Published in final edited form as:

Cancer Lett. 2013 July 10; 335(1): 136–144. doi:10.1016/j.canlet.2013.02.001.

Androgens Regulate TRAIL-induced Cell death in Prostate Cancer Cells via Multiple Mechanisms

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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- κ B 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 two 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].

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Conflict of interest statement

All the authors have no conflict of interest to declare

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- κ B, 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].

Another important 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 μ g/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 μ g/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-I κ B α (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 minutes at 37°C. Cells were stained with 50 μ g/ml propidium iodide for 30 minutes 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 (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA) with a complete protease inhibitor cocktail (Santa Cruz Biotech). Equal amounts of protein (30–50 μ g) 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 μ g 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: CTGTCAAGAGCCTGCCAAGAT; 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 μ l 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 hours 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 hours 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 $\pm$ S.E. 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 250nM 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 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.

3.2 Androgens regulate the expression of key factors in the TRAIL signaling pathway

To understand the mechanisms underlying this biphasic pattern of androgen action, we investigated the effects of androgen on the expression of key players in the TRAIL-induced apoptotic pathway. We studied the expression of the TRAIL receptors: DR4 and DR5. LNCaP cells cultured in androgen-depleted medium expressed substantial amounts of DR4 but not DR5 (Fig. 2A). However, the expression of DR5 was significantly induced by androgen treatment, whereas DR4 expression was only slightly increased. These results indicate that the expression of DR5, but not DR4, is androgen-dependent. Apparently, the action of androgen on DR5 expression is also dose-dependent. Doses of R1881 above 0.1 nM are required for substantial DR5 expression in LNCaP cells (Fig. 2A and 2C). Consistent with these data, previous reports have also reported that androgen deprivation or

anti-androgen treatment decreases the expression of DR5, while androgens increase DR5 expression [18, 25]. To further confirm the action of androgen on the expression of DR5, we ectopically expressed AR in AR-deficient PC3 cells and treated the cells with different concentrations of androgen. Although the basal level of DR5 was relatively high in PC3-AR cells, it is clear that androgen treatment increased the expression of DR5 (Fig. 2B). Given these data, we hypothesize that DR5 mediates the action of androgen on sensitization of LNCaP cells to TRAIL-induced apoptosis.

Adaptor proteins, such as FADD, TRADD and RIP1, are also essential in TRAIL-induced signaling pathways. We therefore evaluated the effects of androgen on the expression of FADD, TRADD and RIP1. Consistent with our previous observation [24], androgen augmented TRADD expression in a dose-dependent manner (Fig. 2A and 2B). More interestingly, in contrast to the increase of TRADD, RIP1 expression in LNCaP cells was decreased by androgen treatment (Fig. 2A). The ratio of RIP1/TRADD went down as R1881 concentration increased, with the most dramatic change occurring around 0.1 nM R1881 (Fig. 2D). In contrast, FADD levels were not significantly affected by androgen treatment. As a control, PSA levels were positively regulated by R1881 in a dose-dependent manner as expected (Fig. 2A).

3.3 Androgens regulate DR5, RIP1 and TRADD via different mechanisms

To further understand how androgen regulates the expression of these proteins, we conducted a time-course experiment to evaluate changes after androgen treatment. As shown in Fig. 3A, upon treatment with 1 nM R1881, the expression of DR5, TRADD and PSA in LNCaP cells gradually increased, and RIP1 expression gradually decreased. However, these changes were not completely synchronized, suggesting different underlying mechanisms for the regulations of these factors.

We also investigated whether these genes are regulated by androgen at the mRNA level. Results from qRT-PCR showed that androgen upregulates both DR5 and TRADD mRNA but has no effect on RIP1 transcripts (Fig. 3 B–D). Pretreatment with the antiandrogen Casodex abolished androgen-induced DR5 and TRADD upregulation, suggesting that androgen transcriptionally regulates the expression of DR5 and TRADD through the androgen receptor, either directly or indirectly. As a control, PSA, a well-known androgen regulated gene, was significantly induced by androgen treatment. Further studies are needed to elucidate the exact mechanisms by which androgen regulates the expression of these factors.

