Vitamin A and apoptosis in prostate cancer

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Abstract

Apoptosis represents an effective way to eliminate cancer cells. Unfortunately, advanced prostate tumors eventually progress to androgen-independent tumors, which are resistant to current therapeutic approaches that act by triggering apoptosis. Vitamin A and its natural and synthetic analogs (retinoids) induce apoptosis in prostate cancer cells in vitro and in animal models, mainly through induction of retinoic acid receptor-β (RARβ). Expression levels of RARβ, however, are significantly reduced in hormone-independent prostate cancer cells. Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) that effectively induces apoptosis of both hormone-dependent and -independent prostate cancer cells in a retinoid receptor-independent manner was identified and has drawn a lot of attention in the field. The apoptotic effect of AHPN requires expression of orphan receptor TR3 (also called nur77 or NGFI-B). Paradoxically, TR3 expression is also induced by androgen and other mitogenic agents in prostate cancer cells to confer their proliferation. The recent finding that TR3 migrates from the nucleus to mitochondria to trigger apoptosis in response to AHPN suggests that the opposing biological activities of TR3 are regulated by its subcellular localization. Thus, agents that induce translocalization of TR3 from the nucleus to mitochondria will have improved efficacy against prostate cancer. TR3, therefore, represents an unexplored molecule that may be an ideal target for developing new agents for prostate cancer therapy.

Endocrine-Related Cancer (2002) 9 87–102

Introduction

Prostate cancer is the most common cancer diagnosed among men in the United States, accounting for 27.5% of all cancer cases in men. It ranks second after lung cancer as the underlying cause of cancer death in US men. Despite aggressive efforts toward earlier detection and treatment, the mortality rate for prostatic carcinoma has steadily increased. The identification of androgens as the major regulator of prostatic epithelial proliferation offered a target for therapeutic intervention. Androgen ablation by surgical gonadectomy or drug treatments that suppress androgen production and action remain the only effective form of therapy for men with advanced disease. Unfortunately, the median duration of response to androgen ablation is less than 2 years, after which the disease will re-emerge in a poorly differentiated, androgen-independent form, which is often fatal. The lack of therapies for this advanced prostate cancer has contributed significantly to the increased mortality rates, and has resulted in the impetus to develop non-androgen-based therapies.

Vitamin A and its natural and synthetic analogs, retinoids, are one of the most investigated classes of chemopreventive drugs for prostate cancer. Early experiments on mouse prostate explant cultures showed that all-trans-retinoic acid (trans-RRA) could both inhibit and reverse the proliferative effects of chemical carcinogens on prostatic epithelium (Lasnitzki & Goodman 1974, Chopra & Wilkoff 1976). Recent studies have demonstrated that retinoids effectively inhibit the growth of prostate cancer cells in vitro and suppress the development of prostate carcinogenesis (Blutt et al. 1997, DiPaola et al. 1997, Campbell et al. 1998, Goossens et al. 1999, McCormick et al. 1999, Pasquali et al. 1999, Richter et al. 1999, Sun et al. 1999b, Urban et al. 1999, Webber et al. 1999, Kelly et al. 2000, Koshiuka et al. 2000, Lotan et al. 2000, Tanabe 2000, Pili et al. 2001). Clinical trials of several retinoids and their combination with other anti-cancer agents have shown significant activities, when retinoids were used in combination with other chemo-therapeutic agents, such as interferon-α and paclitaxel (DiPaola et al. 1997, 1999, Culine et al. 1999, Shalev et al. 2000, Thaller et al. 2000). Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) (also called CD437) (Bernard et al. 1992) was found to potently inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate cancer cells. Thus, these small molecules may serve as prototypes for the...
development of new prostate cancer therapeutic and preventive agents. The recent identification of the molecular targets of retinoid action in prostate cancer cells offers opportunities for the development of novel therapeutic strategies.

Vitamin A signaling pathways

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (Zhang & Pfahl 1993, Kastner et al. 1995, Mangelsdorf & Evans 1995). RARs and RXRs are encoded by three distinct genes (α, β, and γ). In addition, many retinoid receptor isoforms are generated through differential promoter usage, giving rise to a large number of distinct retinoid receptor proteins. To date, there are dozens of receptors which are known to mediate the effect of retinoids. 9-cis RA is a high-affinity ligand for both RARs and RXRs, whereas trans-RA is a ligand for only RARs. Retinoid receptors belong to a large steroid/thyroid receptor superfamily that mediate the biological effects of many hormones, vitamins and drugs. RARs and RXRs act as transcriptional factors to positively or negatively regulate expression of target genes by binding to their response elements (RAREs) located in promoter regions of the target genes (Fig. 1). The physiological role of RARs and RXRs has been extensively studied by knockout experiments (Kastner et al. 1995). Knockout of most of individual RARs activity by homologous recombination appears normal due to redundancy in the function of RARs in vivo. However, knockout of RARα and RARγ as well as RAR double knockouts produces defects that resemble the postnatal vitamin A-deficient syndrome and can be prevented by trans-RA administration, including keratinizing squamous metaplasia of the prostate gland (Kastner et al. 1995).

