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14. ABSTRACT Abnormalities in programmed cell death (apoptosis) machinery play a crucial role in initiation, progression and metastasis of prostate cancer. Therefore, molecules that initiate pro-apoptotic pathways are excellent therapeutic agents in prostate cancer. However, some prostate cancer cells develop resistance to pro-apoptotic agents. In this proposal we examined the regulatory mechanisms of c-FLIP(L), which is an important modulator of apoptosis in prostate cancer.					
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Final Report

INTRODUCTION

Abnormalities in apoptotic machinery play a crucial role in initiation, progression and metastasis of prostate cancer. c-FLIP(L), an anti-apoptotic molecule, has been suggested to play a role in developing resistance to pro-apoptotic agents like tumor necrosis factor (TNF) and TNF related apoptosis inducing ligand (TRAIL). In this proposal we have demonstrated that expression of c-FLIP(L) is necessary and sufficient to account for developing resistance to pro-apoptotic agents like TRAIL. Silencing expression of c-FLIP(L) is adequate to overcome other alternative mechanisms of resistance to TRAIL. Therefore, changing the expression of c-FLIP(L) successfully converts the phenotype of resistant to sensitive prostate cancers in response to pro-apoptotic agents. In addition, the expression of c-FLIP(L) is partially regulated at the transcriptional level.

Two pro-agonist antibodies to the TRAIL receptors have been developed by the Human Genome Science, Inc. (Rockville, MD) designated HGS-ETR1 and HGS-ETR2. HGS-ETR1 and HGS-ETR2 target the DR4 and DR5 TRAIL receptors. Both of these newly developed drugs are presently in phase I and phase II clinical trials in other carcinomas. We have obtained approval from Human Genome Science's scientific committee review board to use HGS-ETR1 and HGS-ETR2 in our prostate cancer studies outlined below. Therefore, knowledge gained from this proposal directly translates to identifying prostate cancer patients who may benefit the most from the pro-apoptotic effects of HGS-ETR1 and HGS-ETR2. This proposal is focused on identifying the molecular mechanisms of resistance to the pro-apoptotic effects of HGS-ETR1 and HGS-ETR2.

This is a final revised report for this award. The issues that the reviewer had raised from the previous report have been corrected.

Prior to this report, an interim report was submitted to the Department of Defense, therefore, there may be some similarities in the data that was reported between the two reports.

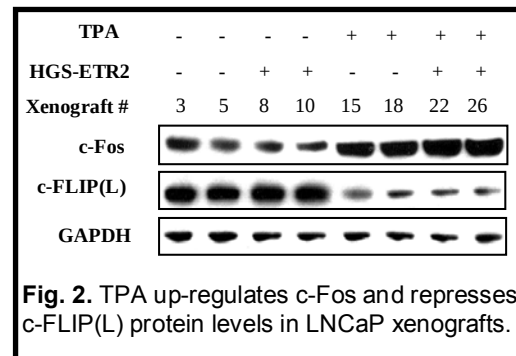
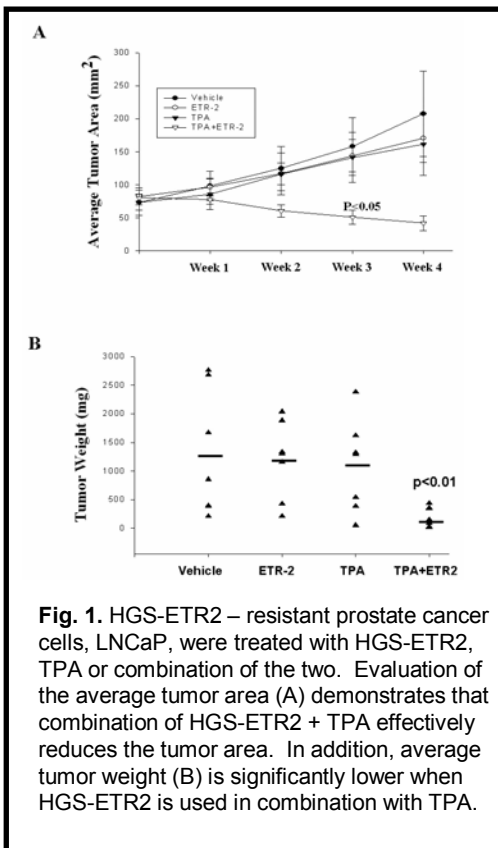
BODY

Specific Aim #1: To examine the efficacy of HGS-ETR1 and HGS-ETR2 in an orthotopic prostate cancer model.

Previously we developed a prostate cancer orthotopic model, and demonstrated efficacy of HGS-ETR2 for TRAIL-sensitive cells (please see last Progress Report). Since our last progress report, we have been correlating our in-vivo findings with in-vitro experiments. We have utilized cell death assays (Annexin V and MTT assays) to demonstrate that PC3 cells are sensitive to HGS-ETR2 therapy. In contrast, PC3 cells are not very sensitive to HGS-ETR1 treatment. Subsequently, we have demonstrated that HGS-ETR2 represses the expression of the anti-apoptotic molecule, c-FLIP(L), a molecular mechanism that is similar to our previous findings using recombinant TRAIL (1).

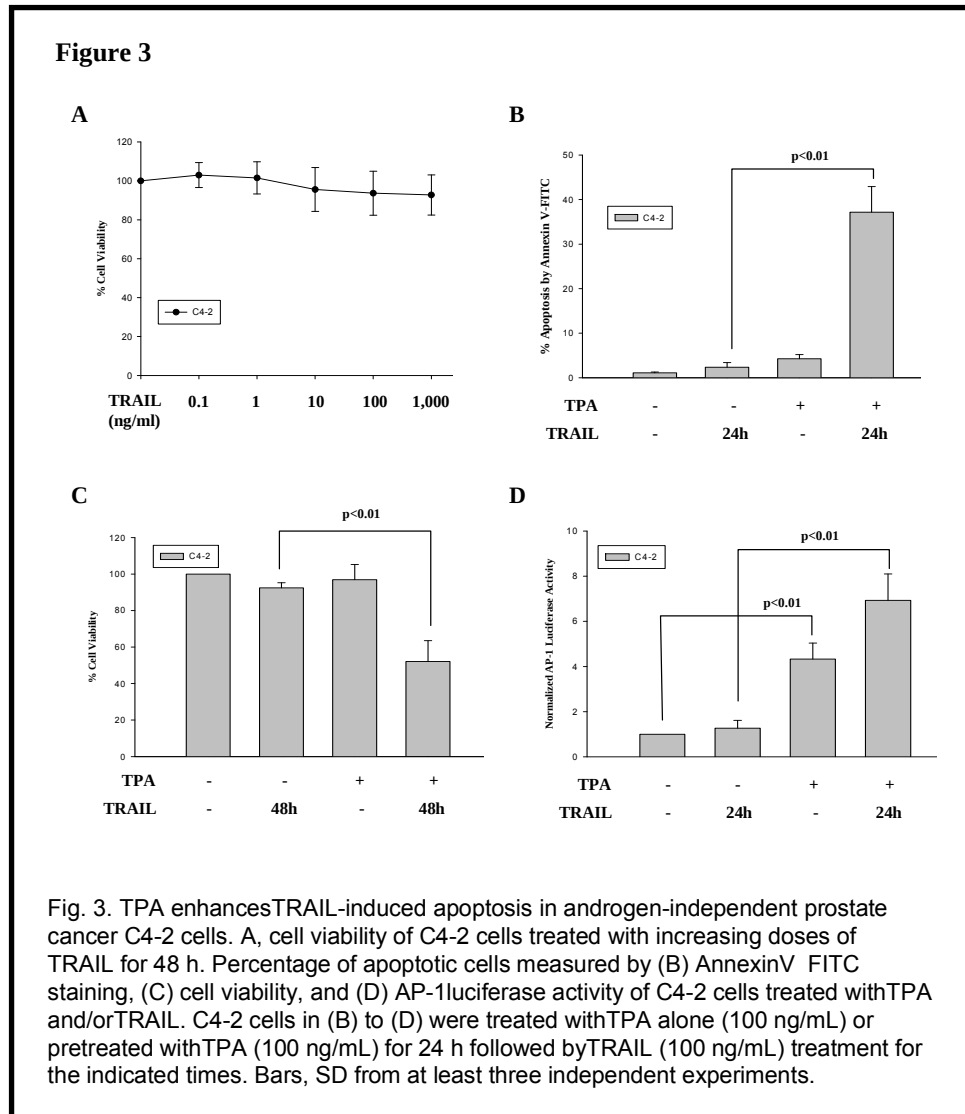
Previously we have shown that c-Fos/AP-1 promotes TRAIL(or HGS-ETR2)-induced apoptosis by repressing the anti-apoptotic molecule c-FLIP(L). We have found that activation of c-Fos is necessary, but insufficient for apoptosis. In this portion of our

studies we investigated whether synthetic induction of c-Fos/AP-1 by low-dose 12-O-Tetradecanoylphorbol-13-acetate (TPA) (2, 3) converts the phenotype of TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype. Recently, we found that HGS-ETR2 when combined with TPA effectively reduces the tumor volume of resistant prostate cancer xenografts. We have examined molecular changes of the xenografts that have been treated with HGS-ETR2 and TPA. We have found that in the presence of TPA, c-Fos is upregulated, while c-FLIP(L) is down-regulated in order to sensitize resistant LNCaP xenografts to the pro-apoptotic effects of HGS-ETR2. Recently, we have demonstrated that c-Fos functions as a pro-apoptotic agent by repressing c-FLIP(L) (4-6). In our in-vivo xenograft studies, we have found that treatment with TPA enhances expression of c-Fos, represses the expression of c-FLIP(L) and sensitizes HGS-ETR2 resistant xenografts (Figs. 1 & 2).



TPA enhances TRAIL-induced apoptosis in androgen-independent prostate cancer cells

Since majority of the morbidity and mortality associated with prostate cancer is secondary to progression from an androgen dependent to an androgen independent state and inefficacy of currently available systemic regimens, we wished to examine whether combination of TPA and TRAIL is effective against TRAIL-resistant androgen independent prostate cancer cells. High dose TPA has been shown to induce apoptosis in the androgen-dependent LNCaP prostate cancer cells, while, TPA is thought to be ineffective in androgen-independent prostate cancer cells (7, 8). Since high dose TPA is associated with tumor progression properties (9), we wished to determine whether low-dose TPA when combined with TRAIL can also sensitize androgen independent

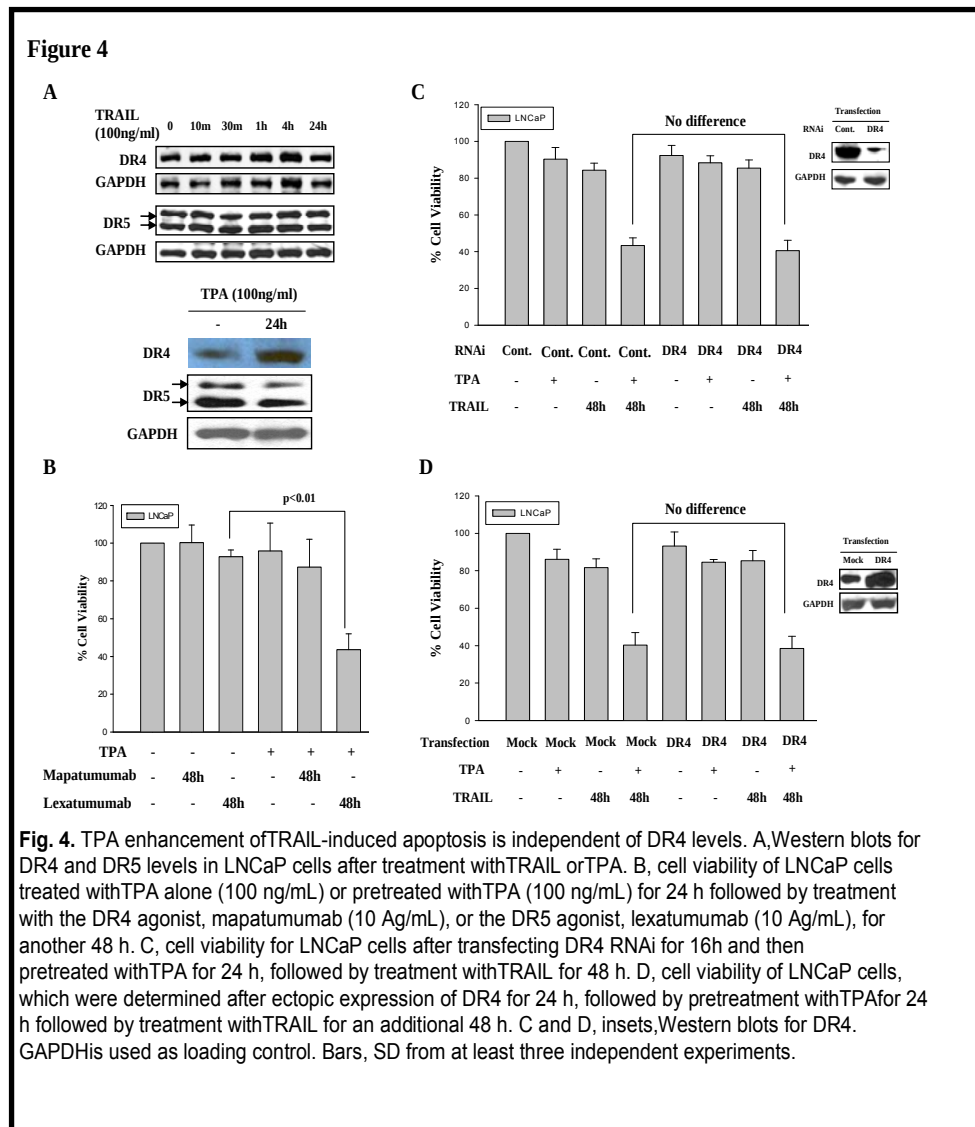


prostate cancer cells that are resistant to TRAIL-induced apoptosis. We found that C4-2 cells, an androgen independent subline of LNCaP cells (10), were resistant to TRAIL-induced apoptosis – a finding similar to the parental LNCaP cells which are androgen dependent (Fig. 3A). Next, we wished to determine whether TPA can enhance the pro-apoptotic properties of TRAIL. We found that combination of TPA with TRAIL converted the phenotype of TRAIL-resistant C4-2 cells to a TRAIL-sensitive phenotype as evidenced by increased apoptotic rate (Fig. 3B) and reduced cell viability (Fig. 3C). In concert with our previous findings in the androgen dependent LNCaP cells, enhancement of TRAIL-induced apoptosis was associated with increased AP-1 activity in the androgen independent C4-2 cells (Fig. 3D). Therefore, low dose TPA is capable of converting the phenotype of TRAIL-resistant prostate cancer cells in both androgen dependent and independent states by increasing AP-1 gene activity.

TPA enhancement of TRAIL-induced apoptosis is independent of DR4 levels

Treatment of LNCaP cells with TRAIL does not alter the protein levels of the DR4 or DR5 TRAIL receptors (Fig. 4A, upper panel). Some investigators have suggested that

TPA enhances the expression of TRAIL receptor DR4 via an AP-1 dependent mechanism (11). However, it is unclear whether increased levels of DR4 are associated with enhancement of TRAIL-induced apoptosis. We, similar to others (11), found that TPA increased TRAIL receptor DR4 levels, but not DR5 levels (Fig. 4A, lower panel). Next we wished to determine whether DR4 and/or DR5 play a functional role in TPA enhanced TRAIL-induced apoptosis. Since recombinant TRAIL activates both DR4 (TRAIL-R1) and DR5 (TRAIL-R2) by promoting trimerization of these cell surface receptors, we utilized fully human monoclonal agonist antibodies specifically targeted against TRAIL-R1 (Mapatumumab) and TRAIL-R2 (Lexatumumab) (12, 13). We found that Mapatumumab alone or in combination with TPA did not enhance TRAIL-induced cell death in LNCaP cells (Fig. 4B). In contrast, Lexatumumab, when combined with TPA promoted cell death and reduced cell viability in the TRAIL-resistant prostate cancer cells (Fig. 4B), which is similar to the results using soluble TRAIL combined with TPA and compatible with our xenograft in-vivo studies (Fig. 1). Further, we examined whether inhibition of DR4 by RNAi (Fig. 4C – inset) or ectopic expression of DR4 (Fig. 4D - inset) would alter sensitivity to TRAIL-induced apoptosis. We found that neither inhibition of DR4 (Fig. 4C), nor increased DR4 levels (Fig. 4D), were associated with TRAIL-induced or TPA-enhanced TRAIL-induced cell death (Fig. 4C and 4D). Conversely, overexpression of DR5 alone sensitized LNCaP cells to TRAIL-induced apoptosis, which was even further enhanced when TRAIL was combined with TPA (data not shown). Therefore, activation of apoptosis through TRAIL receptor 2 (DR5) by Lexatumumab or TRAIL in combination with TPA treatment can promote cell death in TRAIL-resistant LNCaP cells. In contrast, TRAIL receptor 1 (DR4) level is not associated with TPA-enhanced TRAIL-induced apoptosis. Our findings suggest that DR5 mediated pathways are more critical to TPA-enhanced TRAIL-induced apoptosis than the DR4 mediated pathways.



Specific Aim #2: To determine and examine the gene expression profile differences between TRAIL-sensitive (PC3) and TRAIL-resistant (PC3-TR) prostate cancer cells after treatment with HGS-ETR1 or HGS-ETR2. We evaluated the differential gene expression of TRAIL-sensitive and TRAIL-resistant prostate cancer cells using a gene-chip micro-array profile (see appendix for complete manuscript) (1, 14). We have shown that c-Fos is a key regulator of TRAIL-induced (or HGS-ETR2) induced apoptosis. We evaluated the regulatory mechanisms that involve c-Fos in the pro-apoptotic pathway. We have demonstrated for the first time that c-Fos, in addition to its known oncogenic actions, can also function as a pro-apoptotic regulator (6) (see attached manuscript).

We have demonstrated that nuclear c-Fos primes cancer cells to undergo apoptosis, and its expression is necessary but insufficient for TRAIL-induced apoptosis. Spatial and temporal expression of c-Fos is critical for cancer cells to undergo apoptosis after treatment with TRAIL/Apo-2L. We have demonstrated that c-Fos functions as a pro-apoptotic molecule represses the anti-apoptotic gene, c-FLIP(L), by direct binding to c-FLIP(L)'s promoter region (6) (see attached manuscript for details). Future goals of this

proposal are to identify the exact molecular pathways that prime prostate cancer cells to be sensitive to pro-apoptotic agents.

Specific Aim #3: To determine the expression of DR4 and DR5 TRAIL receptors in early and advanced prostate cancer. We have obtained IRB approval to use our prostate tissue cancer bank for assessment of expression of DR4 and DR5 in early and advanced prostate cancer. We evaluated 30 prostate cancer specimens that were paraffin embedded specimens, demonstrated variable intensity of DR4 and DR5 cellular membrane expression without any specific correlation to tumor stage or tumor grade. In a recent report on stage III colon cancer, investigators have found that expression level of DR4 and not DR5 correlates with disease free survival (15). Unfortunately, we have only seen variable expression levels of DR4 and DR5 in human prostate cancer samples and we have not observed any direct correlation between expression of these death receptors and grade or stage of prostate cancer samples.

KEY RESEARCH ACCOMPLISHMENTS

1. In our work we demonstrated that HGS-ETR2 is a better inducer of apoptosis for prostate cancer cells than HGS-ETR1. These findings are based on in-vitro and orthotopic in-vivo experiments that we showed in detail in the following manuscript: Zhang et al, Clinical Cancer Res, 2007;13(23):7181-90. These findings pertain to Specific Aim #1 of the project.
2. In regards to Specific Aim #2, we evaluate the differential gene expression in the prostate cancer cells that were treated with the HGS-related compound, TRAIL. We identified differential expression of c-Fos in the sensitive cells and not the resistant cells. Results from these findings were published in detail in three manuscripts: 1. Zhang et al, Cancer Res 2004;64(19):7086-91; 2. Zhang et al, Cancer Res 2007;67(19):9425-34; 3. Zhang et al, Methods in Enzymology, 2008;446:333-49.
3. In regards to specific aim #3, we have not found any correlation between the degree of DR4 & DR5 expression and tumor stage/grade in over 30 prostate cancer samples that we have evaluated.

OTHER ACCOMPLISHMENTS RELATED TO THIS PROJECT

4. Demonstrated that TPA enhances HGS-ETR2 resistant prostate cancer xenografts by up-regulating c-Fos and repressing c-FLIP(L).
5. Demonstrated that TRAIL receptor DR5 is more functionally important for inducing apoptosis than TRAIL receptor DR4 for TPA enhanced apoptosis.
6. Demonstrated that c-Fos, a well known proto-oncogene, has pro-apoptotic functions. We have shown that localization of c-Fos to the nucleus "primes" prostate cancer cells to undergo apoptosis in response to TRAIL.
7. Demonstrated that c-Fos "primes" prostate cancer cells by repressing the anti-apoptotic molecule c-FLIP(L).

REPORTABLE OUTCOMES

With support we received from DoD, we have published eight manuscripts related to apoptotic pathways and mechanisms of TRAIL-resistance with the current support. The current support has led to obtaining funding from New York Academy of Sciences-Edwin Beer Grant for continuation of the current project.

Below, please find the list of publications that have been generated from CDMRP's support of this project. Although the first listed reference was published one month prior to initiation of funding of this grant, the work had been initiated well before submission of the grant application prior to formal initiation of funding from CDMRP. The CDMRP reviewer raises this issue as a potential problem/issue with the previous final report. However, since the work on this project is a continuum, I feel that the first manuscript listed below is an important contribution to this work.

1. Zhang X, Jin TG, Yang H, Dewolf WC, Khosravi-Far R, Olumi AF. Persistent c-FLIP(L) Expression Is Necessary and Sufficient to Maintain Resistance to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Mediated Apoptosis in Prostate Cancer. *Cancer Res* 2004;64(19):7086-91.
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CONCLUSIONS

Apoptotic pathways are altered in initiation and progression of most cancers, including prostate cancer, therefore, targeting apoptotic pathways for treatment of advanced prostate cancer is a rational approach. TRAIL-agonist compounds, like HGS-ETR2, which are effective against cancer cells but spare normal cells, are ideal agents to fight cancer, because they have minimal associated cytotoxicity. Currently, HGS-ETR2 is in clinical trials for treatment of various malignancies. Therefore, it is important to differentiate between patients who harbor tumors that are sensitive as opposed to those with resistant tumors to pro-apoptotic agents like HGS-ETR2. In our progress report we have shown that TPA sensitizes resistant prostate cancer cells and xenografts to HGS-ETR2 induced apoptosis.

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c-Fos as a Proapoptotic Agent in TRAIL-Induced Apoptosis in Prostate Cancer Cells

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo-2L promotes apoptosis in cancer cells while sparing normal cells. Although many cancers are sensitive to TRAIL-induced apoptosis, some evade the proapoptotic effects of TRAIL. Therefore, differentiating molecular mechanisms that distinguish between TRAIL-sensitive and TRAIL-resistant tumors are essential for effective cancer therapies. Here, we show that c-Fos functions as a proapoptotic agent by repressing the antiapoptotic molecule c-FLIP(L). c-Fos binds the c-FLIP(L) promoter, represses its transcriptional activity, and reduces c-FLIP(L) mRNA and protein levels. Therefore, c-Fos is a key regulator of c-FLIP(L), and activation of c-Fos determines whether a cancer cell will undergo cell death after TRAIL treatment. Strategies to activate c-Fos or inhibit c-FLIP(L) may potentiate TRAIL-based proapoptotic therapies. [Cancer Res 2007;67(19):9425–34]

Introduction

Aberrant apoptotic pathways contribute to initiation and progression of neoplasia; therefore, proapoptotic agents can be used for treatment of various malignancies (1). Although many cancers are sensitive to proapoptotic agents like tumor necrosis factor (TNF), FasL, and TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L (2), some develop resistance and apoptotic stimuli become ineffective (3). Whereas many apoptotic stimuli are associated with severe systemic cytotoxicity, limiting their clinical utility, TRAIL/Apo-2L has the unique feature of inducing apoptosis in cancer cells, with minimal cytotoxicity. Differentiating between cancers that are sensitive to TRAIL-induced apoptosis and cancers that are resistant to TRAIL-induced apoptosis can improve the efficacy of TRAIL-related compounds that are currently in clinical trials (4).

TRAIL-induced apoptosis may involve both extrinsic and intrinsic pathways and can be regulated by many important factors such as nuclear factor- κ B (NF- κ B), Akt, Bcl-2, Bax, XIAP, IAPs, Smac/DIABLO, and c-FLIP (FLICE-like inhibitory protein; ref. 5). We previously showed that expression of the antiapoptotic molecule c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (4). Although expression of c-FLIP(L) can be regulated at the translational and posttranslational levels, we have found that expression of c-FLIP(L) can also

be partially regulated at the transcriptional level. In this report, we show that transcription of *c-FLIP(L)* is repressed by the c-Fos oncoprotein.

Fos proteins are basic region-leucine zipper (bZIP) transcription factors that bind to Jun or other bZIP proteins and create the activator protein 1 (AP-1) dimer complex, which regulates gene expression (6). *c-Fos*, a well-established oncogene, is considered to play a critical role in tumorigenesis, proliferation and transformation, angiogenesis, tumor invasion, and metastasis, and its expression is associated with poor clinical outcomes (6). Therefore, c-Fos has preferentially been considered an antiapoptotic molecule. However, recent evidence suggests that c-Fos may also have a proapoptotic role. The first indication of such proapoptotic function of c-Fos comes from the observation that c-Fos expression preceded apoptosis (7), and is also observed during mammary gland involution (8) and in other systems (9). However, proapoptotic downstream molecular targets of c-Fos are poorly understood.

In this report, we show that c-Fos has a novel proapoptotic function in TRAIL-induced apoptosis. We show that nuclear c-Fos primes cancer cells to undergo apoptosis, and its expression is necessary but insufficient for TRAIL-induced apoptosis. Activation of c-Fos/AP-1 is critical for cancer cells to undergo apoptosis after treatment with TRAIL/Apo-2L. We show that c-Fos, as a proapoptotic molecule, represses the antiapoptotic gene, *c-FLIP(L)*, by direct binding to the promoter region of *c-FLIP(L)*.

Materials and Methods

Materials. Horseradish peroxidase-conjugated secondary antibody (goat-anti-mouse, goat-anti-rabbit, goat-anti-rat antibodies), biotinylated goat-anti-rabbit secondary antibodies, Oct-1 antibody, c-Fos antibody (D1), c-FLIP antibody (G11), c-Jun antibody, Fos B antibody, Fra 1 antibody, Fra 2 antibody, Jun B antibody, Jun D antibody, and c-Fos small interfering RNA (siRNA) were obtained from Santa Cruz Biotechnology, Inc. c-Jun and c-Fos were obtained from Cell Signaling Technology, Inc. Recombinant human TRAIL/TNFSF10 was obtained from R&D Systems, Inc. Monoclonal anti-FLIP antibody (Dava II) was obtained from Apotech Corp. α -Tubulin antibody was purchased from Sigma. Glyceraldehyde-3-phosphate dehydrogenase antibody was from Abcam, Inc. γ -³²P-labeled ATP was purchased from Perkin-Elmer.

Cell culture. PC3, LNCaP, A-498, 786-O, 769-P, MDA-MB231, and MDA-MB453 were obtained from the American Type Culture Collection. PC3-TR is a TRAIL-resistant subline of PC3 cells (10). SN12-PM6 was supplied by Dr. I.J. Fidler, M.D. Anderson Cancer Center (Houston, TX). Cells were cultured as previously described (10).

Cell viability. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Roche Diagnostics) as previously described (10). Cells were then treated with various concentrations of TRAIL.

Apoptosis assay. Apoptosis was detected by using fluorescein-conjugated Annexin V (Annexin V-FITC) kit according to manufacturer's protocol (BD Biosciences).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Aria F. Olumi, Massachusetts General Hospital, 55 Fruit Street, Yawkey Building, Suite 7E, Boston, MA 02114. Phone: 617-643-0237; Fax: 617-643-4019; E-mail: aolumi@partners.org.

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Western blot analyses and immunofluorescence. Western blot and immunofluorescence experiments were carried out as previously described (10, 11). Nuclear extraction was prepared according to the kit from Pierce Biotechnology, Inc. Oct-1 is used as loading control of nuclear extraction. If the Oct-1 amount of whole-cell lysate is at 20% or less than that of the nuclear extraction, nuclear extraction is considered as qualified extraction. Band density was analyzed by GelDoc (Bio-Rad Laboratories).

Luciferase assay. c-FLIP(L) promoter (−1,179 to +281) luciferase structure was provided by Dr. W.S. El-Deiry (University of Pennsylvania, Philadelphia, PA; ref. 12). c-FLIP(L) promoter with the deletion of AP-1(f) site was prepared by our laboratory. Briefly, c-FLIP(L) promoter (−1,700, +300) was cloned from Bac-IP11-536118 (Children's Hospital, Oakland Research Institute, Oakland, CA) with appropriate primers for PCR amplification. The primers used were sense, 5'-CTCGAGTGAACCTGG-GAGGTTAAGGC-3'; antisense, 5'-AGATCTGAGGCAAAGAACCGAAAGC-3', which contained an *Xho*I site and *Bgl*II site, respectively. The PCR products were inserted into pGEMT-Easy vector (Promega Co.). Once the sequence of the construct had been verified, it was subcloned into the PGL3-enhancer vector (Promega) at *Xho*I and *Bgl*II sites. AP-1(f) site binding site was deleted from the above c-FLIP(L)/PGL3-enhancer construct by QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The primers used were as follows: sense, 5'-GAGGCCGAGGCGGGCAAGGACCAG-CAGTTGGAGACCAGCC-3'; antisense, 5'-GGCTGGTCTCCAAGTCTGGT-CCTTGCCCGCCTCGGCCTC-3'. The sequence of "TCACTTGAGG" was deleted and verified by DNA sequencing. Cells were seeded into 24-well plates. When cells reached 80% confluence, both AP-1 luciferase reporter (25 ng/well) and *Renilla* reporter (5 ng/well) from Stratagene or c-FLIP(L) reporter and *Renilla* reporter were cotransfected into cells. In other experiments, when cell reached 70% confluence, c-Fos siRNA, c-Fos, or A-Fos were transfected into cells for 24 h before transfection of luciferase and *Renilla*. Here, *Renilla* served as an internal control for transfection efficiency. After 24 h of transfection, cells were treated with TRAIL (100 ng/mL), and then both attached and floating cells were collected, prepared, and further detected by using Dual-Luciferase Reporter Assay System (Promega). Samples were stored at −20°C until detection. All results represent average of at least three independent experiments ± SD.

Cell extracts and electrophoretic mobility shift assay. Frozen cell pellets were resuspended as described (13). The reactions were made using 10 µg of whole-cell extract and 0.1 to 0.5 ng of ³²P-labeled double-stranded specific oligonucleotides (5,000–25,000 cpm) in the presence of 2 µg of poly(deoxyinosinic-deoxycytidylic acid) (Sigma) in binding buffer [25 mmol/L Tris (pH 8.0); 50 mmol/L KCl; 6 mmol/L MgCl₂; 10% v/v glycerol]. The reaction was incubated at room temperature for 20 min and run on 5% to 7% polyacrylamide 0.5× Tris glycine EDTA. Gels were dried with Bio-Rad gel dryer and imaged using Kodak BioMax MR Film (Fisher Scientific). General AP-1 gel shift oligonucleotide was obtained from Santa Cruz Biotechnology. Wild-type and mutant oligonucleotides with four-tandem repeats of the c-FLIP(L) AP-1(f) site were designed as 5'-ATCACTTGAGGATCACTTGAGGATCACTTGAGGATCACTTGAGG-3' (wild-type) and 5'-ATTGCTTGAGGATTGCTTGAGGATTGCTTGAG-AGGATTGCTTGAGG-3' (mutant). "Co" stands for competing control, using 90% cold prober plus 10% hot prober.

Transfection of c-Fos, A-Fos, and siRNA. Full-length human c-Fos cDNA was provided by Dr. L Shenshedini, University of Toledo, Toledo, OH (14). A-Fos vector was obtained from Dr. Charles Vinson (National Cancer Institute, Bethesda, MD; ref. 15). The plasmids with or without c-Fos or A-Fos were transfected with LipofectAMINE 2000 (Invitrogen Life Technologies). siRNA of c-Fos was transfected into cells by TransMessenger (Qiagen). Nonspecific siRNA was used as control (Qiagen). After transfection with the c-Fos or A-Fos vector for 24 h or c-Fos siRNA for 36 h, the cells were seeded in 96-well plates for cell viability assays or treated with TRAIL.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done by the ChIP Assay Kit (Upstate Cell Signaling Solutions). Cells were cultured in 10-cm dishes, treated with or without TRAIL for 4 h. Cross-linking of DNA and proteins was carried out by adding formaldehyde for final concentration of 1% and incubated for 10 min at

37°C. Both attached and floating cells were collected, washed, and resuspended in 200 µL of SDS lysis buffer for 10 min and then sonicated. Samples were centrifuged for 10 min at 13,000 rpm at 4°C and the supernatant was harvested. Concentration of each sample was quantitated using BCA protein assay. Positive controls were 10% of each DNA sample, which did not included the immunoprecipitation step. The remainder of the samples was equally divided into two groups. The experimental group was immunoprecipitated with specific c-Fos (D-1) antibody, whereas the negative control group was immunoprecipitated with general mouse IgG antibody. After immunoprecipitation, protein-DNA cross-linking was reversed. The isolated DNA was first purified, then amplified by PCR, using specific primers encompassing the c-FLIP(L) AP-1(f) binding site (Genbank). The primers for the experiments in Fig. 4A are 5'-CCTGTGATCCAGCACTTTG-3' (forward primer) and 5'-CACCATGCCCGACTAATTTT-3' (reverse primer).

Xenograft orthotopic implantations and immunohistochemical analysis. Prostate and renal orthotopic implantations were carried out by injection of 1×10^6 cells in either the posterolateral lobe of the prostate or beneath the kidney capsule of athymic nude mice (Charles River Laboratories) at 6 to 8 weeks of age. Mice were implanted with the following cells (untreated group/treated group): PC3 (six of five), PC3-TR (seven of five), LNCaP cells (five of six), SN12-PM6 (eight of eleven), and A-498 (nine of eleven). After implantation of the xenografts 10 weeks for prostate cancer cells and 3 weeks for renal cancer cells, the athymic nude mice were randomly divided into treated and untreated groups and treated with Lexatumumab (TRAIL receptor 2 agonist; Human Genome Science, Inc.) via tail vein twice a week (10 mg/kg), as previously described (16). Four weeks after treatment, all animals were euthanized and xenografts were harvested, and assessed for tumor weight, metastasis, apoptosis, and immunoreactivity for c-Fos and c-FLIP. Tumor tissues were fixed in 10% formalin and embedded in paraffin routinely. Histologic tests and immunohistochemistry were carried out as previously described (11). The dilution of both c-Fos (D1) and c-FLIP (G11) is 1:80. The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. All experiments were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Terminal deoxynucleotidyl transferase-mediated nick end labeling. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was done to assess the apoptotic cells using a commercial kit according to the manufacturer's instruction (Promega). Background reactivity was determined by processing slides in the absence of terminal deoxynucleotidyl transferase (negative control); maximum reactivity was observed by preincubating the tissue sections with DNase I to confirm the quality of the specimen and availability of protocol. Tissue sections were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.) to counterstain the nuclei. At least 200 cells from each sample were captured with a Nikon TE300 microscope and analyzed by counting positive rate. Positive apoptotic rate is defined as the ratio of green staining within nuclear area to the total nuclear staining (blue).

