Acetyl-Keto-β-Boswellic Acid Induces Apoptosis through a Death Receptor 5–Mediated Pathway in Prostate Cancer Cells

Min Lu,¹ Lijuan Xia,¹ Huiming Hua,² and Yongkui Jing¹

¹Division of Hematology/Oncology, Department of Medicine, Mount Sinai School of Medicine, New York, New York and
²Shenyang Pharmaceutical University, Shenyang, China

Abstract

Acetyl-keto-β-boswellic acid (AKBA), a triterpenoid isolated from Boswellia carterri Birdw and Boswellia serrata, has been found to inhibit tumor cell growth and to induce apoptosis. The apoptotic effects and the mechanisms of action of AKBA were studied in LNCaP and PC-3 human prostate cancer cells. AKBA induced apoptosis in both cell lines at concentrations above 10 μg/mL. AKBA-induced apoptosis was correlated with the activation of caspase-3 and caspase-8 as well as with poly(ADP)ribose polymerase (PARP) cleavage. The activation of caspase-8 was correlated with increased levels of death receptor (DR) 5 but not of Fas or DR4. AKBA-induced apoptosis, caspase-8 activation, and PARP cleavage were inhibited by knocking down DR5 using a small hairpin RNA. AKBA treatment increased the levels of death receptor (DR)5 promoter reporter with the mutant CHOP binding site. CAAT/enhancer binding protein homologous protein (CHOP) small hairpin RNA. AKBA treatment increased the levels of PARP cleavage. The activation of caspase-8 was correlated with the up-regulation of CHOP (GADD153) expression. Knocking down CHOP using CHOP small hairpin RNA blocked AKBA-induced apoptosis. AKBA treatment increased the levels of death receptor (DR)5 promoter reporter with the wild type CHOP binding site. These results suggest that AKBA induces apoptosis in prostate cancer cells through a DR5-mediated pathway, which probably involves the induced expression of CHOP.

Introduction

Prostate cancer is the most common form of cancer and is the third leading cause of cancer death of males in the United States (1). Two of the common treatments for prostate cancer, hormone ablation and endocrine therapy, lead to temporary palliation, but relapse frequently occurs 1 to 2 years after initiating treatment (2). The reduction of androgen stimulation in prostatic cells is one of the main treatment strategies for this disease (3). Thus, strategies to find agents that act by different mechanisms are needed to improve prostate cancer therapy.

Decreasing apoptotic cell death seems to be one of the mechanisms of carcinogenesis (4). Two principle types of apoptotic pathways have been described: mitochondria-dependent and death receptor–dependent pathways (5, 6). Many cells undergo a reduction in mitochondria membrane potential before they exhibit signs of nuclear apoptosis after treatment with chemotherapeutic agents (7). However, chemotherapy alone has not been shown to have a beneficial therapeutic effect in prostate cancer patients (8).

Materials and Methods

Reagents. AKBA was isolated from B. carterii Birdw with a purity of 98% as determined by high performance liquid chromatography. JETD-CHO was purchased from CalBiochem. S600125 and TRAIL Receptor 2/Fc Chimera were purchased from Sigma. Z-VAD-CHO, mouse monoclonal anti–caspase-3, and anti–caspase-8 were from BD Biosciences; and rabbit polyclonal anti–DR4, anti–DR5, anti–TRAIL antibodies, and TRAIL ELISA kit were purchased from Alexis Biochemicals. Anti-poly (ADP-ribose) polymerase (PARP) was purchased from Roche. Anti-β-actin, and anti–CHOP (GADD153) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti–Fas antibody was purchased from Upstate. Lipofectamine 2000 was purchased from Invitrogen.
**Cell lines and culture.** The human prostate cancer cell lines, PC-3 and LNCaP, were purchased from the American Type Culture Collection and maintained in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2 $\times$ 10^3) were plated in each well of a 96-well plate and were allowed to adhere and spread for 24 h. Then various concentrations of AKBA were added, and the cells were cultured for 4 days at 37°C. MTT solution (50 $\mu$L of 2 mg/mL) was added per well, and the cultures were continued for an additional 4 h. The medium was aspirated, the cells were dissolved in 200 $\mu$L DMSO, and the absorbance at 570 nm was determined in each well with a 96-well plate reader. The growth of the treated cells was compared with that of untreated cells.

