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Death Receptor-Induced Cell Death in Prostate Cancer

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Abstract Prostate cancer mortality results from metastasis and is often coupled with progression from androgen-dependent to androgen-independent growth. Unfortunately, no effective treatment for metastatic prostate cancer increasing patient survival is available. The absence of effective therapies reflects in part a lack of knowledge about the molecular mechanisms involved in the development and progression of this disease. Apoptosis, or programmed cell death, is a cell suicide mechanism that enables multicellular organisms to regulate cell number in tissues. Inhibition of apoptosis appears to be a critical pathophysiological factor contributing to the development and progression of prostate cancer. Understanding the mechanism(s) of apoptosis inhibition may be the basis for developing more effective therapeutic approaches. Our understanding of apoptosis in prostate cancer is relatively limited when compared to other malignancies, in particular, hematopoietic tumors. Thus, a clear need for a better understanding of apoptosis in this malignancy remains. In this review we have focused on what is known about apoptosis in prostate cancer and, more specifically, the receptor/ligand-mediated pathways of apoptosis as potential therapeutic targets. J. Cell. Biochem. 91: 70–99, 2004.

Key words: apoptosis; death receptors; prostate cancer

APOPTOSIS

Apoptosis or programmed cell death is a cell suicide mechanism that enables multicellular organisms to regulate cell number in tissues and to eliminate unneeded or aging cells as an organism develops. Apoptosis is physiologically normal and an important process for multicellular organisms [Ellis et al., 1991]. However, inappropriate apoptosis is implicated in many human diseases, including cancer [Evan and Vousden, 2001]. Investigation of the mechanisms of apoptosis is one of the hottest areas of modern biology and medicine. Stereotypical morphological changes, as well as biochemical and molecular pathways have been described by many authors [Wyllie, 1997; Kidd, 1998; Hengartner, 2000]. Despite very intensive investigation, many aspects of apoptosis remain poorly understood because of a very high dependence on cell context, the different signals that can induce apoptosis, and the multiple pathways that are involved.

ROLE OF APOPTOSIS IN PROSTATE CANCER

Prostate cancer mortality results from metastasis to the lymph nodes and bone, and progression from androgen-dependent to androgen-independent growth [Isaacs et al., 1994]. It is believed that prostate cancer, like other neoplastic diseases, develops and progresses through an accumulation of genetic alterations. The exact molecular mechanisms underlying the onset and progression of prostate cancer are unknown. Unfortunately, there are limited treatment options available for this disease because chemotherapy and radiotherapy are largely ineffective, and metastatic disease frequently develops even after surgery [Petrylak, 1999; Pisters, 1999; Richie, 1999]. No treatment for metastatic prostate cancer is available that effectively increases patient survival. Inhibition of apoptosis appears to be a critical pathophysiological factor that contributes to the development of prostate cancer. The

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absence of effective therapies reflects in part the lack of knowledge about the molecular mechanisms involved in the development and progression of this disease. Understanding the mechanism(s) of apoptosis inhibition could be the bases for developing more effective therapeutic approaches. Because apoptosis is closely involved in the initiation and progression of human prostate cancer, many chemoprevention and therapeutic regimens attempting to manipulate the apoptosis process have been proposed to aid in treatment.

THE CENTRAL PLAYERS OF APOPTOSIS

Despite the fact that several alternative caspase-independent cell death pathways have recently been described [Leist and Jaattela, 2001], the central players of apoptosis are caspases that are present in the cytosol of all animals and responsible for the cellular changes that occur during apoptosis. Caspases constitute a family of cysteine proteases that cleave substrates at aspartic acid (Asp) residues [Nicholson and Thornberry, 1997; Stennicke and Salvesen, 1998]. Caspases share distinct similarities in amino aside sequence and structure. Despite their shared requirement for cleavage after Asp residues, caspases are highly specific in their substrate preferences [Talanian et al., 1997; Thornberry, 1997]. As is true of most proteases, caspases are synthesized as inactive zymogens. These zymogens are composed of four distinct domains: an N-terminal prodomain of variable size, a large subunit (~20 kDa), a small subunit (~10 kDa) and a linker region between the large and small domains flanked by Asp residues. Classical activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer (Fig. 1). Since caspases both cleave their substrates at Asp residues and are also activated by processing at Asp residues, these proteases can collaborate in proteolytic cascades, in which caspases activate themselves and each other. The caspase cascade amplifies and integrates pro-apoptotic signals, but it cannot explain how the first, most upstream caspase gets activated. Mammalian caspases have been divided into upstream (initiator) and downstream (effector) caspases. Initiator caspases have long prodomains containing structurally related protein modules that physically link these proteases to their

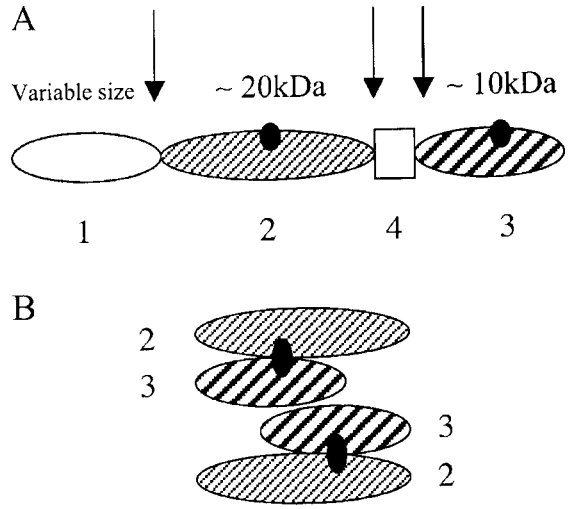


Fig. 1. Schematic illustration of a caspase zymogen (**A**) and active form (**B**). Like other proteases, caspases are synthesized as zymogens. Activation of each caspase is induced by proteolytic cleavage between domains, resulting in the removal of the prodomain and linker region, and assembly of the large and small subunits into an active enzyme. Crystal structure analysis for caspase-1 and -3 shows that active caspases are composed of two heterodimers interacting via small subunits to form a tetramer with two catalytic sites: (1) prodomain–NH_2-terminal domain, which is highly variable in length (23–216 aa) and sequence; (2) large subunit (17–20 kDa); (3) small subunit (9–12 kDa); (4) linker. Arrows show Asp residues for cleavage. Large and small subunits contain residues that are essential for the function of the mature enzyme (shown by black oval).

specific activators. Two types of interaction modules have been detected in the prodomain of initiator caspases: death effector domain (DED) and caspase recruitment domain (CARD) [Boldin et al., 1996; Hofmann et al., 1997] (Fig. 2). Initiator caspases have substrate specificities that are similar to caspase recognition sites present in their own sequence [Thornberry et al., 1997] and can utilize autocatalysis for activation. In addition, it has been also shown that in the case of casapase-9 and -2 proteolytic cleavage between subunits is not necessary for their activation [Renatus et al., 2001; Read et al., 2002]. Optimal caspase recognition sites for initiator caspases are present in the effector caspases sequence, suggesting that these enzymes act downstream of initiator caspases in the proteolytic cascade. Activation of initiator caspases can be also amplified by effector caspases after their activation. Downstream effector caspases cleave multiple substrate proteins (such as PARP, DFF-45, Bcl-2) which protect living cells from apoptosis, destroy cell structural proteins, such as lamin,

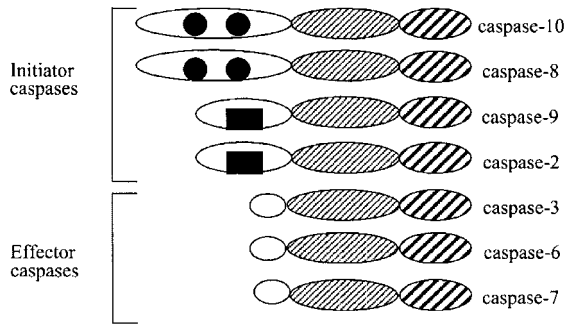


Fig. 2. Apoptosis involved caspases in mammalian cells. The length of the prodomains varies from 22 amino acid residues for caspase-3 and -6 to over 200 in caspase-8 and -10, whereas the active enzymes show high sequence identity and little variation in length. Caspase-8 and -10 contain tandem death effector domains (DED, showed in black circles) that are required for binding to adaptor proteins during death receptor ligation. Caspase-2 and -9 contain a caspase recruitment domain (CARD, black square), which is another protein interacting domain required for assembly of activation complexes. The effector caspases-3, -6, and -7 have very short prodomains with unknown function. The effector caspases are usually activated proteolitically by initiator caspases, whereas initiator caspases are activated through protein–protein interaction.

deregulate protein activity by separating regulatory and catalytic domains, resulting in loss or gain of function (e.g., gelsolin or focal adhesion kinase). Caspases cut off contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis, and disintegrate the cell into an apoptotic body [Thornberry and Lazebnik, 1998].

PATHWAYS OF CASPASE ACTIVATION

Two main pathways leading to caspase activation have been well characterized. The extrinsic pathway is activated by ligand-bound death receptors of the tumor necrosis factor (TNF) receptor family. Different stress pathways cause release of mitochondrial proteins into the cytosol to activate the other apoptosis pathway—the intrinsic pathway. The intrinsic and extrinsic pathways for caspase activation converge on downstream effector caspases. Caspase-8 and -10 are key initiator caspases in the extrinsic pathway. Upon ligand binding, death receptors form membrane-bound signaling complexes (or death-induced-signaling complex: DISC). These complexes then recruit, through adapter proteins, several molecules of procaspase-8 (or -10) resulting in a high local concentration of these zymogens. Under these high concentrations, the low intrinsic protease activity of procasapse-8 (or -10) is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other [Boldin et al., 1996; Muzio et al., 1998]. Activated initiator caspases start the caspase cascade that leads to apoptosis (Fig. 3).

Activation of the intrinsic apoptotic pathway starts from mitochondria. The role of mitochondria in apoptosis is very important and described in several reviews [Green and Reed, 1998; Bossy-Wetzel and Green, 1999; van Loo et al., 2002]. A large variety of different stress signals, such as irradiation, chemical substances, changes in cell homeostasis, engage the mitochondria to release apoptogenic factors such as cytochrome c, Smac/DIABLO, $HtrA_2$/Omi, into the cytosol. Cytochrome c participates in another mechanism of caspase activation used by caspase-9. Unlike other caspases, proteolytic prosessing of procaspase-9 has only a minor effect of the enzyme's catalytic activity. Cytochrome c binds the adapter Apaf-1, forming an "apoptosome" that activates the apoptosis-initiating procaspase-9. The apoptosome is a large complex that might well contain several additional proteins [Cain et al., 2002; Shi, 2002]. In turn, caspase-9 activates effector caspases and induces cell death. Other mitochondrial proteins, Smac/DIABLO and $HtrA_2$/Omi, promote apoptosis by binding to inhibitor of apoptosis proteins (IAPs) and prevent these factors from attenuating caspase activation [Liu et al., 2000; Suzuki et al., 2001]. The IAP family of proteins prevents cell death by inhibiting caspases. IAPs are characterized by the presence of one to three domains known as baculoviral IAP repeat (BIR) domains that are important for their antiapoptoic activity, and also have a RING-finger domain and a CARD domain at the carboxyl terminus. [Deveraux and Reed, 1999; Verhagen et al., 2001; Salvesen and Duckett, 2002]. The effector caspases-3 and -7 and the initiator caspase-9 are among the caspases that are directly inhibited by human IAP family members XIAP, cIAP1, and cIAP2. Different domains in the multi-BIR containing IAPs are responsible for suppression of different caspases. Smac/DIABLO and $HtrA_2$/Omi have a novel tetrapeptide motif that binds the BIR domains of IAPs. IAP antagonists compete with caspases for binding IAPs thus freeing

