated resistance to apoptosis in LNCaP-AI cells. Intrinsic apoptotic pathways can be triggered by UA treatment because c-Jun N-terminal kinase (JNK) is activated and subsequently provokes Bcl-2 phosphorylation and degradation, inducing activation of caspase-9. Although further evaluation is clearly needed, the present results suggest the potential utility of UA as a novel therapeutic agent in advanced prostate cancer. J. Cell. Biochem. 109: 764–773, 2010.

KEY WORDS: URSOLIC ACID; c-JUN N-TERMINAL KINASE; APOPTOSIS; Bcl-2; PROSTATE CARCINOMA

Prostate cancer is the most commonly diagnosed malignancy in older men in the Western world [Jemal et al., 2008]. In the initial stages, prostate cancer cells depend on androgens for growth, and therefore androgen ablation therapy is the standard therapy for the treatment of prostate cancer. However, androgen-independent disease will ultimately prevail in most patients. The progression to androgen-independence marks the most lethal phenotypes of prostate cancer [Gittes, 1991]. There are therapies available to treat hormone-insensitive prostate cancer such as taxane-based chemotherapy. Treatment with docetaxel, one of the taxanes, can prolong life in patients with hormone-refractory prostate cancer (HRPC). Currently docetaxel chemotherapy is the standard treatment for patients with metastatic HRPC. However, a novel therapeutic approach is required because of limited survival benefits afforded by docetaxel chemotherapy [Calabrò and Sternberg, 2007].

Bcl-2, the prototypic member of the Bcl-2 family of proteins, can protect cells from death induced by many stimuli [Martin et al., 1995]. It has been previously reported that cells from androgen-independent prostate cancers express a high level of Bcl-2 and over-expression of Bcl-2 protects prostate cancer cells from undergoing apoptosis upon androgen depletion in vivo [McDonnell et al., 1992; Raffo et al., 1995]. It has also been demonstrated that increased expression of the Bcl-2 gene upon hormone deprivation is involved in the development of androgen-independent growth in prostate

Abbreviations used: HRPC, hormone refractory prostate cancer; UA, ursolic acid; LNCaP-AI, androgen-independent LNCaP; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated protein kinases; p38, p38 MAP kinases; MTT, methyl thiazolyl tetrazolium; FBS, fetal bovine serum; CS-FBS, charcoal-stripped FBS; DMSO, dimethylsulfoxide; PI, propidium iodide; PBS, phosphate buffer solution; DEVD, Asp-Glu-Val-Asp; AFC, 7-amino-4-trifluoromethyl coumarin; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; RT-PCR, reverse transcription polymerase chain reaction; ANOVA, one-way analysis of variance; PS, phosphatidylserine; PBOX-6, 7-[{dimethylcarbamoyl}oxy]-6-(2-naphthyl)pyrrolo-[2,1-*d*] (1,5)-benzoxazepine.

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carcinoma cells in vitro [Raffo et al., 1995]. Bcl-2 over-expression may protect prostate cancer cells from many different apoptotic stimuli, including hormone ablation, radiotherapy, and chemotherapy. Therefore, targeting this survival pathway constitutes a rational approach for the treatment of prostate cancer [Raffo et al., 1995; Yoshino et al., 2006; An et al., 2007].

Ursolic acid (UA), a pentacyclic triterpenoid compound, has been identified in various plants [Liu, 2005]. It has been shown to induce growth arrest and apoptosis in various tumors [Es-saady et al., 1996; Hsu et al., 2004; Aggarwal and Shishodia, 2006]. UA induces apoptosis through multiple pathways, such as inhibition of DNA replication [Choi et al., 2000a], induction of Ca^{2+} release [Baek et al., 1997], caspase activation [Choi et al., 2000a], down-regulation of anti-apoptotic genes [Kassi et al., 2007], as well as inhibition of protein tyrosine kinase [Choi et al., 2000b], and inhibition of NF-kB activity [Hsu et al., 2004]. Kassi et al. [2007] reported UA to induce apoptosis in androgen-sensitive LNCaP cells and in hormone-refractory PC-3 prostate cancer cells associated with a down-regulation of Bcl-2 protein. They found the IC50 for UA to be approximately 32.6 μM for PC-3 cells and 15.7 μM for LNCaP cells. Hormone-refractory prostate cancer cells seemed to be more refractory to UA-induced apoptosis than androgen-sensitive ones. We have generated an androgen-independent LNCaP (LNCaP-AI) prostatic carcinoma cell line from androgen-dependent LNCaP cells by in vitro cell culture [Xu et al., 2009], using a previously described protocol [Lu et al., 1999]. The cell model, including LNCaP and LNCaP-AI cells, resembles prostate cancer progression from the androgen-sensitive to the androgen-independent stage [Karan et al., 2001]. The expression of Bcl-2 was higher in LNCaP-AI cells than in LNCaP cells, making LNCaP-AI cells more refractory to apoptotic stimuli [Lu et al., 1999]. Thus, we used this cell model to investigate the difference between UA-induced apoptosis in androgen-dependent and androgen-independent prostate cancer cells. We wanted to evaluate whether UA can overcome over-expression of Bcl-2-mediated resistance to apoptosis in androgen-independent prostate cancer cells. In addition, we also revealed the molecular mechanisms underlying the apoptosis of prostate cancer cells induced by UA, which had not been fully clarified. Given that UA induces prostate cancer cells apoptosis via regulating the Bcl-2 family [Kassi et al., 2007], it is likely that it interacts with regulators of apoptosis such as the c-Jun N-terminal kinase (JNK) pathway [Maundrell et al., 1997; Deng et al., 2001]. JNK is a member of the mitogen-activated protein kinase (MAPK) family, which also includes extracellular signal-regulated protein kinases (ERK1/2) and p38 MAP kinases (p38) [Schaeffer and Weber, 1999]. Under different experimental conditions JNK has been shown to have positive, negative, or no influence on apoptosis [Verheij et al., 1996; Leppä and Bohmann, 1999; Stadheim and Kucera, 2002]. However, recent studies demonstrated the involvement of the JNK signaling pathway in apoptosis through the phosphorylation and subsequent inactivation of Bcl-2, leading to the activation of its intrinsic signaling pathway [Maundrell et al., 1997; Deng et al., 2001; Sinha-Hikim et al., 2007; Lee et al., 2009].