3.4 Increased DR5 expression contributes to androgen-induced TRAIL sensitization in LNCaP cells

To test whether DR5 plays a role in androgen-induced TRAIL sensitization, we knocked-down the expression of DR4 and/or DR5 in LNCaP cells by siRNAs and measured TRAIL-induced apoptosis either with or without 0.1 nM R1881. Consistent with Fig. 2A, androgen-deprived LNCaP cells express very low levels of DR5, and androgen treatment significantly increased DR5 expression, which correlated with the increase of TRAIL sensitivity (Fig. 4A). Furthermore, depletion of DR5 by siRNAs significantly reduced the augmentation of the TRAIL sensitivity that was induced by androgen treatment.

Wortmannin reduces DR4 expression through a proteolytic mechanism [17]. We observed that DR4 expression was reduced in all samples treated with wortmannin and TRAIL, regardless of the androgen status. However, DR4 reduction caused either by wortmannin or by siRNAs or both, did not change the TRAIL sensitivity, suggesting that DR4 is not critical for TRAIL-induced apoptosis in LNCaP cells (Fig. 4A). Indeed, Zhang and colleagues have

previously suggested that DR4 is dispensable for TRAIL-induced apoptosis in LNCaP cells [26]. Therefore, although DR4 expression was also increased in androgen-treated cells compared to androgen-deprived cells, it is unlikely that DR4 mediates androgen-induced TRAIL sensitization. Similar to that in a variety of other cell lines [27], TRAIL-induced apoptosis in LNCaP cells is selectively DR5-dependent. Taken together, these data indicate that induction of DR5, but not DR4, mediates the sensitization to TRAIL, which could be significantly upregulated by low levels of androgen, such as 0.1 nM R1881.

3.5 Depletion of TRADD sensitizes LNCaP cells to TRAIL-induced cell apoptosis

Both TRADD and RIP1 are adaptor proteins that are critical for transduction of death receptor signaling. They are involved in death receptor-induced apoptosis, as well as NF- κ B activation and MAP kinase activation. However, it is still not clear how they function during TRAIL-induced signaling in prostate cancer cells. Therefore, we depleted TRADD or RIP1 expression by siRNAs and evaluated their roles in apoptosis during TRAIL treatment of LNCaP cells. Interestingly, we found that depletion of TRADD significantly enhanced TRAIL-induced apoptosis (39% vs. 65%, Fig. 5A). In contrast, knocking-down of RIP1 expression did not enhance the TRAIL sensitivity.

To further evaluate the role of TRADD in the action of androgen on TRAIL, we knocked down TRADD expression by TRADD siRNAs in LNCaP cells either with or without 1 nM R1881 and then treated either with or without TRAIL and wortmannin. Consistent with what we observed above, androgen increased DR5 expression and sensitized LNCaP cells to TRAIL in TRADD-competent cells. In TRADD-deficient cells, TRAIL induced more significant cleavage of caspase-3 and caspase-7 (Fig. 5B). Accordingly, TRAIL-induced apoptosis was significantly enhanced by depletion of TRADD (Fig. 5C), suggesting a protective role of endogenous TRADD in TRAIL-induced apoptosis in LNCaP cells.

3.6 Differential role of TRADD and RIP1 in TRAIL-induced NF- κ B activation

We next investigated the potential mechanism by which TRADD protects LNCaP cells from TRAIL-induced apoptosis. A previous report suggested that TRADD is critical for NF- κ B activation, which contributes to the resistance to TRAIL-induced cell death [28]. Thus, we investigated whether TRADD is involved in the regulation of NF- κ B activation. As expected, TRAIL and wortmannin treatment led to a significant reduction of I κ B α in TRADD competent cells, even in the absence of androgen (Fig. 6A, lane 1 and 2). However, in contrast to that in TRADD-competent cells, TRAIL-induced I κ B α reduction was significantly reduced in TRADD deficient cells (Fig. 6A, lane 7 and 8). This effect still existed when cells were treated with 0.1 or 1 nM R1881 (Fig. 6A, lane 10 and 12 VS. lane 4 and 6). Furthermore, use of an NF- κ B luciferase reporter also confirmed that depletion of TRADD hinders TRAIL-induced NF- κ B activation (Fig. 6B). Taken together, these data suggest that TRADD is critical for TRAIL-induced NF- κ B activation, which might contribute to its protective role in TRAIL-induced cell apoptosis. However, we also noticed that depletion of TRADD did not completely block TRAIL-induced NF- κ B activation and that androgen still generally inhibits TRAIL-stimulated NF- κ B activation while increasing TRADD expression (Fig. 6B and data not shown), suggesting that other factors are involved in the regulation of TRAIL-induced NF- κ B activation by androgen.

