Cancer Letters 335 (2013) 136–144

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Androgens regulate TRAIL-induced cell death in prostate cancer cells via multiple mechanisms

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ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form 31 January 2013
Accepted 2 February 2013

Keywords:
Androgen
Prostate cancer
TRAIL
TRADD
Appoptosis

ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising therapeutic agent for prostate cancer because it selectively induces apoptosis in cancer cells but not in normal cells. Previous reports have suggested that androgens regulate TRAIL-induced apoptosis in prostate cancer cells. However, there are discrepancies between these reports of how androgens affect TRAIL-induced cell death. To clarify the role of androgens on TRAIL-induced apoptosis in prostate cancer cells, we investigated the effects of androgen on TRAIL-induced cell death in a dose–response manner. Our results showed that although androgens sensitize LNCaP cells to TRAIL-induced apoptosis, this effect is dose-dependent and biphasic. We found that low levels of androgen are superior to high levels of androgen in term of sensitizing LNCaP cells to TRAIL. We also found that upregulation of DR5 (TRAIL-R2) expression by androgens is critical for sensitizing LNCaP cells to TRAIL. However, low levels of androgen are sufficient to induce DR5 expression and sensitize LNCaP cells to TRAIL-induced cell death. High levels of androgen alter the TRADD/RIP1 ratio, which may contribute to NF-κB activation and sequentially inhibit TRAIL-induced apoptosis.

1. Introduction

Prostate cancer is one of the leading causes of cancer-related death worldwide. When diagnosed at an early stage, most patients elect to undergo surgery or radiation therapy. However, androgen-deprivation therapy is the preferred treatment for advanced-stage disease. None-the-less, following androgen-deprivation therapy most tumors will recur in about 2 years, giving rise to castration-resistant prostate cancer (CRPC). At this stage of the disease, prostate cancers are resistant to androgen-deprivation and incurable. Although CRPC cells are resistant to androgen-deprivation, they are still capable of undergoing apoptosis with appropriate stimuli [1]. Tumor necrosis factor-alpha (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL) are members of the death receptor ligand superfamily and have been suggested as potential anti-prostate cancer agents [2,3].

Because of its low cytotoxicity to normal cells, TRAIL is more promising than TNF-α for cancer therapy. TRAIL-based therapies exhibit selective antitumor activity in a number of cancers, including prostate cancer [4]. Currently, recombinant human TRAIL and human monoclonal anti-TRAIL-receptor antibodies are in phase I and II clinical trials [5].

Endogenous TRAIL triggers apoptotic signaling via receptor-mediated death through its interaction with the death receptors (DRs) on cancer cells [6]. TRAIL initiates programmed cell death upon binding to DR4 (TRAIL-R1) and DR5 (TRAIL-R2) receptors, promotes the recruitment of adaptor proteins, formation of DISC (death inducing signaling complex) and subsequent activation of the caspase cascade [7]. Apoptosis can also be induced by the intrinsic pathway, mediated mitochondrial dysfunction. A link between the extrinsic and intrinsic signaling pathways is mediated by the Bid (BH3-interacting domain death agonist) protein, which is cleaved and activated by caspase-8 [8].

However, some tumor cells are resistant to TRAIL-induced cytotoxicity. Failure to undergo apoptosis has been implicated in resistance of cancer cells to TRAIL surveillance, therefore, contributing to tumor development and progression. Multiple factors might contribute to TRAIL-resistance, including activation of NF-κB, dysregulation of death receptors and decoy receptors and altered expression of pro-apoptotic and/or anti-apoptotic proteins [7,9]. Due to the involvement of multiple factors, it is not surprising that different cell lines of the same type of cancer show differential sensitivity to TRAIL. For instance, the prostate cancer cell lines, PC-3 and DU145, are sensitive to TRAIL-induced cell death, while LNCaP cells are resistant to TRAIL treatment [10]. One of mechanisms by which LNCaP cells resist TRAIL-induced apoptosis is constitutive activation of AKT. Therefore, inhibition of PI3K or AKT sensitizes LNCaP cells to TRAIL treatment [11–16].

