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13. ABSTRACT (Maximum 200 Words)

We assessed the influence of sequential treatment of ionizing radiation followed by TRAIL on intracellular mechanisms of apoptosis of prostate tumor cells *in vitro* and *in vivo*. Prostate normal and cancer cells were exposed to irradiation and TRAIL. Four to six-week old athymic nude mice were injected s.c.with PC-3 tumor cells. Tumor bearing mice were exposed to irradiation and TRAIL, either alone or in combination, and tumor growth, apoptosis and survival of mice were examined. Irradiation significantly augmented TRAIL- induced apoptosis in prostate cancer cells through up-regulation of death receptor DR5, Bax and Bak, and induction of caspase activation. Dominant negative FADD and p53 siRNA inhibited the synergistic interaction between irradiation and TRAIL. The pretreatment of cells with irradiation followed by TRAIL significantly enhanced more apoptosis than single agent alone or concurrent treatment. Irradiation sensitized TRAIL-resistant LNCaP cells to undergo apoptosis. The sequential treatment of xenografted mice with irradiation followed by TRAIL induced apoptosis through activation of caspase-3, induction of Bax and Bak, and inhibition of Bcl-2, and completely eradicated the established tumors with enhanced survival of nude mice. The sequential treatment with irradiation followed by TRAIL can be used as a viable option to enhance the therapeutic potential of TRAIL in prostate cancer.

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INTRODUCTION

Prostate cancer is the most frequently diagnosed malignant neoplasm and the second leading cause of cancer-related deaths in American men (1). It has a low proliferative index and depends on androgens for growth. After castration these tumors regress but later progress to an androgen-independent phenotype that is ultimately lethal for the patient. New therapeutic approaches for the treatment of prostate cancer includes the inhibition of androgen receptors, regulation of apoptosis, blocking of tumor angiogenesis, and inhibition of invasion and metastasis (2, 3). Considerable efforts have been directed recently towards novel pathways that trigger apoptosis selectively in tumor cells irrespective of their androgen receptor status (4). The activation of death receptor pathway induces apoptosis in both androgen-sensitive and -insensitive prostate cancer cells (5). One such ligand of death receptors is a newly discovered cytokine TRAIL (TNF-related-apoptosis-inducing-ligand)/Apo-2L.

TRAIL /Apo-2L is a novel anticancer agent that has been shown to induce apoptosis in a variety of tumor cells of diverse origin both *in vitro* and *in vivo* (6-9). TRAIL can bind to five distinct death receptors of TNF receptor family namely TRAIL-R1/ DR4 (10), TRAIL-R2/DR5 (11, 12), TRAIL-R3 /DcR1/TRID (13, 14), TRAIL-R4 /DcR2/TRUNDD (15), and osteoprotegerin (16). Binding of TRAIL to its receptors DR4 and DR5 results in recruitment of the adaptor protein FADD, which in turn recruits and activates caspase-8 (17, 18). Active caspase-8 transmits signal either by activating downstream caspase-3 or by cleaving Bid to truncated Bid (17). Translocation of tBid to mitochondria triggers Bax and Bak oligomerization, releases mitochondrial proteins, activates downstream caspases and induces apoptosis (19, 20). Caspase-8-mediated Bid processing therefore bridges the extrinsic death receptor-mediated pathway of apoptosis to the intrinsic mitochondrial pathway (20, 21). This provides a mechanism to amplify the execution signal and exacerbate the pace of cell demise. The Bcl-2 family proteins regulate apoptosis by acting mainly at the level of mitochondria (22).

NFκB is a transcription factor that plays an important role in controlling immune and inflammatory responses, cellular proliferation, and adhesion molecules (23-26). NFκB is a heterodimeric or homodimeric complex formed from five distinct subunits, RelA (p65), RelB, c-Rel, NFκB1 (p50) and NFκB2 (p52) (23, 25, 27). RelA (p65), RelB and c-Rel are transcriptionally active members of the NFκB family, whereas p50 and p52 primarily serve as

DNA binding subunits (23-25). The p50 and p52 NFκB subunits are derived from larger precursor products, p105 and p100 respectively, or from differential translation of their mRNAs. The classical form of NFκB, the heterodimer of p50 and p65 subunits, is normally retained in the cytoplasm in association with inhibitor proteins IκBα and IκBβ. When phosphorylated on serine 32 and serine 36, IκBα is degraded by the ubiquitin/26S proteasome pathway, allowing NFκB to translocate to the nucleus and regulate gene expression (23, 25, 26, 28). Recently, NFκB has been implicated in protecting cells from apoptosis (29-32), while much evidence highlights an apparently paradoxical pro-apoptotic role for NFκB (31, 33-35). Based on these studies, it appears that opposite functions of NFκB lies on the expression of its subunits where c-Rel and RelA functions as pro- and anti-apoptotic proteins, respectively. It has recently been shown that TRAIL can activate NFκB (31, 36-38). However, the intracellular signaling pathways responsible for TRAIL receptor-mediated NFκB activation, and the role of distinct subunits of NFκB in TRAIL-induced signaling are unclear.

Akt/PKB is activated in response to activation by many different growth factors, including IGF-I, EGF, basic FGF, insulin, IL-3, IL-6, and macrophage-colony stimulating factor (39). Akt is a serine/threonine protein kinase that has been implicated in mediating a variety of biological responses including inhibiting apoptosis and stimulating cellular growth. There are three mammalian isoforms of this enzymes, Akt1, Akt2, and Akt3 (39). Activation of all three isoforms is similar in that phosphorylation of two sites, one in the activation domain and other in the COOH-terminal hydrophobic motif, are necessary for full activity. For Akt, phosphorylation of T308 in the activation domain by PDK1 is dependent on the products of PI3-K, PIP2 and PIP3. PIP2 and PIP3 bind to the pleckstrin homology domains of Akt and PDK1, which relieves steric hindrance, fully activates PDK1, and translocates Akt to the plasma membrane (40). The mechanism of S473 phosphorylation is less clear. Kinases potentially responsible for S473 phosphorylation include PDK1 (41), integrin-linked kinase (42) and Akt itself (43). Akt activation may also be achieved through PI3-K independent means, either through phosphorylation of Akt by kinases such as PKA (44) or CAM-KK (45), or under conditions of cellular stress (46). Interestingly enough, activation of Akt by PKA or CAM-KK does not appear to require phosphorylation of S473. The relative importance of PI3-K independent and dependent means of Akt activation *in vivo* is unclear. However, once activated, Akt exerts

antiapoptotic effects through phosphorylation of substrates such as Bad (47) or caspase 9 (48) that directly regulates the apoptotic machinery or substrates such as the human telomerase reverse transcriptase subunit (49), forkhead transcription family members (50), or IκB kinases (51) that indirectly inhibit apoptosis.

Previous studies have demonstrated that Akt plays an important role in survival when cells are exposed to different apoptotic stimuli such as growth factor withdrawal, UV irradiation, matrix detachment, cell cycle discordance, DNA damage, and administration of anti-Fas antibody, TGF-β, glutamate, or bile acids (52, 53). Furthermore, Akt was found to be overexpressed in some gastric adenocarcinomas, and breast, ovarian, prostate and pancreatic cancers (54). However, the role of Akt in tumor cell survival and resistance to cancer therapy has not been well studied in any tumor system.

The PTEN tumor suppressor gene is inactivated by mutations in many types of tumors including endometrium, brain, and prostate (55-58). PTEN is a lipid phosphatase that can dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP3) (59). Through direct regulation of PIP3 levels, PTEN negatively regulates the PI3K signaling pathway, which transduces extracellular growth regulatory signals to intracellular mediators of growth and cell survival (60). Accordingly, inactivation of PTEN due to mutations in tumors led to increased activity of Akt (61, 62), whereas reintroduction of PTEN suppressed Akt activity (63). Finally, much of the ability of PTEN to regulate the cell cycle and induce apoptosis appears to be mediated via its ability to regulate Akt enzymatic activity (62, 63).

The objectives of this project were to investigate:

(i) the intracellular mechanisms of interactive effects of irradiation and TRAIL on apoptosis.

(ii) Whether the sequential treatment with irradiation followed by TRAIL induced more apoptosis than concurrent treatment with irradiation and TRAIL.

(iii) the requirement of p53 in doxorubicin, irradiation and TRAIL induced apoptosis.

(iv) the involvement of PTEN-Akt pathway in TRAIL induced apoptosis.

(v) the activation of MAP kinases after TRAIL treatment.

(vi) the specific roles of NFκB subunit in TRAIL-induced apoptosis.

BODY

We have attached a manuscript entitled "Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer *in vitro* and *in vivo*: intracellular mechanisms". The methods and materials, and related data are shown in the attached manuscript. The intracellular mechanisms by which irradiation enhances the apoptosis inducing potential of TRAIL are presented in this paper.

In addition, the data for other experiments are given below.

Regulation of genes by TRAIL

In this proposal, our studies will be limited to the analyses of genes involved in apoptosis. We proposed to use various methods such as RPA, Western blotting and microarray techniques to measure gene expressions. We have already presented the data on regulation of gene expression (apoptosis-related genes) by chemotherapeutic drugs, irradiation and TRAIL. The microarray data dealing with changes in gene expressions are shown in the Table 1. We compared the expression of genes in prostate cancer DU-145 cell treated with or without TRAIL for 16 h. Total RNA was isolated from each group. The α-^{32}P-dATP labeled cDNAs were hybridized onto Atlas human cancer cDNA microarray filters (Clontech). The data were analyzed using software from Clontech. The more than two-fold changes in gene expression are shown in the Table 1.

Table. 1. Comparison of Gene Expression Profiles in DU-145 cells. Cells were treated with or without TRAIL (25 nM) for 16 h. At the end of incubation, total RNAs were extracted, labeled and hybridized onto Atlas human cancer cDNA microarray filters (Clontech). Comparison of gene expression profiles obtained from control and treatment filters are shown.

Gene code	Expression	Protein/gene
A01d	UP	jun-B
A02c	Down	myb proto-oncogene; c-myb
A13m	Down	retinoblastoma-binding protein 1 (RBP1) isoform I + isoform II
A14d	Down	epidermal growth factor receptor
B04d	Down	Bruton's tyrosine kinase (BTK)

B05j	Up	rho6 protein
B07e	Up	rho-associated coiled-coil containing protein kinase p160ROCK
B07i	Up	serine/threonin protein phosphatase 2B catalytic subunit gamma
B09i	Up	protein tyrosine phosphatase sigma
B12j	Up	p21-rac2; small G protein
B12m	Up	guanylate kinase (GMP kinase)
C10d	Up	inhibitor of apoptosis protein1 (HIAP1; API1) + IAP homolog C
C11a	Up	caspase & rip adaptator with death domain (CRADD)
D01g	Down	neural cell adhesion molecule; CALL
D01h	Down	desmocollin 2A/2B precursor (DSC2); desmosomal glycoprotein II/III
D01k	Down	urokinase-type plasminogen activator receptor GPI-anchored form precursor (U-PAR)
D02c	Down	lymphocyte antigen
D02e	Down	integrin beta 6 precursor (ITGB6)
D02h	Down	delta catenin
D02k	Down	nuclear hormone receptor
D03c	Down	natural killer cell protein 7 (NKG7); protein GIG1
D03d	Down	cadherin 11 precursor (CDH11); osteoblast-cadherin (OB-cadherin)
D03g	Down	cadherin 4 (CDH4)
D03j	Down	interleukin-6 receptor alpha subunit precursor (IL-6R-alpha; IL6R)
D03k	Down	low-affinity nerve growth factor receptor (NGF receptor; NGFR)
D04e	Down	fibronectin receptor alpha subunit (FNRA); integrin alpha 5 (ITGA5)
D04g	Down	cadherin 6 precursor (CDH6); kidney cadherin (K-cadherin)
D04h	Down	erythropoietin receptor (EPOR)
D05c	Down	natural killer cell anitgen CD94
D05f	Down	integrin alpha 9 (ITGA9); integrin alpha-RLC
D05g	Down	cadherin 12 (CDH12); brain cadherin precursor (Br-cadherin)
D05h	Down	platelet-derived growth factor receptor beta subunit (PDGFRB); CD140B antigen
D05i	Down	fibroblast growth factor receptor 3 precursor (FGFR3)
D06a	Down	guanine nucleotide-binding protein G(Y) alpha 11 subunit (GNA11; GA11)
D06f	Down	integrin beta 1 (ITGB1)
D06g	Down	cadherin 13 (CDH13)
D06h	Down	keratinocyte growth factor receptor (KGFR)
D07e	Down	integrin alpha 1 (ITGA1); laminin & collagen receptor
E01n	Up	unrearranged immunoglobulin V(H)5 pseudogene
E03c	Up	macrophage-stimulating protein (MSP)
E03k	Up	placental protein 11 precursor (PP11)
E04g	Up	interleukin-12 beta subunit precursor (IL-12B)
E04h	Up	G protein-coupled receptor kinase 6 (GPRK6; GRK6)
E04i	Up	PDGF associated protein
E04m	Up	IgG receptor FC large subunit P51 precursor (FCRN)
E05g	Up	interleukin-12 alpha subunit precursor (IL-12A)
E05j	Up	plasminogen precursor (PLG)
E05k	Up	oxytocinase; cystinyl aminopeptidase
E07l	Down	ubiquitin-activating enzyme E1-related protein
E08i	Down	matrix metalloproteinase 7 (MMP7); matrilysin
E09i	Down	matrix metalloproteinase 8 (MMP8)
E09k	Down	ubiquitin C-terminal hydrolase 6;
E10f	Down	interleukin-6 precursor (IL-6)

E10i	Down	matrix metalloproteinase 9 (MMP9)
E10k	Down	chymotrypsin-like protease CTRL-1 precursor
E11g	Down	Wnt-13
E11h	Down	lunatic fringe
E11i	Down	matrix metalloproteinase 12 (MMP12); metalloelastase
E12h	Down	Wnt-2; int-1 related protein (IRP)
E12j	Down	tissue inhibtor of mettaloproteinase 4 (TIMP4)
E12l	Down	T cell receptor variable region
E12n	Down	collagen 6 alpha 2 subunit (COL6A2)
E13m	Down	immunoglobulin alpha 1 heavy chain constant region
F03a	Up	collagen 16 alpha 1 subunit precursor (COL16A1)
F10h	Up	dual-specificty A-kinase anchoring protein 1
F10i	Up	arginine/serine-rich splicing factor 7
F10k	Down	G-alpha interacting protein (GAIP)
F13d	Up	adenylate kinase isoenzyme 1 (AK1)

The requirement of p53 for doxorubicin, irradiation and/or TRAIL-induced apoptosis

We examined whether p53 is required for doxorubicin, irradiation and/or TRAIL-induced apoptosis (Fig. 1). Recently developed RNA interference (RNAi) technology has successfully been used to inhibit p53 function. In this technique, short interfering (si) double stranded RNAs are introduced to *in vitro* cultured cells resulting in degradation of specific mRNAs and a functional knock-out of a particular gene. P53 siRNA inhibited apoptosis induced by doxorubicin or irradiation in the presence or absence of TRAIL in LNCaP cells (Fig. 9). These data suggest that p53 is required for doxorubicin, irradiation and/or TRAIL induced apoptosis in LNCaP cells.

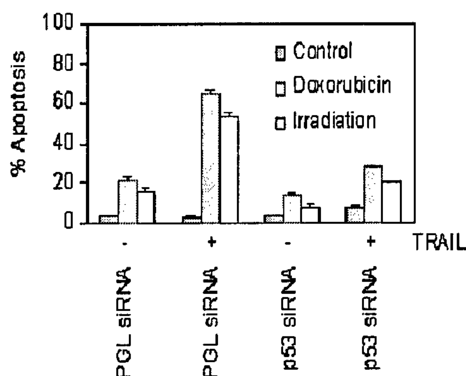


Fig. 1. Involvement of p53 in doxorubicin, irradiation and/or TRAIL-induced apoptosis. LNCaP cells were transfected with either control (PGL siRNA) or p53 siRNA plasmid in the presence of lipofectamine. Culture medium was replaced after 24 h of transfection, and cells were exposed to doxorubicin (1 μM) or irradiation (5 Gy) in the presence or absence of TRAIL (50 nM). Apoptosis was measured by DAPI staining.

Effects of PI-3 kinase inhibition on Akt activity and apoptosis on prostate cancer cells

Since Akt is an important regulator of various intracellular events in prostate tumor progression, we explored whether increased Akt activity may be involved in TRAIL resistance. To elucidate the mechanisms controlling the resistance to LNCaP cells to the cytotoxic effects of TRAIL, Akt activity was measured by immunoblotting using phospho-specific Ser^{473} Akt antibody. The data revealed the highest expression of constitutively active Akt in LNCaP cells, moderate expression in PC-3 and TSU-Pr1, and the lower expression in PC-3M and DU-145 (Fig. 2A). By comparison, normal prostate cells do not express constitutively active Akt. Total Akt levels in LNCaP, PC-3, PC-3M, TSU-Pr1, DU-145, and PrEC did not change. We further confirmed the constitutive activation of Akt by kinase assay (Fig. 2B). The kinase assay revealed the differential expression of constitutively active Akt with LNCaP cells expressing the highest and DU-145 cells expressing the lowest activity (Fig. 2B), similar to observations revealed by the Western blot analysis (Fig. 2A).