In the present work, we report the anti-proliferative effects of UA on androgen-dependent LNCaP and androgen-independent LNCaP-AI prostate cancer cells to be similar. Furthermore, the signaling cascade following JNK activation and Bcl-2 phosphorylation and degradation plays a pivotal role in UA-induced apoptosis in the two prostate cancer cell lines.

MATERIALS AND METHODS

CELL CULTURE AND METHYL THIAZOLYL TETRAZOLIUM (MTT) ASSAY

Human prostate cancer LNCaP cells, provided by Prof. Klaus Jung (Department of Urology, University Hospital Charité, Humboldt University, Germany), were routinely maintained in RPMI 1640 (Gibco, MD) containing 10% fetal bovine serum (FBS) (Gibco). The LNCaP-AI cell subline was derived from LNCaP cells by Xu et al. [2009] and was routinely maintained in phenol-red free RPMI-1640 (Gibco) medium containing 10% charcoal-stripped FBS (CS-FBS, Biological Industries, M.P. Ashrat, Israel).

Tumor cell growth in the absence of androgens was assessed by the methyl thiazolyl tetrazolium (MTT) colorimetric assay, as described by Lu et al. [1999]. Briefly, after being cultured in phenol-red free RPMI-1640 medium containing 10% CS-FBS for 1 week, LNCaP or LNCaP-AI cells (5×10^3/well) were seeded in 96-well culture plates in phenol-red free RPMI-1640 medium containing 10% CS-FBS. The cells were incubated for 3, 5, 7, and 9 days. At the end of the incubation, 20 μl MTT (5 mg/ml; Sigma–Aldrich) was added for 4 h at 37°C. After incubation, the supernatants were removed carefully and 150 μl dimethylsulfoxide (DMSO; Sigma–Aldrich, MO) was added to each well. After 10 min incubation and vibration, the absorbance was read at a test wavelength of 490 nm.

To evaluate the effect of UA on prostate cancer cells, the MTT colorimetric assay was performed as described above. A stock solution (10^{-2} M) of UA (Sigma–Aldrich) was prepared in sterilized DMSO and stored at 4°C. The 6×10^3 LNCaP or LNCaP-AI cells were plated in 96-well culture plates and allowed to adhere for 12 h. The cells were treated with UA (100, 80, 50, 20, 10, and 1 μM) for 48 h. As a control, DMSO was added to the culture at a concentration of 1:1,000. The number of viable cells was expressed as the ratio of the number of viable cells with UA treatment to that without treatment. All experiments were repeated in triplicate.

ANNEXIN V-FITC/PROPIDIUM IODIDE (PI) DOUBLE STAINING

Morphological changes of apoptotic cells were determined by fluorescence microscopy using an annexin V-FITC apoptosis detection kit (Abcam, MA). Prostate cancer cells (6×10^4/well) were plated in six-well culture plates and incubated for 12 h before treatment. After treatment with 50 μM UA or DMSO (1:1,000) for 48 h, cells were washed twice with phosphate buffer solution (PBS) and stained for 10 min at room temperature with annexin V-FITC/PI. The cells were observed under fluorescence microscope (NIKON, Tokyo, Japan) by an observer blinded to the cell treatment.

ANNEXIN V-FITC/PI FLOW CYTOMETRIC ANALYSIS

Prostate cancer cells (5×10^5/well) were plated in six-well plates and incubated for 12 h before treatment. After treatment with 50 μM UA or DMSO (1:1,000) for 48 h, cells were collected by centrifugation. Cells were suspended in 500 μl annexin V binding buffer, 5 μl annexin V-FITC and 5 μl PI, and incubated at room temperature for

5 min in the dark. Approximately 20,000 cells were analyzed using a FACS flow cytometer (Becton Dickinson, NJ). In some experiments, cells were pretreated for 2 h with the indicated JNK inhibitor SP600125 or p38 inhibitor SB203580 (Merck Biosciences Ltd., Nottingham, UK) before UA treatment (50 μM for 48 h) and assessment of apoptosis. Apoptotic cells were defined as annexin V-FITC positive with or without PI positive, as described by Deeb et al. [2004].

CASPASE ACTIVITY MEASUREMENT

Cellular caspase activity (3, 8, and 9) was detected using caspase-3/CPP32, caspase-8/FLICE, and caspase-9/Mch6 fluorometric assay kits (BioVision, CA). The assays for caspase-3, caspase-8 and caspase-9 activity are based on the cleavage of the following substrate: Asp-Glu-Val-Asp (DEVD)-7-amino-4-trifluoromethyl coumarin (AFC), Ile-Glu-Thr-Asp (IETD)-AFC, and Leu-Glu-His-Asp (LEHD)-AFC, respectively. After treatment, cells were lysed and cell samples were centrifuged at 10,000*g* for 20 min. The protein concentration of the resultant supernatant was measured using the bicinchoninic acid method [Smith et al., 1985]. An aliquot of 50 μg protein from each cell lysate was incubated with 2 μl of fluorescently-labeled caspase substrate (1 mmol/L) at 37°C for 45 min. The release of AFC was measured with a fluorometric plate reader (Molecular Devices, CA) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. In some experiments, cells were pretreated for 2 h with the indicated JNK inhibitor SP600125 before UA treatment (50 μM for 48 h) and assessment of caspase activity. Experiments were performed in triplicate. The fold increase in caspase activity was determined by comparing the release of AFC from the apoptotic sample to that from the control [Alikhani et al., 2003].

QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated using Trizol reagent (TaKaRa, Dalian, China). Once isolated, 1 μg of total RNA was reverse transcribed using superscript (TaKaRa). Intron-spanning primers were either obtained from published literature [Wang et al., 2008] or designed using the Primer3 Output program, and synthesized by TaKaRa as follows: Bcl-2: forward primer 5′-AGTTCGGTGGGGTCATGTGTG-3′, reverse primer 5′-CTTCAGAGACAGCCAGGAGAAATC-3′. GAPDH: forward primer 5′-CGGAGTCAACGGATTTGGTCGTATTGG-3′, reverse primer 5′-GCTCCTGGAAGATGGTGATGGGATTTCC-3′. The primers yielded PCR products of 200 (Bcl-2) and 221 (GAPDH) nucleotides. Quantitative PCRs for indicated genes were also carried out. Using SYBR Green/Fluorescein PCR Master Mix (TaKaRa), 0.5 μl cDNA was amplified using real-time PCR with a BioRad MyiQ thermocycler and SYBR green detection system (BioRad, CA). Samples were run in triplicate to ensure amplification integrity. The standard PCR conditions were: 95°C for 2 min, then 40 cycles at 95°C, 15 s; 60°C, 15 s; and 72°C, 20 s. The melting curve of a product is sequence-specific and can be used to distinguish nonspecific from specific PCR products. Relative gene expression quantifications were calculated according to the comparative cycle threshold (Ct) method using GAPDH as an internal standard, and using the $2^{-\Delta Ct}$ formula [Livak and Schmittgen, 2001].

WESTERN BLOT ANALYSIS

Cell extraction and Western blot were carried out as previously described [Fan et al., 2000]. Cells were lysed in 25 mM HEPES (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.3 M NaCl, 0.2% SDS, 0.5% sodium deoxycholate, protease inhibitors (50 μg/ml leupeptin, 10 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin), and phosphatase inhibitors (20 mM β-glycerophosphate, 1 μM okadaic acid, 50 mM NaF, and 1 mM Na_3VO_4). Equal amounts of protein (30 μg/lane) were separated using SDS-PAGE and transferred to nitrocellulose membranes. Following probing with a specific primary antibody and horseradish peroxidase-conjugated secondary antibody (1:3,000, Santa Cruz Biotechnology, CA), all immunoblots were visualized by chemiluminescence (Thermo Scientific Inc., IL). The following first antibodies were used: anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-JNK, anti-phospho-JNK (Thr183/Tyr185), anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-phospho-c-Jun (Ser63) (Cell Signaling Technology, MA), anti-Bcl-2, anti-phospho-Bcl-2(Ser70) and anti-β-actin (Santa Cruz). β-actin served as a loading control. MAPK activity was assessed as previously described [Gee et al., 2002]. Briefly, after detection of phospho-ERK1/2, phospho-JNK, and phospho-p38, membranes were stripped, blocked, and incubated with anti-ERK1/2, anti-JNK and, anti-p38 antibodies for the detection of total ERK1/2, JNK, and p38 levels. MAPK activity was expressed as the ratio of the phosphorylated MAPK band to that of the total MAPK band.

STATISTICAL ANALYSIS

Statistical analysis was performed using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered significant. Data are expressed as the mean ± SE.

RESULTS

UA INDUCES APOPTOSIS IN LNCAP AND LNCAP-AI CELLS

To show the effect of androgen ablation on LNCaP and LNCaP-AI cell growth, the tumors were cultured in medium containing 10% CS-FBS for different times and the MTT assay was performed. As shown in Figure 1A, androgen ablation inhibits LNCaP cell proliferation. However, LNCaP-AI cell can proliferate in the absence of androgen. The results showed that the LNCaP-AI cells we established grew in an androgen-independent fashion, similar to that reported by Lu et al. [1999].

To examine whether UA has any effect on the number of viable LNCAP and LNCAP-AI cells, the cells were treated with the indicated concentrations of UA for 48 h. The number of viable cells was assessed by MTT assay. Treatment with UA resulted in a dose-dependent reduction in the number of viable cells in the two cell lines (Fig. 1B). As shown in Figure 1B, the anti-proliferative effects of UA on LNCaP and LNCaP-AI prostate cancer cells were similar. Because, treatment of the cells with 50 μM of UA had a significant effect on cell viability, we used this concentration for further analysis.

In order to examine whether the anti-proliferative effect of UA on LNCAP and LNCAP-AI cells involved apoptosis, morphological changes of apoptotic cells were determined by fluorescence

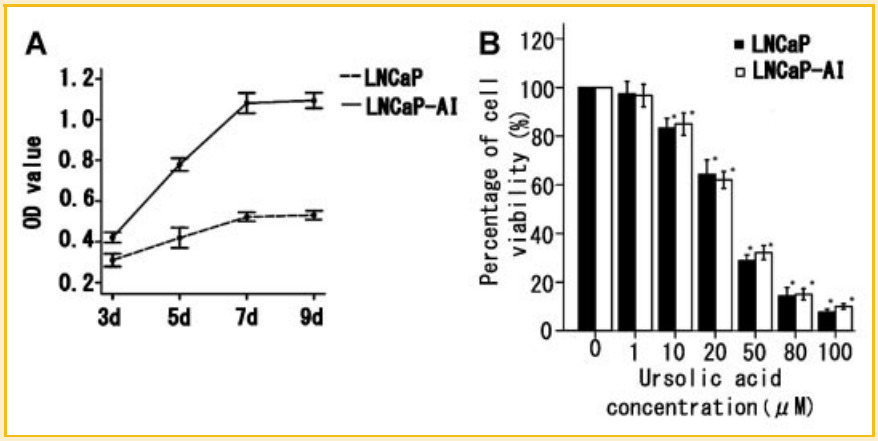

Fig. 1. MTT assay. A: The effect of androgen ablation on LNCaP and LNCaP-AI cell growth was determined by the MTT assay, as described in Materials and Methods Section. B: The effect of UA on proliferation of prostate cancer LNCaP and LNCaP-AI cells was determined by the MTT assay, as described in Materials and Methods Section. Results represent the means from three independent experiments ($^{*}P < 0.05$).