**Apoptosis assay.** DNA fragmentation was determined by labeling DNA strand breaks with terminal deoxynucleotidyl transferase (TUNEL; ref. 28). The TUNEL assay was performed according to the manufacturer's instructions (PharMingen). Cells were analyzed by flow cytometry using a FACScan. All data were collected, stored, and analyzed by LYSIS II software (Becton Dickinson).

**Mitochondria membrane potential assay.** The mitochondrial membrane potential was determined with a Mitochondrial Membrane Sensor kit as described by the manufacturer (Clontec Laboratories, Inc.). Briefly, cells were seeded in 6-well plates for 24 h before AKBA treatment. After treatment, the cells were washed with serum-free medium and incubated with 1 mL diluted BD Mitosensor reagent at 37°C in a humidified, 5% CO2 incubator for 15 to 20 min. Then the cells were gently rinsed with 1× PBS and examined under a microscope using a band-pass filter. The Mitosensor reagent aggregates in the mitochondria of healthy cells that were fluorescence orange. When mitochondrial membrane potentials are altered, the Mitosensor reagent does not accumulate in the mitochondria and remains as monomers in the cytoplasm where it fluoresces green (27).

**Plasmid preparation for shRNA and transfection.** The DR5 and LacZ pENTR/U6 shRNA entry vector were constructed following the description of the instruction manual (Invitrogen). The LacZ shRNA was used as a control. In brief, two complementary ssDNA oligonucleotides were synthesized and annealed with an encoding shRNA of DR5 or LacZ, then the double-stranded oligo was cloned into the pENTR/U6 vector. The vectors were used to transfect LNCaP and PC-3 cells with Lipofectamine 2000. The relative levels of DR4 and DR5 in shRNA-transfected LNCaP cells were analyzed by Western blot analysis. The target sequence of DR5 was AGA\text{AGCCTTGTGC}TCTGTCGTC.

**Luciferase assay.** The pGL3/DR4 (−1773/+63) promoter reporter plasmid was obtained from Dr. S-Y Sun (Emory University School of Medicine, Atlanta, GA; ref. 29). Plasmids of pDR5/−1188, pDR5/−437, and pDR5/mi CHOP were obtained from Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan; ref. 30). The pCH110 plasmid that codes β-galactosidase was cotransfected into PC-3 and LNCaP cells using Lipofectamine 2000 (31). The relative levels of DR4 and DR5 in shRNA-transfected LNCaP cells were analyzed by Western blot analysis. The target sequence of DR5 was AGA\text{AGCCTTGTGC}TCTGTCGTC.

**Reverse transcription-PCR.** RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and Moloney leukemia virus reverse transcriptase (Promega) following standard protocols. Primers used in these experiments were as follows: DR5, 5′-ATGAGATCGTGAGTATCTTGCAGC-3′ and 5′-TGACCACCTTATCATGACATGCTGTC-3′; and β-actin, 5′-ATCCAGAAAATCCTTCAACATC-3′ and 5′-CTGCGTGTGGATCCACATCTGCT-3′. After reverse transcription, the cDNA product was amplified by PCR with three units of Taq DNA polymerase (Promega) and 2.5 mmol/L Mg2+, using standard protocols at annealing temperature of 55°C. Western blot analysis was done as described previously (32), and the contents of TRAIL in the medium were determined using TRAIL ELISA kit (Alexis Biochemicals) following the protocol.

**Results**

AKBA inhibits cell growth and induces apoptosis in both LNCaP and PC-3 cells in an androgen-independent fashion. The cell growth inhibitory effects of AKBA in LNCaP and PC-3 cells were determined using the MTT assay. Both LNCaP and PC-3 cells were responsive to AKBA treatment (Fig. 1A). AKBA at 10 μg/mL inhibited nearly 80% of cell growth in both cell lines after treatment for 4 days. The apoptotic effects of AKBA in both cell lines were determined using the TUNEL assay. AKBA induced apoptosis at a concentration of 20 μg/mL after 24 h of treatment (Fig. 1B). Because both LNCaP (androgen receptor–positive) and PC-3 (androgen receptor–negative) cells are sensitive to AKBA apoptosis induction, it suggests that AKBA inhibits cell growth and induces apoptosis through a pathway independent of androgen receptor activity.