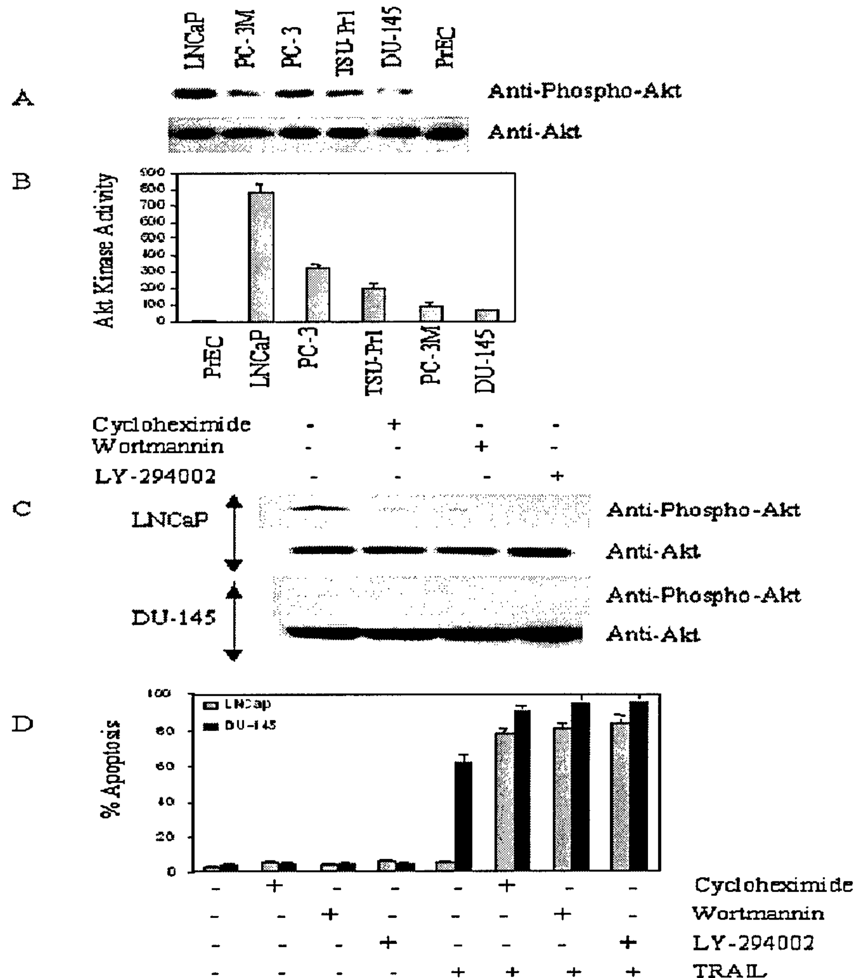


Fig. 2. Constitutive active Akt in prostate cancer cells. (A), Immunoblotting with anti-phospho-Akt, or anti-Akt antibody. (B) Measurement of constitutively active Akt in prostate normal and cancer cells by kinase assay. (C) Inhibition of phospho-active Akt in LNCaP and DU-145 cells by PI-3 kinase inhibitors (200 nM wortmannin, and 20 μM LY-294002) and protein synthesis inhibitor (10 μM cycloheximide). LNCaP and DU-145 cells were treated with wortmannin (200 nM), LY-294002 (20 μM) or cycloheximide (10 μM) in the presence or absence of TRAIL (50 ng/ml) for 24 h. The Western blots were performed with anti-phospho Akt antibody or anti-Akt antibody. (D) LNCaP and DU-145 cells were treated with wortmannin (200 nM), LY-294002 (20 μM) or cycloheximide (10 μM) in the presence or absence of TRAIL for 24 h, and apoptosis was measured by DAPI staining. Data represent mean ± SE.

We next examined whether inhibition of PI-3 kinase by LY-294002 and wortmannin, or protein synthesis by cycloheximide can inhibit constitutively active Akt (Fig. 2C). Treatment of LNCaP cells with LY-294002 (20 μM), wortmannin (200 nM), or cycloheximide (10 μM) for 8 h reversed the high constitutive activity of Akt (Fig. 2C). Since LNCaP cells possess a high constitutively active Akt, we sought to examine whether down-regulation of constitutively active Akt makes LNCaP cells sensitive to TRAIL. LNCaP and DU-145 cells were pre-treated with LY-294002 (20 μM), wortmannin (200 nM), or cycloheximide (10 μM) for 45 min, followed by treatment with TRAIL (50 ng/ml), and apoptotic nuclei were then counted by DAPI staining (Fig. 2D). TRAIL alone induced apoptosis in DU-145 cells, but had no effect on LNCaP cells. Pretreatment of LNCaP cells with the inhibitors of PI3- kinase (wortmannin or LY-294002) or protein synthesis (cycloheximide) sensitizes LNCaP cells to TRAIL. Wortmannin, LY-294002, and cycloheximide further enhanced the effects of TRAIL on apoptosis in DU-145 cells, which express low levels of constitutively active Akt. Thus, a high constitutively active Akt level in LNCaP cells renders them resistance to TRAIL.

TRAIL-mediated apoptosis occurs at the level of BID cleavage following caspase-8 activation in LNCaP cells.

Our earlier work has demonstrated that the formation of Death Inducing Signaling Complex (DISC) is essential for TRAIL-induced signaling, which require an adaptor protein FADD. Furthermore, formation of DISC is essential for activation of caspase-8. To rule out the

possibility of defects in caspase-8, we compared the caspase-8 activity between LNCaP and DU-145 cells (Fig. 3A). There was no difference in TRAIL-induced caspase-8 activity between LNCaP and DU-145 cells. Pre-incubation of cells with wortmannin or LY-294002 did not affect TRAIL-induced caspase-8 activity. This data suggest that resistance of LNCaP cells to TRAIL is not due to defects in caspase-8 activation.

Treatment of cells with TRAIL results in BID (a BH3-domain-only molecule) cleavage at its amino terminus as demonstrated previously by our laboratory. Cleavage of cytosolic p22 BID by caspase-8 generates a truncated p15 BID (tBID) fragment that translocates to the mitochondria. Immunodepletion of tBID from subcellular fractions argues that tBID is required for cytochrome c release from the mitochondria. We therefore examined the effects of TRAIL on BID cleavage in LNCaP and DU-145 cells (Fig. 3B). While ineffective alone, TRAIL along with wortmannin or LY-294002 cleaved BID in both LNCaP and DU-145 cells.

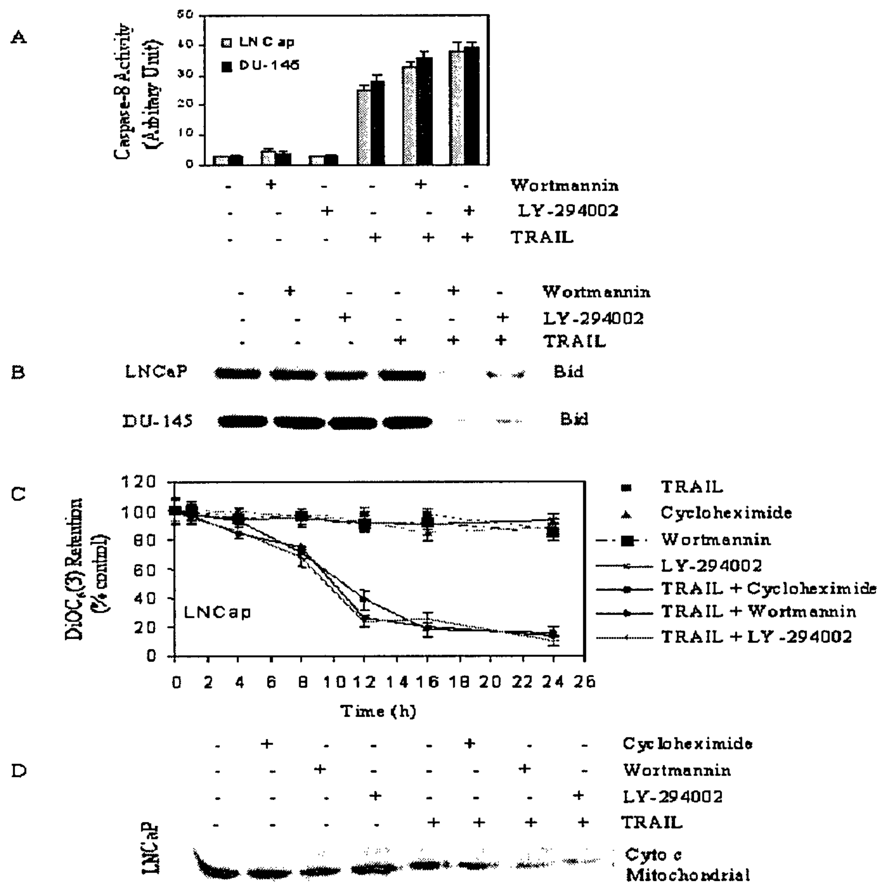


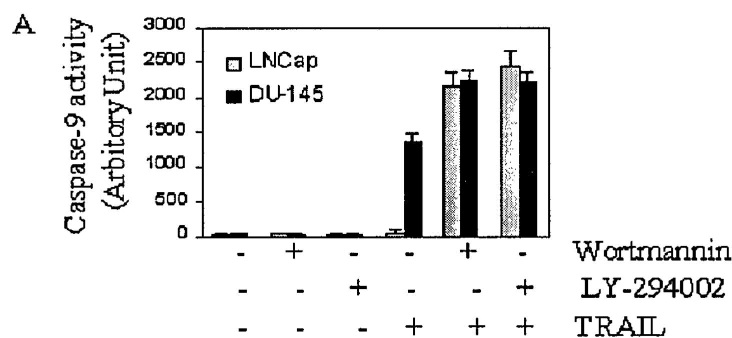
Fig. 3. Regulation of caspases-8 activity, Bid cleavage, mitochondrial membrane potential and cytochrome c release by wortmannin plus TRAIL or LY-294002 plus TRAIL. (A) LNCaP and DU-145 cells were treated with wortmannin (200 nM) or LY-294002 (20 μM) in the presence or absence of TRAIL (50 ng/ml) for 18 h. Caspase-8 activity was measured as per manufacturer's directions (Clontech). (B) LNCaP and DU-145 cells were treated with wortmannin (200 nM) or LY-294002 (20 μM) in the presence or absence of TRAIL (50 ng/ml) for 18 h. Bid cleavage was measured by the Western blot analysis. (C) Combination of TRAIL with wortmannin, LY-294002 and cycloheximide induce drop in mitochondrial membrane potential. LNCaP cells were treated with wortmannin (200 nM), LY-294002 (20 μM) or cycloheximide (10 μM) in the presence or absence of TRAIL (50 ng/ml) for various time points. Mitochondrial membrane potential was measured with $DiOC_6(3)$ dye. (D) Combination of TRAIL with wortmannin, LY-294002 and cycloheximide induce cytochrome c release. LNCaP cells were treated with wortmannin (200 nM), LY-294002 (20 μM) or cycloheximide (10 μM) in the presence or absence of TRAIL (50 ng/ml) for 24 h. Cells were mechanically lysed and separated into mitochondrial and cytosolic S100 fractions. The amounts of cytochrome c retained in the mitochondrial fractions were determined by the Western blot analysis.

Mitochondria appear to play a key role in apoptosis. During apoptotic cell death, the early events that occur are mitochondrial depolarization and the loss of cytochrome c from the mitochondrial intermembrane space. The fluorescent dye $DiOC_6(3)$ localizes to the mitochondria and the mitochondrial permeability transition (MPT) reduces the accumulation of $DiOC_6(3)$ as a consequence of the loss in mitochondrial membrane potential ($\Delta\psi_m$). If the high constitutive activity of Akt causes TRAIL resistance in LNCaP cells, then Akt may block apoptosis either upstream or downstream of mitochondrial dysfunction such as $\Delta\psi_m$ and cytochrome c release from the mitochondria. Since TRAIL-induced BID cleavage in LNCaP cells occurs in the presence of wortmannin or LY-294002, we sought to investigate mitochondrial dysfunction by measuring $\Delta\psi_m$. Incubation of cells with TRAIL, cycloheximide, wortmannin, or LY-294002 alone had no effect on $\Delta\psi_m$ in LNCaP cells (Fig. 3C). However, TRAIL in combination with cycloheximide, wortmannin, or LY-294002, caused a rapid decrease in $\Delta\psi_m$ in LNCaP cells (Fig. 3C). In contrast, treatment of DU-145 cells with TRAIL alone caused a significant drop in $\Delta\psi_m$ (data not shown). Since opening of the MPT pores causes

releases of cytochrome c from mitochondria, we sought to examine the levels of cytochrome c retained in mitochondria in LNCaP cells treated with various drug combinations (Fig. 3D). TRAIL, cycloheximide, wortmannin, or LY-294002 alone had no effect on cytochrome c release from mitochondria to cytosol in LNCaP cells (Fig. 2D). However, the combination of TRAIL with cycloheximide, wortmannin, or LY-294002 resulted in loss of cytochrome c from the mitochondria (Fig. 3D).

TRAIL-induced activation of caspase-9 and caspase-3 in DU-145 cells but not in LNCaP cells.

Mitochondrial dysfunction appears to be essential for the formation of apoptosomes (a complex consisting of cytochrome c, Apaf-1 and ATP), which in turn activate caspase-9 and caspase-3. We therefore sought to examine the activation of caspase-9 and caspase-3 in cells treated with TRAIL in the presence and absence of wortmannin or LY-294002. Treatment of LNCaP and DU-145 cells with wortmannin or LY-294002 had no effect on caspase-9 and caspase-3 activity (Fig. 4A and B). As expected, TRAIL-induced caspase-9 and caspase-3 activity in DU-145 cells but not in LNCaP calls. By comparison, TRAIL further enhances the activation of caspase-9 and caspase-3 when combined with LY-294002 or wortmannin (Fig. 4A and B).



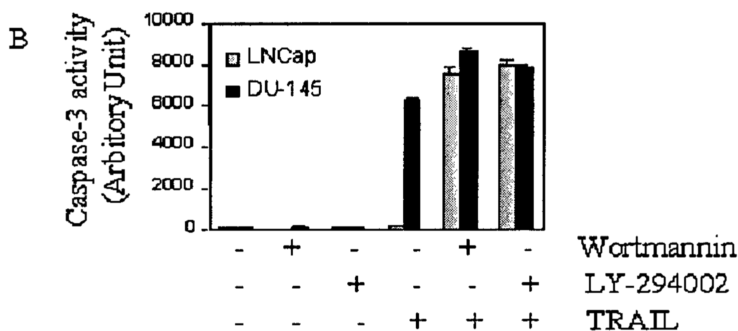


Fig. 4. Regulation of caspase-9 and caspase-3 activity by wortmannin plus TRAIL or LY-294002 plus TRAIL. (A) LNCaP and DU-145 cells were treated with wortmannin (200 nM) or LY-294002 (20 μM) in the presence or absence of TRAIL (50 ng/ml) for 18 h. Caspase-9 activity was measured as per manufacturer's directions (Oncogene Research Products). (B) LNCaP and DU-145 cells were treated with wortmannin (200 nM) or LY-294002 (20 μM) in the presence or absence of TRAIL (50 ng/ml) for 18 h. Caspase-3 activity was measured as per manufacturer's directions (Calbiochem). The data represent mean ± SE.

Attenuation of constitutively active Akt by dominant negative Akt (DN-Akt) or PTEN sensitizes LNCaP cells to TRAIL.

If Akt is the only signaling molecule for TRAIL resistance in LNCaP cells, then down-regulation of Akt by dominant negative Akt (DN Akt) will sensitize cells to TRAIL. We, therefore, used the genetic approach to down-regulate Akt activity in LNCaP cells with dominant negative Akt and examined the effects of TRAIL on apoptosis and BID cleavage (Fig. 5 A and B). LNCaP cells were transiently transfected with empty vector, WT-Akt, CA-Akt or DN-Akt and treated with or without TRAIL (50 nM). Transfection of LNCaP cells with empty vector, WT-Akt or CA-Akt had no effect on apoptosis, whereas transfection of cells with DN-Akt makes LNCaP cells sensitive to TRAIL (Fig. 5A). By comparison to LNCaP cells, transfection of empty vector or WT-Akt in DU-145 cells had no significant difference on TRAIL-induced apoptosis, whereas transfection of CA-Akt abrogated TRAIL-induced apoptosis (Fig. 5A). In addition, transfection of DN-Akt in DU-145 cells slightly enhanced TRAIL-induced apoptosis compared to empty vector or WT-Akt transfected cells. Furthermore, downregulation of Akt activity by DN-Akt in LNCaP cells results in BID cleavage in response to TRAIL treatment (Fig. 5B), suggesting inhibition of constitutively active Akt in LNCaP cells is sufficient to induce apoptosis by TRAIL via BID cleavage.