microscopy using annexin V-FITC and PI double staining. The phospholipid-binding protein annexin V has a high affinity for phosphatidylserine (PS), a negatively charged membrane phospholipid located on the inner surface of the plasma membrane of living cells [Fadok et al., 1992]. PS is translocated to the outer side of the plasma membrane and becomes accessible for staining with annexin V in apoptotic cells [Deeb et al., 2004]. The LNCaP and LNCaP-AI cells that are considered viable are annexin V-FITC and PI negative. Apoptotic cells were annexin V-FITC positive, with or without PI staining. Figure 2 shows that UA induced typical morphological changes of apoptosis in the two cell lines.

ACTIVATION OF JNK BY UA

The MAPK family is involved in the regulation of cell proliferation and apoptosis [Chang and Karin, 2001]. We therefore investigated whether UA induced activation of ERK1/2, JNK and, p38 in LNCAP and LNCAP-AI cells. The results showed that 50 μM UA efficiently induced JNK phosphorylation, but not ERK1/2 or p38 phosphorylation (Fig. 3A). The best-studied target of JNK is c-Jun [Mechta-Grigoriou et al., 2001]. JNK phosphorylation of c-Jun at serines 63 and 73 promotes the activation of this transcription factor [Dérijard et al., 1994]. We found that 50 μM of UA treatment for 2 h induced an increase in the phosphorylation of c-Jun (Ser 63) in both prostate

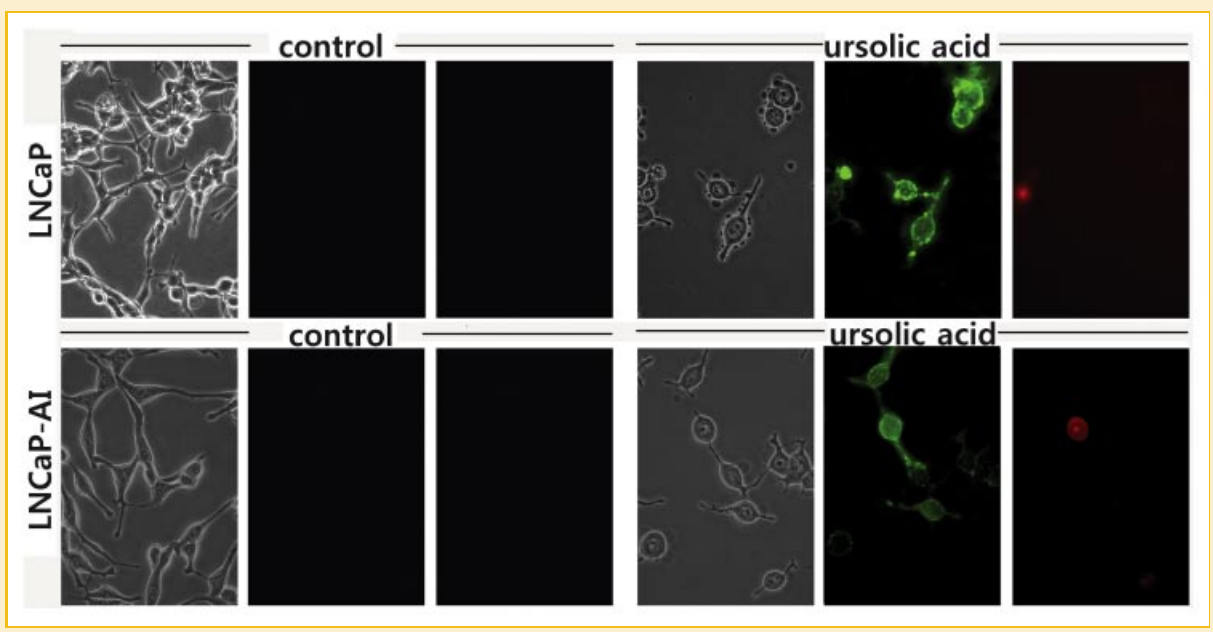

Fig. 2. UA treatment induced typical apoptotic morphological changes in prostate cancer LNCaP and LNCaP-AI cells. Annexin V-FITC and PI double stained cells were examined by fluorescence microscopy, as described in Materials and Methods Section. Microscopic evaluation of the cells showed that most of the control cells were negative for both annexin V and PI, indicating live cells with cellular integrity. Apoptotic cells, identified as those which were annexin V positive, appeared green in color with or without PI positive staining (red color) (400×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