Results

AP-1 activity is increased in TRAIL-sensitive cancer cells and c-Fos translocates to the nucleus. Prostate cancer cells have variable sensitivity to recombinant human TRAIL/Apo-2L. We found that PC3 cells were very sensitive to TRAIL/Apo-2L, whereas PC3-TR and LNCaP cells were very resistant to TRAIL/Apo-2L (Fig. 1A). Previously, we have shown that c-FLIP(L), but not c-FLIP(s), is necessary and sufficient to maintain resistance to TRAIL/Apo-2L (4). We also found that expression of c-FLIP(L) is partially regulated at the transcriptional level. To explore the transcription factors that regulate *c-FLIP(L)* and may play an important role in differentiating between TRAIL-sensitive and TRAIL-resistant cancer cells, we analyzed the putative promoter and regulatory regions of *c-FLIP(L)* by using the Alibaba 2.1 software (17). We

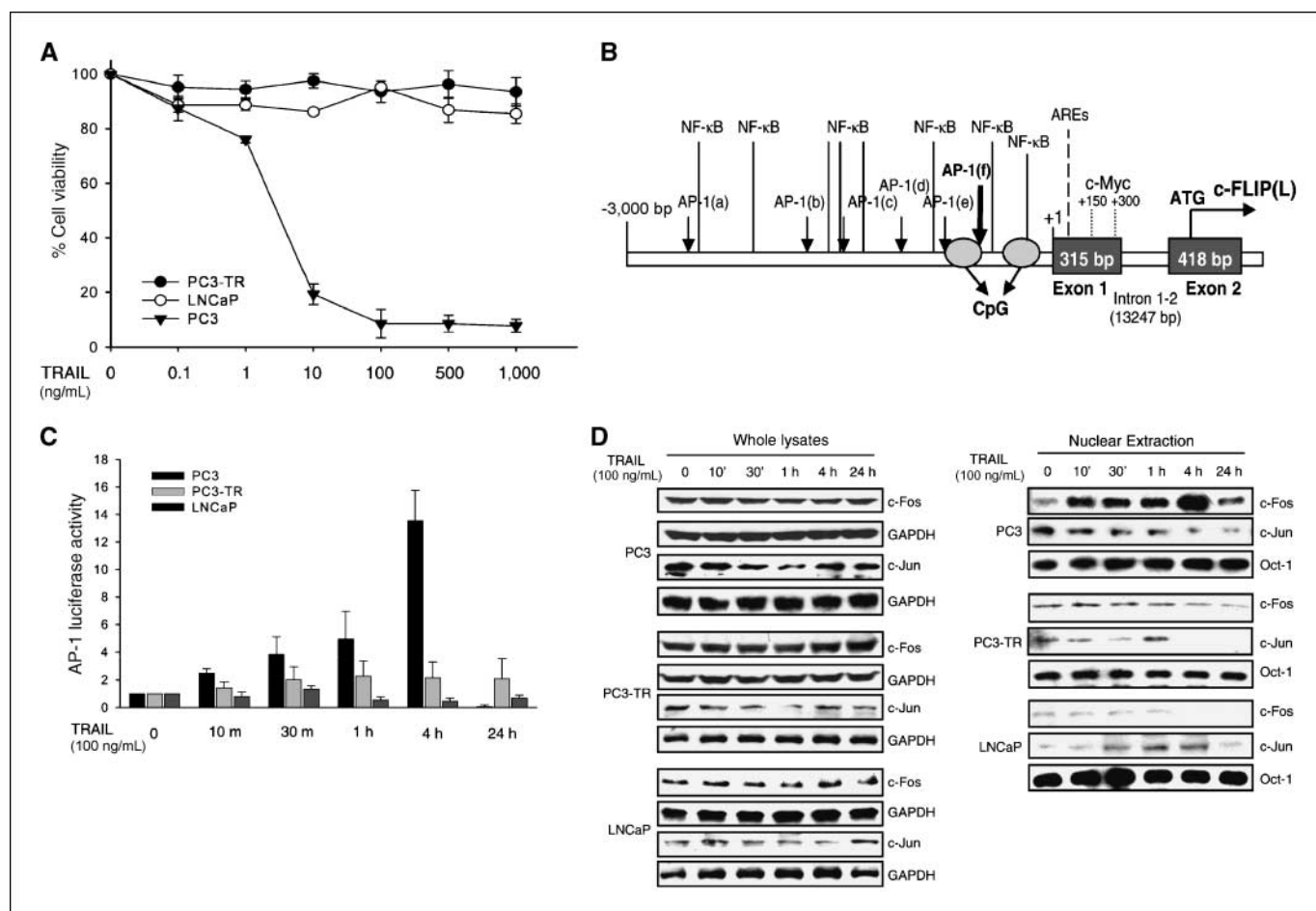


Figure 1. Nuclear c-Fos and AP-1 are up-regulated in TRAIL-sensitive cancer cells after TRAIL treatment. **A**, cells were treated with TRAIL/Apo-2L with different doses for 24 h and assayed for death by the MTT assay. [A is the same as the one used in our prior work (10); it is only shown here to demonstrate to the current readers our model of TRAIL-sensitive and TRAIL-resistant prostate cancer cells]. **B**, potential binding sites for putative transcriptional factors in the *c-FLIP(L)* promoter and regulatory region. Putative AP-1, NF- κ B, and ARE sites are indicated. **C**, time course of AP-1 luciferase activity after TRAIL/Apo-2L in prostate cancer cells. **D**, c-Fos and c-Jun whole-cell lysate (*top*) and nuclear (*bottom*) protein levels after TRAIL treatment at different time points. Oct-1 is used as loading control for nuclear extracts. Bars, SD from at least three independent experiments.

analyzed the possible transcription factor binding sites that spanned 17,000 bp of the *c-FLIP(L)* gene, including 3 kb upstream of exon1, introns 1 to 2, and exon 2 region before the ATG start codon (Fig. 1B). We identified potential binding sites for NF- κ B (18, 19), androgen response elements (20) and Myc (12), all of which have been shown to regulate expression of *c-FLIP(L)*. In addition to identifying known transcription factors that regulate *c-FLIP(L)*, we also found multiple potential AP-1 binding sites in the *c-FLIP(L)* regulatory region (Fig. 1B). Because AP-1 family of proteins are important transcription factors, we hypothesized that AP-1 might be an important regulator of *c-FLIP(L)*; it may play a key role in mediating cell response to TRAIL-induced apoptosis.

To determine whether AP-1 has any direct transcriptional activity in regulating TRAIL-induced apoptosis, we examined for AP-1 activity and DNA binding in TRAIL-sensitive (PC3) and TRAIL-resistant (PC3-TR and LNCaP) cells. We found that the activity of AP-1 significantly increased in the TRAIL-sensitive PC3 cells; however, AP-1 activity did not significantly change in the TRAIL-resistant PC3-TR and LNCaP cells (Fig. 1C). However, the increased AP-1 activity in the TRAIL-sensitive cells were temporal; 24 h after TRAIL treatment, no more AP-1 activity was detected because majority of the cells have undergone apoptosis by this time

(Fig. 1C; ref. 4). In addition, in the TRAIL-sensitive PC3 cells, we found increased AP-1 DNA binding as determined by electrophoretic mobility gel shift assay (EMSA), a finding that was not observed in the TRAIL-resistant PC3-TR or LNCaP cells (data not shown). These results show that there is increased AP-1 activity and DNA binding in the TRAIL-sensitive cells, but not in the TRAIL-resistant cells.

Because AP-1 family of proteins is composed of dimers between several family members, we wished to determine which member(s) were mainly responsible for the increased AP-1 activity in the TRAIL-sensitive PC3 cells after TRAIL treatment. Total protein whole-cell lysate Western blot analysis did not reveal any differences in protein levels of the two major components of AP-1, c-Fos and c-Jun (Fig. 1D, *top*), and other AP-1 family members (Fos B, Jun B, Jun D, Fra1, and Fra2; data not shown) in either TRAIL-sensitive (PC3) or TRAIL-resistant (PC3 and LNCaP) cancer cells after TRAIL/Apo-2L treatment. Because AP-1 is a well-known transcription factor, we examined the nuclear levels of its different family member proteins. Surprisingly, we found that expression of nuclear c-Fos was increased rapidly in the TRAIL-sensitive PC3 cells by Western blot (Fig. 1D, *bottom*) and immunofluorescence (Supplementary Fig. S1). In contrast, in TRAIL-resistant cells, we

found that nuclear c-Fos had either no significant change in PC3-TR cells or was decreased in LNCaP cells (Fig. 1D, bottom). Nuclear c-Fos was increased in the PC3 cells after 10 min of TRAIL/Apo-2L treatment and reached its peak after 4 h; however, nuclear c-Fos levels decreased at 24 h after treatment because majority of PC3 cells were dead at this time point. In addition, there was no increase in other nuclear AP-1 member proteins, including c-Jun, Fos B, Jun B, Jun D, Fra1, and Fra2 in the TRAIL-sensitive PC3 cells (Fig. 1D and data not shown). Therefore, the change in nuclear c-Fos protein levels (Fig. 1D) correlated with AP-1 activity levels (Fig. 1C) in the TRAIL-sensitive PC3 cells.

Although c-Fos nuclear translocation occurred rapidly 10 min after TRAIL treatment in the TRAIL-sensitive PC3 cells, we found that the TRAIL-sensitive PC3 cells undergo apoptosis 60 min after TRAIL treatment as determined by Annexin V-FITC flow cytometric assay (data not shown). Therefore, c-Fos nuclear translocation may precede initiation of apoptosis in the TRAIL-sensitive cells, and is maintained after they have undergone apoptosis.

To assure that the increased nuclear c-Fos we observed was not a PC3 cell line or prostate cancer-specific phenomenon, we evaluated other TRAIL-sensitive and TRAIL-resistant renal and

breast cancer cells. We found that expression of nuclear c-Fos was also increased before initiation of apoptosis in the TRAIL-sensitive renal (A-498) and breast (MDA-MB231) cancer cells, but not in the TRAIL-resistant renal 786-O and breast cancer cells MDA-MB453 (data not shown). Therefore, elevated nuclear c-Fos seems to occur before the cells undergo apoptosis, and it is a common finding in different cancer cell types sensitive to TRAIL-induced apoptosis.

c-Fos “primes” cancer cells to undergo apoptosis. We found that increased expression of nuclear c-Fos in the TRAIL-sensitive PC3 cells correlated with increased AP-1 luciferase activity. However, it is unclear if increased c-Fos/AP-1 merely reflects a stress response (21), or if activated c-Fos has a direct role in regulating apoptosis. Silencing expression of nuclear c-Fos by siRNA reduced AP-1 activity (Fig. 2A) and changed PC3 cells from TRAIL-sensitive to a more TRAIL-resistant phenotype (Fig. 2B). In addition, ectopic expression of a dominant-negative form of AP-1, A-Fos (15), also reduced the AP-1 activity in the PC-3 cells (Fig. 2C), and converted the PC3 cells from a TRAIL-sensitive to a TRAIL-resistant phenotype (Fig. 2D). These data show that c-Fos primes cancer cells to undergo cell death, and nuclear localization of c-Fos is necessary for TRAIL-induced apoptosis.

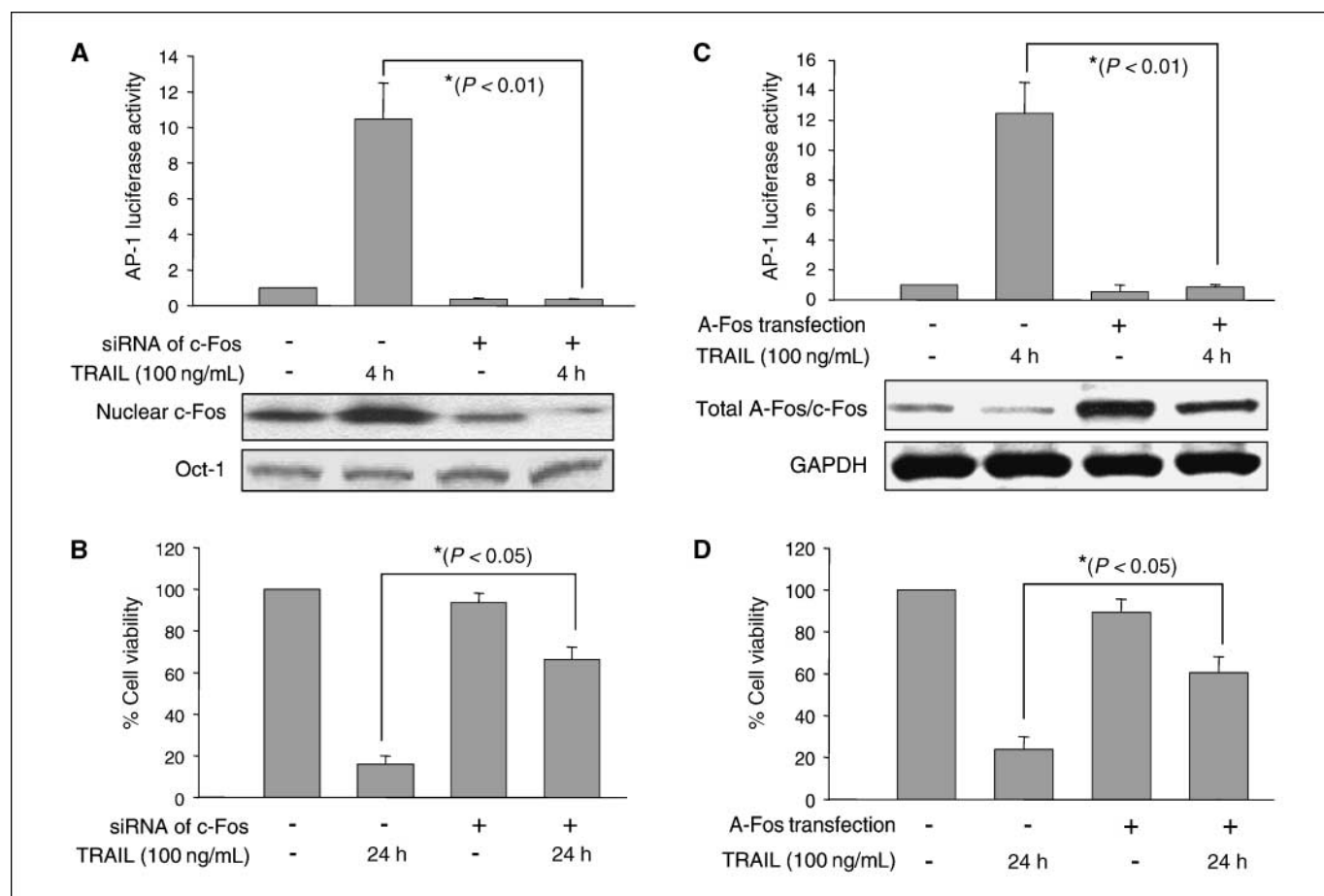


Figure 2. Inhibition of c-Fos/AP-1 converts TRAIL-sensitive PC3 cells to a more TRAIL-resistant phenotype. **A**, nuclear c-Fos protein levels and AP-1 luciferase activity in PC3 cells after using c-Fos siRNA in the absence or presence of TRAIL. Oct-1 is loading control for nuclear extracts. **B**, cell viability assays, with control nonspecific siRNA (-) or with c-Fos siRNA (+), before and after TRAIL treatment. **C**, AP-1 luciferase activity and protein expression of c-Fos/A-Fos transfection of dominant-negative AP-1, A-Fos, and TRAIL treatment. Note that c-Fos antibody recognizes both A-Fos and c-Fos. **D**, cell viability assays with A-Fos ectopic expression. *, significant differences between control and experimental samples. **B**, SD of at least three replicate experiments. c-Fos transfection (-) or A-Fos transfection (-) refer to empty vector control transfections.

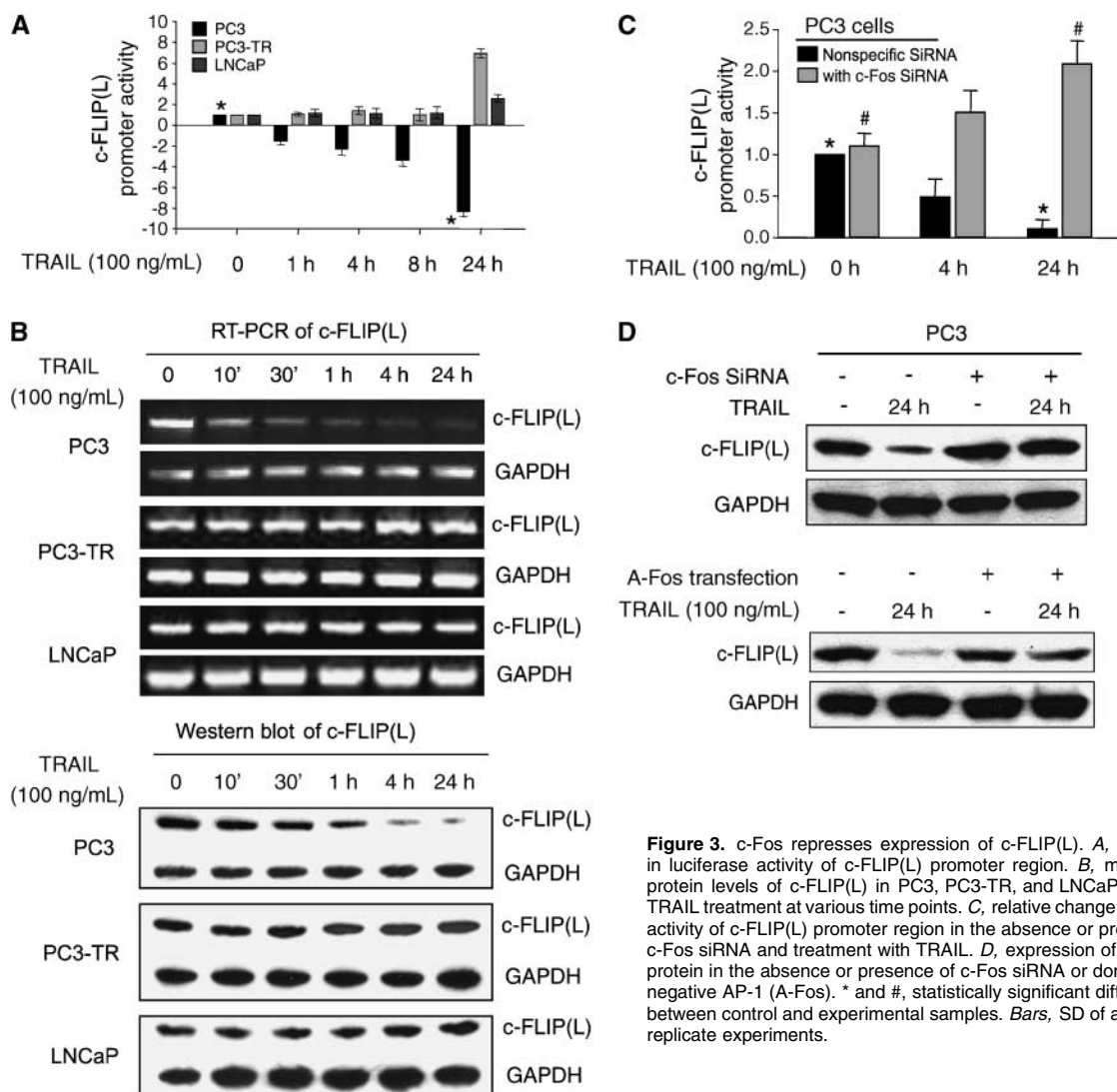


Figure 3. c-Fos represses expression of c-FLIP(L). **A**, fold change in luciferase activity of c-FLIP(L) promoter region. **B**, mRNA and protein levels of c-FLIP(L) in PC3, PC3-TR, and LNCaP cells after TRAIL treatment at various time points. **C**, relative change in luciferase activity of c-FLIP(L) promoter region in the absence or presence of c-Fos siRNA and treatment with TRAIL. **D**, expression of c-FLIP(L) protein in the absence or presence of c-Fos siRNA or dominant negative AP-1 (A-Fos). * and #, statistically significant difference between control and experimental samples. Bars, SD of at least three replicate experiments.

c-Fos represses the expression of c-FLIP(L) directly. c-Fos/AP-1 increases in the TRAIL-sensitive PC3 cells after TRAIL/Apo-2L treatment (Fig. 1C and D), whereas previously we have found that c-FLIP(L) expression in PC3 cells decreases in a time- and dose-dependent manner after TRAIL/Apo-2L treatment (4). The inverse correlation between c-Fos and c-FLIP(L) protein levels in the TRAIL-sensitive cells, and the presence of multiple AP-1 binding sites in *c-FLIP(L)* regulatory region (Fig. 1B), prompted us to examine whether the c-Fos/AP-1 in TRAIL-sensitive cells may affect the expression of the antiapoptotic molecule, c-FLIP(L). After treatment with TRAIL/Apo-2L, *c-FLIP(L)* promoter luciferase activity was reduced in the TRAIL-sensitive PC3 cells, whereas *c-FLIP(L)* promoter luciferase activity was potentiated in the TRAIL-resistant PC3-TR and LNCaP cells (Fig. 3A). The reduced *c-FLIP(L)* luciferase activity correlated with reduced c-FLIP(L) mRNA and protein levels in the TRAIL-sensitive PC3 cells. However, c-FLIP(L) mRNA and protein levels in the TRAIL-resistant PC3-TR and LNCaP cells were unchanged before and after TRAIL treatment (Fig. 3B). Silencing expression of c-Fos by

siRNA potentiated the c-FLIP(L) promoter luciferase activity after treatment with TRAIL/Apo-2L in the TRAIL-sensitive PC3 cells, whereas the c-FLIP(L) promoter luciferase activity was reduced when c-Fos expression was not silenced (Fig. 3C). Moreover, protein levels of c-FLIP(L) were maintained when c-Fos expression was either reduced by siRNA (Figs. 2A and 3D) or its AP-1 activity was suppressed by a AP-1 dominant-negative A-Fos (Figs. 2C and 3D).

To further determine whether c-Fos has any direct transcriptional activity in regulating *c-FLIP(L)*, we examined the potential AP-1 binding sites in the putative *c-FLIP(L)* regulatory region [17,000 bp upstream of the c-FLIP(L) ATG start codon; Fig. 1B]. We identified and examined binding of c-Fos to 14 AP-1 binding sites in the putative *c-FLIP(L)* regulatory region via ChIP assays, which included six AP-1 binding sites before exon 1 (designated sites "a" through "f" in Fig. 1B) and eight within introns 1 to 2. We only detected binding of c-Fos protein to the *c-FLIP(L)* AP-1(f) site (Figs. 1B and 4A). ChIP assays showed that binding of c-Fos to the *c-FLIP(L)* AP-1(f) site increased in the TRAIL-sensitive PC3

cells, whereas c-Fos binding to the *c-FLIP(L)* AP-1(f) site was reduced in the TRAIL-resistant PC3-TR and LNCaP cells after treatment with TRAIL/Apo-2L. To confirm the importance of c-Fos/AP-1 binding AP-1(f) site on regulating c-FLIP(L) expression, we deleted this AP-1(f) site in our *c-FLIP(L)* promoter luciferase reporter. We found that deletion of the *c-FLIP(L)* AP-1(f) site abolished the ability of c-Fos to suppress c-FLIP(L) expression (Fig. 4B). To further determine whether AP-1 binding to the *c-FLIP(L)* AP-1(f) site was specific to this AP-1(f) DNA sequence, we designed a wild-type and mutant oligonucleotide with four-tandem repeats of the AP-1(f) binding site. EMSA showed that binding to the wild-type AP-1(f) sequence was increased in the TRAIL-sensitive PC3 cells after treatment with TRAIL/Apo-2L, whereas there was minimal to no binding to the wild-type AP-1(f) site in the TRAIL-resistant PC3-TR and LNCaP cells (Fig. 4C). In contrast, binding to the mutant AP-1(f) site was abolished, regardless of whether the cells were TRAIL-sensitive or TRAIL-resistant. These data further confirm that c-Fos protein binds to *c-FLIP(L)* AP-1(f) site, represses expression of *c-FLIP(L)* gene, and sensitizes cancer cells to undergo TRAIL-induced apoptosis. Deletion and mutations of the *c-FLIP(L)* AP-1(f) promoter region abrogates the ability of c-Fos to repress the antiapoptotic molecule, c-FLIP(L).

Ectopic expression of c-Fos represses c-FLIP(L) and sensitizes TRAIL-resistant cancer cells. Next, we wished to determine whether ectopic expression of c-Fos can alter TRAIL-induced apoptosis in resistant prostate cancer cells. c-Fos was ectopically expressed in TRAIL-resistant PC3-TR and LNCaP cells. Concomitant with increased c-Fos protein levels in both PC3-TR and

LNCaP cells, c-FLIP(L) levels were reduced by half (Fig. 5A), AP-1 activity was increased (Fig. 5B), and cell viability was decreased (Fig. 5C). These data suggest that c-Fos represses the expression of the antiapoptotic molecule, c-FLIP(L). In addition, ectopic expression of wild-type c-Fos in PC3-TR and LNCaP cells was associated with increased c-Fos protein levels and enhanced AP-1 activity, which led to nuclear localization of c-Fos (data not shown), but did not promote cell death (Fig. 5C). Therefore, the TRAIL-resistant cells (LNCaP and PC3-TR) were sensitized to TRAIL when c-Fos was ectopically expressed. We conclude that nuclear localization of c-Fos by itself is necessary but insufficient to promote apoptosis in cancer cells.

c-Fos translocates to the nucleus in orthotopic *in vivo* xenografts. To determine whether nuclear expression of c-Fos that we observed *in vitro* in the TRAIL-sensitive cancer cells is also found in *in vivo* models, we orthotopically implanted prostate cancer cells (PC3, PC3-TR, and LNCaP) and renal cancer cells (SN12-PM6, A-498) in the posterolateral lobe of the prostate and under the kidney capsule of athymic nude mice, respectively. After orthotopic implantation of the xenografts (11), the athymic mice were treated with a TRAIL receptor 2 agonist antibody (Lexatumumab), which is currently in clinical trials for advanced malignancies (4). Lexatumumab has equivalent sensitivity and resistance profiles and induces similar downstream signaling molecules as recombinant TRAIL (16). Orthotopically implanted xenografts were treated for 4 weeks with i.v. Lexatumumab, and primary tumors were harvested and assessed for tumor weight, TUNEL staining, and expression of c-Fos and c-FLIP(L). The rate of tumor formation in the TRAIL-sensitive and TRAIL-resistant xenografts

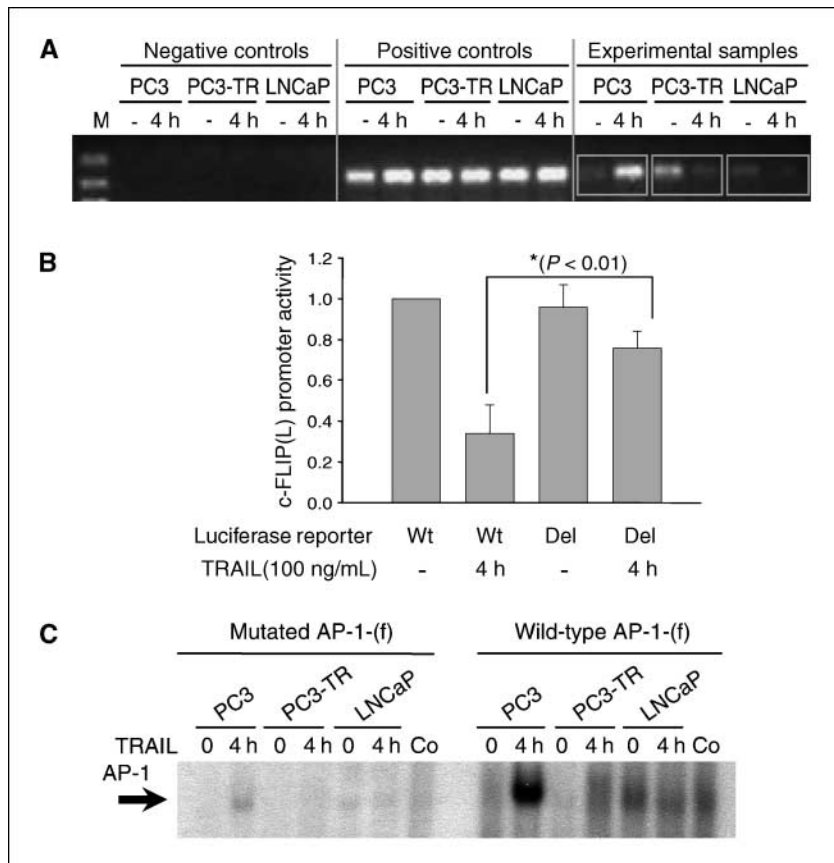


Figure 4. c-Fos represses expression of c-FLIP(L) by direct binding to its promoter region. **A**, AP-1(f) binding to c-FLIP(L) promoter analyzed by CHIP assay. Cells were treated with TRAIL (100 ng/mL) for 4 h. **B**, c-FLIP(L) promoter luciferase activity after deletion of AP-1(f) in the presence and absence of TRAIL/Apo-2L. *Wt* and *Del*, wild-type c-FLIP(L) promoter luciferase reporter and deletion of AP-1(f) site from the reporter, respectively. **C**, EMSA of AP-1 using a wild-type or mutated four-tandem oligonucleotide of the c-FLIP(L) AP-1(f) binding site as probes. *Co*, competing control. *, statistically significant difference between indicated groups. Bars, SD of at least three replicate experiments.

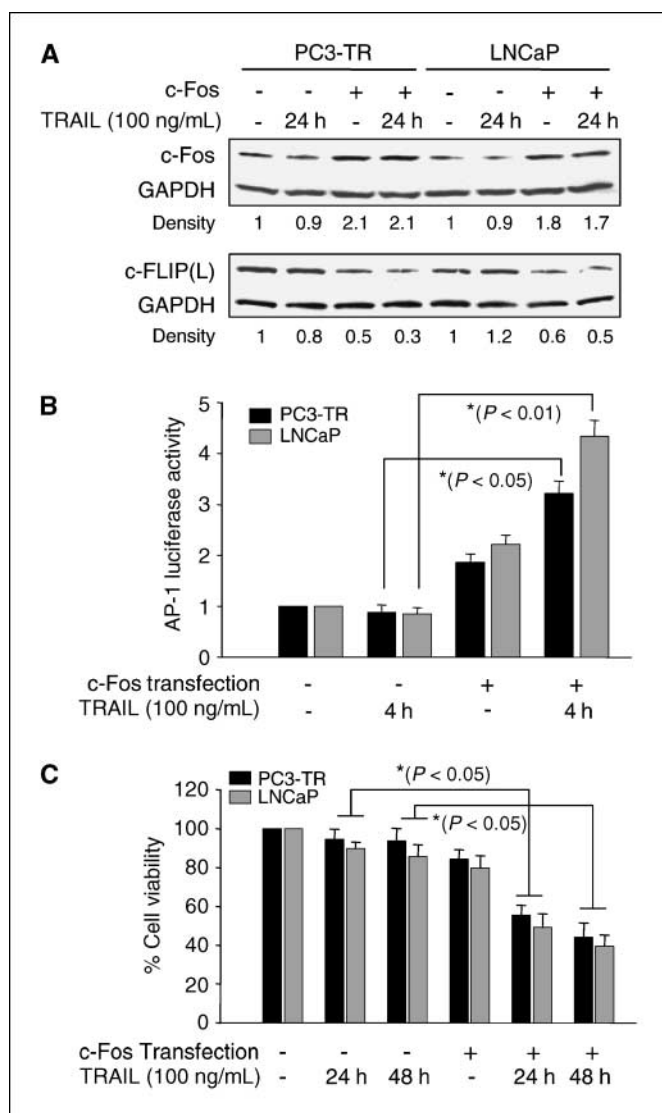


Figure 5. Ectopic expression of c-Fos sensitizes TRAIL-resistant PC3-TR and LNCaP cells. **A**, expression of c-Fos and c-FLIP(L) in PC3-TR and LNCaP cells in the absence or presence of c-Fos transfection. Numbers, band intensity. AP-1 luciferase activity (**B**) and cell viability (**C**) of PC3-TR and LNCaP cells with empty vector (–) or with c-Fos (+) ectopic expression, and treatment with TRAIL. *, significant difference between indicated groups. Bars, SD of at least three replicate experiments.

was equivalent, and there was no significant difference in the body weight of the animals in the treated and untreated groups (Supplementary Table S1). However, TRAIL-sensitive xenografts had a much higher apoptotic rate ($P < 0.01$) and significantly decreased tumor burden ($P < 0.05$) when the animals were treated with Lexatumumab compared with the control groups. In contrast, the apoptotic rates were very low and there was no significant difference of tumor burden in TRAIL-resistant PC3-TR and LNCaP xenografts with or without treatment (Supplementary Table S1). Similar results were also observed in renal cancer SN12-PM6 and A-498 xenografts (22). Next, we found that expression of nuclear c-Fos was pronounced in the TRAIL-sensitive PC3, SN12-PM6, and A-498 xenografts after treatment, but not in the TRAIL-resistant PC3-TR and LNCaP xenografts (Fig. 6). These findings suggest that increased nuclear expression of c-Fos is found not only in TRAIL-

sensitive *in vitro* models, but also in orthotopic *in vivo* models after treatment with TRAIL receptor agonist compounds. Potentially, nuclear localization of c-Fos could be used to identify human cancers that are sensitive to TRAIL-induced apoptosis.

Discussion

Because the AP-1 family member protein c-Fos plays a crucial role in a variety of biological processes, identifying the downstream targets of c-Fos has significant implications in understanding of normal development, inflammation, and oncogenesis (6). In this report, we show that c-Fos, in addition to its well-known oncogenic function, has a novel proapoptotic function in TRAIL-induced apoptosis. c-Fos exerts its proapoptotic function by repressing c-FLIP(L). We define Fos-dependent priming (FDP) as increased expression of nuclear c-Fos after treatment with TRAIL/Apo-2L. Clinical implications of these results include the possibility of using FDP as a marker in cancer patients being treated with proapoptotic agents. The presence of FDP may identify tumors that are sensitive to proapoptotic stimuli, whereas lack of FDP identifies resistant tumors.

Caspases are important modulators of apoptosis (for review, see ref. 23). Activation of specific death domain receptors, like DR4 and DR5 by their ligand, TRAIL, promotes formation of death-inducing signaling complex (DISC). DISC recruits an adaptor molecule, Fas-associated death domain (FADD), which in turn interacts with and activates caspase-8 and/or caspase-10, leading to initiation of the extrinsic proapoptotic signaling pathway. Because of its sequence homology with caspase-8, c-FLIP(L) has been shown to competitively inhibit the interaction between FADD and caspase-8, and thus inhibits the initiation of proapoptotic stimuli. We and others (4, 24, 25) have shown that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. Here, we show that c-Fos represses expression of c-FLIP(L). Therefore, for a cancer cell to undergo apoptosis after TRAIL treatment is dependent on a feedback loop mechanism as determined by activation of c-Fos and c-FLIP(L) level.

c-Fos is one of the immediate-early response and inducible transcription factors. Its level is increased after many stress stimuli, including some proapoptotic stimuli. For example, c-Fos levels have been increased after chemotherapy (26), UV radiation (27, 28), and TNF- α exposure (29). Few studies have noted increased levels of c-Fos after TRAIL treatment (30, 31); however, the function of c-Fos in these biological settings have not been clearly defined. Although, c-Fos usually acts as a transcriptional activator, it has been shown that it can also function as a transcriptional suppressor. For example, c-Fos can negatively regulate its own expression (32, 33), or other molecules like inducible nitric oxide synthase, TNF- α , and IL-12 (34, 35). Our report shows for the first time the role of c-Fos as a repressor of the antiapoptotic molecule, c-FLIP(L).