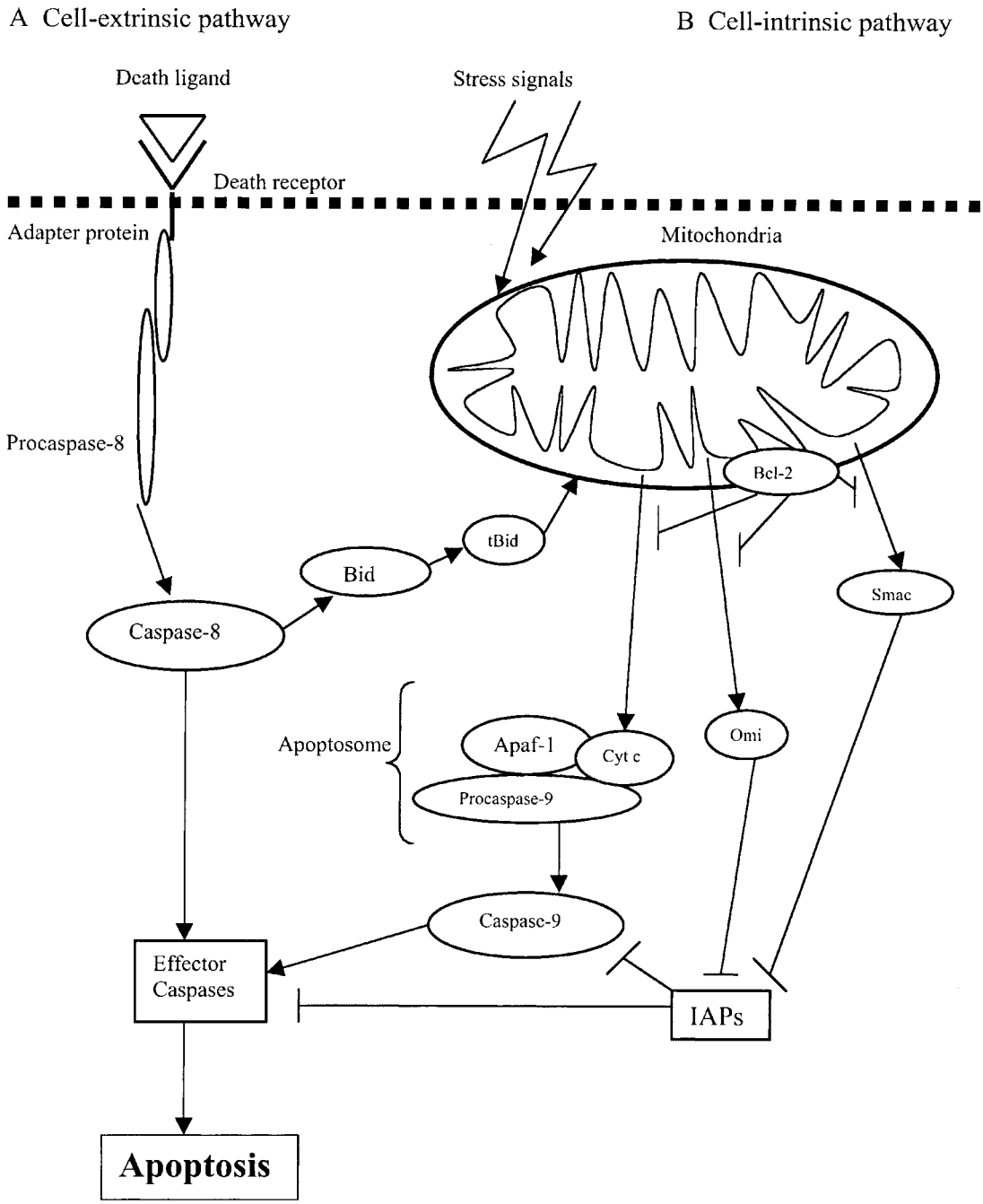


Fig. 3. Pathways of caspase activation. **A**: The cell-extrinsic pathway is activated in vivo by death ligands that engage death receptors, resulting in adapter proteins recruitment and activation of initiator caspases-8 or -10 that start the caspase cascade and cell death. Caspase-8 also cleaves Bid, which induces translocation of truncated Bid (tBid) to mitochondria and initiate the cell-intrinsic pathway. **B**: The cell-intrinsic pathway is activated by diverse stress signals which cause release of cytochrome c. Cytochrome c (Cyt c) together with the adaptor protein Apaf-1 participate in "apoptosome" formation and activation of caspase-9 that in turn activate effector caspases and cell death. In addition, stimulated mitochondria can release other apoptotic factors such as Smac/DIABLO and $HtrA_2$/Omi that bind inhibitors of apoptosis (IAPs). Bcl-2 can prevent release of mitochondrial factors.

caspases from the grip of the IAPs and promoting apoptosis.

The abilities of the intrinsic pathway to induce apoptosis is highly dependent on involvement of the Bcl-2 family proteins [Brown, 1997; Adams and Cory, 1998]. Bcl-2 family proteins are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability that is required for release of cytochrome

c and other apoptogenic proteins. More then 15 members of this family have been identified in mammalian cells. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) and can be divided into three groups (Fig. 4). The various Bcl-2 family members can dimerize with one another, with one monomer antagonizing or enhancing the function of the other. In this way the ratio of inhibitors to activators in a cell may determine the propensity of the cell to undergo to apoptosis. Another attractive mechanism to regulate dimerization of Bcl-2 family members is phosphorylation. For example, Bad, an anti-apototic member of the Bcl-2 family, is phosphorylated by a kinase that can be activated by growth factor engagement. Phosphorylated Bad loses its ability to bind Bcl-X_L an antiapoptotic family member. Instead, phosphorilated Bad binds to 14-3-3, a protein that can interact with several signaling enzymes. The dissociated Bcl-X_L can then execute its antiapoptotic functions.

Bcl-2, one of the best characterized antiapoptotic proteins of this family, localizes in the mitochondrial outer membrane, endoplasmic reticulum (ER) and nuclear envelope, and may register damage to these components by modifying the flux of small molecules of proteins [Reed, 1997; Green and Reed, 1998]. Another important member of the Bcl-2 family (Bid) is crucial for death receptor-mediated apoptosis in many cell systems. Activation of caspase-8 leads to cleavage Bid and the truncated form of Bid (tBid) localizes to mitochondria and facilitates the release of apoptogenic proteins (Fig. 3).

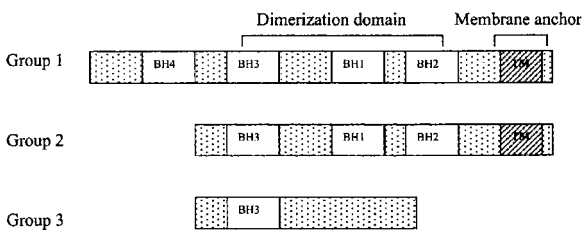


Fig. 4. Bcl-2 family members. Three groups have been identified. Group 1 is an anti-apoptosis group of proteins, such as Bcl-2 and Bcl-X_L. BH1 to BH4 are conserved sequence motifs. Group 2 proteins are proapoptotic and lack a functional BH4 domain. This group includes Bax and Bak. Group 3 is a large group of proapoptotic molecules such as Bid and Bad. This group contains only the BH3 domain and except for this domain, group 3 proteins are unrelated to Bcl-2. [Brown, 1997; Deveraux and Reed, 1999]. BH1, BH2, and BH3 domains are responsible for homo- or hetero-dimerization. The TM domain is important for localization to membrane structure.

However, the precise mechanism of tBid action is unknown [Esposti, 2002].

MULTIPATHWAY REGULATORS

Pathways of apoptotic events are closely dependent on cell context. There are many apoptosis-modulating proteins that may impact several apoptosis pathways simultaneously, possibly at multiple points. A simplified scheme of apoptosis regulation is shown in Figure 5. For example, transcription factors, such as NF-κB, AP-1, p53 can regulate expression of many genes involved in apoptosis on different levels. For example, Rel/NF-κB transcription factors have been shown to play an important role in the regulation of the apoptotic program, either as inducers or as inhibitors of apoptosis [Barkett and Gilmore, 1999; Lin et al., 1999]. Whether Rel/NF-κB promotes or inhibits apoptosis appears to depend on the specific cell type and the type of inducer. In mammals, there are five known members of the NF-κB family-p50/p105 (NF-κB1), p65/RelA, c-Rel, RelB and p52/p100 (NF-κB2)—each encoded by a unique gene. NF-κB proteins bind to specific DNA binding site as hetero- or homodimers. In normal cells, NF-κB is localized in the cytoplasm as an inactive complex bound by inhibitors known as IκBs. Various signals can lead to phosphorylation and then degradation of the IκBs with the resultant translocation of the active Rel/NF-κB into the nucleus [Henkel et al., 1993; Brown et al., 1995; Baldwin, 1996; Karin, 1999] where NF-κB regulates gene expression. Regulation by NF-κB has been described for more than 150 genes including genes of some death receptors and death ligands, and other transcriptional factors such as c-myc, junB, and p53. In addition, NF-κB can regulate expression of many anti-apoptotic proteins such as Bcl-2 family members and IAPs [Pahl, 1999]. Another transcriptional factor, AP-1, has also been implicated in the process of apoptosis [Wisdom, 1999; Shaulian and Karin, 2002]. AP-1 is a homo- or heterodimer composed mainly of basic region-leucine zipper proteins that belong to the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) families. In addition, Jun can dimerize with partners such as JDP1, ATF-2, ATF-3 [Wisdom, 1999; Shaulian and Karin, 2001]. The consequence of AP-1 activation is cell type specific. While it may promote apoptosis in some cell types, it is required for the survival

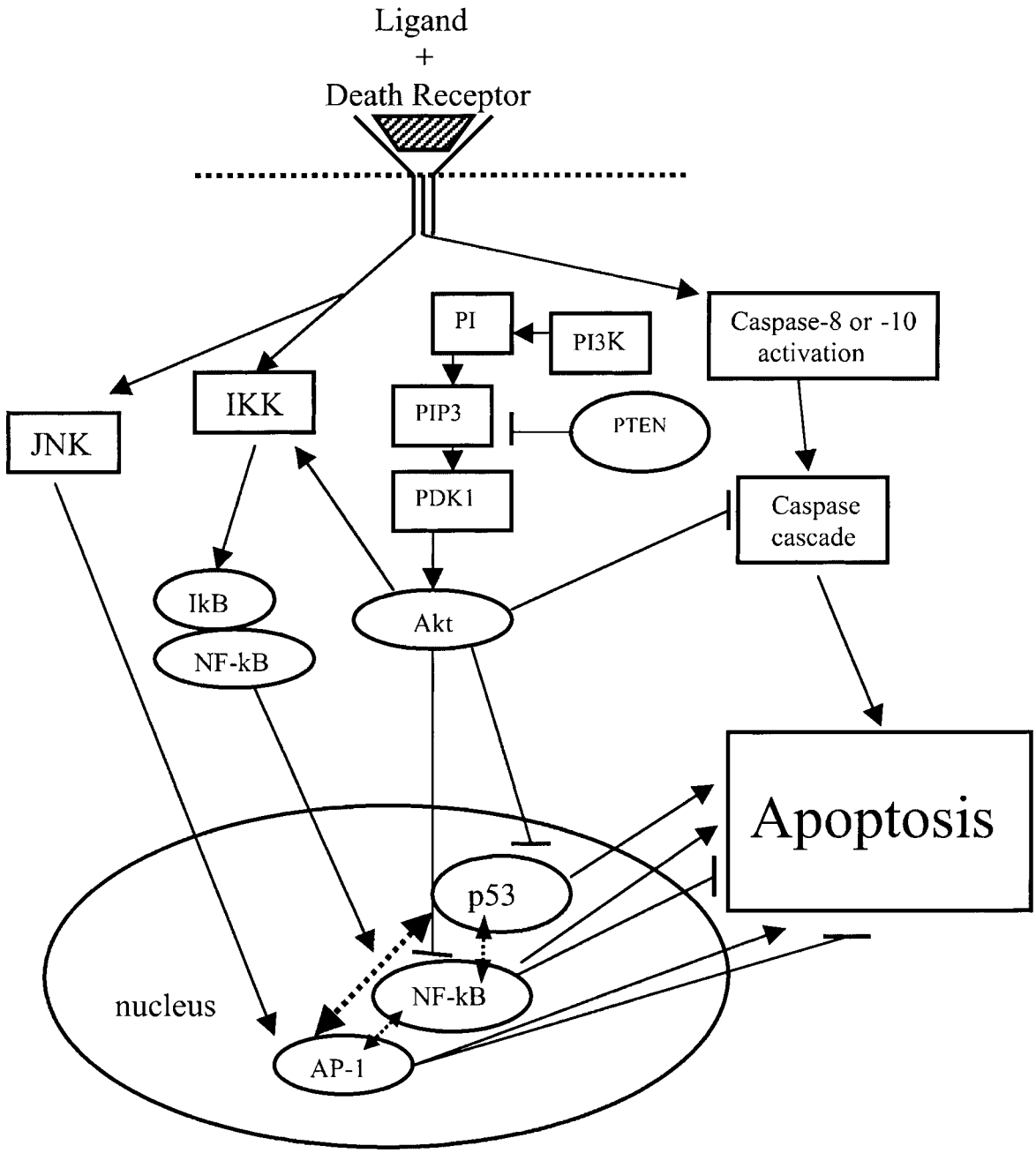


Fig. 5. Crosstalk of multipathway apoptosis regulators. Stimulation of death receptors by their ligands leads to recruitment and activation of caspase-8 or -10, activation of effector caspases, and apoptosis. At the same time, ligand–receptor ligation starts several other signaling pathways that regulate apoptosis. Activation of IKK leads to phosphorylation and degradation of inhibitors of NF-κB and translocation of NF-κB to the nucleus. NF-κB activates transcription of more than 150 genes including antiapoptotic and proapoptotic genes. Activation of JNK leads to AP-1 activation that also regulates transcription of proapoptotic as well as antiapoptotic genes. Multiple signals lead to activation of p53-strong proapoptotic factor. p53, NF-κB, and AP-1 effect each other's activation by different mechanisms (shown by spot lines). Death receptor signaling also leads to phosphorylation of phosphoinositol (PI) by PI3K and activation of phosphoinositide-dependent kinase-1 (PDK1) that phosphorylate and activate Akt. Akt has multiple roles in cell signaling. For example, Akt can activate IKK and induce NF-κB activation but also can decrease the transactivation ability of p65 of NF-κB and inhibit NF-κB. In addition, Akt prevents caspase activation and controls the level of p53, activation of Akt can be prevented by PTEN.

