[Frontiers in Bioscience 11, 1549-1568, May 1, 2006]

APO2 ligand/tumor necrosis factor-related apoptosis-inducing ligand in prostate cancer therapy

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1. ABSTRACT

Prostate cancer is one of the most common cancers in men and is the second leading cause of cancer-related death in the USA. Many anti-tumor agents against prostate cancer cells have been developed, but their unacceptable systemic toxicity to normal tissues usually limits their use in the clinic. Apo2 ligand (Apo2L), also called Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), is one of several members of the TNF gene superfamily that induces apoptosis through engagement of death receptors. This protein has generated tremendous enthusiasm as a potential tumor-specific cancer therapeutic because, as a stable trimer, it selectively induces apoptosis in many transformed cells, but not in most normal cells. In this review we discuss its potential use in prostate cancer therapy, the mechanisms by which induces apoptosis or that underlie resistance to it, and strategies for sensitization to overcome them. Conventional chemotherapeutic and chemopreventive drugs, irradiation, and other therapeutic agents, such as histone deacetylase inhibitors or retinoids can sensitize Apo2L/TRAIL-resistant cells and tumors. Investigating the apoptotic effects of Apo2L/TRAIL, a unique tumor-specific cell death ligand, now in clinical trials, alone or in combination may not only help in understanding its antineoplastic role in prostate carcinoma but may also provide insights into basic mechanisms of apoptosis.

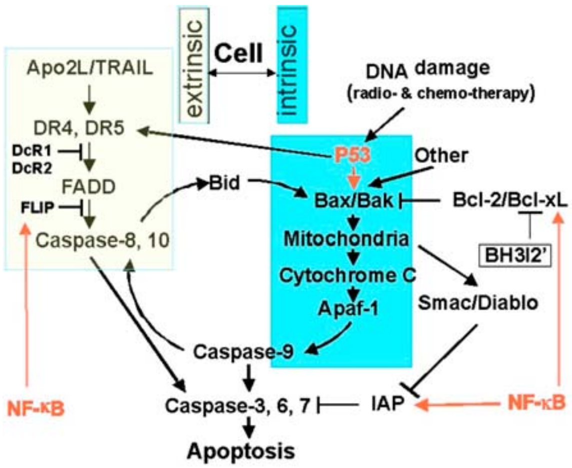

Figure 1. Schematic model of cell-extrinsic and cell-intrinsic pathways of apoptosis. The apoptosis signaling pathways can be activated through the cell-extrinsic and cell-intrinsic pathways. The extrinsic pathway engages Apo2L/TRAIL through DR4 and DR5, with it's effect being prevented by the DcR1 and DcR2 decoys and FLIP. The intrinsic pathway requires mitochondrial localization and activation of Bax and Bak that can be prevented by anti-apoptotic Bcl-2 family proteins or pharmacologic inhibitors, such as BH3I-2'. Crosstalk between the extrinsic and intrinsic pathways requires cleavage of Bid. p53, once activated following DNA damage, controls expression of critical apoptotic genes, such as Bax, and DR5. In contrast, NF-kappaB controls expression anti-apoptotic genes, such as Bcl-xL, Bcl-2, FLIP, and the inhibitors of apoptosis (IAP) (modified from 1, with permission).

2. INTRODUCTION

2.1. Receptors that bind Apo2L/TRAIL

Apo2 ligand [Apo2L; also named Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)] is one of several members of the TNF gene superfamily that induces apoptosis through engagement of death receptors (1). Apo2L/TRAIL acts on five receptors, two of which contain cytoplasmatic "death domains" and signal apoptosis, and the other three that act as "decoys": death receptor 4 (DR4) (2), death receptor 5 (DR5) (3, 4), decoy receptor 1 (DcR1) (4), decoy receptor 2 (DcR2) (5), and osteoprotegerin (OPG) (6). DcR2 has a truncated, non-functional cytoplasmatic death domain, while DcR1 and OPG lack a cytosolic region and are anchored to the plasma membrane through a glycophospholipid moiety. OPG was discovered first to bind TNF superfamily member RANKL, but later found to also bind Apo2L/TRAIL.

The Apo2L/TRAIL gene, that spans approximately 20-kb and contains five exons, contains a promoter region with several putative transcription factor-binding sites (7). Experiments with reporter constructs indicated transcriptional regulation by signal transducers and activators of transcription STAT (7, 8), NF-kappaB (9-11) IRF-1 (12), IRF-3 (13), forkhead (14, 15), with FKHRL1 and FKHR having been examined in prostate carcinoma (14).

Expression of several of the Apo2L/TRAIL receptors can be regulated during therapy, mostly in a p53-dependent manner, by irradiation and a variety of therapeutic agents (3, 16). A recent study also revealed aberrant methylation of DcR1 or DcR2 in: prostate cancer (60%), primary breast cancer (70%), primary lung cancer (31%), bladder cancer (42%), cervical cancer (100%), ovarian cancer (43%), primary malignant mesothelioma (63%), lymphoma (41%), leukemia (26%), and multiple myeloma (56%). Methylation of DR4 and DR5 was found rarely in any of the tumor types examined, with methylation of all these 4 receptors being uncommon in non-malignant tissues. It seems that aberrant methylation, however, was the cause for silencing of DcR1 and DcR2 expression (17).

2.2. Apoptosis activation: Apo2L/TRAIL as a critical trigger of the extrinsic apoptosis pathway

Apo2L/TRAIL acts on the cell-extrinsic signaling pathway, inducing apoptosis through activation of the DR4 and/or DR5 receptors (Figure 1). Similar to FasL, Apo2L/TRAIL initiates apoptosis upon binding to its cognate death receptors and inducing the recruitment of specific cytoplasmic proteins to the intracellular death domain of the receptor, which form the death-inducing signaling complex (DISC) (18). In cells that express both DR4 and DR5, these receptors can form heterocomplexes (19). DR4 and DR5 each can recruit and activate Caspase-8 (19) and Caspase-10 through the Fas associated death domain (FADD) adaptor protein domain. Caspase-10 is recruited to and activated at the native Apo2L/TRAIL and CD95 DISC in a FADD-dependent manner and can functionally substitute for Caspase-8 (20). These apical caspases will further activate the effector caspases, such as Caspase-3, Caspase-6, and Caspase-7 (21). Activated effector caspases can cleave a number of cellular proteins, such as PARP [poly(ADP-ribose) polymerase] (22), resulting in apoptosis. Apo2L/TRAIL acts independently of p53, making it a potentially effective weapon against chemoresistant or radioresistant tumors (1, 23).

Apoptosis can be activated by the intrinsic pathway, which implicates mitochondria and the Bcl-2 family of apoptotic proteins, and the extrinsic pathway, that involves the death receptors. The link between the cell-extrinsic and cell-intrinsic signaling pathways is mediated by the proapoptotic Bcl-2 family protein Bid, which is cleaved and activated by Caspase-8. Active Bid then further activates Bax or Bak and thus amplifies apoptosis induction through the cell-intrinsic pathway. The precise mitochondrial membrane damage produced, for example, by the translocation of activated Bax to mitochondria, is still unclear. Subcellular relocalization is usually accompanied by conformational changes of Bax or Bak, together with their full insertion into mitochondrial membranes as homo-oligomerized multimers, resulting in the formation of large protein-permeable pores. This results in the release of apoptogenic factors, caspase-activating or caspase-independent death effectors, such as cytochrome *c*, Smac/DIABLO, Omi/HtrA2 serine proteases, apoptosis inducing factor (AIF), and EndoG (21, 24, 25). In the cytosol, cytochrome *c* binds to Apaf-1 in a dATPdependent manner, causing its oligomerization (26). Apaf-1 then recruits Caspase-9, which once activated, in turn, stimulates the effector caspases (26, 27). In some cell lines, death receptor engagement of the cell-extrinsic pathway is sufficient to induce apoptosis; however, in most cell types, apoptosis requires amplification of the cell-extrinsic pathway through the cell intrinsic pathway (28). Finally, apoptotic bodies are formed, recognized by specialized phagocytes and neighboring cells and cleared by phagocytosis (29, 30).

3. Apo2L/TRAIL-INDUCED APOPTOSIS IN PROSTATE CELLS

Caspase-8 activation is necessary, but not sufficient, for Apo2L/TRAIL-mediated apoptosis and is presumably blocked downstream of Caspase-8 by the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, in LNCaP cells (31). In androgen-independent PC-3 and DU-145 cells, treatment with Apo2L/TRAIL caused a rapid apoptotic cell death, whereas TNF-alpha was ineffective unless it was used in the presence of the protein synthesis inhibitor cycloheximide. The induction of apoptosis by Apo2L/TRAIL in PC-3 cells was mediated by DR4 and the downstream caspases (32).