We postulate that posttranslational modifications of c-Fos may determine whether cancer cells are sensitive or resistant to TRAIL-induced apoptosis. In our *in vitro* and orthotopic *in vivo* studies, we showed that nuclear translocation of c-Fos and repression of c-FLIP(L) gene is an important process in promoting TRAIL-induced apoptosis in cancer cells. Cellular localization and activation of c-Fos can depend on its phosphorylation, protein stability, and other chaperone proteins. Recent work has suggested that phosphorylation of c-Fos, which is an important determinant of its activity and expression, is tightly regulated by a variety of kinases such as mitogen-activated protein kinase (36), FRK (37),

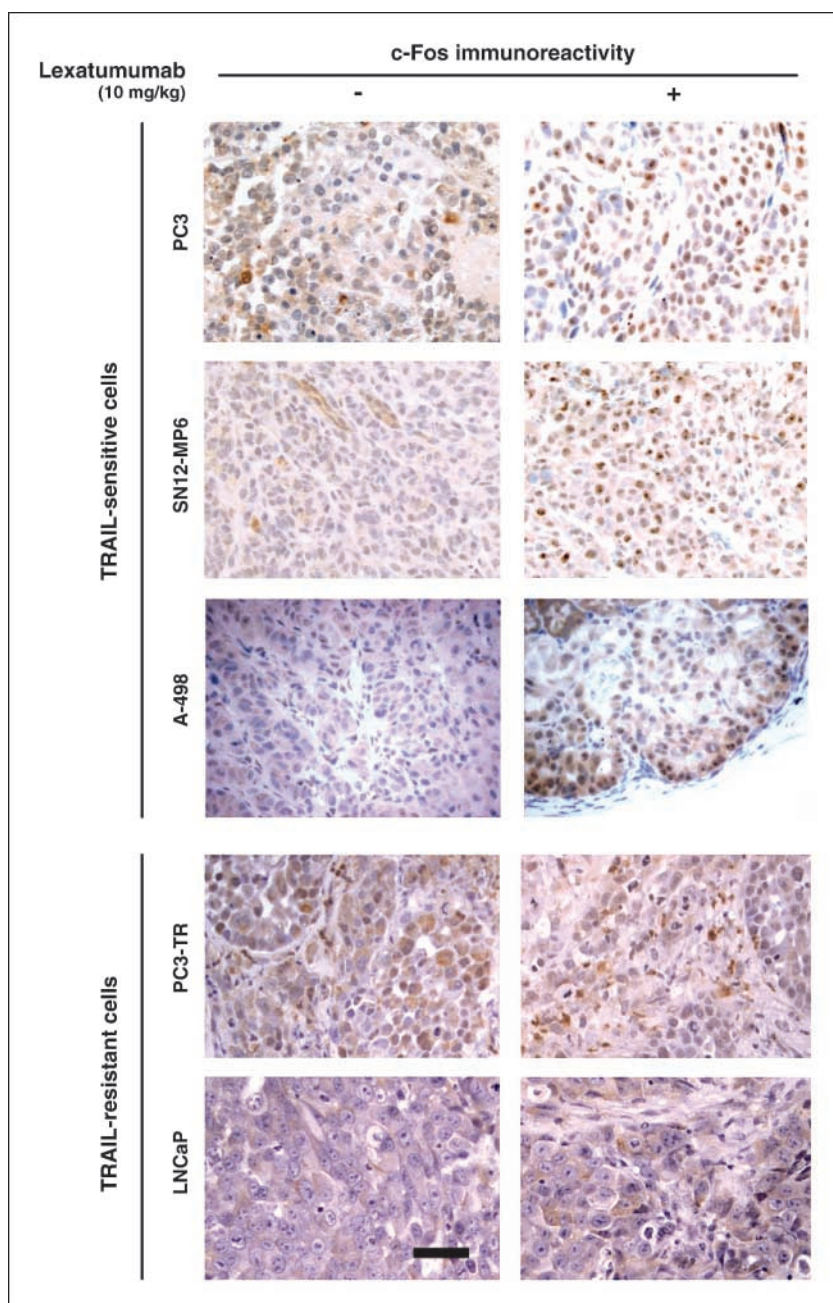


Figure 6. Immunohistochemical analysis of orthotopically implanted prostate cancer cells (PC3, PC3-TR, and LNCaP) and renal cancer cells (SN12-PM6 and A-498). Representative immunohistochemical images of c-Fos without (–) or with (+) Lexatumumab treatment in TRAIL-sensitive or TRAIL-resistant prostate cancer and renal cancer xenografts. Bar, ~ 50 μ m.

RSK2 (38), CKII (39), and PDK1 (40). Protein stability of c-Fos, another regulator of its physiologic function, has been shown to be dependent on its COOH-terminal PEST3 domain, which modulates c-Fos proteasome-mediated degradation (41). Associated proteins in the form of chaperone proteins or heterodimers can also regulate c-Fos structure and function. As a follow-up study to the current report, we have recently reported that the proteasome inhibitor, MG-132, sensitizes TRAIL-resistant cancer cells by up-regulating AP-1 activity (22). Therefore, up-regulation of AP-1 and sensitization to TRAIL-induced apoptosis is another example of necessary mechanisms that may serve an important function in overcoming resistance to TRAIL-induced apoptosis. Therefore, we believe that the posttranslational modifications of c-Fos can significantly affect its ability to regulate *c-FLIP(L)* gene expression

and TRAIL-induced apoptosis, and it is an area under investigation in our laboratory.

The c-FLIP family of proteins is homologous to pro-caspase-8 (for review, see ref. 23). Both c-FLIP(L) and c-FLIP(s), and perhaps the newly detected c-FLIP(r) (42), can bind to the DED domains of FADD and caspase-8 and regulate apoptosis through their interference with the recruitment of caspase-8 to FADD. Most reports suggest that c-FLIP(L) has an antiapoptotic role, largely due to results from experiments using ectopic expression of c-FLIP(L). Moreover, *c-FLIP(L)*^{–/–} mouse embryonic fibroblasts are more sensitive to proapoptotic agents, which strongly suggests that c-FLIP(L) has an antiapoptotic function (43). However, some recent reports suggest that c-FLIP(L) may have a dual function, a proapoptotic function at low physiologic concentrations, and an

antiapoptotic function at high cellular concentrations (44). In accordance with the role of c-FLIP(L) as an antiapoptotic molecule, we have found that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. Meanwhile, our observations found that level of c-FLIPs were too low to be detected by Western blot in prostate and renal cancer cell lines we used, which did not support that c-FLIPs might play an important role in regulating TRAIL sensitivity in these cells. Although regulation of c-FLIP(L) can occur at the translational and posttranslational levels, we found that transcriptional regulation of c-FLIP(L) may also affect cancer cell sensitivity to TRAIL-induced apoptosis (10). Other investigators have shown that NF- κ B (18, 19), c-Myc (12), nuclear factor of activated T cells (45), and even androgen receptor response elements (20) may regulate expression of c-FLIP(L) through direct or indirect mechanisms. Here, we show that c-Fos directly binds the AP-1(f) site of the *c-FLIP(L)* gene (Fig. 4A), represses expression of c-FLIP(L), and promotes TRAIL-induced apoptosis. In contrast, deletion of the AP-1(f) site abrogates binding of c-Fos, leading to enhancement of *c-FLIP(L)* gene expression and resistance to TRAIL-induced apoptosis. The AP-1(f) site lies within a CpG island (Fig. 1B); therefore, methylation patterns in this site may regulate the direct interaction between c-Fos protein and the *c-FLIP(L)* gene.

Some limitations of our study is that we only investigated the effect of c-Fos/AP-1 on c-FLIP(L). It is likely that c-Fos regulates other apoptosis-related molecules besides c-FLIP(L) to alter cell sensitivity to TRAIL-induced apoptosis. Our rationale for investigating the interaction between c-Fos and c-FLIP(L) stems from

prior reports demonstrating up-regulation of c-Fos in cancer cells after TRAIL treatment (30, 31), presence of multiple c-Fos/AP-1 binding sites in the putative promoter region of c-FLIP(L) (Fig. 1B), and our previous work suggesting that c-FLIP(L) could be regulated transcriptionally in cancer cells after TRAIL treatment (10). In addition, we have not investigated the mechanism that c-Fos is translocated from the cytoplasm to the nucleus. Chaperon proteins, alterations in c-Fos phosphorylation, or changes in c-Fos protein stability are all potential mechanism that may play a role in translocation of c-Fos from the cytoplasm to the nucleus in TRAIL-sensitive cells. Currently, these areas are under active investigation in our laboratory.

In conclusion, we have shown that c-Fos has a proapoptotic function by repressing the antiapoptotic molecule, c-FLIP(L). FDP is necessary but insufficient for TRAIL-induced apoptosis. We believe that presence of FDP identifies cancers that are sensitive, while lack of FDP identifies cancers that are resistant to TRAIL-induced apoptosis.

Acknowledgments

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Low-Dose 12-*O*-Tetradecanoylphorbol-13-Acetate Enhances Tumor Necrosis Factor – Related Apoptosis-Inducing Ligand – Induced Apoptosis in Prostate Cancer Cells

Xiaoping Zhang, Wenhua Li, and Aria F. Olumi

Abstract **Purpose:** Previously, we have shown that c-Fos/activator protein-1 (AP-1) promotes tumor necrosis factor (TNF) – related apoptosis-inducing ligand (TRAIL) – induced apoptosis by repressing the antiapoptotic molecule c-FLIP(L). In this study, we investigated whether synthetic induction of c-Fos/AP-1 by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) converts the phenotype of TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype *in vitro* and *in vivo*. **Experimental Design:** Low-dose TPA was used to determine whether LNCaP prostate cancer cells could be converted to a TRAIL-sensitive phenotype in *in vitro* and *in vivo* studies. We also assessed whether TPA enhancement of TRAIL-induced apoptosis varies between androgen-sensitive and androgen-insensitive prostate cancer cells and evaluated the role of TRAIL receptors, DR4 and DR5, in TPA-enhanced TRAIL-induced apoptosis. **Results:** We show that the combination of TRAIL with low-dose TPA has no effect on nonmalignant prostate epithelial cells; however, TPA up-regulates most AP-1 proteins and AP-1 activity, reduces c-FLIP(L), and potentiates TRAIL-induced apoptosis. We show that the combination of TPA + TRAIL is effective in promoting apoptosis in both hormone-sensitive LNCaP and hormone-insensitive LNCaP-C4-2 prostate cancer cells. Although TPA enhances the TRAIL-receptor 1 (DR4) level, sensitization of prostate cancer cells seems to be more dependent on TRAIL-receptor 2 (DR5) than TRAIL-receptor 1 levels. *In vivo* xenograft experiments suggest that TPA elevates the expression of c-Fos and reduces c-FLIP(L). Combination of TPA with TRAIL-receptor 2 agonist antibody, lexatumumab, effectively increases apoptosis and reduces LNCaP xenograft tumor burden. **Conclusions:** TPA, when combined with the proapoptotic agent TRAIL, is effective in changing the phenotype of some TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype.

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL/Apo2L) has a unique selectivity for triggering apoptosis in many cancer or transformed cells, but with minimal to no effect on most normal cells (1–3); therefore, it is associated with minimal cytotoxicity (4). Recently, TRAIL-like ligands and agonist TRAIL-receptor monoclonal antibodies have entered phase I and II clinical trials with a very limited

cytotoxic profile when used systemically in a variety of cancers (5, 6). Therefore, TRAIL-receptor agonists are new proapoptotic pharmaceutical agents with great potential as new cancer therapeutic agents. Although many cancer cells undergo TRAIL-mediated apoptosis, some are resistant to TRAIL. Therefore, we have been investigating mechanisms to overcome TRAIL resistance in cancer cells so that TRAIL-associated compounds can be used effectively in clinical trials.

TRAIL interacts with specific death domain receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), to induce intracellular cytoplasmic formation of the DISC (death-inducing signaling complex; ref. 7). Following the formation of DISC at the intracellular plasma membrane, proapoptotic signals are initiated by caspase-8, which can further activate downstream proapoptotic molecules and subsequent programmed cell death, which may also activate the mitochondrial mediated proapoptotic pathways via cleavage of Bid (8). A key inhibitor of death receptor signaling is c-FLICE-like inhibitory protein (c-FLIP; refs. 9, 10). c-FLIP shows a high level of homology to caspase-8 and caspase-10, but has no protease activity and prevents the formation of a competent DISC by binding to the FADD adaptor protein and competing off caspase-8 (10). We have previously shown that expression of c-FLIP long form (c-FLIP(L)), not c-FLIP short form (c-FLIP(s)), is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis in

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prostate cancer cells (11). More recently, we have shown that c-Fos, one of the two major components of activator protein-1 (AP-1) family member proteins, has a novel proapoptotic function by priming prostate cancer cells to undergo TRAIL-induced apoptosis (12, 13). We have found that the up-regulation of c-Fos/AP-1 is necessary but insufficient for cancer cells to be sensitive to TRAIL-induced apoptosis. We have also found that c-Fos/AP-1 functions as a proapoptotic molecule by directly repressing the antiapoptotic gene, *c-FLIP(L)*. This finding suggests that strategies to potentiate c-Fos/AP-1 activation and/or inhibit c-FLIP(L) may enhance the efficacy of TRAIL for treatment of various malignancies.

12-O-Tetradecanoylphorbol-13-acetate (TPA) has been shown to activate protein kinase C (PKC) and c-jun-NH₂-kinase pathways and the AP-1 proteins (e.g., c-Fos and c-Jun) to promote differentiation, cell cycle arrest, and apoptosis in a variety of cell model systems (14–16). TPA can directly mediate expression of AP-1 genes via serum response element sites at their promoters (16). In addition, TPA can activate PKC and mitogen-activated protein kinases, which will directly or indirectly activate AP-1 proteins and their functions (16). Therefore, TPA can strongly induce the expression of AP-1 proteins. Recently, high-dose TPA alone has been shown to promote apoptosis in androgen-dependent prostate cancer cells (17) and enhance the therapeutic effects of radiation in LNCaP prostate cancer cells (18, 19). More importantly, TPA has been used in a variety of clinical trials to potentiate the effect of chemotherapy (15, 20–22). Therefore, TPA has the potential of enhancing the therapeutic effects of some systemic agents for the treatment of various malignancies.

Because we have found that the activation of c-Fos/AP-1 is necessary for cancer cells to undergo TRAIL-induced apoptosis and TPA is a strong inducer of c-Fos/AP-1, we hypothesized that TPA might sensitize TRAIL-resistant prostate cancer cells to undergo apoptosis after TRAIL treatment. In the present study, we show that TRAIL or a TRAIL-R2 agonist antibody, combined with low-dose TPA, up-regulates AP-1 proteins and its activity, reduces c-FLIP(L) levels, and potentiates apoptosis in TRAIL-resistant LNCaP cells in *in vitro* and *in vivo* experiments. Therefore, TPA, when combined with the proapoptotic agent TRAIL, is effective in changing the phenotype of some TRAIL-resistant prostate cancers to a TRAIL-sensitive phenotype.

Materials and Methods

Materials. Recombinant human TRAIL/TNFSF10 was obtained from R&D System Inc. TRAIL-receptor 1 (DR4) agonist monoclonal antibody, mapatumumab, and TRAIL-receptor 2 (DR5) agonist monoclonal antibody, lexatumumab, were obtained from Human Genome Sciences, Inc. Antibodies to c-Fos, Fos B, Fra-1, Fra-2, Jun B, Jun D, DR4, DR4 RNAi, and horseradish peroxidase-conjugated secondary antibodies (goat-anti-mouse, goat-anti-rabbit, and goat-anti-rat antibodies) were obtained from Santa Cruz Biotechnology, Inc. Antibody to c-Jun was from Cell Signaling. Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Abcam. Antibodies to c-FLIP and DR5 were from Apotech Corp. TPA was from Sigma.

Cell culture material, cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, apoptosis by Annexin V-FITC, Western blot assays, transfection of plasmids, and RNAi and luciferase assay. References for material and techniques can be found from our previously published works (5, 12, 13). DR4, DR5, and their vector pcDNA3.1

were from Dr. Roya Khosravi-Far's laboratory (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA).

Establishment of mouse s.c. xenograft and experimental design. LNCaP cells 1×10^6 were mixed with Matrigel (BD Biosciences) according to manufacturer's protocol and injected into the posterior trunk of each athymic nude mice (Charles River Laboratories). Nine weeks after injection, 28 mice with visible tumor mass were randomly divided into four groups (each has seven mice): vehicle group, lexatumumab (Lexa) group, TPA group, and TPA + Lexa group, and treated with saline, TPA (100 ng/g), Lexa (10 µg/g), and TPA (100 ng/g) + Lexa (10 µg/g) via tail vein twice a week, respectively. Mouse body weight and tumor area were measured twice a week. Four weeks after treatment, all animals were euthanized, and xenografts were harvested and assessed for tumor weight, apoptosis [terminal nucleotidyl transferase-mediated nick end labeling (TUNEL)], and Western blot for c-Fos and c-FLIP. Tissue samples for Western blot analysis were preserved in liquid nitrogen and then prepared in radioimmunoprecipitation assay buffer with 2% SDS. All experiments were approved by the Institutional Animal Care and Use Committee at our institution.

TUNEL labeling to assess the apoptotic cells was done using a commercial kit according to the manufacturer's instruction (Promega Co.). Background reactivity was determined by processing slides in the absence of terminal deoxynucleotidyl transferase (negative control); maximum reactivity was observed by preincubating the tissue sections with DNase I to confirm the quality of the specimen and availability of protocol. Tissue sections were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.) to counterstain the nuclei. At least three microscopic views (each with at least 200 cells) from each sample were captured with a Nikon TE300 microscope and analyzed by counting positive rate. Positive apoptotic rate is defined as the ratio of green staining within the nuclear area to the total nuclear staining (blue).

Statistical analysis. GraphPad Instat software (version 3.0) was used for all statistical analyses. For apoptosis, cell viability, tumor area, and tumor volume assessments, Kruskal-Wallis test (nonparametric ANOVA) was used to compare each treatment group.

Results

Low-dose TPA sensitizes LNCaP cells to TRAIL-induced apoptosis. Although PC3 prostate cancer cells are sensitive to TRAIL-induced apoptosis, LNCaP cells and nontumorigenic and immortalized BPH-1 cells (benign prostatic hyperplasia cells) are resistant to the proapoptotic effects of TRAIL (Fig. 1A). Because high-dose TPA can induce apoptosis in androgen-dependent prostate cancer cells (17, 23), we focused on identifying a low dose of TPA, which does not directly induce apoptosis as a single agent. We found that TPA at 100 ng/mL did not induce cell death in LNCaP or BPH-1 cells (Fig. 1B). However, at higher concentrations (1,000 to 10,000 ng/mL), TPA induced cell death in LNCaP prostate cancer cells, but not the nontumorigenic transformed BPH-1 cells (Fig. 1B).

Next, we examined whether a nontoxic dose of TPA (100 ng/mL) can enhance the efficacy of TRAIL-induced apoptosis. We found that TPA combined with TRAIL significantly increased apoptosis in the TRAIL-resistant LNCaP cells as shown by Annexin V (Fig. 1C) and cell viability (Fig. 1D) assays. However, TPA, combined with TRAIL, did not significantly affect the cell viability of BPH-1 cells (Fig. 1D). Therefore, a nonproapoptotic dose of TPA can enhance TRAIL-induced apoptosis in LNCaP cells, but not in nonmalignant transformed prostate cells like BPH-1.

c-Fos/AP-1 is required for TPA enhancement of TRAIL-induced apoptosis in LNCaP cells. To assess whether low-dose TPA can

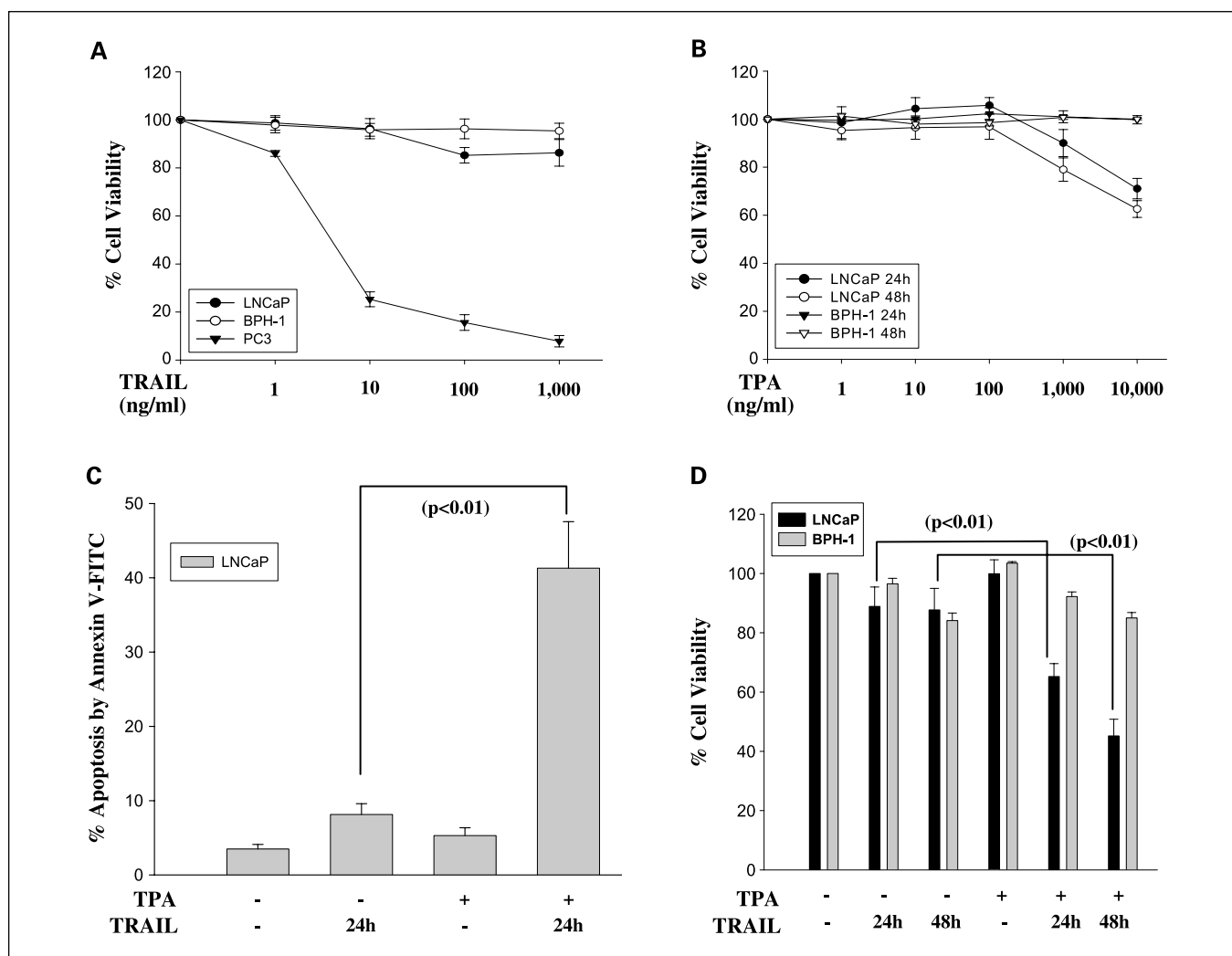


Fig. 1. Low-dose TPA enhances TRAIL-induced apoptosis in LNCaP cells. *A*, cell viability of PC3, LNCaP, and BPH-1 cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were treated with TRAIL for 24 h. *B*, cell viability of LNCaP and BPH-1 cells treated with different doses of TPA for 24 h and 48 h. *C*, percentage of apoptotic cells was measured by Annexin V – FITC staining. *D*, cell viability of LNCaP and BPH-1 cells. Cells in (*C*) and (*D*) were treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h and then treated with TRAIL (100 ng/mL) for the indicated times. Bars, SD from at least three independent experiments.

induce the expression of AP-1 family members and AP-1 activity, we examined for protein expression and AP-1 activity in LNCaP cells. We found that TPA alone or TPA combined with TRAIL increased not only the expression of c-Fos, one of the two major members of AP-1 family, but also other AP-1 family members like Fos B, Fra-1, Fra-2, Jun B, and Jun D (Fig. 2A). However, c-Jun protein levels, another major AP-1 family member protein, were not changed significantly. In accordance with our immunoblot findings (Fig. 2A), TPA alone or TPA combined with TRAIL increased AP-1 activity (Fig. 2B). Therefore, these results suggest that TPA increases the level of some AP-1 proteins and enhances AP-1-related gene activity.

Because c-Fos and c-Jun are two major components of the AP-1 complex (24) and the expression of c-Jun was not changed before and after TPA treatment (Fig. 2A), we determined whether the induction of c-Fos/AP-1 by TPA was required for TRAIL-induced apoptosis in resistant prostate cancer cells. We used a dominant negative form of c-Fos/AP-1, A-Fos (25), to inhibit c-Fos/AP-1. We found that A-Fos inhibited TPA-induced

AP-1 activity (Fig. 2C, top, two rightmost columns). In conjunction with reduced AP-1 activity, TPA did not enhance the ability of TRAIL to promote apoptosis when the activity of c-Fos/AP-1 was inhibited by the dominant negative A-Fos (Fig. 2C, bottom, rightmost column). Therefore, the activity of c-Fos/AP-1 is necessary for TPA to enhance TRAIL-induced apoptosis.

In our previous studies, we have shown that c-Fos/AP-1 was necessary for cancer cells to be sensitive to TRAIL-induced apoptosis (12, 13). Furthermore, we have shown that one of the major mechanisms for c-Fos/AP-1 to promote apoptosis is through cytoplasmic to nuclear translocation and direct inhibition of c-FLIP(L) gene via binding to its promoter region (12, 13). Similarly, in the current study, we also found that the combination of TPA and TRAIL reduced both mRNA of c-FLIP(L) and protein levels (Fig. 2D), which confirms that the regulation of c-FLIP(L), at least partially, occurs at the transcriptional level. It is noteworthy that although TPA alone increased AP-1 activity by 3-fold (Fig. 2B), it did not reduce c-FLIP(L) levels (Fig. 2D). However, TPA combined with TRAIL

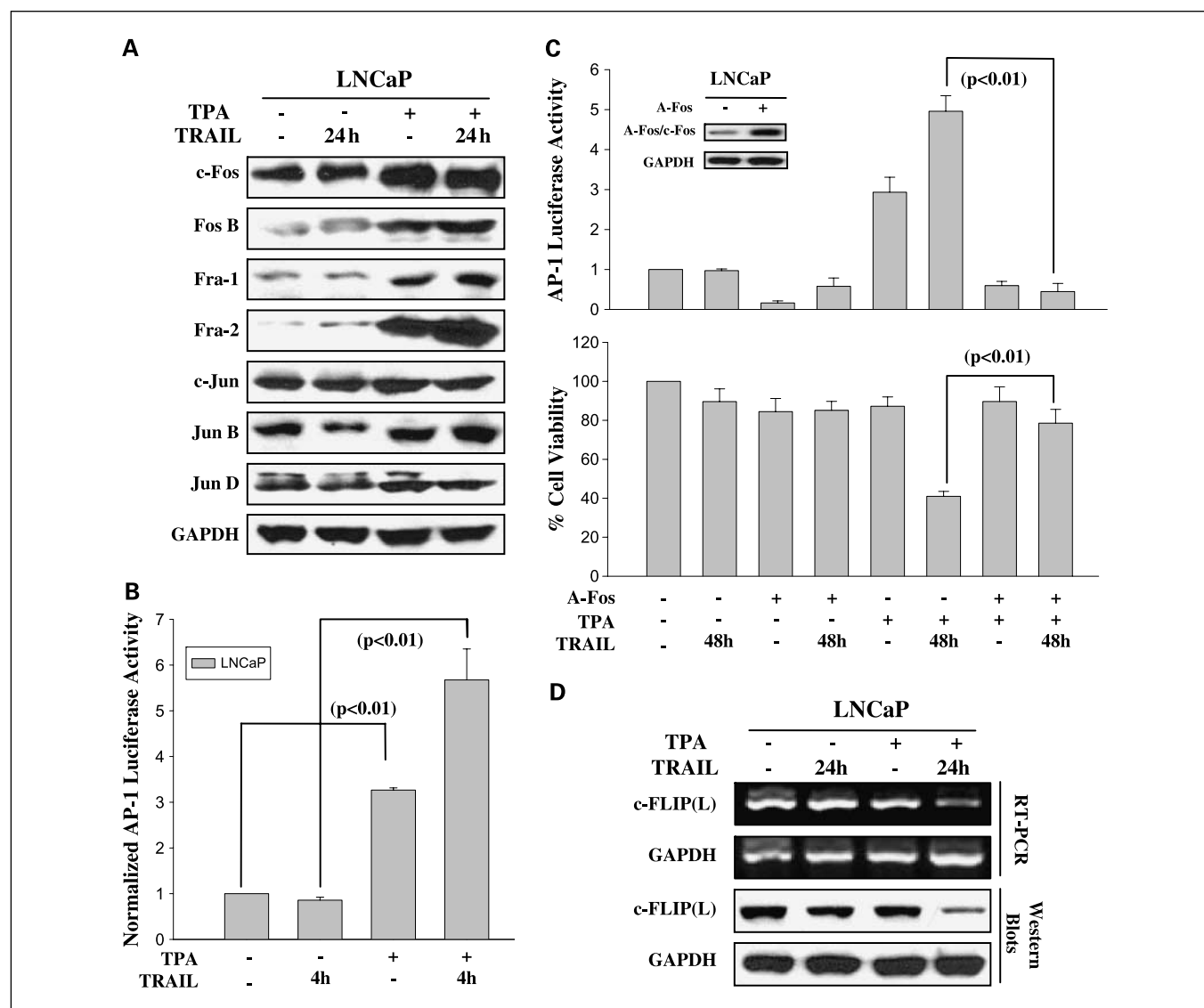


Fig. 2. TPA-induced AP-1 activity is necessary for TRAIL-induced apoptosis in resistant prostate cancer cells. **A**, TPA increases the levels of several AP-1 family-related proteins. **B**, AP-1 luciferase activity is increased in LNCaP cells treated with TPA. **C**, top, AP-1 luciferase activity; bottom, cell viability assays. Top inset, Western blot represents expression of A-Fos and c-Fos after mock or A-Fos transfections. Note that the antibody recognizes both A-Fos (dominant negative) and endogenous c-Fos. Cells were transfected with A-Fos for 24 h before TPA and TRAIL treatments. **D**, reverse transcription-PCR and Western blots for c-FLIP(L). LNCaP cells treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h then treated with TRAIL (100 ng/mL) for the indicated time. GAPDH represents loading control for mRNA or proteins. Bars, SD from at least three independent experiments.

increased AP-1 activity more and reduced the expression of c-FLIP(L). Therefore, the activation of c-Fos/AP-1 is necessary to sensitize TRAIL-resistant prostate cancer cells but insufficient to promote apoptosis. We believe that the repression of c-FLIP(L) by c-Fos is one of the molecular mechanisms that promotes TRAIL-induced apoptosis (11–13).

TPA enhances lexatumumab-induced apoptosis in LNCaP cells in vivo. To determine whether TPA can enhance TRAIL-induced apoptosis *in vivo*, we used a TRAIL receptor agonist monoclonal antibody, lexatumumab, that is currently in clinical trials for treatment of various malignancies (5, 6). Lexatumumab induces apoptosis via similar pathways as recombinant TRAIL (26, 27). We found that lexatumumab had equivalent sensitivity toward TRAIL-resistant (LNCaP) and TRAIL-sensitive (PC3) cells as recombinant TRAIL (Figs. 1A

and 3A). Therefore, using lexatumumab as opposed to recombinant TRAIL for our *in vivo* experiments is more clinically relevant at the current time (28).

Nine weeks after LNCaP xenograft implantation, 28 mice with equivalent tumor sizes (Fig. 3B, week 0) were randomly divided into four treatment groups (7 mice per group): vehicle (saline), lexatumumab (Lexa, 10 µg/g), TPA (100 ng/g) or TPA + Lexa groups. Each treatment group was treated twice per week for 4 weeks via tail-vein injection with the indicated regimen. Before initiating therapy, the average tumor area for each group was 73.3 ± 12.0 , 82.6 ± 12.8 , 73.1 ± 19.2 , and 80.4 ± 5.5 mm² for the vehicle, Lexa, TPA, and TPA + Lexa groups, respectively (Fig. 3B; *P* value, not significant). However, on weeks 3 and 4 after initiating therapy, the group treated with TPA + Lexa had a much lower tumor area than any of

the other treatment groups (mean tumor area for week 3: $50.6 \pm 10.6 \text{ mm}^2$; $P < 0.05$; for week 4: $41.7 \pm 11.2 \text{ mm}^2$, $P = 0.01$; Fig. 3B). The average tumor weight of the animals treated with vehicle, Lexa, or TPA was comparable, whereas the median tumor weight of the animals treated with TPA + Lexa was 5-fold lower as compared with the other groups [Tumor weights (median $\pm$ SE): vehicle $856 \pm 415 \text{ mg}$; Lexa $1,306 \pm 257 \text{ mg}$; TPA $1,297 \pm 305 \text{ mg}$; TPA+ Lexa $150 \pm 65 \text{ mg}$; Fig. 3C, $P < 0.05$].

To examine whether reduced tumor volume and weight in TPA + Lexa-treated animals is associated with increased apoptosis rate, the xenografts were analyzed for TUNEL staining. We found that the TPA + Lexa-treated group had a significantly higher rate of TUNEL staining compared with other treatment groups (Fig. 4A and Supplementary Fig. S1).

Because our *in vitro* studies have shown that TPA promotes AP-1-related proteins, we evaluated the expression of c-Fos in the xenografts from different treatment groups. Similar to our *in vitro* findings, we found that c-Fos protein levels were elevated in xenografts that were exposed to TPA or TPA + Lexa (Fig. 4B). In our previous studies, we have shown that c-Fos binds and represses the transcription of the antiapoptotic protein, c-FLIP(L) (11–13). Therefore, we wished to evaluate protein levels of c-FLIP(L) in the xenografts. We found that c-FLIP(L) protein levels were reduced in the animals that were treated with either TPA or TPA + Lexa (Fig. 4B). Although c-FLIP(L) levels were reduced in the TPA-treated group, the xenograft volume and weight were not significantly affected in the TPA-treated group, a finding that is different from our *in vitro* studies (Fig. 2D), suggesting that reduction of c-FLIP(L) alone was not sufficient to affect tumor burden *in vivo*. To investigate whether the differences in c-FLIP(L) expression *in vitro* and *in vivo* are not secondary to using TRAIL (for the *in vitro* studies) and the TRAIL receptor agonist, lexatumumab (for the *in vivo* studies), we used lexatumumab in combination with TPA in *in vitro* studies. We found that both lexatumumab and TRAIL, when used alone or in combination with TPA, had similar effects on AP-1 activity and c-FLIP(L) levels (Figs. 2B and D and 4C). Therefore, these results underscore the differences between *in vitro* and *in vivo* studies (29).

Next, we evaluated the possible toxicity of our treatments in the animals. Because lexatumumab is a humanized monoclonal antibody, we did not expect that it would activate the mouse TRAIL receptor and lead to side effects in the mouse. However, to examine the possible side effects of TPA, animals were weighed twice per week during the study period. We did not observe any difference between the total body weight of the animals in either the vehicle-treated or any of the other treatment groups. In addition, at the completion of the study when the mice were euthanized, liver, lung, kidney, heart, and brains of the mice were harvested and assessed by H&E microscopic evaluation. We did not observe any evidence of toxicity or necrotic changes in any of the organs examined (Supplementary Fig. S1). Therefore, in TRAIL-resistant prostate cancer cell, LNCaP, the combination of low-dose TPA with the TRAIL receptor 2-agonist antibody, lexatumumab, can dramatically decrease tumor area and weight *in vivo* and significantly increase apoptosis by increasing c-Fos levels and reducing c-FLIP(L) levels.