of others. The exact function of AP-1 is dependent on its composition, posttranslational modifications and presence of interacting proteins. It has been reported that AP-1 may regulate apoptosis by regulation of death ligands and death receptor expression via the AP-1 binding sites in their respective promoters [Eichhorst et al., 2001; Guan et al., 2002]. AP-1 can regulate control cell survival and death through its ability to regulate the expression and function of cell cycle regulators, such as cyclin D, p21 (WAF1) and p53 [Shaulian and Karin, 2001]. p53 plays a major role in modulating the apoptotic response in tumor cells following different signals [Vousden, 2000]. p53 can directly engage each of the major apoptosis pathways in the cell, stimulating both death receptor signaling and mitochondrial perturbation, including cytochrome c release. Signaling from the death receptors such as DR5 and Fas is increased by p53-dependent transcriptional activation of receptors [Bates and Vousden, 1999]. Transcriptionally independent activities of p53 induce relocalozation of death receptors from the Golgi to the cell surface [Bennett et al., 1998]. Recent reports have described p53 transcriptional targets that encode proteins localized to the mitochondria and effect mitochondrial membrane potential [Oda et al., 2000a,b]. In addition, p53 itself can localize to mitochondria and contribute to apoptosis by modulate signaling in mitochondria [Marchenko et al., 2000]. The level of p53 can be controlled by another apoptosis multipathway regulator-Akt (PKB) [Mayo and Donner, 2002]. Akt is an important regulator of cell proliferation and survival. There are three mammalian isoforms of this enzyme, Akt1, Akt2, and Akt3 [Chan et al., 1999]. Activation of all three isoforms is similar and requires the phosphorylation of two sites. Activation of PI3-kinase and Akt promotes cell survival through multiple mechanisms. Akt directly phosphorylates multiple protein targets of relevance to apoptosis, suppressing cell death within the intrinsic pathway. In addition to acting as a kinase for many substrates involving in these processes, Akt forms complexes with other proteins that act as modulators of Akt activity and function [Brazil et al., 2002]. Amplification of genes encoding Akt isoforms has been found in several types of human cancers [Hill and Hemmings, 2002]. Pathological elevation in Akt activity is common occurrence in tumors due to loss of the tumor suppressor PTEN (phosphatase and tensine homolog), also known as MMAC1. PTEN is able to dephosphorylate the lipid second messenger phosphotidilinositol (PI) 3,4,5-phosphate and negatively regulates survival signaling through the phospatidylinositol-3 kinase (PI3K)/Akt pathway.

DEATH RECEPTORS

Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily. The name, tumor-necrosis factor, underscores an historical connection between the TNF gene superfamily and cancer therapy. More then an hundred years ago it was noted that some patients with cancer had spontaneous regression of their tumors after acute bacterial infection. By 1944, Shear isolated a factor from gram-negative bacteria called endotoxin or lipopolysaccharide (LPS) that induced tumor necrosis after injection into tumor-bearing mice Shear et al. [1944]. In 1962 W. O'Malley with colleagues showed that serum from LPS-treated mice could also induce tumor necrosis in tumor bearing mice [O'Malley et al., 1962]. This observation was confirmed later by other investigators and LPS-induced serum factor was named tumor necrosis factor (TNF). The TNF receptor (TNFR) was shown to be expressed by mammalian cells years later, and led to the discovery of a superfamily of transmembrane proteins. In fact, two TNF gene families are now recognized, both as superfamilies, with 18 ligands and 28 receptors [Ashkenazi, 2002]. The ligands are expressed by cells as homotrimeric type-II transmembrane proteins, except LT-α, which is directly secreted as a soluble protein. The extracellular, carboxy-terminal region of many of the TNF-superfamily ligands is proteolytically processed by metalloproteases into a soluble protein.

The TNF-family receptors are type I membrane proteins. The basic signaling unit of the TNFRs superfamily consists of three receptors bound by a trimeric ligand molecule. Recent evidence indicates, however, that TNF-RI and Fas pre-associate in the plasma membrane as homooligomeres, independently of ligand; the association occurs through an amino-terminal region called the pre-ligand association domain (PLAD) [Locksley et al., 2001]. This implies that, at least for some TNF-superfamily members, the ligand might not actually induce

receptor oligomerization, suggesting that the pre-associated oligomeric receptor complex undergoes conformation changed in manner that facilitates signaling. Members of TNF receptor family contain an extracellular ligand-binding domain of one to six cysteine-rich repeated subdomains. Additionally, the death receptors contain a homologous cytoplasmic sequence termed the "death domain" (DD). Death domains mediate interaction of death receptors with DD-containing adaptor proteins. The adaptors contain additional sequence modules that mediate binding to intracellular effector enzymes. One adaptor, called FADD (Fas-associated death domain), activates specific caspases that initiate apoptosis. Another adaptor, called TRADD (TNFR-associated death domain), stimulates protein kinases that control phosphorylation cascades to induce activation of transcription factors. Alternatively TRADD can induce apoptosis through FADD [Ashkenazi and Dixit, 1998]. Thus, death receptors can transmit two distinct signaling cascades, leading either to cellular apoptosis or activation of NF-κB [Hsu et al., 1996; Ponton et al., 1996; Schneider et al., 1997]. The signal transduction machinery that couples a subset of these receptors to initiate cell death or NF-κB activation cascades is very dependent on cell type.

A subfamily of death receptors that contain DDs and that can transmit death signals after death ligand binding, include: CD95/Fas, DR3, TNF-RI, TRAIL-R1, TRAIL-R2, DR6, EDAR. Receptors that bind the same ligands but do not have or have truncated DDs can not transmit apoptotic signals (Table I). The best-characterized death receptors are CD95/Fas and TNF-R1 [Ashkenazi and Dixit, 1998]. TRAIL receptors have been intensively investigated in the last several years.

SIGNALING BY CD95L (FAS LIGAND)

Binding of CD95L to CD95 (Fas receptor) induces a series of protein-protein interactions that lead to assembly of a death-inducing signaling complex (DISC) at the cytoplasmic DD of CD95. Upon ligation, CD95/Fas recruits the adapter protein FADD (Fas-associated death domain) through their DDs (Fig. 6A). FADD also contains a "death effector domain" (DED) that binds to an analogous domain of procaspase-8 leading to self-activation of caspase-8, which in turn activates downstream effector caspases and induces apoptosis. CD95 signals can also trigger pathways that lead to NF-κB and AP-1 activation. For example, it has been shown that a caspase-8-like inhibitory protein (FLIP) can interact with TNF-receptor associated factors 1 and 2 (TRAF-1 and TRAF-2), as well as with the kinases RIP and Raf-1, resulting in the activation of the NF-κB and MAPK signaling pathways [Kataoka et al., 2000]. CD95L also binds to the decoy receptor DcR3, which does not have DD and cannot transmit signals. Overexpression of DcR3 can prevent CD95-induced apoptosis [Roth et al., 2001; Tsuji et al., 2003].

SIGNALING BY TNF-α

TNF-α binds TNF-RI inducing association of receptor DDs with TRADD (Fig. 6B). TRADD functions as a platform adapter that recruits several signaling molecules to the activated receptor: FADD, TRAFs, and receptor-interaction protein (RIP). FADD recruits caspase-8 and initiates an apoptosis caspase cascade in a manner similar to CD95. Signals through other adaptor proteins, such as TRAFs and RIP, lead to activation of NF-κB and JNK/AP-1. TRAFs and RIP activate the NF-κB-inducing kinase

TABLE I. Death Ligands and Their Receptors*

Death domain containing receptor	Non-death domain receptor	Death ligand
Fas (CD95, Apo1)	DcR3	CD95L (FasL, Apo1L)
TNFRI (p55, CD120a)	TNFRII (p75, CD120b)	TNFα, LTα
DR3 (Apo3, WSL-1, TRAMP, LARD)	DcR3	Apo3L (TWEAK, TL1A)
TRAIL-R1 (DR4)	TRAIL-R3 (DcR1, TRID, LIT)	TRAIL
TRAIL-R2 (DR5, Apo2, TRICK2, KILLER)	TRAIL-R4 (DcR2, TRUNDD)	(Apo2L)
	OPG	
DR6		?
EDAR		EDA (A1)

*Synonyms are given in parentheses [see Ashkenazi, 2002].

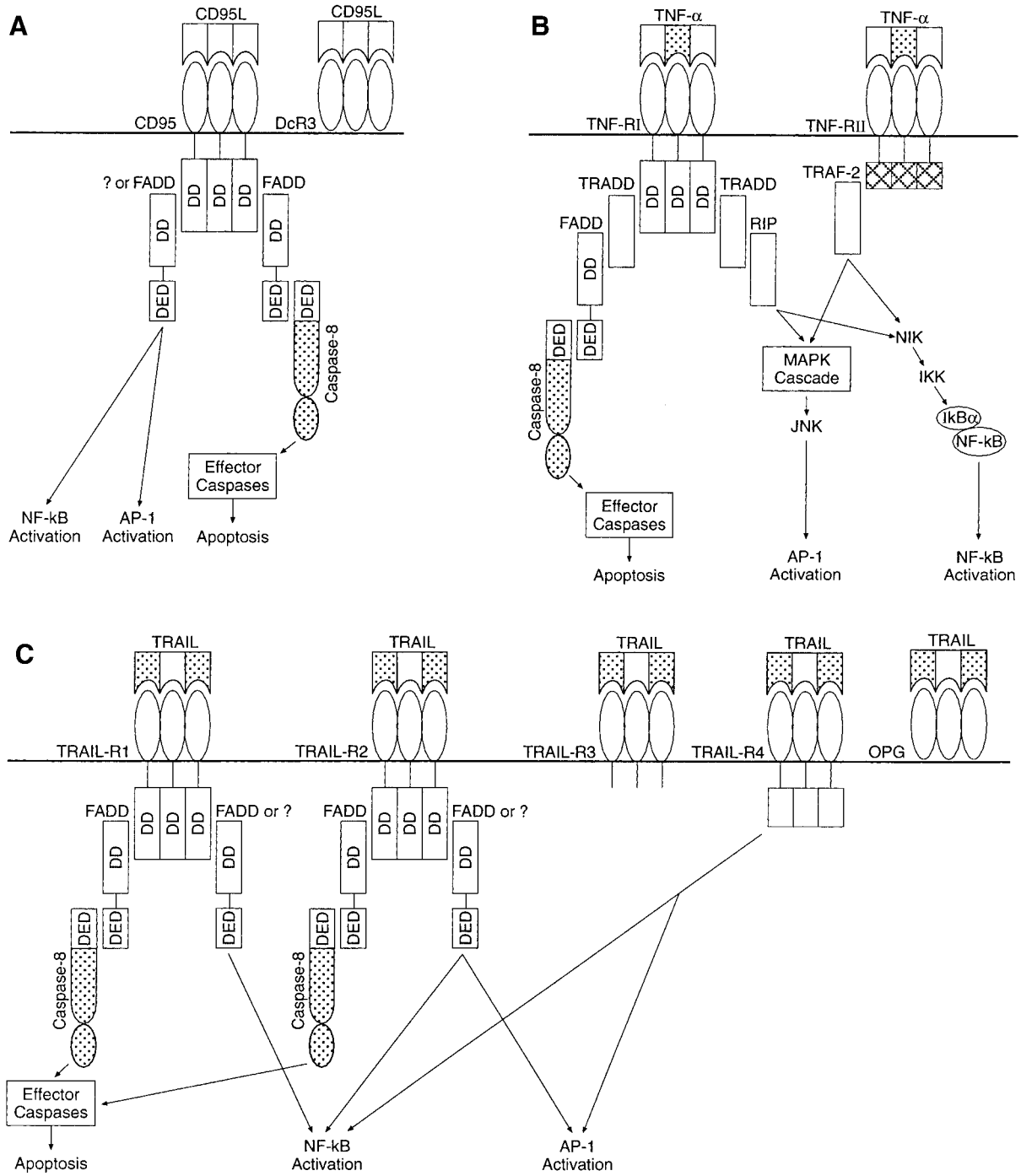


Fig. 6. Signaling by CD95L (**A**), TNF-α (**B**), TRAIL (**C**). See text for details.