Human prostate normal epithelial cells (PrEC) were found to be resistant to Apo2L/TRAIL-induced apoptosis (33, 34). However, one study suggested that Apo2L/TRAIL is capable of inducing apoptosis in PrEC, as efficiently as in some tumor cell lines (35). PrEC were found to contain fewer Apo2L/TRAIL DcR1 and DcR2 receptors. This finding was interpreted as an inability to block the Apo2L/TRAIL-triggered apoptotic signal: lack of decoy receptors in these cells was suggested to be responsible for the ability of these untransformed normal cells to respond to Apo2L/TRAIL. This result suggests the possible link between the unusual sensitivity of PrEC to Apo2L/TRAIL and their deficiency in anti-apoptotic decoy receptors and the possibility that Apo2L/TRAIL could be a useful treatment for the early stages of prostate cancer, because the majority of prostate cancers are derived from epithelial cells (35). However, these results have not been reproduced with well-characterized trimeric Apo2L/TRAIL preparations and therefore have to be interpreted with caution.

3.1. Role of Bcl-2 family members

A critical step in the cell-intrinsic pathway is the activation and translocation of the Bcl-2 family member Bax to the mitochondria, leading to the dissipation of the mitochondrial transmembrane potential and cytochrome *c* release to the cytosol. This facilitates assembly of the Apaf-1 apoptosome with recruitment and activation of Caspase–9, as an initiator caspase, and subsequently, the effector caspases. Multi-domain pro-apoptotic members of the Bcl-2 family such as Bax, or its homologue Bak, contain 3 Bcl-2 homology domains (BH1-3). These proteins are counteracted by the anti-apoptotic family members Bcl-2 or Bcl-xL, that contain an additional BH4 domain. A subset of the Bcl-2 family proteins contain only the BH3 domain, such as Bid, Bik, Bim, NOXA, and PUMA. BH3-domain-only proteins interact with the anti-apoptotic Bcl-2 family members to block their function or, alternatively, with pro-apoptotic Bcl-2 family members to augment their activity (1).

Experiments with LNCaP-derived C4-2 cells in which Bax expression was knocked-down by small inhibitory RNA molecules (siRNA) demonstrated Bax requirement for Apo2L/TRAIL-mediated apoptosis, even though Bak was expressed in these cells (36). Also, DU-145 prostate carcinoma cells that have lost Bax protein expression due to mutation, failed to release cytochrome *c* and to activate Caspase-3 and Caspase-9 when exposed to Apo2L/TRAIL (37). Similar findings were reported in a colon carcinoma cell line that carries a Bax gene deletion or selected for Bax mutation (38). Mitochondrial depolarization, cytochrome *c* release, activation of Caspase–9, and effector caspases were prevented in Bax-deficient cells. Thus, in these cells, the intrinsic pathway was required for Apo2L/TRAIL-mediated apoptosis, with Bax being essential for induction of the mitochondrial events. These findings in human cells (36-38), recently confirmed by several other reports, were surprising since, based on the mouse knock-out studies, it has been suggested that Bax and Bak play redundant roles, and inactivation of both molecules is required to fully disrupt apoptotic signaling by death receptors.

In PC-3 prostate carcinoma cells it was shown that Bcl-xL has a more important role in Apo2L/TRAIL-induced apoptosis compared to Bcl-2, as down-regulation by siRNA-mediated knockdown of Bcl-xL, but not Bcl-2, markedly amplified Apo2L/TRAIL-induced apoptosis. Knockdown of Bcl-xL and administration of Apo2L/TRAIL significantly synergized in dissipation of mitochondrial membrane potential (MMP), release of cytochrome *c*, activation of Caspase-9, and Caspase-3, and consequently, apoptotic cell death. In contrast, knockdown of Bcl-2 did not affect any of these activities (39).

3.2. Role of NF-kappaB

NF-kappaB acts as a survival factor by protecting tumor cells from Apo2L/TRAIL, TNF-alpha, radiotherapy, and chemotherapy (10, 11, 40, 41). NF-kappaB has been reported to induce expression of FLIP, Bcl-xL, Bcl-2, and XIAP, which are considered to be responsible for its protection against cell death. LNCaP cells express constitutively active nuclear NF-kappaB, which was suggested to mediate resistance of LNCaP cells to Apo2L/TRAIL, by inhibition of caspases and Bid activation. Inhibiting NF-kappaB activation, confers enhanced sensitivity of these tumor cells to Apo2L/TRAIL (42, 43). Androgen dependent cells (LNCaP and LAPC4), in comparison with those independent of androgen (DU-145), have lower levels of basal NF-kappaB activity and seem to be more sensitive to proteasome inhibition (e.g. by bortezomib) (44). Interestingly, NF-kappaB is also found in mitochondria of prostatic carcinoma cells, where it is thought to regulate mitochondrial genome-encoded mRNA levels in response to Apo2L/TRAIL treatment. Apo2L/TRAIL affects DNA binding activity of mitochondria-associated NF-kappaB, but does not change the amount of NF-kappaB subunit p65 in mitochondria, which suggests activation of mitochondrial NF-kappaB without additional translocation of NF-kappaB subunits to mitochondria (45).

3.3. Other forms of cell death

The natural occurrence of both Apo2L/TRAIL-induced apoptotic and necrotic signaling mechanisms within tumor cells has been suggested. Transfection of murine Apo2L/TRAIL and transduction of a recombinant adenovirus encoding the murine Apo2L/TRAIL cDNA (Ad5-mTRAIL) in two murine tumor cell lines, TRAMP-C2 (prostate adenocarcinoma) and Renca (renal adenocarcinoma), has indicated that mApo2L/TRAIL can also kill tumor cells by inducing necrosis (46).

Other types of cell death, such as autophagy and mitotic catastrophe have been also implicated in the cytotoxic response to chemotherapeutic drugs of epithelial cells, such as those of prostate or breast. Such alternative modes of cell death have been suggested also for Apo2L/TRAIL used alone or in combination with other treatments in different prostate cancer cell types. Recently, it was shown that treatment with exogenous Apo2L/TRAIL induces extensive autophagy in monolayer and 3D cultures of MCF-10A mammary epithelial cells (47). Autophagy is a bulk-protein-degradation process characterized by the formation of double-membrane vacuoles, called autophagic vacuoles, which deliver cytoplasmic contents and organelles to the lysosome for destruction. Increasing evidence suggests that autophagy contributes to programmed cell death (48). By contrast, mitotic catastrophe is a form of cell death resulting from the failure of mitosis. DNA damage induces mitotic catastrophe in mammalian cells, which results in the formation of cells with two or more nuclei (49-51). Recently, it was shown that the DNA damage-induced mitotic catastrophe can be mediated by the Chk1-dependent mitotic exit DNA damage checkpoint (52), although this phenomenon has been observed primarily in p53-deficient cells and has not been reported for Apo2l/RTRAIL-treated cells.

4. PROSTATE CANCER MODELS FOR DETERMINING SENSITIVITY TO APO2L/TRAIL-INDUCED APOPTOSIS

4.1 Apo2L/TRAIL variants

The extrinsic pathway of apoptosis may be engaged with either Apo2L/TRAIL or agonisitc antibodies directed against the receptors. Some variation between the effectiveness of Apo2L/TRAIL against tumors in the published reports is likely caused by the use of various recombinant versions of human Apo2L/TRAIL. One version contains Apo2L/TRAIL amino acids 114-281, which were fused to an amino-terminal polyhistidine tag (53). A second version contains Apo2L/TRAIL amino acids 95-281, which were fused amino terminally to a modified yeast Gal-4 leucine zipper that promotes trimerization of the ligand (54). A third version contains residues 95-281, which were fused to an amino-terminal Flag epitop tag, crosslinked to anti-Flag antibodies enhancing its activity against certain cell lines. (8, 55) A fourth, recombinant version of the ligand, which is preferred for clinical applications, is now tested in Phase I clinical trials. This variant contains amino acids 114-281 of human Apo2L/TRAIL without any added exogenous sequences and is therefore the least likely to be immunogenic in human patients. The production of this variant was optimized by adding Zn and reducing agent to the cell culture media and extraction buffers, and by formulation of the purified protein at neutral pH (56, 57). Besides using the recombinant ligand, which has a limited stability and therefore biological effectiveness, one might envision alternative modalities of expression of Apo2L/TRAIL for therapeutic purposes, such as its expression using a gene therapy approach. Another approach is to engage the death receptors DR4 or DR5 directly, with agonistic antibodies (58, 59). However, while effective, there have been few studies directed towards understanding the mechanism of apoptosis using this approach.

4.2 Cell lines

There are over 200 human prostate cancer cell lines and derivative sublines used in prostate cancer research. An online database of these prostate cancer cell lines is freely accessible via the World Wide Web, at http://www.CaPCellLines.com (60, 61). (Table 1) summarizes the most widely used prostate cancer cell lines. While some of these prostate cancer cell lines are androgen-dependent, such as LNCaP and LAPC4, others are androgen-independent, such as DU-145 and PC-3, with LNCaP-derived C4-2 being characterized as androgen hypersensitive (89). Many of these cell lines are clonal derivatives, which were selected for acquired biological activities (e.g. androgen independence) or stable expression of exogenous genes.