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0304-3835/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2013.02.001

Another import factor that affects TRAIL sensitivity in LNCaP cells is the level of androgen. However, previous reports are inconsistent in describing the action of androgen on TRAIL-induced cell death. Some have suggested that androgens sensitize LNCaP cells to TRAIL [17,18], while others have shown protective effects of androgens on TRAIL-induced apoptosis [19–21]. Here we report that the effects of androgens on TRAIL-induced apoptosis depend on the dose of androgen and demonstrate a biphasic pattern. Our studies indicate that androgens may impact TRAIL-induced apoptosis through multiple factors, including receptors, adaptors and inhibitors of apoptosis (IAPs). Thus, the final readout of androgen action on TRAIL-induced apoptosis depends on the balance of these factors.

2. Materials and methods

2.1. Cell culture

LNCaP and PC3 cells were purchased from the American Type Tissue Collection (Manassas, VA). PC3-AR cells were developed by ectopically expressing androgen receptor (AR) in PC3 cells. Cells were cultured in RPMI 1640 medium (Invitrogen, San Diego, CA) containing 9% fetal bovine serum (Invitrogen), 100 units/ml streptomycin and 0.25 μg/ml amphotericin B (Invitrogen). In experiments assessing androgen effects, cells were seeded in RPMI 1640 medium containing 5% charcoal-stripped serum (CSS), 100 units/mL streptomycin and 0.25 μg/ml amphotericin B.

2.2. Reagents and chemicals

SiRNA-A (no target siRNA), RIP siRNA (h2) and TRADD siRNA were purchased from Santa Cruz Biotech. SiGenome SMART pool directed DR4 and DR5 were purchased from Thermo Scientific. Plasmid constructs for full-length AR (h5HBhAR) has been described [22,23]. Wortmannin and TRAIL were purchased from CHEMICON (Temecula, CA). Bicalutamide (Casodex) was provided by AstraZeneca (Wilmington, DE) and methyltrienolone (R1881) was purchased from DuPont (Boston, MA). The following antibodies were used in this study: anti-ERK2, anti-AR and anti-RIP1 (k-20) (Santa Cruz Biotech); anti-α-Tubulin, anti-survivin, anti-IAP1, anti-XIAP, anti-cleaved-PARP, anti-cleaved-caspase −3 and 7 (Cell Signaling); anti-TRADD and anti-IκBα (BD Transduction Lab); anti-FADD (BD Pharmingen).

2.3. Analysis of cell apoptosis by flow cytometry

Treated or untreated cells grown in 6-well plates were harvested by trypsin digestion at indicated time points (including floating and adhesive cells). After washing with PBS, the cells were fixed in 70% ice-cold ethanol. Cells were recovered by centrifugation at 1000*g* for 5 min at 4 °C, washed, and treated with RNase for 30 min at 37 °C. Cells were stained with 50 μg/ml propidium iodide for 30 min at room temperature, and analyzed in a FACScan flow cytometer (BD Biosciences). For each experiment, a minimum of 20,000 cells were counted and the percentage of cells at sub-G1 was analyzed by Cellquest® (BD Biosciences).

2.4. Western blotting

Whole-cell lysates were prepared in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) with a complete protease inhibitor cocktail (Santa Cruz Biotech). Equal amounts of protein (30–50 μg) were loaded onto 10% NuPage Bis-Tris gels (Invitrogen), and electrophoresis was performed according to the manufacturer's instructions. Proteins were blotted onto nitrocellulose membranes (Bio-Rad). Blots were probed with the primary antibodies overnight at 4 °C and washed with TBS-T. These blots were incubated with the second antibodies and visualized using the Enhanced Chemiluminescent kit (Amersham Biosciences).