TPA enhances TRAIL-induced apoptosis in androgen-independent prostate cancer cells. Because a majority of the morbidity

and mortality associated with prostate cancer is secondary to the progression from an androgen-dependent to an androgen-independent state and inefficacy of currently available systemic regimens (30), we examined whether the combination of TPA and TRAIL is effective against TRAIL-resistant androgen-independent prostate cancer cells. High-dose TPA has been shown to induce apoptosis in the androgen-dependent LNCaP

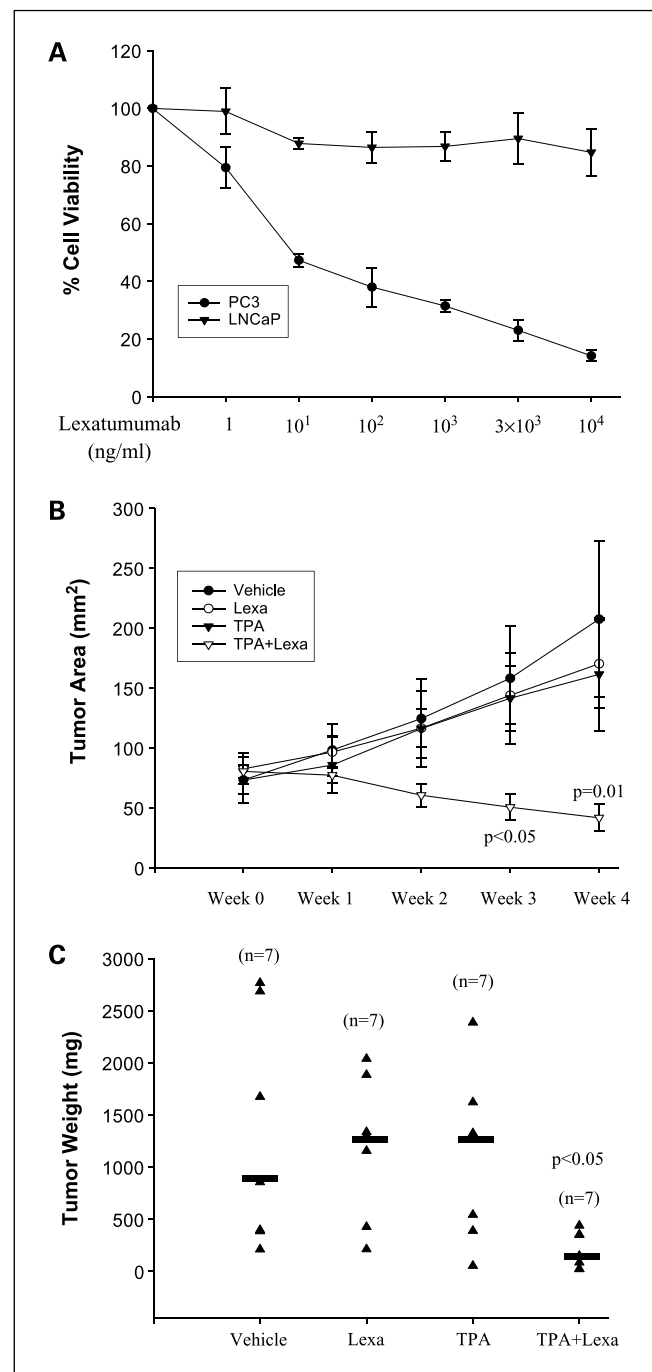


Fig. 3. Combination of TPA and lexatumumab reduces tumor burden in LNCaP xenografts. **A**, cell viability of PC3 and LNCaP cells after treatment with lexatumumab (Lexa). Bars, SD. **B**, average tumor area during the 4-week treatment in vehicle, Lexa, TPA and TPA + Lexa groups. Week 0, tumor sizes before initiating therapy. **C**, scatter plot of tumor weight. Triangle, tumor weight of a xenograft; black bars, median weight for the tumors in each group ($n = 7$ per group). Bars, SD. P values represent comparison of the TPA + Lexa group to the other treatment arms.

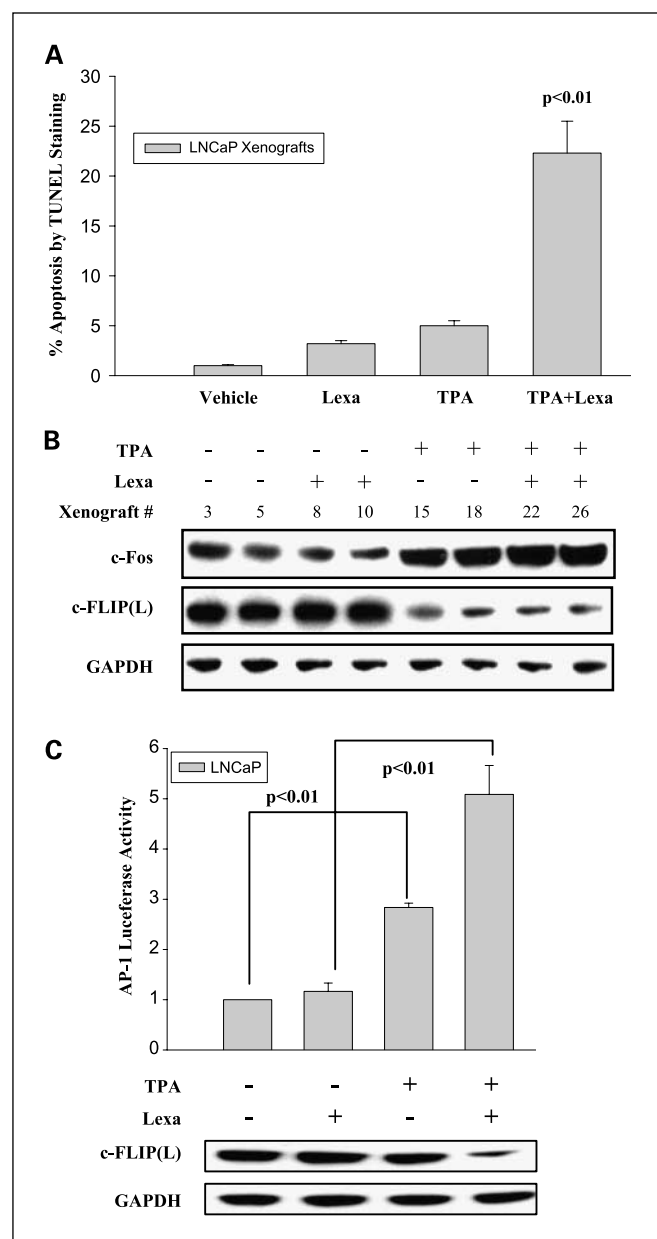


Fig. 4. Combination of TPA and lexatumumab increases apoptosis in LNCaP xenografts. **A**, TUNEL assay; percentage of apoptotic cells was measured by evaluating three randomly selected microscopic fields at 40 $\times$ magnification. Bars, SD. *P* value is measured by comparisons between TPA + Lexa group and other treatment groups. **B**, Western blot analysis of c-Fos and c-FLIP(L) in LNCaP tumor xenografts. **C**, *in vitro* AP-1 activity and c-FLIP(L) protein levels for LNCaP cells. Cells were pretreated with TPA (100 ng/mL) for 24 h and then treated with lexatumumab (10 μ g/mL) for 4 h when assessing AP-1 activity levels and for 24 h when assessing for c-FLIP(L) protein levels.

prostate cancer cells, whereas TPA is thought to be ineffective in androgen-independent prostate cancer cells (17, 18). Because high-dose TPA is associated with tumor progression properties (15, 21), we determined whether low-dose TPA, when combined with TRAIL, can also sensitize androgen-independent prostate cancer cells that are resistant to TRAIL-induced apoptosis. We found that C4-2 cells, an androgen-independent subline of LNCaP cells (31), were resistant to TRAIL-induced apoptosis, a finding similar to the parental LNCaP cells, which

are androgen dependent (Figs. 1A and 5A). Next, we determined whether TPA can enhance the proapoptotic properties of TRAIL. We found that the combination of TPA with TRAIL converted the phenotype of TRAIL-resistant C4-2 cells to a TRAIL-sensitive phenotype as evidenced by increased apoptotic rate (Fig. 5B) and reduced cell viability (Fig. 5C). In concert with our earlier findings in the androgen-dependent LNCaP cells (Fig. 2B), the enhancement of TRAIL-induced apoptosis was associated with increased AP-1 activity in the androgen-independent C4-2 cells (Fig. 5D). Therefore, low-dose TPA is capable of converting the phenotype of TRAIL-resistant prostate cancer cells in both androgen-dependent and androgen-independent states by increasing AP-1 gene activity.

TPA enhancement of TRAIL-induced apoptosis is independent of DR4 levels. Treatment of LNCaP cells with TRAIL does not alter the protein levels of the DR4 or DR5 TRAIL receptors (Fig. 6A, *top*). Some investigators have suggested that TPA enhances the expression of TRAIL receptor DR4 via an AP-1-dependent mechanism (32). However, it is unclear whether increased levels of DR4 are associated with the enhancement of TRAIL-induced apoptosis. Similar to others (32), we found that TPA increased TRAIL receptor DR4 levels, but not DR5 levels (Fig. 6A, *bottom*). Next, we determined whether DR4 and/or DR5 play a functional role in TPA-enhanced TRAIL-induced apoptosis. Because recombinant TRAIL activates both DR4 (TRAIL-R1) and DR5 (TRAIL-R2) by promoting the trimerization of these cell surface receptors, we used fully human monoclonal antibodies specifically targeted against TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab; refs. 5, 6). We found that mapatumumab alone or in combination with TPA did not enhance TRAIL-induced cell death in LNCaP cells (Fig. 6B). In contrast, lexatumumab, when combined with TPA, promoted cell death and reduced cell viability in the TRAIL-resistant prostate cancer cells (Fig. 6B), which is similar to the results using soluble TRAIL combined with TPA (Fig. 1D) and compatible with our xenograft *in vivo* studies (Fig. 3B and C). Furthermore, we examined whether the inhibition of DR4 by RNAi (Fig. 6C, *inset*) or ectopic expression of DR4 (Fig. 6D, *inset*) would alter sensitivity to TRAIL-induced apoptosis. We found that neither inhibition of DR4 (Fig. 6C) nor increased DR4 levels (Fig. 6D) were associated with TRAIL-induced or TPA-enhanced TRAIL-induced cell death (Fig. 6C and D). Conversely, overexpression of DR5 alone sensitized LNCaP cells to TRAIL-induced apoptosis, which was even further enhanced when TRAIL was combined with TPA (Supplementary Fig. S2). Therefore, the activation of apoptosis through TRAIL receptor 2 (DR5) by lexatumumab or TRAIL, in combination with TPA treatment, can promote cell death in TRAIL-resistant LNCaP cells. In contrast, TRAIL receptor 1 (DR4) level is not associated with TPA-enhanced TRAIL-induced apoptosis. Our findings suggest that DR5-mediated pathways are more critical to TPA-enhanced TRAIL-induced apoptosis than the DR4-mediated pathways.

Discussion

Prostate cancer is the second leading cause of death in men, accounting for 232,900 new cases annually (33). Typically, localized prostate cancer is treated effectively with surgery or radiotherapy and for carefully selected cases with watchful waiting (34). However, advanced hormone refractory prostate

cancer is fatal and accounts for 30,350 deaths annually (33). The proapoptotic agent, TRAIL, has great potential as an antitumor agent because it selectively induces apoptosis in cancer cells (1–3). Although many cancer cells are sensitive to TRAIL-induced apoptosis, some develop resistance. Many groups have been investigating the synergistic effects of different drugs in combination with TRAIL to overcome the resistance developed by cancer cells (35–42). Previously, we have shown that the activation of c-Fos/AP-1 is a necessary component for cancer cells to undergo TRAIL-induced apoptosis (12, 13). Therefore, in the present study, we investigated whether the activation of c-Fos/AP-1 by a synthetic compound, TPA, may convert the TRAIL-resistant prostate cancer cells to become TRAIL sensitive. We show that TRAIL combined with low-dose TPA effectively sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis *in vitro* and *in vivo*. Low-dose TPA sensitizes TRAIL-resistant prostate cancer cells by up-regulating the AP-1 family proteins and AP-1 activity. Moreover, the combination of TRAIL with low-dose TPA enhances cell death in androgen-dependent and androgen-independent prostate cancers, but not in nonmalignant transformed BPH-1 cells.

From our current and past experience (11), TRAIL sensitivity does not seem to be related to androgen dependency of prostate cancer cells. For example, we have found that some androgen-independent cells such as PC3 and DU145, cells are sensitive to TRAIL, whereas other androgen-independent cells such as C4-2 are resistant to TRAIL (ref. 11; Fig. 5). Conversely, the androgen-dependent LNCaP and CWR22 cells are resistant to TRAIL (Fig. 1 and data not shown). Therefore, TPA can enhance TRAIL-induced apoptosis in resistant prostate cancer cells regardless of their androgen dependency state. Clinically, this is an important distinction because low-dose TPA can potentially enhance the proapoptotic activity of TRAIL in prostate cancer patients in androgen-dependent and androgen-independent states, therefore making this combination therapy more widely available for prostate cancer patients with advanced disease.

TRAIL binds to at least five cell surface receptors: DR4, DR5, and three decoy receptors DcR1, DcR2, and osteoprotegerin (43). Only binding to DR4 or DR5 initiates TRAIL-induced apoptosis. When TRAIL and TRAIL receptors were initially identified, it was logical to suspect that expression levels of TRAIL receptors may contribute greatly to TRAIL sensitivity

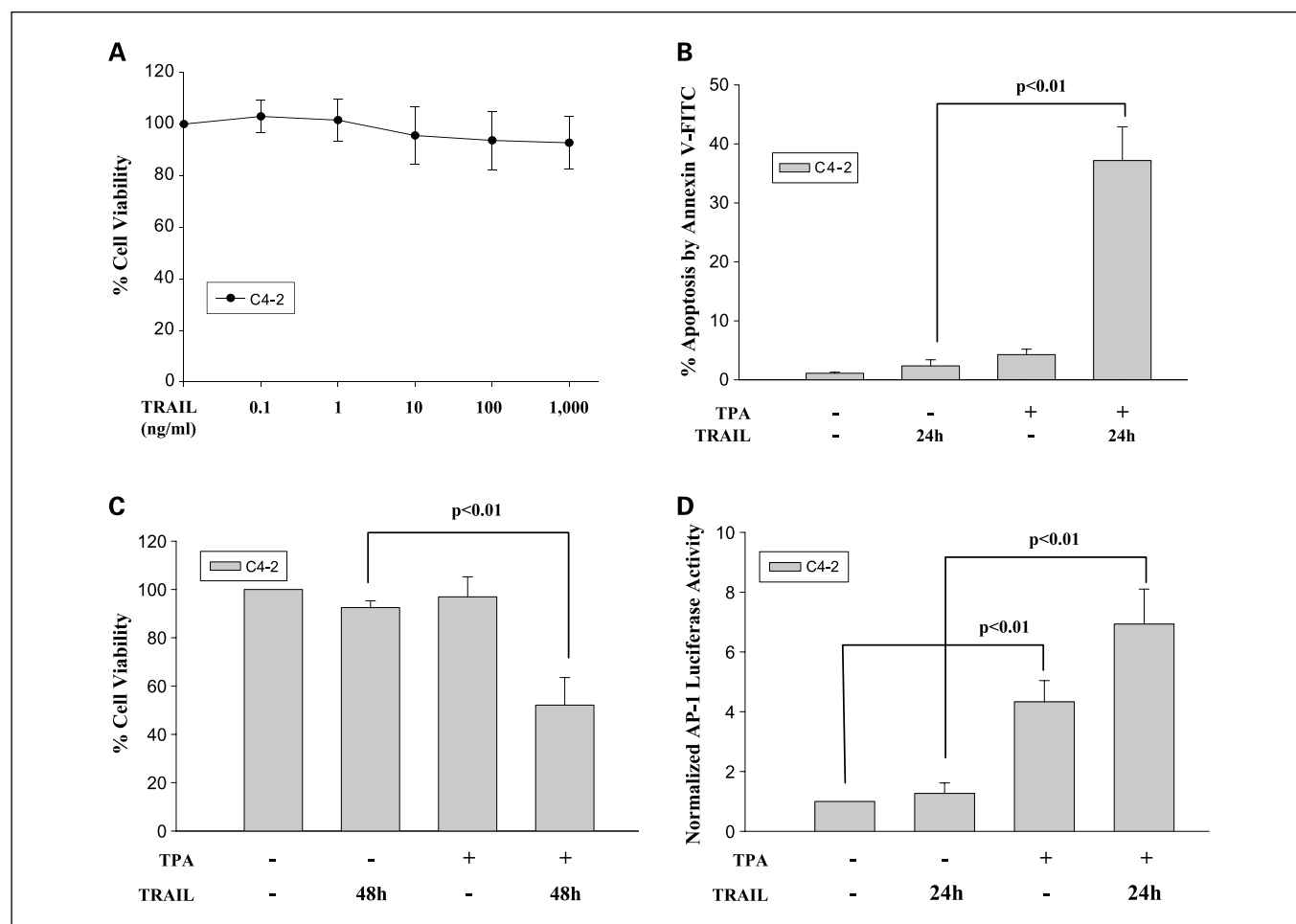


Fig. 5. TPA enhances TRAIL-induced apoptosis in androgen-independent prostate cancer C4-2 cells. *A*, cell viability of C4-2 cells treated with increasing doses of TRAIL for 48 h. Percentage of apoptotic cells measured by *(B)* Annexin V – FITC staining, *(C)* cell viability, and *(D)* AP-1 luciferase activity of C4-2 cells treated with TPA and/or TRAIL. C4-2 cells in *(B)* to *(D)* were treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h followed by TRAIL (100 ng/mL) treatment for the indicated times. Bars, SD from at least three independent experiments.

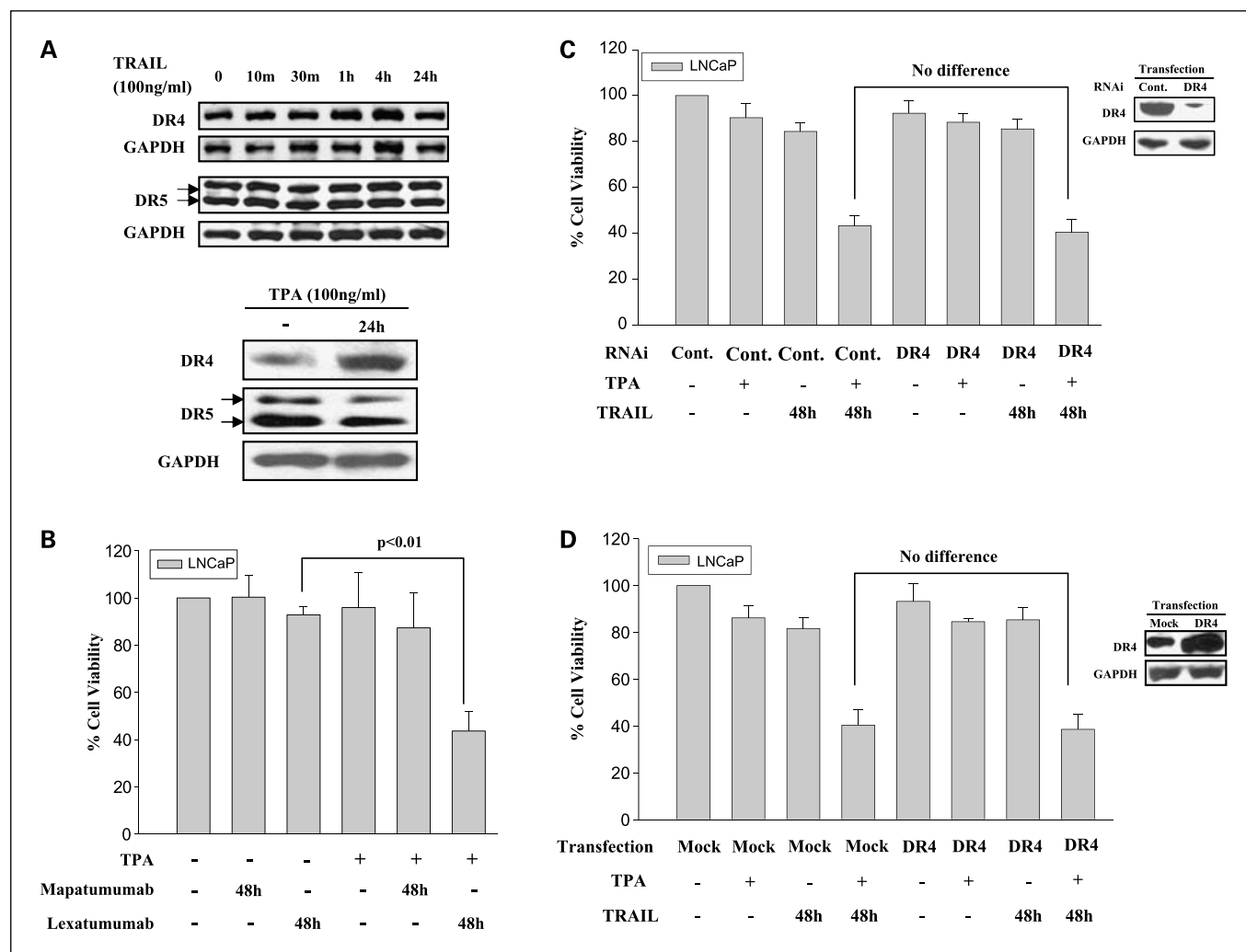


Fig. 6. TPA enhancement of TRAIL-induced apoptosis is independent of DR4 levels. **A**, Western blots for DR4 and DR5 levels in LNCaP cells after treatment with TRAIL or TPA. **B**, cell viability of LNCaP cells treated with TPA alone (100 ng/mL) or pretreated with TPA (100 ng/mL) for 24 h followed by treatment with the DR4 agonist, mapatumumab (10 μ g/mL), or the DR5 agonist, lexatumumab (10 μ g/mL), for another 48 h. **C**, cell viability for LNCaP cells after transfecting DR4 RNAi for 16 h and then pretreated with TPA for 24 h, followed by treatment with TRAIL for 48 h. **D**, cell viability of LNCaP cells, which were determined after ectopic expression of DR4 for 24 h, followed by pretreatment with TPA for 24 h followed by treatment with TRAIL for an additional 48 h. **C** and **D**, insets, Western blots for DR4. GAPDH is used as loading control. Bars, SD from at least three independent experiments.

(44). However, later studies have shown that there are no significant associations between TRAIL sensitivity and expression level of TRAIL receptors (45). Recently, it has been shown that TPA enhances the expression of DR4 by increasing AP-1 binding at the DR4 promoter region (32); however, it has been unclear whether increased DR4 levels by TPA can potentiate the sensitivity of prostate cancer cells to TRAIL-induced apoptosis. In this study, we showed that TRAIL treatment alone does not change DR4 and DR5 levels (Fig. 6A), and we also confirmed that TPA increased DR4 protein levels, as has been shown by others (32). However, we found that increased DR4 levels alone are not associated with enhancing TRAIL-induced apoptosis because ectopic expression of DR4 in LNCaP cells did not potentiate TRAIL-induced apoptosis or TPA-enhanced TRAIL-induced apoptosis (Fig. 6D). To investigate whether DR4 or DR5 may be more important for TRAIL-induced apoptosis when combined with TPA, we used human monoclonal agonist antibodies, map-

tumumab and lexatumumab, which target TRAIL-R1 and TRAIL-R2, respectively. We found that lexatumumab (TRAIL-R2 agonist) and not mapatumumab (TRAIL-R1 agonist) is capable of promoting cell death when combined with low-dose TPA. In addition, ectopic expression of DR5 sensitizes LNCaP cells to TRAIL-induced apoptosis particularly when combined with TPA (Supplementary Fig. S2). Therefore, TRAIL-R2 cell surface receptor pathway is preferentially activated in this prostate model system during TPA-enhanced TRAIL-induced apoptosis. Our findings are further supported by a recent report that DR5 has a greater contribution to TRAIL-induced apoptosis than DR4 (46).

Many approaches have been employed to overcome TRAIL resistance in cancer cells, notably by combination therapy of TRAIL with chemotherapy or radiotherapy (40–42). A concern about using TPA is its potential toxicity and tumor-promoting properties. Some studies have shown that TPA can act as a tumor promoter in skin tumorigenesis with relatively high

concentrations (2.5 nmol/L; refs. 47, 48). In other studies, TPA has been shown to promote apoptosis as a single agent in androgen-dependent prostate cancer cells at concentrations of 10 to 50 nmol/L (17, 18, 23), concentrations that are 60- to 300-fold higher than the one used in our *in vitro* and *in vivo* studies (i.e., 100 ng/mL for *in vitro* studies or 100 ng/g for *in vivo* studies is equal to 0.162 nmol/L). In addition, TPA has been used successfully in patients with refractory hematologic malignancies in phase I clinical trials in China and the United States (15, 21, 22). In our study, we specifically focused on using a low dose of TPA to minimize its potential toxicity. Assessing the animals in our *in vivo* studies by body weight and histology of multiple different organs did not show any grossly

detectable toxic effects. In addition, recent clinical trials that have used lexatumumab for treatment of various malignancies as a single agent have not been associated with any significant toxicity (5, 6, 28).

In conclusion, we show that TPA activates AP-1 family of proteins and enhances TRAIL-induced apoptosis in both androgen-dependent and androgen-independent prostate cancer cells. In our system, the activation of the DR5 TRAIL receptor may play a more important role than activation of the DR4 TRAIL receptor. Further studies are required to determine whether the combination of TPA with TRAIL agonist compounds is suitable for patients with advanced prostate cancer.

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MG-132 Sensitizes TRAIL-Resistant Prostate Cancer Cells by Activating c-Fos/c-Jun Heterodimers and Repressing c-FLIP(L)

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent because it induces apoptosis in cancer cells but not in normal cells. Unfortunately, some cancer cells develop resistance to TRAIL-induced apoptosis. Therefore, it is clinically relevant to determine the molecular mechanisms that differentiate between TRAIL-sensitive and TRAIL-resistant tumors. Previously, we have shown that the antiapoptotic molecule cellular-FLICE-inhibitory protein long isoform [c-FLIP(L)] is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. We have found that c-FLIP(L) is transcriptionally regulated by the activator protein-1 (AP-1) family member protein c-Fos. Here, we report that MG-132, a small-molecule inhibitor of the proteasome, sensitizes TRAIL-resistant prostate cancer cells by inducing c-Fos and repressing c-FLIP(L). c-Fos, which is activated by MG-132, negatively regulates c-FLIP(L) by direct binding to the putative promoter region of the *c-FLIP(L)* gene. In addition to activating c-Fos, MG-132 activates another AP-1 family member, c-Jun. We show that c-Fos heterodimerizes with c-Jun to repress transcription of c-FLIP(L). Therefore, MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating the AP-1 family members c-Fos and c-Jun, which, in turn, repress the antiapoptotic molecule c-FLIP(L). [Cancer Res 2007;67(5):2247–55]

Introduction

Prostate cancer is the second leading cause of cancer death in American men accounting for 232,900 new cases and 30,350 deaths annually (1). In a majority of cases, early-stage prostate cancer can be treated effectively with surgery or radiotherapy. However, advanced hormone refractory metastatic prostate cancer can be a fatal disease without effective treatment.

Cell surface death receptor ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has attracted attention to cancer therapy not only because of its ability to effectively kill cancer cells but also because it has little effect on normal cells; therefore, TRAIL has minimal cytotoxicity (2). TRAIL induces apoptosis by binding to DR4 and DR5, two related death receptors, causing the formation of a death-inducing signaling complex, which includes the receptors, the adaptor protein FADD, and caspase-8 (3, 4). Autoactivated caspase-8 initiates the apoptotic

executing caspase cascade and subsequent programmed cell death (extrinsic cell death pathway). Activation of Bid to its truncated form, tBid, leads to the release of cytochrome *c* from the mitochondria, which then activates the mitochondrial-mediated proapoptotic pathway (intrinsic cell death pathway; ref. 5).

Although many cancers undergo TRAIL-induced apoptosis, some develop resistance (6). Cellular sensitivity for TRAIL-induced apoptosis can be modulated at several levels. Inducing the expression of DR5 can enhance TRAIL signaling and overcome TRAIL resistance in cancer cells (7, 8). TRAIL-induced apoptosis can also be modulated at the mitochondrial level by the proapoptotic molecules Bax and Bak and the antiapoptotic molecule Bcl-2 (9). Cellular-FLICE-inhibitory protein (c-FLIP) is another class of important intracellular antiapoptotic molecules, which can block the apoptotic signaling pathway of TRAIL-induced apoptosis. c-FLIP protein homologues interrupt apoptotic signaling by competing with caspase-8 for binding to the DED domains of FADD and also regulate apoptosis through their interference with the recruitment of caspase-8 to FADD (4, 10, 11). The levels of intracellular c-FLIP, therefore, may determine the sensitivity of cancer cells to apoptosis triggered by TRAIL (12, 13). The c-FLIP family of proteins, c-FLIP(L), c-FLIP(s), and perhaps the newly detected c-FLIP(r) (14), can bind to the DED domains of FADD and caspase-8 and regulate apoptosis through their interference with the recruitment of caspase-8 to FADD.

We have shown in the past that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (6). Intracellular c-FLIP(L) can be regulated at either the transcriptional, translational, or posttranslational levels. Expression of c-FLIP(L) has been shown to be modulated by nuclear factor- κ B (NF- κ B; refs. 15, 16), Akt (17, 18), c-Myc (13), p53 (19), and E3-ubiquitin ligase (20). We have found that transcriptional repression of c-FLIP(L) by the AP-1 family member protein c-Fos is critical in modulating resistance and sensitivity of cells in TRAIL-induced apoptosis.¹

To sensitize TRAIL-resistant cancer cells, proteasome inhibitors have been combined with TRAIL in a variety of different cancer models. For example, the proteasome inhibitor PS-341 has been shown to help overcome TRAIL resistance in colon and bladder cancer cells (21–25). Another proteasome inhibitor, MG-132, has a potent antitumor function and has been shown to sensitize resistant cancer cells to the proapoptotic effects of TRAIL (7, 8, 26, 27). In this study, we examined the mechanism that MG-132 sensitizes prostate cancer cells to TRAIL-induced apoptosis. We show that MG-132 sensitizes TRAIL-resistant

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prostate cancer cells by up-regulating the AP-1 family proteins c-Fos and c-Jun, which, in turn, repress the antiapoptotic molecule c-FLIP(L). c-Fos/c-Jun heterodimers bind to the c-FLIP(L) promoter, repress its transcriptional activity, and reduce c-FLIP(L) mRNA and protein levels. These findings suggest that elevated c-Fos and c-Jun can play an important role in determining whether a cell is responsive or resistant to the proapoptotic effects of TRAIL.

Materials and Methods

Chemicals and antibodies. Recombinant human TRAIL/TNFSF10 was obtained from R&D Systems, Inc. (Minneapolis, MN). Proteasome inhibitor MG-132 was obtained from EMD Calbiochem (La Jolla, CA). Antibodies were obtained from the following sources: horseradish peroxidase-conjugated secondary antibody (goat anti-mouse, goat anti-rabbit, and goat anti-rat antibodies), Oct-1 (C-21), c-Fos (D1), Fos B (C-11), Fra-1 (N-17), Fra-2 (L-15), JunB (N-17), Jun D (329), and c-Fos small interfering RNA (siRNA) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). c-Jun, phospho-c-Jun (Thr91), and c-Fos antibodies were obtained from Cell Signaling (Beverly, MA). Monoclonal c-FLIP(L) antibody (Dava II) was obtained from Apotech Corp. (San Diego, CA). Phospho-c-Fos (T232) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Abcam, Inc. (Cambridge, MA).

Cell lines. PC3 and LNCaP prostate cancer cell lines and HEK 293T cells were from the American Type Culture Collection (Manassas, VA). BPH-1 (benign prostatic hyperplasia cells immortalized with SV40 large T antigen) cells were provided by Dr. Simon Hayward (Vanderbilt University, Nashville, TN; ref. 28). PC3-TR was a TRAIL-resistant subline established from parental PC3 cells by TRAIL treatment selection (6).

Cell viability and apoptosis assays. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method (Cell TITER 96 Aqueous Assay, Promega, Madison, WI). Cells cultured in 96-well plates were treated with TRAIL and/or MG-132 for 24 h. MTS substrates were added and incubated for 2 h at 37°C. Absorbance was measured at 490 nm. Viability of control cells treated with DMSO was set at 100%, and absorbance of wells with medium and without cells was set at zero.

For apoptosis assays, cells were washed with PBS and resuspended in binding buffer from Sigma Chemical Co. (St. Louis, MO), and stained with FITC-conjugated Annexin V (Roche Diagnostic Co., Indianapolis, IN) and propidium iodide for 15 min at room temperature. Annexin V fluorescence was determined with a FACScan flow cytometer, and the membrane integrity of the cells was simultaneously assessed by the propidium iodide exclusion method.

Cell extracts and Western blot analysis. Cells were harvested for total cell lysates with radioimmunoprecipitation assay buffer [RIPA; 1% NP40, 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, and 0.1% SDS] containing a mixture of protease inhibitors [cocktail 1×, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 20 mmol/L, 40 mmol/L NaF, and 3 mmol/L Na₂VO₄]. After sonication for 15 s, cell debris was discarded by centrifugation at 12,000 × g for 10 min at 4°C, and the protein concentration was determined by bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rockford, IL). The procedure for the nuclear protein extraction was carried out according to the manufacturer's instructions (NE-PER nuclear and cytoplasmic extraction reagents kit, Pierce Biotechnology). Cells were swollen with hypotonic buffer and then disrupted. The cytoplasmic fraction was removed, and the nuclear protein was released from the nuclei by a high-salt buffer. The lysate was boiled for 10 min and frozen at -80°C. Western blot was carried out as previously described (6).

Semiquantitative reverse transcription-PCR analysis. Total RNA was isolated with the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA yield and purity were evaluated by measuring A_{260}/A_{280} and agarose gel electrophoresis. Reverse transcription-PCR (RT-PCR) was done using a Superscript One-Step RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA). The total

RNA (0.4 µg) was used in RT-PCR of 25 µL reaction system. cDNA synthesis was done at 50°C for 30 min using the following cycle temperatures and times: denaturation at 94°C for 50 seconds, annealing at 56°C for 50 seconds, and polymerization at 72°C for 2 min (total number of cycles, 30) with a final extension at 72°C for 10 min. In each reaction, the same amount of GAPDH was used as an internal control. The primers used for PCR were as follows: c-FLIP(L), 5'-GTCTGCTGAAGTCATCCATCAG-3' (forward) and 5'-CTTATGTGTAGGAGAGGATAAG-3' (reverse); c-Fos, 5'-GAATAAGATGGCTGCAGCCAAATGC-3' (forward) and 5'-AAGGAAGACGTGTAAGCAGTGCAGC-3' (reverse); and GAPDH, 5'-TCCACCACCCTGTTGCTGTA-3' (forward) and 5'-ACCACAGTCCATGCCATCAC-3' (reverse). The PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed.

Luciferase assay. c-FLIP(L) promoter luciferase structure was kindly provided by Dr. W.S. El-Deiry (University of Pennsylvania, Pennsylvania, PA; ref. 13). Cells were seeded into 24-well plates. When cells reached 50% to 80% confluence, both AP-1 luciferase reporter (25 ng/well) and *Renilla* reporter (5 ng/well) from Stratagene (La Jolla, CA) or c-FLIP(L) promoter and *Renilla* reporter were cotransfected into cells. In other experiments, c-Fos siRNA or full-length human c-Fos cDNA plasmid was transfected into cells for 24 h before transfection of luciferase and *Renilla*. *Renilla* acted here as an internal control for transfection efficiency. After 24 h of transfection, cells were treated with TRAIL (100 ng/mL). Thereafter, cells were collected, prepared, and further detected by using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Samples were stored at -20°C until detection. All results represent an average of at least three independent experiments ± SD.

Transfection with c-Fos vector or c-Fos siRNA. A full-length human c-Fos cDNA, provided by Dr. L. Shemshadini (University of Toledo, Toledo, OH), was cloned into a pSG5 vector (29). Plasmids with or without c-Fos were transfected with LipofectAMINE 2000 (Invitrogen Life Technologies). siRNA of c-Fos was then transfected into cells by TransMessenger Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. After transfection with the c-Fos vector for 24 h or the c-Fos siRNA for 36 to 48 h, the cells were seeded in 96-well plates for cell viability assays or treated with TRAIL for Western blot assays.