Different prostate cancer cell lines, LNCaP, LNCaP C4-2, LNCaP-Bcl-2, DU-145, PC-3, PC3AR, PC3Neo, PC3Bcl-2, PC-3M, PC3-TR, PC-93, LAPC4, CL-1, ALVA-31, DuPro, CWR22Rv1, PPC-1, and DU-145 respond with different sensitivities to Apo2L/TRAIL. For example, ALVA-31, PC-3, and CWR22Rv1 are highly sensitive to apoptosis induced by Apo2L/TRAIL, while PPC-1 is moderately sensitive, with LNCaP, LNCaP-derived C4-2, DuPro, and PC3-TR being the most resistant (33, 34, 36, 66-69, 74). Most of the studies have shown that DU-145 cells are sensitive to Apo2L/TRAIL-induced apoptosis (33, 34, 66, 67) or moderately sensitive (68). However, there are also studies which concluded that the DU-145 cell line is resistant to Apo2L/TRAIL-induced apoptosis (69). These discrepancies are likely to be based on differences in experimental conditions used, particularly the nature of the Apo2L/TRAIL preparation (see 4.1).

Other cell lines were also used in the past for prostate cancer research. Two are particularly important to mention: TSU-Pr1, which was found to be resistant (33, 69), moderately sensitive (66) or sensitive (34) to Apo2L/TRAIL-induced apoptosis and JCA-1, described as being moderately sensitive (66, 69). However, recently, it has been pointed out that these two cell lines seem to be derivatives of the T24 bladder carcinoma cells and

Table 1 Commonly used human prostate cancer cell lines: characteristics and responsiveness to Apo2L/TRAIL

Cell line	Origin	Characteristics	Sensitivity to Apo2L/TRAIL
DU-145	Initiated from a brain metastasis of a prostatic carcinoma from a 69-year-old male Caucasian; was the first prostate cancer cell line to be established in tissue culture (60, 62).	Number of chromosomes: 46 to 143; androgen independent; express low levels of PAP and fail to express AR, PSA, or hK2; also express CK-7, 8, 18, & 19, but fail to express CK-5 & 14 (63, 64); s.c. injections into nude mice produces tumors phenotypically & genotypically similar to parental cells (65).	**sensitive** (33, 34, 66, 67) **moderately sensitive** (68) **resistant** (69)
LNCaP	Isolated from a needle aspiration biopsy of a lymph node metastatic lesion from a 50-year-old white man (70).	Highly aneuploid, chromosome number: 33-91; androgen dependent; androgen receptor (AR) and estrogen receptor are expressed, but AR contains a T877A mutation, which results in a somewhat promiscuous response to steroids (71, 72); express CK-8 and 18, and have a wtTP53 (73); the original line formed tumors in ~ 50% of nude mice when injected s.c. (71).	**resistant** (33, 66-69, 74)
C4-2 (LNCaP subline)	It was established by co-injecting the C4 line with human MS osteosarcoma cells into castrated hosts and harvesting cells from the resultant tumor (75).	Chromosomal markers similar to those of parental LNCaP cells and distinct from those of the MS bone stromal cell line; androgen hipersenzitive; consistently metastasize to lymph nodes and bones when injected s.c. or orthotopically into intact or castrated mice (60, 76, 89).	**resistant** (36)
PC-3	Initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian (77).	Are 100% aneuploid; express CKs 5, 8, & 18, and contain a frameshift mutation in TP53 that results in a premature stop codon; androgen independent; injected s.c into athymic nude mice form tumors that are 1-3 cm in diameter within 60 days (60, 73, 78); neither PC-3 nor its sublines express the RNA or protein of AR, PSA or hK2; express normal levels of PAP, and CK-8 and 18, and fail to express CK-5 and 15 (79).	**sensitive** (33, 34, 66-69, 74)
PC-3M (PC-3 subline)	Established from a PC-3 xenograft; is more aggressive than the parental xenograft (87, 88).	About 75% of cells have 60 to 61 chromosomes, the rest being pentaploid or hexaploid; androgen independent (79); form tumors in mice.	**sensitive** (67)
ALVA-31	From a biopsy specimen of primary tumor obtained during prostatectomy (80); same origin, derivative from, or contaminated by PC-3 (60, 73, 81).	Chromosome number: 24 -12; androgen dependent; homozygous deletion at D10S541 & an identical TP53 gene mutation with PC-3 and PPC-1 (73, 81); forms tumors in mice.	**sensitive** (34, 66)
PPC-1	Derived from transurethral resection of the prostate in a 67-year-old black male patient with advanced stage D2 cancer. (82); same origin, derivative from, or contaminated by PC-3 (60, 83-85).	Abnormal karyotype, hypotetraploid cells (82); exhibit relaxed growth factor requirements and anchorage independent growth, and they are highly tumorigenic in nude mice (86).	**moderately sensitive** (69)

therefore are not of prostatic origin (90). Even after this paper was published, TSU-Pr1 and JCA-1 continued to be used in prostate cancer research by several laboratories (60), with the number of articles using them for prostate cancer research continuing to increase. From 6 articles on TSU-Pr1 published between January 2003-March 2004 (60), the number increased to 13, with 5 articles using JCA-1, between January 2003-August 2005. Surprisingly, even though their significance is in question, most of these reports have been published in quite reputable journals.

When treated with 100 ng/ml Apo2L/TRAIL, we estimate that less then 10-15% killing was observed in Apo2L/TRAIL resistant prostate cancer cell lines LNCaP, DU-145, and DuPro, around 20% killing in Apo2L/TRAIL slightly sensitive PPC-1 cancer cell line and 30% and 50% killing in Apo2L/TRAIL sensitive cancer cell lines PC-3 and CWR22Rv1, respectively (69). Also, re-expression of the androgen receptor (AR) in PC-3 cells (PC-3AR) reduced survival to ~ 41%, as compared to ~ 82% observed in the PC-3Neo controls (43).

4.3. Preclinical models

The most widely used preclinical model is that of xenografts of human prostate cancer cells, mostly introduced subcutaneously (s.c.) in athymic mice. Mostly, PC-3 (91), LNCaP-derived C4-2 (36), and DU-145 (92) human prostate tumor xenografts, grown in athymic nude mice, were used for determining sensitivity *in vivo* to Apo2L/TRAIL-induced apoptosis, when Apo2L/TRAIL was used alone or in combination with chemotherapeutic drugs, such as paclitaxel, etoposide, doxorubicin, camptothecin (or its derivative CPT-11; irinotecan), or with X-rays (Ray and Almasan, unpublished data). As shown in (Table 2) and further discussed in section 6.7, some of these treatments were quite effective in reducing tumor burden and sometimes leading to complete cure of the animals (36).

Other mouse models can also be effective for testing Apo2L/TRAIL efficiency and for analyzing the molecular modifications and signal transduction pathways *in vivo*. Use of mouse models, in which distinct genetic lesions can be correlated with the tumor phenotype, would not only help to understand the biochemical pathways responsible for the different prostate cancers, but also provide valuable systems in which to test pathway-targeted therapies.

5. MECHANISMS OF RESISTANCE OF PROSTATE CANCER CELLS TO APO2L/TRAIL

A wide-range of molecular mechanisms have been attributed to the cellular resistance to apoptotic stimuly. These include: elevated Akt activity, lack of active lipid phosphatase PTEN, expression of eNOS, constitutively active NF-kappaB, androgen deprivation (in androgen dependent prostate cancer cells), persistent c-FLICE-inhibitory protein c-FLIPL expression, a mechanism involving GSK-3beta activation, XIAP expression, c-Jun N-terminal kinase (JNK) activation, Bcl-xL and Bcl-2 overexpression, PKCeta and OPG levels.

Surprisingly**,** some studies concluded that the susceptibility of various prostate cancer cell types to Apo2L/TRAIL-induced apoptosis did not appear to correlate with the levels of the Apo2L/TRAIL death receptor DR4 or DR5, decoy receptors DcR1 and DcR2, Flame-1, Bax, Bak, or the IAP family of proteins (34, 68). However, it has been shown that while mitochondrial response to Apo2L/TRAIL is limited in LNCaP cells, mitochondria from these cells are capable of responding to apoptotic stimuli (93).

5.1. Bcl-2 and Inhibitors of Apoptosis Proteins

The Bcl-2 and inhibitors of apoptosis (IAP) family of proteins are the best characterized molecular determinants for the mechanism of resistance to various apoptotic stimuli. In PC-3 prostate carcinoma cells, it was shown that Bcl-xL has a more important role in Apo2L/TRAIL-induced apoptosis compared to Bcl-2. Down-regulation of Bcl-xL, but not Bcl-2, markedly amplified Apo2L/TRAIL-induced apoptosis (38). However, PC-3 cells were shown to be sensitive to Apo2L/TRAIL treatment, whereas derivative PC-3 cells overexpressing Bcl-2 were resistant. Bcl-2 overexpression did not affect Caspase-8 activation, however it did change the processing pattern of Caspase-3. Bcl-2 overexpression inhibited the activation of mitochondrial localized Caspase-2, Caspase-7, and Caspase-9 and abrogated Apo2L/TRAIL-induced cytochrome *c* release and dissipation of mitochondrial membrane potential (94).