The tumor suppressor gene PTEN is quite often inactivated in primary human prostate cancers, particularly in the more advanced cancers, and in human prostate cancer xenografts and cell lines including PC-3 and LNCaP. These studies suggest that downstream target of the PI3-kinase pathway, such as AKT that is negatively regulated by PTEN, may be increasingly activated with prostate tumor progression. We, therefore, transfected LNCaP cells with either empty vector or PTEN cDNA and incubated in the presence or absence of TRAIL (Fig. 5C).

Transfection of LNCaP cells with PTEN cDNA resulted in induction of apoptosis (Fig. 5C) and cleavage of BID by TRAIL (Fig. 5D). These data confirmed our previous findings that constitutively active Akt is involved in the resistance of LNCaP cells to TRAIL.

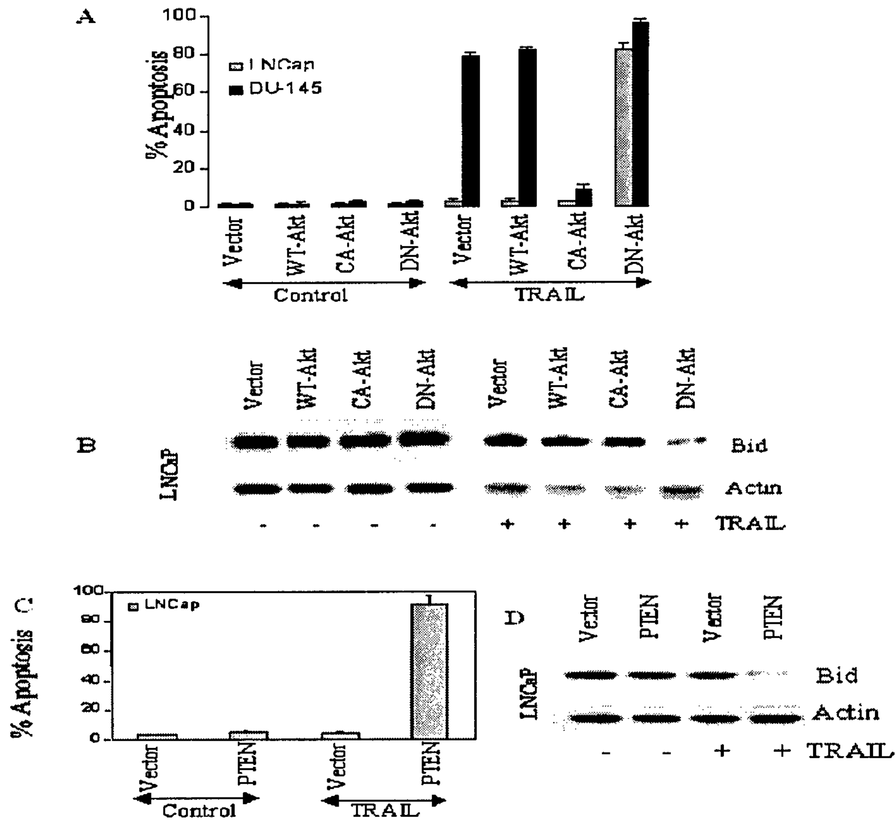


Fig. 5. Overexpression of dominant negative Akt sensitizes LNCaP cells to TRAIL. (A) LNCaP cells were transiently transfected with empty vector, WT-Akt, CA-Akt or DN-Akt. The culture medium was changed and cells were treated with or without TRAIL (50 ng/ml). Apoptosis was measured after 24 h. (B) TRAIL induces BID cleavage in LNCaP cells transfected with DN-Akt. LNCaP cells were transiently transfected with empty vector, WT-Akt, CA-Akt or DN-Akt. The culture medium was changed and cells were treated with or without TRAIL (50 ng/ml) for 24 h. The cells were mechanically lysed and crude proteins were isolated. The amount of BID cleaved was determined by the Western blot analysis. The same blot was reprobed with anti-actin antibody to determine whether equal amount of proteins were loaded. (C) Overexpression of PTEN in LNCaP cells causes apoptosis induced by TRAIL. LNCaP cells were transiently

transfected with empty vector or plasmid containing PTEN cDNA for 24 h. The culture medium was changed and cells were treated with or without TRAIL (50 ng/ml) for 24 h. Apoptosis was measured by DAPI staining. (D), Transfection of cells with PTEN cDNA sensitizes LNCaP cells to Bid cleavage. LNCaP cells were transiently transfected with empty vector or plasmid containing PTEN cDNA for 24 h. The culture medium was changed and cells were treated with or without TRAIL (50 ng/ml) for 24 h. Bid cleavage was measured by the Western blot analysis. The same blot was reprobed with anti-β-actin antibody to determine whether equal amount of proteins were loaded.

Overexpression of constitutively active Akt in PC-3M cells confers TRAIL resistance.

Since TRAIL treatment alone can kill PC-3M cells owing to low levels of constitutively active Akt, we wished to investigate the effects of the overexpression of constitutively active Akt on TRAIL-induced drop in mitochondrial membrane potential and apoptosis. Transfection of empty vector, WT-Akt, CA-Akt and DN-Akt was performed in PC-3M cells, and Akt activity was measured by kinase assay (Fig. 6A). As expected, group transfected with CA-Akt cDNA expressed active form of Akt, whereas activation (phosphorylation) of Akt was not observed in groups transfected with empty vector, WT-Akt and DN-Akt. We next examined the effects of WT-Akt, CA-Akt and DN-Akt on TRAIL-induced apoptosis as measured by the nucleosome ELISA assay (Fig. 6B). PC-3M cells were transfected with empty vector, WT-Akt, CA-Akt and DN-Akt, and treated with or without TRAIL for 24 hr. TRAIL-promoted apoptosis in cells transfected with empty vector, WT-Akt and DN-Akt. In contrast, transfection of CA-Akt cDNA in PC-3M cells inhibited TRAIL-induced apoptosis. We next examined the effects of WT-Akt, CA-Akt and DN-Akt on TRAIL-induced drop in $\Delta\psi_{m.}$ TRAIL-induced drop in $\Delta\psi_m$ in PC-3M cells transfected with empty vector, WT-Akt or DN-Akt (Fig. 6C). In contrast, transfection of CA-Akt cDNA in PC-3M cells inhibited TRAIL-induced drop in $\Delta\psi_{m.}$ These data suggest that an increased Akt activity due to overexpression of CA-Akt in PC-3M cells makes them resistance to TRAIL. This effect is similar to LNCaP cells, which express high levels of constitutively active Akt and are resistant to TRAIL.

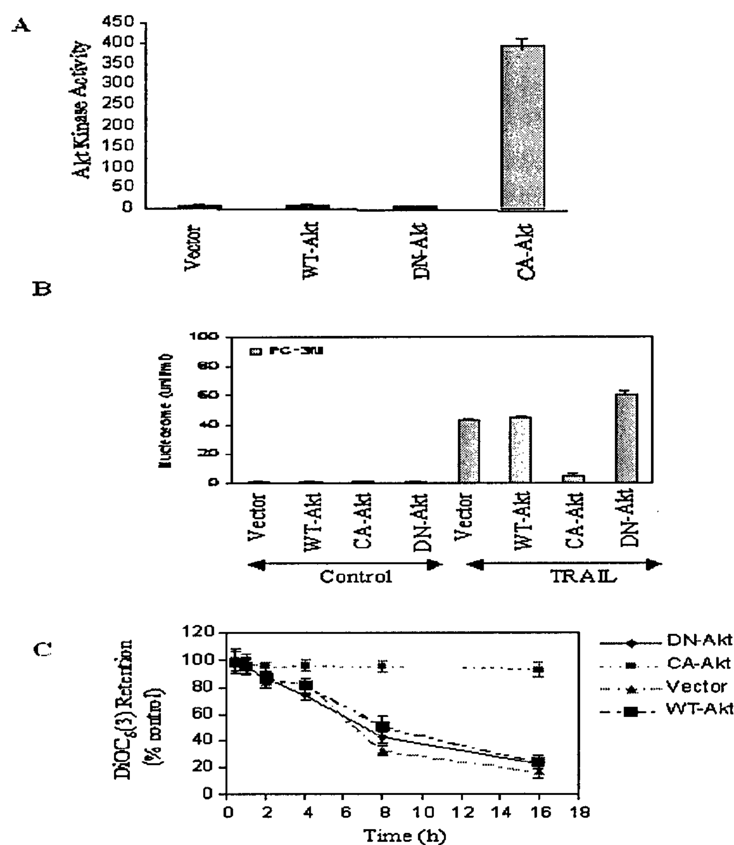


Fig. 6. Effects of overexpression of constitutively active Akt on apoptosis and mitochondrial membrane potential. (A) PC-3M cells were transiently transfected with wild type Akt, dominant negative Akt (DN-Akt), constitutively active Akt (CA-Akt) or empty vector. Akt activity was measured by kinase assay as described in material and methods (B) Overexpression of constitutively active Akt makes PC-3M cells resistant to TRAIL-induced apoptosis. PC-3M cells were transfected with empty vector, WT-Akt, CA-Akt or DN-Akt. Cells were treated with or without TRAIL (50 ng/ml) for another 24 h and apoptosis was measured by nucleosome ELISA assay. The data represent mean ± SE of three experiments. (C) TRAIL causes mitochondrial depolarization that is not prevented by overexpresssion of CA-Akt. PC-3M cells were transfected with empty vector, WT-Akt, CA-Akt or DN-Akt for 24 h. Cells were treated with or without TRAIL (50 ng/ml) for another 24 hr and apoptosis was measured. During the last 30 min of treatment, $DiOC_6(3)$ was added. An aliquot of cells was used for the determination of cell-associated $DiOC_6(3)$ fluorescence. The data represent mean ± SD of three independent experiments.

Effects of MAP kinases on TRAIL-induced apoptosis

Mitogen-activated protein kinases (MAPKs) transduce signals from the cell membrane to the nucleus in response to a variety of different stimuli and participate in various intracellular signaling pathways that control a wide spectrum of cellular processes, including cell growth, differentiation, and stress responses. In contrast to p42MAPK/extracellular signal-regulated kinase (ERK2) and p44MAPK/ERK1, which are activated by mitogenic stimuli, p46 c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p54JNK/SAPK and p38MAPK are activated by inflammatory cytokines and cellular stresses. In fact, the stimulation of JNK was a prerequisite for cell death under various conditions, and a blockade of JNK activation resulted in the prevention of cell death. MAPKs can be dephosphorylated and inactivated by dual-specificity phosphatases. These findings imply that under certain circumstances JNK may function in an intracellular signaling pathway, leading to cell death. Once activated, JNK/SAPK phosphorylates several transcription factors including c-Jun, ATF-2, and ELK-1, thereby regulating gene expression.

We therefore examined whether TRAIL induces activation of MAP kinases. TRAIL induces activation of JNK, ERK and p38 MAP kinases in a time dependent manner (Fig. 7). Exposure of cells with irradiation also induced activation of JNK, ERK and p38. Further studies are underway to determine the role of MAP kinase in TRAIL induced apoptosis.

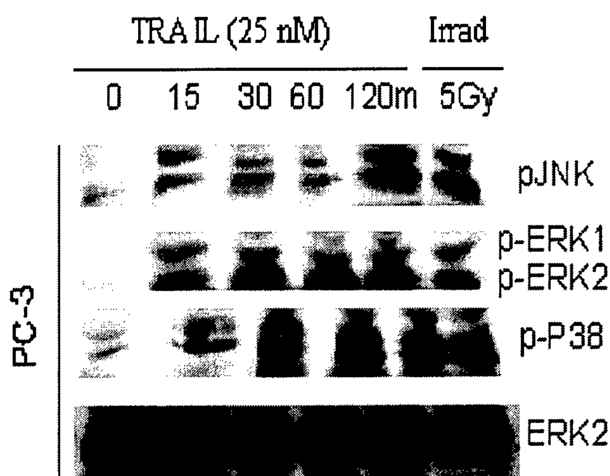


Fig. 7. Effects of TRAIL on activation of MAP kinases. PC-3 cells were treated with TRAIL for 0, 15, 30, 60, and 120 min. Cells were harvested and the activation of JNK, ERK and P38 was measured by the western blotting using phospho-specific antibodies. Irradiation (Irrad) was used as a positive control.

Effects of RelA (p65), p50 and c-Rel subunits on TRAIL-induced NFκB DNA binding activity and apoptosis

Recent studies have shown the involvement of NFκB in the regulation of apoptosis. In cancer cells, NFκB is constitutively active, and therefore may change the apoptotic response of TRAIL. In order to determine whether NFκB plays a role in TRAIL-induced apoptosis, we first examined the activation of NFκB in prostate cancer LNCaP cells (Fig. 8A). LNCaP cells were treated with TRAIL for 0, 20, 40, and 60 min. Translocation of NFκB to nucleus was examined by EMSA (Electrophoretic Mobility Shift Assay). The NFκB binding activity was increased in a time-dependent manner in LNCaP cells after treatment with TRAIL, reaching a maximum at 120 min (Fig. 8A).

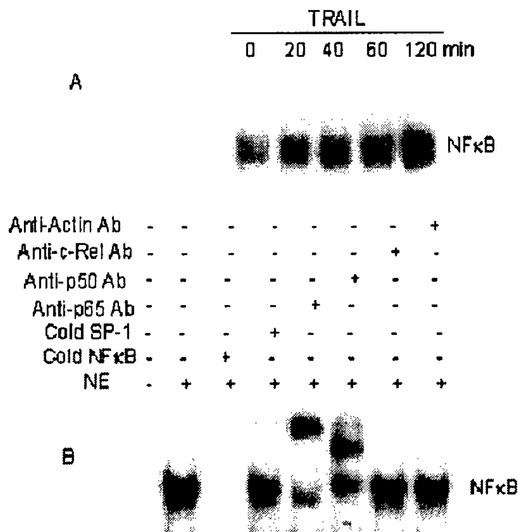


Fig. 8. NFκB-DNA binding activity in prostate cancer cells. (A), LNCaP cells were treated with TRAIL (25 nM) for 20, 40, 60, and 120 min. After the incubated period, cells were harvested and nuclear extracts were prepared. Nuclear extracts were analyzed for NFκB binding with its consensus sequence by EMSA. (B), Involvement of various subunits of NFκB in TRAIL-induced NFκB activation. LNCaP cells were treated with TRAIL (25 nM) for 2 h. Nuclear extracts were incubated in the presence or absence of antibodies against anti-p65, anti-p50, and anti-c-Rel to determine the presence of NFκB subunits in the nuclear extract by super shift assay. An anti-β-actin antibody was used as a control. Cold NFκB and SP-1 probes (50 X) were used as competitors.

To further identify which NFκB subunit(s) contribute to enhanced DNA binding activity, we performed supershift assay with specific antibodies against different subunits of NFκB, i.e. RelA (p65), p50 and c-Rel (Fig. 8 B). Treatment of LNCaP cells with TRAIL enhanced the NFκB-DNA binding activity. Incubation of nuclear extracts with unlabelled consensus NFκB oligonucleotide completely abolished DNA binding, suggesting the specificity of NFκB bands. Antibodies specific for the p65, and p50 subunits of NFκB caused significant supershift. By comparison, no significant supershift was observed with anti-c-Rel antibody, probably because

of the nature of antibody or absence of this subunit of NFκB in the nucleus. An anti-β-actin antibody was used as a control.

We next examined the transcriptional activation of NFκB by luciferase assay. LNCaP cells were transiently transfected with κB-luciferase reporter plasmid construct and the NFκB transactivity was measured by the luciferase assay (Fig. 9A). Transfection of LNCaP cells with super repressor mIκBα, p65, p50, and c-Rel had no effect NFκB transactivity. Transfection of LNCaP cells with p65, p50 and c-Rel induced NFκB transactivity after TRAIL treatment, whereas neo and mIκBα had no effect on luciferase activity after TRAIL treatment.