Immunoprecipitation and immunoblotting. Cells were lysed at 4°C for 30 min in RIPA lysis buffer containing protease inhibitors. Lysates were centrifuged at 12,000 × g at 4°C for 10 min to remove insoluble materials. The supernatants were then collected, and the total protein was determined using the BCA assay (Pierce). Supernatants of equal amounts of protein were incubated at 4°C overnight with either c-Fos antibody or IgG control antibody. Protein A-Sepharose was added and incubated at 4°C for 1 to 4 h. The immunocomplexes were washed thrice in cold lysis buffer. The bound proteins were eluted from the column in preheated sample buffer [50 mmol/L Tris-HCl (pH 6.8), 50 mmol/L DTT, 1% SDS, 0.005% bromophenol blue, and 10% glycerol] and denatured by boiling for 5 min. The immunoprecipitates and whole lysate proteins were then subjected to 4% to 12% SDS-PAGE. Immunoblot analysis was done with the indicated antibodies.

Cell extracts and electrophoretic mobility shift assay. Frozen cell pellets were resuspended in 4 volumes of lysis buffer: 20 mmol/L HEPES (pH 7.9); 0.2 mmol/L EDTA; 0.2 mmol/L EGTA; 10% glycerol; 10 mmol/L Na molybdate; 2 mmol/L Na PPI; 2 mmol/L Na orthovanadate; 0.5 mmol/L spermidine; 0.15 mmol/L spermine; 50 µmol/L *N*-tosyl-L-phenylalanine chloromethyl ketone; 25 µmol/L *N*-α-p-tosyl-L-lysine chloromethyl ketone; 1 µg/mL each of aprotinin, pepstatin A, and leupeptin; 0.5 mmol/L benzamidate; 1 mmol/L DTT; and 0.5 mmol/L PMSF. KCl was added to 400 mmol/L final, and the extracts were incubated at 4°C for 30 min and centrifuged at 10,000 × g for 5 min. The supernatant contained the whole-cell extracts. The reactions were made using 3 µL of whole-cell extract and 0.1 to 0.5 ng of ³²P-labeled double-stranded specific oligonucleotides (5,000–25,000 cpm) and run on 5% to 7% polyacrylamide gels containing 0.5× Tris glycine EDTA. Gels were dried with Bio-Rad gel dryer (Bio-Rad, Hercules, CA) and imaged using Kodak BioMax MR Film (Fisher Scientific, Atlanta, GA). General AP-1 gel shift oligonucleotide was obtained from Santa Cruz Biotechnology. Wild-type oligonucleotides of the c-FLIP(L)

AP-1(f) site was designed as 5'-ATCACTTGAGGATCACTTGAGGATCAC-TTGAGGATCACTTGAGG-3'.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done using the ChIP Assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY). PC3-TR cells were cultured in 10-cm dishes and treated with TRAIL and/or MG-132 for 4 h. Cross-linking of DNA and proteins were fixed by adding formaldehyde directly to the culture medium to a final concentration of 1% and incubated for 10 min at 37°C. Cells were collected and washed with PBS that contained protease inhibitors. Harvested cells were resuspended in 200 μ L of SDS lysis buffer for 10 min. Cell lysates were sonicated and samples were centrifuged at 12,000 $\times g$ for 10 min at 4°C, and the supernatant was harvested. Concentration of each sample was quantitated by the BCA technique (Pierce Biotechnology). Positive controls were 10% of each DNA sample, which did not include the immunoprecipitation step. The remainder of the samples was divided equally into two groups. The experimental group was immunoprecipitated with specific c-Fos (D-1) antibody, whereas the negative control group was immunoprecipitated with the general IgG antibody. After eluting protein-DNA from antibody, protein-DNA cross-linking was reversed by heating at 65°C for 4 h. The isolated genomic DNA was first purified by phenol/chloroform extraction and ethanol precipitation. Then, the DNA was amplified by PCR, using specific primers encompassing the region containing the AP-1(f) binding site according to the human c-FLIP(L) sequence (Genbank). The conditions were as follows: primers 5'-CCTGTGATCCCAGCACTTGG-3' (forward) and 5'-CAC-CATGCCGACTAATTTT-3' (reverse); denaturation at 94°C for 30 seconds; annealing at 56°C for 45 seconds; polymerization at 72°C for 30 seconds, for 25 cycles. Finally, PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Results

MG-132 sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis. Although PC3 cells are sensitive to TRAIL-induced apoptosis, PC3-TR and LNCaP cells are resistant to the proapoptotic effects of TRAIL (Fig. 1A; ref. 6). Combination of TRAIL with MG-132 sensitizes resistant prostate cancer cells, PC3-TR, and LNCaP, to undergo apoptosis (Fig. 1B and C). Because TRAIL is more effective against cancer cells than benign immortalized cells (2), we wished to determine whether the effect of MG-132 + TRAIL is specific to cancer cells or whether immortalized but nontumorigenic cells undergo cell death. Nontumorigenic and immortalized 293T (human embryonic kidney) and BPH-1 (benign prostatic hyperplasia) cells were treated with MG-132, TRAIL, or in combination with MG-132 + TRAIL. We found that neither treatment as single agents nor combination of treatments promoted cell death in the immortalized noncancerous cell lines (Fig. 1D). These data suggest that MG-132 is capable of sensitizing cancerous cells, but not benign transformed cells, to undergo TRAIL-induced apoptosis.

Combination of TRAIL and MG-132 represses c-FLIP(L) and induces c-Fos. The antiapoptotic protein c-FLIP(L) plays an important role in TRAIL sensitivity of cancer cells. We have shown in the past that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (6). In addition, we have found that in TRAIL-sensitive cancer cells, the antiapoptotic molecule c-FLIP(L) is repressed by the AP-1 family protein c-Fos, a mechanism that is lacking in TRAIL-resistant cancer cells.¹ Because MG-132 sensitizes resistant cancer cells to undergo TRAIL-induced apoptosis, we wished to determine whether the ability of c-Fos to repress c-FLIP(L) is restored in the presence of MG-132.

c-FLIP(L) protein and mRNA levels were maintained when the resistant PC3-TR cells were treated with either TRAIL or MG-132

alone (Fig. 2A). However, the combination of MG-132 + TRAIL led to reduction of the antiapoptotic molecule c-FLIP(L) at the mRNA level and protein levels, as shown by the semiquantitative RT-PCR and Western blot analyses (Fig. 2A). The c-FLIP(L) mRNA level was noticeably reduced 12 h after treatment with MG-132 and TRAIL.

Because we have found that up-regulation of the AP-1 family protein c-Fos is necessary for TRAIL-induced apoptosis, we examined whether c-Fos levels are up-regulated in the presence of MG-132. We found that c-Fos protein and mRNA levels were increased in the presence of MG-132 alone (Fig. 2B), a condition that does not promote cell death in resistant prostate cancer cells (Fig. 1B and D). However, combination of MG-132 and TRAIL sensitizes prostate cancer cells to undergo cell death while promoting c-Fos levels at the mRNA and protein levels (Fig. 2B). Decrease in c-FLIP(L) and increase in c-Fos protein levels are observed in a time-dependent (Fig. 2C) and dose-dependent (data not shown) manner. Because c-Fos is a well established transcription factor (30–32), we determined whether there is any significant change in nuclear c-Fos levels in the presence of MG-132. We found that nuclear c-Fos, and more specifically phosphorylated nuclear c-Fos, was increased when treated with MG-132 or MG-132 + TRAIL (Fig. 2D). This result shows that MG-132 sensitizes resistant prostate cancer cells to undergo apoptosis (Fig. 1) by repressing expression of c-FLIP(L) and promoting expression of c-Fos. Similar to our previous results, increased expression of c-Fos in response to MG-132 does not induce cell death; it only primes resistant prostate cancer cells to undergo apoptosis (Figs. 1 and 2).

Combination of TRAIL and MG-132 increases AP-1 activity and decreases c-FLIP(L) promoter activity. Because the combination of MG-132 and TRAIL reduces the expression of c-FLIP(L) and enhances the expression of the AP-1 family member c-Fos (Fig. 2), we wished to determine whether there is any direct interaction between the transcription factor AP-1/c-Fos and the antiapoptotic molecule c-FLIP(L). First, we examined the luciferase AP-1 activity in the resistant PC3-TR cells. We found that MG-132 alone or combination of MG-132 + TRAIL significantly enhanced the AP-1 activity in the resistant PC3-TR cells after 24 h of treatment (Fig. 3A). Enhancement of AP-1 activity was particularly pronounced when MG-132 was combined with TRAIL (Fig. 3A). c-FLIP(L) promoter activity was not significantly changed in the presence of MG-132; however, the combination of MG-132 + TRAIL led to significant reduction of the c-FLIP(L) promoter activity (Fig. 3B). This result, again, suggests that the proteasome inhibitor, MG-132 alone, sensitizes resistant prostate cancer cells to undergo apoptosis by enhancing AP-1 activity, which only in the presence of the proapoptotic agent TRAIL will lead to repression of the c-FLIP(L) antiapoptotic molecule (Fig. 2).

Next, we examined whether inhibition of c-Fos by siRNA can affect c-FLIP(L) promoter activity. To ensure that our siRNA was functioning as expected, AP-1 activity and c-Fos protein levels were assessed in the presence or absence of c-Fos siRNA. We found that AP-1 activity and c-Fos protein levels were reduced in the PC3-TR cells in the presence of c-Fos siRNA (Fig. 3C). In addition, inhibition of c-Fos by siRNA led to increased c-FLIP(L) promoter activity (Fig. 3C).

Next, we wished to examine the effect of inhibiting c-Fos by siRNA on c-FLIP(L) promoter activity when treated with MG-132 and TRAIL. The luciferase activity in the control groups were normalized (Fig. 3D). Then, we examined the luciferase activity when the cells were treated with MG-132, TRAIL, or MG-132

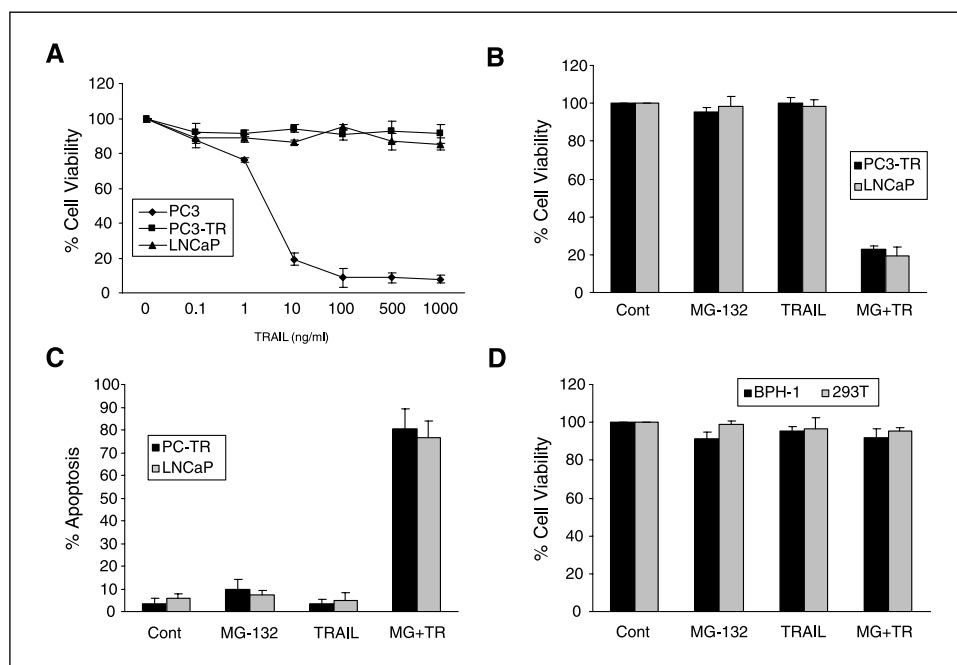


Figure 1. Cell viability and apoptosis assays after treatment with TRAIL and/or MG-132. *Points and columns*, average of at least three independent experiments; *bars*, SD. *A*, sensitivity of prostate cancer cells to TRAIL. Cell viability was evaluated by MTS assay after treatment for 24 h with increasing concentrations of TRAIL. Viability of untreated cells was set at 100%. *B*, TRAIL-resistant prostate cancer cells, PC3-TR or LNCaP cells, were treated with MG-132, TRAIL, or TRAIL (100 ng/mL) + MG-132 (1 μ M) for 24 h. Cell viability was evaluated by MTS. *C*, fluorescence-activated cell sorting analysis for apoptosis after treatment with MG-132 (1 μ M), TRAIL (100 ng/mL), or combination of the two for 24 h. Apoptosis was assessed by FITC-conjugated Annexin V and propidium iodide staining for 15 min at room temperature. The percentage of apoptotic cells was determined by Annexin V–stained positive cells. *D*, cell viability after exposure of nonmalignant benign prostatic hyperplasia (BPH-1) or HEK 293T cells to MG-132 (1 μ M), TRAIL (100 ng/mL), or combination of the two did not induce cell death after 24 h of treatment.

+ TRAIL. We found that c-FLIP(L) promoter luciferase activity did not differ significantly from the controls when the cells were treated with MG-132 or TRAIL alone. c-FLIP(L) promoter activity decreased in the cells that were treated with MG-132 + TRAIL (Fig. 3D, *last two columns*). However, c-Fos siRNA rescued and promoted c-FLIP(L) promoter activity when the cells were treated with MG-132 + TRAIL (Fig. 3D, *last two columns*). In addition, c-Fos siRNA helped maintain the expression of c-FLIP(L) protein (Fig. 4A). To determine whether inhibition of c-Fos by siRNA had any

functional role, we examined the cell viability of PC3-TR cells. As previously shown, we found that combination of MG-132 + TRAIL sensitized prostate cancer cells to undergo apoptosis. However, when c-Fos was inhibited by siRNA, PC3-TR cells became more resistant to cell death than controls when treated with MG-132 + TRAIL (Fig. 4B, *last two columns*).

In contrast, ectopic expression of c-Fos (Fig. 4C, *inset*) increased AP-1 activity and c-Fos protein level as expected, but also led to reduction of c-FLIP(L) promoter activity (Fig. 4C). Ectopic

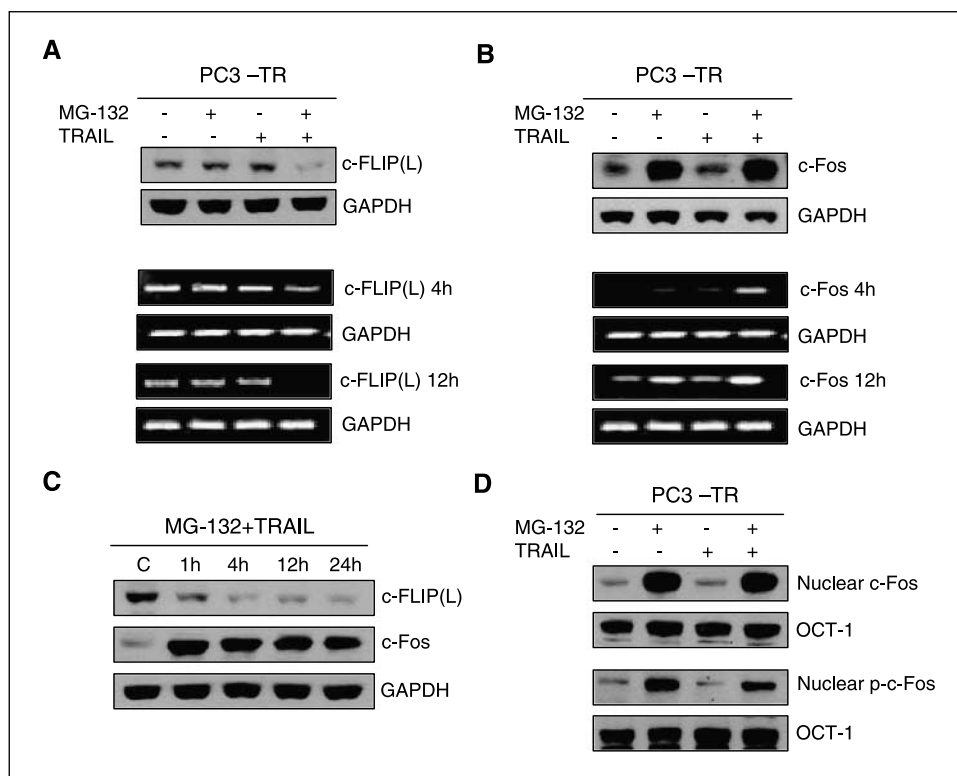
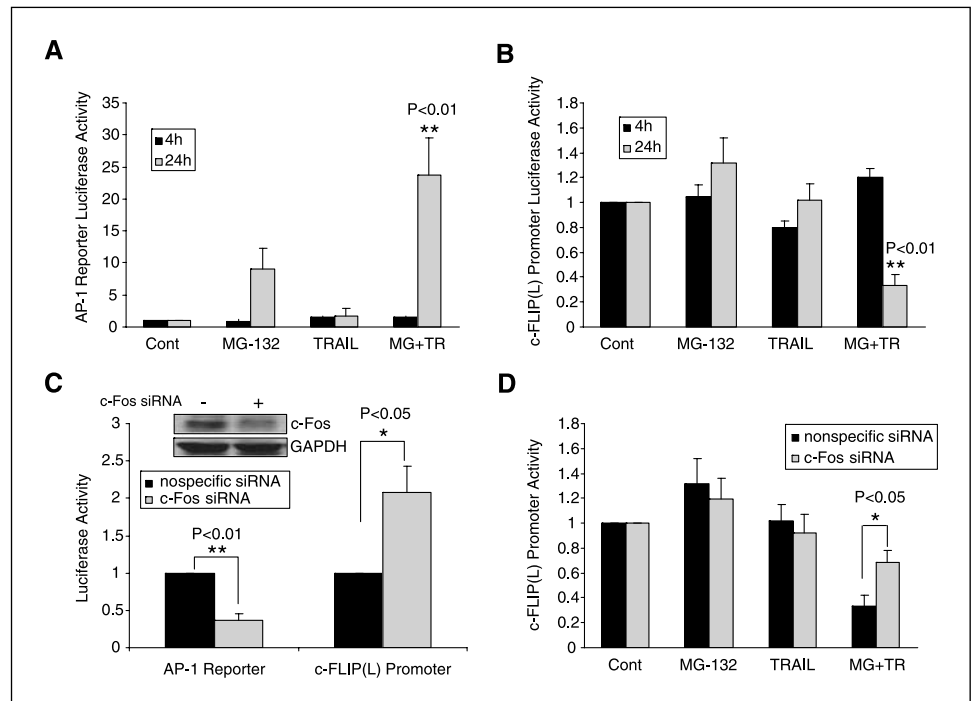


Figure 2. TRAIL combined with MG-132 represses c-FLIP(L) and induces c-Fos. *A* and *B*, c-FLIP(L) (*A*) and c-Fos (*B*) expression by Western blot (*top*) and semiquantitative RT-PCR analysis (*bottom*). PC3-TR cells were treated with MG-132 (1 μ M), TRAIL (100 ng/mL), or MG-132 combined with TRAIL. Western blot results represent 24 h after treatment. GAPDH is used as loading control. *C*, protein expression of c-FLIP(L) and c-Fos. PC3-TR cells were treated with MG-132 (1 μ M) + TRAIL (100 ng/mL) for different times (1, 4, 12, and 24 h). *D*, Western blot analysis of nuclear c-Fos and phosphorylated c-Fos (p-c-Fos) after treatment with MG-132 (1 μ M), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h. Oct-1 is used as loading control of nuclear extracts.

Figure 3. MG-132 combined with TRAIL increases the AP-1 activity and decreases the c-FLIP(L) promoter activity. Columns, means from four independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$. A, AP-1 reporter luciferase activities (A) and c-FLIP(L) promoter luciferase activities (B) in PC3-TR cells after treatment with MG-132 (1 $\mu\text{mol/L}$), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 4 or 24 h. Luciferase activity of control samples without treatment were set at 1, and fold increase or fold decrease are represented accordingly. C, PC3-TR cells were transfected with c-Fos siRNA for 48 h and then assessed for AP-1 reporter luciferase activities or c-FLIP(L) promoter luciferase activities. Western blot shows successful reduction of c-Fos after siRNA-c-Fos treatment. D, c-FLIP(L) promoter luciferase activities. PC3-TR cells were transfected with c-Fos siRNA for 48 h, and then treated with MG-132 (1 $\mu\text{mol/L}$), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h.



expression of c-Fos and reduced c-FLIP(L) promoter activity are associated with sensitizing resistant prostate cancer cells to undergo TRAIL-induced apoptosis (data not shown). Therefore, MG-132 enhances the c-Fos activity, reduces c-FLIP(L) promoter activity, and sensitizes prostate cancer cells to undergo apoptosis.

MG-132 up-regulates AP-1 activity by increasing nuclear translocation of c-Fos/c-Jun and their interaction. AP-1 family transcription factors are dimeric protein complexes composed of heterodimers between Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and activating transcription factor (ATF) family gene products, which convert extracellular signals into changes of specific target gene expression (31, 32). Because we found that the AP-1 activity of PC3-TR cells are increased in response to MG-132 (Fig. 3A), we wished to examine whether any other AP-1 family members, besides c-Fos (Fig. 2), plays a key role in sensitizing cancer cells and regulating promoter activity of c-FLIP(L) during TRAIL-induced apoptosis. We found that MG-132 increased levels of c-Jun protein in PC3-TR cells, whereas there was no significant change in the protein levels of other AP-1 members (FosB, JunB, JunD, Fra-1, and Fra-2; Fig. 5A). In particular, nuclear levels of total c-Jun and phospho c-Jun were significantly increased (Fig. 5B).

c-Fos functions as a transcription factor by heterodimerizing with c-Jun and other AP-1 family members (31, 32). To determine whether the increased c-Fos and c-Jun nuclear levels after MG-132 treatment are associated with direct interactions between c-Fos and c-Jun, immunoprecipitation experiments between c-Fos and c-Jun were done. We found that direct interactions between c-Fos and c-Jun were increased in PC3-TR cells when the cells were exposed to MG-132. Similar results were obtained when MG-132 was combined with TRAIL. However, TRAIL alone did not enhance c-Fos/c-Jun interactions (Fig. 5C). Similar results were obtained when c-Jun antibody was used for the immunoprecipitation experiments (Fig. 5C). Therefore, the proteasome inhibitor MG-132 enhances c-Fos and c-Jun levels, enhances direct interactions

between c-Fos and c-Jun, and presumably promotes heterodimerization and transcriptional activity.

c-Fos and c-Jun bind to the c-FLIP(L) promoter region. To determine whether increased protein levels of c-Fos and c-Jun in response to MG-132 is associated with increased DNA binding, electrophoretic mobility shift assay (EMSA) and EMSA supershift assays were done. We found that AP-1 DNA binding is increased in the presence of MG-132, TRAIL, or MG-132 + TRAIL. However, we observed supershift bands for c-Fos and c-Jun particularly when the cells were treated with MG-132, demonstrating the specificity of binding of these AP-1 family member proteins in response to MG-132 (Fig. 6A).

Because c-Fos and c-Jun DNA binding is increased in response to treatment of cells with MG-132 and c-Fos represses the antiapoptotic molecule c-FLIP(L), we wished to determine if c-Fos and c-Jun specifically bind to the c-FLIP(L) putative promoter region. Previously, we examined 14 potential AP-1 binding sites upstream and within the first intron of c-FLIP(L) coding region (Fig. 6B). We found binding of c-Fos only to the AP-1-(f) site (see Fig. 6B) in the putative promoter region of c-FLIP(L).¹ We have found that in prostate cancer cells that are sensitive to TRAIL-induced apoptosis, mutations or deletions to the AP-1-(f) site abrogates binding of c-Fos, increases c-FLIP(L) promoter activity, and converts the phenotype of TRAIL-sensitive prostate cancer cells to become TRAIL resistant. Therefore, in our current model, with TRAIL-resistant prostate cancer cells that are sensitized by MG-132, we wished to determine whether there is increased binding of c-Fos and/or c-Jun at the AP-1-(f) site of c-FLIP(L). ChIP experiments were done to determine direct binding of c-Fos and c-Jun at the AP-1-(f) site. There was no significant binding of either c-Fos or c-Jun to the AP-1-(f) site of c-FLIP(L) without treatment or with TRAIL treatment alone. However, in the presence of MG-132, both c-Fos and c-Jun showed enhanced binding to the c-FLIP(L) AP-1-(f) site (Fig. 6C). These data show that MG-132 sensitizes resistant prostate cancer cells

to proapoptotic effects of TRAIL by enhancing c-Fos and c-Jun interactions and transcriptionally repressing the expression of c-FLIP(L) by binding to the AP-1(f) site of *c-FLIP(L)*.

Discussion

TRAIL has great potential as an antitumor agent because it can selectively induce apoptosis in cancer cells, yet spare most normal cells. Although many cancer cells are sensitive to TRAIL-induced apoptosis, some develop resistance. Many groups have been investigating the synergistic effects of different drugs in combination with TRAIL to overcome the resistance developed by cancer cells (8, 20, 21, 33–38). In the present study, we showed that TRAIL combined with the proteasome inhibitor MG-132 could effectively sensitize TRAIL-resistant prostate cancer cells to undergo

apoptosis. Moreover, this combined treatment did not induce death in nonmalignant cell (BPH-1 and HEK 293T; Fig. 1). MG-132 sensitizes TRAIL-resistant prostate cancer cells by up-regulating the AP-1 family proteins c-Fos and c-Jun, which, in turn, repress the antiapoptotic molecule c-FLIP(L). As for the other well studied c-FLIP isoform, c-FLIP(s), we have not found it to be expressed in our prostate cancer cells. Therefore, the effects of MG-132 on c-FLIP(s) was not examined in our study.

Proteasome inhibitors are attractive cancer therapeutic agents because they can regulate apoptosis-related proteins (e.g., TRAF2, BAX, IAP, and p53 proteins; refs. 12, 23–25). PS-341 has been approved by the Food and Drug Administration for treatment of patients with multiple myeloma, and many clinical trials are ongoing to examine the efficacy of PS-341 for treatment of other malignancies (39, 40). MG-132 is another small-molecule proteasome inhibitor, and numerous reports have shown that MG-132 inhibits NF- κ B activation through stabilization of the inhibitor of κ B/NF- κ B complex, as well as prevention of nuclear translocation of NF- κ B (41, 42). Other mechanisms that MG-132 sensitize cancer cells include increased expression of mitogen-activated protein kinase and activation of c-Jun-NH₂-kinase (26, 27) or up-regulation of death receptor DR5 and Bik accumulation (7, 8, 26, 27).

In the current study, we determined whether TRAIL-resistant prostate cancer cells, which are sensitized by MG-132, have changes in the AP-1/c-Fos and c-FLIP(L) signaling pathway. In the presence of MG-132, we found that inhibition of c-Fos by siRNA led to up-regulation of c-FLIP(L) promoter activity, and, conversely, ectopic expression of c-Fos reduced c-FLIP(L) promoter activity (Fig. 3). After priming the resistant prostate cancer cells by MG-132 to undergo apoptosis, we showed that the AP-1 family members c-Fos and c-Jun directly bind to the *c-FLIP(L)* AP-1(f) site (Fig. 6) after treatment with TRAIL.

In TRAIL-sensitive prostate cancer cells, we have found that the AP-1 family member proteins only bind to the AP-1(f) site of the *c-FLIP(L)* promoter region and none of the other putative AP-1 binding sites in the putative promoter region of *c-FLIP(L)*. Deletions and mutations at the *c-FLIP(L)* AP-1(f) site abrogate binding of c-Fos to the *c-FLIP(L)* promoter and maintain expression of c-FLIP(L) promoter activity.¹ The current study shows that treatment of resistant prostate cancer cells with MG-132 potentiates binding of c-Fos and c-Jun proteins to the *c-FLIP(L)* AP-1(f) site (Fig. 6B and C). Although binding of c-Fos/c-Jun to the putative promoter region of *c-FLIP(L)* after treatment with MG-132 may be necessary, it is not sufficient to reduce c-FLIP(L) mRNA and protein levels (Fig. 2). Therefore, addition of TRAIL to MG-132 induces other factors to repress c-FLIP(L) levels and potentiate cell death.

The AP-1 transcription factor is composed of protein dimers between the Jun, Fos, and ATF family members. The predominant forms of AP-1 in most cells are Fos/Jun heterodimers, which have a high affinity for binding to an AP-1 site. The regulation of these transcription factors is critical in determining the response to various physiologic and environmental stimuli (31, 32). In addition to c-Fos, we found that c-Jun was another AP-1 family protein that was activated by MG-132. c-Jun protein levels increased in the TRAIL-resistant cancer cells after treatment with MG-132. More specifically, MG-132 promoted expression of nuclear c-Jun protein and heterodimerization with c-Fos and binding to the c-FLIP(L) promoter (Fig. 5). These results suggest that c-Fos/c-Jun heterodimers may act concomitantly to down-regulate c-FLIP(L) expression and sensitize resistant cancer cells to undergo TRAIL-induced apoptosis.

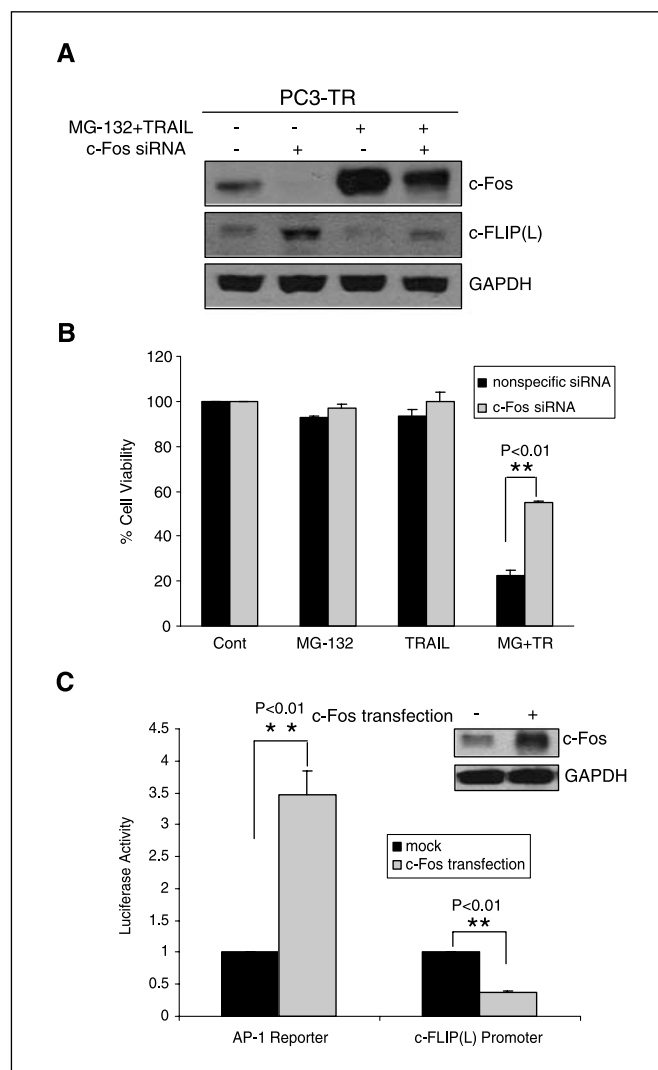
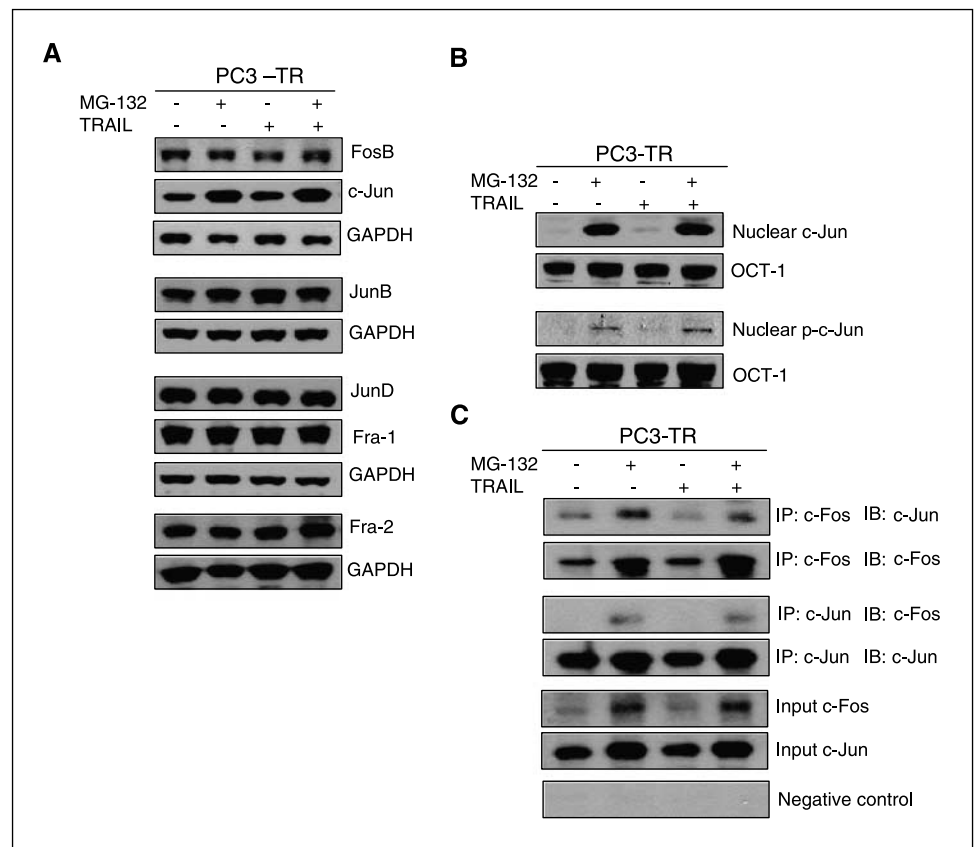


Figure 4. A, c-Fos and c-FLIP(L) protein levels of PC3-TR cells were assayed by Western blot after cells were transfected with c-Fos siRNA for 48 h and then treated with MG-132 (1 μ M/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h. B, cell viability of PC3-TR cells transfected with c-Fos siRNA or nonspecific siRNA for 48 h and then treated with MG-132 (1 μ M/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h (MG+TR). Cell viability was evaluated by MTS assay. Viability of untreated cells was set at 100%. C, AP-1 reporter and c-FLIP(L) promoter luciferase activity analysis. Ectopic expression of c-Fos for 48 h in PC3-TR cells was done, and then cells were transfected with AP-1 reporter or c-FLIP(L) promoter plasmids and *Renilla* for another 24 h.

Figure 5. MG-132 up-regulates AP-1 activity by increasing nuclear c-Fos and c-Jun and their heterodimerization. **A**, Western blot analysis of AP-1 member proteins (FosB, c-Jun, JunB, JunD, Fra-1, and Fra-2). PC3-TR cells were treated with MG-132 (1 μ mol/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h. **B**, Western blot analysis of nuclear protein for total c-Jun and phosphorylated c-Jun in PC3-TR cells. **C**, immunoprecipitation (IP) assay between c-Fos and c-Jun protein. PC3-TR cells were treated with MG-132 (1 μ mol/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 4 h. Equal-quantity whole-cells lysates were immunoprecipitated using c-Fos or c-Jun antibody. The resulting immune complex was subjected to Western blot analysis. Input was 2% of the total from each sample of immunoprecipitation.