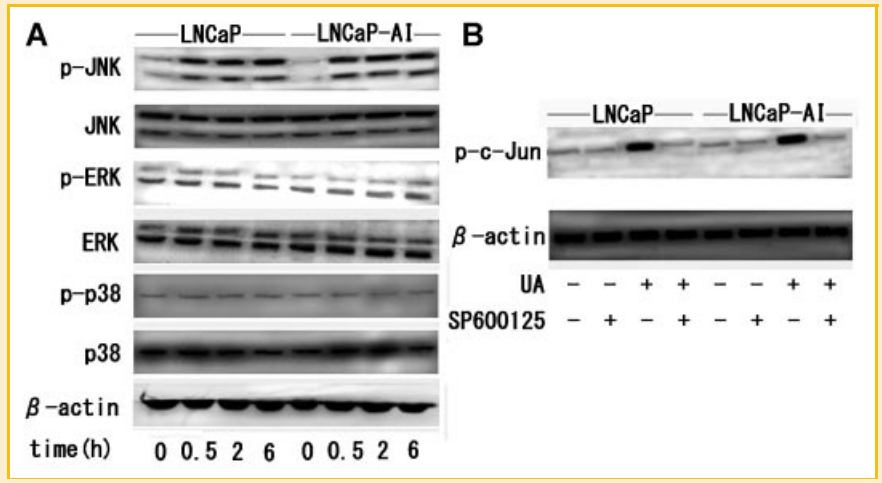

Fig. 3. The effect of UA on MAPKs signaling pathways. A: The effect of UA on the activity of ERK1/2, JNK, and p38. Western blots were carried out using antibodies against phospho-ERK1/2, total ERK1/2, phosphor-JNK, total JNK, phosphor-p38, and total p38, as described in Materials and Methods Section. MAPK activity is expressed as the ratio of phosphorylated MAPK band to that of the total MAPK band. β-actin served as the loading control. B: The effect of SP600125 on the UA-induced activation of JNK. Western blots were carried out using antibodies against phospho-c-Jun (Ser63), as described in Materials and Methods Section. β-actin served as the loading control.

cancer cell lines. We further examined the effect of the JNK-specific inhibitor SP600125 on the activation of JNK induced by UA. SP600125 is a small molecular inhibitor that exhibits high specificity for JNK [Weston and Davis, 2002]. Prostate cancer cells were pretreated with 10 μM SP600125 for 2 h before treatment with 50 μM UA for an additional 2 h. SP600125 pretreatment completely prevented UA-induced c-Jun phosphorylation (Fig. 3B). These results suggest that UA activates JNK but not ERK1/2 and p38 in the two cell lines.

JNK IS POTENTIALLY INVOLVED IN UA-INDUCED APOPTOSIS

LNCaP or LNCaP-AI cells were treated with 50 μM UA for 48 h and the percentages of apoptotic cells, as determined with annexin V-FITC/PI flow cytometric analysis, were 74.3 ± 3.6% and 72.1 ± 2.4%, respectively (Fig. 4). We next examined the involvement of JNK in apoptosis induced by UA. LNCaP or LNCaP-AI cells were pretreated with 10 μM SP600125 for 2 h, followed by 50 μM UA for an additional 48 h, and the percentages of apoptotic cells were reduced to 8.6 ± 1.7% and 9.1 ± 2.7%, respectively (Fig. 4). In order to demonstrate specificity of SP600125, we used p38 inhibitor SB203580 as a negative control. SB203580 is a highly specific p38 inhibitor that does not significantly inhibit JNK or ERK [Cuenda et al., 1995]. We found that pretreatment with 10 μM SB203580 for 2 h did not influence UA-induced apoptosis in LNCaP or LNCaP-AI cells (Fig. 4).

The caspase cascade is crucial for apoptotic signal transduction [Budihardjo et al., 1999]. Individual caspase activation was therefore assessed using caspase fluorometric assay kits. Activities of caspase-

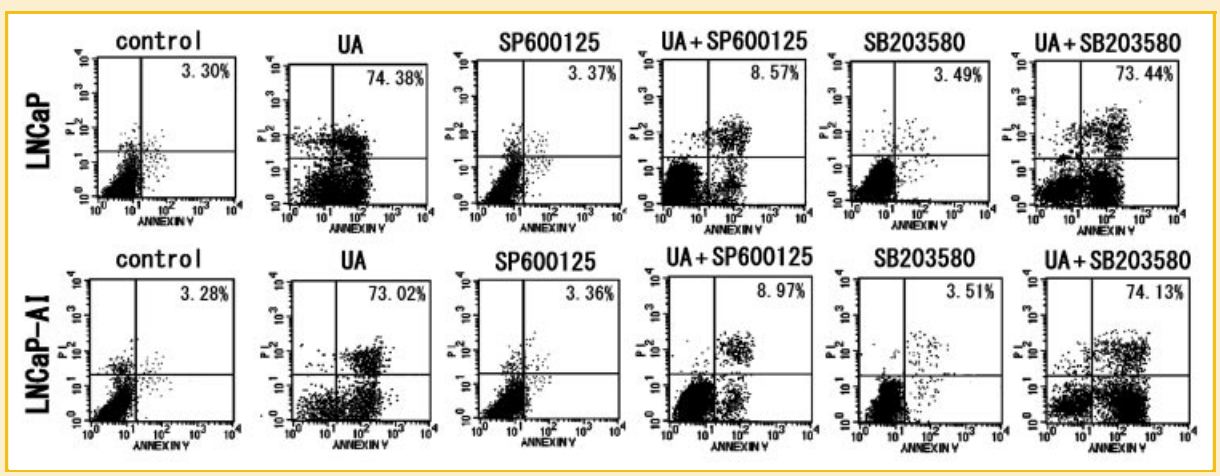

Fig. 4. The effect of SP600125 or SB203580 on UA-induced apoptosis in LNCaP or LNCaP-AI cells was measured using the annexin V-FITC apoptosis detection kit, as described in Materials and Methods Section. Viable cells do not bind annexin V-FITC and PI as reflected in the lower left-hand quadrant of the dot plot. Apoptotic cells are annexin V-FITC positive, with or without PI positivity, as shown in the right-hand quadrant.