RXRs form heterodimers with many nuclear receptors including RARs, thyroid hormone receptor (TR), vitamin D receptor and peroxisome proliferator-activated receptor (PPAR) (Zhang & Pfahl 1993, Kastner et al. 1995, Mangelsdorf & Evans 1995), thereby mediating diverse endocrine signaling pathways. The function of RXRs, however, is more restricted. The role of ligands in the regulation of retinoid receptor function is complex. RAR/RXR is activated mainly through binding of RAR with its ligand, although there are some situations where binding of both the RAR and RXR components with their respective ligands can contribute to the activity of the RAR/RXR heterodimers (Zhang & Pfahl 1993, Kastner et al. 1995, Mangelsdorf & Evans 1995). The retinoid binding to RXRs is required for the activation of RXR homodimers and certain RXR heterodimers, such as TR3/RXR and PPARγ/RXR (Zhang et al. 1992b, Kastner et al. 1995, Mangelsdorf & Evans 1995). Unliganded retinoid receptors can act as negative transcription factors by binding to the RAREs of retinoid target genes, and recruit receptor corepressors, such as NcoR (Xu et al. 1999), leading to histone deacetylation and formation of an inactive chromatin structure preventing transcription. Binding of retinoids to their receptors induces receptor conformational changes that serve as switches by releasing the receptor corepressors and by facilitating the recruitment of receptor co-activators, such as CBP (Xu et al. 1999). Several of the co-activator proteins have histone acetylase activity that contributes to the formation of an active chromatin structure and results in the transcription of target genes.

In addition to their direct effects on transcription, liganded RAR can modulate the activity of other transcriptional factors, such as AP-1 (Pfahl 1993). Activated retinoid receptors can inhibit the activity of AP-1, thereby regulating the expression of AP-1 target genes. The inhibition of AP-1 activity is linked to the anti-proliferative effects of retinoids, and appears to be separable from their direct activation of transcription of retinoid-target genes. Synthetic retinoids that specifically inhibit AP-1 activity without activating transcription have been developed (Fanjul et al. 1994, Chen et al. 1995, Li et al. 1996). These AP-1-specific retinoids can inhibit cell proliferation in vitro.

Recent evidence indicating that the cytoplasmic action of several hormone receptors represents an important mechanism for regulating their biological function has accumulated. The proapoptotic effect of the orphan receptor TR3 (also known as nur77 and NGFI-B) does not require its transcriptional regulation because TR3 with its DNA-binding domain deleted is still capable of inducing apoptosis (Li et al. 2000). In contrast, the cytoplasmic action of TR3, through its mitochondrial targeting, is essential for its apoptotic activity (Li et al. 2000). The glucocorticoid receptor was also found to reside on mitochondria (Scheller et al. 2000), while differentiation of PC12 phaeochromocytoma cells is accompanied by nuclear export of NGFI-B (Katagiri et al. 2000). Estrogen receptors and androgen receptors trigger cell proliferation through their interaction with Src or phosphatidylinositol-3-OH kinase in the cytoplasm (Migliaccio et al. 2000, Simoncini et al. 2000, Kousteni 2001).

Apoptotic signalings

Apoptosis, also known as programmed cell death, is an evolutionarily conserved and indispensable process during normal embryonic development, tissue homeostasis and regulation of the immune system (Fisher 1994, Steller 1995, White 1996). The apoptotic process can be initiated by several different stimuli, including growth factor withdrawal, DNA damage, deregulation of the cell cycle or ligation of death receptors (Fisher 1994, Steller 1995, White 1996). These different apoptotic stimuli induce diverse early signaling events, which then converge by activating a common central biochemical pathway that is responsible for the execution of apoptosis. Execution of apoptosis is primarily mediated by caspases, a family of cysteine proteases with
Figure 1 Retinoid signaling. Retinoid receptors are ligand-dependent transcription factors. (A) Schematic representation of retinoid receptor. The receptor may be divided into five regions (A, B, C, D, E and F) based on structural and functional similarities among members of the steroid/thyroid hormone receptor superfamily. DNA binding domain (DBD), ligand binding domain (LBD) and transactivation domains (AF-1 and AF-2) are indicated. (B) Mechanism of action of retinoid receptors. Trans-RA or 9-cis RA enter cells directly from the circulation, and bind to DNA-bound RAR or RXR, thereby eliciting a transcriptional response.