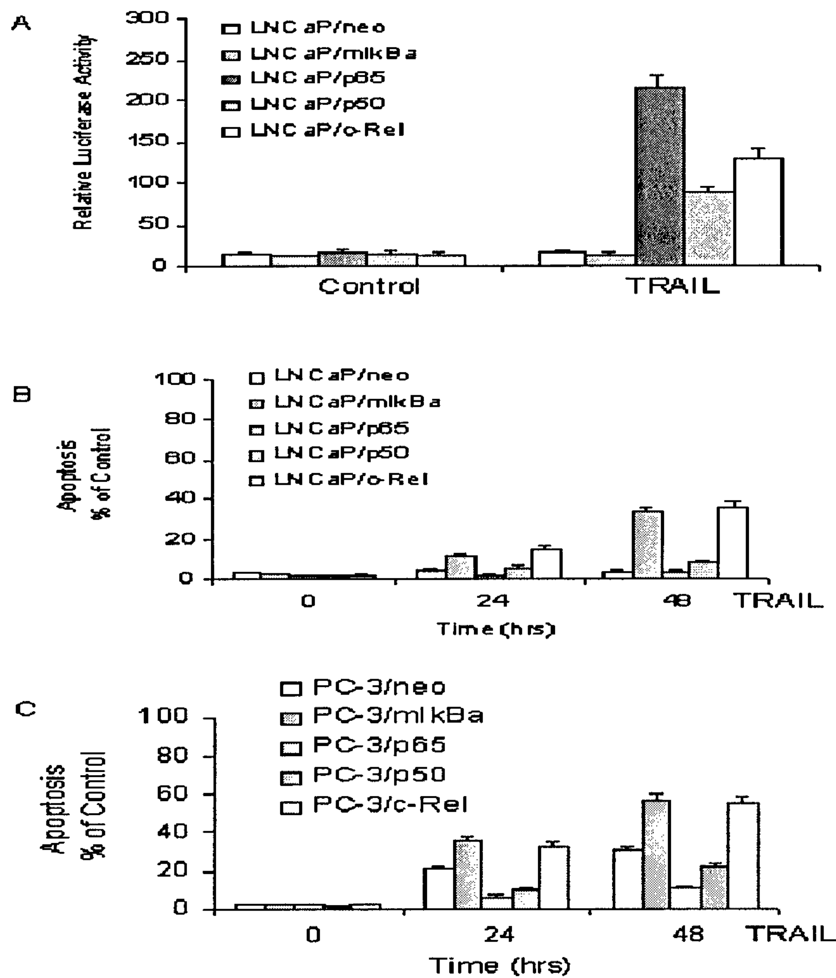


Fig. 9. Effect of different subunits of NFκB on transcriptional activity and apoptosis of prostate cancer cells to TRAIL. (A), LNCaP cells were transfected with different subunits of NFκB and co-transfected with NFκB/luciferase reporter plasmid and a CMV4 promoter-driven β-gal expression plasmid. The luciferase activity was assayed for NFκB activation and normalized to β-gal. (B and C), Effects of various subunits of NFκB on TRAIL-induced apoptosis. LNCaP and PC-3 cells were transfected with various subunits of NFκB (neo, mIκBα, p65, p50 and c-Rel) with control plasmid (pCMV-LacZ) encoding β-galctosidase (β-Gal) enzyme, and were treated with TRAIL (25 nM) for 0, 24 and 48 h. More than 85% of cells were transfected, and there were no significant differences in transfection efficiency among groups. After incubation, cells were harvested, fixed and stained with DAPI to measure apoptosis. Data represent mean ± SE of three independent experiments.

The physiological role of NFκB in cell survival and apoptosis is not clear. NFκB has been found to act as an anti- or a pro-apoptotic factor. The opposing effects of NFκB on cell survival and apoptosis may be due to differential expression of its subunits. We therefore sought to examine the specific role of NFκB subunits on TRAIL-induced signaling. LNCaP and PC-3 cells were transiently transfected with plasmid cDNAs containing different subunits of NFκB (RelA/p65, p50, and c-Rel), and a mutant NFκB inhibitory protein IκBα (Ser32/Ser36) termed as mIκBα (Fig. 9 B and C). Transfected cells were treated with or without TRAIL (25 nM) for 24 or 48 h, fixed, and stained with DAPI. TRAIL induced apoptosis in PC-3 cells, but not in LNCaP cells. The mutant IκBα (Ser32/Ser36) expression slightly enhanced the apoptotic response to TRAIL in both LNCaP and PC-3 cells. Overexpression of RelA (p65) subunit significantly attenuated TRAIL-induced apoptosis in PC-3 cells at 24 and 48 hrs. By comparison, transfection of cells with c-Rel enhanced apoptosis in both cell types after TRAIL treatment. Overexpression of p50 subunit slightly inhibited apoptosis after TRAIL treatment. These results suggest that RelA (p65) subunit of NFκB blocks, whereas c-Rel enhances TRAIL-induced apoptosis.

KEY RESEARCH ACCOMPLISHMENTS

- Irradiation significantly augmented TRAIL- induced apoptosis in prostate cancer cells through up-regulation of DR5, Bax and Bak, and induction of caspase activation. Dominant negative FADD and p53 siRNA inhibited the synergistic interaction between irradiation and TRAIL. The pretreatment of cells with irradiation followed by TRAIL significantly enhanced more apoptosis than single agent alone or concurrent treatment. Furthermore, irradiation sensitized TRAIL-resistant LNCaP cells to undergo apoptosis (please see the attached manuscript).

- We demonstrate that Akt is activated in LNCaP cell line. LNCaP cells use Akt for survival because when PI3-K inhibitors are added or kinase-dead Akt transfected, LNCaP cells undergo apoptosis. Manipulating Akt activity alters sensitivity to TRAIL, transfecting constitutively active Akt into PC-3M cells that have low endogenous Akt activity increases resistance to TRAIL; alternatively, adding a PI3-K inhibitor or transfecting kinase-dead Akt into cells with high levels of Akt activity results in dramatic sensitization to these modalities. These data show that targeting a specific kinase that promotes survival such as Akt can change the apoptotic potential of prostate cancer cells resulting in greater efficacy of TRAIL *in vitro*.

- TRAIL induces activation of JNK, ERK and p38 MAP kinases. Further studies are under way to examine the specific role of these kinase in TRAIL signaling.

- The transcription factor NFκB is constitutively active in cancer cells. Down-regulation of NFκB by mIκBα sensitizes cells to undergo apoptosis. RelA (p65) subunit of NFκB blocks, whereas c-Rel enhances TRAIL-induced apoptosis. The dual function of NFκB, as an inhibitor or activator of apoptosis, depends on the relative levels of RelA and c-Rel subunits. Thus, NFκB activity may play an important role in tumor progression, and down-regulation of RelA or up-regulation of c-Rel represents a possible therapeutic target for the treatment of cancer.

REPORTABLE OUTCOMES

Presentations

1. Singh, T.R., S. Shankar, and R. K. Srivastava. Clinical Significance of TRAIL in cancer. 8th World Congress on Advances in Oncology, and 6th International Symposium on Molecular Medicine. Athens, Greece, October 16-18, 2003.
2. Chen, X., T.R. Singh, S. Shankar, and R.K. Srivastava. *In vivo* regulation of apoptosis and angiogenesis by histone deacetylase inhibitors and TRAIL in human prostate cancer. 94th Annual Meeting of the American Association for Cancer Research, Washington, D.C, July 11-14, 2003.
3. Singh, T.R., S. Shankar, and R.K. Srivastava. The role NFκB in mitochondria during apoptosis. 95th Annual Meeting of the American Association for Cancer Research. Orlando, Florida, March 27-31, 2004.

Publication

1. Shankar, S., T.R. Singh, and R.K. Srivastava. 2004. Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer *in vitro* and *in vivo*: intracellular mechanisms. Prostate (in press).

CONCLUSIONS

- TRAIL can be used to kill prostate cancer cells. However, some prostate cancer cell lines are resistant to TRAIL.
- TRAIL activates JNK, ERK and p38 MAP kinases.
- TRAIL can kill prostate cancer cells harboring wild type p53 or mutated p53.
- Treatment with irradiation is an effective approach to sensitize prostate cancer cells to TRAIL-induced apoptosis.
- Downregulation of AKT sensitizes TRAIL-resistant cancer cells to undergo apoptosis.
- The ratio between RelA (p65) and c-Rel subunits will determine whether activation of NFκB will trigger apoptotic or survival signal. In addition, up-regulation of RelA (p65) subunit of NFκB can enhance TRAIL resistance, whereas up-regulation of c-Rel can enhance TRAIL sensitivity. Thus, regulation of NFκB subunits may be a novel strategy for cancer therapy.

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APPENDICES

1. I have attached a manuscript entitled "Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer *in vitro* and *in vivo*: intracellular mechanisms".

Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer *in vitro* and *in vivo*: intracellular mechanisms

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Keywords: TRAIL, apoptosis, irradiation, Bax, p53, Bak, Bcl-2, prostate cancer, death receptors

Abbreviations: TRAIL, tumor necrosis factor related apoptosis-inducing ligand; Apo-2L, Apo2 ligand; IAP, inhibitors of apoptosis protein; FasL, Fas ligand; DR, death receptor; DcR, decoy receptor

Running Title: Interactive effects of irradiation and TRAIL on apoptosis

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ABSTRACT

Background: We assessed the influence of sequential treatment of ionizing radiation followed by TRAIL on intracellular mechanisms of apoptosis of prostate tumor cells *in vitro* and *in vivo*.

Methods: Prostate normal and cancer cells were exposed to irradiation and TRAIL. Four to six-week old athymic nude mice were injected s.c.with PC-3 tumor cells. Tumor bearing mice were exposed to irradiation and TRAIL, either alone or in combination (TRAIL after 24 h of irradiation), and tumor growth, apoptosis and survival of mice were examined. Expressions of death receptors, Bcl-2 family members and caspase were measured by Western blotting, ELISA and ribonuclease protection assay; tumor cellularity was assessed by H& E staining; inhibition of p53 was performed by RNA interference (siRNA) technology, and apoptosis was measured by annexin V/propidium iodide staining, and terminal deoxynucleotidyltransferase-mediated nick end labeling assay.

Results: Irradiation significantly augmented TRAIL- induced apoptosis in prostate cancer cells through up-regulation of DR5, Bax and Bak, and induction of caspase activation. Dominant negative FADD and p53 siRNA inhibited the synergistic interaction between irradiation and TRAIL. The pretreatment of cells with irradiation followed by TRAIL significantly enhanced more apoptosis than single agent alone or concurrent treatment. Furthermore, irradiation sensitized TRAIL-resistant LNCaP cells to undergo apoptosis. The sequential treatment of xenografted mice with irradiation followed by TRAIL induced apoptosis through activation of caspase-3, induction of Bax and Bak, and inhibition of Bcl-2, and completely eradicated the established tumors with enhanced survival of nude mice.

Conclusion: The sequential treatment with irradiation followed by TRAIL can be used as a viable option to enhance the therapeutic potential of TRAIL in prostate cancer.

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignant neoplasm and the second leading cause of cancer-related deaths in American men (1). It has a low proliferative index and depends on androgens for growth. After castration these tumors regress but later progress to an androgen-independent phenotype that is ultimately lethal for the patient. New therapeutic approaches for the treatment of prostate cancer includes the inhibition of androgen receptors, regulation of apoptosis, blocking of tumor angiogenesis, and inhibition of invasion and metastasis (2, 3). Considerable efforts have been directed recently towards novel pathways that trigger apoptosis selectively in tumor cells irrespective of their androgen receptor status (4). The activation of death receptor pathway induces apoptosis in both androgen-sensitive and -insensitive prostate cancer cells (5). One such ligand of death receptors is a newly discovered cytokine TRAIL (TNF-related-apoptosis-inducing-ligand)/Apo-2L.

TRAIL /Apo-2L is a novel anticancer agent that has been shown to induce apoptosis in a variety of tumor cells of diverse origin both *in vitro* and *in vivo* (6-9). TRAIL can bind to five distinct death receptors of TNF receptor family namely TRAIL-R1/ DR4 (10), TRAIL-R2/DR5 (11, 12), TRAIL-R3 /DcR1/TRID (13, 14), TRAIL-R4 /DcR2/TRUNDD (15), and osteoprotegerin (16). Binding of TRAIL to its receptors DR4 and DR5 results in recruitment of the adaptor protein FADD, which in turn recruits and activates caspase-8 (17, 18). Active caspase-8 transmits signal either by activating downstream caspase-3 or by cleaving Bid to truncated Bid (17). Translocation of tBid to mitochondria triggers Bax and Bak oligomerization, releases mitochondrial proteins, activates downstream caspases and induces apoptosis (19, 20). Caspase-8-mediated Bid processing therefore bridges the extrinsic death receptor-mediated pathway of apoptosis to the intrinsic mitochondrial pathway (20, 21). This provides a mechanism to amplify the execution signal and exacerbate the pace of cell demise. The Bcl-2 family proteins regulate apoptosis by acting mainly at the level of mitochondria (22).

We have recently shown that several prostate cancer cell lines were sensitive to TRAIL, whereas LNCaP cells were resistant (7). Recent studies have shown that irradiation up-regulates death receptors mainly TRAIL-R2/DR5 in human prostate cancer

cells *in vitro.* Therefore, irradiation may enhance the apoptosis inducing potential of TRAIL in TRAIL-sensitive cells, and sensitize TRAIL-resistant LNCaP cells by up-regulating death receptors and activating caspases, so that successive treatment with TRAIL would result in apoptosis. The aims of present study were to investigate (i) the intracellular mechanisms of interactive effects of irradiation and TRAIL on apoptosis, and (ii) whether the sequential treatment with irradiation followed by TRAIL could be used as a preclinical model for prostate cancer. Because PC-3 cell line shows a high metastatic potential after orthotopic implantation into nude mice, we used this clinically relevant model to evaluate the interactive effects of irradiation and TRAIL on tumor growth and survival. We demonstrate that (i) irradiation and TRAIL induce Bax and Bak, activate caspases and enhance apoptosis through up-regulation of DR5, and, (ii) sequential treatment of mice with irradiation followed by TRAIL reduces tumor growth by inducing apoptosis and inhibiting the number of proliferating cells, and enhances the survival of tumor-bearing mice. Thus, sequential treatment with irradiation followed by TRAIL may be beneficial for the treatment of prostate cancer.

MATERIALS AND METHODS

Reagents. Antibodies against caspase-3, caspase-8 and poly ADP ribose polymerase (PARP), and ELISA kits for DR4 and DR5 were purchased from Biosource International, Inc. (Camarillo, CA). Anti-caspase-3 and anti-caspase-7 antibodies for immunofluorescence studies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Bax and Bak were purchased from Santa Cruz (Santa Cruz, CA). Anti-tubulin antibody was purchased from Oncogene Research (Cambridge, MA). Enhanced chemiluminescence (ECL) western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL). Lipofectamine reagent was from Invitrogen Life Technologies (Carlsbad, CA). TRAIL was synthesized as described earlier (23). The caspase inhibitors z-IETD-fmk (z-Ile-Glu-Thr-Asp-fluoromethylketone), and z-VAD-fmk (CBZ-Val, Ala, Asp-fluoromethylketone) were purchased from Enzyme System Products (Livermore, CA, USA). RNase protection assay (RPA) kit was purchased from PharMingen (San Diego, CA). Fluorochrome

conjugated secondary antibodies were purchased from Sigma Chemical Co (St Louise, MO). Cell death detection kit (TUNEL) was purchased from Roche Applied Sciences (Indianapolis, IN).

Cells and Culture Conditions. Human prostate normal (PrEC) and cancer (PC-3, DU-145 and LNCaP) cells were obtained from the Clonetics (Gaithersburg, MD) and American Type Culture Collection (Manassas, VA), respectively. PrEC cells express wild type p53. PC-3 cells are null for p53 gene, and express Bcl-2 gene. DU145 cells express mutated p53 and are null for Bcl-2 gene. LNCaP cells express wild type p53 and Bcl-2. PrEC cells were grown in PrECM medium (Clonetics). Cancer cells were grown in RPMI 1640 supplemented with D-glucose, HEPES buffer, 2mM L-glutamine, 1% penicillin-streptomycin mixture, and 10% fetal bovine serum. All cells were grown in tissue culture dishes at 37°C with 5% CO_2.

Western Blot Analysis. Cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM sodium orthovanadate, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 2 μg/ml aprotinin). Lysates were sonicated for 10 s, centrifuged for 20 min at 10,000 X g and stored at -70 ^{0}C. Equal amounts of lysate protein were run on 10% SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were blocked with 6% nonfat dry milk in TBST buffer (20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 0.01% Tween 20) and incubated with primary antibody in TBST containing 1% bovine serum albumin overnight at 4 ^{0}C. Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody and ECL reagents.

RNase Protection Assay (RPA). Total RNAs were extracted using Trizol™ reagent (Life Technologies Inc, Gaithersburg, MD). The RNase Protection Assay was performed as per manufacture's instructions (PharMingen). Briefly, probe set including the DNA templates was used for T7 RNA polymerase-directed synthesis of [^{32}P]UTP-labeled antisense RNA probes. 2 μg of RNA was incubated with α-^{32}P-UTP labeled single-stranded RNA probes overnight at 56^{0}C, and treated with RNase for 45 min at 30^{0}C. The RNA-RNA complexes were resolved by electrophoresis in 6% denaturing polyacrylamide gels and analyzed by autoradiography.

Analysis of DR4 and DR5 by ELISA. Cells were treated with TRAIL and/or irradiation. At the end of incubation period, cells were harvested and washed twice with ice-cold PBS. Cells were lysed in extraction buffer for 30 min on ice with vortexing at 10 min intervals. The extracts were centrifuged at 13,000 rpm for 10 min at 4^0C. Lysates were aliquoted, and assayed for DR4 and DR5 proteins by ELISA as per manufacture's directions (Biosource International, Inc.).