(NIK), which in turn activates the inhibitor of IκB kinase complex, IKK. IKK phosphorylates IκB leading to degradation of IκB and allowing NF-κB to move to the nucleus to activate transcription [Wallach et al., 2002]. The pathway from TRAF-2 and RIP to JNK involves a cascade that includes the mitogen-activated protein (MAP) kinases MEKK1 (MAP/Erc kinase kinase-1), JNKK (JNK kinase), c-Jun N-terminal kinase (JNK) [Ichijo, 1999], as well as AP-1 activation. TNF-α also binds TNF-RII, typical member of the non-death domain-containing subgroup of the TNF receptor family. TNF-RII itself does not induce apoptosis, but

may play an important role in the regulation of apoptosis through TNF-RI. Several investigators have reported that TNF-RII potentiates TNF-RI-mediated apoptosis [Declercq et al., 1998; Weiss et al., 1998; Pimentel-Muinos and Seed, 1999]. TNF-RII binds TRAF-2 and signals activation of NF-κB and AP-1.

SIGNALING BY TRAIL

TRAIL interacts with five cellular receptors that form a distinct subgroup within the TNFR superfamily. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) have cytoplasmic DDs and induce apoptosis (Fig. 6C). Some earlier investigations described involvement of TRADD, TRAFs and RIP in TRAIL receptor DISC formation, and FADD-independent pathway activation of caspases from receptors suggesting another adaptor protein binding to activated TRAIL receptors [MacFarlane et al., 1997; Sheridan et al., 1997]. However, these reports were based on overexpression of TRAIL receptor in cells. A number of reports have described that FADD-DN blocks apoptosis induction through TRAILRs [Chaudhary et al., 1997; Schneider et al., 1997]. According to these authors TRAIL receptor DISC formation is very similar to that of the Fas receptor. Analysis of the native TRAIL death-inducing signaling complex (DISC) also revealed ligand-dependent recruitment of FADD and caspase-8. Differential precipitation of ligand-stimulated TRAIL receptors demonstrated that FADD and caspase-8 were recruited to TRAIL-R1 and TRAIL-R2 independently of each other. [Bodmer et al., 2000; Sprick et al., 2000]. It has also been shown that TRAIL induced homomeric and heteromeric complexes of TRAIL-R1 and TRAIL-R2, and stimulated recruitment of FADD and caspase-8, and caspase-8 activation in non-transfected cells. TRADD and RIP, which bind TNF-R1, do not bind DR4 and DR5. Thus, TRAIL and CD95L(FasL) initiate apoptosis through similar mechanisms, and FADD may be a universal adaptor for death receptors [Kischkel et al., 2000; Sprick et al., 2000]. TRAIL-R1 and TRAIL-R2 are also able to induce cascade signals for NF-κB and AP-1 activation [Jeremias and Debatin, 1998; Hu et al., 1999; MacFarlane, 2003]. By contrast, TRAIL-R4 (DcR2) contains an incomplete DD and so is unable to transduce a death signal but can signal NF-κB and AP-1 activation [Degli-Esposti et al., 1997; Hu et al., 1999]. TRAIL-R3 (DcR1), lacks a cytoplasmic domain, and is bound to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor and does not mediate apoptosis upon ligation. In addition to these four membrane-associated receptors, the secreted TNFR homologue (osteoprotegerin) OPG can also bind TRAIL, albeit with lower affinity [reviewed in MacFarlane, 2003]. Transfection of TRAIL-sensitive target cells with either TRAIL-R3 or -R4 indicated that they could act as "decoy" receptors by competing with TRAIL-R1 and -R2 for binding to TRAIL. However, using monoclonal antibodies specific for each of the membrane-bound TRAIL receptors, no correlation was found between TRAIL resistance and TRAIL-R3/-R4 expression in several cellular systems [reviewed in Walczak and Krammer, 2000]. Thus, under native conditions the physiological function of the non-death-inducing TRAIL receptors, namely TRAIL-R3, -R4, and OPG, is still not understood.

DEATH RECEPTORS CAN INITIATE BOTH EXTRINSIC AND INTRINSIC PATHWAYS

Engagement of death receptors with their ligands can initiate both extrinsic and intrinsic pathways. Recently, two types of cells were defined which differ in the kinetics of caspase activation [Scaffidi et al., 1998; Scaffidi et al., 1999]. In so-called type I cells, the death signal is propagated by a caspase cascade initiated by the activation of a large amount of caspase-8 at the DISC, followed by a rapid cleavage of caspase-3 and other caspases, which in turn cleave vital substrates in the cell. In type II cells, a DISC is formed, but the caspases cascade cannot be propagated directly and has to be amplified via mitochondria. In these cells caspase-8 cleaves the Bcl-2 family member Bid and tBid translocates to and activates the mitochondrial pathway. Mitochondria are activated in both type I and II cells but are not strictly necessary for the death of type I cells. It is possible that a special characteristic at the receptor level lead to these differences.

DEATH RECEPTORS EXPRESSION AND APOPTOSIS IN PROSTATE CELLS

The screening of human prostatic cell lines ALVA 31, DU145, JCA1, LNCaP, ND1 and PC3,

for expression of Fas antigen showed that all lines examined reacted with anti-Fas mAb [Rokhlin et al., 1997]. The levels of Fas cell surface expression in these cell lines were approximately the same as in the T-cell line Jurkat, which is extremely sensitive to Fas-mediated apoptosis. All of the human prostate tissues studied were found to express Fas in both benign and malignant basal and secretory epithelial cells. Thus, Fas expression on cell lines reflects the expression of Fas on human tumors. However, anti-Fas mAb induced apoptosis in only two of six cell lines examined. There was no correlation between sensitivity to anti-Fas mAb effects and cell surface expression of Fas antigen [Rokhlin et al., 1997]. One of the mechanisms of Fas-mediated apoptosis resistance is the expression of a truncated Fas receptor lacking the intracellular death-signaling domain [Cascino et al., 1996]. Resistance might be also be due to the presence of soluble forms of Fas, which are produced by alternative splicing and do not contain the TM domain [Papoff et al., 1996]. However, in the prostate cancer cell lines examined, expressions of truncated variants of the DDs or a soluble form of Fas were not found [Rokhlin et al., 1997]. At the same time, anti-Fas mAb could induce apoptosis in resistant cell lines in the presence of cycloheximide suggesting that the resistance to Fas-mediated apoptosis is critically dependent on protein synthesis and that the apoptotic machinery involved is already in place. This has led to the important conclusion that the intrinsic propensity to undergo Fas-mediated apoptosis could be a target for therapeutic intervention. Investigation of Fas- and TNF-mediated apoptosis in cell hybrids between resistant (DU145 and JCA1) and sensitive (ALVA31 and PC3) cell lines indicated that resistance to Fas- and TNF-mediated apoptosis dominates over sensitivity in cell hybrids and suggest that resistance might be regulated by an apoptosis suppressor factor (or factors) acting in resistant but not in sensitive cells [Rokhlin et al., 1997a]. Treatment of sensitive prostate cancer cell lines with anti-Fas mAb leads to activation of caspase-8 and then effector caspase-7 (and to a lesser extent caspase-3) [Rokhlin et al., 1998], Bid-cleavage, release of cytochrome c, and activation of caspase-9 [Gewies et al., 2000]. It is interesting to note that induction of the mitochondrial pathway (cytochrome c release, caspase-9 activation) by anti-Fas mAb was noted in both sensitive and resistant to Fas cell lines. Activation of caspase-8 is necessary but not sufficient to complete Fas-mediated apoptosis in PC3 cells without activation of the mitochondrial pathway. In addition, caspase-3 activation after Fas-receptor ligation involves two steps and is dependent on mitochondrial activation [Guseva et al., 2002].

TRAIL and its receptors were identified in the rat ventral prostate at both protein and mRNA levels and were immunolocalized in prostatic epithelial cells [Vindrieux et al., 2002]. Several groups have shown that TRAIL induces apoptosis in many transformed cells. However, its pro-apoptotic effect is minimal in benign cells, which is promising for cancer therapy. [French and Tschopp, 1999; Walczak et al., 1999; Baetu and Hiscott, 2002; Kim and Seol, 2003]. Androgen-independent PC3 cells were highly sensitive to TRAIL treatment. TRAIL receptor ligation in PC3 activates both extrinsic and intrinsic pathways. Caspases-2, -3, -7, -8, and -9, Bid processing, dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$), and cytochrome c release were all detected, and the mitochondrial pathway is critical for TRAIL-induced apoptosis in PC3 [Rokhlin et al., 2001]. TRAIL also induces DISC formation and caspase-8 activation in androgen-dependent LNCaP cells. However, caspase-8 activation is necessary but not sufficient for TRAIL-mediated apoptosis and is presumably blocked downstream of caspase-8 by the PI3K/Akt pathway [Chen et al., 2001; Rokhlin et al., 2002a]. Expressions of TNF receptors on prostate cancer cells were also shown but did not correlate with sensitivity to TNF [Nakajima et al., 1996].

Thus, death receptors are expressed on prostate cancer cells but unfortunately the expression of these receptors does not correlate with sensitivity of these cells to ligand or antibody mediated cell death. However, the combination of death receptor ligands with other drugs could potentially initiate apoptosis of prostate tumors, as discussed later.

ROLE OF Bcl-2 FAMILY MEMBERS IN PROSTATE CANCER

Eighteen years ago Bcl-2 was discovered as an oncoprotein implicated in human follicular lymphoma. Bcl-2 is an anti-apoptotic mediator that has been involved in the molecular biology

of a wide range of human cancers [Tsujimoto et al., 1985]. The Bcl-2 family proteins play important roles in the regulation of apoptosis. Bcl-2 inhibits apoptosis against various toxic stresses through stabilization of membrane potential, blocking the release of apoptosis inducer proteins such as cytochrome c [Kluck et al., 1997] and AIF [Susin et al., 1999] from mitochondria into the cytoplasm and thus inhibits the subsequent apoptosis-executing signaling events. Overexpression of Bcl-2 and Bcl-X_L probably occurs in more than half of all cancers [Amundson et al., 2000] rendering tumor cells resistant to myriad apoptotic stimuli, including most cytotoxic anticancer drugs [Reed, 2003]. Thus, Bcl-2 has been associated with resistance to anti-cancer therapies that take action by inducing apoptosis in various clinical conditions.

When distribution of Bcl-2 was analyzed in benign prostate tissue by the use of monospecific antibodies, it was found in basal cells of the prostatic epitelium and was undetectable in secretory cells. This dissimilarity correlated with the different level of differentiation of these two kinds of cells [Hockenbery et al., 1991]. Importantly, although no Bcl-2 protein has been detected in benign prostatic hyperplasia [Hockenbery et al., 1991], positive staining for Bcl-2 was detected in prostatic intraepithelial neoplasia [Colombel et al., 1993], which is considered by many as the putative precursor of human prostate cancer [Abate-Shen and Shen, 2000].

Bcl-2 expression was initially associated with androgen-independent prostate cancer by McDonnell et al. who evaluated Bcl-2 expression immunohistochemically [McDonnell et al., 1992]. These authors found that all androgen-independent carcinomas were positive for Bcl-2 expression, while 70% of androgen-dependent samples were virtually negative for Bcl-2 staining, and the other 30% showed only week staining. Similar results have been reported by Colombel and colleagues [Colombel et al., 1993]. Krajewska et al. also showed that increased levels of Bcl-2, Bcl-X correlated with prostate cancer, and suggested that these survival factors associate with a hormone-insensitive, metastatic phenotype [Krajewska et al., 1996]. Despite the obvious association between Bcl-2 levels and prostate cancer progression and that some bone metastasis in hormonally treated patients were negative for Bcl-2, the upregulation of Bcl-2 is apparently not the exclusive mechanism for tumor progression [Denmeade et al., 1996]. McConkey et al. [1996] demonstrated that a variant of the prostatic carcinoma cell line LNCap was characterized by increased expression of Bcl-2 and decreased level of Bax that correlated with the resistance to apoptosis when compared to the parental variant of the same cell line. In the same way, high Bcl-2 expression and low levels of Bax expression have been correlated with the poor therapeutic response of prostate cancer to radiotherapy [Mackey et al., 1998] indicating that Bcl-2 overexpression has direct clinical consequences [Gurumurthy et al., 2001].

It was recently reported that the upregulation of Bcl-2 is mediated by the NF-κB transcriptional regulation of the bcl-2 p2-promoter in LNCaP cells under TNF-α treatment [Catz and Johnson, 2001]. Interestingly, TNF-α induction of Bcl-2 protein expression in these cells was only observed in the absence of androgens, while inhibition of NF-κB in the presence of hormone completely ablated Bcl-2 expression [Catz and Johnson, 2001]. It has also been reported by Nakashima and coauthors that prostate cancer patients experiencing a relapse in disease after hormone ablation therapy have increased serum TNF-α levels compared with untreated patients or patients in remission [Nakashima et al., 1998]. Therefore, the increase in Bcl-2 expression in response to TNF-α in the absence of hormone is significant to the disease state [Catz and Johnson, 2003].