AKBA activates caspase-8 and caspase-3 in both LNCaP and PC-3 cells. To investigate the mechanisms of AKBA-induced apoptosis, the levels of caspase-8 and caspase-3 proteins as well as their cleave fragments were determined. AKBA at a concentration of 20 μg/mL decreased the levels of caspase-8 and caspase-3 protein and resulted in the formation of cleave fragments (the activated forms of both enzymes; Fig. 2A). Correlated with the activation of caspase-3, one of its substrate, PARP, was cleaved. Caspase-8 has been found to activate caspase-3 either directly or indirectly through a mitochondrial-mediated pathway (33). The mitochondria membrane potential (MMP) was determined in PC-3 cells after AKBA treatment using Mitosensor Reagent. Mitosensor Reagent accumulated in the mitochondria and fluorescenced orange in untreated cells and cells treated with AKBA at 10 μg/mL.
AKBA at 20 μg/mL decreased the MMP and blocked the MitoSensor Reagent accumulation in the mitochondria fluorescing green (Fig. 2B). The decrease of MMP and the cleavage of PARP due to AKBA treatment were completely inhibited by pretreatment with a caspase-8 inhibitor, IETD-CHO, or a general caspase inhibitor, Z-VAD-CMK (Fig. 2C and D). These data suggest that the activation of caspase-8 plays an important role in AKBA-induced apoptosis.

**AKBA increases the levels of TRAIL and DR5 in both LNCaP and PC-3 cells.** Caspase-8 is activated by a death receptor-mediated pathway (33). To determine the pathways of the AKBA-induced caspase-8 activation, the levels of DR4, DR5, TRAIL, and Fas were determined using Western blot analyses. The levels of TRAIL and DR5, but not DR4, were increased after AKBA treatment. AKBA increased the levels of TRAIL protein after AKBA treatment as short as 2 h, and then the levels of TRAIL protein remained constant until 24 h (Fig. 3). The levels of Fas were transiently increased after AKBA treatment for 2 to 4 h and then decreased to the basal levels at 8 h in PC-3 cells and at 24 h in LNCaP cells (Fig. 3). The levels of DR4 were not changed after AKBA treatment for 24 h (Fig. 3). To determine whether AKBA induced apoptosis through ligation activation of DR5 by released TRAIL in AKBA-treated cells, a TRAIL receptor FC chimeric protein that block DR5 activation by TRAIL was used, and it was found that TRAIL receptor FC chimeric protein did not attenuate AKBA-induced apoptosis in both PC-3 and LNCaP cells (data not shown). In addition, the levels of TRAIL in the cultured medium of cells treated by AKBA were not increased (data not shown). These data suggest that AKBA may induce apoptosis in prostate cancer cells through the increased levels of DR5 protein independent of TRAIL ligation activation.

**Knockdown of DR5 attenuates AKBA-induced apoptosis in LNCaP and PC-3 cells.** To determine the role of DR5 in AKBA-induced apoptosis, DR5 was knocked down using shRNA. DR5 shRNA selectively knocked down DR5 protein but not DR4 protein in LNCaP cells (Fig. 4A). The basal as well as the AKBA-induced levels of DR5 were decreased by the transfection of DR5 shRNA. Correlated with the decrease in the levels of DR5 protein by DR5 shRNA, the cleavages of caspase-8, caspase-3, and PARP due to AKBA treatment were inhibited. Using TUNEL assay, it was found that AKBA-induced apoptosis in PC-3 cells was completed inhibited by DR5 shRNA (Fig. 4B). These data suggest that the induced expression of DR5 protein indeed plays a pivotal role in AKBA-induced apoptosis.

**Up-regulation of DR5 by AKBA correlates with the increased expression of CHOP.** DR5 has been found to be regulated through a nuclear factor-κB (NF-κB), a p53, and/or a
c-Jun-NH2-kinase (JNK)-mediated pathway (34–38). To determine if NF-κB plays a role in AKBA-mediated DR5 up-regulation, the p65 nuclear translocation and the levels of IκB protein were determined in both LNCaP and PC-3 cells. AKBA at 20 μg/mL did not induce p65 nuclear translocation nor did it decrease the levels of IκB protein (Fig. 5A). To determine if p53 is involved in AKBA-induced apoptosis, the levels of p53 and its phosphorylated form were determined in both cell lines. LNCaP cells, but not PC-3 cells, express p53 (Fig. 5B). The levels of p53 and phosphorylated p53 were weakly up-regulated by AKBA treatment in LNCaP cells but not in PC-3 cells (Fig. 5B). To determine if the JNK pathway is involved in DR5 regulation, the levels of phosphorylated c-jun were analyzed in both cell lines. The levels of phosphorylated c-jun were increased with AKBA treatment, suggesting that JNK is activated. It has been found that JNK up-regulates CHOP, and DR5 is a CHOP-responsive gene (30, 36, 39). Thus, the levels of CHOP were determined in both cell lines after AKBA treatment, and it was found that the levels of CHOP protein were increased in both cell lines after AKBA treatment (Fig. 5B). JNK inhibitor SP600125 attenuated the induction of CHOP and DR5 proteins as well as PARP cleavage in both cell lines (Fig. 5C). These data suggest that AKBA increases the levels of DR5 protein through pathways involving JNK activation and CHOP induction.