The role of X-linked inhibitor of apoptosis (XIAP) in apoptosis resistance was demonstrated by overexpression of Smac/DIABLO, which inhibited IAPs and sensitized the cells to Apo2L/TRAIL. The earliest and the most pronounced change induced by actinomycin D (ActD) in prostate cancer cells was down-regulation of XIAP. Both of these treatments sensitized prostate cancer cells to Apo2L/TRAIL-induced apoptosis (95-97). Moreover, persistent c-FLIPL expression was shown to be necessary and sufficient to maintain resistance to Apo2L/TRAIL-mediated apoptosis in prostate cancer. In contrast to sensitive cells, Apo2L/TRAIL-resistant LNCaP and PC3-TR (an Apo2L/TRAIL-resistant subpopulation of PC-3) cells showed increased c-FLIPL mRNA levels and maintained steady protein expression of c-FLIPL after treatment with Apo2L/TRAIL (74).

5.2. Survival factors

In addition to known apoptosis modulators, survival factors play a critical role in the prostate cancer cellular response to various therapeutics. Of these, androgen signaling plays key roles in the development and progression of prostate cancer.

Phosphorylation of the androgen receptor (AR) by Akt results in a decrease in its transcriptional activity, which is associated with a decrease in the ability of AR to interact with ARA70 (98). Also, phosphorylation-dependent ubiquitination and degradation of AR by Akt require Mdm2 E3 ligase activity (99). In contrast, stimulation of MAPK by overexpression of the ErB2/Her2/Neu proto-oncogene, a member of the epidermal growth factor (EGF) family, is postulated to enhance AR-dependent transcription through phosphorylation of AR (100). The protein kinase A (PKA) activation was shown to activate AR in the absence of the androgen (101). Recently, it was shown that FOXO3a (forkhead), a PI3K/Akt downstream substrate, induces directly AR gene expression (102). By inhibiting PI3K/AKT activity, PTEN negatively regulates phosphorylation of MDM2 and its subsequent nuclear translocation, thus augmenting the level and function of p53 (103). P53 impacts on expression of several Bcl-2 family members (25,104): BH3 family members (Noxa, Puma, Bik), and BH1-3 family members (Bax, Bak), with a transcription-independent role reported in apoptosis through its mitochondrial translocation. Akt inhibits apoptosis by phosphorylating the Bad component of the Bad/Bcl-xL complex. Phosphorylated Bad binds to 14-3-3 causing dissociation of the Bad/Bcl-xL complex and allowing cell survival (105,106). Akt and p21-Ras, an Akt activator, induce phosphorylation of pro-Caspase-9, thus inhibiting its activity (107).

Table 2. Treatments which sensitize prostate cancer cells to Apo2L/TRAIL-induced apoptosis

Cotreatment agent/Refs.	Mechanism of action alone/in cotreatment with Apo2L/TRAIL	Cell line
Akt inhibitors: Akt-I-1 & Akt-I-1,2 (144)	Akt-I-1 inhibits Akt1 while Akt-I-1,2 inhibits both Akt1 and Akt2; block phosphorylation of Akt at Thr308 & Ser473 and of Akt substrates; promote Apo2L/TRAIL-induced apoptosis in LNCaP cells.	LNCaP
Amiloride; potassium-sparing diuretic (134)	Promotes dephosphorylation of Akt, PI3K & PDK-1 kinases along w/PTEN & PP1alpha phosphatases; augments Apo2L-induced apoptosis by inhibiting phosphorylation of kinases/phosphatases associated w/ the PI3K-Akt pathway.	DU-145
Anthracenediones: Actinomycin D (ActD); (95, 131)	Decreases XIAP and increases Bcl-xL/-xS levels; w/Apo2L showed a synergistic effect; pretreatment followed by Apo2L, TNF-alpha, or anti-Fas CH-11 monoclonal antibody (not in reverse order), induced apoptosis.	CL-1, LNCaP, DU-145, PC-3
Anthracyclines: Doxorubicin (Adriamycin, Rubex) topoisomerase II inhibitor (33, 69, 125-129)	Decreases levels of c-FLIPS but not Bcl-2, Bcl-xL, & XIAP; augments Apo2L-induced apoptosis through up-regulation of DR4, DR5, Bax, Bak, & caspase activation (PC-3, DU-145, LNCaP & PC-3 xenografts); w/ Apo2L has a synergistic effect on LNCaP, LNCaP-Bcl-2, PC-3, & PC93, but not on normal prostatic stromal cells, partially blocked by Bcl-2 expression.	PC-3 (*in vitro* & *in vivo),* DuPro,PPC1, DU145,PC93, LNCaP/-Bcl2
Bisindolylmaleimides derivates (II, VIII & IX): mostly Bis IX (150)	Bis IX is potent inducer w/Apo2L, TNF-alpha, & agonistic anti-Fas antibody; induces p53 accumulation in LNCaP w/o induction of p53-responsive genes p21 & Mdm2; DNA binding activity was prevented by ActD, suggesting that ActD and Bis IX have similar mechanisms of interaction with DNA.	LNCaP
Chimeric Bcl-xL antisense oligonucleotides (38)	With Apo2L, knockdown of Bcl-xL (but not of Bcl-2) significantly synergized in dissipation of MMP, release of cytochrome *c*, activation of Caspase-9 & -3.	PC-3
Curcumin (42)	Sensitizes to Apo2L by inhibiting NF-kappaB (suppression of Ikappa-Balpha)	LNCaP
DJ-1 expression silencing using siRNA (159)	Silencing of DJ-1 expression in PC-3 cells, which express a high but constitutive level of DJ-1.	PC-3
Etoposide (VP-16), topoisomerase II inhibitor (33, 127)	Augmented Apo2L-induced apoptosis through up-regulation of DR4, DR5, Bax, Bak, and caspases activation; apoptosis induced w/Apo2 was partially abrogated by overexpression of Bcl-2.	LNCaP, PC-3 (*in vitro* & *in vivo)*
GSK-3beta inhibitors: (122) lithium chloride, SB216763	GSK-3beta suppression sensitizes to Apo2L-induced apoptosis; dependent on Caspase-8 activity but independent of NF-kappaB.	PC-3, DU-145, LNCaP
Histone deacetylase inhibitors: Trichostatin A (TSA), Sodium butyrate (SB), Suberoylanilide hydroxamic acid (SHA) & Depsipeptide (147-149)	TSA induces Caspase-9 activation, release of cytochrome *c* & Smac from mitochondria; DU-145 overexpressing Bcl-2 were resistant; w/Apo2L, TNF-alpha, or anti-Fas Ab did not increase the level of histone acetylation, but induced the release of acetylated histones from chromatin into the cytosol; SB & SHA sensitize to Apo2L independent of p53, in p53 wild-type (A549) -null (PC-3), w/Apo2L synergized; Depsipeptide enhances the effect of Ad5-Apo2L	ALVA-31, PC-3, DU-145
Hydroxyurea (RR inhibitor) (Ray *et al*, unpublished)	C4-2 cells were recruited to S-phase, which sensitized them to Apo2L induced apoptosis (See also CPT-11, low-dose).	C4-2
Hypoxia (152)	Increased Apo2L-induced PARP cleavage, activation of Caspase-8 & 3, not 9.	Du145,LNcaP
Indole-3-carbinol (153)	Induced DR4 & DR5 expression at both transcriptional and translational levels.	LNCaP
Inhibitors of BH3-mediated dimerization to Bcl-xL: (BH3I-2') (Ray *et al*, Apoptosis, in press)	Disrupts the interactions mediated by the BH3 domain between pro and anti-apoptotic members of the Bcl-2 family, liberating more Bax/Bak to act on mitochondrial membrane; w/ Apo2L synergistically induces apoptosis in C4-2 through both the extrinsic and intrinsic apoptotic pathways of apoptosis.	C4-2
Ligands of PPAR gamma): 15d-PGJ2, ciglitazone, troglitazone; triterpenoids CDDO & CDDO-Me (154)	PPAR (peroxisome proliferator-activated receptor) gamma selectively reduces levels of FLIP; both PPARgamma agonists and antagonists displayed these effects, regardless of the levels of expression and even in the presence of a dominant-negative mutant.	PPC-1
Low extracellular pH (155)	With Apo2L enhances the association of truncated Bid with Bax.	DU145
Low glucose concentration (152, 156)	Augmented Apo2L-induced PARP cleavage & activation of Caspase-8, -3, and -9; elevates ceramide that may cause dephosphorylation of Akt and maintain dephosphorylation of Akt in the presence of Apo2L, down-regulates FLIP; enhances Apo2L-cytotoxicity, inversely related to levels of FLIP, but not DR5.	DU-145, LNCaP
Metabolic inhib.: emetine, anisomycin, cycloheximide, puromycin (67,123)	Activation of JNK; inhibition of JNK activation resulted in protection against apoptosis induced by w/Apo2L; w/Apo2L sensitized PC-3, that was inhibited by expressing Bcl-2.	PC-3, LNCaP
Methylseleninic acid (MSA) (157)	Downregulates expression of FLIP; blocked Apo2L-mediated BAD phosphorylation at Ser112 and Ser136 in DU-145; w/Apo2L activates the mitochondrial pathway-mediated amplification loop.	LNCaP, DU-145
NF-kappaB inhibition by mutated IkappaB (43)	Reduced response to Apo2L is modulated by NF-kappaB-mediated inhibition of caspases and Bid activation.	PC3AR, PC3Neo
Mifepristone, an antiprogestin (138, 139)	Added before Apo2L, induced significant apoptosis through Caspase-8 activation and Bid; induces expression of death receptors *in vitro* & in xenografts in LNCaP, but not in C4-2.	C4-2, LNCaP
Nitric oxide donors (e.g. DETANONOate) (158)	Sensitizes to Apo2L via inhibition of constitutive NF-kappaB activity, Bcl-xL expression, cytochrome c and Smac release, Caspase-3 & -9 activation.	DU-145, PC3, CL-1, LNCaP
NOS inhibitor (34)	Endothelial nitric oxide synthase (eNOS), an Akt substrate, was highly	LNCaP