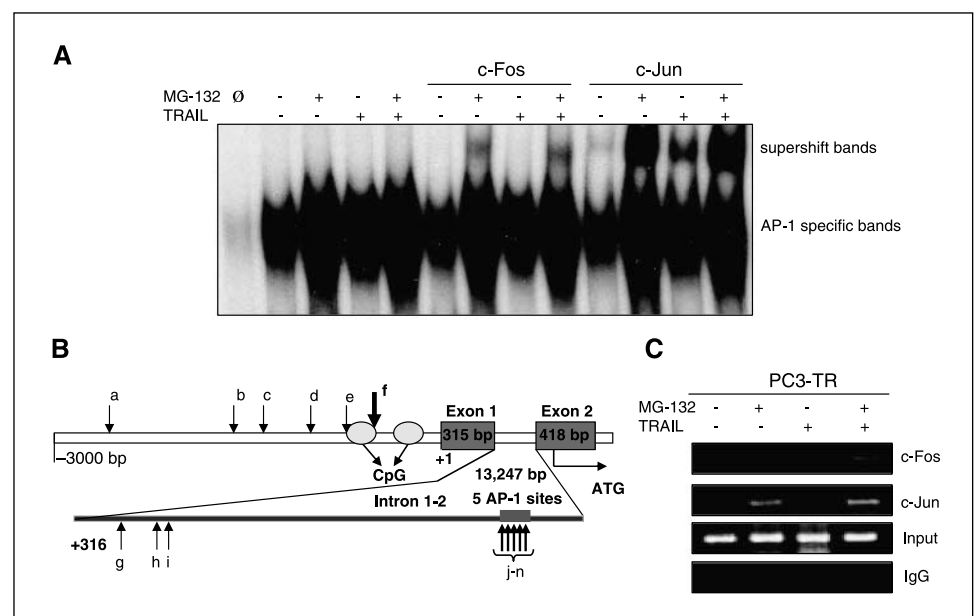


Activation of AP-1 family members by other tumor necrosis factor (TNF) family signaling pathways, besides TRAIL, has been implicated. For example, the TNF receptor member receptor activator of NF- κ B ligand (RANKL) is a key regulator of bone homeostasis. RANKL induces expression of c-Fos, an important step in bone development. To maintain proper balance in bone development, c-Fos activates its own inhibitor, IFN- β , to reduce RANKL signaling. Thus, an autoregulatory mechanism involving

c-Fos, the TNF receptor family member RANKL, and IFN- β play a crucial role in bone development (43). In the present study, we identified a similar autoregulatory mechanism that involves c-Fos/c-Jun heterodimerization in TRAIL-resistant cancer cells.

We postulate that posttranslational modifications of AP-1 family member proteins, particularly c-Fos and c-Jun, play an important role in determining whether cancer cells are sensitive or resistant to TRAIL-induced apoptosis. Cellular localization and activation of

Figure 6. AP-1 binding to c-FLIP(L) promoter was analyzed by EMSA and ChIP assay. **A**, EMSA and EMSA supershift assay for c-Fos and c-Jun binding to DNA. **B**, AP-1 binding sites of the putative regulatory region of c-FLIP(L) before the ATG start codon. **C**, AP-1 binding to "f" site of c-FLIP(L) putative promoter region was analyzed by ChIP assay. Negative controls are samples using nonspecific IgG. Positive controls are whole-cell lysates without the immunoprecipitation step, and experimental samples include the ChIP assay using c-Fos or c-Jun antibodies.



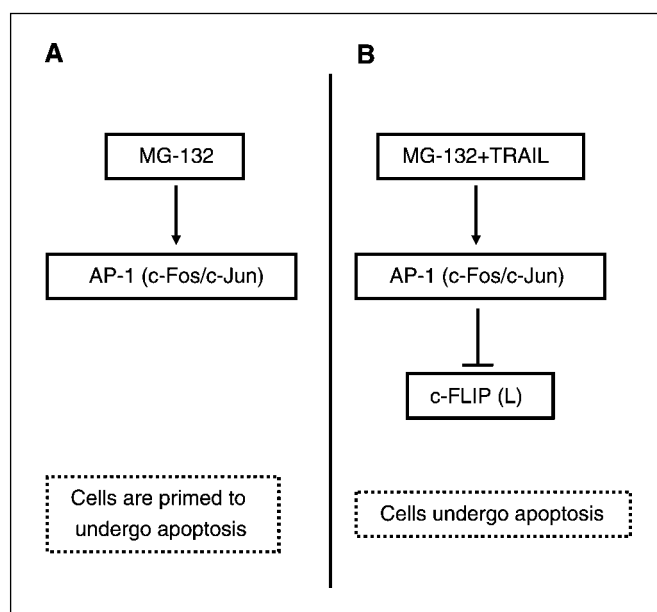


Figure 7. Model for MG-132 priming and sensitization of TRAIL-resistant cancer cells. Cells are primed after exposure to MG-132 by up-regulation of AP-1 (c-Fos/c-Jun) but do not undergo cell death (A). However, combination of MG-132 and TRAIL primes cancer cells and promotes apoptosis of TRAIL-resistant prostate cancer cells (B).

c-Fos and c-Jun can depend on their phosphorylation, protein stability, and other chaperone proteins. Recent work has suggested that phosphorylation of c-Fos, which is an important determinant of its activity and expression, is tightly regulated by a variety of kinases (20, 44–48). Protein stability of c-Fos, another regulator of its physiologic function, has been shown to be dependent on its COOH-terminal PEST3 domain, which modulates the proteasome-mediated degradation of c-Fos (49). Associated proteins in the form of chaperone proteins or heterodimers can also regulate c-Fos structure and function. Therefore, we believe that c-Fos and c-Jun posttranslational modifications can significantly affect its

ability to regulate *c-FLIP(L)* gene expression and TRAIL-induced apoptosis, and it is an area under investigation in our laboratory.

One limitation of our study is that MG-132 is a general proteasome inhibitor and can affect many different molecular pathways. Noting this limitation, we focused our attention on the effect of MG-132 on the AP-1-related protein c-Fos. Because our prior work has suggested that c-Fos, and not other AP-1 protein family members, is an important modulator of c-FLIP(L) protein, we primarily focused our attention on the effects of c-Fos and c-FLIP(L). However, our present results suggest that sensitization of TRAIL-resistant cancer cells by MG-132 lead to increased levels of c-Jun, as well as to c-Fos, a finding not seen in TRAIL-sensitive cells. In particular, we showed that DNA binding of c-Jun to potential AP-1 sites after treatment with TRAIL may be more pronounced than binding of c-Fos to potential AP-1 sites (Fig. 6). Our future studies will determine whether c-Fos and c-Jun have an equal or disproportionate effect on transcriptional regulation of c-FLIP(L) and modulation of TRAIL-induced apoptosis in cancer cells that are sensitized by the proteasome inhibitor MG-132.

In summary, we show that MG-132 primes and sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis by activating the AP-1 family member proteins c-Fos and c-Jun (Fig. 7A). Combination of MG-132 with TRAIL in TRAIL-resistant prostate cancer cells promotes cell death by increased heterodimerization of c-Fos/c-Jun and direct repression of the c-FLIP(L) antiapoptotic molecule (Fig. 7B). Therefore, we report a new regulatory pathway by which MG-132 sensitizes cancer cells for apoptosis, and combination of TRAIL with proteasome inhibitors may be an effective strategy for treating TRAIL-refractory tumors.

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Repression of NF- κ B and activation of AP-1 enhance apoptosis in prostate cancer cells

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TNF α and TRAIL, 2 members of the tumor necrosis factor family, share many common signaling pathways to induce apoptosis. Although many cancer cells are sensitive to these proapoptotic agents, some develop resistance. Recently, we have demonstrated that upregulation of c-Fos/AP-1 is necessary, but insufficient for cancer cells to undergo TRAIL-induced apoptosis. Here we present a prostate cancer model with differential sensitivity to TNF α and TRAIL. We show that inhibition of NF- κ B or activation of AP-1 can only partially sensitize resistant prostate cancer cells to proapoptotic effects of TNF α or TRAIL. Inhibition of NF- κ B by silencing TRAF2, by silencing RIP or by ectopic expression of I κ B partially sensitized resistant prostate cancer. Similarly, activation of c-Fos/AP-1 only partially sensitized resistant cancer cells to proapoptotic effects of TNF α or TRAIL. However, concomitant repression of NF- κ B and activation of c-Fos/AP-1 significantly enhanced the proapoptotic effects of TNF α and TRAIL in resistant prostate cancer cells. Therefore, multiple molecular pathways may need to be modified, to overcome cancers that are resistant to proapoptotic therapies.

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Key words: apoptosis; TRAIL; TNF- α ; mechanisms of resistance

TNF (tumor necrosis factor) family members regulate a variety of biological processes such as cell development, differentiation, tumorigenesis, cell proliferation, cell survival and/or apoptosis.¹ Among TNF members, tumor necrosis factor α (TNF α) and tumor necrosis factor-related apoptosis ligand (TRAIL) are 2 cytokines that possess strong antitumor activity. Both TNF α and TRAIL are capable of inducing cell death in cancer cells. However, TNF α is associated with significant cytotoxicity, which limits its clinical utility.¹ In contrast, TRAIL promotes apoptosis in cancer cells with limited damage to normal cells; therefore, it is associated with minimal cytotoxicity—making TRAIL an ideal anti-cancer agent from the TNF family members.^{2,3} Although many cancers are sensitive to TNF α - and TRAIL-induced apoptosis, some develop resistance.

Both TNF α and TRAIL induce apoptosis through activating specific receptors. TNF α activates TNF receptor 1 (TNFR1)⁴ and TNF receptor 2 (TNFR2),⁵ whereas TRAIL activates DR4 (TRAIL-R1), DR5 (TRAIL-R2) and three other decoy receptors.⁶ Although TNF α - and TRAIL-induced apoptosis share many common intracellular pathways, there are some distinguishing differences between the two. TRAIL interacts with specific death domain receptors, DR4 and DR5, to rapidly induce intracellular cytoplasmic formation of the DISC (death inducing signaling complex).^{3,7,8} DISC formation may involve the recruitment of caspase-8, FADD, TRADD, TRAFs and RIP to the death domain of the activated receptor to induce the extrinsic apoptosis pathway.⁹

In contrast to DISC formation by TRAIL-induced apoptosis, DISC formation induced by TNF α involves 2 sequential signaling complexes.¹⁰ Complex I consists of TNFR1, TRAF2, RIP and the adaptor TRADD, and it may rapidly activate NF- κ B, thereafter increasing the expression of the anti-apoptotic molecule, c-FLIP, a homologous and competitive inhibitor of caspases 8/10.¹⁰ In a second step, caspase 8/10 and FADD can be recruited into the released complex I of TRAF2, RIP, TRADD and death domain, and assembled complex II. Complex II transduces signals of cell death when complex I fail to activate NF- κ B.

NF- κ B is a transcription factor that regulates death-domain-mediated apoptosis.¹¹ NF- κ B subunits, RelA/p65, cRel, RelB,

NF- κ B1/p50 and NF- κ B2/p52, can form homodimeric or heterodimeric complexes. NF- κ B is sequestered in the cytoplasm by its specific inhibitor I κ B. I κ B can be phosphorylated by I κ B kinase and quickly degraded via proteasome-mediated pathway, resulting in the rapid nuclear translocation of NF- κ B and activation of NF- κ B. Therefore, proteasome inhibitors such as MG132 can inhibit NF- κ B activity by suppressing the degradation of I κ B.¹² NF- κ B and its important modulators, TRAF2 and RIP, in TNF α -induced apoptosis have been well characterized.¹³ However, the effects of NF- κ B on TRAIL signaling remain controversial—while some reports suggest that NF- κ B activation protects cells from TRAIL-induced apoptosis,^{14–16} others suggest that NF- κ B may promote apoptosis.¹⁷ These discrepancies indicate that the role of NF- κ B in TRAIL-induced apoptosis is unclear; and other important pathways are to be considered when evaluating the true NF- κ B function in regulating TRAIL-induced apoptosis.

Previously, we have found that TRAIL-induced apoptosis can be regulated by c-Fos,^{18,19} a member of the AP-1 transcriptional factors.²⁰ We have found that c-Fos, has a novel proapoptotic function in TRAIL-induced apoptosis in addition to its well-known oncogenic function. We have demonstrated that up-regulation of c-Fos/AP-1 is necessary, but insufficient for cancer cells to undergo TRAIL-induced apoptosis.¹⁹

In this study we identified a prostate cancer cell model with differential sensitivity to TNF α - and TRAIL-induced apoptosis. We demonstrate that in order for prostate cancer cells to be sensitive to TNF α or TRAIL, cancer cells reduce NF- κ B activity and/or increase AP-1 activity. In resistant cancer cells, inhibition of NF- κ B alone or activation of AP-1 alone can only partially sensitize cancer cells to TNF α or TRAIL. However, concomitant inhibition of NF- κ B and activation of AP-1 significantly sensitizes prostate cancer cells to TRAIL- or TNF α -induced apoptosis. Therefore, multiple molecular pathways may be modified, to overcome cancers that are resistant to proapoptotic therapies.

Material and methods

Materials

Recombinant human TRAIL/TNFSF10 and TNF- α /TNFSF1A were obtained from R&D System (Minneapolis, MN). Antibodies to RIP and c-Fos, Horseradish peroxidase-conjugated secondary antibodies (goat-anti-mouse, goat-anti-rabbit, goat-anti-rat antibodies) were obtained from Santa Cruz Biotechnology (Santa

Additional Supporting Information may be found in the online version of this article.

[†]Xiaoping Zhang and Xu Huang contributed equally to this work.

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Cruz, CA). Antibodies to TRAF2 and GAPDH were obtained from Abcam (Cambridge, MA). Antibodies to I κ B and cleaved PARP were obtained from Cell signaling Technology, (Danvers, MA).

Cell culture material, cell viability, Western Blot assays and apoptosis

PC3 and LNCaP were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). PC3-TR is a TRAIL-resistant subline of PC3 cells that was derived in our laboratory.²¹ Cells were grown in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 10% FBS and 5% penicillin at 37°C with 5% CO₂. Cell viability was determined by MTT method (Roche Diagnostics, Indianapolis, IN) and Western Blot assays were carried out as previously described.²¹

Flow cytometry was used to assess the sub-G1 DNA population of cells undergoing apoptosis. Cells were harvested, washed twice with cold phosphate-buffered saline and fixed with cold 70% ethanol for at least 1 hr at 4°C. Cells were washed twice with DNA extraction buffer (192 mM Na₂HPO₄, 4 mM citric acid, pH 7.8). Cells were incubated with propidium iodide (PI; 50 μ g/ml, Molecular Probes, Eugene, OR) and RNase A at room temperature for 30 min before analyzed by flow cytometry. All results were from at least triplicate experiments.

Transfection of plasmids and siRNA and luciferase assay

pNF- κ B luciferase (25 ng/well) and pAP-1 luciferase reporter plasmids (25 ng/well) were purchased from Stratagene (La Jolla, CA). Renilla Luciferase Reporter was purchased from Promega (Madison, WI). TRAF2-specific siRNA and RIP-specific siRNA were purchased from Santa Cruz Biotechnology. Negative control siRNA was purchased from Qiagen (Valencia, CA). pCMV-I κ B-M plasmid was purchased from BD Biosciences (Franklin Lakes, NJ). Full length human c-Fos cDNA was provided by Dr. L Shemshadini, University of Toledo, OH.²² The plasmids and siRNA were transfected as previously described.^{18,19,21}

Luciferase assay

Cells were seeded into 24-well plates. When the cells were 80% confluent, both AP-1 luciferase reporter (25 ng/well) and Renilla reporter (5 ng/well) from Stratagene (La Jolla, CA) or NF- κ B reporter (25 ng/well) and Renilla reporter from Stratagene (La Jolla, CA) were cotransfected into cells. Here, Renilla served as an internal control for transfection efficiency. After 24 hr of transfection, cells were treated with TRAIL (100 ng/ml) or TNF α (100 ng/ml) for 4 hr, then both were attached and floating cells were collected, prepared and further detected by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Samples were stored at -20°C until detection. All results represent average of at least 3 independent experiments $\pm$ SD.

Cell extracts and electrophoretic mobility shift assay (EMSA)

Frozen cell pellets were resuspended in 4 volume of lysis buffer: 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol, 10 mM Na molybdate, 2 mM Na pyrophosphate, 2 mM Na orthovanadate, 0.5 mM spermidine, 0.15 mM spermine, 50 μ M TPCK, 25 μ M TLCK, 1 μ g/mL each of aprotinin, pepstatin A and leupeptin, 0.5 mM benzamide, 1 mM DTT and 0.5 mM PMSF. KCl was added to 400 mM final, and the extracts were incubated at 4°C for 30 min and centrifuged at 10,000g for 5 min. The supernatant contained the whole cell extracts. The reactions were made using 3 μ l of whole cell extract and 0.1–0.5 ng of ³²P-labeled double-stranded specific oligonucleotides (5,000–25,000 cpm) and run on 5–7% polyacrylamide gels containing 0.5 $\times$ Tris glycine EDTA. Gels were dried with Bio-Rad gel dryer (Hercules, CA) and imaged using Kodak BioMax MR Film (Fisher Scientific, Atlanta, GA). General AP-1 gel shift oligonucleotide was obtained from Santa Cruz Biotechnology. The reaction containing 90%

nonlabeled and 10% ³²P-labeled oligonucleotides probe was used as control.

Results

Prostate cancer cell lines have differential sensitivity to TRAIL- and TNF α -induced apoptosis

To evaluate the sensitivity of prostate cancer cells to proapoptotic agents, LNCaP, PC3 and PC3-TR cells, a subline of PC3 cells which is resistant to TRAIL treatment,²¹ were treated with TRAIL or TNF α in time-dependent and dose-dependent experiments. We found that PC3 cells were very sensitive to TRAIL, whereas, LNCaP and PC3-TR cells were resistant to TRAIL-induced apoptosis even with long exposures of 24, 48 and 72 hr (Fig. 1a and data not shown). In contrast, LNCaP cells were sensitive to TNF α in a dose- and time-dependent manner whereas PC3 and PC3-TR cells were resistant at 24, 48 and 72 hr of exposure (Fig. 1b and data not shown). The results indicate that PC3 cells are sensitive to TRAIL, but resistant to TNF α -induced apoptosis. In contrast, LNCaP cells are sensitive to TNF α -induced apoptosis but resistant to TRAIL, whereas PC3-TR cells are resistant to both TRAIL- and TNF α -induced apoptosis. As a marker of apoptosis, cleaved PARP products was used to detect differential response of these cells to TRAIL or TNF α -induced apoptosis. Cleaved PARP was dramatically increased in TRAIL-sensitive PC3 cells or TNF α -sensitive LNCaP cells after treatment for 4 and 24 hr. However, cleaved PARP was only slightly increased in TRAIL-resistant or TNF α -resistant cells (Fig. 1c). These findings provide an *in vitro* prostate cancer model with differential sensitivity to TRAIL and TNF α , and enabled us to investigate common and differential proapoptotic pathways for TRAIL- and TNF α -induced apoptosis.

Resistance of prostate cancer cells to TRAIL or TNF α correlate with increased NF- κ B and decreased AP-1 activities

NF- κ B is a key regulator of TNF α -induced apoptosis,²³ and we have recently demonstrated that c-Fos/AP-1 activity is necessary, but insufficient for cancer cells to undergo TRAIL-induced apoptosis.^{18,19} Therefore, we wished to evaluate the role of both NF- κ B and AP-1 activities in mediating TRAIL-induced and TNF α -induced apoptosis in prostate cancer cells. We found that NF- κ B activity increased after 1 hr of TRAIL treatment in PC3-TR and LNCaP cells, which are both resistant to TRAIL-induced apoptosis. After 24 hr of TRAIL treatment, the NF- κ B activity was nearly 4-fold higher compared to the basal levels in the TRAIL-resistant cells. In contrast, in the TRAIL-sensitive PC3 cells NF- κ B activity maintained at the same level and then decreased to 30% of basal level after 24 hr of TRAIL-treatment because most cells died at 24 hr treatment (Fig. 2a).

In a similar fashion, NF- κ B activity in TNF α -resistant cell lines, PC3 and PC3-TR, increased as soon as 30 min after TNF α treatment and reached 10-fold higher than basal level after 48 hr of TNF α treatment. In contrast, NF- κ B activity in LNCaP cells, which are sensitive to TNF α -induced apoptosis and have very low basal NF- κ B activity,^{24,25} was maintained at the same level even after 48 hr of treatment (Fig. 2b).

Given our prior findings that the AP-1 family members play a critical role in regulating TRAIL-induced apoptosis in prostate cancer cells,^{18,19} we wished to evaluate the role of AP-1 family members in regulating both TRAIL- and TNF α -induced apoptosis. Luciferase reporter assay for AP-1 activity demonstrated that the AP-1 activity was dramatically increased after treatment with TRAIL in TRAIL-sensitive PC3 cells, but not in TRAIL-resistant PC3-TR and LNCaP cells, even though the baseline AP-1 activity was very low in LNCaP cells (Supporting Data S1). However, after 24 hr of the treatment, the AP-1 activity was barely detectable in PC3 cells, mostly because majority of the PC3 cancer cells were dead at this point of time (Fig. 2c).

AP-1 activity also increased in the TNF- α sensitive LNCaP cells after treatment with TNF α , but to a lesser degree than the

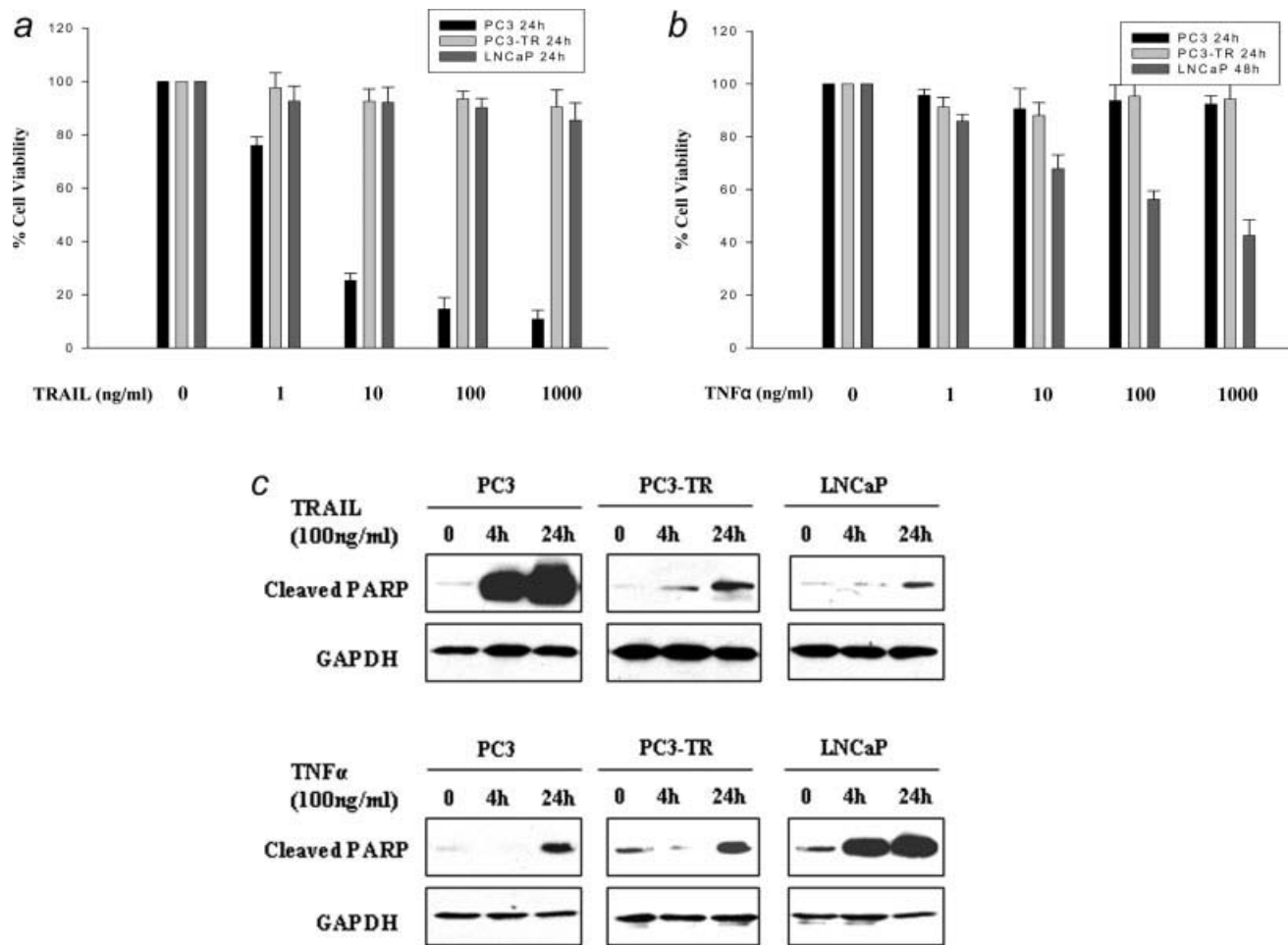


FIGURE 1 – Prostate cancer cell lines PC3, PC3-TR and LNCaP have differential sensitivity to TRAIL and TNF α . PC3, PC3-TR and LNCaP cells were treated with TRAIL (a) or TNF α (b) with increasing concentrations and different time-course. Cell viability was measured by the MTT method after 24 and 48 hr of treatment. Error bars (SD) represent results of at least 3 independent experiments. (c) PARP cleavage product protein levels in PC3, PC3-TR and LNCaP cells after treatment with TRAIL (100 ng/ml) or TNF α (100 ng/ml) for 4 and 24 hr.

TRAIL-sensitive prostate cancer cells after TRAIL treatment (Figs. 2c and 2d). Treatment with TNF α had no significant effect on AP-1 luciferase activity in the TNF α -resistant cells (PC3 and PC3-TR).

Table I summarizes our initial findings, which demonstrates that either downregulation of NF- κ B or upregulation of AP-1 activities are important components for cancer cells to be sensitive to TNF α - or TRAIL-induced apoptosis. Therefore, we postulate that decreased NF- κ B activity and/or increased AP-1 activity may be important for sensitization of prostate cancer cells to TRAIL and TNF α treatments.

Silencing TRAF2 or RIP suppresses NF- κ B activation and sensitizes PC3 and PC3-TR cells to TNF α or TRAIL treatments

TRAF2 and RIP, two critical components of DISC,²⁶ play a critical role in activation of NF- κ B. Therefore, we evaluated the protein levels for TRAF2 and RIP in PC3 (sensitive to TRAIL but resistant to TNF α) and PC3-TR (resistant to both TRAIL and TNF α) cells. The PC3-TR cells are a subline of PC3 cells that were generated in our laboratory,²¹ and the two cell lines serve as a good model to investigate the molecular differences and similarities between TRAIL and TNF α sensitivity in cancer cells. We found that in PC3 cells, which are sensitive to TRAIL-induced apoptosis, the protein levels of both TRAF2 and RIP decreased and became undetectable after 4 hr of treatment with TRAIL. In con-

trast, the protein levels of TRAF2 and RIP were maintained in PC3 cells after treatment with TNF α , even after 24 hr of treatment (Fig. 3a). In PC3-TR cells, which are resistant to both TRAIL and TNF α , protein levels for TRAF2 and RIP were maintained after either TRAIL or TNF α treatments (Fig. 3a). We also observed modest reduction of TRAF2 and RIP in LNCaP cells (sensitive to TNF α and resistant to TRAIL) after TNF α treatment (data not shown). These results suggest that reduction of TRAF2 and RIP levels, two important NF- κ B modulators, correlate with whether prostate cancer cells will undergo apoptosis after treatment with TRAIL and TNF α .

To examine the effects of TRAF2 and RIP on NF- κ Bs activity and the effect they may exert on prostate cancer cells, we silenced the expression of TRAF2 and RIP in PC3 and PC3-TR cells. As shown previously, NF- κ B activity was induced in PC3 cells after treatment with TNF α , while silencing TRAF2 and/or RIP effectively suppressed NF- κ B activation by 2- to 4-folds (Fig. 3b, top panel). Similarly in PC3-TR cells, the activation of NF- κ B in response to TRAIL and TNF α treatment was suppressed by silencing TRAF2 or RIP expression (Figs. 3c and 3d, top panel). Therefore, TRAF2 and RIP function as NF- κ B activators in prostate cancer cells in response to TRAIL or TNF α treatments.

Next, we wished to determine whether suppression of NF- κ B by silencing TRAF2 and/or RIP can affect the prostate cancer cells' response to exposure to apoptotic agents. We found that both PC3 and PC3-TR cells were more prone to undergo apoptosis

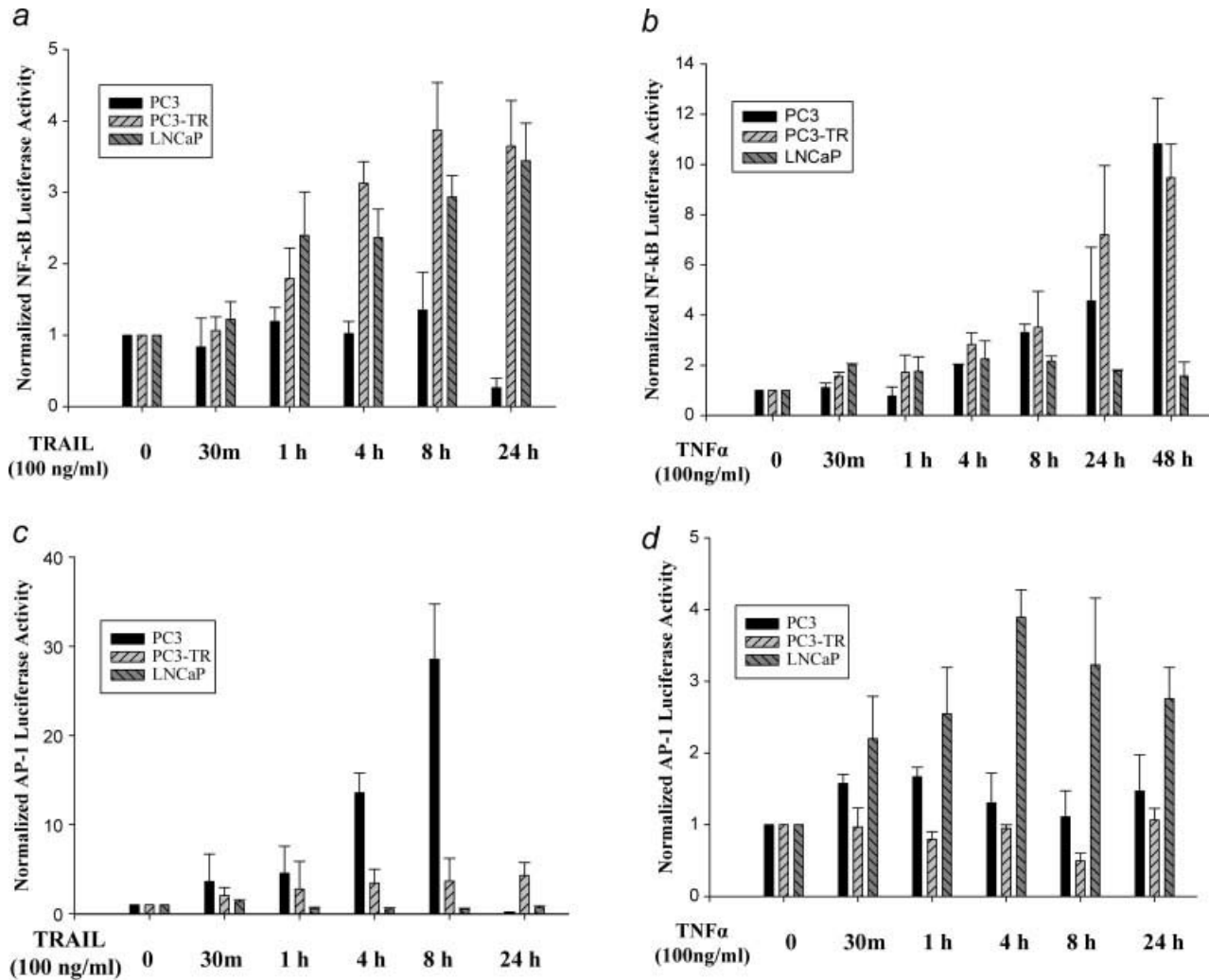


FIGURE 2 – Alterations in NF- κ B and AP-1 activities correlate with sensitivity to TRAIL and TNF α in prostate cancer cells. NF- κ B (*a* and *b*) and AP-1 (*c* and *d*) luciferase activities in PC3, PC3-TR and LNCaP cells in response to TRAIL (100 ng/ml) (*a* and *c*) and TNF α (100 ng/ml) (*b* and *d*). Error bars (SD) represent results of at least 3 independent experiments.

TABLE 1 – SUMMARY OF CHANGES OF AP-1 AND NF- κ B ACTIVITIES AND SENSITIVITY TO TRAIL AND TNF α

	PC3		PC3-TR		LNCaP	
	TRAIL	TNF α	TRAIL	TNF α	TRAIL	TNF α
NF- κ B	↓	↑	↑	↑	↑	NC*
AP-1	↑	NC	NC	NC	NC	↑
Sensitivity	S	R	R	R	R	S

↑: Increased activity; ↓: decreased activity; NC: no change; S: sensitive; R: resistant; * Basal level is very low.

when treated with either TNF α or TRAIL (Figs. 3*b*, 3*c* and 3*d*, bottom panels). Silencing both TRAF2 and RIP did not have an additive effect in reducing the NF- κ B activity or enhancing apoptosis, suggesting that TRAF2 and RIP function in *series* and have equivalent effects on NF- κ B activity and sensitization of prostate cancer cells to undergo apoptosis.

I κ B partially sensitizes resistant cancer cells to TRAIL and TNF α -induced apoptosis

Modulation of TRAF2 and RIP can affect NF- κ Bs activation, and affect prostate cancer cells' response to TNF α - and TRAIL-induced apoptosis (Fig. 3). Therefore, we wished to determine whether direct inhibition of NF- κ B could modulate prostate cancer

cells' sensitivity to TNF α and TRAIL. To inhibit NF- κ B, we ectopically expressed, I κ B, an inhibitory subunit of NF- κ B.¹¹ pCMV-I κ B α -M expresses I κ B α with a point mutation which abrogates I κ B α s ability to be phosphorylated and subsequently degraded.²⁷ After ectopic expression of pCMV-I κ B (Figs. 4*a* and 4*b* – Western Blots), NF- κ Bs activity was reduced in PC3 and PC3-TR cells and could not be activated when the cells were treated with TNF α or TRAIL (Figs. 4*a* and 4*b* – upper panels). Subsequently, PC3 cells were treated with TNF α , and PC3-TR cells were treated with TRAIL or TNF α . The cells that expressed pCMV-I κ B α -M were partially sensitized to TNF α (PC3 and PC3-TR cells) and TRAIL (PC3-TR cells) by demonstrating a lower percentage of viable cells (Figs. 4*a* and 4*b* – lower panels). Therefore, although NF- κ B is a key modulator of apoptosis in cancer cells,²³ inhibition of NF- κ B activity only partially sensitizes prostate cancer cells toward proapoptotic agents. Next, we wished to investigate other key regulators of TNF α -induced and TRAIL-induced apoptosis, which may work in parallel with NF- κ Bs activities.