**AKBA increases the mRNA levels of DR5 and activates the DR5 promoter through a CHOP-mediated pathway.** To determine if AKBA up-regulate DR5 protein transcriptionally, the levels of DR5 mRNA were determined using reverse transcription-PCR (RT-PCR). The levels of DR5 mRNA were increased in both LNCaP and PC-3 cells after AKBA treatment (Fig. 6A). The activation of DR4 and DR5 promoter reporters by AKBA treatment were determined. The basal level of promoter activity of DR5 was higher than that of DR4 after transfection into both cell lines. AKBA treatment significantly increased the activity of the DR5 promoter reporter, but not that of the DR4 promoter reporter, in both cell lines (Fig. 6B). To test if the activation of the DR5 promoter reporter is mediated through CHOP, the DR5 promoter with a mutant CHOP binding site was used. AKBA activated the DR5 promoter reporter with the wild-type CHOP binding site but did not activate DR5 promoter reporter with the mutant CHOP binding site (Fig. 6C). These data suggest that AKBA up-regulates DR5 at a transcriptional level, and that CHOP plays an important role in the AKBA-induced DR5 expression.

**Discussion**

The death receptor–mediated apoptotic pathway has been proposed as a therapeutic target in prostate cancer (11, 40). Agents that could activate this pathway should have therapeutic potential in prostate cancer treatment. It has been found that several...
natural products do induce apoptosis in prostate cancer cells, at least in part, by activation of the death receptor–mediated pathway (13, 14, 41, 42). Boswellic acids, including AKBA and BAA isolated from gum resin of B. serrata and B. carterii Birdw., have been found to have apoptosis induction ability in cancer cells (20, 24, 26). Although AKBA has been reported to induce apoptosis in several tumor cell lines, the mechanisms of its action are not clear. In this study, we found that AKBA induced apoptosis through the death receptor–mediated pathway in prostate cancer cells.

By using androgen receptor–positive LNCaP and androgen receptor–negative PC-3 cells, we found that AKBA inhibited cell growth and induced apoptosis in both cell lines (Fig. 1). These data suggest that AKBA may not act through interfering with the androgen signaling pathway. Because AKBA induced apoptosis in both cell lines, the apoptosis induction ability of AKBA should be,

at least, one of its mechanisms of inhibiting prostate cancer cell growth. p53 has been shown to play an important role in apoptosis induction (43). LNCaP cells express p53, but PC-3 cells do not (Fig. 5B). Although AKBA increased the levels of phospho-p53 in LNCaP cells, AKBA equally induced apoptosis in both cell lines without induction of p53 in PC-3 cells. These data suggest that apoptosis induction of AKBA, at least in PC-3 cells, is through a p53-independent pathway.