RIP1 is another adaptor protein that plays an important role in death receptor-induced NF- κ B activation. To investigate the role of RIP1 in TRAIL-induced NF- κ B activation, we knocked-down RIP1 expression combined with or without TRADD depletion and monitored the NF- κ B activity with a NF- κ B p65 luciferase reporter. As expected, depletion of TRADD decreased TRAIL-induced NF- κ B activation, confirming a critical role of TRADD in this pathway. Surprisingly, knock-down of RIP1 greatly increased NF- κ B activity, suggesting an

inhibitory role of RIP1 in TRAIL-induced NF- κ B activation in LNCaP cells (Fig. 6B). It is still not clear why TRADD and RIP1 have contradictory roles in TRAIL-induced NF- κ B activation. Zheng and colleagues previously suggested that TRADD and RIP1 compete for binding to TNF receptors [29]. Similar mechanism may also exist in TRAIL-induced cell signaling. Given the fact that androgen increases TRADD expression and decreases RIP1 expression in LNCaP cells (Fig. 2A and 2D), it is very likely that androgen-induced changes in the TRADD/RIP1 ratio might contribute to the protection of LNCaP cells from TRAIL-induced apoptosis by facilitating NF- κ B activation.

4. Discussion

Androgens and the AR are important for the growth and survival for prostate cancer cells. Generally, androgens protect prostate cells from cell apoptosis induced by a variety of agents, including death receptor ligands [30]. However, previous studies have been inconclusive with regards to the action of androgen on TRAIL-induced apoptosis [17–21]. In this study, we demonstrated that androgens sensitize LNCaP cells to TRAIL-induced apoptosis in a dose-dependent manner and this action presents with a biphasic pattern. Specifically, we found that low levels of androgen are superior to high levels of androgen in term of sensitizing LNCaP cells to TRAIL. This finding may help to conciliate the inconsistent observations from previous reports.

Thus, an important question is: how do androgens potentiate TRAIL-induced apoptosis? We believe that upregulation of DR5 is a key factor. Although both DR4 and DR5 can bind to TRAIL and trigger downstream signaling [31], our data suggest that DR5 is more important than DR4 in TRAIL-induced apoptosis in LNCaP cells. First, androgen-deprived LNCaP cells express reasonable levels of DR4 but are highly resistant to TRAIL-induced apoptosis (Fig. 1A and 2A). On the other hand, PC3-AR cells express a relatively high level of DR5 and are sensitive to TRAIL (Fig. 2B and supplemental Fig. 2). Second, knocking-down DR5, but not DR4, blocks TRAIL-induced apoptosis in LNCaP cells (Fig. 4). Other evidence comes from the treatment with wortmannin, which reduces DR4 expression, and promotes, instead of inhibits, TRAIL-induced apoptosis [17]. In PC3-AR cells DR5 expression is not dependent on the presence of androgens (Fig. 2B). Therefore, PC3-AR cells are sensitive to TRAIL and treatment of androgen reduces TRAIL-induced apoptosis (supplemental Fig. 2). However, in LNCaP cells DR5 expression is androgen-dependent (Fig. 2A and supplemental Fig. 3). Androgen-deprived cells lack DR5 expression, therefore, resist to TRAIL treatment. Thus, androgens upregulate DR5 expression in LNCaP cells, which sensitize cells to TRAIL-induced apoptosis.

However, our data also indicate that low levels of DR5 are sufficient to mediate TRAIL-induced apoptosis. Higher levels of DR5 are not necessarily correlated with more significant apoptosis. Dose-response data indicate that 0.1 nM R1881 induced only a moderate level of DR5 expression but rendered maximal TRAIL-induced apoptosis (Fig. 1A, 2A and supplemental Fig. 3). Indeed, further increases of DR5 with high doses of androgen are associated with a decline in cell sensitivity to TRAIL. Taken together, our data demonstrate that low levels of androgen are sufficient to induce moderate levels of DR5 expression and sensitize LNCaP cells to TRAIL-induced apoptosis. Although high levels of androgen further increase DR5 expression, TRAIL-induced apoptosis declines, suggesting that some pro-survival factors are induced by high levels of androgen.