Estimation of Bcl-2 family members protein levels in prostate cancer cell lines has shown that DU145 does not express Bcl-2, where ALVA 31, JCA1, LNCaP, ND1 and PC3 cell lines does not display any consistent differences in the expression of Bcl-2, Bcl-X, and Bak. PCR analysis has also shown that prostate cancer cell lines expressed approximately the same levels of Bcl-X_L and Bcl-X_S isoforms [Rokhlin et al., 1997]. In contrast, Bax protein was not detected in DU145, whereas Bax was expressed in the other cell lines [Rokhlin et al., 1997]. Others have also reported that PC3 and LNCaP express Bcl-2 and Bax, but DU145 does not [Haldar et al., 1996]. It is hard to explain how differences in the expression of Bcl-2 family proteins modulate sensitivity to apoptogenic factors. As was described above, PC3 cells are sensitive to Fas and TRAIL. Bcl-2 overexpression in these cells prevents apoptosis induced by

Fas or TRAIL [Rokhlin et al., 2001; Guseva et al., 2002]. It is interesting, that caspases-8 and -3 were detected only in the cytosolic fraction of PC3, but caspases-2, -7, and -9 were found both in cytosolic and mitochondrial fractions. Bcl-2 overexpression did not affect caspase-8 activation although it did change the processing pattern of caspase-3. At the same time, Bcl-2 overexpression inhibited the activation of mitochondrial localized caspases-2, -7, and -9. Bcl-2 also abrogated TRAIL-induced cytochrome c release and dissipation of ($\Delta\Psi_m$). Thus, in contrast to lymphoid cells, Fas- and TRAIL-induced apoptosis in PC3 depends both on mitochondrial integrity and caspase activation [Rokhlin et al., 2001].

The pathways that are activated in response to TRAIL in prostate cells have been investigated [Griffith et al., 2000; Yu et al., 2000; Chen et al., 2001; Nesterov et al., 2001; Nimmanapalli et al., 2001; Rokhlin et al., 2001]. Several reports confirm that inhibition of TRAIL-induced apoptotic signals occurs at the level of mitochondria [Chen et al., 2001; Munshi et al., 2001; Nesterov et al., 2001; Rokhlin et al., 2001]. Munshi et al. [2001] exposed PC3, DU145, and LNCaP cells to TRAIL in the presence of cycloheximide, which acted as a sensitizer. Apoptosis was induced rapidly in PC3 and DU145 control cells, whereas induction in LNCaP required 24 h. All cell line variants expressing Bcl-2 were resistant to the apoptotic effects of TRAIL. Treatment with subtoxic concentrations of actinomycin D (ActD) significantly sensitized tumor cells (CL-1, DU145, and PC3) to TRAIL-mediated apoptosis. Treatment with TRAIL alone, although it was insufficient to induce apoptosis, resulted in loss of mitochondrial membrane potential and release of cytochrome c from the mitochondria into the cytosol in the absence of significant caspase activation. The earliest and most pronounced change induced by ActD was down-regulation of X-linked inhibitor of apoptosis (XIAP) and up-regulation of Bcl-X_L/Bcl-X_S proteins [Ng et al., 2002].

LNCaP, which is resistant to TRAIL-induced apoptosis, becomes sensitive to TRAIL after overexpression of full-length, wild-type BAD (BAD WT). TRAIL induces caspase-dependent cleavage of BAD WT that results in generation of a M(r) 15,000 protein. LNCaP stably expressing truncated BAD (tBAD) and cells expressing mutated BAD at the caspase cleavage site were less sensitive to TRAIL treatment when compared to LNCaP expressing BAD WT. Cytochrome c and Smac/DIABLO release from mitochondria into cytosol was found after TRAIL treatment only in cells overexpressing BAD WT. Furthermore, differences in phosphorylation of serine residues for BAD WT and tBAD were identified. BAD WT was phosphorylated at positions S136 and S155, whereas tBAD was phosphorylated at positions S112, S136, and S155. LNCaP stably expressing BAD mutated at serine 112 to alanine was less sensitive to TRAIL treatment when compared to LNCaP expressing BAD WT. Lastly, recombinant BAD cleaved by caspase-3 is a more potent inducer of cytochrome c and Smac/DIABLO release than BAD WT. In summary, BAD-mediated sensitivity of LNCaP to TRAIL depends on the phosphorylation status of BAD WT and tBAD [Taghiyev et al., 2003].

In summary, involvement of the mitochondrial pathway in receptor-mediated apoptosis in prostate cancer cells makes Bcl-2 family member proteins important players. Detection of Bcl-2 family member proteins expression levels in pathologic samples might help to individualize therapeutic strategies and could have important implications to their success.

ROLE OF NF-κB ACTIVATION IN PROSTATE CANCER

There is accumulating evidence that NF-κB has an important role in tumorigenesis, tumor progression and chemotherapy resistance [Foo et al., 1999; Baldwin, 2001; Karin et al., 2002]. The initial evidence came from the retroviral-encoded gene v-Rel that causes aggressive tumors in chickens. Other transforming viral proteins such as HTLV-1 Tax can activate NF-κB. Mutations in NF-κB and IκB families proteins were documented in many lymphoid neoplasms [Fracchiolla et al., 1993; McKeithan et al., 1997; Rayet and Gelinas, 1999]. NF-κB contributes to oncogenises and resistance to chemotherapy by its ability to suppress cell death pathways [Beg et al., 1995; Mayo and Baldwin, 2000]. However, there is also evidence in the literature that NF-κB can play a proapoptotic role, for example by regulating death receptors and death ligands expression [Kasibhatla et al., 1998; Kasibhatla et al., 1999; Kasof et al., 2001; Shetty et al., 2002]. Thus, the exact role of NF-κB activation depends on cell context.

It has been shown in several reports that NF-κB is constitutively activated in androgen-independent prostate cancer cell lines PC3, DU145, and JCA1 [Palayoor et al., 1999; Gasparian et al., 2002], and that this activation may be important for the support of their androgen-independent status [Gasparian et al., 2002]. It is known that NF-κB and the androgen receptor inversely regulate each other by competing for coactivators. Another possible mechanism of androgen receptor regulation is by up-regulation of IκB [Keller et al., 1996]. The constitutive activation of NF-κB in prostate tumor cells may increase expression of anti-apoptotic proteins, thereby decreasing the effectiveness of anti-tumor therapy and contributing to the development of the malignant phenotype [Palayoor et al., 1999]. In addition, constitutive activation of NF-κB has been detected in human prostate cancer tissues [Gasparian et al., 2002; Suh et al., 2002]. Lessard et al. also showed NF-κB nuclear localization in prostate cancer tissues and suggested that it might be used to help predict response to treatment [Lessard et al., 2003]. Interestingly, high invasive PC3 cells showed greater constitutive NF-κB activity than the low invasive PC3 cells [Lindholm et al., 2000], suggesting that increased NF-κB activity may contribute to prostate cancer invasion.

Constitutive NF-κB activation in different cancers can be connected with altered expression of IκBs as well as mutations in IκB genes in tumor cells [Emmerich et al., 1999; Rayet and Gelinas, 1999]. However, mutant IκBs or changes in IκBs expression has not been shown for prostate cancer. Increased NF-κB activity in prostate cancer includes activation of signaling pathways leading to phosphorylation of IκB or NF-κB subunits. For example, increased expression or processing of NF-κB subunits and NF-κB phosphorylation was constitutively increased in high invasive PC3 cells compared with the low invasive PC3 [Lindholm et al., 2000]. It is important to mention that amplification, overexpression and rearrangements of most genes coding for Rel/NF-κB factors have been found in hematopoetic tumors and could underlie constitutive NF-κB activation [Rayet and Gelinas, 1999]. However, in solid tumors and cell lines derived from solid tumors overexpression of p50 and p52 have been found [Rayet and Gelinas, 1999]. These proteins have low transactivation activity, thus the biological role of p50 and p52 homodimers appears to be ambiguous [Budunova et al., 1999]. The participation of p65 in solid tumors is controversial. p65 exhibits strong transactivation potential and alteration of p65 in solid tumors has been only rarely reported [Rayet and Gelinas, 1999]. However, p65/p50 NF-κB complexes with the highest transactivation potential among other NF-κB dimers are specific for prostate cancer and occurred without p65 or p50 overexpression in androgen-independent prostate cancer cell lines [Gasparian et al., 2002]. It has been reported that in androgen-independent prostate cancer cell lines including CL2 cells derived from LNCaP cells, IκB was heavily phosphorylated and IKK was aberrantly activated. The mechanism of constitutive activation may be correlated with constitutive activation of IKKs and consequently, a faster IκB turnover [Gasparian et al., 2002].

It is well described that death receptor signals can lead to apoptosis and NF-κB activation at the same time. For example, TNF-α is one of the most potent activators of NF-κB and can induce high NF-κB activation in minutes in many cell types, including prostate cancer cell lines [Mukhopadhyay et al., 2001; Gasparian et al., 2002]. The role of NF-κB activation in TNF-α-induced apoptosis remains controversial. Several reports show involvement of NF-κB activation in TNF-α induced apoptosis, and indicate that cell death induced by TNF-α is negatively regulated by NF-κB in different cell types and in mice [Beg and Baltimore, 1996; Van Antwerp et al., 1996; Dhanalakshmi et al., 2002]. Sumitomo et al. [1999] showed that NF-κB activation results in the resistance of DU145 and PC-3 to TNF-α. Another report indicated that TNF-α-mediated apoptosis is NF-κB independent in human breast carcinoma MCF7 cells [Cai et al., 1997]. The proapoptotic effects of NF-κB activation were also shown in LNCaP after simultaneous treatment with TNF-α and irradiation [Kimura and Gelmann, 2002] where activation of NF-κB is involved in activation of serine proteases.

The signals induced by TRAIL receptors are not as well understood as those of TNFR1. Although several studies indicate that TRAIL can induce NF-κB activation, [Chaudhary et al., 1997; Schneider et al., 1997; Jeremias and Debatin, 1998] one group has reported contradictory results [Pan et al., 1997]. The role of NF-κB in TRAIL-induced apoptosis is controversial. In one report, inhibition of NF-κB could

not protect from TRAIL-induced apoptosis [Hu et al., 1999]. However, other reports suggest that NF-κB activation protects cells from apoptosis induced by TRAIL [Jeremias and Debatin, 1998; Goke et al., 2000]. According to our unpublished data, TRAIL induces NF-κB activation in TRAIL resistant LNCaP cells and decreases constitutive activation of NF-κB in TRAIL-sensitive PC3 cell line supporting an antiapoptotic role of NF-κB activation. TRAIL treatment induced NF-κB activation in LNCaP cells to a lower extent and at a later time compared to TNF treatment and suggests that different components of the DISCs of TNF and TRAIL receptors might be involved in the activation of NF-κB after ligation of death receptors with TNF-α or TRAIL. Fas-mediated NF-κB activation has been also reported [Ponton et al., 1996; Dudley et al., 1999] and this activation appears to protect cells from Fas-mediated cell death [Dudley et al., 1999]. We have not observed activation of NF-κB in LNCaP cells after treatment with anti-Fas antibody.

It has been proposed that inhibition of NF-κB may prove to be therapeutic in certain leukemias or lymphomas where NF-κB appears to play a unique survival role [Bargou et al., 1997] such dramatic effects in most solid tumors is unlikely, since they express other antiapoptotic factors. However, there is some evidence that blocking NF-κB activity suppresses tumor growth and metastasis of human prostate cancer by inhibiting angiogenesis and invasion [Palayoor et al., 1999]. Thus, the combination of TNF-α and NF-κB inhibitors could be an effective approach to TNF-α-resistant human prostate cancer.

Inhibition of NF-κB activation shows a great promise for treating of prostate cancer [Kikuchi et al., 2003]. Several dietary chemopreventive compounds, including flavanoids, curcumin, resveratrol, are known to block NF-κB activation [Holmes-McNary and Baldwin, 2000; Yamamoto and Gaynor, 2001]. Agents that initiate or increase death receptor-induced apoptosis in prostatic cancer cell lines by inhibition of NF-κB are shown in Table II. However, it has been difficult to develop NF-κB inhibitors that act specifically in cancer cells. Learning more about the complicated process of NF-κB regulation should lead to better therapeutic approaches to target specific cell types [Karin et al., 2002].