RNAi Technology. Sequences of oligonucleotides for inhibition of p53 and the PGL3 control siRNA have been described (24). The sequence of the p53 oligonucleotide is: 5' r(CUACUUCCUGAAAACAACG)d(TT) 3', 5' r(CGUUGUUUUCAGGAAGUAG)d(TT)3'. siRNA oligos were transfected using the transmessenger transfection reagent (Qiagen). In preliminary studies, cells were treated with 10-100 μM siRNA according to the manufacturer's instructions in order to determine the optimum concentration to downregulate the target gene. In later studies, cells were transfected with 100 μM oligos for 3 h, after which the cells were grown for a further 48 h in normal medium, then exposed to irradiation (5 Gy). The medium was replaced with fresh medium and the cells were grown for 48 h. The expression of death receptors and apoptosis were measured by ELISA and flowcytometry.

Antitumor Activity of Irradiation and TRAIL. Balb/c *nu/nu* mice (six weeks old) were maintained according to University of Maryland animal care guidelines under pathogen-free conditions and on a normal ad libitum diet. PC-3 (3×10^6 in 100 μl) (25) were implanted subcutaneously into the right flak of nude mice. After about 4 weeks, tumors were of sufficient size to conduct the study. Animals bearing tumors were randomized (seven per group) before treatment. Tumor growth was followed by measurements of tumor diameters with a sliding caliper two times a week. The tumor volume (TV) was calculated according to the formula: $TV = L \times W^2/2$, where L and W are the major and minor dimensions, respectively. After tumor formation (100 mm^3), mice were injected i.p. with vehicle (80% propylene glycol - 20% PBS), exposed to irradiation (5 Gy), TRAIL (15 mg/kg), TRAIL after 24 h of irradiation (5 Gy). In combination treatments, irradiation was given 24 h prior to TRAIL treatment because our goal was to induce death receptors DR4 and/or DR5 by irradiation so that successive treatment with TRAIL would result in enhanced apoptosis of tumor cells. The treatments

were carried out once a week for 3 weeks after tumor formation. Mice survival was monitored daily.

Caspase-3 and Caspase-7 Activity *in Situ*. Tumors were excised from mice, fixed with 10% formalin, embedded in paraffin and sectioned. Slides were washed with PBS-T three times for 5 min each, and blocked with 3% BSA in PBST for 1 h at room temperature. Sectioned were incubated with fluorescent primary antibody in 3% BSA in PBST and propidium iodide (0.5 μg/ml) with gentle shaking overnight at 4^0C. Sections were washed thrice for 10 min each with PBST and once with PBS. Slides were mounted with Gelvatol and visualized under the fluorescence microscope.

TUNEL and H & E Staining. Tumor tissues were excised and fixed with 10% formalin, embedded in paraffin and sectioned. Tissue sections were either stained for H & E (overall morphological observations) or labeled with the TUNEL reaction mixture. In brief, paraffin-embedded tissue slides were dewaxed and rehydrated by heating at 60^0C followed by washing in xylene and rehydration through a graded series of ethanol and double distilled water. Tissue sections were incubated for 20 min at 37^0C with proteinase K working solution (15 μg/ml in 10 mM Tris/HCl, pH 7.4) followed by rinsing twice with PBS. After air-drying the slides, 50 μl TUNEL reaction mixture was added on sample and covered with lid, and slides were incubated for 60 min at 37 ^{0}C in a humidified atmosphere in the dark. Slides were washed thrice with PBS, air-dried, mounted and visualized with a fluorescence microscope.

Immunofluorescence Analysis of Bax. In order to study the translocation of Bax to mitochondria, PC-3 cells were cultured on the coverslips and exposed to irradiation (5 Gy). After 24 h, cells were washed with PBS twice, fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton-X100, and blocked with 5% BSA in PBS for 30 minutes. The cells were incubated with anti-Bax antibody (1:200 dilutions) for 2 h, washed with PBS, and further incubated with secondary antibody conjugated with texas red (1:2000 dilution) for one hr. During secondary antibody incubation, the cells were also incubated with DAPI (0.5 μg/ml) and mitotracker green (50 nM). The cells were then washed and mounted with immuno-mount solution and visualized by fluorescence microscopy (Nikon, Japan).

Assessment of Apoptosis by Annexin V Staining. Cells were resuspended in 100 μl of staining solution (containing annexin V fluorescein and propidium iodide in a HEPES buffer; Annexin-V-FLUOS Staining Kit; Boehringer Mannheim). After incubation at room temperature for 20 min, cells were analyzed by flow cytometry.

Statistical Analyses. The mean and SEM were calculated for each data point. Differences between groups were analyzed by one or two way ANOVA. Differences in the rates of complete tumor inhibitions or survivors were validated by χ^2 test. Survival curves were drawn according to Kaplan-Meier analysis. Significant differences among groups were calculated at $P < 0.05$.

RESULTS

Synergistic effects of irradiation and TRAIL in prostate normal and cancer cells *in vitro*. Since irradiation is commonly used in combination therapy to treat prostate cancer patients, we sought to examine whether irradiation enhances the therapeutic potential of TRAIL in human prostate normal (PrEC) and cancer (PC-3, DU-145, LNCaP) cells. We first determined the interactive effects of irradiation and TRAIL on apoptosis in prostate normal and cancer cells (Table 1). Irradiation induced apoptosis with varying sensitivity in prostate cancer cells, but not in normal PrEC cells at 24 or 48 h of treatment. PC-3 and DU-145 cells were sensitive to TRAIL, whereas LNCaP and PrEC cells were resistant to TRAIL. Concurrent treatments of PC-3, DU-145 and LNCaP cells with irradiation plus TRAIL for 24 or 48 h induced significantly more apoptosis than exposure to single agent alone. Concurrent treatments of normal PrEC cells with irradiation plus TRAIL also slightly induced more apoptosis than exposure to single agent alone.

We and others have reported that chemotherapeutic drugs induced apoptosis partly by up-regulation of death receptors, we therefore sought to examine whether pretreatment of cells with irradiation followed by TRAIL induced more apoptosis than the concurrent treatment with irradiation and TRAIL. The pretreatment of cells with irradiation may increase the apoptosis inducing potential of TRAIL by up-regulating death receptors DR4 and/or DR5 in TRAIL-sensitive cells, and may sensitize TRAIL-resistant LNCaP cells. PC-3, DU-145, LNCaP and PrEC cells were pretreated with

irradiation for 24 h, followed by TRAIL treatment for another 24 h (Table 1). Interestingly, the pretreatment with irradiation followed by TRAIL increased the apoptosis inducing potential of TRAIL in TRAIL responsive PC-3 and DU-145 cells, and sensitized TRAIL-resistant LNCaP cells. This sequential treatment slightly induced more apoptosis in normal PrEC cells compared to concurrent treatment.

In order to understand the mechanism of this synergistic interaction, reverse sequence of exposure was used in which cells were pretreated with TRAIL for 24 h, followed by exposure with irradiation for additional 24 h. Reverse sequence of exposure (TRAIL followed by irradiation) has resulted in significantly less apoptosis than the sequential treatment of cells with irradiation followed by TRAIL. The data on apoptosis were comparable to clonigenic survival assay (data not shown). These studies suggest that sequential treatment with irradiation followed by TRAIL can be used to enhance the apoptosis inducing potential of TRAIL in TRAIL-sensitive cells, and to sensitize TRAIL-resistant cancer cells.

Effects of Irradiation on Caspase-8, DR4 and DR5. Recent studies have shown that TRAIL induces apoptosis by binding to death receptors and activating caspases (17, 26). Since irradiation and TRAIL interact together in inducing apoptosis, we sought to examine whether this interaction is due to up-regulation of death receptors DR4 and DR5, and caspase-8. We therefore examined the effects of irradiation on the regulation of caspase-8, DR5 and DR4 in prostate normal and cancer cells by RNase Protection Assay. Prostate normal (PrEC) and cancer (DU-145, PC-3 and LNCaP) cells were treated with irradiation for various time points (0 to 24 h), and RPAs were performed after isolation of total RNA (Fig. 1A). Irradiation has no effect on the expression of caspase-8, DR4 and DR5 expression in PrEC cells. By comparison, irradiation significantly induced the expression of caspase-8 and DR-5 in DU-145 and LNCaP cells, but slightly in PC-3 cells. There was no significant change in the expression of DR4 due to irradiation in all the cell lines tested.

We next used ELISA to confirm the induction of death receptors by irradiation (Fig. 1B). Prostate normal and cancer cells were exposed to irradiation and TRAIL alone for 24 or 48 h, and the expressions of DR4 and DR5 proteins were measured. Irradiation

had no significant effect on the expression of DR4 protein in PrEC, DU-145, PC-3 and LNCaP cells (Fig. 1B). The expression of DR5 protein was induced by irradiation in all the prostate cancer cell lines, but not in normal PrEC cells (Fig. 1C). By comparison, TRAIL slightly induced DR4 and DR5 expressions in PC-3 and DU-145 cells, but not in PrEC and LNCaP cells. These data suggest that the up-regulation of death receptors by TRAIL may increase the susceptibility of the cells to TRAIL-induced apoptosis.

We next examined whether p53 is required for irradiation-induced (Fig. 2). Recently developed RNA interference (RNAi) technology has successfully been used to inhibit p53 function. In this technique, short interfering (si) double stranded RNAs are introduced to *in vitro* cultured cells resulting in degradation of specific mRNAs and a functional knock-out of a particular gene. Transfection of p53 RNAi has no effect on DR4 protein (Fig. 2A), whereas it significantly inhibited irradiation-induced DR5 expression in LNCaP cells (Fig. 2B). P53 siRNA also inhibited irradiation-induced apoptosis in LNCaP cells (Fig. 2C). These data suggest that p53 is required for irradiation-induced DR5 expression and apoptosis of LNCaP cells.

Irradiation Enhances TRAIL-induced Apoptosis Through Caspase Activation. Activation of caspase-8 is essential for the formation of active TRAIL-DISC and subsequent apoptosis. Caspase-8 can activate downstream caspase-3 directly or through intrinsic pathway involving mitochondria (27, 28). Since irradiation augment TRAIL-induced apoptosis, we sought to examine the mechanism of this interaction by measuring caspase-3 and caspase-8 activation by ELISA in LNCaP and PC-3 cells. Irradiation induced caspase-3 activity, but had no effect on caspase-8 activity in both cells (Fig. 3 A and B). On the other hand, TRAIL induced caspase-3 and caspase-8 activities in PC-3 cells, but not in LNCaP cells. Interestingly, the combination of irradiation followed by TRAIL significantly enhanced more caspase-3 and caspase-8 activities in TRAIL-sensitive PC-3 and TRAIL-resistant LNCaP cells than single agent alone.

To identify whether the augmentation of TRAIL-induced apoptosis by irradiation was mediated through induction of death receptor and subsequent activation of caspase and apoptosis, we measured caspase activity and apoptosis in the presence of caspase inhibitors. LNCaP and PC-3 cells were exposed to irradiation, TRAIL or their

combination in the presence or absence of the caspase-8 inhibitor (z-IETD-fmk) or control peptide (z-FA-fmk) (Fig. 3C). TRAIL induced caspase-8 activity in PC-3 cells, but not in LNCaP cells. Irradiation has no effect on caspase-8 activity in both cell types. The caspase-8 inhibitor (z-IETD-fmk) significantly inhibited caspase-8 activity in PC-3 cells exposed to TRAIL alone or irradiation plus TRAIL. The control peptide had no effect on caspase-8 activity. TRAIL induced apoptosis in PC-3 cells but not in LNCaP cells (Fig. 3D). By comparison, irradiation induced apoptosis in both cell types. Irradiation induced apoptosis was blocked by a pan caspase inhibitor (z-VAD-fmk) but not by the caspase-8 inhibitor (z-IETD-fmk) in LNCaP and PC-3 cells. TRAIL induced apoptosis was completely inhibited by both z-IETD-fmk and z-VAD-fmk in PC-3 cells. However, irradiation plus TRAIL induced apoptosis was completely blocked by z-VAD-fmk, but partially inhibited by z-IETD-fmk in both cell types. These data suggest that caspase activation plays a significant role in the synergistic interaction among irradiation and TRAIL.

We next measured the cleavage of caspase-3 and PARP in DU-145, PC-3 and LNCaP cells by the Western blot analysis (Fig. 4). TRAIL induced caspase-3 and PARP cleavage in TRAIL-sensitive DU-145 and PC-3 cells, but not in TRAIL-resistant LNCaP cells. By comparison, irradiation significantly induced caspase-3 and PARP cleavage in DU-145 and PC-3 cells, but slightly in LNCaP cells. The combination of irradiation and TRAIL also cleaved caspase-3 and PARP in both TRAIL-sensitive (DU-145 and PC-3 cells) and TRAIL-resistant LNCaP cells. These data suggest that irradiation potentiates the cleavage of caspase-3 and PARP in these cells.

Effects of Irradiation and/or TRAIL on the Expression of Bax, Bak and Bcl-2 Proteins. Bcl-2 protects cells from a wide range of cytotoxic insults, including cytokine deprivation, UV- and γ-irradiation, and chemotherapeutic drugs (9, 29-31), whereas Bax and Bak act as pro-apoptotic proteins (19, 20, 32). Since Bax, Bak and Bcl-2 proteins play major roles in apoptosis, we examined whether the effects of TRAIL and/or irradiation were mediated by these proteins (Fig. 5A). Exposure of PC-3 cells with TRAIL or irradiation resulted in induction of pro-apoptotic Bax and Bak, and inhibition of anti-apoptotic Bcl-2 protein. The combination of irradiation and TRAIL has no further

effect on the induction of Bax and Bak. By comparison, the combination of irradiation and TRAIL completely inhibited Bcl-2 expression.

In healthy cells, Bax is predominantly located in the cytoplasm. In cells undergoing apoptosis, Bax translocates to the mitochondria to disrupt mitochondrial functions to induce apoptosis (19, 33). We therefore examined the effects of irradiation on Bax translocation from cytoplasm to mitochondria by immunofluorescence (Fig. 5B). In control cells, there was no translocation of Bax into mitochondria (mitochondria = green color, nucleus = blue color, Bax = red color). By comparison, irradiation induced translocation of Bax from cytoplasm into the mitochondria (translocation of Bax into mitochondria = yellow color). These data suggest that translocation of Bax into mitochondria is one of the mechanisms of enhancing apoptosis inducing potential of TRAIL.

The Role of Dominant Negative FADD in Apoptosis. FADD acts as an adaptor protein in death receptor-induced apoptosis (34, 35); where it links death receptor with caspase-8 or caspase-10 (17, 36). Expression of DED (death effector domain) deletion mutant containing only the C-terminus of FADD (FADD-DN) functions as a dominant negative in death receptor mediated apoptotic signaling because of its association with the activated receptor and its inability to recruit caspase-8 (34). Conversely, overexpression of a full-length FADD is pro-apoptotic, due to the inherent ability of the DED to oligomerize and form a complex that functionally resembles an activated receptor (34, 37). In order to assess the involvement of death receptor pathway on the interactive effects of irradiation and TRAIL on apoptosis, we blocked the death receptor signaling by FADD-DN (Fig. 6). As expected, TRAIL induced apoptosis in PC-3/Neo cells, but not in LNCaP/Neo cells. By comparison, irradiation induced apoptosis in both PC-3 and LNCaP cells. Combined treatment of irradiation with TRAIL enhanced apoptosis in PC-3/Neo cells, and sensitized TRAIL-resistant cells LNCaP/Neo cells. Overexpression of FADD-DN inhibited TRAIL-, and irradiation plus TRAIL-induced apoptosis. Similarly, FADD-DN inhibited the synergistic interaction between irradiation and TRAIL in LNCaP cells. FADD-DN had no effect on irradiation-induced apoptosis in PC-3 and LNCaP cells. These data suggest that signaling pathway activated by irradiation and

TRAIL are distinct, however, irradiation potentiates the effects of TRAIL by enhancing the activity of death receptor pathway.

Antitumor Activity of Irradiation with TRAIL *in vivo*. Since sequential treatment of PC-3 cells with irradiation followed by TRAIL enhanced apoptosis in a synergistic manner *in vitro*, we sought to validate this treatment combination *in vivo*. Irradiation has been shown to induce apoptosis in cancer cells with varying sensitivity (38, 39). According to our *in vitro* data, synergistic effect of TRAIL and irradiation was observed when TRAIL was added 24 h after irradiation (Table 1). After tumor formation, mice were exposed to irradiation, TRAIL or irradiation followed by TRAIL as described in methods and materials. Treatment of mice with irradiation and TRAIL alone inhibited tumor growth and enhanced survival (Fig. 7 A and B). Interestingly, sequential treatment with irradiation followed by TRAIL administration synergistically inhibited tumor growth and enhanced survival of mice. Toxicity was not observed in the liver and brain tissues of mice as measured by H & E staining. These data suggest that the sequential treatment of prostate cancer patients with irradiation followed by TRAIL could be a better treatment option that the single agent alone.