	expressed only in LNCaP; NOS inhibitor sensitized LNCaP cells to Apo2L.	
PI-3 kinase inhibitors: Wortmannin (W) and LY-294002 (LY) (31, 66, 67, 119, 143)	LY suppresses constitutive Akt activity and sensitizes LNCaP to Apo2L; blocks constitutive Akt activity, phosphorylation & decreases IAP-2, the total amount of TRAIL-R1, but not TRAIL-R2 and the amount of TRAIL-R1 precipitated by Apo2L; w/Apo2L accelerated processing of Caspase-8 and activation of Caspase-2, -3, -7, -8, -9, dissipation of MMP, release of cytochrome *c* and cleavage of PARP, Akt, p21/WAF1, and MDM2.	LNCaP
PKCeta (chimeric antisense oligonucleotides against PKCeta) (116)	Synergized w/Apo2L, activating Caspase-3 and internucleosomal DNA fragmentation; augmented Apo2L-induced MMP dissipation & cytochrome *c* release: PKCeta acts upstream of mitochondria.	PC-3
Platinum Antitumor Agents: Cisplatin (127)	Overcomes resistance to Apo2L by triggering caspases activation; when used w/Apo2L, apoptosis was partially abrogated by Bcl-2.	LNCaP & PC-3
Proteasomes inhibitors: Bortezomib/Velcade (formerly PS-341); (proteasome & NF-kB inhibitor) (44, 135-137).	Increased Bik, Bim, DR4, and DR5 expression, but not Bax, Bak, Caspase-3, -8, c-FLIP or FADD; markedly sensitizes resistant cells to Apo2L, irrespective of Bcl-xL overexpression; p21-mediated Cdk inhibition promotes Apo2L sensitivity via Caspase-8 activation; w/Apo2L synergy in androgen-dependent (AD) LNCaP and LAPC4 cells and androgen independent (AI) in DU-145, but antagonism in CL1 cell line (AI); is active in AD & AI cell lines, although AD cells, w/lower levels of basal NF-kappaB activity, are sensitive.	LNCaP, LAPC4, DU-145, CL-1
PTEN – adenovirus mediated expression of PTEN (115).	Suppressed constitutive Akt activation in LNCaP and enhanced apoptosis induced by Apo2L, anti-Fas, & TNF-alpha by facilitating Caspase-8 and BID activation through a FADD-dependent pathway.	LNCaP
Pyrimidine Analogs: 5-FU, Gemcitabine (37, 131)	5-FU w/ Apo2L causes Bax not Bak mediated synergistic induction of apoptosis through the mitochondrial pathway; Gemcitabine is synergistic w/Apo2L in CL-1 cells.	DU-145, CL-1
Retinoids: CD 437 (151)	Increases expression of c-Myc, c-Jun, c-Fos, DR4, DR5, and Fas; synergistic w/Apo2L or w/FasL.	DU-145, PC-3, LNCaP
RNase L activators: 2',5'-oligoadenylate (133)	Prostate cancer cells were sensitive to apoptosis from the combination of 2',5'-oligoadenylate with either Apo2L/TRAIL or topoisomerase I inhibitors.	DU-145
Taxanes: Paclitaxel (33, 68)	Increased DR4 and/or DR5 (< 8-fold) but not DcR1 & DcR2 levels; sequential co-treatment of PC-3 (*in vitro* & *in vivo*), DU-145, and LNCaP w/Apo2L induced more apoptosis than Apo2L alone and was associated with up-regulation of DR-4,-5 ,Bax, Bak, Caspase-8, -3 & Bid activation, cyt *c* release.	PC-3, DU-145, LNCaP, PC-3 (*in vitro* & *in vivo)*
Topoisomerase I inhibitors: camptothecin and its derivates CPT-11 (irinotecan) and topotecan; (33, 36, 132 & Ray, Almasan, unpublished data)	CPT augmented Apo2L-induced apoptosis through upregulation of DR4, DR5, Bax, and Bak, & caspase activation; w/Apo2L achieves tumor control in cells and tumors through Bcl-2 family proteins and caspases activation; w/Apo2L caused S-phase checkpoint activation through the ATM/Chk2- and ATR/Chk1-Cdc25A pathways and inhibition of Cdk2-associated kinase activity; recruitment in S-phase by low-dose CPT-11 or hydroxyurea, sensitized C4-2 cells to Apo2L; topotecan upregulates TRAIL-R1 and TRAIL-R2 and downregulates survivin.	DU-145, LNCaP, C4-2 (*in vitro* & *in vivo),* PC-3 (*in vitro* & *in vivo*)
Transfection w/ Smac cDNA (96)	With Apo2L sensitizes CL-1, by enhancing the release of Smac/DIABLO from mitochondria and decreasing IAP family proteins (XIAP, c-IAP1, and c-IAP2).	CL-1
Vinca Alkaloids: Vinblastine,Vincristine (33)	Augmented Apo2L/TRAIL-induced apoptosis through up-regulation of DR4, DR5 and caspase activation.	PC-3, DU145, LNCaP
X rays (91, 92, 141, 142)	5 Gy up-regulated expression of DR5 & Fas; w/Apo2L and w/Fas Ab was independent of Tp53 status in DU-145; cross-sensitization w/Apo2L entirely depends on Bax proficiency (Bak being not sufficient) and induced apoptosis through upregulation of DR5, Bax, Bak and caspase activation (PC-3), while dominant negative FADD and p53 siRNA inhibited the synergistic interaction; w/Apo2L or w/adenovirus-driven Apo2L expression in PC-3 and DU-145 xenografts respectively, have a synergistic action in induction of apoptosis.	LNCaP, PC-3 (*in vitro* & *in vivo),* DU-145 (*in vitro* & *in vivo)*
XIAP antisense oligos (97)	With Apo2L enhanced potency ~12-13-fold.	DU-145

NF-kappaB is another survival factor that acts by protecting tumor cells from Apo2L/TRAIL, TNF-alpha, radiotherapy, and chemotherapy. The role of NF-kappaB in Apo2L/TRAIL-induced apoptosis (10, 11, 40-44) and its role in the resistance of prostate cancer cells to Apo2L/TRAIL, was discussed above in subsection 3.2. Also NF-kappaB was shown to increase expression of survival gene products, such as IAPs, Bcl-2, and Bcl-xL (108-111). Active Akt phosphorylates IKKalpha, which promotes activation of its heterodimeric partner, IKKbeta in the IKKalpha/IKKbeta complexes. The IKK complex phosphorylates IkappaB, thereby promoting its dissociation from NF-kappaB. Dissociation of IkappaB permits NF-kappaB to enter into the nucleus, where it binds DNA and activates genes that promote immunity and cell survival (112-114). Transcription factors, such as AR, FOXO3A, p53, or NF-kappaB translocate to the nucleus where they bind to response elements located in the promoters of transcription target genes. For example, a dimeric AR will be activated by androgens and, following its nuclear translocation, will bind to an androgen-responsive element (ARE). All these interactions representing the regulation of prostate cell biology and Apo2L/TRAIL-induced apoptosis are summarized in (Figure 2).