2.5. Real time quantitative RT-PCR

Total RNA from cultured cells was isolated by Trizol® (Invitrogen) according to the manufacturer's instruction. cDNA was prepared from 5 μg total RNA using the SuperScript III first strand synthesis system (Invitrogen) following the manufacturer's instruction. qRT-PCR was performed with SYBR green PCR Master Mix (Applied Biosystems) on an Applied Biosystems System Sequence Detector 7700HT. All reactions were assessed for quality by examination of both amplification and dissociation curves. Results were normalized to GAPDH level. The following primers were used in this study: TRADD forward primer: CGCATACCTGTTTGTGGAGTC; TRADD reverse primer: CGGTGGATCTTCAGCAATCTG; RIP1 forward primer: TGGGAAAGCACTGGAAAAC; RIP1 reverse primer: GTCGATCCTGGAACACTGGT; DR5 forward primer: CGTCCGCATAAATCAGCA; DR5 reverse primer: CAGAGCAGACTCAGCTGA; PSA forward: AGGCCTTCCCTGTACACCAA; PSA reverse: CTGTCAGAGCCTGCCAAGAT; Human GAPDH primers were purchased from Applied Biosystems.

2.6. Transfection by electroporation

Transfection by electroporation was performed as described previously [24]. Briefly, cells were mixed with plasmids and/or siRNAs in 400 μl of RPMI 1640 medium and transferred into a 4-mm cuvette (BTX Inc., San Diego, CA). Cells were electroporated with a BTX T820 square wave electroporator (BTX Inc., San Diego, CA). Transfection efficiency was monitored 12 h after transfection with enhanced green fluorescent protein (EGFP) by examining aliquots of cells under a Zeiss fluorescence microscope with a wavelength of 488 nm. Routinely, transfection efficiency of 60–80% was achieved.

2.7. Luciferase assay

For luciferase assay, cells were transfected by electroporation as described above. Cells were harvested and lysed with the Passive Lysis Buffer at 48 h after transfections. The luciferase activities in cell lysates were determined by a luciferase reporter assay system (Promega, Madison, WI) and were normalized to per microgram protein. All experiments were performed at least three times.

2.8. Statistical analysis

Data are expressed as mean ± SE and are the result of at least three separate experiments. Statistical analysis was performed using Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Androgen action on TRAIL-induced cell death is dose-dependent and biphasic

Earlier reports showed that androgen-dependent LNCaP cells are TRAIL-resistant due to increased AKT activity caused by PTEN deletion; therefore inhibition of AKT signaling by either wortmannin or LY294002 greatly sensitizes LNCaP cells to TRAIL [11,13]. This sensitization might be due to promotion of BID cleavage and/or down-regulation of the inhibitor of apoptosis protein 2 (IAP-2) [11,16]. Therefore, in our studies we used 250 nM wortmannin to sensitize LNCaP cells to TRAIL-induced apoptosis. Consistent with previous reports, 100 ng/ml TRAIL alone induced no significant cleavage of caspase-7, but pretreatment with wortmannin inhibited the constitutively activated AKT and enabled the activation of TRAIL-induced caspase-cascade (Supplemental Fig. S1).

Because previous reports have been inconsistent with respect to the action of androgen on TRAIL-induced cell death, we conducted a dose–response experiment to test whether the dose of androgen is critical for its action on TRAIL-induced apoptosis. Interestingly, the effect of androgen on TRAIL-induced apoptosis exhibits a biphasic pattern in LNCaP cells (Fig. 1A). LNCaP cells were mostly resistant to TRAIL when the androgen level was extremely low (R1881 $\leqslant$ 0.01 nM), but exhibited a maximal sensitivity to TRAIL when treated with a low level of androgen (0.1 nM R1881). However, as the androgen level was increased further, TRAIL-induced apoptosis was paradoxically reduced. Consistently, western blotting also indicated that in the absence of androgen TRAIL abrogates c-FLIP expression but induces a limited cleavage of c-PARP. The TRAIL-induced apoptosis is more significant with 0.1 nM R1881 treatment than either lower or higher doses (Fig. 1B).

Taken together, these data indicate that the effect of androgen on TRAIL-induced apoptosis is dose-dependent. Low levels of androgen appear to be superior to high levels of androgen in term of sensitizing LNCaP cells to TRAIL-induced apoptosis.