Since the combination of irradiation and TRAIL was effective in reducing tumor growth and enhancing survival of mice, we sought to examine the caspase-3 and caspase-7 activities, Bax expression and apoptosis in tumor tissues derived on day 17 from xenografted nude mice (Fig. 8A). The activation of caspase-3 and caspase-7, and induction of Bax were measured by immunohistochemistry. Apoptosis was examined by TUNEL and H & E staining. Irradiation and TRAIL alone induced caspase-3 and caspase-7 activities, Bax expression and apoptosis (TUNEL and H & E staining) in tumor tissues derived from xenografted mice compared to tissues derived from control mice (Fig. 8 A). Sequential treatment of nude mice with irradiation followed by TRAIL induced significantly higher caspase-3 and caspase-7 activities, Bax induction and apoptosis compared to single treatment alone. Increased caspase-3 and caspase-7 activities, Bax expression and tumor cell apoptosis correlated with reduction in tumor volume as shown above. Furthermore, cell proliferation index in tumor tissues (as measured by Ki-67 staining) was significantly lower in combination treatment than the

single agent alone (data not shown). These data suggest that irradiation and TRAIL induce apoptosis through activation of multiple caspases.

We next examined whether the effects of irradiation on tumor regression was due to induced expression of DR4, DR5, Bax and Bak *in vivo* by the Western blot analysis. Irradiation induced expression of DR5, Bax and Bak, but had no effect on DR4 expression in tumor homogenates taken from tumors at day 17 (Fig. 8B). By comparison, treatment of mice with TRAIL resulted in the induction of DR4, DR5, Bax and Bak in tumor tissues. The combination treatment of mice with irradiation and TRAIL had no further effects on the expression of DR4, DR5, Bax and Bak. Thus, the up-regulation of DR5, Bax and Bak, and subsequent activation of caspase-3 and caspase-7 by irradiation is responsible for enhancing the ability of TRAIL to regress tumor in nude mice.

DISCUSSION

Here, we show that ionizing radiation induces death receptor DR5, and subsequent treatment with TRAIL causes apoptosis in an additive or synergistic manner in TRAIL-sensitive cells, and sensitize TRAIL-resistant prostate cancer cells. Based on molecular analysis of death signaling pathways several new approaches have been developed to increase the efficacy of TRAIL. Although several studies have demonstrated the synergistic interactions of irradiation and TRAIL *in vitro* (40, 41), this is clinically relevant study where we have demonstrated the molecular mechanisms of sequential treatment with irradiation followed by TRAIL on tumor cell apoptosis *in vitro* and *in vivo*. The sequential treatment of mice with irradiation followed by TRAIL resulted in enhanced caspase-3 activity and apoptosis in tumor cells, which is accompanied by a regression of tumor growth and an enhancement of survival of xenografted mice. Induced expression Bax and Bak, and down-regulation of Bcl-2 were also observed and these proteins are likely to have played significant roles in tumor cell apoptosis. Our data show that the synergistic interaction with irradiation and TRAIL will involve multiple proteins such as DR5, caspase-3, caspase-7, caspase-8, Bax, Bak, and FADD.

Sequential treatment of cells with irradiation followed by TRAIL produces more apoptotic response than the concurrent treatment, indicating that the pretreatment with irradiation allow cells to become more sensitive to TRAIL due to up-regulation of DR5, activation of the pro-apoptotic factors Bax and Bak, and down-regulation of Bcl-2. Irradiation also sensitizes TRAIL-resistant LNCaP cells by up-regulation of DR5 and activation of caspase -3, and -8. Similarly, Several studies have shown that pretreatment of cells with chemotherapeutic drugs up-regulate DR4 and/or DR5 expressions, thereby enhancing TRAIL-induced apoptosis of prostate cancer cells *in vitro* (42, 43). Thus, an increase in DR5 levels by irradiation will enhance the sensitivity of cells to TRAIL.

Multiple genes are involved in irradiation-induced apoptosis. Androgen-sensitive (LNCaP) and -insensitive (PC-3 and DU-145) cells can be killed by the combination of irradiation and TRAIL. We have demonstrated that cells harboring both wild type (LNCaP) and mutant p53 (PC-3) (44) can be killed by irradiation and TRAIL, suggesting the involvement of p53-independent pathways in regulation of apoptosis. The up-regulation of DR5 mRNA expression by p53 has been demonstrated (45), and dominant negative version of p53 has been shown to block irradiation-induced DR5 expression (41). In certain cell types, irradiation induces apoptosis through the p53 directed *de novo* synthesis of the death agonist Bax (46, 47), indicates involvement of mitochondrial caspase pathway. p53-independent regulation of DR5 gene has also been demonstrated (45). Thus, the combination of irradiation and TRAIL can be used to target prostate cancer cells harboring wild type and mutant p53. Other possible candidates that may regulate the expression of DR5 gene are NFκB, AP-1 and SP-1 (48, 49). We have recently demonstrated that the RelA/p65 subunit of NFκB acts as a survival factor by inhibiting expression of DR4/DR5 and caspase-8 and up-regulating cIAP1 and cIAP2, whereas c-Rel acts as a proapoptotic factor by enhancing DR4, DR5 and Bcl-Xs, and inhibiting cIAP1, cIAP2 and survivin after TRAIL treatment (50). Thus, the dual function of NFκB, as an inhibitor or activator of apoptosis, may be cell type specific where relative levels of RelA and c-Rel subunits determine the ultimate cell fate.

The observation that irradiation can induce death receptors, and that low doses of irradiation can cooperate synergistically with TRAIL in enhancing apoptosis may have implications for clinical therapy. Radiation induces caspase-8, caspase-3, and caspase-7

activation, leading to proteolytic cleavage of cellular proteins such as poly(ADP-ribose) polymerase (47). In our study, TRAIL-induced apoptosis required caspase-8, whereas it was not essential for irradiation-induced apoptosis. Activation of death receptors by TRAIL also induced cleavage and activation of caspase-8 and -3, similar to our previous findings in breast, prostate and lung cancer (7-9, 17), indicating that irradiation may mediate its apoptotic effects not only through increased levels of the DR5 receptors, but also through caspase cascade by directly engaging mitochondria. The combination of irradiation and TRAIL had no further effects on the expression of death receptors, Bax and Bak, and cleavage of caspase-3 and PARP, yet there was significantly more apoptosis than single agent alone. It raises the possibility that TRAIL will increase the susceptibility of the cells to TRAIL induced apoptosis. Bax and Bak are widely distributed, and function mainly at the mitochondria (19, 20, 51). Inactivation of Bax affected apoptosis only slightly and disruption of Bak had no discernible effect, but inactivation of both genes dramatically impaired apoptosis in many tissues (19, 20, 51, 52). So, the presence of either Bax or Bak seems to be essential for apoptosis in many cell types. Bax is cytosolic monomer in healthy cells, but it changes conformation during apoptosis, integrates into the outer mitochondrial membrane and oligomerizes (53-55). In healthy cells, Bak is an oligomeric integral mitochondrial membrane protein, but it too changes conformation during apoptosis and might form larger aggregates (53, 54, 56). Bax and Bak oligomers are believed to provoke or contribute to the permeabilization of the outer mitochondrial membrane, allowing efflux of apoptogenic proteins (57). Irradiation and TRAIL induced activation of Bax and Bak, suggesting that both agents can amplify apoptotic signals at the level of mitochondria. The simultaneous activation of Bax and Bak may have synergistic effects on the activation caspase-3 and apoptosis.

Bid seems to promote death by activating Bax and Bak, and it might also inactivate pro-survival relatives (58). Bid might act by inducing Bax and Bak to oligomerize and form pores in the mitochondrial membrane, but the oligomers do not contain Bid (56), which seems to form homotrimers in the membrane (59). Bid activation indicates the requirement for caspase amplification in the TRAIL-DR pathway by a mechanism reported to take place through translocation of activated Bid to mitochondria, oligomerization of Bax and Bak, facilitating cytochrome c release and

activation of caspase-9 and then caspase-3 and/or caspase-7 leading to apoptosis (17, 21, 60). Synergistic activation of caspase-3 by irradiation and TRAIL appears to be one of the mechanisms of enhanced apoptosis both *in vitro* and *in vivo*.

Most cytotoxic agents including irradiation are now thought to kill cells predominantly by trigerring their apoptosis program. Overexpression of Bcl-2 renders tumor cells refractory to diverse therapeutic drugs and radiation, *in vivo* as well as *in vitro*, and selection for drug resistance in cancer cells is often accompanied by upregulation of Bcl-2 (61, 62). Downregulation of Bcl-2 can trigger apoptosis in cancer cells, and enhances the therapeutic potential of anticancer drugs and irradiation. Phase III clinical trials with antisense Bcl-2 deoxynucleotides are underway (63). RNAi using small RNA duplexes, delivered as synthetic oligonucleotides or expressed from vectors, seems more promising than antisense oligonucleotides (64).

In conclusions, we have demonstrated that the ionizing radiation enhances the therapeutic potential of TRAIL in TRAIL-sensitive cells, and sensitize TRAIL-resistant cells. The interactions of irradiation and TRAIL involve multiple genes such as DR5, caspase-8, caspase-3, caspase-7, Bax, Bak and Bcl-2. The observed synergy between TRAIL and therapeutic doses of irradiation can form the basis for developing strategies for pharmacological intervention, with potential for clinical application. By understanding the mechanisms exploited by the cancers, new therapeutic targets are being developed. We anticipate that fresh diagnostic measures and additional therapeutic options targeted at the specific defect will soon be added to armamentarium in our efforts to thwart unregulated cancer cells growth. In particular, the sequential treatment of prostate cancer patients with irradiation followed by TRAIL could be a better option than single agent alone.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Effects of irradiation on the expression of caspase-8, DR4, and DR5 in prostate normal and cancer cells. (A), Effects of irradiation on gene expression of caspase-8, DR4 and DR5 as measured by ribonuclease protection assay (RPA). PrEC, DU-145, PC-3 and LNCaP cells were exposed to irradiation (5 Gy) and cells were harvested at 0, and 24 h. Total RNA was used in to measure the expression of caspase-8, DR4, and DR5 by RPA. L32 and GAPDH were shown as house-keeping genes. The mRNA levels were shown as fold change (x) relative to untreated control cells. All the data represent values obtained by normalizing levels of mRNAs of treated cells to those of untreated control cells and the L32 control. (B and C), Effects of irradiation and TRAIL on the expression DR4 and DR5 proteins in prostate normal and cancer cells. Prostate normal (PrEC) and cancer (DU-145, PC-3 and LNCaP) cells were exposed to irradiation (5 Gy) or treated with TRAIL (50 nM), and cells were harvested at the end of 24 or 48 h. Expressions of DR4 and DR5 proteins were measured by ELISA as per manufacturer's instructions (Biosource International). Con = Control, Irra = Irradiation, TR = TRAIL. * = significantly different from control, $P < 0.05$.

Fig. 2. Involvement of p53 in irradiation-induced death receptor induction and apoptosis. Cells were transfected with either control (PGL siRNA) or p53 siRNA plasmid in the presence of lipofectamine. Culture medium was replaced after 24 h of transfection, and cells were exposed to irradiation (5 Gy). The expressions of DR4 and DR5 were measured by ELISA at 24 h (A and B), and apoptosis by annexin-V-FITC staining at 48 h (C). * = significantly different from control, $P < 0.05$.

Fig. 3. Involvement of caspase(s) in irradiation and/or TRAIL-induced apoptosis. (A and B), Effects of irradiation and TRAIL on the activities of caspase-3 (A) and caspase-8 (B) in LNCaP and PC-3 cells. Cells were exposed to irradiation (5 Gy) in the presence or absence of TRAIL (50 nM) for 24 h. At the end of incubation period, cells were harvested to measure the caspase-3 or -8 activities by ELISA. * = significantly different from control, $P < 0.05$. (C), Cells were pretreated with 50 μM z-FA-fmk (control peptide) or z-IETD-fmk (caspase-8 inhibitor) for 2 h followed by exposure with irradiation in the presence or absence of TRAIL (50 nM) for 24 h. Cells were harvested to measure the

caspase-8 activity by ELISA. Data represents mean ± SE. * = significantly different from control, $P < 0.05$. (D), Cells were pretreated with 50 μM z-FA-fmk (control peptide), z-VAD-fmk (pan inhibitor) or z-IETD-fmk (caspase-8 inhibitor) for 2 h followed by exposure with irradiation in the presence or absence of TRAIL (50 nM) for 24 h. Cells were harvested to measure apoptosis by DAPI staining. Data represents mean ± SE. * = significantly different from control, $P < 0.05$.

Fig. 4. Effects of TRAIL and /or irradiation on caspase-3 and PARP cleavage in prostate cancer cells. Prostate cancer DU-145 (A), PC-3 (B), and LNCaP (C) cells were exposed to irradiation (5 Gy) followed by treatment with or without TRAIL (50 nM) for 24 h. Cells were harvested and the cleavage of PARP and caspase-3 was measured by the Western blot analysis. PARP antibody recognizes the whole PARP (uncleaved), whereas caspase-3 antibody recognizes the procaspase-3 and the cleaved fragment. Anti-tubulin antibody was used as a loading control.

Fig. 5. Effects of irradiation and TRAIL on Bcl-2 family member. (A), Effects of TRAIL and /or irradiation on Bax, Bak and Bcl-2 expression. Prostate cancer PC-3 cells were exposed to irradiation (5 Gy) followed by treatment with or without TRAIL (50 nM) for 24 h. Cells were harvested and the expressions of Bax, Bak, and Bcl-2 were measured by the Western blot analysis. Anti-tubulin antibody was used as a loading control. (B), Immunofluorescence analysis of localization of Bax to mitochondria in PC-3 cells exposed to irradiation (5 Gy) for 24 h. Red color indicates Bax, green color indicates mitochondria, blue color indicates nucleus, and yellow color indicates the localization of Bax into the mitochondria (green and red = yellow). Note the presence of yellowish mitochondria in cells exposed to irradiation.

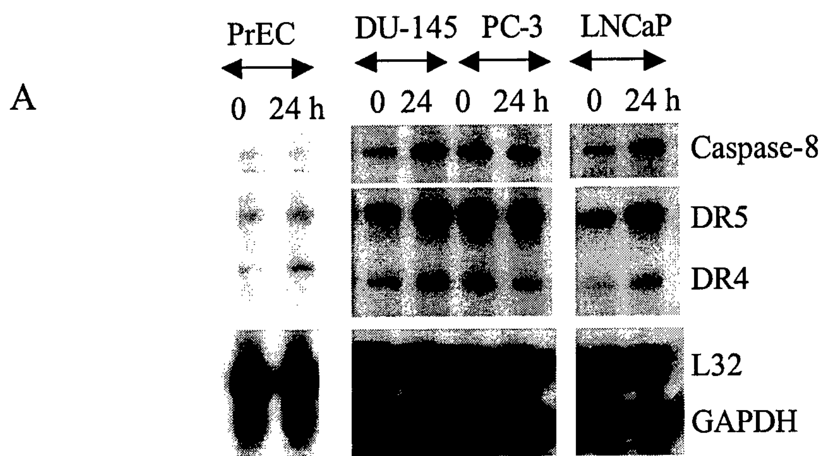
Fig. 6. Involvement of FADD in irradiation and/or TRAIL-induced apoptosis of prostate cancer cells. (A), PC-3 cells were transiently transfected with either control vector (PC-3/Neo) or a vector expression dominant negative FADD (PC-3/DN-FADD). Culture medium was replaced after 24 h of transfection, and cells were exposed to irradiation (5 Gy) followed by treatment with TRAIL (50 nM). After 48 h, cells were harvested to measure apoptosis by DAPI staining. * = significantly different from control, $P < 0.05$. (B), LNCaP cells were transiently transfected with either control vector (LNCaP/Neo) or a vector expression dominant negative FADD (LNCaP/DN-FADD). Culture medium was

replaced after 24 h of transfection, and cells were exposed to irradiation (5 Gy) followed by treatment with TRAIL (50 nM). After 48 h, cells were harvested to measure apoptosis by DAPI staining. * = significantly different from control, $P < 0.05$.

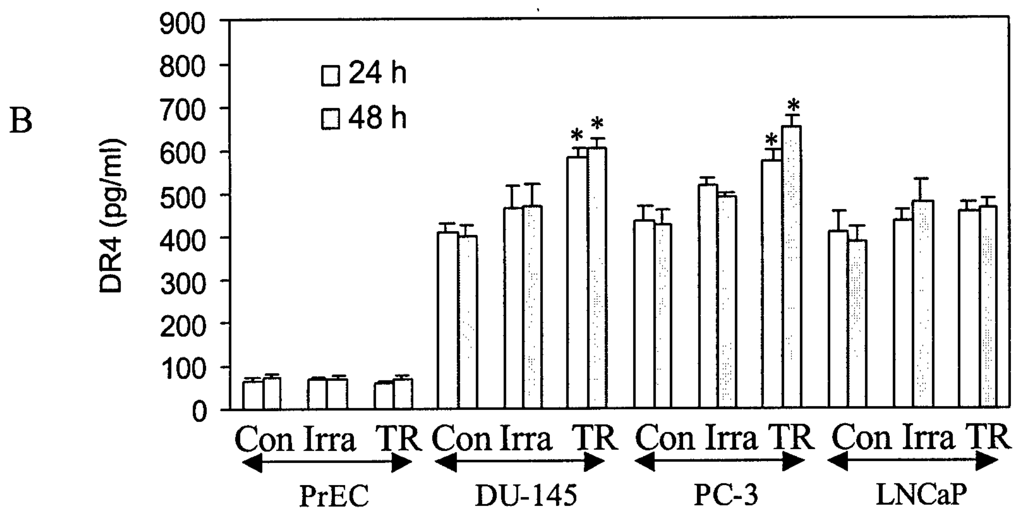
Fig. 7. Effects of pretreatment of mice with irradiation followed by TRAIL on tumor growth and survival. (A), Interactive effects of irradiation with TRAIL on tumor volumes at different times after tumor cell transplantation in mice. Prostate cancer PC-3 (3×10^6) cells were injected into the flank of Balb c nude mice. After tumor formation ($100\ mm^3$), mice were injected i.p. with vehicle, exposed to irradiation (5 Gy), injected with TRAIL, and exposed to irradiation (5 Gy) followed by TRAIL administration (24 h after irradiation). Treatments were given once per week for three weeks. Tumor volume was measured weekly. Data represent mean ± SE. (B), Kaplan-Meier analysis of survival of mice bearing prostate cancer xenografts. Mice were treated with vehicle alone, irradiation alone, TRAIL alone or irradiation followed by TRAIL (24 h after irradiation) as described in A. Survival was monitored daily.