There are two distinctly different pathways, the extrinsic and intrinsic pathways, transducing the death signals to caspase-mediated apoptotic machinery (Nunez et al. 1998). The extrinsic pathway involves activation of the superfamily of the tumor necrosis factor receptors (TNFR) or CD95 (Fas), by binding to their respective ligands, which in turn recruit procaspase-8 and -10 to membrane-associated signaling complexes, resulting in their activation (Fig. 2). Activation of these upstream caspases is sufficient to directly activate effector caspases such as caspase-3, -6 and -7, or indirectly induce apoptosis by cleaving Bid involved in the release of mitochondrial cytochrome c. The intrinsic pathway is activated directly by various forms of cellular stress that trigger mitochondrial release of cytochrome c into the cytosol. Cytosolic cytochrome c then binds to, and triggers oligomerization of the CED-4 homolog Apaf-1. The resulting ‘apoptosome’ recruits and activates procaspase-9 which, in turn, recruits and activates effector caspases, such as caspase-3 and possibly caspase-7 (Fig. 2). Additionally, the caspases can be activated by granzyme B, a major serine protease in cytotoxic lymphocyte granules (Shi et al. 1992). Once the effector caspases are activated, these enzymes cleave a number of cellular polypeptides leading to disassembly of key structural components of the nucleus and cytoskeleton, inhibition of DNA repair, replication, and transcription, and activation of endonucleases that irreversibly damage the genome (Fisher 1994, White 1996).

Members of the Bcl-2 family are known to modulate apoptosis in different cell types in response to various stimuli (Adams & Cory 1998, Reed 1998). Some members act as antiapoptotic proteins, such as Bcl-2 and Bcl-XL, whereas others function as proapoptotic proteins, such as BAX and BAK. Proapoptotic and antiapoptotic members can heterodimerize and seemingly titrate one another’s function. Many Bcl-2 family proteins reside on the mitochondrial outer membrane (Adams & Cory 1998, Reed 1998). Bcl-2 prevents mitochondrial disruption and the release of cytochrome c from mitochondria, while Bax and Bak create pores in mitochondrial membranes and induce cytochrome c release. In addition, most proapoptotic proteins antagonize antiapoptotic proteins through heterodimerization with them (Adams & Cory 1998, Reed 1998). Caspase-dependent apoptosis can also be regulated by members of the inhibitors of apoptosis (IAP) protein family. IAPs suppress apoptosis by physically interacting with and inhibiting the catalytic activity of caspases (Deveraux & Reed 1999). In apoptotic cells, the caspase inhibition by IAPs is negatively regulated by a mitochondrial protein Smac/DIABLO, which is released from the
Zhang: Vitamin A and apoptosis in prostate cancer

**Figure 2** Apoptosis pathways. In the extrinsic pathway, ligation of death receptors activates initiator caspase-8 through the adaptor molecule Fadd. In the intrinsic pathway, cytochrome c (Cyto c) is released from mitochondria in response to a variety of death stimuli and binds to Apaf-1 to activate caspase-9. Active caspase-8 or -9 then activates effector caspases, such as caspase-3, resulting in morphological features of apoptosis. Caspase-8 also activates Bid, which then targets mitochondria to induce cytochrome c release, providing a link between the two pathways.

mitochondrial intermembrane space into the cytosol upon apoptotic stimuli (Du et al. 2000, Verhagen et al. 2000).

**Apoptosis and prostate cancer development**

Impaired apoptosis is involved in tumor initiation and progression, since apoptosis normally eliminates cells with increased malignant potential such as those with damaged DNA or aberrant cell cycling (Fisher 1994, Thompson 1995). Most prostate cancer cells have a protracted history of development, suggesting that prostate cancer cells must have evolved various mechanisms to subvert the apoptotic program (Bruckheimer & Kyprianou 2000). Impaired apoptosis signaling and extended cell survival seem to be closely associated with prostate tumor initiation, metastasis and progression to the androgen-insensitive state (Coffey et al. 2001). Increased levels of Bcl-2 are associated with emergence of an androgen-independent phenotype and overexpression of Bcl-2 can facilitate multistep prostate carcinogenesis in an animal model (Bruckheimer et al. 2000). Proapoptotic Bax contains a polymorphism in an unstable microsatellite causing a frameshift in androgen-independent DU145 cells (Rampino et al. 1997).

Recent studies have indicated a crucial role of the PTEN tumor suppressor in the regulation of prostate cancer development. PTEN catalyzes dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate and antagonizes signaling pathways that rely on PI3K activity (Wu et al. 1998). PTEN is frequently inactivated in primary human prostate cancers, particularly in the more advanced cancers (Ittmann 1998), in human prostate xenografts and in cell lines (Li et al. 1997, Vlietstra et al. 1998, Whang et al. 1998). Release of the negative regulation of the PI3K pathway by PTEN may activate the cell survival kinase Akt during prostate tumor progression (Stambolic et al. 1998). Indeed, activated Akt regulates a number of intracellular events implicated in prostate tumor progression and androgen independence. Disruption of PTEN leads to suppression of apoptosis (Stambolic et al. 1998), due to inactivation of Bad (Datta et al. 1997) or caspase-9 (Cardone et al. 1998) by Akt. The disruption can also accelerate cell cycle progression (Sun et al. 1999a), through suppression of AFX/Forkhead transcription factor activity by Akt (Brunet et al. 1999, Kops et al. 2000)
Androgen ablation and apoptosis

Androgen withdrawal is the primary choice of therapy for men with advanced prostate cancer, and it generally leads to regression of the disease. It is believed that apoptosis is mainly responsible for the regression of prostate cancer cells (Buttyan et al. 2000) and increased levels of apoptosis were indeed observed in human prostate cancer cells after androgen withdrawal (Denmeade et al. 1996, Reuter 1997, Montironi et al. 1998). However, in the CWR22 human prostate cancer xenograft model it was shown that the regression was due to cell cycle arrest rather than to apoptosis (Agus et al. 1999). It remains to be further investigated as to what degree that apoptosis is involved in tumor regression and how the process is regulated.