TABLE II. Agents That Inhibit Constitutive or Death Receptor Induced-NF-κB Activation in Prostate Cancer Cells and Sensitize Cells to Apoptosis

Agent	Reference
Zn	Uzzo et al. [2002]
Selenium compounds	Gasparian et al. [2002a]
Pyrrolidinethiocarbamate	Gunawardena et al. [2002]
Genistein	Li and Sarkar [2002]
Silibinin	Dhanalakshmi et al. [2002]
Curcumin	Mukhopadhyay et al. [2001]
Ibuprofen	Palayoor et al. [1999]

ROLE OF p53 IN PROSTATE CANCER APOPTOSIS

p53 is a regulator of genotoxic stress that plays an important role in DNA damage response, DNA repair, cell cycle regulation and in triggering apoptosis after cell injury. p53 regulates the expression of a variety of apoptosis-related genes that affect both the intrinsic (Bax, Noxa, Puma, Bid, Bcl-2, Bcl-XL) and extrinsic (Fas, TRAIL-R2, PIDD, DcR1, DcR2) pathways [reviewed by Reed, 2003]. Moreover, p53 is the most commonly mutated gene in human malignancy which makes p53 a major potential target for gene-specific therapy of cancer [Vogelstein et al., 2000]. A fundamental role of p53 in prostate cancer progression was found in the mouse prostate reconstitution (MPR) model [Thompson et al., 1995]. Prostate cancer was identified in 100% of heterozygous and homozygous p53 mutant MPRs with metastatic deposits in 95% of the mice, but no metastasis were found in wild-type p53 mice. In addition, the authors noted that the pattern of metastasis was remarkably similar to that in human prostate cancer. It is noteworthy that in many cases lack of p53 mutations does not mean that p53 is functionally active in tumors. For example, in neuroblastomas, p53 is sequestered in the cytosol [Moll et al., 1996], and in sarcomas and cervical carcinomas it is inactivated by Mdm2 and E6 proteins, respectively, [Miller et al., 1996; Rapp and Chen, 1998]. It remains unclear whether p53 in prostate cancer is functionally active and how it affects the biology of these tumors.

TNF-α has been shown to induce apoptosis and accumulation of p53 in various cell types, including the ME-180 cell line [Donato and Perez, 1998], rat glioma cells [Yin et al., 1995], ovarian cancer cells [Gotlieb et al., 1994], and in

human breast carcinoma MCF-7 cells [Jeoung et al., 1995]. To gain insight into the possible interrelation between TNF-α-induced apoptosis and p53 pathway in LNCaP cells, we investigated the effect of functional inactivation of p53 on cellular responses to TNF-α treatment. We switched off p53 function by transfection of LNCaP with genetic suppressor element 56 (GSE-56) [Ossovskaya et al., 1996] that resulted in the generation of the LN-56 subline. This subline differs from LNCaP by only a single genetic alteration specifically targeting the function of p53. The established system allowed us to demonstrate the involvement of p53 in TNF-α-induced apoptosis and to investigate the mechanisms of p53-dependent apoptosis.

LN-56 cells became resistant to TNF-α and this resistance was accompanied by the lack of p21/WAF1 upregulation that could be responsible for the observed effects of p53 suppression on TNF-α-dependent apoptosis. In fact, it has been reported that low levels of p21/WAF1 can convert normal cell cycle arrest into apoptotic cell death [Canman et al., 1995; Waldman et al., 1996]. It has also been shown that p21/WAF1 was increased but selectively cleaved by caspases during p53-dependent apoptosis in ML-1 cells (human myeloblastic leukemia), induced by γ-irradiation [Gervais et al., 1998]. The authors reported that proteolysis of p21/WAF1 abolished its interaction with proliferation cell nuclear antigen (PCNA). They suggested that the cleavage of p21/WAF1 might interfere with DNA repair, causing prolonged presence of DNA lesions that may trigger apoptosis. Interestingly, p21/WAF1 accumulation and proteolysis has been also found in response to TNF-α treatment in ME-180 cells and analysis of a TNF-α-resistant subline indicated that this process might be p53-dependent [Donato and Perez, 1998].

We also observed p53-dependent elevation of p21/WAF1 levels that was accompanied by caspase-dependent p21/WAF1 proteolysis during TNF-α-mediated apoptosis in LNCaP, but not in LN-56. p53 dependence of proteolytic cleavage in LNCaP was shown for another important growth controlling protein, Rb. We have previously reported caspase-mediated interior cleavage of Rb in Fas-sensitive prostatic carcinoma cell lines, PC3 and ALVA31 [Rokhlin et al., 1998]. We also observed Rb proteolysis in LNCaP after TNF-α treatment, and, in addition, the levels of Rb proteolysis were found to be p53-dependent. Interior cleavage of Rb has been previously reported in Jurkat during DNA-damage-induced apoptosis [Fattman et al., 1997]. Gottlieb and Oren found cleavage of Rb in the IL-3-dependent lymphoid cell line DA-1 during apoptosis, induced by IL-3 withdrawal [Gottlieb and Oren, 1998]. They have shown that depletion of IL-3 resulted in caspase-mediated Rb cleavage and occurred preferentially in the cells which expressed functional p53. Importantly, their data suggest that p53 itself appears to play a role in Rb cleavage, possibly by controlling caspase activation. Our data suggest that Rb proteolysis may play an important role at the late stages of apoptosis but does not play a crucial role during the early events of apoptosis in LNCaP. Thus, p53 appears to control both the early and late events of apoptosis in LNCaP, apparently interacting with p21/WAF1 and Rb, respectively.

It has been found that 80–85% of tumor-derived alterations in the *p53* gene are missense point mutations localized within the DNA-binding domain of the protein [Soussi et al., 2000]. The majority of p53 mutant tumors express a single mutant allele of p53 (see p53 mutation database: http://www.iarc.fr/p53/Index.html). However, p53 mutation databases contain several cases in which two different p53 mutations are coexpressed in one tumor. The biological significance of coexpression of two different mutants in one tumor has not been analyzed and became the subject of our work [Gurova et al., 2003]. As a model, we chose the prostatic carcinoma cell line DU145 that is resistant to Fas-mediated apoptosis [Rokhlin et al., 1997] and carries different mutations in the two alleles of p53, resulting in the coexpression of two mutant polypeptides with different amino acid substitutions (Pro to Leu in 223, and Val to Phe in 274 codons [Isaacs et al., 1991]. We characterized the biological properties of each of the DU145-derived mutants in comparison with wild-type p53 after transduction into several p53-deficient cell lines, separately and in combination so we could investigate whether there is a form of cooperation between the mutants creating additional selective advantages to cancel cells. Both mutants showed some properties that closely resemble wild type p53. However, these relatively "weak" mutants create a p53 protein with new structural and functional properties when co-expressed in one cell. Synergism of the DU145-derived mutants

was revealed in their effect on cell sensitivity to Fas-mediated apoptosis, the most unusual property of these mutants. Whereas each of them alone had some, albeit weak, suppressive effect on the Fas sensitivity of transduced prostatic cell line PC3, that is itself p53-negative and Fas-sensitive, the combination of p53–274(Phe) and p53–223(Leu) caused a strong anti-Fas effect possibly acting by down-regulating Fas receptor (FasR) expression. Two major conclusions of this work are the generation of p53 protein with new properties from a combination of two rather weak mutants and the strong inhibition of Fas-mediated apoptosis by the resulting p53 protein. Gain-of-function has been reported for many tumor-derived p53 mutants [Blandino et al., 1999; Blagosklonny, 2000]. Mutant p53 can acquire alterations in their transactivation capabilities and increase cell resistance to cytotoxic drugs even when transduced on a p53-deficient background. Down-regulation of FasR expression accompanied by resistance to Fas-mediated apoptosis is a new type of gain-of-function by p53 mutants. Although each of the DU145-derived mutants possesses weak anti-Fas property, their combined expression generates p53 protein with much stronger anti-Fas activity, providing an unusual example of gain-of-function that presumably results from the combination of two mutant p53 subunits in one protein.

In addition to the role of p53 in TNF- and Fas-mediated apoptosis in prostate tumors, p53 apparently regulates the expression of prostate specific antigen (PSA) [Gurova et al., 2002]. By using cDNA microarray gene expression profiling, we found a fourfold increase in the PSA mRNA level in LNCaP. The p53 pathway was suppressed by a dominant-negative p53 mutant (GSE-56). Consistently, p53 suppression either by GSE-56 or by dominant-negative mutant p53–175His caused a 4–8-fold increase in secretion of PSA protein in culture medium, suggesting that PSA gene expression is under negative control of p53. The inhibitory effect of wild type p53 in transient transfection experiments was undetectable in the presence of trychostatin A, suggesting the involvement of histone deacetylation in negative regulation of PSA promoter activity. Since LNCaP is considered as an adequate in vitro model of hormone-dependent prostate cancer, we can presume that the results obtained in these cells may reflect regulation of PSA in naturally occurring tumors. Thus, it appears that one of the most useful diagnostic prostate tumor markers is, in fact, a tissue specific indicator of p53 inactivation in prostate cells. Being dependent on p53 inactivation, elevated production of PSA may therefore be indicative for the ongoing selection of p53-deficient cell variants with the broken control of apoptosis. In fact, the loss of functional p53 by LNCaP is accompanied not only by elevated PSA secretion but also by acquisition of high tumorigenecity and resistance to TNF-α-mediated apoptosis [Rokhlin et al., 2000].

ROLE OF Akt IN PROSTATE CANCER

The serine/threonine kinase Akt is an important regulator of cell proliferation and survival. Akt has a wide range of cellular targets, and the oncogenecity of Akt arises from activation of both proliferative and anti-apoptotic signaling [Chan et al., 1999]. Furthermore, Akt contributes to tumor progression by promoting cell invasiveness and angiogenesis. These observations establish Akt as an attractive target for cancer therapy. A cellular inhibitor of Akt, termed carboxyl-terminal modulator protein, reverts the phenotype of viral Akt-transformed cells, suggesting that a specific Akt inhibitor will be useful in the treatment of tumors with elevated Akt activity. Since inhibition of Akt activity induces apoptosis in a range of mammalian cells, Akt inhibition may be effective, in combination with other anticancer drugs, for the treatment of tumors with other mutations [Hill and Hemmings, 2002].

Akt is activated via the PI3K pathway that has emerged as a critical pathway for cell survival in prostate cells. Expression of all three Akt isoforms has been found in normal prostate and tumor tissues [Zinda et al., 2001]. The PTEN tumor suppressor, a phosphatase for the lipid product of PI3K, specifically inhibits the activation of Akt. PTEN expression is lost in more than 50% of the advanced prostate cancers, through methylation-mediated transcriptional silencing or PTEN deletion [Ittmann, 1998; Whang et al., 1998]. The loss in PTEN function is associated with constitutive activation of Akt and hyperactivation of Akt has also been found in prostate cancer xenografts and cellular models of prostate cancer, such as LNCaP [Vlietstra et al., 1998; Whang et al.,

1998; Chen et al., 2001]. In addition, functional loss of PTEN and subsequent activation of the Akt may be a particularly potent signal involved in prostate cancer progression to androgen-independence and therefore presents a series of potential targets for therapy of advanced androgen-independent prostate cancer [Graff, 2002].

Activation of the Akt pathway can suppress the apoptotic response, undermine cell cycle control and selectively enhance the production of key growth and survival factors. Thus many proteins and intracellular signaling pathways can influence these biological processes. In reality, activated Akt regulates a number of intracellular targets implicated in cell growth and apoptosis. For instance, Akt may phosphorylate (and inactivate) the pro-apoptotic proteins such as Bad [Datta et al., 1997] and caspase-9 [Cardone et al., 1998] and inhibits the activity of DEVD-targeted caspases without changing the steady-state levels of Bcl-2 and Bcl-X_L. Akt also inhibits apoptosis and the processing of procaspases to their active forms by delaying mitochondrial changes in a caspase-independent manner. Akt can activate NF-κB-mediated transcriptional pathway through activation of IKK [Ozes et al., 1999; Romashkova and Makarov, 1999; Madrid et al., 2001]. PTEN has been shown to impair TNF-induced activation of Akt and IKK and NF-κB DNA binding and transactivation in DU145 [Gustin et al., 2001], underscoring the possible importance of PTEN loss in disregulation of NF-κB activity in prostate cancer. Adenovirus-mediated expression of PTEN completely suppresses constitutive activation of Akt in LNCaP cells and PTEN expression sensitizes cells to death receptor-induced apoptosis [Yuan and Whang, 2002]. PTEN-mediated apoptosis was associated with caspase-3 and -8 activation and involves the FADD dependent pathway. PTEN also affects the mitochondrial pathway of apoptosis because Bcl-2 overexpression also blocked PTEN-mediated apoptosis [Yuan and Whang, 2002]. Akt activation is sufficient to inhibit the release of cytochrome c from mitochondria and the alterations in the inner mitochondrial membrane potential. Several groups observed that inhibition of PI3K/Akt pathway sensitizes cells to apoptosis after death receptor stimulation. For example, Chen et al. [2001] showed that modulation of Akt activity by pharmacological or genetic approaches alters the cellular responsiveness to TRAIL. We also observed that TRAIL can transiently activate caspase-8 in LNCaP but does not induce apoptosis. Inhibition of PI3K/Akt pathway by wortmannin resulted in caspase cascade activation, caspase-dependent proteolysis of p21/WAF1, MDM2 and Akt itself and leads to cell death [Rokhlin et al., 2002a]. Thus, constitutive activation of Akt is an important regulator of TRAIL sensitivity in prostate cancer.