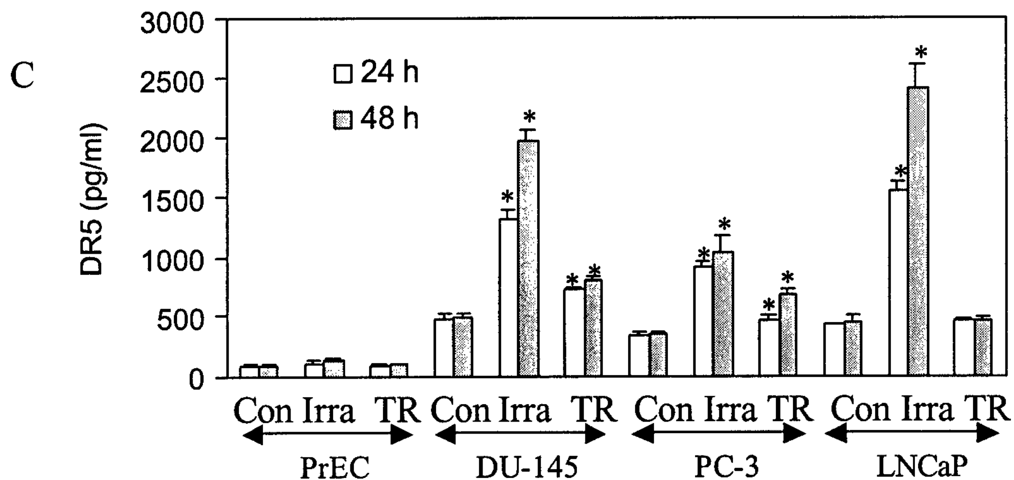
Fig. 8. Effects of irradiation and TRAIL on death-related proteins and apoptosis *in vivo*. Mice were exposed to irradiation alone, TRAIL alone, and irradiation followed by TRAIL administration (24 h after irradiation) as described in Materials and Methods. Mice were sacrificed on day 17, and tumors were extracted, frozen or fixed in buffered formalin. (A), Activation of caspase-3, and caspase-7, induction of Bax and apoptosis in formalin-fixed tumor tissues derived from PC-3 cells xenografted mice. Apoptosis was assessed by TUNEL and H & E staining. (B), Expressions of DR4, DR5, Bax, and Bak in tumor tissues derived from xenografted mice on day 17. Tumor extracts were concentrated, and protein expressions were measured by the Western blot analysis. Tubulin was used as a loading control.

FIG 1



	PrEC 0	PrEC 24 h	DU-145 0	DU-145 24	PC-3 0	PC-3 24 h	LNCaP 0	LNCaP 24 h
Cas-8 (x)	1	1	1	3.1	1	1	1	2.8
DR5 (x)	1	1.2	1	3.5	1	1.5	1	3.2
DR4 (x)	1	1	1	1.3	1	1	1	1.1





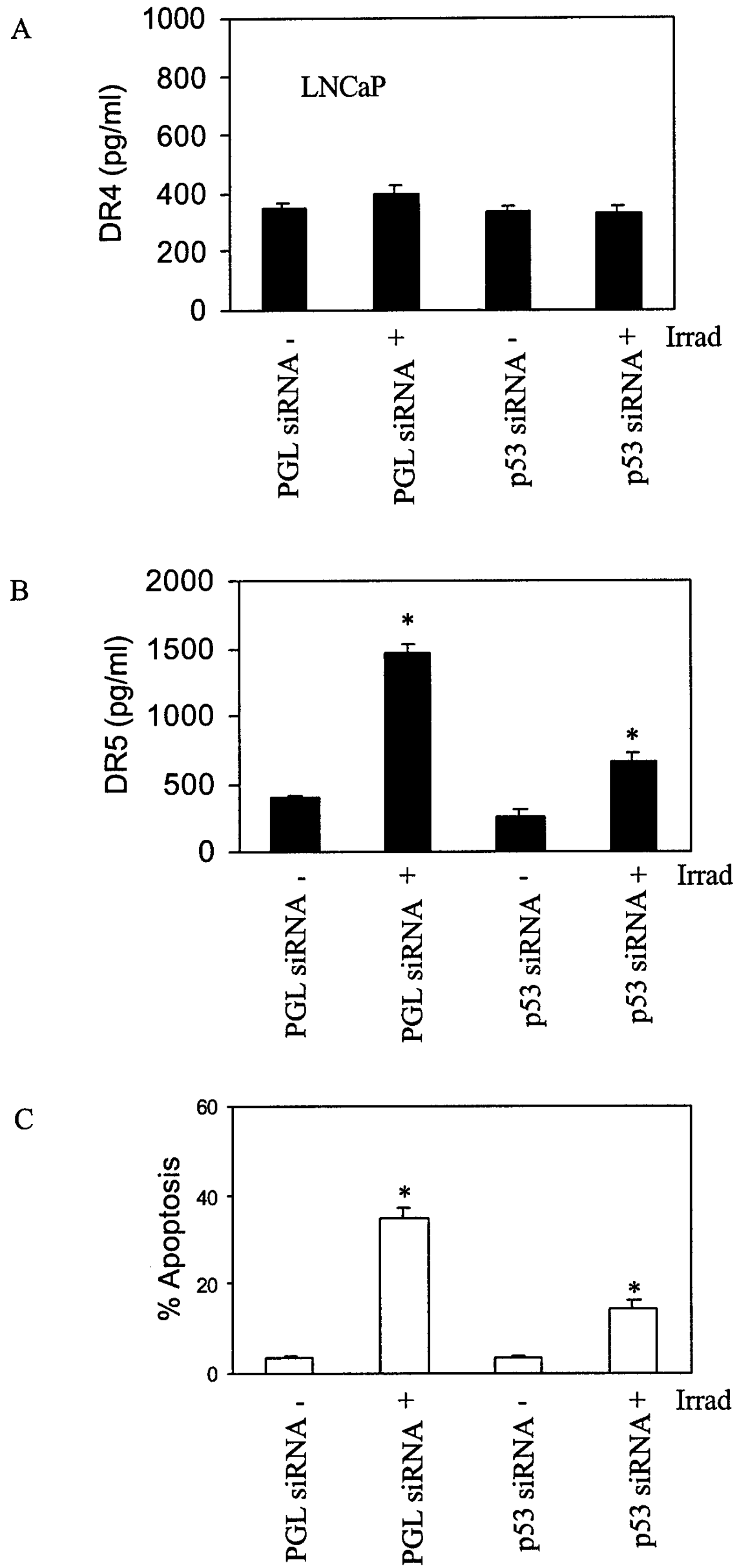


FIG. 2
A
LNCaP
DR4 (pg/ml)
1000
800
600
400
200
0
Irrad
PGL siRNA -
PGL siRNA +
p53 siRNA -
p53 siRNA +
B
DR5 (pg/ml)
2000
1500
1000
500
0
*
*
Irrad
PGL siRNA -
PGL siRNA +
p53 siRNA -
p53 siRNA +
C
% Apoptosis
60
40
20
0
*
*
Irrad
PGL siRNA -
PGL siRNA +
p53 siRNA -
p53 siRNA +

FIG. 3

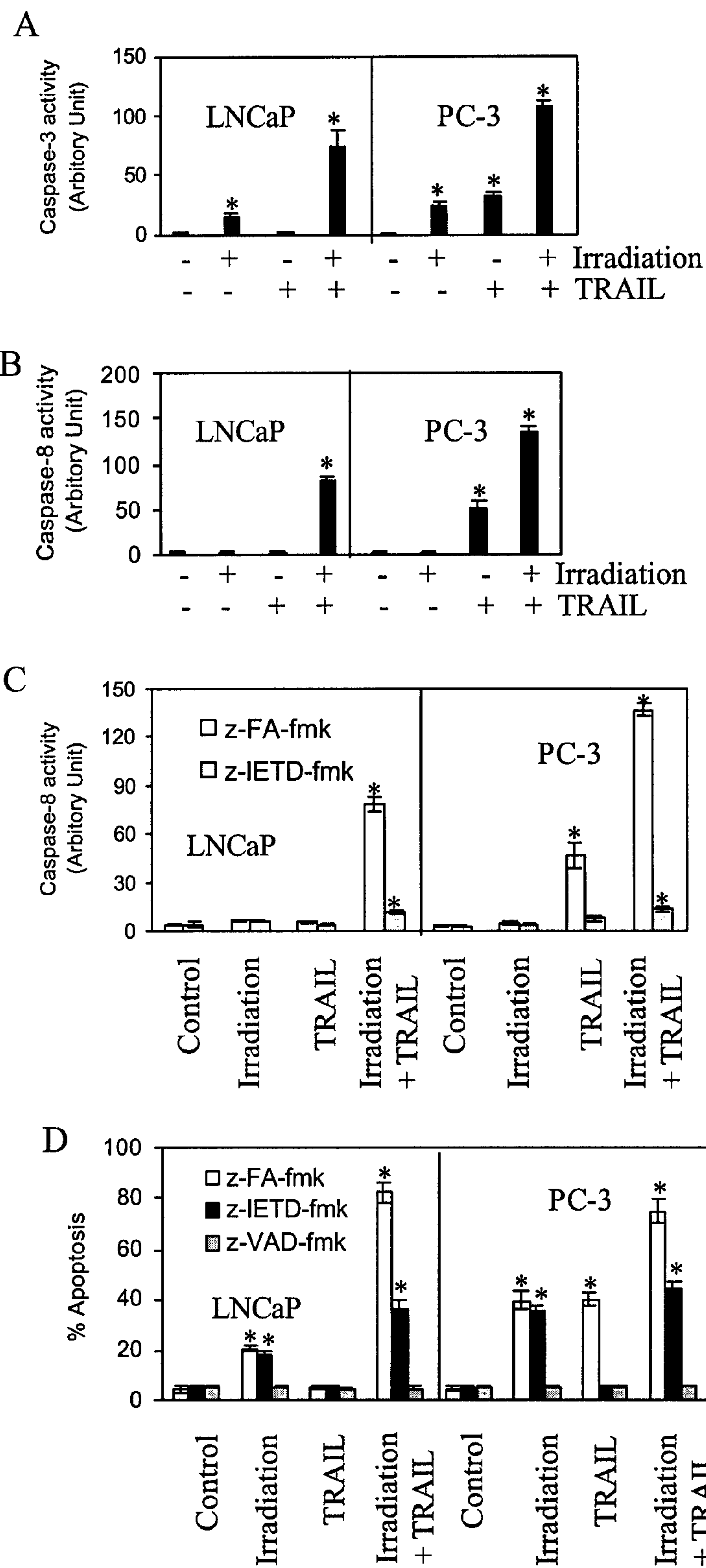


FIG. 4

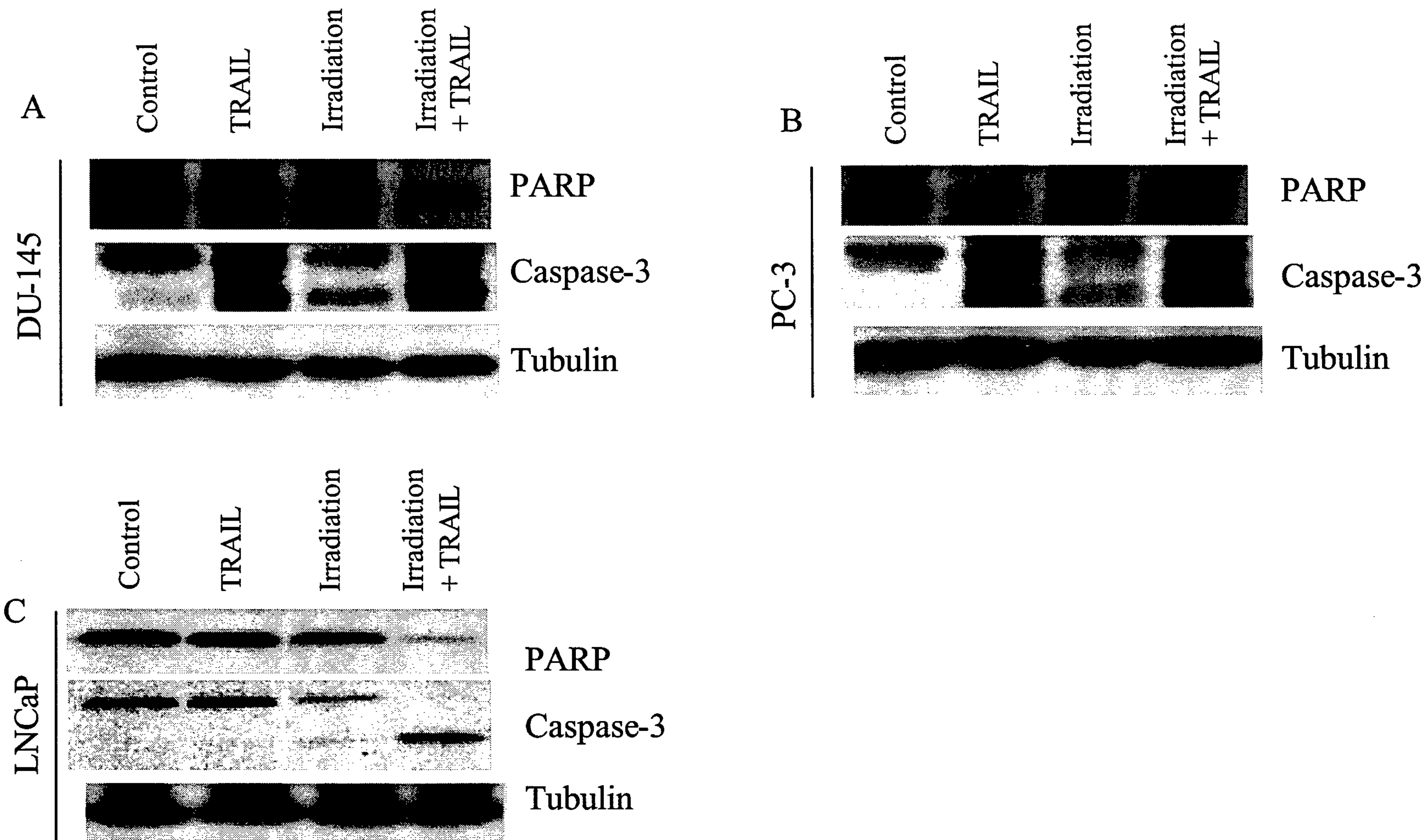
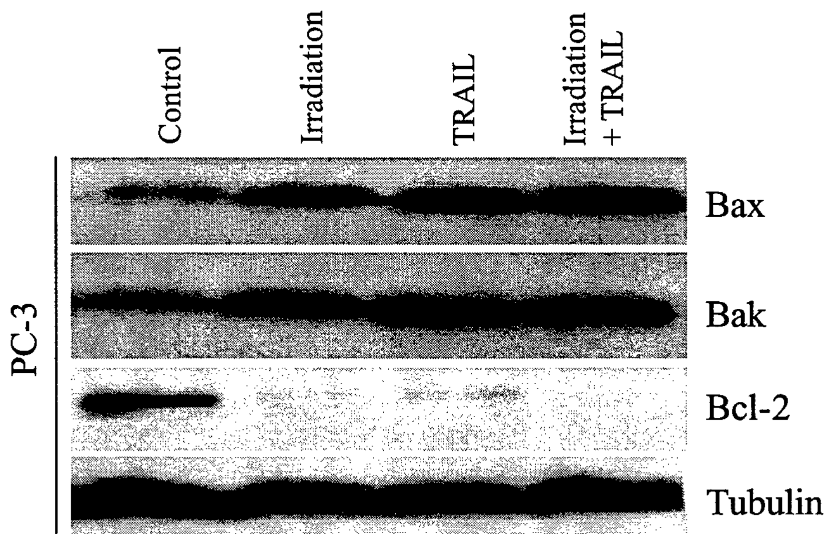