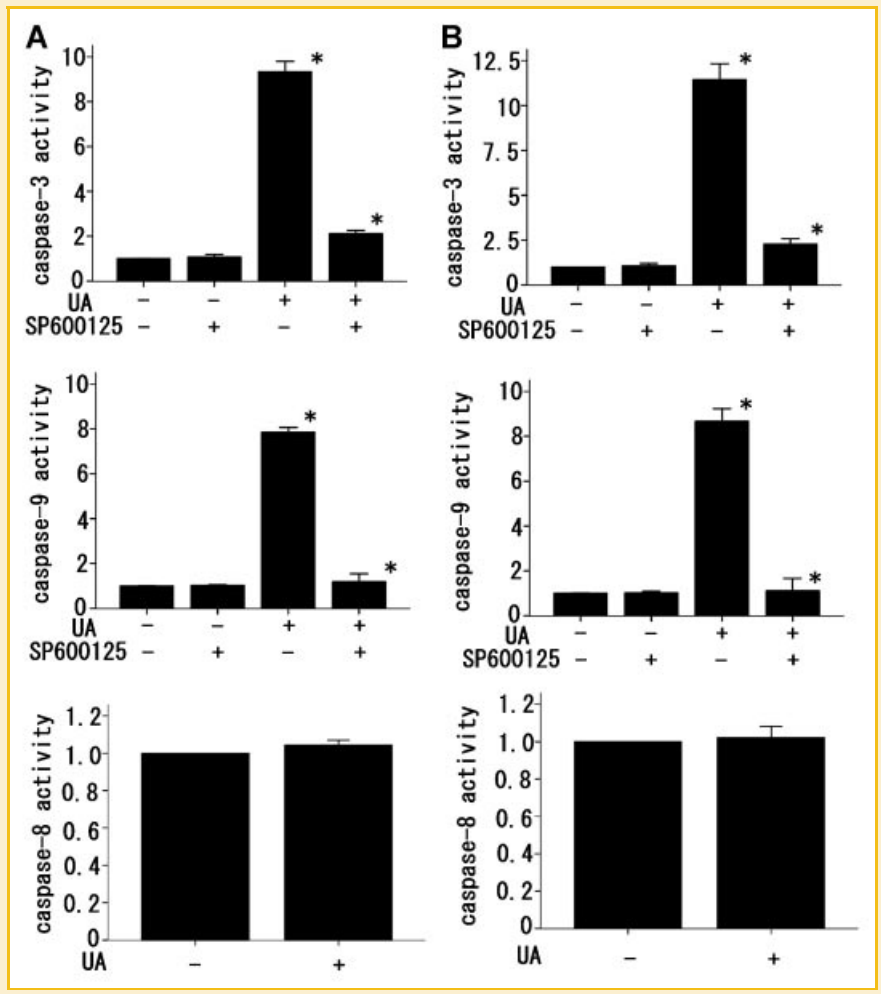

Fig. 5. The effect of UA on caspase activation was assessed using the caspase fluorometric assay kits, as described in Materials and Methods Section. The results shown here are representative of three independent experiments ($^{*}P < 0.05$). A: The effect of UA on caspase-3, caspase-9,and caspase-8 activation in LNCaP cells. B: The effect of UA on caspase-3, caspase-9, and caspase-8 activation in LNCaP-AI cells.

3 and caspase-9 were shown to be significantly increased after UA treatment for 48 h in both LNCaP and LNCaP-AI cells. However, UA did not induce activation of caspase-8 (Fig. 5A,B). The effects of JNK inhibition on caspase activation were also assessed. We found that inhibiting JNK by adding SP600125 completely blocked caspase-9 and caspase-3 activation in LNCaP or LNCaP-AI cells after UA treatment (Fig. 5A,B). Thus, these results strongly suggest that JNK performs a crucial function in UA-induced apoptosis in the two cell lines, accompanied by the activation of caspases-9 and caspase-3.

JNK ACTIVATION BY UA INDUCES PHOSPHORYLATION OF Bcl-2

As the apoptotic response to UA treatment was found to be principally mediated by JNK activation, we attempted to evaluate the contribution of the proapoptotic or antiapoptotic factors affected by JNK. Bcl-2 family members are well known to be important proapoptotic or antiapoptotic regulators. Previous studies have shown that phosphorylation of Bcl-2 mediates Bcl-2 inactivation, and Bcl-2 phosphorylation can also lead to Bcl-2 degradation [Cheng et al., 2001; Ruvolo et al., 2001; Yanamadala et al., 2007]. Recently it has been shown that the JNK-mediated phosphorylation of Bcl-2 induces apoptosis [Maundrell et al., 1997; Deng et al., 2001; Sinha-Hikim et al., 2007; Lee et al., 2009]. Furthermore, the Ser70 site of Bcl-2 is a specific phosphorylation site for JNK [Deng et al., 2001]. We therefore examined whether UA treatment could induce phosphorylation of Bcl-2 at the Ser70 site and reduce Bcl-2 expression. We found that androgen-independent LNCaP-AI cells express much higher protein and mRNA levels of Bcl-2 than the parental LNCaP cells. But the protein level of Bax in LNCaP-AI cells is similar to that in parental LNCaP cells (Fig. 6A,B). Bcl-2 was shown to be phosphorylated at the Ser70 site after 50 μM of UA treatment for 6 h in both LNCaP and LNCaP-AI, and the Bcl-2 protein levels decreased slightly. SP600125 was able to block this phosphorylation (Fig. 6C), suggesting the involvement of JNK regulation in Bcl-2 phosphorylation. After 50 μM of UA treatment for 48 h, the Bcl-2 expression level was significantly reduced in both LNCaP and LNCaP-AI cells. On the other hand, SP600125 prevented UA-induced Bcl-2 down-regulation. However, UA treatment did not affect the expression of Bax in the two cell lines (Fig. 6A). To establish whether the UA-stimulated loss of Bcl-2 was mediated by changes in gene expression, quantitative RT-PCR was performed for