How do high levels of androgen promote cell survival? NF- κ B activation and upregulation of anti-apoptotic factors are the most likely answers. Although NF- κ B activation is only weakly induced by TRAIL, it has been well demonstrated that NF- κ B activation is critical for inhibition of apoptosis [32, 33]. NF- κ B induces expression of c-FLIP, Bcl-xL, Bcl-2,

xIAP and other pro-survival factors [7]. We previously showed that TRADD is critical for TNF- α -induced NF- κ B activation in prostate cancer cells [24]. Here we demonstrate that TRADD is also important for TRAIL-induced NF- κ B activation. TRADD has been considered to be a pro-apoptotic protein, because overexpression of TRADD leads to cell apoptosis, while TRADD deficiency blocks TNF- α -induced cell death [34, 35]. However, recent studies have suggested that TRADD in fact could be either pro-survival or pro-apoptotic, depending on the cellular milieu [28, 36]. Indeed, the role of TRADD in LNCaP cells is most likely pro-survival, because TRADD deficiency reduces NF- κ B activation and enhances TRAIL-induced apoptosis (Fig. 5A–C and 6B).

Another interesting finding in our study was that RIP1 deficiency increases TRAIL-induced NF- κ B activation (Fig. 6B). The role of RIP1 in TRAIL-induced NF- κ B activation and apoptosis is largely undefined in prostate cells. We suspect that TRADD and RIP1 may compete with each other for binding to receptors or FADD, and that the ratio of TRADD/RIP1 is critical for balancing cell survival and death. Given the fact that androgens increase TRADD expression but decrease RIP1 expression, it is very likely that androgen-induced increase of the TRADD/RIP1 ratio actually contributes to cell resistance to TRAIL-induced apoptosis.

Androgens also upregulate a variety of anti-apoptotic proteins, including c-FLIP and survivin [20, 37, 38]. Survivin is a member of the inhibitors of apoptosis protein (IAP) family and has an important role in inhibition of apoptosis. Aberrant expression of survivin has been found in various types of cancer including prostate cancer, and targeting survivin is considered to be a promising therapy for cancer [39]. Consistent with a previous report [38], we observed that androgens enhance the expression of survivin, but not IAP1 or XIAP, in a dose dependent manner (Fig. 6A and supplemental Fig. 3 and 4). Therefore, it is likely that androgens inhibit TRAIL-induced apoptosis through upregulation of survivin. However, the dose-response data indicate that only a low level of androgen is required for survivin expression. Its expression peaked at 0.1 nM R1881 and decreased with exposure to higher levels of androgen treatment (supplemental Fig. 3 and 4). This means that survivin expression is positively, but not negatively, correlated with TRAIL-induced cell apoptosis. Therefore, although survivin is upregulated by androgens and might contribute to inhibition of cell apoptosis, apparently, it is not a major player in the regulation of TRAIL-induced apoptosis in prostate cancer cells. More studies are needed to elucidate the contribution of survivin to androgen-mediated cell survival.

In summary, we found that androgens dynamically regulate TRAIL-induced apoptosis in LNCaP cells in a dose-dependent manner. Generally, androgens sensitize LNCaP cells to TRAIL-induced apoptosis, but this effect is dose-dependent and biphasic. Low levels of androgen greatly sensitize cells to TRAIL, probably via upregulation of DR5 expression, which plays a pivotal role in TRAIL-induced apoptotic signaling. High levels of androgen have less potential to sensitize LNCaP cells to TRAIL than low levels of androgen, even though DR5 expression is high. We hypothesize that androgen-induced changes in the TRADD/RIP1 ratio contribute to inhibition of apoptosis via enhancement of NF- κ B activation. The knowledge that TRAIL sensitivity is maximized in the presence of a low level of androgen may provide clues for improving TRAIL efficacy in prostate cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Lucy J. Schmidt for assistance to this study. The work was supported by NIH grants CA121277, CA91956, CA15083, CA125747, DK65236 and the T.J. Martell Foundation.

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Figure 1. Androgen action on TRAIL-induced cell death is dose-dependent and biphasic
(A) LNCaP cells were cultured in CSS medium and treated with various doses of R1881 for 48 hours as indicated. Cells were pre-treated with 250 nM wortmannin for 1 hour, followed by 100 ng/ml TRAIL for 16 hours. Cell apoptosis was assessed by flow cytometry with propidium iodide staining. The percentage of cells at sub-G1 is the results of three independent experiments and presented as mean $\pm$ SE. **(B)** LNCaP cells were grown in CSS medium and treated with 0, 0.1 or 1 nM R1881 for 48 hours and followed with or without 100 ng TRAIL and 250 nM wortmannin for 6 hours. Cell lysates were prepared and equal

amounts of protein were analyzed by Western blotting. Equal protein loading was demonstrated by immunoblotting for ERK2.