It has been shown that elevated Akt activity protects LNCaP cells from Apo2L/TRAIL-induced apoptosis, and that the PI-3 kinase/Akt pathway may inhibit apoptotic signals by inhibiting processing of BID (66, 67). LNCaP cells displayed a high Akt activity, with endothelial

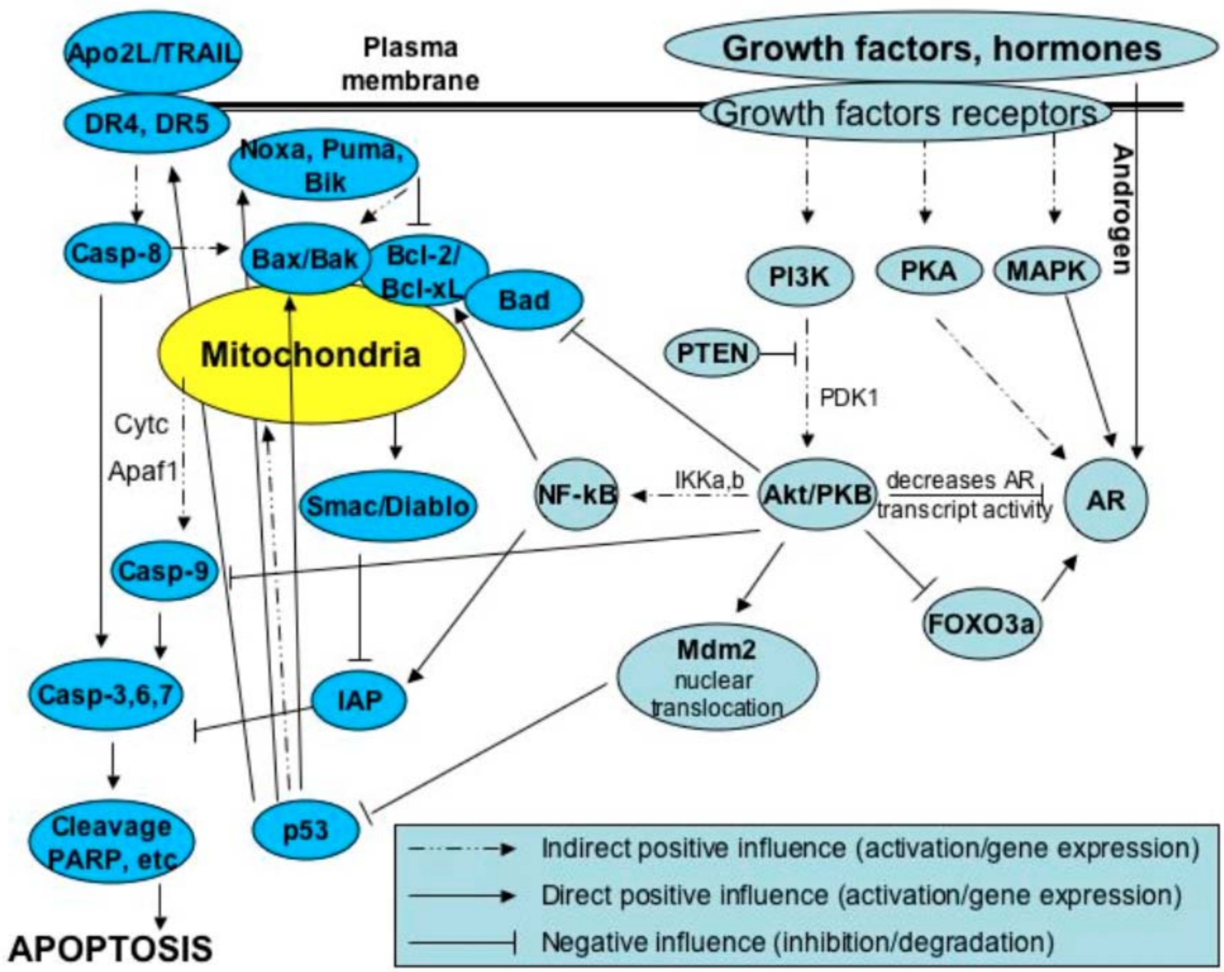

Figure 2. Survival factors regulate prostate cell biology and Apo2L/TRAIL-induced apoptosis. Akt decreases AR transcriptional activity and AR expression by inhibiting FOXO3a and promoting phosphorylation-dependent ubiquitination and degradation of AR. In contrast, stimulation of MAPK is postulated to enhance AR-dependent transcription through phosphorylation of AR and protein kinase A (PKA); PKA was shown to activate AR in the absence of androgen. PTEN inhibits PI3K/AKT activity and negatively regulates phosphorylation of MDM2 and its subsequent nuclear translocation, thus augmenting the level and function of p53, which impacts on expression of several Bcl-2 family members. Akt inhibits apoptosis by phosphorylating the Bad component of the Bad/Bcl-xL complex. Akt and p21-Ras, an Akt activator, induce phosphorylation of pro-Caspase-9, thus inhibiting its activity. Also NF-kappaB (NF-kB) was shown to increase expression of survival gene products, such as IAPs, Bcl-2, and Bcl-xL. Transcription factors, such as AR, FOXO3a, p53, or NF-kappaB translocate to the nucleus where they bind to response elements located in the promoters of their transcription target genes.

nitric oxide synthase (eNOS), one of the Akt substrates, being highly expressed in LNCaP but not in other cells. Inhibition of eNOS activity by NOS inhibitor sensitized LNCaP cells to Apo2L/TRAIL. Conversely, PC-3 cell clones stably expressing eNOS were resistant to Apo2L/TRAIL-induced apoptosis (34). Overexpression of constitutively active Akt in PC-3M cells, which express very low levels of constitutively active Akt, restored resistance to Apo2L/TRAIL (67).

The PTEN tumor suppressor is frequently mutated in human tumors, including those of the prostate. Loss of PTEN function is associated with constitutive survival signaling through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway**.** Adenovirus-mediated expression of PTEN completely suppressed constitutive Akt activation in LNCaP prostate cancer cells and enhanced apoptosis induced by a broad range of apoptotic stimuli, such as Apo2L/TRAIL, TNF-alpha, and agonistic antibodies against Fas (115). Down-regulation of protein kinase C (PKC)eta also potentiates the cytotoxic effects of exogenous Apo2L/TRAIL in PC-3 prostate cancer cells (116).

Cancer-derived OPG was also suggested to be an important survival factor in hormone-resistant tumor cells, as a strong negative correlation was seen between the levels of OPG and the capacity of Apo2L/TRAIL to induce apoptosis in prostate cancer cells, which produced increased levels of OPG endogenously (117). OPG has also been involved in bone remodeling, where it acts as an inhibitor of osteoclastogenesis. Metastasis to the skeleton occurs in around 70% of patients with advanced prostate cancer, suggesting that the bone microenvironment may provide factors that favor the growth and survival of prostate cancer cells. It seems that, at least a part of the survival advantage gained by prostate cancer cells in colonizing bone may be caused by the production of OPG by tumor-associated stromal cells (118).

5.3. Androgen dependence

Androgens play major roles in promoting the development and progression of prostate cancer. Androgens regulate apoptosis induced by TNFR family ligands (Apo2L/TRAIL & TNF-alpha) using multiple signaling pathways in androgen dependent prostate cancer cells. Apo2L/TRAIL-DISC formation and sensitivity to Apo2L/TRAIL treatment are androgen-dependent, as LNCaP cells remained resistant to treatment with Apo2L/TRAIL after androgen deprivation even in the presence of the PI3K/Akt pathway inhibitor Wortmannin (119). It was suggested that in androgen-deprived LNCaP cells, Apo2L/TRAIL and TNF-alpha stimulate cell growth and activate the mitogenic and antiapoptotic signaling pathways involving NF-kappaB, STAT3, PI3K, and beta-catenin (120). However, another study using the LNCaP cell line concluded that 5-dihydrotestosterone inhibited apoptosis induced by Apo2L/TRAIL or TNF-alpha in a dose-dependent manner. There was a direct, androgen-dependent correlation between the levels of activated Akt and caspases activation after treatment with Apo2L/TRAIL and TNF-alpha. It was also suggested that there are regulatory mechanisms of p53 expression by androgen at the gene and protein levels and that there is a mutual regulation of expression between p53 and the androgen receptors (121).

5.4. Other factors

Of the many other potential modulators of apoptosis resistance, it is worth mentioning GSK-3beta and JNK. GSK-3beta suppression sensitizes prostate cancer cells to Apo2L/TRAIL-induced apoptosis that was dependent on Caspase-8 activity, but independent of NF-kappaB activation, suggesting that a mechanism involving GSK-3beta activation may be responsible for Apo2L/TRAIL resistance in prostate cancer cells (122). On the other hand, inhibition of JNK activation resulted in protection against Apo2L/TRAIL. Conversely, activation of JNK sensitized PC-3 cells to Apo2L/TRAIL-induced apoptosis by translation inhibitors, in cells that are otherwise Apo2L/TRAIL-resistant. Nevertheless, in addition to JNK activation, other aspects of translation inhibition such as the suppressed activity of IAPs, or activation of other signal transduction pathways, could also be involved (123). However, another study has shown that treatment of PC-3 cells with Apo2L/TRAIL activated JNK1. Surprisingly, inhibition of JNK1 activation by its dominant-negative mutant had little effect on Apo2L/TRAIL-induced apoptosis (32), indicating that JNK1-independent factors are involved in apoptosis of these cells.