Progression to androgen independence after androgen-deprivation therapy is a multifactorial process by which cells acquire the ability to proliferate in the absence of androgens. Altered expression of apoptotic-regulatory genes likely plays some role in the development of hormone resistance of prostate cancer (Howell 2000). In the LNCaP prostate tumor model, adjuvant treatment with antisense Bcl-2 oligonucleotides after castration delays progression to androgen independence (Gleave et al. 1999). Androgen-independent prostate cancer cells also show resistance to apoptosis induction by chemotherapeutic agents and radiotherapy (Bruckheimer & Kyprianou 2000, Szostak & Kyprianou 2000). Overexpression of Bcl-2 and Bcl-XL is found in many androgen-independent cell lines and may be responsible for resistance to apoptosis (Bruckheimer & Kyprianou 2000, Coffey et al. 2001, Li et al. 2001), and antisense Bcl-2 oligonucleotides sensitize prostate cancer cells to the apoptotic effect of chemotherapeutic agents (Leung et al. 2001).

Retinoids and prostate cancer apoptosis

Growing evidence suggests that induction of apoptosis is a major mode of cell death in response to most cancer chemopreventive and chemotherapeutic agents (Fisher 1994, Thompson 1995, Bruckheimer & Kyprianou 2000). Retinoids exert potent apoptotic effects both in development and in cancer cells (Nagy et al. 1998). Retinoid-induced teratogenesis is associated with craniofacial malformations due to excessive apoptosis in the region (Sulik et al. 1988), while the limb malformations induced by retinoids are also associated with excessive cell death in the apical ectodermal ridge (Sulik & Dehart 1988). Retinoids regulate the development of the central nervous system in part through their apoptotic effect (Alles & Sulik 1990, 1992).

Induction of apoptosis by retinoids has been observed in various prostate cancer cells in vitro and in vivo. Trans-RA induces apoptosis of normal and malignant epithelial prostate cells (Pasquali et al. 1999), and it strongly enhances the apoptotic effect of docetaxel in DU-145 and LNCaP prostate cancer cells (Nehme et al. 2001). The combination of trans-RA and organic arsenical melarsoprol synergistically induces apoptosis of DU-145 and PC-3 cells in vitro and in immunodeficient mice (Koshiuka et al. 2000). The synthetic retinoid N-(4-hydroxyphenyl) retinamide (4HPR) is known to induce apoptosis in various malignant cells (Nagy et al. 1998). 4HPR also induces apoptosis of androgen-dependent and -independent cells (Sun et al. 1999b, Webber et al. 1999). The combination of 13-cis RA and phenylbutyrate synergistically induces apoptosis of several human and rodent prostate carcinoma cell lines (Pili et al. 2001).

The molecular mechanisms by which retinoids induce apoptosis of prostate cancer cells remain largely unknown. Induction of apoptosis of prostate cancer cells by several retinoids appears to be associated with down-regulation of Bcl-2 expression (DiPaola & Aisner 1999, DiPaola et al. 1999, Pasquali et al. 1999, Nehme et al. 2001), induction of insulin-like growth factor-binding protein-3 (IGFBP-3) (Goossens et al. 1999) and tissue transglutaminase (Pasquali et al. 1999), an enzyme that accumulates in cells undergoing apoptosis. Interestingly, RXRα was found to interact with IGFBP-3, and IGFBP-3-induced apoptosis was abolished in RXRα-knockout cells. It is likely that RXRα/IGFBP-3 interactions modulate the effects of IGFBP-3 on apoptosis (Liu et al. 2000).

RARβ and retinoid responses

The involvement of retinoid receptors in mediating proapoptotic effects of retinoids is complex, since some retinoids may act in a retinoid receptor-independent manner. However, many studies have suggested a crucial role of RARβ in the modulation of retinoid-induced apoptosis of prostate cancer cells. RARβ is up-regulated during apoptosis induced by the combination of phenylbutyrate and 13-cis RA in human and rodent prostate carcinoma cell lines and prostate tumors in the xenograft model (Pili et al. 2001), suggesting that RARβ expression may mediate the growth-inhibitory effect of retinoids. RARβ was also induced during trans-RA-induced apoptosis of prostate cancer cells (Pasquali et al. 1999). The expression of RARβ in 4HPR-treated prostate tissue was slightly higher than in the placebo-treated group (Lotan et al. 2000). Interestingly, introduction of RARβ in RARβ-negative prostate cancer cells resulted in increased sensitivity to the growth-inhibitory effect of retinoids and vitamin D (Campbell et al. 1998).