Figure 2. Androgens regulate the expression of key factors in the TRAIL signaling pathway (A) LNCaP cells were cultured in CSS medium and treated with various doses of R1881 for 48 hours. Cell lysates were prepared and equal amounts of protein were analyzed by Western blotting with according antibodies. (B) PC3 cells were transfected with AR by electroporation. Cells were cultured in CSS medium for 24 hours. Medium was then removed and cells were washed by PBS. CSS medium was refilled and cells were treated with various doses of R1881 for 48 hours. Cell lysates were prepared and equal amounts of protein were analyzed by Western blotting with according antibodies. (C) LNCaP cells were treated as (A) and DR5 expression was quantitated from three experiments. (D) LNCaP cells were treated as (A). TRADD and RIP1 expression were quantitated from three independent Western blots and the ratio of TRADD/RIP1 was visualized.

Figure 3. Androgens regulate DR5, RIP1 and TRADD via different mechanisms

(A) LNCaP cells were cultured in CSS medium and treated with 1 nM R1881. Cells were harvested and lysed in RIPA buffer at time points as indicated. Equal amounts of protein were analyzed by Western blotting with according antibodies. (B–E) LNCaP cells were pre-treated with or without 10μM Casodex for 1 hr followed by ETOH or 1 nM R1881 for 48 hrs. Relative levels of DR5, RIP1, TRADD and PSA were measured by qRT-PCR using GAPDH as an internal control. Results are representative of three independent experiments and presented as mean ± SE. *, $P < 0.05$;

A

Figure 4. Increased DR5 expression contributes to androgen-induced TRAIL sensitization in LNCaP cells

(A) LNCaP cells were transfected with si-DR4 or si-DR5 and treated with or without 0.1 nM R1881 for 48 hours as indicated. Cells were then treated with 250nM wortmannin for 1 hour followed by 100 ng/ml TRAIL for 16 hours. Cells were collected and fixed in 70% ETOH. The percentage of cells at sub-G1 was measured by flow cytometry with propidium iodide staining. Results are representative of three independent experiments and illustrated as mean $\pm$ SE. *, $P < 0.05$ (upper). DR4, DR5 and tubulin expression is shown by Western blotting accordingly (bottom).

Figure 5. Depletion of TRADD sensitizes LNCaP cells to TRAIL-induced cell apoptosis
 (A) LNCaP cells were transfected with indicated siRNAs and cultured in medium containing 9% FBS. Cells then were treated with or without 250 nM wortmannin and 100 ng/ml TRAIL for 16 hours. Cells were collected and analyzed by flow cytometry with propidium iodide staining. Results are representative of three independent experiments and illustrated as mean $\pm$ SE. *, $P < 0.05$ (B) LNCaP cells were transfected with no target siRNA or TRADD siRNA and treated with 1 nM R1881 for 48 hours. Cells were pretreated with 250 nM wortmannin for 1 hour, followed by 100 ng/ml TRAIL for 6 hours. Cell lysates were analyzed by Western blotting by according antibodies. (C) LNCaP cells were treated as (B) except TRAIL treatment was extended to 16 hours. Apoptotic rate was measured by sub-G1 staining as described in (A). Results are representative of three independent experiments and illustrated as mean $\pm$ SE. *, $P < 0.05$

Figure 6. Differential roles of TRADD and RIP1 in TRAIL-induced NF- κ B activation

(A) LNCaP cells were transfected with no target siRNA-A or si-TRADD and treated with indicated concentrations of R1881 for 48 hours. Cells were pre-treated with 250 nM wortmannin for 1 hour, followed by 100 ng/ml TRAIL for 6 hours. Cell lysates were prepared, and equal amount of total protein was analyzed by Western blotting with according antibodies. (B) LNCaP cells were transfected with 2 μ g NF- κ B p65-luc plasmids and 100 nmol siRNAs as indicated. Transfected cells were treated with PBS or 100 ng/ml TRAIL for 24 hours and cell lysates were analyzed for luciferase activity. The luciferase units in each sample were normalized to 1 microgram total protein. Results are representative of three independent experiments and illustrated as mean $\pm$ SE. *, $P < 0.05$; **, $p < 0.01$ (upper). Cell lysates were also analyzed by Western blotting. RIP1 and TRADD expression is shown at the bottom.