In addition, inhibition of PI3K/Akt pathway by wortmannin affected TRAIL-DISC function in LNCaP [Rokhlin et al., 2002b]. We have found that TRAIL and wortmannin together accelerated processing of caspase-8 on the DISC and apparently the release of caspase-8 from the DISC into the cytoplasm. Surprisingly, we found that wortmannin alone induced caspase activity and decreased the amount of TRAIL-R1 recruited to the DISC as well as the total amount of TRAIL-R1. Interestingly, the wortmannin-induced decrease of TRAIL-R1 was found to be unique to LNCaP; TRAIL-R1 was unchanged in PC3 and DU145 after wortmannin treatment. Moreover, the levels of two other receptors, Fas and TRAIL-R2, were also unchanged in all three cell lines tested. Thus, wortmannin affects only TRAIL-R1 and only in LNCaP. This unique feature of TRAIL-R1 in LNCaP is not due to a mutation in the TRAIL-R1. Therefore, the decreased level of TRAIL-R1 under wortmannin treatment is dependent on an unknown factor(s) that is induced (or suppressed) only in LNCaP. It is conceivable that caspase activation may be one of the mechanisms since wortmannin treatment activated caspases in LNCaP but not in PC3 and DU145. However, caspase activation in LNCaP after treatment with TRAIL did not result in decreased levels of TRAIL-R1. These data pieces have prompted us to hypothesize that wortmannin, in addition to caspase activity, could induce activity of some other protease(s) that results in proteolysis of TRAIL-R1. We have found that only TPCK, inhibitor of chymotrypsin-like proteases, and Z-VAD, a pan-caspase inhibitor, protected TRAIL-R1. Obviously, TPCK-like proteases, but not caspases, could play a leading role in TRAIL-R1 proteolysis since caspase activation under TRAIL treatment did not result in TRAIL-R1 proteolysis. A key question is whether a specific sequence in TRAIL-R1, but not in TRAIL-R2, may be targeted by chymotrypsin-like

proteases. There is a sequence ValCysPheTrpArgLeuGlyLeuLeuArgGly (220–230 aa) in the middle of TRAIL-R1, but not in TRAIL-R2, and this region is rich with hydrophobic amino acids that could be a potential target for chymotrypsin-like proteases.

Thus, the PI3K pathway has a prominent role in protecting prostate cancer cells from TNF and TRAIL-induced apoptosis and suppression of Akt function markedly enhances cytokine-induced apoptosis responses [Chen et al., 2001]. TRAIL in combination with agents that down-regulate Akt activity could be potentially used for prostate cancer treatment.

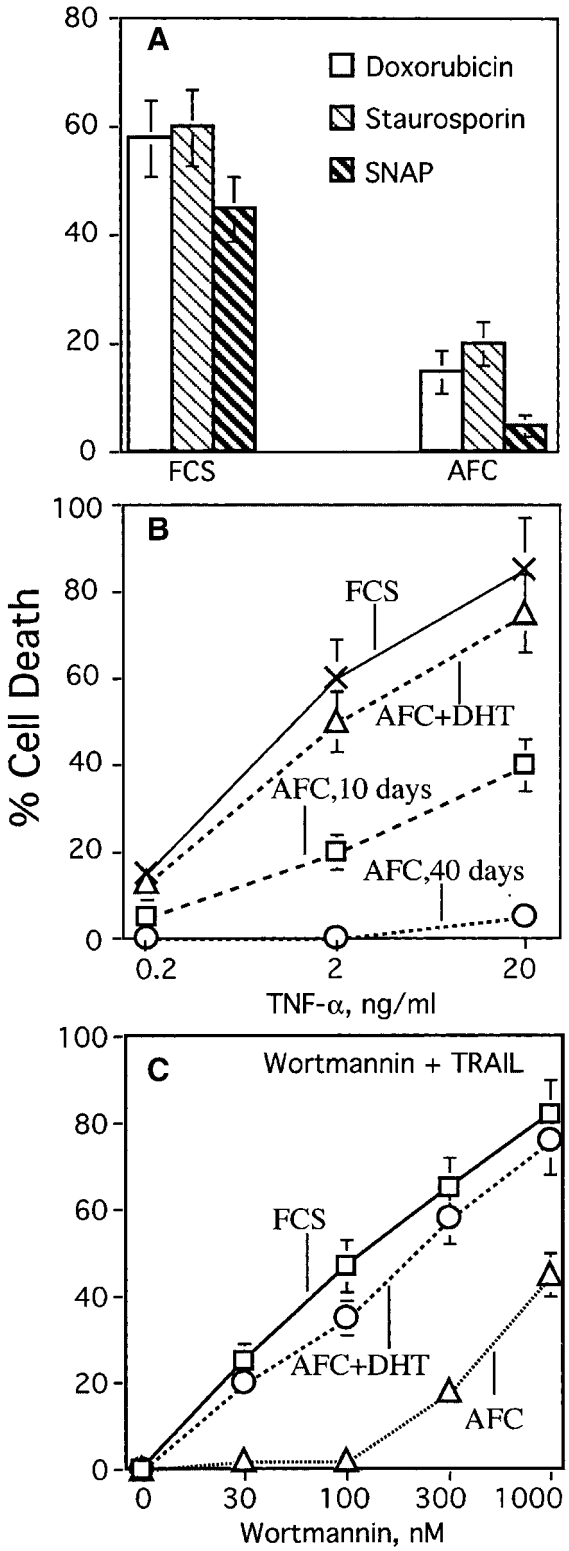
ANDROGEN REGULATION OF TRAIL RECEPTOR EXPRESSION AND APOPTOSIS IN PROSTATE CANCER

A major problem with treatment of prostate cancer is the development of resistance to therapy, particularly androgen-independent disease. Prostatic cancer cells require androgen for growth at early stages and androgen withdrawal induces apoptosis in an androgen-dependent prostate cancer. During cancer progression these cells typically lose their dependency on androgen, and androgen ablation becomes ineffective [Lin et al., 2001]. As has been recently reviewed by Litvinov et al. [2003], androgen ablation therapy is not curative because of the accumulation of molecular changes inducing gain of function in the AR signaling pathway that results in activation of novel AR-dependent signaling pathway but without requiring androgen ligand binding. It has also been recently shown that AR possesses an intrinsic androgen-independent transcriptional activity [Huang et al., 2002] and this ligand-independent activity was observed when AR was highly expressed. Androgen withdrawal has been shown to result in an increase of PI3K/Akt pathway activity, which supports survival after androgen ablation [Murillo et al., 2001]. We have also found that androgen deprivation increased the levels of Akt protein and phospho-active Akt, and that this could be reversed in the presence of dehydrotestosterone (DHT) [Rokhlin et al., 2002b]. These data indicate that the regulation of Akt activity is androgen-dependent. It has recently been shown that Akt phosphorylates the androgen receptor at Ser-210 and Ser-790 and results in suppression of androgen receptor transactivation [El-Deiry, 2001]. Taken together, these data suggest that there is the cross-talk between these two signaling pathways and that it is finely regulated.

In order to investigate the functional significance of androgen withdrawal in apoptosis, we assessed apoptosis in LNCaP cultured both in normal medium and in androgen free conditions (AFC) in response to treatment with TNF-α, SNAP (nitric oxide donor), doxorubicin, staurosporin, and TRAIL in the presence of wortmannin. As shown in Figure 7A, LNCaP was more resistant to effect of doxorubicin, staurosporin, and SNAP tested in the absence of steroid compared to LNCaP cultured in normal medium and DHT was capable to restore sensitivity of LNCaP to TNF-α-induced apoptosis (Fig. 7B). The same results were obtained in the case of treatment with TRAIL in the presence of wortmannin: cells become resistant to treatment after androgen withdrawal and DHT completely restored their sensitivity (Fig. 7C). Thus, androgens may play a critical role in sensitivity of LNCaP to apoptosis induced by a variety of agents.

It is noteworthy that LNCaP became resistant to TRAIL treatment even in the presence of the PI3K/Akt inhibitor wortmannin after culturing in AFC. This indicates that PI3K/Akt pathway is not the only pathway that determines the resistance to apoptosis in AFC. To unravel the nature of other factors mediating the apoptosis resistance in AFC, we investigated the caspase activation cascade and DISC formation after treatment with TRAIL in the presence/absence of wortmannin.

Caspase activity was not detected in cells cultured in AFC, but was restored to a small extent in the presence of 10 pM DHT and sharply induced in the presence of 1 nM DHT [Rokhlin et al., 2002b] that indicate caspase activation is androgen-dependent after treatment of cells with wortmannin and TRAIL. Examination of the TRAIL-DISC assembly in both TRAIL-resistant (LNCaP) and TRAIL-sensitive (PC3) cell lines showed DISC formation in the both LNCaP and PC3. TRAIL–DISC of FADD, caspase-8, TRAIL-R1, and TRAIL-R2. CRADD, RIP, TRADD, and FLIP were not detected as associated with the DISC, although these proteins were easily detected in lysates of LNCaP and PC3 cells. These results further suggest that the differences between LNCaP and PC3 in their sensitivity to TRAIL treatment



are not dependent on the efficiency of DISC formation and are presumably mediated by downstream signaling events.

The next question we asked was whether androgen withdrawal and treatment with wortmannin affect TRAIL-DISC formation. We have shown that when LNCaP was cultured in media supplemented with FCS, recruitment of FADD and caspase-8 to the DISC was dependent on TRAIL-induced receptor aggregation since FADD and caspase-8 were not found upon addition of biotynilated (biot) TRAIL directly to cell lysates. Treatment with wortmannin did not induce DISC formation. To examine TRAIL–DISC formation after androgen withdrawal, LNCaP was cultured in AFC and then treated with biot-TRAIL in the absence/presence of wortmannin.

Interestingly, we have found that FADD and caspase-8 were not recruited to the DISC in AFC and this inability to form a TRAIL–DISC is determined by dramatic decreases of the levels of TRAIL-R1 and TRAIL-R2 in LNCaP after culturing in AFC (Fig. 8A). Western blot analysis showed that the levels of both receptors were 7–10 times lower after culturing in AFC. At the same time the expression of FADD and caspase-8 were unchanged. In addition, treatment with wortmannin did not affect TRAIL–DISC formation. The real-time quantitative RT-PCR has showed that the expression of TRAIL-R1 gene was unchanged after culturing of LNCaP in AFC and expression of TRAIL-R2 gene was decreased by twofold. Thus, the decreased levels of TRAIL-R1 and TRAIL-R2 are mediated by an unknown, androgen-dependent factor(s) at the translational (or posttranslational) level. Some members of the TNF receptor family are associated with lipid rafts that are

Fig. 7. Cell death of LNCaP is androgen-dependent after different treatments. **A**: LNCaP was cultured for 10 days in AFC and then cells were treated in 96-well plates for 48 h with doxorubicin (0.5 μg/ml), staurosporin (0.4 μM), or SNAP (0.5 mM). The same treatments were performed with LNCaP cultured in medium supplemented with FCS. **B**: LNCaP was cultured either in medium with FCS or in AFC for 10 and 40 days. Cells were also cultured in AFC for 20 days and then cultured for an additional 20 days in AFC supplemented with 10 pM DHT. Cells were then plated in 96-well plates and treated for 48 h with different concentrations of TNF-α. **C**: LNCaP was cultured in medium supplemented with FCS or in AFC (for 14 days) in the absence of DHT or in the presence of DHT. Cells were then plated in 96-well plates and treated for 24 h with different concentrations of wortmannin in the presence of TRAIL (100 ng/ml). Cell death was estimated by calcein assay.