The involvement of RARβ is also implicated by the finding that its expression mediates prostatic ductal branching morphogenesis in response to retinoids (Aboessef et al. 1997). The expression of RARβ and RXRβ was significantly reduced in malignant prostate compared with normal prostates (Lotan et al. 2000). In contrast, RARα, RARγ, RXRα and RXRγ were expressed in both normal and prostate tumor tissues (Lotan et al. 2000). RARβ was also selectively lost in DU-145 and PC-3 androgen-independent prostate cancer cell lines while RARα, RARγ and RXRα were well expressed (Campbell et al. 1998, Sun et al. 1999b). These observations suggest that loss of RARβ is associated with prostate carcinogenesis. The fact that reduced RARβ was observed in the normal tissue adjacent to the tumor suggests that this change is an early event in prostate carcinogenesis (Lotan et al. 2000). Similar changes were also observed in head and neck cancer (Xu et al. 1994).

How RARβ exerts its potent tumor-suppressive effects remains to be elucidated. A recent study demonstrated that RARβ can potently inhibit AP-1 activity (Lin et al. 2000b) and induce apoptosis of various cancer cells. The proapoptotic effect of RARβ was implicated in the finding that the expression of RARβ in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (Dolle et al. 1989, Mendelsohn et al. 1991, Ruberte et al. 1991, Kochhar et al. 1993, Soprano et al. 1993a,b). In our previous study, we observed that trans-RA-induced apoptosis in ZR-75-1 breast cancer cells is mediated by RARβ (Liu et al. 1996). Inhibition of RARβ activity by the expression of RARβ anti-sense RNA reduced the number of apoptotic cells, whereas trans-RA-induced apoptosis was only observed in hormone-independent cells when RARβ was introduced and expressed in the cells (Liu et al. 1996).

The mechanism that causes loss of RARβ in prostate cancer is not clear. It is unlikely that lack of RARβ expression is due to structural abnormalities of the RARβ gene (Gebert et al. 1991), but possibly because of changes in transcription. Expression of RARβ is highly induced by trans-RA through a RARE (βRARE) present in its promoter (Hoffmann et al. 1990, Sucov et al. 1990, de The et al. 1990), which is activated by RAR/RXR heterodimers in response to retinoids (Zhang et al. 1992a). Vitamin A serum levels are lower in patients with prostate cancer (Reichman et al. 1990). In addition, prostate cancer tissues have five to eight times less trans-RA than normal prostate or benign prostate (Pasquale et al. 1996). Reduced levels of retinoids in prostate cancer tissue may contribute to loss of RARβ expression. Interestingly, RARβ cannot be induced by exogenous retinoids in androgen-independent prostate cancer cells, despite expression of RARs and RXRs in these cells (Sun et al. 1999b). Similar observations were also made in other cancer, such as lung cancer, cells which express RARs and RXRs, but fail to express RARβ in response to retinoids (Zhang et al. 1994). These observations argue against the involvement of reduced retinoid levels in inhibiting RARβ expression, and also demonstrate that expression of RARs and RXRs is not sufficient to render RARβ expression responsive to trans-RA. Thus, factors other than RARs and RXRs are required for the effect of trans-RA on inducing RARβ expression, and these may be lost in cancer cells. Recently, we found that expression of the orphan receptor COUP-TF is positively correlated with RARβ induction and growth inhibition by trans-RA in various cancer cell lines and it is underexpressed in many RARβ-negative cancer cell lines (Wu et al. 1997b, Lin et al. 2000a). Further studies demonstrated that COUP-TF is required for trans-RA to induce RARβ expression, growth inhibition and apoptosis in cancer cells (Lin et al. 2000a). The effect of COUP-TF is likely due to its transactivation of the RARβ promoter through its binding to a DR-8 element present in the promoter, resulting in enhanced interaction of RARα with its co-activator CBP (Lin et al. 2000a). Thus, COUP-TF induces RARβ promoter transcription by acting as an accessory protein for RARα to recruit its co-activator. Whether lack of COUP-TF expression is responsible for loss of RARβ in androgen-independent prostate cancer cells remains to be illustrated. Methylation of the RARβ promoter was recently reported to contribute to RARβ inactivity (Sirchia et al. 2000), suggesting a possibility of hypermethylation of the RARβ promoter in prostate cancer cells.