Activation of AP-1 sensitizes resistant prostate cancer cells to TRAIL- and TNF α -induced apoptosis

In addition to NF- κ Bs role in regulating apoptosis in cancer cells, we have found that c-Fos/AP-1 represses the anti-apoptotic gene, c-FLIP(L) and primes prostate cancer cells to undergo

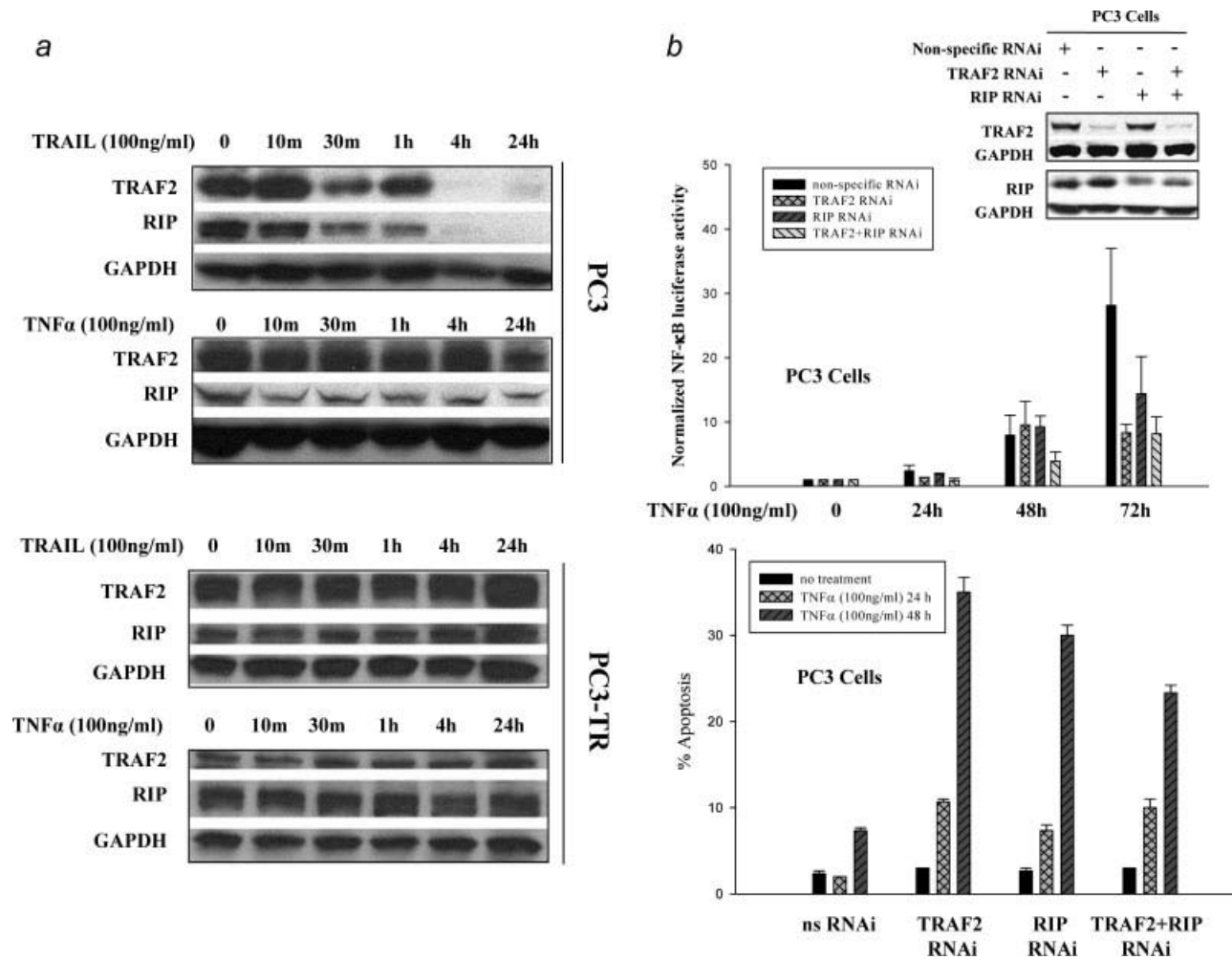


FIGURE 3 – TRAF2 and RIP as modulators of NF- κ B and regulators of TRAIL- and TNF α -induced apoptosis. (a) Reduction of TRAF2 and RIP protein levels correlated with sensitivity to TRAIL- and TNF α -induced apoptosis. (b) Silencing TRAF2, RIP or both reduced NF- κ B activity (top panel) and partially sensitized PC3 cells to TNF α (bottom panel). Western blots were performed with TRAF2- and RIP-specific antibodies to detect the efficiency of RNAi. Nonspecific siRNA was used as negative control. GAPDH was used as loading control.

TRAIL-induced apoptosis.¹⁹ We have demonstrated that activation of c-Fos/AP-1 is necessary but insufficient for TRAIL-induced apoptosis. In addition, we have demonstrated that AP-1 is activated in sensitive prostate cancer cells after treatment with either TRAIL or TNF α (Fig. 2). Here we wished to determine whether activation of c-Fos/AP-1 could affect TRAIL- and/or TNF α -induced apoptosis. We found that ectopic expression of wild type c-Fos led to increased expression of c-Fos and AP-1 activity and converted TRAIL-resistant prostate cancer cells (PC3-TR and LNCaP) and TNF α -resistant prostate cancer cells (PC3 and PC3-TR) to a more sensitive phenotype, respectively (Figs. 5a and 5b).

To test whether inhibition of c-Fos/AP-1 activity can alter the phenotype of TRAIL-sensitive (PC3) and TNF α -sensitive (LNCaP) prostate cancer cells, we inhibited the AP-1 activity by a dominant negative form of c-Fos/AP-1 (*i.e.* A-Fos).²⁸ We found that ectopic expression of A-Fos reduced AP-1 luciferase activity in PC3 and LNCaP cells after TRAIL and TNF α treatments, respectively (Figs. 5c and 5d, top panels). Reductions in AP-1 activity were associated with changing the phenotype of TRAIL-sensitive (PC3) and TNF α -sensitive (LNCaP) cells to a more resistant phenotype (Figs. 5c and 5d, bottom panel).

This data demonstrates that AP-1 activity has a direct role in mediating TRAIL and TNF α apoptotic-mediated responses. Therefore, manipulating AP-1 activity can alter sensitivity of

prostate cancer cells to proapoptotic agents like TRAIL and TNF α .

Concomitant reduction of NF- κ B and increase of AP-1 activities sensitize a significant portion of prostate cancer cells to TRAIL- or TNF α -induced apoptosis

Our results showed that both NF- κ B and AP-1 activity play essential roles in modulating sensitivity of prostate cancer cells to TRAIL- and TNF α -induced apoptosis. Suppressing NF- κ B or increasing AP-1 activities alone partially sensitizes the cells to TRAIL- and TNF α -induced apoptosis. We postulate that combination of decreased NF- κ B and increased AP-1 activities could potentiate the proapoptotic effects of TRAIL and TNF α . Therefore, I κ B α -M and c-Fos were simultaneously introduced into prostate cancer cells to repress NF- κ B and activate AP-1, respectively, in TRAIL-resistant (PC3-TR and LNCaP) or TNF α -resistant (PC3 and PC3-TR) prostate cancer cells. Concomitant expression of I κ B α -M and c-Fos (Fig. 6a) led to a much higher percentage of apoptosis (TRAIL treatment in PC3-TR and LNCaP cells; TNF α treatment in PC3 and PC3-TR cells) (Figs. 6b and 6c) than manipulating either gene family alone (Figs. 4 and 5). These results suggest that simultaneous activation of AP-1 and inhibition of NF- κ B can significantly enhance the efficacy of proapoptotic agents like TRAIL and TNF α .

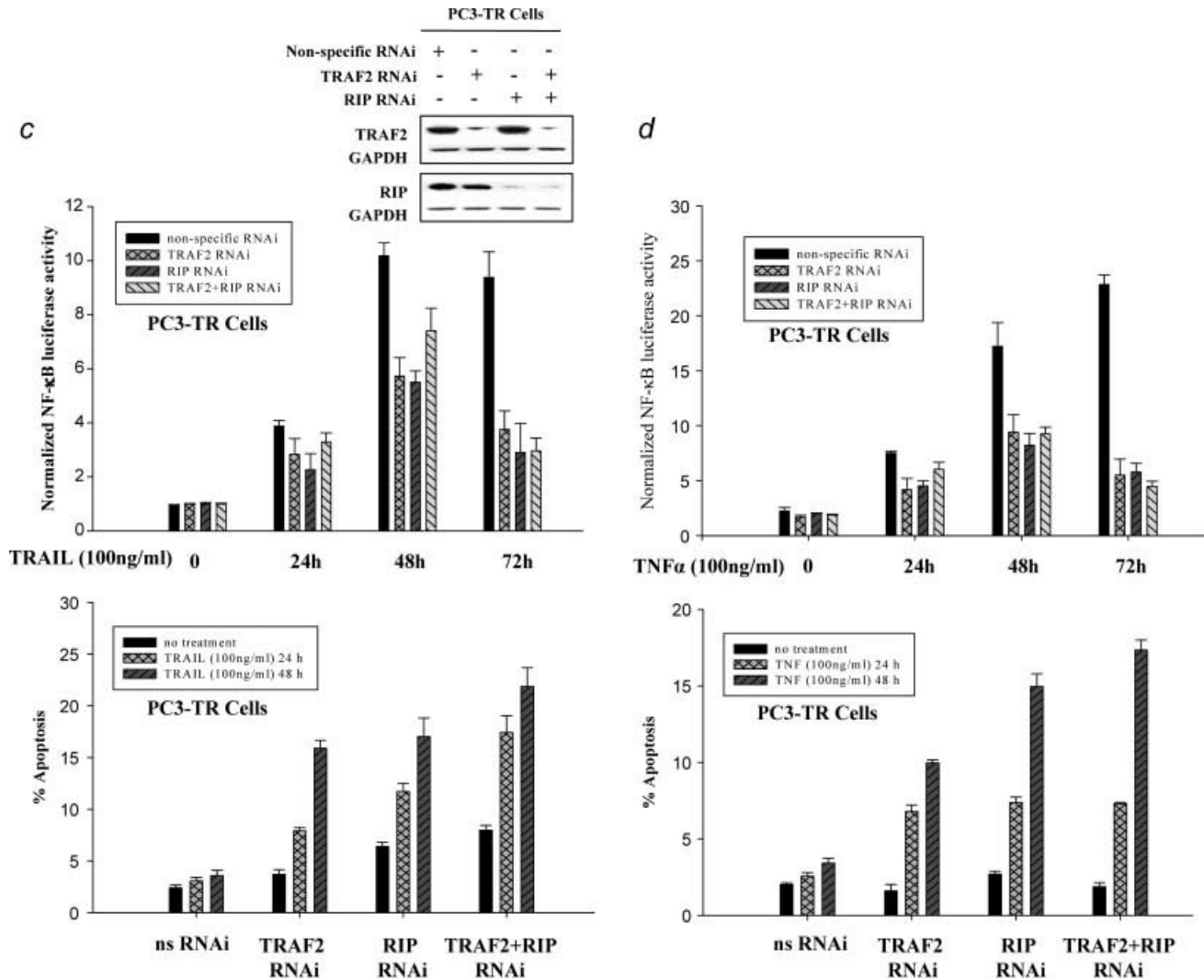


FIGURE 3 – CONTINUED. (c) Silencing TRAF2, RIP or both reduced NF- κ B activity (top panel) and partially sensitized PC3-TR cells to TRAIL (bottom panel). Western blots were performed to detect the efficiency of RNAi. (d) Silencing TRAF2, RIP or both reduced NF- κ B activity (top panel) and partially sensitized PC3-TR cells to TNF α (bottom panel). Apoptosis is measured by flow cytometric analysis of the sub-G1 population. Error bars (SD) are results of at least 3 independent experiments.

Discussion

Although TRAIL and TNF α share many similar intracellular pathways, TNF α is associated with significant cytotoxicity, limiting TNF α s clinical utility as a cancer therapeutic agent. Therefore, identifying similarities and differences between TRAIL- and TNF α -induced apoptosis can help differentiate between proapoptotic signals, which may be responsible for the cytotoxicity that is associated with some proapoptotic agents. Here, we have identified a prostate cancer model where prostate cancer cells are differentially sensitive to TRAIL- and TNF α -induced apoptosis. We show that reduction of NF- κ B activity or enhancement of AP-1 activity alone can partially sensitize resistant prostate cancer cells to proapoptotic effects of TRAIL or TNF α . However, concomitant reduction of NF- κ B and enhancement of AP-1 activities sensitize a high percentage of resistant prostate cancer cells to TRAIL- or TNF α -induced apoptosis.

NF- κ B is a key transcription factor that suppresses TNF α -induced apoptosis. Although the precise basis for this signaling pathway continues to be explored, many studies have suggested that NF- κ B-mediated cell survival may be closely related to its downstream anti-apoptotic genes such as c-FLIP,²⁹ Bcl-2, IAPs,

XIAP and Survivin.³⁰ In our prostate cancer model, NF- κ B activation serves as a survival signal, which protects cells from apoptosis whether induced by TNF α or TRAIL. In the presence of TNF α and TRAIL, NF- κ B activity increased in TRAIL-resistant (PC3-TR and LNCaP) and TNF α -resistant (PC3-TR and PC3) cancer cells (Figs. 2a and b). On the other hand, NF- κ B activity was decreased or maintained at low level in TRAIL-sensitive (PC3) and TNF α -sensitive (LNCaP) cells (Figs. 2a and 2b).

We tested the expression of the well-documented NF- κ B activating proteins TRAF2 and RIP in different prostate cancer cell lines after TRAIL and TNF α treatments. Although, some investigators have suggested that TRAF2 may have little role in TRAIL-induced NF- κ B activation,³¹ our present prostate cancer model suggests that silencing TRAF2 leads to reduction of NF- κ B activity and partially sensitizes prostate cancers to TRAIL-induced apoptosis (Figs. 3c, 3d and 3e). This finding suggests that in addition to regulating TNF α -induced apoptosis, TRAF2 also plays an important role in TRAIL-induced apoptosis by regulating activation of NF- κ B in prostate cancer cells. In addition to TRAF2, we found that RIP, another important regulator of DISC formation,²⁶ regu-

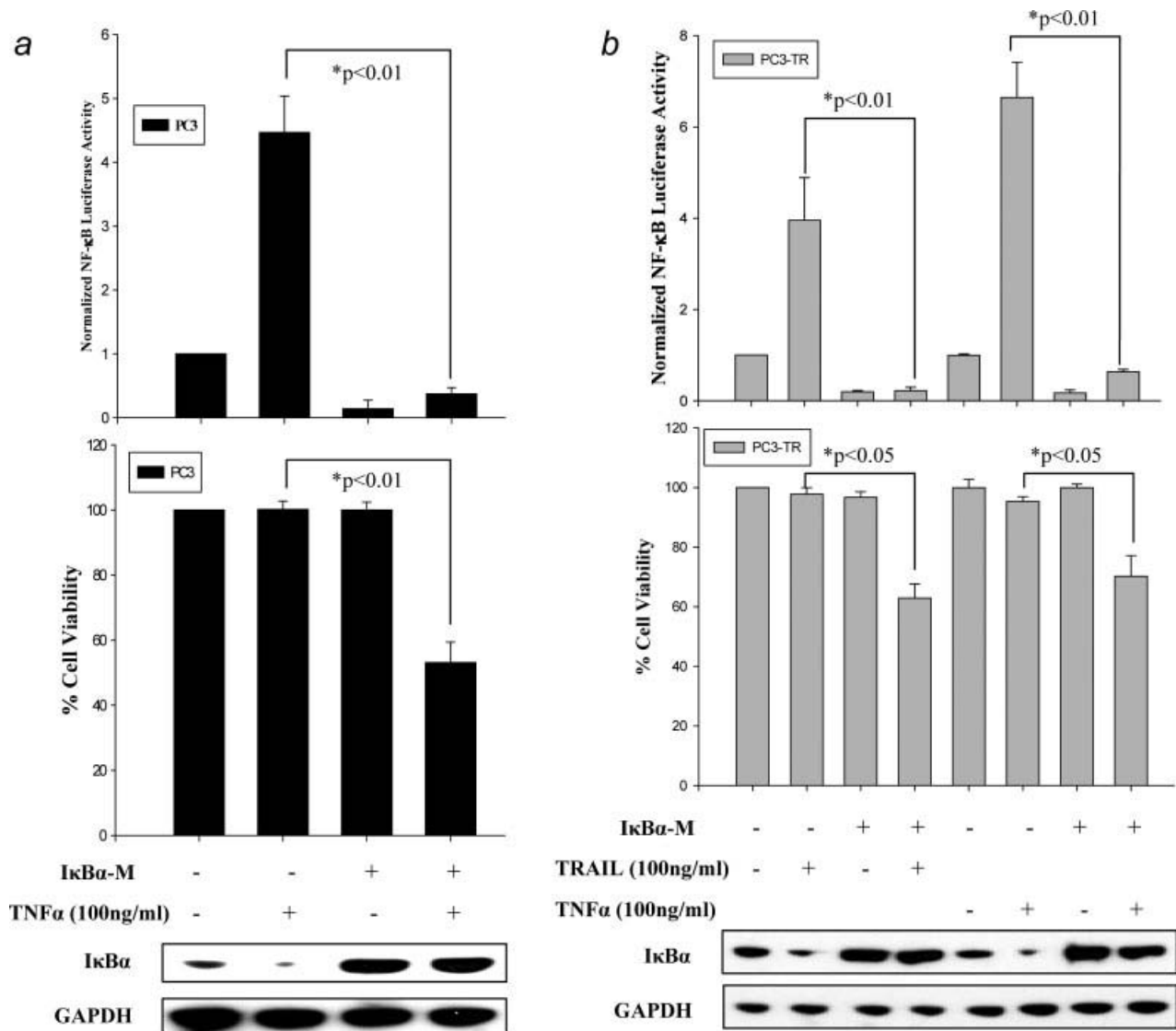


FIGURE 4 – Inhibition of NF- κ B activity by I κ B α -M sensitizes prostate cancer cells to TRAIL- and TNF α -induced apoptosis. (a) NF- κ B luciferase activity (top panel), cell viability (middle panel) of PC3 cells with ectopic expression of I κ B α -M or control vector (Western blot – bottom panel) with or without TNF α treatment for 24 hr. (b) NF- κ B luciferase activity (top panel), cell viability (middle panel) of PC3-TR cells with ectopic expression of I κ B α -M or control vector (Western blot – bottom panel) with or without TRAIL or TNF α treatment for 24 hr. Transfection of empty vector was used as control (–). Cell viability was measured by MTT assay. Error bars (SD) are results of at least 3 independent experiments. “*” Refers to statistically significant differences between indicated groups.

lates NF- κ B and sensitivity of prostate cancer cells to TRAIL and TNF α (Fig. 3).

The relationship and interactions between TRAF2 and RIP for activation of NF- κ B activation are controversial. Our results showed that simultaneous silencing of TRAF2 and RIP did not have any added benefit in reducing activity of NF- κ B, or enhancing cell death, as compared to silencing of TRAF2 or RIP alone (Figs. 3c, 3d and 3e). These results suggest that TRAF2 and RIP regulate activation of NF- κ B in “series”, and not in a “parallel” cell signaling pathway—a finding that is consistent with previous reports that TRAF2-mediated ubiquitination leads to the activation of downstream kinases including RIP.³² However, NF- κ B activation mediated by TRAF2 and RIP may be different between TRAIL- and TNF α -induced apoptotic pathways. After TNF binds to its receptor, RIP, TRADD and TRAF2 form complex I. Binding of TRAF2 to TNF DISC is TRADD dependent and complex I can activate NF- κ B directly.³³ In contrast, TRAIL-induced formation of DISC complex I requires FADD

but not TRADD, whereas DISC complex II recruits RIP and TRADD.^{10,33}

The precise mechanism of dual regulation of NF- κ B and AP-1 is under investigation in our lab. We postulate that c-FLIP, a key anti-apoptosis molecule, may play an important role in mediating both NF- κ B and AP-1 related pathways in prostate cancer cells. We have shown in the past that c-FLIP(L) is an important regulator of TRAIL-induced apoptosis,^{21,34} and its expression is negatively regulated by c-Fos/AP-1.¹⁹ Because c-FLIPs expression can be induced by NF- κ B,²⁹ and in this study, and our prior work we have shown that AP-1s activity is required for cancer cells to be sensitive to TNF α and TRAIL, c-FLIP(L) may function as a common gene for cross-coupling NF- κ B and AP-1 activities in prostate cancer cells undergoing apoptosis. Because there are 3 functional c-FLIP isoforms: c-FLIP(L),³⁵ c-FLIP(s)³⁶ and FLIP(R)³⁷; each isoform may function differently in mediating the cross-talk between the NF- κ B and AP-1 related pathways.

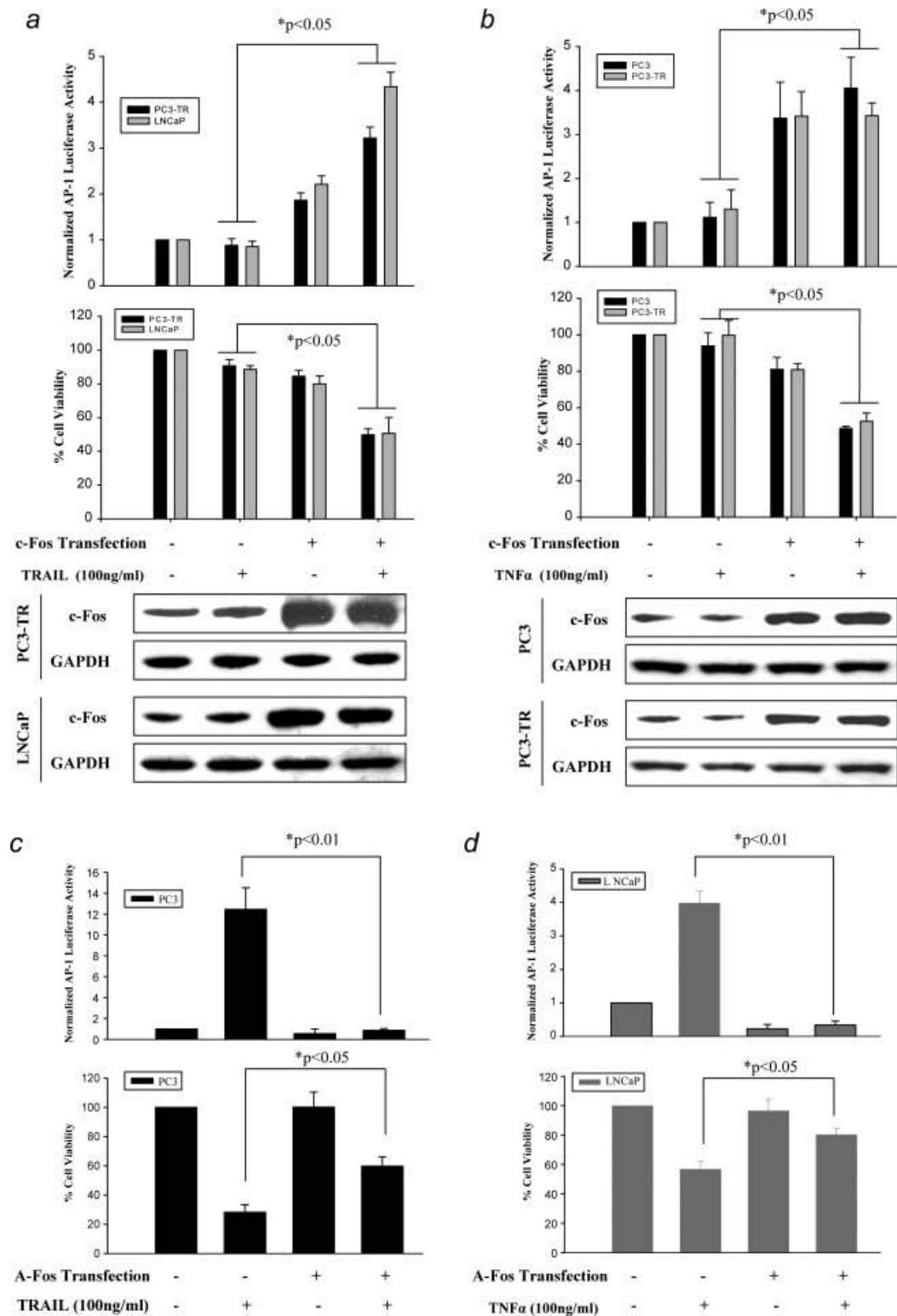


FIGURE 5 – Increased AP-1 activity partially sensitizes resistant prostate cancer cells to TRAIL or TNF α , whereas inhibition of AP-1 activity partially reduced sensitivity to TRAIL and TNF α in prostate cancer cells. (a) Ectopic expression of c-Fos (bottom panel), increased AP-1 activity (top panel) and partially sensitized TRAIL resistant PC3-TR and LNCaP cells to TRAIL (middle panel). (b) Ectopic expression of c-Fos (bottom panel) increased AP-1 activity (top panel) and partially sensitized TNF α resistant PC3 and PC3-TR cells to TNF α (middle panel). (c) Ectopic expression of A-Fos (an AP-1/c-Fos dominant negative) inhibited AP-1 activity (top panel) and partially reduced TRAIL-induced cell death (bottom panel) in PC3 cells. (d) Ectopic expression of A-Fos inhibited AP-1 activity (top panel) and partially reduced TNF α -induced cell death (bottom panel) in LNCaP cells. “–” Indicates transfection of empty vector was used as control. Error bars (SD) are results of at least 3 independent experiments. “*” Refers to statistically significant differences between indicated groups.

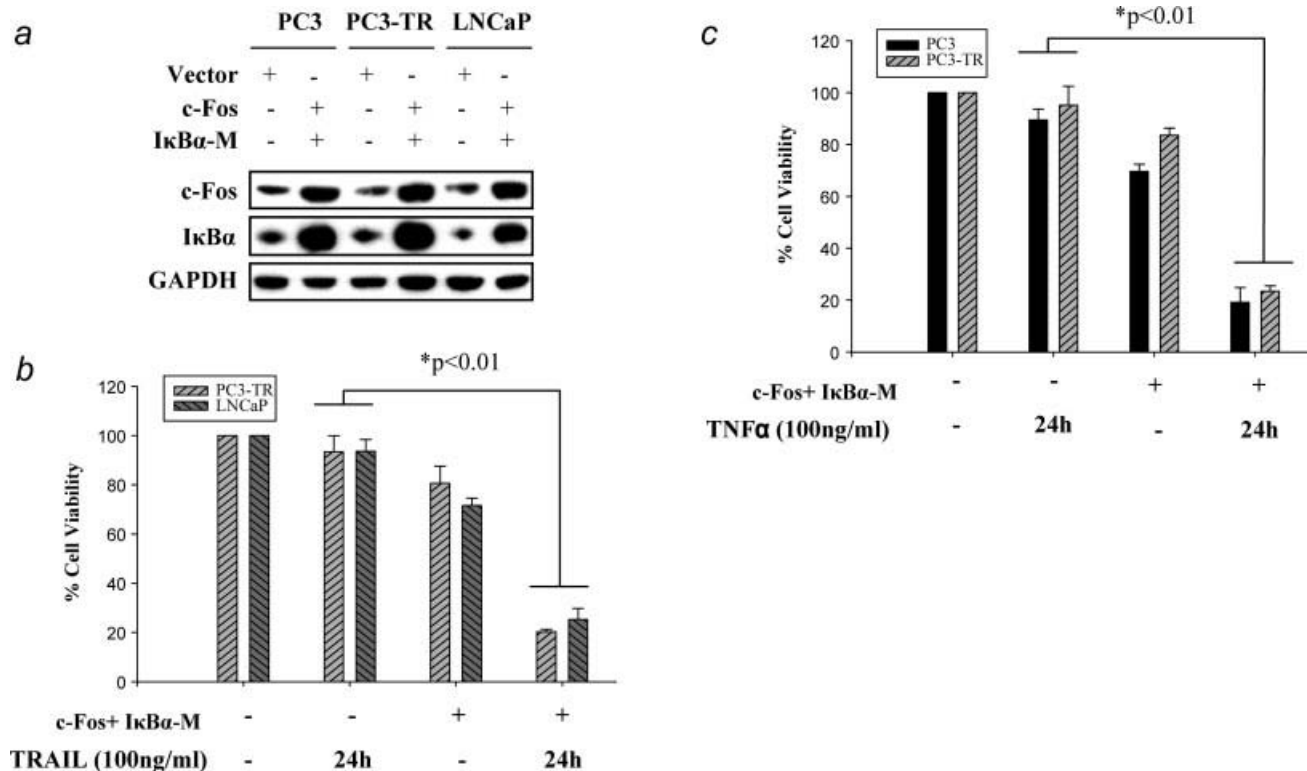


FIGURE 6 – Concomitant inhibition of NF- κ B and potentiation of AP-1 activities markedly changes TRAIL- or TNF α -resistance in prostate cancer cells. (a) Simultaneous ectopic expression of c-Fos and I κ B α -M in prostate cancer PC3, PC3-TR and LNCaP cells. (b) TRAIL-induced cell death in PC3-TR and LNCaP cells before and after cotransfection of c-Fos and I κ B α -M. (c) TNF α -induced cell death in PC3 and PC3-TR cells before and after cotransfection of c-Fos and I κ B α -M. Error bars (SD) are results of at least 3 independent experiments. “*” Refers to statistically significant differences between indicated groups.

Although TRAIL- and TNF α -induced apoptosis may share some common pro-apoptotic pathways,³⁸ Other important pathways such as Akt³⁹ and mitogen-activated protein kinases (MAPKs)⁴⁰ may differentially regulate TRAIL and TNF α signaling. We believe that regulation of TRAIL and TNF α signaling requires cross talks between multiple regulatory signaling networks, some of which include NF- κ B and AP-1. For example, it has been suggested that AP-1 may directly regulate NF- κ B by direct interactions with the NF- κ B subunit, p65.^{41,42} Other examples of potential cell signaling cross talks include Akt induction of NF- κ B by phosphorylating I κ B.⁴³ Yet, another example is NF- κ Bs ability to inhibit JNK and promote c-FLIP(L) to promote survival signals.⁴⁴ To balance NF- κ Bs survival signals, JNK has been shown to activate the E3 ubiquitin ligase, ITCH, thereby inhibiting c-FLIP(L) and potentiating proapoptotic signals.⁴⁴

In addition to the extrinsic pathway which is mediated by death receptors such as TNF and TRAIL receptors, the intrinsic pathway is regulated largely by the Bcl-2 family. Most epithelial cancer cells including prostate cancer are type II cells. The apoptosis process in these cells utilizes both intrinsic and extrinsic pathways. We have found that inhibition of caspase 8/10 (extrinsic pathway) and caspase 9 (intrinsic pathway) and caspase 3 can completely block TRAIL-induced apoptosis in PC3 cells (Zhang and Olumi, unpublished data). Many groups have shown the important role of Bcl-2 family members in mediating TRAIL or TNF α signaling pathways. However, the role of c-Fos/AP-1 in relation to Bcl-2 activity remains to be explored. Because c-Fos is translocated from the cytoplasm to the mitochondria after TRAIL treatment,⁴⁵ it is possible that c-Fos/AP-1 activities may also play an important role in mediating the intrinsic apoptotic pathway and the Bcl-2 machinery.

Because normal development, growth and malignant progression of prostate cells are all dependent on androgens, one may postulate that androgens or the androgen-receptor may affect interactions in TRAIL- and TNF α -induced apoptosis. In fact, it has been shown that the c-FLIP(L) promoter region contains multiple androgen-response element sequences.⁴⁶ In our model system, we evaluated the hormone-dependent LNCaP cells to a series of hormone-independent LNCaP sub-lines.⁴⁷ We did not find any difference of sensitivity to TRAIL-induced apoptosis, c-Fos or c-FLIP(L) expression between the hormone-dependent or the hormone-independent sub-lines (data not shown). Therefore, our preliminary studies does not suggest that androgen-dependence of prostate cancer cells may play a key role in TRAIL-induced apoptosis.

Conclusion

Our study demonstrates that concomitant reduction of NF- κ B and enhancement of AP-1 activity potentiates the proapoptotic effects of TRAIL- and TNF α -induced apoptosis. Therefore, multiple molecular pathways, such as NF- κ B and AP-1, may need to be modulated to overcome TRAIL or TNF α resistance for cancer therapies.

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Novel Targeted Pro-Apoptotic Agents for the Treatment of Prostate Cancer

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Purpose: We reviewed and highlighted novel targeted apoptotic mediated therapies that can be used to treat prostate cancer. **Materials and Methods:** A comprehensive review of the peer reviewed literature in the area of apoptosis was performed with special emphasis on apoptotic mediated pathways with promising novel targeted therapies that can be used for patients with prostate cancer.

Results: The apoptotic pathway can be classified into 2 separate broad categories, including the extrinsic and the intrinsic pathways. Targeting the extrinsic or intrinsic mediated pathway holds promise for developing novel agents for treating prostate cancer. We discuss apoptosis related molecules and therapies, as categorized by 1) targeting apoptosis pathway for antitumor treatment, 2) targeting apoptosis regulators for antitumor treatment and 3) drugs that potentiate pro-apoptotic agents.

Conclusions: Defining the molecules responsible for apoptosis and their intricate molecular interactions will help guide us in developing drugs with less toxicity for appropriately selected patients with prostate cancer and other malignancies. Because neoadjuvant and adjuvant clinical trials are under way using novel pro-apoptotic agents for prostate cancer, it is imperative for urologists to be active members of the clinical research team and become familiar with the molecular pathways, and potential benefits and toxicities associated with these novel agents.

Key Words: prostate, prostatic neoplasms, apoptosis, drug therapy, apoptosis regulatory proteins

Prostate cancer is one of the most commonly diagnosed cancers in the United States with approximately 234,460 individuals diagnosed in 2006, of whom approximately 27,350 died of the disease.¹ In contrast to localized prostate cancer, which can be treated effectively with surgery, radiation therapy or in select cases with watchful waiting, advanced hormone independent prostate cancer is unresponsive to conventional hormonal therapy and it accounts for the majority of deaths. Although androgen suppression could induce apoptosis in prostate cancer,² it is not considered a targeted pro-apoptotic therapy. Despite ablative androgen therapy prostate cancer can progress to a hormone independent state, which accounts for most of the morbidity and mortality associated with prostate cancer. Therefore, newer therapies are required to treat prostate cancers that are unresponsive to hormonal therapies. As newer pro-apoptotic therapies enter clinical trials for treating patients with prostate cancer, it is imperative for urologists to be active members of the clinical research team, become familiar with apoptotic molecular pathways and be

aware of the potential side effects of new cancer therapy agents.

Apoptosis, also known as programmed cell death, has critical roles in the development, homeostasis maintenance and host defense in multicellular organisms. It is characterized by DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage. Initiation and progression of many urological diseases, including renal failure, lupus nephritis, urological normothermic ischemia and most importantly tumors in urological organs, have been associated with dysregulated apoptotic pathways.

Unlike normal prostate tissue, where the rate of cell proliferation and cell death is well balanced, the rate of proliferation of metastatic prostate cancer cells is approximately 15-fold higher than that in normal prostate cells, while there is no significant difference in the rate of cell death between normal and cancerous tissues.³ Therefore, targeting apoptosis pathways can be an excellent therapeutic strategy for many malignancies, including prostate cancer.

Apoptosis occurs through extrinsic and intrinsic apoptotic pathways (fig. 1). The final step involved in each pathway is a caspase cascade, which cleaves regulatory and structural molecules, leading to cell death.⁴ In addition to components involved directly in the apoptosis pathways, a number of molecules function as apoptosis modulators, which regulate apoptosis at the transcriptional, translational and post-translational levels. Exploring the regulatory molecules in the apoptosis pathways will help advance novel targeted therapies for prostate cancer.

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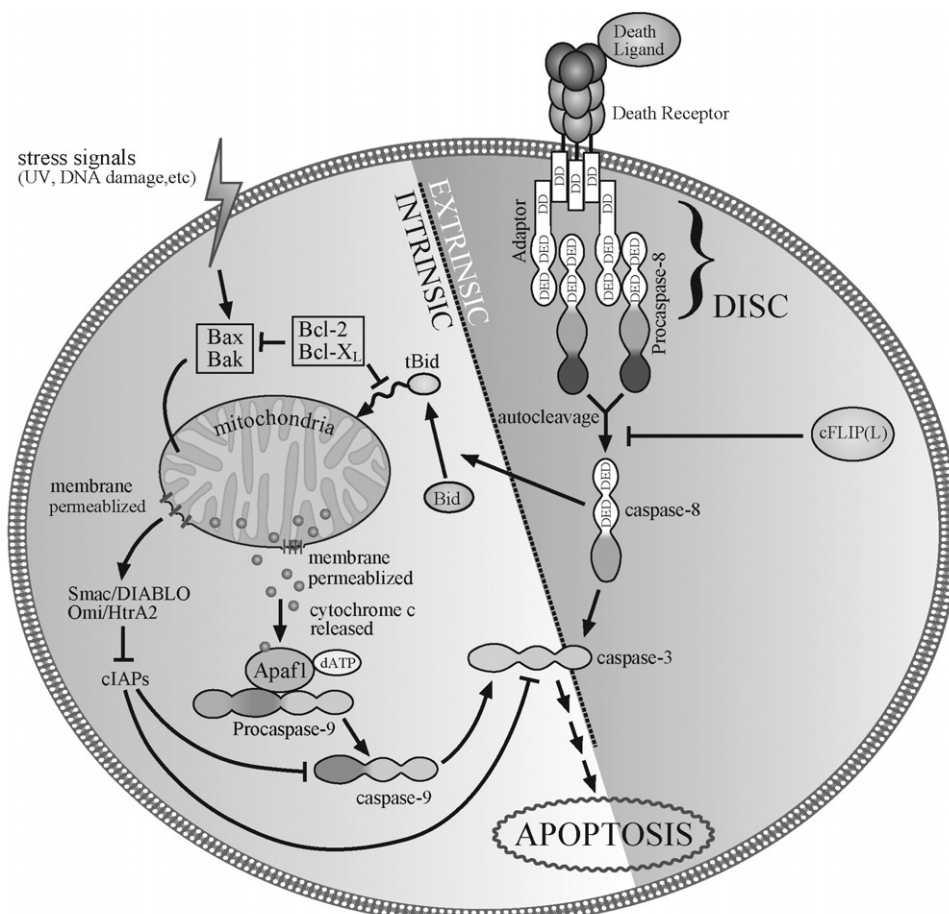


FIG. 1. Extrinsic and intrinsic apoptotic pathways. Arrows indicate activation, induction and translocation. Bars indicate inhibition and blockage. *UV*, ultraviolet light. *tBid*, truncated BID molecule. *Apaf1*, apoptotic protease activating factor 1. *ATP*, adenosine triphosphate.