FIG. 5



B

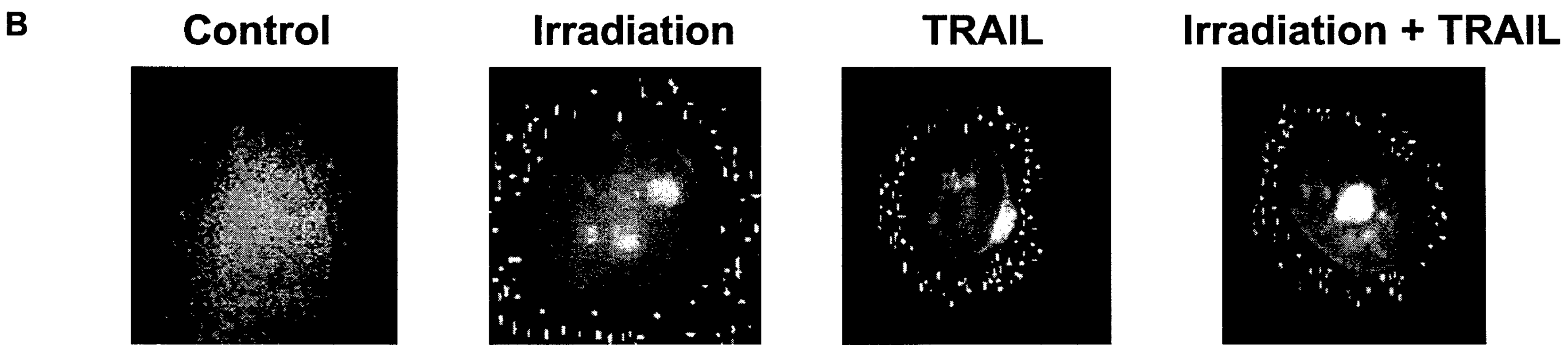
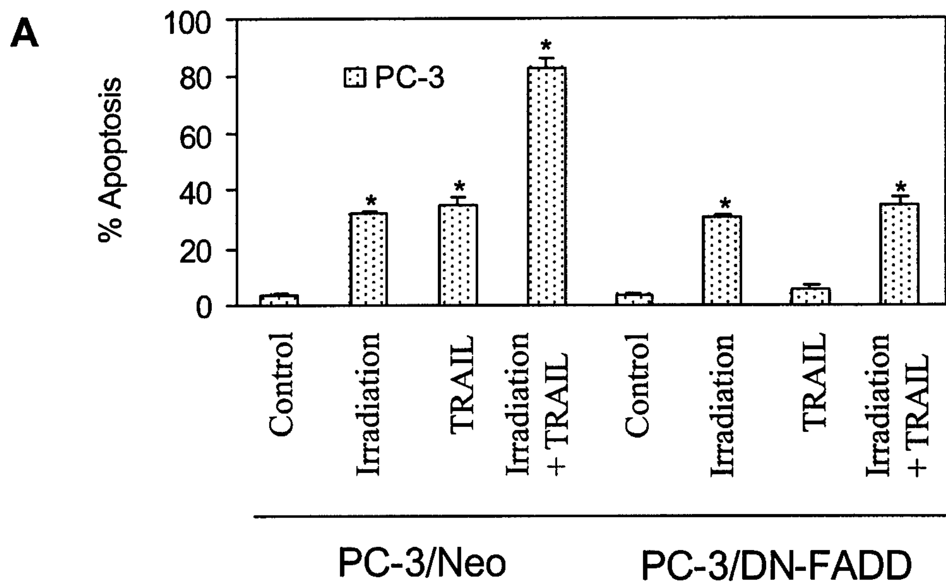


FIG. 6



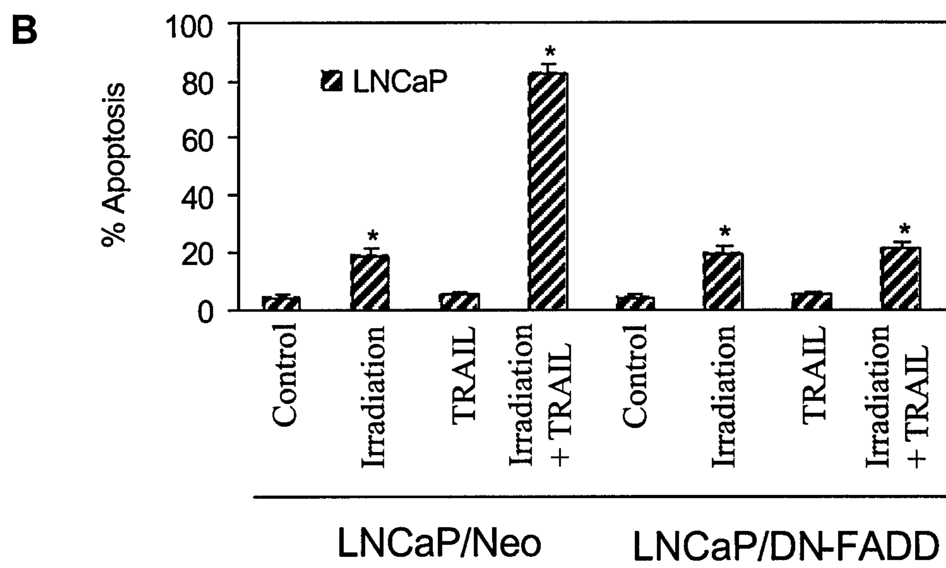


FIG. 7

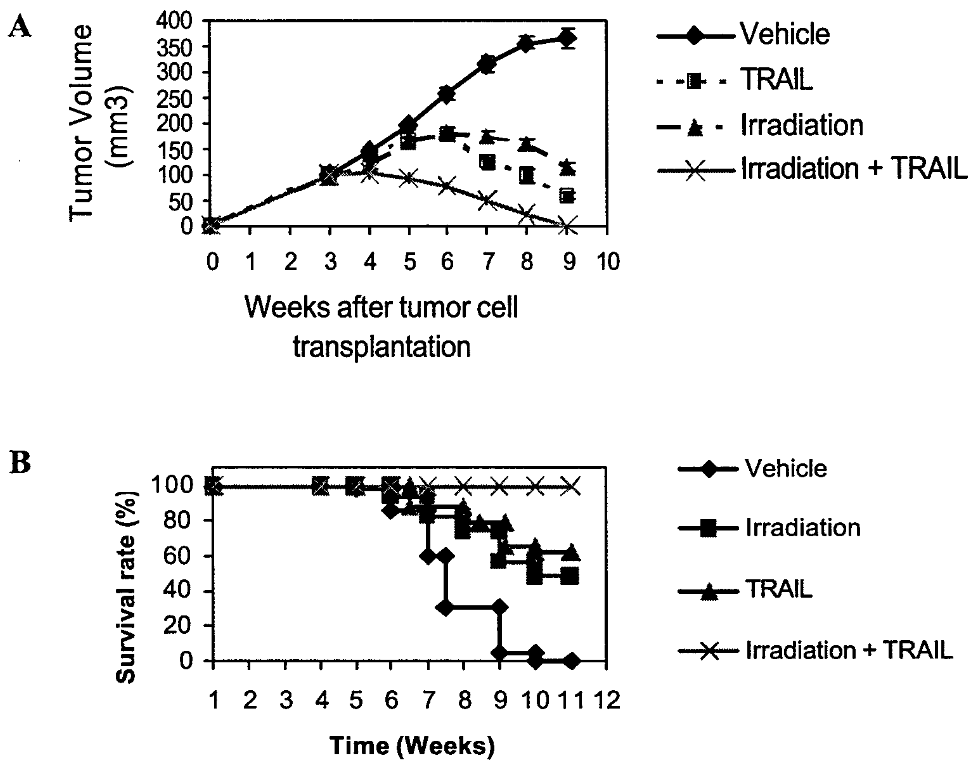
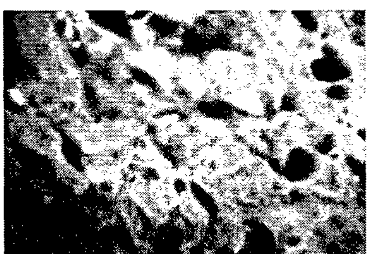
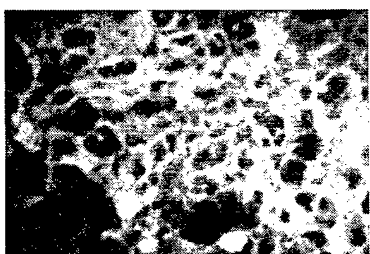
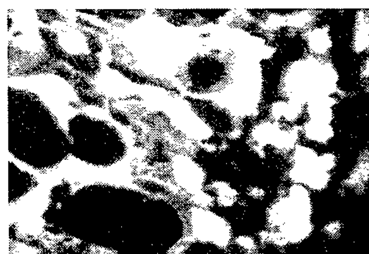
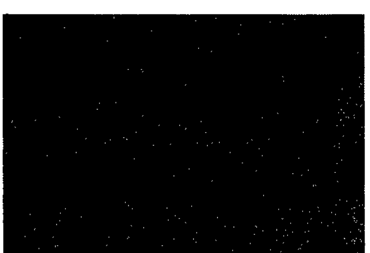
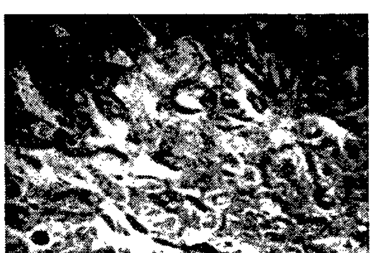
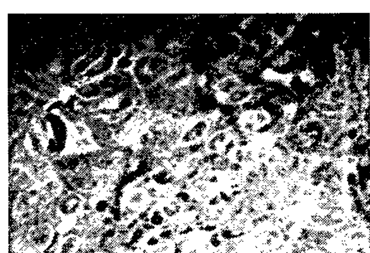
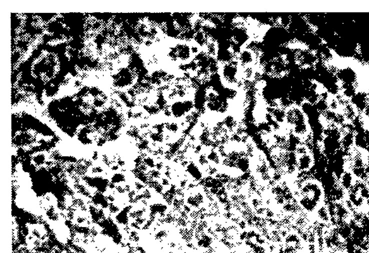
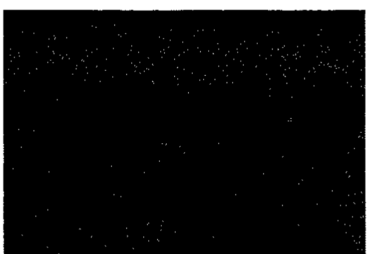
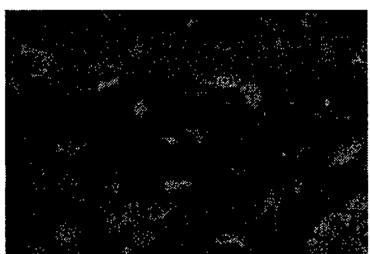


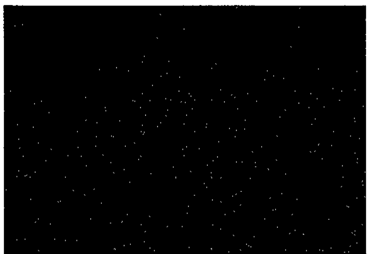
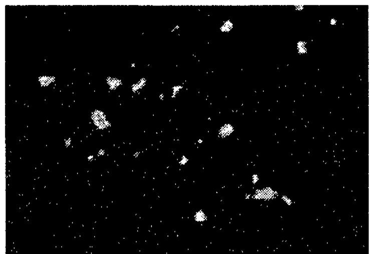
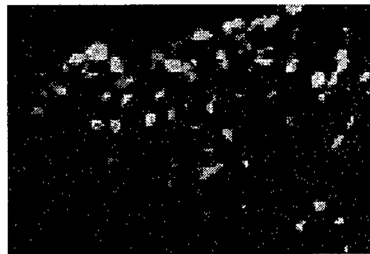
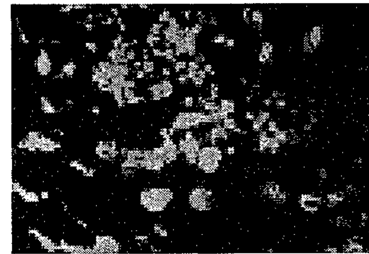
FIG. 8

A

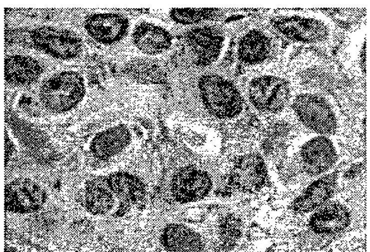
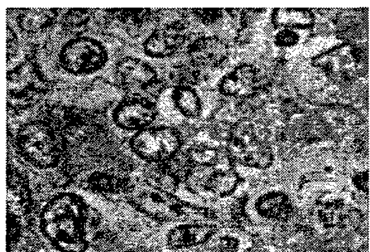
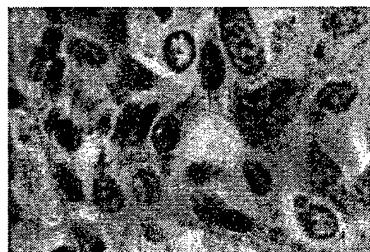
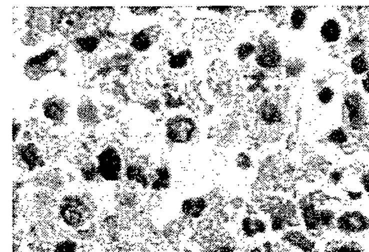





FIG. 8

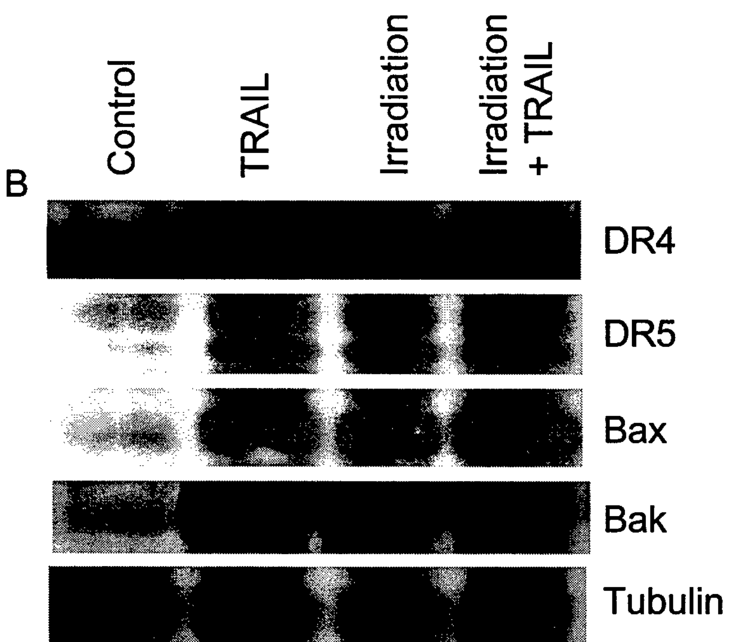


Table 1. Irradiation enhances apoptosis inducing potential of TRAIL, and sensitizes TRAIL resistant cells. Cells were exposed to irradiation (5 Gy) for 24 h, followed by treatment with or without TRAIL (0.1 μM) for another 24 h. Alternatively, cells were treated with TRAIL (0.1 μM) for 24 h, followed by treatment with or without irradiation (5 Gy) for 24 h. At the end of incubation period, apoptosis was measured by annexin V-FITC and PI staining. * = significantly different from control, $P < 0.05$.

	PC-3	DU-145	LNCaP	PrEC
Control	3.2 ± 0.1	3.6 ± 0.2	3.4 ± 0.2	2.9 ± 0.4
Irradiation (5 Gy), 24h	17.4 ± 0.2 *	16.9 ± 1.2 *	4.6 ± 0.5	3.2 ± 0.6
Irradiation, 24h→24h	36.4 ± 1.4 *	24.3 ± 2.1 *	19.3 ± 1.2 *	3.5 ± 0.3
TRAIL (0.1 μM), 24h	24.7 ± 1 *	16 ± 1.2 *	4.6 ± 0.4	3.1 ± 0.3
TRAIL, 24h→24h	38.8 ± 0.5 *	23.7 ± 1.4 *	6.4 ± 0.9	3.5 ± 0.4
Irradiation + TRAIL, 24h (Concurrent treatment)	35.6 ± 2.5 *	31.7 ± 1.6 *	19.6 ± 0.8 *	5.2 ± 0.4
Irradiation + TRAIL, 48h (Concurrent treatment)	50.5 ± 1.2 *	46.6 ± 1.8 *	35.4 ± 1.2 *	6.4 ± 0.4 *
Irradiation, 24h→TRAIL, 24h (Sequential treatment)	92.2 ± 3.2 *	75.6 ± 2.9 *	63.1 ± 2.9 *	9.2 ± 0.4 *
TRAIL, 24h→Irradiation, 24h (Sequential treatment)	51.3 ± 1.4 *	42.8 ± 1 *	33.8 ± 1.7 *	6.2 ± 0.3 *