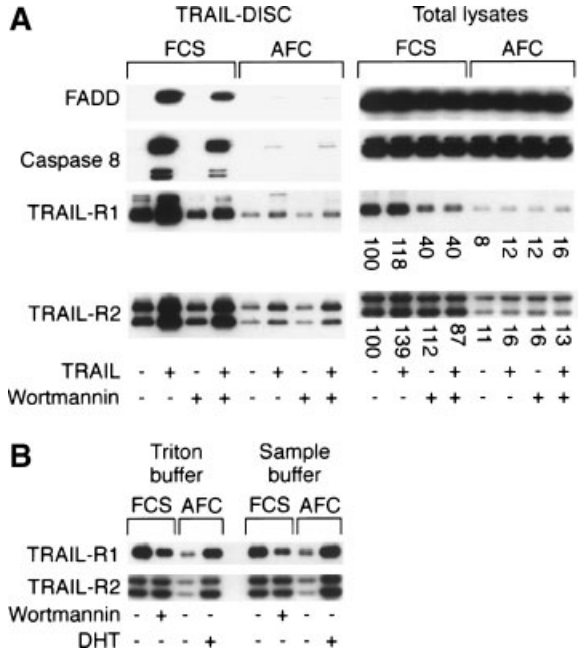


Fig. 8. Androgen withdrawal results in prevention of TRAIL-DISC assembly and decreased levels of TRAIL-R1 and -R2. **A**: LNCaP cells were cultured in media supplemented with FCS or in AFC for 7 days. Biot-TRAIL was added to the lysates in the untreated control (–). Cells were treated either with wortmannin alone (1 μM) for 16 h, biot-TRAIL alone (200 ng/ml) for 1 h, or simultaneously with both agents. TRAIL-bound protein complexes were precipitated and examined by western blot analysis. Total lysates were used as positive control (**right panels**). The number below TRAIL-R1 and -R2 bands indicates the relative intensity of band, compared to reference (untreated cells in FCS), which is taken as 100. **B**: LNCaP cells were cultured in media supplemented with FCS or in AFC for 7 days in the absence of DHT or in the presence of 100 nM of DHT. Cells were lysed either in 1% Triton X-100 buffer or in 2× Laemmli's sample buffer. The expression of TRAIL-R1 and -R2 was examined by Western blot analysis. [Copyright Lands Bioscience 2002: Rokhlin et al., 2002b.]

known to be insoluble in nonionic detergents such as Triton X-100 [Cottin et al., 2002]. Therefore, we investigated whether decreased level of TRAIL-R1 and -R2 in AFC are determined by translocation of these receptors to lipid rafts. To answer this question, we dissolved cells either in 1% Triton X-100 lysis buffer or in 2× Laemmli's sample buffer containing 10% SDS. It has been found that the decreased levels of TRAIL receptors were observed in both buffers (Fig. 8B). However, experiments with dissolved cells either in 1% Triton X-100 lysis buffer or in 2× Laemmli's sample buffer containing 10% SDS showed decreased levels of TRAIL receptors in both buffers (Fig. 8B) and indicate that the localization of the TRAIL receptors in lipid rafts is not responsible for the decrease in receptor expression after treatment. Finally, we observed that DHT restored the capacity of cells to form the TRAIL–DISC that was completely lost in AFC. Thus, this data suggests that the levels of TRAIL receptors and TRAIL-DISC formation are androgen-dependent.

The most important finding in our study is that the capacity of LNCaP cells to form TRAIL–DISC was completely abrogated after androgen withdrawal. As reviewed by Ashkenazi, the main physiological role of TRAIL is apparently the induction of apoptosis in tumor cells [Ashkenazi, 2002]. Therefore, the decreased levels of TRAIL receptors after androgen withdrawal seems to play an important role in survival of tumor cells after onset of androgen ablation. It is conceivable that metastatic androgen-independent cancer cells arise as a subpopulation that has acquired the capacity to downregulate the expression of death receptors and therefore to escape the antitumor surveillance by immune cells. Our findings suggest that, if downregulation of TRAIL receptors after androgen ablation occurs in prostate cancer, identification of drugs that will upregulate TRAIL receptors expression after androgen deprivation may prove to be therapeutically useful.

BISINDOLYLMALEIMIDES POTENTIATE TNFR FAMILY-MEDIATED CELL DEATH IN HUMAN PROSTATIC CARCINOMA CELL LINES

It has been suggested in many studies that combined treatment with chemotherapy agents and apoptosis-inducing ligands is a more effective strategy for cancer treatment [El-Deiry, 2001; Ashkenazi, 2002; Reed, 2002]. This approach is especially promising in the case of TRAIL treatment since TRAIL does not damage normal cells. There are several studies that describe the effect of drugs in combination with TRAIL treatment on prostate tumor cell death. Nimmanapalli et al. showed that treatment with paclitaxel increased TRAIL-R1 and -R2 protein (but not mRNA) levels without affecting the protein levels of other death receptors and ligands, such as DcR1, DcR2, TRAIL, Fas, and Fas ligand [Nimmanapalli et al., 2001]. The authors found that sequential treatment of PC3, DU145, and LNCaP with paclitaxel followed by TRAIL induced significantly more apoptosis

than TRAIL treatment alone. In another study [LaVallee et al., 2003], upregulation of TRAIL-R2 in PC3 was found under treatment with 2-methoxyestradiol, a natural metabolite of estradiol, which is currently in several clinical trials under the name of Panzem. Pretreatment with doxorubicin has been shown to sensitize several prostate cancer cell lines to TRAIL-induced apoptosis [Kelly et al., 2002]. Doxorubicin did not affect the protein levels of Bax, Bcl-2, Bcl-X, and TRAIL receptors but levels of c-FLIP was found to be decreased which apparently predisposes cells to TRAIL-induced apoptosis. In another study from this laboratory, malignant but not normal prostate cells were found to be selectively killed by doxorubicin in combination with antibody to TRAIL-R1 [Voelkel-Johnson, 2003].

We investigated the effect of bisindolylmaleimides on prostate cancer cells apoptosis in combination with different death receptor ligands: TNF-α, Fas (using agonistic anti-Fas receptor antibody IPO-4) and TRAIL. Bisindolylmaleimide (Bis) derivatives were originally produced as protein kinase C (PKC) inhibitors [Toullec et al., 1991; Bit et al., 1993; Jacobson et al., 1995]. However, subsequent studies showed that Bis VIII induced Fas- and TNF-α-mediated apoptosis in an astrocytoma cell line and in lymphoid cells independently of the inhibition of PKC [Zhou et al., 1999]. Several Bis derivatives (I, II, III, IV, and VIII) have been found to facilitate Fas- and TRAIL-mediated apoptosis in human dendritic cells [Willems et al., 2002]. Bis VIII has also been described to potentiate TNF-α-, Fas-, and TRAIL-mediated apoptosis in Jurkat cells [Meng et al., 2002]. Recently, it has been reported that Bis VIII in combination with agonistic Abs to TRAIL-R2 enhanced apoptosis through the MKK4/JNK/p38 kinase and mitochondrial pathways [Ohtsuka and Zhou, 2002].

Based on our own previous work in receptor-ligand induced cell death [Rokhlin et al., 1997, 1998, 2000, 2001, 2002a], it was of great interest to investigate whether Bis's could modulate the resistance of human prostatic carcinoma cells to TNF receptor family-induced apoptosis. We investigated the effect of four Bis derivatives (I, II, VIII, and IX) in human prostatic carcinoma cell lines and found that Bis IX was the most potent inducer of apoptosis under simultaneous treatment with TNF-α, agonistic anti-Fas mAb, and TRAIL [Rokhlin et al., 2002]. Bis IX synergistically induced caspase activity in combination with apoptosis-inducing ligands and converted the phenotype of cell lines from apoptosis-resistant to –sensitive. Bis IX induced p53 accumulation in LNCaP, which expresses wt p53, that was not accompanied by the induction of p53-responsive genes, *p21/WAF1* and *Mdm2*. Moreover, the induction of p21/WAF1 and Mdm2 by doxorubicin was abrogated by simultaneous treatment with Bis IX. These effects apparently result from general inhibition of transcription by Bis IX. We have shown by Northern blot analysis that the transcription activity of the hygromycin gene after transient transfection of pcDNA3.1-Hygro plasmid in 293 and HeLa cells was inhibited by Bis IX in a dose-dependent manner. Moreover, DNA-binding activity of Bis IX was prevented by actinomycin D, suggesting that actinomycin D and Bis IX have similar mechanisms of interaction with DNA. In addition, we found that actinomycin D and Bis IX induced caspase activity to the same extent during TRAIL-mediated apoptosis. In summary, these results suggest that Bis IX potentiates TNF receptor family-mediated cell death in part as an inhibitor of transcription.

Bis IX was found to induce time-dependent apoptosis in combination with TNF-α, TRAIL, and anti-Fas Ab. Using human prostatic carcinoma cell lines DU145 and LNCaP that are resistant to treatment with TNF family death-inducing ligands, we have shown that different, albeit still unidentified, inhibitory factors are responsible for the resistance to TRAIL-, Fas-, and TNF-α-mediated apoptosis. Our data also suggest that the turnover of apoptosis suppressor factors is much faster in DU145 compared to LNCaP [Rokhlin and Cohen, 2003].

Earlier, we described that LNCaP is sensitive to TNF-α treatment and that TNF-α-mediated apoptosis in LNCaP is p53-dependent [Rokhlin et al., 2000]. However, as can be seen from Figure 7 and in Rokhlin et al. [2002b], LNCaP became resistant to TNF-α treatment after culturing in androgen-free conditions (AFC) and it is important that Bis IX induce the sensitivity to TNF-α treatment in LNCaP cultured in AFC. In the presence of 4 μM of Bis IX the sensitivity of LNCaP was similar to LNCaP cultured in FCS when cells were treated by 20 ng/ml of TNF-α [Rokhlin et al., 2002].

Interestingly, Bis IX became fluorescent in the cells with excitation at 485 nm and emission

at 645 nm and was observed in the mitochondrial fraction but not in the cytosol. Further analysis revealed that Bis IX interacts with cardiolopin, which is known to be a mitochondrial specific phospholipid. We further found that Bis IX is freely distributed both within cells and extracellular medium and acts as a reversible apoptosis-inducing agent. We have also previously described that human prostatic carcinoma cell line PC3 was sensitive to treatment with anti-Fas agonistic Ab (IPO-4) and TRAIL and overexpression of Bcl-2 in PC3 converted these cells from sensitive to resistant [Rokhlin et al., 2001]. Since Bis IX binds to mitochondria and acts as inhibitor of transcription, we examined whether Bis IX could overcome the Bcl-2-dependent resistance to Fas- and TRAIL-mediated apoptosis. We have found that Bis IX synergistically increased cell death of PC3-Bcl-2 under simultaneous treatment with Bis IX in a dose-dependent manner, and different concentrations of anti-Fas mAb or TRAIL [Rokhlin and Cohen, 2003].

It is well known that an effective therapy for androgen-independent prostate cancer is urgently needed. There are a limited number of agents that are cytotoxic to prostate tumor cells. However, these agents typically induce death in proliferating cells. Bis IX, or other Bis derivatives, may be good candidates for treatment of prostate tumor cells for the following reasons: (1) Bis IX is capable of potentiating TNF receptor family induced apoptosis in LNCaP even after androgen withdrawal when cells stop proliferating; (2) The effect of Bis IX is p53-independent: Bis IX potentiated TNF receptor family induced apoptosis not only in LNCaP that expresses wild type p53, but also in PC3, which is p53 null and in DU145 that expresses two different p53 mutants; (3) Bis IX can overcome Bcl-2-dependent resistance to Fas- and TRAIL-mediated apoptosis; (4) Inhibition of transcription by Bis IX in normal cells should not have significant consequences. Zhou et al. [1999] support this point of view. In their experiments, rats were injected with 250 μg Bis VIII every other day for five doses without side effects. In addition, they showed that Bis VIII in vivo did not have a substantial effect on activated T cells from Fas-deficient *lpr*/*lpr* mice. Moreover, Bis VIII selectively potentiated apoptosis in activated T cells while having little effect on non-activated T cells. Ohtsuka and Zhou [2002] have also shown that Bis VIII synergistically increased apoptosis induced by agonistic antibodies (TRA-8) to TRAIL-R2 in several tumor cell lines including DU145 and PC3. They have also detected that combined treatment with Bis VIII and TRA-8 resulted in nearly complete tumor regression upon treatment of human 1321N1 astrocytoma cells in SCID mice. (5) The reversibility of Bis IX-mediated apoptosis could potentially be advantageous for combination therapy that includes TRAIL. Since the protein(s) that are responsible for mediating resistance to TRAIL-induced apoptosis have different half-lives in different tumor cells, any successful treatment will require administration of multiple doses in order to convert the vast majority of the tumor cells from TRAIL-resistant to -sensitive. The reversible and time-dependent effect of Bis IX determines its low toxicity [Rokhlin and Cohen, 2003], and should allow for the development of a flexible therapeutic regime with, hopefully, minimal side effects. The published and our own data indicate that Bis derivatives are multifunctional agents that are capable of overcoming the resistance of tumor cells to TNF family death ligands in different cell types.

CONCLUSIONS

As noted at the outset, apoptosis is a realistic therapeutic target for prostate cancer, particularly in later stages of the disease. This, however, is predicated on understanding mechanisms that underlie apoptosis in prostate cancer. A significant body of knowledge has been accumulated, however, significant gaps remain. Our work, as well as that of others, suggests combination therapy, e.g., apoptosis-inducing agents combined with small molecules targeting specific and critical resistance pathway intermediates may be the best approach. One such molecule, Bis IX, shows promise but has not been extensively studied in vivo, let alone in early phase clinical trials. In addition, our observation about androgen regulation of apoptosis, e.g., TRAIL-mediated cell death, will need further investigation. Clearly, androgens play a critical role in prostate cancer and evolution to androgen independent disease is a fundamental aspect of this disease. Therefore, studies aimed at understanding these intersecting pathways are critical to the development of new therapeutic regimens. And, there is reason to be optimistic. Our understanding of such

pathways continues to increase and it is reasonable to anticipate bench to bedside translation in the near future.

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