6. SENSITIZATION OF PROSTATE CANCER CELLS TO APO2L/TRAIL-INDUCED APOPTOSIS

Many prostate cancer cells, such as C4-2, are quite resistant to treatment, particularly when a non-tagged, Zn-bound recombinant trimeric version of Apo2L/TRAIL is used. We have previously shown that CPT-11 (36) and BH3I-2' (Ray, Bucur, and Almasan, Apoptosis, 10 (6), in press) sensitize LNCaP-derived C4-2 human prostate cancer cells to Apo2L/TRAIL-induced apoptosis. However, these Apo2L/TRAIL resistant prostate cancer cells can be made sensitive to Apo2L/TRAIL-induced apoptosis, using multiple strategies. The information containing the type of treatment, the mechanism of action, alone or following co-treatment (*in vitro* and *in vivo*) with Apo2L/TRAIL, the type of prostate cancer cell lines used, and key references are summarized in (Table 2).

6.1. Conventional radiation and chemotherapeutic drugs

6.1.1. Anthracyclines: doxorubicin

Anthracycline antibiotics are extensively used in conventional cancer chemotherapy of solid tumors and hematological malignancies (124). Of these, doxorubicin has the broadest spectrum of activity. Doxorubicin decreases the levels of c-FLIP and augments Apo2L/TRAIL-induced apoptosis through up-regulation of DR4, DR5, Bax and Bak, and caspase activation (33, 69, 125-129).

6.1.2. Anthracenediones: actinomycin D

Anthracenediones differ from anthracyclines by the lack of the glycoside substituents. Actinomycins were the first antibiotics isolated from the culture broth of *Streptomyces* by Waksman and Woodruff in 1940, and have activity against gram-positive and gram-negative bacteria and some fungi. Toxicity precluded their use as anti-infectious agents (130). Actinomycin D (ActD) decreases XIAP, increases Bcl-xL/xS levels and acts synergistically with Apo2L/TRAIL to induce apoptosis in CL-1, LNCaP, DU-145, and PC-3 human prostate cancer cell lines (95, 131).

6.1.3. Pyrimidine analogs: 5-fluorouracil, gemcitabine

Pyrimidine analogs have been used in the treatment of diseases as cancer, psoriasis, fungal and viral infections. 5-fluorouracil (5-FU), a thymidylate synthase inhibitor (37) and gemcitabine, a ribonucleotide reductase inhibitor (131) were shown to sensitize prostate cancer cells to Apo2L/TRAIL-induced apoptosis.

6.1.4. Platinum antitumor compounds: cisplatin

The platinum antitumor agents are complexes of platinum with ligands that can be displaced by nucleophilic (electron-rich) atoms to form strong bonds with covalent characteristics. Thus, like the alkylating agents, the platinum agents form strong chemical bonds with thiol sulfurs and amino nitrogens in proteins and nucleic acids

(130). Cisplatin in combination with Apo2L/TRAIL overcomes resistance to Apo2L/TRAIL by triggering caspase activation (127).

6.1.5. Taxanes: paclitaxel

Structurally, the taxanes are complex esters consisting of a 15-member taxane ring system linked to an unusual four-member oxetan ring. Although the taxanes affect microtubules, they are substantially different from the Vinca alkaloids in terms of their principal mechanisms of action, pharmacology, clinical indications, and toxicology (130). Paclitaxel sensitizes PC-3 both *in vitro* and *in vivo*, as well as LNCaP, and DU-145 human prostate cancer cell lines to Apo2L/TRAIL-induced apoptosis, by up-regulation of DR4, DR5, Bax and Bak, cytochrome *c* release, and activation of Caspase-8, Caspase -3, and Bid (33, 68).

6.1.6. Vinca alkaloids: vinblastine and vincristine

The Vinca alkaloids are naturally occurring or semisynthetic nitrogenous bases extracted from the pink periwinkle plant *Catharanthus roseus* G. Don. (130). Vincristine and vinblastine augment Apo2L/TRAIL-induced apoptosis through up-regulation of DR4, DR5, and caspase activation (33).

6.1.7. Other chemotherapeutic agents

Topoisomerase I is an enzyme involved in DNA replication and RNA transcription. Camptothecin (33) and its derivates, such as CPT-11 (irinotecan) (36) and topotecan (132) are topoisomerase I inhibitors, which were shown to be very efficient in combination with Apo2L/TRAIL, in prostate cancer cells (36). Interestingly, apoptosis induced by CPT-11 in combination with Apo2L/TRAIL could be prevented in cells lacking the hereditary prostate cancer 1 (HPC1) allele, that maps to the RNASEL gene encoding a protein (RNase L), that has been implicated in the antiviral activity of interferons in DU-145 cells. The RNase L-deficient cells were highly resistant to apoptosis by combination treatments with camptothecin, topotecan, or SN-38, and Apo2L/TRAIL. An inhibitor of c-Jun NH(2)-terminal kinases reduced apoptosis induced by treatment with either the RNase L activator 2',5'-oligoadenylate or the combination of camptothecin and TRAIL, thus also implicating c-Jun NH(2)-terminal kinase in the apoptotic signaling pathway. These findings indicate that RNase L integrates and amplifies apoptotic signals generated during treatment of prostate cancer cells with, 2-5A, topoisomerase I inhibitors, and Apo2L/TRAIL (133).

As summarized in (Table 2), experiments were also performed with other therapeutics, such as amiloride (134), bortezomib/velcade, formerly PS-341 (proteasome & NF-kappaB inhibitor) (44, 135-137), etoposide (VP-16) (33, 127), and mifepristone (an antiprogestin) (138, 139). A natural compound, curcumin (diferuloyl - methane; E100 when used as a food additive), was effective by suppressing Ikappa-B alpha phosphorylation (42).

6.1.8. X-rays

Radiation, when used alone, does not typically rely on death receptors to execute the apoptotic program (140), although that may be the case, at least partially, in certain cell types (55). However, most cancer cell lines are Type II and therefore mediate death receptor signaling though the mitochondrial pathway (36). X-rays, while ineffective in many prostate cancer cell lines, when used as a single agent, were shown to augment Apo2L/TRAIL-induced apoptosis through upregulation of DR5, Bax, and Bak, and caspase activation, in PC-3 and LNCaP cells. Treatment of xenografted PC-3 followed by Apo2L/TRAIL–induced apoptosis through activation of Caspase-3, induction of Bax and Bak, and inhibition of Bcl-2 (LNCaP and PC-3 *in vitro* & PC-3 *xenografts*). Also, combination of ionizing radiation and adenovirus-driven TRAIL expression overcame human prostate cancer cell resistance to Apo2L/TRAIL in cell culture *in vitro* and in DU-145 human prostate tumor xenografts *in vivo* (91, 92, 141, 142).

6.2. Compounds that act on Bcl-2 family proteins

Bcl-2 family of proteins represent important components of the intrinsic pathways of apoptosis (104). Modulation of the expression and/or their activity can represent an important strategy of sensitizing prostate cancer cells to Apo2L/TRAIL-induced apoptosis. Use of BH3I-2', that disrupts the interaction between the anti-apoptotic and pro-apoptotic members of the Bcl-2 family (Ray, Bucur, and Almasan, Apoptosis, 10 (6), in press), and transfection with second-generation chimeric antisense oligonucleotides against Bcl-xL, which effectively downregulate Bcl-xL (38), combined with Apo2L/TRAIL, were shown to have a synergistic effect in prostate cancer cells.

6.3. Inhibitors of cell survival factors

As described in sections 3.2 and 5.2, NF-kappaB is an important factor in the resistance of prostate cancer cells to Apo2L/TRAIL-induced apoptosis. Curcumin (diferuloyl-methane) sensitizes prostate cancer cells to Apo2L/TRAIL by inhibiting nuclear NF-kappaB through suppression of IkappaB-Kinase alpha phosphorylation (42). NF-kappaB inhibition by adenoviral infection of mutated Ikappa B was also shown to be efficient in sensitization to Apo2L/TRAIL-induced apoptosis (43).

PI-3 kinase pharmacologic inhibitors suppress constitutive Akt activity. Thus LY-294002 (66, 67) and Wortmannin (31, 66, 67, 119, 143) were shown to be efficient in combination with Apo2L/TRAIL in killing prostate cancer cells. Akt-I-1 and Akt-I-1,2 block phosphorylation of Akt at Thr308 and Ser473, reduce the levels of active Akt in cells, block the phosphorylation of known Akt substrates and promote Apo2L/TRAIL-induced apoptosis in LNCaP cells (144). Amiloride was also shown to promote Apo2L/TRAIL effectiveness by dephosphorylating Akt, PI3K, and PDK-1 kinases along with the PTEN and PP1alpha phosphatases (134). Adenovirus-mediated expression of PTEN suppressed constitutive Akt activation in LNCaP and enhanced apoptosis induced by Apo2L/TRAIL, anti-Fas, and TNF-alpha (115).