The anti-cancer effects of conventional retinoids appear to be limited to androgen-dependent prostate cancer cells, whereas the more aggressive, androgen-independent prostate
cancer cells are refractory (Campbell et al. 1998). Loss of RARβ induction by trans-RA may be responsible for diminishment of trans-RA activities in androgen-independent prostate cancer cells. Induction of RARβ by classical retinoids, such as trans-RA, is mediated by activation of RAR/RXR heterodimers which bind to the βRARE (Zhang et al. 1992a). Unfortunately, this pathway appears to be impaired in androgen-independent prostate cancer cells. It is therefore important to identify alternative pathways that activate the RARβ promoter. Recent studies have demonstrated that RXR-selective retinoids represent promising agents for the prevention and treatment of cancer. 9-cis RA has demonstrated significant anti-proliferative and/or differentiating activity in in vitro models of breast cancer (Anzano et al. 1994, Rubin et al. 1994, Gottardis et al. 1996b), leukemia and lymphoma (Gottardis et al. 1996b), lung cancer (Guzey et al. 1998), and head and neck cancer (Giannini et al. 1997). Its activity was also observed in prostate cancer cells (Blutt et al. 1997, McCormick et al. 1999). Combination of 9-cis RA and 1,25-dihydroxyvitamin D3 synergistically inhibited the growth of LNCaP (Blutt et al. 1997, McCormick et al. 1999). McCormick et al. (1999) conducted a chemoprevention study to evaluate the activity of 9-cis RA as an inhibitor of prostate carcinogenesis in animals, and observed that continuous dietary administration of 9-cis RA before MNU administration reduced cancer incidence in the dorsolateral + anterior prostate. Similarly, the dosage levels of 9-cis RA reduced the incidence of cancer in all accessory sex glands (McCormick et al. 1999). RXR-selective retinoids were more effective than trans-RA at inhibiting mammary carcinogenesis in animals (Anzano et al. 1994), and RXR-selective retinoid LGD 1069 inhibited the growth of established breast tumors (Gottardis et al. 1996a, Bischoff et al. 1998).

How RXR ligands effectively inhibit the growth of cancer cells has not been established. Through its binding to RXR, RXR ligands may indirectly influence a wide range of functions, which are regulated by other nuclear receptors that heterodimerize with RXR (Zhang & Pfahl 1993, Kastner et al. 1995, Mangelsdorf & Evans 1995). In our previous studies (Wu et al. 1997a), we observed that inhibition of cancer cell growth by RXR-selective retinoids was associated with induction of RARβ expression in estrogen-independent MDA-MB231 cells and lung cancer cells (Wu et al. 1997a), suggesting that induction of RARβ expression contributes to the growth-inhibitory effects of these retinoids. Furthermore, we observed that their effect on RARβ induction is in part mediated through TR3/ RXR heterodimers which bind to the βRARE (Wu et al. 1997a). Thus, RXR ligands may exert their potent anti-cancer activity through inducing RARβ expression in cancer cells that are resistant to classical retinoids (Fig. 3). Thus, specific ligands for the RXR receptor may have significant activity as inhibitors of carcinogenesis in the prostate, whereas retinoids whose binding is limited to RAR may be inactive.

AHPN and its analogs: potent apoptotic inducers of prostate cancer cells

The sensitivity of prostate cancer cells to apoptosis-inducing effects of retinoids diminishes during the progression of prostate tumors. Androgen-independent derivatives of LNCaP cells were more resistant than their parental androgen-dependent LNCaP cells to apoptotic effects of trans-RA. In addition, malignant prostate cancer cells showed resistance to radiotherapy and chemotherapy. This has been the major challenge in the therapy of prostate cancer. Thus, retinoids capable of inducing apoptosis of advanced malignant prostate cancer cells are expected to be suitable agents for prostate cancer treatment.

Recently, a new class of synthetic retinoids related to AHPN (also called CD437) (Bernard et al. 1992) has been found to potent inhibit the growth and induce apoptosis of both androgen-dependent and -independent human prostate carcinoma cells (Liang et al. 1999, Lu et al. 1999, Li et al. 2000, Sun et al. 2000). When the growth-inhibitory and apoptosis-inducing effects of trans-RA and AHPN were compared in androgen-dependent and -independent prostate cancer cell lines, AHPN significantly inhibited the growth and induced apoptosis of androgen-independent prostate cancer cell lines, while trans-RA had little effect on these cells (Sun et al. 2000). A synthetic retinoid, CD-271, which is related to AHPN and selectively activates the RARγ subtype in a given context, also shows increased anti-proliferative activity against prostate cancer cells over trans-RA (Lu et al. 1999). Interestingly, AHPN was more effective in killing androgen-independent cells such as DU-145 and PC-3 than the androgen-dependent LNCaP cells (Sun et al. 2000). Thus, AHPN may be representative of a novel class of compounds suitable for treatment of androgen-independent prostate cancer. AHPN was also identified to be a potent apoptotic inducer in many different types of cancers, including lung (Sun et al. 1997, 1999c,d,e, Adachi et al. 1998b, Li et al. 1998), cervical (Oridate et al. 1997), ovarian (Langdon et al. 1998), melanoma (Schadendorf et al. 1995, 1996), leukemia (Hsu et al. 1997, Gianni & de The 1999, Mologni et al. 1999) and neuroblastoma (Meister et al. 1998). The apoptotic effect of AHPN is independent of retinoid receptor expression, indicating that its activity is not restricted by lack of RARβ in prostate cancer cells.