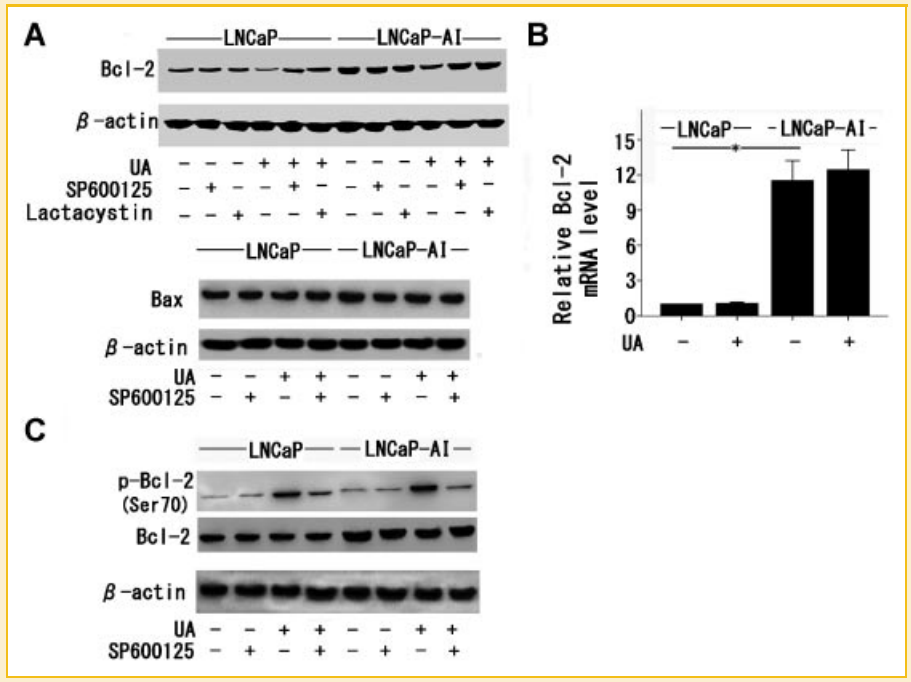

Fig. 6. The effect of UA on Bcl-2, Bax and phosphorylated Bcl-2 expressions. A: The effect of UA treatment for 48 h on protein levels of Bcl-2 and Bax was assessed by Western blot, as described in Materials and Methods Section. β-actin served as the loading control. B: The effect of UA treatment for 6 h on mRNA levels of Bcl-2 was assessed by quantitative RT-PCR, as described in Materials and Methods Section. The results represent the means from three independent experiments ($^{*}P < 0.05$). C: The effect of UA treatment for 6 h on protein levels of phosphorylated Bcl-2 and Bcl-2 was assessed by Western blot, as described in Materials and Methods Section. β-actin served as the loading control.

each condition. We found that UA treatment for 6 h did not have an effect on the mRNA expression level of Bcl-2 (Fig. 6B). As Yanamadala et al. [2007] revealed that Bcl-2 phosphorylation induced Bcl-2 degradation through the proteasome pathway, we further investigated whether UA-induced Bcl-2 phosphorylation led to Bcl-2 degradation through the proteasome pathway. LNCaP and LNCaP-AI cells were cultured with 50 μM of UA in the presence or absence of 10 μM lactacystin (Sigma–Aldrich), which is a specific proteasome inhibitor [Fenteany et al., 1995]. We found lactacystin to be nearly completely inhibited the UA-induced loss of Bcl-2 (Fig. 6A). Taken together, these results support the notion that UA-mediated Bcl-2 phosphorylation leading to Bcl-2 down-regulation through the proteasome pathway is required for the activation of JNK in UA-induced apoptosis.

DISCUSSION

We established an androgen-independent prostate cancer cell line, LNCaP-AI, from the androgen-dependent cell line LNCaP using prolonged androgen deprivation culture, as previously described [Xu et al., 2009]. In this study, we found LNCaP and LNCaP-AI cells to be similarly sensitive to UA treatment. We also demonstrated that JNK may play a crucial role in UA-induced apoptosis in the two prostate cancer cell line cells probably via inducing Bcl-2 phosphorylation and degradation and activating caspase-9.

Currently, three basic apoptotic signaling pathways have been established: mitochondria, endoplasmic reticulum, and death receptor [Daniel, 2000]. In general, caspase-driven cascades can be activated by multiple pathways, including through a membrane death-receptor pathway that triggers the initiator caspase-8 activation or through the mitochondrial pathway that relies on cytochrome *c* release and thus activates the initiator caspase-9 [Green and Reed, 1998; Kim, 2002]. In the present study, we found that UA treatment in LNCaP and LNCaP-AI cells activated caspase-9 along with caspase-3, but not caspase-8, implying that the UA-induced apoptosis in these cells exhibits intrinsic pathway dependence.

Some other studies have shown that apoptosis can be induced through MAPK pathways in some cancer cells [Chang and Karin, 2001]. In this study, we find that UA treatment in LNCaP and LNCaP-AI cells induces the activation of JNK, but not ERK1/2 and p38, in a time-dependent manner. Additionally, we showed that SP600125, a specific inhibitor of JNK activation, partly protects these cells from UA-induced apoptosis and completely blocks caspase-9 activation after UA treatment. However, SB203580, a specific p38 inhibitor, did not influence the UA-induced apoptosis in LNCaP and LNCaP-AI cells. The results suggested that UA induces apoptosis in both cell lines via JNK activation.