Caspase activation plays pivotal roles in both mitochondria and death receptor–mediated pathways (44). AKBA treatment activates caspase-3 and caspase-8 and results in PARP cleavage (Fig. 2). Caspase-8 has been shown to play an important role in both mitochondria- and death receptor–mediated pathways (45). AKBA induces the activation of caspase-8 activity and decreases MMP (Fig. 2). Because AKBA-induced apoptosis and decrease in MMP are both diminished after pretreatment with the caspase-8 inhibitor IETD-CHO, activated caspase-8 seems to be a key factor in the pathway involved in AKBA-mediated apoptosis process (Fig. 2). The activation of caspase-8 has been found to be mediated by the increased expression of a death receptor and/or ligation of a receptor with its ligand (6). It has been reported that boswellic acid activated caspase-8 independent of Fas/FasL interaction in colon cancer cells (46). Consistent with this report, we observed that Fas was only transiently induced after AKBA treatment in PC-3 cells (Fig. 3B). These data suggest that Fas would not be a key factor in mediating AKBA-induced apoptosis. Previously, we found that BAA increased the levels of both DR4 and DR5 expression in leukemia cells (27). We reported here that the levels of DR5 protein but not of DR4 protein were induced by AKBA treatment in both prostate cancer cell lines (Fig. 3). The induced expression of DR5 correlates with AKBA-induced apoptosis. Knockdown of DR5 blocked AKBA-induced activities of caspase-8 and caspase-3, PARP cleavage, and apoptosis (Fig. 4). Interestingly, because intracellular TRAIL levels are also increased after AKBA treatment (Fig. 3), TRAIL ligation to DR5 may also participate in AKBA-induced apoptosis. To prove this possibility, we have determined the levels of TRAIL in the medium of cells treated by AKBA and the blockade of TRAIL Receptor 2/Fc Chimera on AKBA-induced apoptosis. It was found that the levels of TRAIL were not increased in medium of cells treated with AKBA, and that TRAIL Receptor 2/Fc Chimera did not block AKBA-induced apoptosis. These data suggest that the up-regulation of DR5 protein but not TRAIL ligation plays an important role in AKBA-induced apoptosis.

Because AKBA increases the levels of DR5 mRNA in both LNCaP and PC-3 cells, it suggests that AKBA up-regulates DR5 at a transcriptional level (Fig. 6A). DR5 can be regulated by several factors including p53, NF-κB, JNK, and CHOP (34–38). Because AKBA induces the expression of DR5 in PC-3 cells, which do not express p53, it is possible that AKBA induces DR5 expression through a p53-independent pathway, at least in PC-3 cells. Inhibition of NF-κB activation has been thought to play a role in the apoptosis induction and anti-inflammatory effects of AKBA (18, 23, 26). Surprisingly, AKBA at concentrations, which increased the levels of DR5 protein, did not induce p56 nuclear translocation nor decrease the levels of IκBα protein (Fig. 5). Thus, it seems that the induced expression of DR5 by AKBA may not be mediated by the inhibition of NF-κB activation.

Recently, CHOP has been considered to play an important role in regulating DR5 expression, and a CHOP binding site has been identified in DR5 promoter (36). CHOP was induced by AKBA treatment in both cell lines that was correlated with the
induction DR5 protein (Fig. 5C). AKBA activated a DR5 promoter reporter but did not activate a DR5 promoter reporter with a mutant CHOP binding site (Fig. 6). These data suggest that AKBA induces DR5 expression via a CHOP-mediated pathway. Several agents, such as AKBA, have been shown to up-regulate DR5 expression through an induction of CHOP expression (30, 47, 48). The mechanisms of CHOP activation by these agents are unclear. Our study suggests that CHOP could be activated by a JNK-mediated pathway. AKBA increased the levels of phosphorylated c-jun, and that a JNK inhibitor SP600125 attenuated AKBA-mediated expression of both CHOP and DR5 protein as well as the PARP cleavage in prostate cancer cells (Fig. 5C). In summary, our data indicate that AKBA induces apoptosis through the up-regulation of DR5, which may be mediated by JNK-activated CHOP in prostate cancer cells.

Acknowledgments

Received 8/2/2007; revised 11/12/2007; accepted 12/19/2007.

Grant support: R21AT001539 from NIH/NCCAM.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Figure 6. AKBA increases the levels of DR5 mRNA and activates the DR5 promoter reporter in LNCaP and PC-3 cells. A, the levels of DR5 mRNA. LNCaP and PC-3 cells were treated with AKBA at 10 and 20 μg/mL for 24 h. The mRNA levels of DR5 were determined using RT-PCR as described in Materials and Methods. B, the activities of DR5 and DR4 promoter reporters. C, the activities of DR5 promoter reporters with wild-type or a mutant CHOP binding site. pG3/DR5/–1818, pG3/DR4/–1772, pG3/DR5/–437, or pG3/DR5/mtCHOP were cotransfected with pCH110 into LNCaP and PC-3 cells for 24 h and then treated with AKBA for 24 h. Cells were collected for measuring luciferase and β-galactosidase activities. β-galactosidase activity was used for normalization of transfection efficiency.