Orphan receptor TR3: a regulator of both survival and apoptosis of prostate cancer cells

AHPN-induced apoptosis may involve p53-dependent and -independent as well as caspase-dependent and -independent pathways (Adachi et al. 1998a, Fontana et al. 1998, Hsu et al. 1999, Marchetti et al. 1999, Zhang et al. 1999, Zhang

Endocrine-Related Cancer (2002) 9 87–102

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Zhang: Vitamin A and apoptosis in prostate cancer

**Figure 3** Signaling pathways for RARβ induction. βRARE in the RARβ promoter is essential for induction of RARβ by retinoids. The βRARE can be activated by RAR/RXR heterodimer in response to RAR ligands. Alternatively, it can be activated by RXR ligands through a TR3/RXR heterodimer that also binds to the βRARE.

2000). Expression of a variety of apoptosis-associated genes, such as cJun, cFos, c-Myc, p21, Bcl-2, Bax, DR4, DR5 and Fas can be regulated by AHPN in a cell type-specific manner. Their role in AHPN-induced apoptosis, however, remains to be determined. We have recently demonstrated that the expression of TR3 is required for AHPN-induced apoptosis in human prostate cancer cells (Li et al. 2000). TR3 message was also highly induced by AHPN in LNCaP cells (Li et al. 2000). The apoptotic effect of the AHPN analog MM11453 was completely abolished in LNCaP cells stably expressing TR3 antisense RNA (Li et al. 2000).


TR3 plays a critical role in regulating both proliferation and apoptosis of prostate cancer cells. Levels of TR3 are dramatically induced by androgen (Uemura & Chang 1998) and epidermal growth factor (Li et al. 2000) in LNCaP prostate cancer cells. Interestingly, TR3 is also rapidly induced in LNCaP cells in response to apoptotic stimuli, including AHPN (Li et al. 2000), calcium ionophore, etoposide (VP-16) (Uemura & Chang 1998, Li et al. 2000) and phorbol ester (Young et al. 1994, Li et al. 2000) and in the ventral prostate of animals by androgen ablation (Uemura & Chang 1998). Expression of TR3 antisense RNA significantly inhibits apoptosis induced by these agents (Li et al. 1998, Uemura & Chang 1998). Because of its potent effects in regulating cellular proliferation and apoptosis, TR3 may play a role in the development or progression of prostate cancer. In fact, TR3 is more highly expressed in prostate cancer areas than in adjacent normal or benign prostate hypertrophic tissue (Uemura & Chang 1998). TR3 is also highly expressed in lung cancer cell lines (Wu et al. 1997b). The role of TR3 in cancer development is further indicated by the finding that TR3 is involved in a chromosomal translocation identified in extra-skeletal myxoid chondrosarcoma (Labelle et al. 1995, 1999).

How TR3 exerts opposing biological activities was poorly understood. Similar to other members of the steroid/thyroid/retinoid receptor superfamily, it was believed that TR3 functioned in the nucleus as a transcriptional factor to regulate gene expression necessary to alter the cellular phenotype in response to various stimuli. TR3 response elements (NBRE or NurRE) have been identified (Wilson et al. 1990, Philips et al. 1997). In addition, TR3 can heterodimerize with RXR (Forman et al. 1995, Perlmann & Janson 1995, Wu et al. 1997a) and COUP-TF (Wu et al. 1997b). The observations that over-expression of TR3 in cancer cells confers retinoid resistance by modulating transcriptional
regulation of retinoids (Wu et al. 1997b) and that the TR3 fusion protein identified in extra-skeletal myxoid chondrosarcoma is about 270-fold more active than the native receptor in transactivation (Labelle et al. 1995, 1999) suggests that TR3 may mediate cell proliferation through its transcriptional regulation.

Much less was known about the mechanism by which TR3 functions to regulate apoptosis. TR3 might be involved in the apoptotic process by regulating expression of certain apoptosis-associated genes (Liu et al. 1994, Woronicz et al. 1994, 1995, Weih et al. 1996, Cheng et al. 1997). Unfortunately, no comprehensive characterization of its target genes was achieved. By using a variety of approaches, we recently demonstrated that TR3-dependent apoptosis of LNCaP prostate cancer cells does not require its DNA binding and transactivation, but is associated with translocation of this protein from the nucleus to mitochondria, where it resides on the outer mitochondrial membrane and induces cytochrome c release (Li et al. 2000). These results reveal a novel mechanism by which a nuclear transcriptional factor translocates to mitochondria to initiate apoptosis (Fig. 4). Translocation of TR3 between the nucleus and the cytoplasm represents a new mechanism for cross-talk between different signaling pathways (Fig. 4). This exciting finding, together with the observations that TR3 is associated with cancer cell proliferation by acting as a nuclear transcriptional factor, demonstrates that the opposing biological activities of TR3 are regulated by its subcellular localization. These data suggest a new approach of eliminating prostate cancer cells by inducing cytoplasmic localization of TR3. AHPN analogs and other agents that specifically induce TR3 mitochondrial localization will effectively induce apoptosis of prostate cancer cells that express TR3. Interestingly, TR3 is induced by androgen or growth factors through nuclear action of TR3. Thus, AHPN and related analogs may be potent inhibitors of androgen and growth factor action in prostate cancer cells.