6.4. Histone deacetylase inhibitors

Histone deacetylase inhibitors are a new class of antineoplastic agents currently being evaluated in clinical trials. Regulation of gene expression is mediated by several mechanisms, such as DNA methylation, ATP-dependent chromatin remodeling, and post-translational modifications of histones, which include the dynamic acetylation and deacetylation of epsilon-amino groups of lysine residues present in the tail of core histones (145). The enzymes responsible for the reversible acetylation/ deacetylation processes are histone acetyltransferases and histone deacetylases, respectively (146). Depsipeptide (FR901228) (147), sodium butyrate (148), suberoylanilide hydroxamic acid (148), and trichostatin A (TSA; also an antifungal antibiotic) (148, 149) were all shown to sensitize resistant prostate cancer cells to Apo2L/TRAIL-induced apoptosis.

6.5. Metabolic inhibitors: blocking protein synthesis

Metabolic inhibitors, such as protein synthesis inhibitors, were shown to be efficient in combination with Apo2L/TRAIL. These compounds include cycloheximide, anisomycin, emetine, harringtonine, and puromycin, protein translation inhibitors that were shown to lead to activation of JNK (67, 123).

6.6. Other types of treatment

A variety of other types of treatments, based on pharmacologic or molecular approaches, were also shown to sensitize prostate cancer cells to Apo2L/TRAIL-induced apoptosis. Pharmacologic inhibitors included bisindolylmaleimides derivates (II, VIII, and IX) (150), the syntetic retinoid CD 437 {6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid} (151), GSK-3beta inhibitors: lithium chloride and SB216763 (122), hydroxyurea (Ray and Almasan, unpublished data), hypoxia (152), indole-3-carbinol, a phytochemical produced in fruits and vegetables (153), ligands of peroxisome proliferator-activated receptor-gamma (PPAR gamma): 15d-PGJ2, ciglitazone, troglitazone, triterpenoids CDDO & CDDO-Me (154). Physiological factors, such as low extracellular pH (155) and low glucose concentration (152, 156) were also critical. Other effective agents were methylseleninic acid (157), nitric oxide donors {e.g. (Z)-1-[2-(2-aminoethyl)-N-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate (DETANONOate)} (158) and NOS inhibitors (34). Finally, RNA-interference-mediated silencing of DJ-1 expression (159), transfection with second-generation chimeric antisense oligonucleotides against PKCeta (116), with a Smac/DIABLO cDNA, encoding a neutralizing inhibitor of IAPs (96), and XIAP antisense phosphorodiamidate morpholino oligomer (97) were also efficient.

6.7. Sensitization to Apo2L/TRAIL-induced apoptosis *in vivo*

Most of the compounds described above and in (Table 2) were tested *in vitro* cell cultures of prostate cancer cell lines. Only few of them were also tested *in vivo*, in athymic nude mice with, for example, C4-2, PC-3, and DU-145 human tumor xenografts. Testing *in vivo* is important as we have shown previously a striking difference between expression and activation of apoptotic regulators *in vitro* and *in vivo*, with the most significant difference being Bak expression in C4-2 cells. Apo2L/TRAIL and CPT-11 achieve prostate cancer tumor control *in vivo* and *in vitro* through regulation of Bcl-2 family proteins and potent activation of caspases. Bcl-2 family proteins play an important role in induction of apoptosis by the combination treatment in established C4-2 tumor xenografts. Moreover, they indicate that, even in similar cells, under different biological conditions, different Bcl-2 family members may be responsible for inducing apoptosis (36). This clearly supports recent findings on the distinct roles of the pro-apoptotic multidomain Bcl-2 protein family homologues Bax and Bak.

The sequential treatment of PC-3 tumor xenografted in mice with chemotherapeutic drugs (paclitaxel, etoposide, doxorubicin, and camptothecin) followed by Apo2L/TRAIL-induced Caspase-3 activity and apoptosis, inhibited angiogenesis, completely eradicated the established tumors, and enhanced survival of mice. They augmented Apo2L/TRAIL-induced apoptosis in cancer cells through up-regulation of DR4, DR5, Bax, and Bak, and caspase activation (33). Treatment of PC-3 tumor xenografted mice with irradiation followed by Apo2L/TRAIL, induced apoptosis through activation of Caspase-3, induction of Bax and Bak, and inhibition of Bcl-2, and completely eradicated the established tumors with enhanced survival of nude mice (16). Combination of doxorubicin and Apo2L/TRAIL is more effective in growth inhibition of PC-3 xenografts *in vivo* than either agent alone and could present a novel treatment strategy against hormone-refractory prostate cancer. The intracellular mechanism by which doxorubicin enhances the effect of Apo2L/TRAIL on PC-3 xenografts may be through reduced expression of c-FLIP (129).

Finally, administration *in vivo* of AdFlt-TRAIL at the site of tumor growth, in combination with radiation treatment, also produced significant suppression of the growth of DU-145 human prostate tumor xenografts, in athymic nude mice (92).

7. PERSPECTIVES

Prostate cancer represents a major public health challenge, being the most common malignancy in North American and European men and the second leading cause of cancer-related death in American men (160). Apo2L/TRAIL, now in clinical trials, is emerging as a powerful inducer of apoptosis. Alone, or in combination with other treatments, its use may represent an important strategy for treatment of prostate cancer.

Most of the studies using Apo2L/TRAIL have indicated that activating apoptosis by engaging the death receptors through their ligation confers a tumor selective apoptotic induction and may select for outgrowth of resistant tumors, such as those that accumulate Bax mutations (1, 38). However, the Apo2L/TRAIL-initiated signal may be quite weak in many tumor cells, including most prostate cancer cell lines. Use of combination treatments, particularly with DNA-damaging agents that activate the intrinsic pathway of apoptosis, often leads to their synergistic action and effective tumor control. Conversely, use of conventional radiotherapy or chemotherapy will eventually select for recurrent, therapy-resistant tumor cells that have lost critical apoptotic effectors, such as the tumor suppressor p53. Targeting death receptors, such as that mediated by Apo2L/TRAIL might be a useful therapeutic strategy because it does not require p53 and thus may circumvent their resistance to conventional therapeutics.

Resistance to radiotherapy or chemotherapy, that often is associated also with androgen deprivation therapy in the case of prostate carcinomas, represents a severe clinical problem (161). As death receptors can induce apoptosis independently of p53, their targeting may represent a useful therapeutic strategy, especially in cells in which the p53-response pathway has been inactivated. Recombinant soluble Apo2L/TRAIL induces apoptosis in many cancer cell lines, regardless of their p53 status. In tumors that retain some responsiveness to conventional therapy, death receptor engagement in combination with chemotherapy or radiation might lead to synergistic apoptosis activation, as well as reduce the probability that tumor cells acquire resistance to either type of treatment. In addition to the combination treatments, that have been shown to be effective in inducing apoptosis in prostate cancer cells, there are other combinations that probably will show a good result in these types of cells, for example Apo2L/TRAIL in combination with other therapeutic agents that were shown to have a synergistic effect in other types of cancer cells.

Many of the results presented in this review have been obtained *in vitro,* using cell cultures. However, there can be significant differences between the response of different types of cells grown *in vitro* and *in vivo*, even when they are subjected to the same treatment. For example, we showed that the combination of Apo2L/TRAIL plus CPT-11 exerts antitumor activity both *in vitro* and *in vivo*. However, there was a striking difference between expression and activation of apoptotic regulators *in vitro* and *in vivo*, with the most significant difference being induction of Bak expression in xenografts, indicating that different molecular means may be used by the same cells under different biological conditions to activate apoptosis. (36). Also, *in vivo,* there is a paracrine/ autocrine loop involving prolactin (PRL) within the human prostate. PRL had no significant effect on the proliferation of PC-3, DU-145 and LNCaP, but inhibited Apo2L/TRAIL-induced apoptosis in PC-3 cells, possibly via enhanced Akt/PKB phosphorylation (162). Another example is represented by OPG in metastasis of the bone. It seems that, at least a part of the survival advantage gained by prostate cancer cells in colonizing bone may be caused by the production of OPG by tumor-associated stromal cells (117, 118).

Much progress has been made in elucidating the mechanism of Apo2L/TRAIL-induced apoptosis, the resistance of some prostate cancer cells to Apo2L/TRAIL, and how prostate cancer cells can be sensitized. Nevertheless, there are still some obstacles to overcome, to make Apo2L/TRAIL treatment a viable strategy in prostate cancer therapy, as it has not been yet effective with therapeutic agents that are most commonly used for the standard therapy of the prostate.

8. ACKNOWLEDGEMENTS

Drs Octavian Bucur and Subrata Ray contributed equally to this work. This work was supported by research grants from the National Institutes of Health (CA81504 and CA82858 to AA) and the Romanian Ministry of Education and Research (OB).

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Key Words: Apo2L, TRAIL, Cytokine, TNF, Prostate, Apoptosis, Cell Death, Review

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