It has been previously reported that JNK participates in apoptotic signaling via phosphorylation-dependent control of Bcl-2 [Maundrell et al., 1997; Deng et al., 2001; Sinha-Hikim et al., 2007; Lee et al., 2009], and the specific phosphorylation site of Bcl-2 for JNK is

the Ser70 site [Deng et al., 2001]. The Bcl-2 family includes Bcl-2 and Bax, and Bcl-2 can form heterodimers with the pro-apoptotic protein Bax. On the one hand, the relative proportions of Bcl-2 to Bax determine cell viability during apoptotic stimuli. An increased ratio of Bcl-2 to Bax promotes cell survival, whereas an increased ratio of Bax to Bcl-2 protein promotes cell apoptosis [Oltvai et al., 1993]. On the other hand, Bcl-2 phosphorylation results in the release of the pro-apoptotic protein Bax from the Bcl-2/Bax complex, which subsequently induces apoptosis [Haldar et al., 1996]. Therefore, it is possible that phosphorylation of Bcl-2 by UA alters the dimerization properties of Bax, thereby promoting apoptosis.

As previous studies have revealed that Bcl-2 phosphorylation can also lead to Bcl-2 degradation [Cheng et al., 2001; Yanamadala et al., 2007], we investigated the effect of UA treatment on protein levels of Bcl-2 in LNCaP and LNCaP-AI cells. We found that UA treatment for 6 h slightly decreased protein levels of Bcl-2. After treatment with UA for 48 h, the Bcl-2 protein levels decreased significantly. These results showed that UA causes time-dependent down-regulation of Bcl-2 protein levels. However, the UA-induced loss of Bcl-2 was nearly completely inhibited in cells treated with the JNK inhibitor SP600125. We also investigated whether the UA-stimulated loss of Bcl-2 was mediated by changes in gene expression using quantitative RT-PCR. UA treatment for 6 h did not decrease the mRNA expression of Bcl-2, so we conclude that UA did not influence the transcription of the Bcl-2 gene. We further studied what causes Bcl-2 degradation. Previous study has showed that Bcl-2 phosphorylation is able to induce Bcl-2 degradation through the proteasome pathway [Yanamadala et al., 2007]. Proteasomes are the major intracellular machinery for protein degradation and they degrade 70–90% of cellular proteins [Rock et al., 1994]. Lactacystin is a specific proteasome inhibitor that can repress major peptidase activities of proteasomes [Fenteany et al., 1995]. In our study, pretreatment with lactacystin was able to nearly inhibit UA-induced degradation of Bcl-2 in LNCaP and LNCaP-AI cells. These results suggest that UA-induced Bcl-2 phosphorylation via JNK activation leads to Bcl-2 degradation via the proteasome pathway. It also explained why UA induces a time-dependent decrease in Bcl-2 protein levels. However, the mechanism by which Bcl-2 phosphorylation targets the Bcl-2 protein for degradation by the proteasome needs further study. We also found the protein levels of Bax in LNCaP-AI cells to be similar to those in parental LNCaP. UA treatment for 48 h did not increase the expression of Bax in either cell line. This finding suggests that the increased Bax/Bcl-2 ratio also contribute to the activation of caspase-9 and induction of apoptosis.

Thus, our findings in the present study may suggest that UA induces Bcl-2 phosphorylation and degradation along with activating caspase-9 via JNK regulation, and consequently induces apoptosis in LNCaP and LNCaP-AI cells.

Previously, many studies have showed the over-expression of Bcl-2 to protect prostate cancer cells from many different apoptotic stimuli [Raffo et al., 1995; Yoshino et al., 2006; An et al., 2007]. Lu et al. [1999] found that the over-expression of Bcl-2 in androgen-independent LNCaP-AI cells made them more resistant to apoptotic stimuli than the parental LNCaP cells. We also found the Bcl-2 levels in LNCaP-AI cells to be higher than those in LNCaP cells in our study. Although LNCaP-AI cells express a much higher level of Bcl-2 than the parental LNCaP cells do, UA induces apoptosis in LNCaP and LNCaP-AI cells with similar potency. This finding suggests that UA can overcome Bcl-2-mediated resistance to apoptosis via activation of JNK in prostate cancer cells. Mc Gee et al. [2004] also found the same phenomenon, specifically that the novel pyrrolo-1,5-benzoxazepine compound 7-[{dimethylcarbamoyl}oxy]-6-(2-naphthyl)pyrrolo-[2,1-*d*] (1,5)-benzoxazepine (PBOX-6) could induce apoptosis in Bcl-2-overexpressing cancer cells. Apoptosis was induced with similar potency in wild-type T leukemic CEM cells and cells overexpressing Bcl-2 after PBOX-6 treatment. They found that PBOX-6 induction of phosphorylation and inactivation of Bcl-2 via JNK activation abrogated the protective effect of Bcl-2. The precise reason why activation of JNK is able to overcome Bcl-2-mediated resistance to apoptosis is not clear. We found that the degree of UA-induced activation of JNK in LNCaP cells was similar to that in LNCaP-AI cells (Fig. 3). Consistent with this finding, UA induced similar levels of Bcl-2 phosphorylation and degradation in both cells (Fig. 6). We presumed that the similar change in Bcl-2 levels might destroy the balance between proapoptotic and antiapoptotic pathways and thus induce apoptotic cascades with similar potency in both cell lines. However, there might be other pathways by which UA overcomes the overexpressing Bcl-2-mediated resistance to apoptosis in LNCaP-AI cells, and these need further study.

In summary, we have demonstrated that UA effectively induces apoptosis, probably via JNK-mediated Bcl-2 phosphorylation and degradation in both the androgen-independent human prostate cancer cell line LNCAP-AI and parental androgen-dependent LNCaP cells. UA can overcome Bcl-2-mediated resistance to apoptosis in LNCaP-AI cells. These findings suggest that UA-induced apoptosis may be a potential therapeutic approach in prostate cancer and further study is warranted.

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