**Prospective**

Induction of apoptosis is an effective way to eliminate cancer cells. The acquisition of resistance toward apoptosis during prostate tumor progression is perhaps the major obstacle in the treatment of prostate cancer. Retinoids inhibit the growth and induce apoptosis of prostate cancer cells in vitro and prevent prostate carcinogenesis in animals, suggesting that retinoids are promising agents for the prevention and treatment of human prostate cancer. However, the apoptotic effect of classical retinoids diminishes in androgen-independent prostate cancer cells, and clinical trials using conventional retinoids have not demonstrated significantly beneficial effects. Loss of RARβ may contribute to retinoid resistance in advanced prostate cancer cells. Alternative approaches to induce RARβ expression may render prostate cancer cells sensitive to apoptotic effects of retinoids. In vitro and animal studies have suggested that RXR ligands are effective inhibitors of prostate carcinogenesis and they are capable of inducing RARβ expression through alternative approaches, such as TR3/RXR heterodimers. Elucidation of their mechanisms of action will provide valuable information, allowing design and identification of a new generation of synthetic retinoids that are likely to be more effective in the prevention and treatment of prostate cancer.

Synthetic retinoids related to AHPN effectively induce apoptosis of both androgen-dependent and -independent prostate cancer cells, indicating that these retinoids represent a new class of drugs that have therapeutic value for the treatment of prostate cancer. The clinical potential of this class of retinoids and their new generation needs to be explored.

Modern biology has suggested that cancer drug discovery based on molecular differences between tumor and normal cells is a new and feasible approach. With an improved understanding of apoptotic processes in prostate cancer cells, many potential new targets for therapy can be discovered. The illustration that orphan receptor TR3 mediates the apoptotic effect of AHPN analogs in prostate cancer cells suggests that TR3 is an ideal target for cancer drug development. Levels of TR3 are induced by androgen and growth factor in prostate cancer cells as well as by androgen ablation and may be necessary to support proliferation of prostate cancer cells. Thus, TR3 can mediate opposing biological activities, cell death and survival (Fig. 4). The unique property of TR3 provides an excellent opportunity to develop novel drugs targeted at TR3. Agents such as AHPN and its analogs that specifically induce mitochondrial localization of TR3 will convert TR3 from a cancer cell-promoting (adverse effect) to a cancer cell apoptosis-inducing (beneficial effect) molecule.

Cellular localization of TR3 defines its biological function. How TR3 is translocated from the nucleus to the cytoplasm and targets mitochondria in response to apoptotic stimuli is unclear. This information is essential for developing retinoids that induce mitochondrial localization of TR3. The fact that TR3 mitochondrial targeting is regulated by various stimuli, including TPA, calcium ionophore and growth factors (Li et al. 2000), which are known to act through membrane signaling pathways involving various kinases and phosphatases, suggests that phosphorylation of TR3 may play a crucial role in regulating TR3 subcellular activities.

The observation that TR3 can heterodimerize with RXR (Forman et al. 1995, Perlmann & Jansson 1995, Wu et al. 1997a) suggests that RXR and its ligands are likely involved in the regulation of TR3-dependent apoptotic pathways. This is supported by previous observations that RXR and its ligand 9-cis-RA inhibit activation-induced apoptosis of T-cells and thymocytes (Yang et al. 1993, 1995a,b, Bissonnette et al. 1995, Szondy et al. 1998), in which TR3 plays a role (Liu et al. 1994, Woronicz et al. 1994, 1995). RXR, through its heterodimerization with TR3, may be required...
Zhang: Vitamin A and apoptosis in prostate cancer

**Figure 4** TR3-dependent cell survival and cell death pathways. TR3 induced by survival stimuli, such as growth factors, functions in the nucleus through either its homodimerization or heterodimerization with RXR or COUP-TF to regulate expression of genes involved in cell proliferation. In contrast, TR3 induced by death stimuli, including AHPN, may undergo a conformational change, which is required for its export to the cytoplasm, where it resides on mitochondria. On mitochondria, TR3 regulates mitochondrial activities, resulting in release of cytochrome c (cyto c) into the cytosol.

for cytoplasmic localization of TR3 or for its mitochondrial targeting. Illustrating the molecular mechanisms by which RXR and its ligands regulate TR3-dependent apoptotic pathways in prostate cancer cells will provide additional modes to regulate apoptosis of prostate cancer cells and new treatment approaches for prostate cancer.

**Acknowledgements**

We thank L Frazer for the preparation and Siva Kumar Kolluri and Sharon James for the critical reading of the manuscript. This work is in part supported by grants to the author from the National Institutes of Health, the US Army Medical Research and Material Command and The Charlotte Geyser Foundation.

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Zhang: Vitamin A and apoptosis in prostate cancer


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