EXTRINSIC PATHWAY

The extrinsic pathway of apoptosis is also known as the cytoplasmic or DR pathway. Extracellular agents such as TRAIL and TNF induce the extrinsic pathway through the activation of specific DRs distributed on the plasma membrane (figs. 1 and 2). Components involved in the extrinsic pathway include DRs, adaptor molecules, regulatory complex DISC and caspases. Specific DRs become activated by their specific ligands and as a result they form dimer/trimer complexes (fig. 1). Within seconds the activated DRs recruit adaptor molecules, which further transmit the death signals to DISC, where the 2 adaptor proteins FADD and TNF receptor-associated death domain have been shown to recruit caspase-8 or 10 to activate the extrinsic apoptotic pathway.

In addition to recruiting pro-apoptotic molecules, adaptor proteins can also recruit anti-apoptotic molecules, of which TRAF2 and receptor interacting protein have been shown to initiate anti-apoptotic signals by activating NF- κ B, leading to cancer cell survival. Our unpublished data indicate that TRAF2 and receptor interacting protein are regulated differentially in response to TRAIL and TNF in sensitive and resistant prostate cancers.

The activation of pro-apoptotic stimuli from the extrinsic mediated pathway also leads to cleavage of the pro-apoptotic molecule BID. The truncated BID molecule enters the mitochondria, activates the intrinsic apoptotic pathway and

functions as a bridge between the extrinsic and intrinsic mediated apoptotic pathways (fig. 1).

INTRINSIC PATHWAY

The apoptotic intrinsic pathway is also known as the mitochondrial pathway. The Bcl-2 family of proteins is the most important modulator of the intrinsic apoptosis pathway.⁵ The Bcl-2 superfamily consists of pro-apoptotic and anti-apoptotic members, and the interaction and overall balance between the 2 groups of Bcl-2 families could determine the fate of a cell.⁵ Over expressed in advanced prostate cancer, anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L are currently under investigation as important targets for prostate cancer treatment.

When activated by apoptotic signals, mitochondria undergo 2 major changes, including 1) permeabilization of the outer membrane and 2) reduction of the inner membrane potential, resulting in the release of cytochrome c, Smac/Diablo and apoptosis inducing factor from the outer membrane of the mitochondria. After it is released in the cytosol cytochrome c interacts with apoptotic protease, activating factor 1 and leading to the recruitment of procaspase-9 to form a complex termed the apoptosome. The apoptosome in turn cleaves caspase-3 and 7, resulting in the activation of the caspase cascade and apoptosis. Smac/Diablo and Omi/

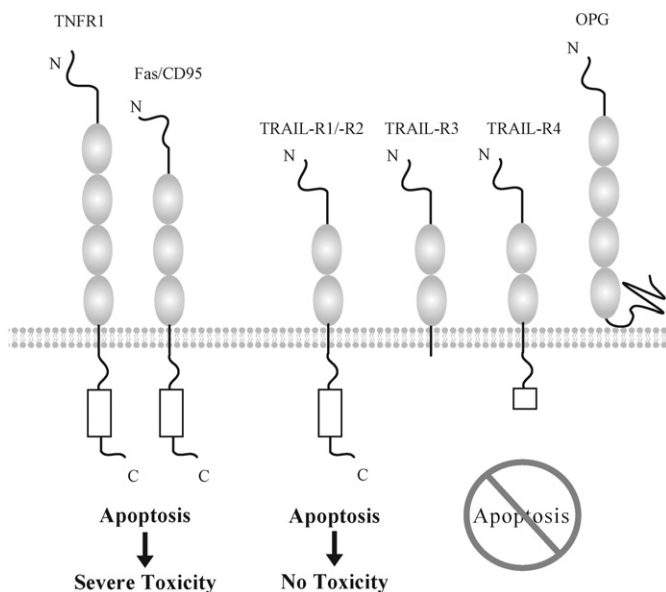


FIG. 2. Apoptosis related consequence of DR mediated death signal transduction. Activation of TNF receptor 1 and Fas/CD95 leads to apoptosis with severe toxicity, while activation of TRAIL-R1 and R2 by TRAIL induces apoptosis without toxicity in normal (N) cells. Decoy receptors OPG and TRAIL-R3 do not induce apoptosis for lacking cytoplasmic domains. C, cancer.

HtrA2 have been shown to inactivate endogenous inhibitors of caspases such as IAPs, thus, promoting mitochondria related apoptosis (fig. 1).⁶

CASPASE CASCADE

As the final executioner of apoptosis, caspase activity is important for the extrinsic and intrinsic mediated apoptotic pathways. As a family of intracellular proteases, caspases can be grouped into inflammatory caspases (caspase-1, 4, 5 and 11 to 14), initiator caspases (caspase-2 and 8 to 10) and effector caspases (3, 6 and 7).⁷ As 30 to 50 kDa proteins that act as inactive precursor zymogens, procaspases are recruited to the DISC, where they undergo autocleavage at the internal proteolytic site and become activated caspases in response to pro-apoptotic signals.⁷ Activated initiator caspases further activate the downstream caspase cascade (extrinsic pathway) or provoke the release of cytochrome c (intrinsic pathway) to transmit the death signal to effector caspases (fig. 1). The extrinsic and intrinsic pathways converge on caspase-3, which cleaves a series of proteins such as nuclear lamins, gelsolin, fodrin and poly(adenosine diphosphate-ribose) polymerase, leading to cell cycle arrest, inactivation of DNA repair and anti-apoptotic proteins, and the direct disassembly and reorganization of cell structures.⁷ In fact, the loss of caspase-1 and 3 has been associated with prostate cancer progression.⁸

Caspase activity could be regulated by a number of factors, of which caspase inhibitors are being extensively studied. IAP and c-FLIP(L) are anti-apoptotic proteins known for their role in caspase inactivation. IAP and c-FLIP(L) are potential therapeutic targets for prostate cancer, as described. We discuss potential targeted therapies aimed at the extrinsic or intrinsic apoptosis mediated pathways. Although some pro-apoptotic signals may activate the 2 pathways simultaneously, we differentiated and localized the

pro-apoptotic signaling pathways that are important for prostate cancer therapy.

DRUGS TARGETING THE EXTRINSIC APOPTOTIC PATHWAY

c-FLIP(L): TRAIL

Although pro-apoptotic and anti-apoptotic roles have been reported,⁹ c-FLIP(L) is considered mainly an anti-apoptotic factor involved in the extrinsic pathway. c-FLIP(L) is homologous to procaspase-8 but it lacks the catalytic domain required for caspase functions. Therefore, c-FLIP(L) competitively inhibits the activation of procaspase-8 and as a result it acts as a major anti-apoptotic molecule in resistant cancers.¹⁰ Loss of c-FLIP(L) expression has been reported to be essential for castration induced apoptosis in the prostate gland and enhanced c-FLIP(L) expression has been associated with prostate cancer progression to the androgen resistant stage.¹⁰ The pro-apoptotic molecule TRAIL targets and significantly lowers the expression of c-FLIP(L), while inducing the apoptotic pathway.¹¹ However, persistent c-FLIP(L) expression leads to the generation of cancer cells that are resistant to TRAIL induced apoptosis.¹¹ TRAIL belongs to the TNF superfamily and it shares with TNF common components involved in apoptosis induction. While the clinical usefulness of TNF is limited by its severe cytotoxicity, TRAIL triggers apoptosis in tumor cells while sparing normal cells, making TRAIL an ideal anticancer therapy agent. The combination of chemotherapeutic agents such as doxorubicin has been shown to effectively decrease c-FLIP(L) expression, thus, sensitizing prostate cancer cells to TRAIL induced apoptosis.¹²

TRAIL Receptor Agonists: HGS-ETR1, HGS-ETR2, HGS-TR2J and 15d-PGL₂

The activation of TRAIL receptors leads to the induction of death inducing signals from the plasma membrane to the cytoplasm. TRAIL has 5 known specific receptors, including the 2 agonistic receptors TRAIL-R1 and R2, and the 3 decoy receptors TRAIL-R3, TRAIL-R4 and OPG. TRAIL-R3 and R4 cannot transmit apoptotic signal because they lack or have a truncated death domain. OPG, a secreted TNF receptor homologue involved in bone homeostasis, may also act as a decoy receptor of TRAIL (fig. 2). Some groups have suggested that the excessive presence of TRAIL decoy receptors on the normal cell surface may account for the minimum toxicity of TRAIL when used systemically.¹³ On the other hand, TRAIL-R1 and R2, also known as DR4 and DR5, respectively, are functional DRs and excellent targets for antitumor therapy. Two forms of agonistic human monoclonal antibodies, HGS-ETR1 and HGS-ETR2, which activate TRAIL-R1 and R2, respectively, have been developed. HGS-ETRs are reported to mimic the activity of TRAIL. Preclinical animal studies have demonstrated that TRAIL receptor agonist antibodies such as HGS-ETRs can induce apoptosis in tumor cells and inhibit tumor growth in a broad range of human malignancies. Phase II clinical trials with HGS-ETRs as a single agent and phase Ib clinical trials of HGS-ETRs in combination with chemotherapeutic agents are under way for treating various solid malignancies.¹⁴

Other forms of TRAIL receptor agonist compounds are HGS-TR2J¹⁵ and 15-d-PGJ₂.¹⁶ While HGS-TR2J was designed to have high affinity for TRAIL-R2 receptor, directly increasing receptor activity, 15-d-PGJ₂ enhances the expression of TRAIL-R2 receptor by indirect means. A mechanism by which 15-d-PGJ₂ increases TRAIL-R2 receptor levels is by increasing the stability of TRAIL-R2 mRNA. However, 15-d-PGJ₂ also enhances apoptosis in prostate cancer cells by other means, including increasing caspase activity, promoting Bid cleavage and activating the intrinsic apoptotic pathway.¹⁶

DRUGS TARGETING THE INTRINSIC APOPTOTIC PATHWAY

Bcl-2 Family

Members of the Bcl-2 family are major regulators of intrinsic mediated apoptosis. They also bridge signals from the extrinsic to the intrinsic pathway. The Bcl-2 family of proteins includes pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic members include Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk, and anti-apoptotic members include Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Mcl-1. Members of the Bcl-2 family usually form homodimers or heterodimers to exert their functions. They are characterized by the Bcl-2 family homology domains BH1 to 4, which mediate dimer forming interactions (fig. 3).¹⁷ The BH1 and BH2 domains are important for regulating anti-apoptotic function, while the BH3 domain is important for regulating pro-apoptotic functions.¹⁷ The BH4 domain is mostly present in anti-apoptotic proteins and it is important for death repression.¹⁷

Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope. Bcl-2 prevents the release of cytochrome c from mitochondria. Since Bcl-2 antagonizes the effects of most cancer therapeutic agents, a major attractive target is the inhibition of Bcl-2 or the activation of the pro-apoptotic molecule Bax.

Antisense oligonucleotides against Bcl-2, BH3 inhibitors and Bcl-2 post-translational modulators are currently available to induce apoptosis in cancer cells, including prostate carcinoma, through the Bcl-2 family mediated intrinsic pathway.¹⁸

Antisense oligonucleotides against Bcl-2 family members. Antisense agents are short oligonucleotides designed to complement target RNA molecules and inhibit the translation of specific proteins. Antisense molecules against Bcl-2 family members have been generated for cancer therapy. An example is G313 (Genasense®), an antisense phosphothiorate oligonucleotide that targets and suppresses Bcl-2 ex-

pression, which is currently in clinical trials.¹⁹ By inhibiting the function of Bcl-2, G313 increases the efficiency of many anticancer drugs.¹⁹ More specific to prostate cancer, a group of antisense oligonucleotides that target Bcl-2 plus Bcl-X_L was recently developed, which was found to successfully induce apoptosis and enhance chemosensitivity in AIPC cells.²⁰ Therefore, future clinical trials will determine whether antisense Bcl-2 therapies against prostate cancer would enhance the efficacy of other therapies or whether they may directly function as pro-apoptotic cancer therapy agents.

BH3 inhibitors. The development of BH3 inhibitors is based on the findings that BH3 mediated interaction between pro-apoptotic and anti-apoptotic Bcl-2 family members is important for regulating apoptosis. In many cancers BH3-only proteins such as Bad and Bid are bound at the BH3 domains by the anti-apoptotic group of Bcl-2 family members, thereby decreasing their activity as pro-apoptotic proteins.

In many cancers anti-apoptotic members, including Bcl-2 and Bcl-X_L, are over expressed, while pro-apoptotic BH3-only protein signaling is decreased. BH3 inhibitors in the form of peptides and small molecule inhibitors have been designed to mimic the function of BH3-only proteins. For example, cpm-1285, ABT-737 and BH3I-2' are examples of newly developed BH3 inhibitors that were designed to decrease the activity of Bcl-2 anti-apoptotic members, while potentiating the activity of Bcl-2 family pro-apoptotic members. BH3 inhibitors have been shown to enhance the therapeutic effects of chemotherapeutic agents such as paclitaxel²¹ or enhance the activity of other pro-apoptotic molecules such as TRAIL in prostate cancer cells.¹⁸ Hydrocarbon stapled modifications of the BH3 domain have been used to enhance the pro-apoptotic efficacy of BH3 helix targeted agents.^{22,23}

Bcl-2 post-translational modulator. Since post-translational modulation such as protein phosphorylation affects protein-protein interactions, targeting the interaction between Bcl-2 family members through post-translational modulation is another promising strategy. As a semisynthetic taxoid analogue, docetaxel phosphorylates Bcl-2, thus, disrupting the interaction between Bcl-2 and Bax, and inducing apoptosis in hormone dependent and independent prostate cancer.²⁴ As a single agent or when combined with estramustine, a metabolite of capecitabine, docetaxel has been studied in phase III trials with proven efficacy against prostate cancer.²⁴

IAP: 1396 Family and Embelin

IAP is a family of caspase inhibitors that share BIR domains. Eight family members have been identified in humans, of which XIAP is the most potent caspase inhibitor among IAPs. XIAP contains 3 BIR domains. It has been reported that BIR2 of XIAP inhibits the activation of caspases-3 and 7, while BIR3 inhibits caspase-9. It has been documented that the small molecules 1396-11, 12, 22 and 34, which bind specifically to BIR2, restore the activity of caspase-3 and 7, and induce apoptosis in several tumor cell lines.²⁵ Preclinical studies have indicated that 1396-12 is not lethal to normal hematopoietic cells in short-term cytotoxicity evaluation.²⁶ In addition, embelin, a cell permeable,

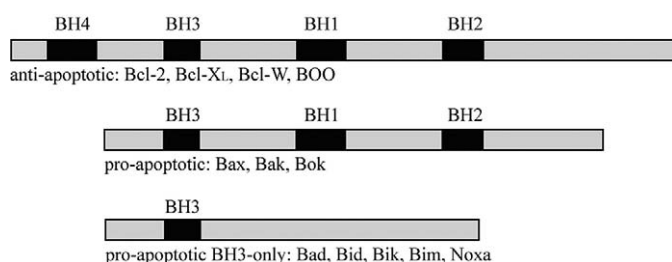


FIG. 3. BH domains of Bcl-2 family members

small molecular inhibitor of XIAP, has been reported to bind to BIR3 of XIAP and induce apoptosis in prostate cancer cells with a minimal effect on normal prostate epithelial and fibroblast cells.²⁷ Therefore, XIAP inhibitors are attractive agents for treating various malignancies, including prostate cancer,²⁷ due to their property of inducing apoptosis in cancer cells without associated cytotoxicity.²⁶

In addition to synthesized XIAP antagonists, endogenous antagonists of XIAP, including Smac, have been identified. The ratio of XIAP to Smac expression increases during tumor progression, making Smac another potential target for therapy.

Smac: Smac-Mimic

The anti-apoptotic function of XIAP is balanced by pro-apoptotic molecules such as Smac, Omi/HtrA2 and XAF1. For example, Smac is secreted from mitochondria during apoptosis and it interacts with the BIR domains of IAP by its consensus AVPI sequence, thus, blocking the apoptosis inhibitory effect of IAP.²⁸ Therefore, Smac mimicking agents have been generated to counterbalance the function of XIAP.²⁸ A small molecule (Smac-mimic), which has Smac N-terminal AVPI residues, was recently developed and found to sensitize human cancer, including prostate cancer, to TRAIL and TNF α induced apoptosis.²⁹

DRUGS TARGETING APOPTOSIS MODULATOR

Some apoptotic related molecules are not directly involved in the apoptosis pathway. Instead, they modulate specific components of the apoptotic molecules. Targeting these apoptosis modulators could achieve effective therapeutic results in inducing apoptosis without major cytotoxicity.

PI3K-Akt Pathway and

mTOR: CCI-779, RAD001 and AP23573

Akt is an anti-apoptotic serine-threonine kinase that has been found to be constitutively active in prostate cancer (fig. 4).³⁰ Akt is translocated to the plasma membrane by PI3K mediated PIP3 production. PTEN has been shown to decrease Akt activity and a loss of PTEN has been documented in prostate cancer, leading to the up-regulation of Akt.³⁰ Activated Akt phosphorylates and inactivates the pro-apoptotic proteins Bad and caspase-9, while activating the anti-apoptotic molecule NF- κ B. As a downstream component of the PI3K-Akt pathway, mTOR suppresses the activity of the pro-apoptotic Bcl-2 family molecule BAD and shuts off PI3K activation by a negative feedback loop mechanism (fig. 4). mTOR is targeted and inhibited by rapamycin, an important antitumor agent. A few rapamycin analogues, including CCI-779, RAD001 and AP23573, are currently in clinical trials and have shown promising results, particularly for renal cell carcinoma.³¹ Clinical trials with mTOR inhibitors are also under way in advanced prostate cancer.³² However, since the anti-apoptotic Bcl-2 family members are commonly activated in hormone independent prostate cancer, it is possible that the mTOR inhibitors may need to be combined with other chemotherapeutic agents for efficacious results.

NF- κ B: Silibinin and Velcade®

NF- κ B is a well known transcription factor that is involved in a number of biological processes and constitutively activated in most advanced prostate cancers.³³ NF- κ B is normally sequestered as inactive in cytoplasm by its inhibitor I κ B. When I κ B is phosphorylated by IKK α and degraded by proteasome, NF- κ B is released into the nucleus and regulates the transcription of target genes. While NF- κ B can have dual pro-apoptotic and anti-apoptotic functions, most

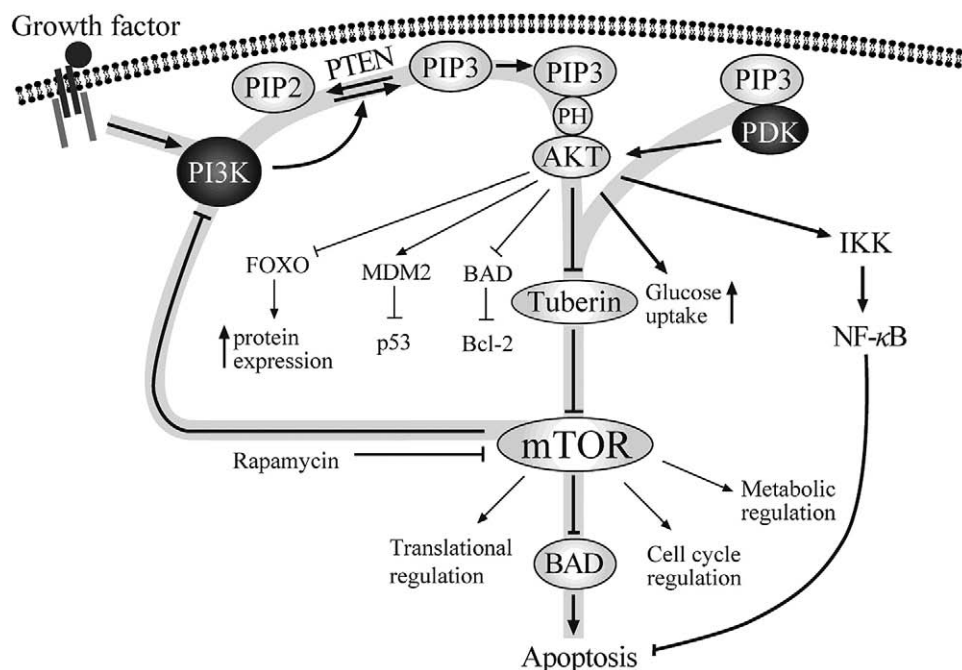


FIG. 4. PI3K-Akt pathways. Arrows indicate activation, induction and translocation. Bars indicate inhibition and blockage. Gray lines indicate major PI3K-Akt pathway and negative feedback.

investigators have found that NF- κ B functions as an anti-apoptotic factor by inducing the transcription of many anti-apoptotic proteins, such as TRAF1, TRAF2, Bcl-2, Bcl-X_L and cIAP.

Several agents that suppress the constitutive activation of NF- κ B are being studied for the putative chemoprevention of prostate cancer. Silibinin has been reported to inhibit NF- κ B activity in prostate cancer cells by decreasing IKK α activity.³⁴ The proteasome inhibitor PS-341 (Velcade), which is a novel boronic acid dipeptide that inhibits 26S proteasome activity, has been reported to induce apoptosis in prostate cancer cells in vitro, partly by abrogating NF- κ B activation.³⁵

COX-2: Nonsteroidal Anti-Inflammatory Drugs

Normally expressed at a low level but boosted during tumor progression, COX-2 is the rate limiting enzyme that catalyzes prostaglandin and thromboxane synthesis, and it is involved in inflammatory pathways.³⁶ COX-2 over expression in prostate cancers leads to decreased apoptosis, increased angiogenesis, increased tumor invasiveness and decreased immune surveillance.³⁷ Several COX-2 inhibitors, such as celecoxib, rofecoxib and valdecoxib, are currently available as antitumor medication for prostate cancer.³⁷ However, a recent Food and Drug Administration report has suggested that COX-2 selective nonsteroidal anti-inflammatory drugs could be associated with possible cardiovascular events and life threatening gastrointestinal bleeding. Therefore, it is unclear whether COX-2 inhibitors will be used to a great degree for prostate cancer as preventive agents or compounds for the active treatment of prostate cancer.

Proteasome Inhibitors: Velcade and MG-132

Proteasomes degrade several pro-apoptotic proteins and tumor suppressors, including Bax, c-Jun N-terminal kinase, p53 and I κ B. Proteasome inhibition increases the level of proteasome substrate, therefore, enhancing the ability of the cell to undergo apoptosis.

Bortezomib (former name PS-341 and commercial name Velcade) was the first proteasome inhibitors to enter clinical trials for advanced multiple myeloma and other malignancies. Bortezomib specifically binds the 20S proteasome complex and inhibits its activity, leading to the inhibition of NF- κ B and the activation of pro-apoptotic pathways, including caspase-8 and 3.³⁵ Bortezomib sensitizes prostate cancer cells to TRAIL induced apoptosis.³⁸ Clinical trials to examine the efficacy of bortezomib for prostate cancer are expected in the near future.

MG-132 is another proteasome inhibitor that has been shown to potentiate the activity of pro-apoptotic agents such as TRAIL.³⁹ Molecular mechanisms by which MG-132 potentiates the effect of TRAIL induced apoptosis are the stabilization of the TRAIL-R2 level and the repression of anti-apoptotic Bcl-2 family members.⁴⁰ Our studies have demonstrated that MG-132 enhances the effects of TRAIL induced apoptosis by repressing the expression of the anti-apoptotic molecule c-FLIP(L). Therefore, proteasome inhibitors hold great promise to improve the efficacy of targeted pro-apoptotic drugs for patients with prostate cancer.³⁹

Despite the antitumor efficacy of the targeted pro-apoptotic agents discussed, 2 major issues cause concern, including toxicity and resistance. New approaches are being developed to address these questions.

OVERCOMING TOXICITY

The toxicity associated with apoptosis inducing treatment includes unintended killing of normal cells and activating inflammatory mediators. Several apoptosis inducing therapeutic agents for prostate cancer, such as TNF α , are associated with high toxicity when used systemically.

To overcome the toxicity associated with pro-apoptotic drugs several groups are developing agents in prodrug forms. For metastatic prostate cancer the prodrugs can be designed to bind prostate specific antigen and human glandular kallikrein-2, which are prostate specific molecules, with the hope of targeting prostate cancer tissues and minimizing the systemic side effects of apoptosis inducing agents.⁴¹

OVERCOMING RESISTANCE TO PRO-APOPTOTIC DRUGS

Although many cancers are sensitive to pro-apoptotic drugs, some develop resistance. Cancer cells can develop resistance to pro-apoptotic agents such as TRAIL at many levels from DR to intracytoplasmic regulators (see Appendix).⁴² Overcoming drug resistance is a major challenge in all fields of cancer therapy. Molecular factors attributable to the cellular resistance of apoptotic stimuli in prostate cancers include increased Akt activity, the lack of active lipid phosphatase PTEN, constitutively active NF- κ B androgen deprivation, persistent c-FLIP(L) expression, XIAP expression, c-Jun N-terminal kinase activation, and Bcl-xL and Bcl-2 over expression.⁴³ To overcome apoptotic resistant cancer cells combination therapies with CDDO, HDACI, IMiD and thalidomide may serve as good strategies to sensitize resistant cancer cells to pro-apoptotic agents.

CDDO and CDDO-Im

CDDO is a derivative of oleanolic acid, which is a naturally occurring triterpenoid. The semisynthetic triterpenoid has been found to suppress the inflammatory enzymes inducible nitric oxide synthase and COX-2. Furthermore, an imidazole derivative of CDDO, namely CDDO-Im, is 5 times more potent than CDDO as an antitumor agent in vivo and in vitro. CDDO and CDDO-Im induce apoptosis in tumor cells by activating the extrinsic and intrinsic pathways selectively in tumor cells.⁴⁴ Others have reported that CDDO compounds may sensitize cells to pro-apoptotic agents by down-regulating c-FLIP(L) or up-regulating the cell surface TRAIL receptors TRAIL-R1 and R2.⁴⁵

HDACI

Epigenetic changes, otherwise known as changes that are not coded in the DNA sequence, have been shown to modulate gene expression and have an important role in tumor initiation and progression.⁴⁶ One of the epigenetic changes that modulates gene expression is histone acetylation. Generally increased histone acetylation leads to increased transcription and gene expression. HDAC removes acetyl groups from histone proteins and altered HDAC activity has been

identified in several cancers.⁴⁶ HDACIs have been shown to induce cell cycle arrest and apoptosis in cancers with little associated toxicity.⁴⁶ The mechanism of HDACI induced apoptosis involves the activation of caspase, and/or the induction of cleavage and activation of Bid.⁴⁷ The HDACI AN-7 has been reported to have in vivo and in vitro antitumor activity in prostate cancer.⁴⁸ In addition to AN-7, a number of HDACIs, including suberoylanilide hydroxamic acid and the benzamide derivative MS275, are in preclinical and clinical development stages.⁴⁸ Suberoylanilide hydroxamic acid has been shown to sensitize resistant prostate cancers to TRAIL by activating TRAIL-R2, up-regulating caspase-3 and inducing Bid truncation.⁴⁹ Future clinical trials will define the role of HDACIs as single agents or their possible use in combination with other drugs for prostate cancer treatment.

IMiDs and Thalidomide

In addition to inhibiting IL-6, IMiD and thalidomide enhance pro-apoptotic signals by up-regulating caspase activities, inhibiting NF- κ B and down-regulating anti-apoptotic proteins such as cIAP and c-FLIP.⁵⁰ Thalidomide has been included in phase II studies with docetaxel for AIPC treatment.⁴³ Patients with AIPC who were treated with the combination of thalidomide and docetaxel showed a 53% response by decreased prostate specific antigen compared with 37% in the docetaxel-only arm. Median progression-free survival was 5.9 months for the combination with a survival of 25.9 months compared with 3.7 and 14.7 months, respectively, for docetaxel alone. Therefore, the combination of IMiDs and thalidomide may have a role in patients with advanced prostate cancer in a hormone refractory state.

CONCLUSIONS

Dysregulated apoptotic pathways have an important role in the initiation and progression of prostate cancer. Inhibiting anti-apoptotic molecules or potentiating pro-apoptotic molecules can serve as an excellent treatment strategy for prostate cancer. One challenge will be to identify agents with limited toxicity and maximal therapeutic efficacy. Another challenge will be to identify the proper patient population that would benefit the most from novel agents targeting the apoptotic pathways. Defining the apoptotic mediated signal transduction pathways and the intricate molecular interactions will help guide us in developing drugs with less toxicity for appropriately selected patients with prostate cancer and other malignancies. As neoadjuvant and adjuvant clinical trial are designed for newly developed pro-apoptotic mediated therapies, it is important for urologists to be active participants in clinical research teams by becoming familiar with the molecular pathways and the potential toxicities for patients enrolled in clinical trials.

ACKNOWLEDGMENTS

HGS-ETR1 and HGS-ETR2 were developed at Human Genome Science, Inc., Rockville, Maryland.

APPENDIX

Major Mechanisms of Resistance to TRAIL Induced Apoptosis⁴²

Molecules	Mechanisms
DRs	Low levels of DR4 and DR5, and/or high levels of decoy receptors
DISC assembly	
FADD	FADD deficiency due to mutation or deletion
Caspases	Down-regulation or absence of caspase-8/10
c-FLIP	Up-regulation or increased stability of c-FLIP(L)
Bcl-2 family	Increase in the ratio of anti-apoptotic and pro-apoptotic Bcl-2 family proteins
IAPs, Smac/Diablo	Up-regulation of IAPs, such as cIAP1, cIAP2, XIAP, NAIP, survivin and BRUCE, and reduced level or release of Smac/Diablo
NF- κ B	Promotes expression of antiapoptotic proteins, such as Bcl-2, Bcl-XL, cIAP and c-FLIP(L)
Akt	Activates multiple pathways such as mTOR, NF- κ B, Bcl-2, and inhibits, FOXO and p53
Mitogen-activated protein kinases	Activates extracellular regulated kinase 1/2

Abbreviations and Acronyms

AIPC	=	androgen independent prostate cancer
BIR	=	baculovirus IAP repeat
CDDO	=	2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid
c-FLIP	=	cellular FLICE-inhibitory protein
c-FLIP(L)	=	c-FLIP (long)
COX-2	=	cyclooxygenase-2
DISC	=	death-inducing signaling complex
DR	=	death receptor
FADD	=	Fas associated protein with death domain
HDAC	=	histone deacetylase
HDACI	=	HDAC inhibitor
IAP	=	inhibitor of apoptosis proteins
I κ B	=	inhibitor of NF- κ B
IKK	=	I κ B kinase
IMiD	=	immunomodulatory drug
mTOR	=	mammalian target of rapamycin
NF- κ B	=	nuclear factor- κ B
OPG	=	osteoprotegerin
PI3K	=	phosphatidylinositol 3-kinase
PSA	=	prostate specific antigen
PTEN	=	phosphatase and tensin homolog deleted on chromosome ten
Smac	=	second mitochondria-derived activator of caspases
TNF	=	tumor necrosis factor
TRAF	=	TNF receptor-associated factor
TRAIL	=	TNF-related apoptosis-inducing ligand

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OVERCOMING RESISTANCE TO TRAIL-INDUCED APOPTOSIS IN PROSTATE CANCER BY REGULATION OF c-FLIP

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Abstract

Using Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL) for cancer therapy is attractive, because TRAIL is effective against cancer cells without inducing significant cytotoxicity, making it an ideal cancer drug. However, some cancer cells evade TRAIL-induced apoptosis and become resistant. We have been investigating the molecular mechanisms that differentiate between TRAIL-resistant and TRAIL-sensitive prostate cancer cells. We have found that transcriptional regulation of the anti-apoptotic molecule, c-FLIP(L), can regulate sensitivity of cancer cells to TRAIL. We have found that c-Fos, represses expression of c-FLIP(L), and promotes TRAIL-induced apoptosis.

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Identifying molecular mechanisms that differentiate between sensitive and resistant cancer cells will help improve pro-apoptotic cancer therapies.

1. INTRODUCTION

Apoptosis is a cellular response that regulates important processes such as homeostasis, immunosurveillance, and elimination of unwanted cells. It has become clear that most, but not all, types of apoptosis require activation of a class of cysteine proteases, the caspases. There are two major signaling pathways of apoptosis: the extrinsic pathway and intrinsic pathway. The extrinsic pathway activates executive caspase-8 and caspase-10 through death receptors on the cellular surface, whereas intrinsic pathway activates caspase-9 by releasing cytochrome *c* and activating other mediators from mitochondria.

Caspases are activated in a hierarchical order. Activated caspase-8 triggers the extrinsic apoptotic pathway by directly activating effectors such as caspase-3 and caspase-7. Caspase-8 can also initiate the intrinsic apoptotic pathway through the activation of Bid (Sinicrope and Penington, 2005; Suliman *et al.*, 2001). Both pathways lead to the activation of caspase-3 and eventual apoptotic cell death (Suliman *et al.*, 2001).

Shortly after the identification of caspase-8 and caspase-10, a structurally related protein was cloned independently by nine groups and, therefore, originally had eight different names (Tschopp *et al.*, 1998). The most widely used name is c-FLIP, shortened from cellular FLICE inhibitor protein (FLICE: FADD-like interleukin-1 β -converting enzyme).

2. STRUCTURE AND MOLECULAR MECHANISMS OF c-FLIP IN APOPTOSIS

c-FLIP is a human cellular homolog of viral FLICE-inhibitory proteins(v-FLIPs) (Thome *et al.*, 1997) and contains tandem death-effector domains and caspase-like domain similar to pro-caspase-8 and pro-caspase-10 but lacks amino acid residues that are critical for caspase activity, most notably the cysteine in the catalytic center (Chang and Yang, 2000; Irmeler *et al.*, 1997). When ligands such as TNF α , FasL, or TRAIL interact with specific death domain receptors, the interaction will induce intracellular cytoplasmic formation of the DISC (death inducing signaling complex) (Bodmer *et al.*, 2000; Changet *et al.*, 2006; Kischkel *et al.*, 2000; Micheau and Tschopp, 2003; Schneider *et al.*, 1997; Sheridan *et al.*, 1997; Sprick *et al.*, 2000). DISC formation involves recruitment of caspase-8/10 through

an adaptor protein (FADD, TRADD, and RIP) to the death effector domain (DED) of the activated receptor (Pan *et al.*, 1997; Zhang *et al.*, 2005). c-FLIP protein homologs interrupt apoptotic signaling by competing with caspase-8 for binding to the DED domains of FADD and also regulate apoptosis through their interference with the recruitment of caspase-8 to FADD (Irmeler *et al.*, 1997; Medema *et al.*, 1997; Wajant *et al.*, 2000).

c-FLIP mRNA gives rise to multiple protein isoforms. There are three protein isoforms: a long c-FLIP form (c-FLIP[L]) (Irmeler *et al.*, 1997), a short c-FLIP form (c-FLIP[s]) (Irmeler *et al.*, 1997; Krueger *et al.*, 2001a; 2001b), and a third recently identified form, called FLIP(R) (Golks *et al.*, 2005). All three isoforms contain DED domains and can, therefore, remain bound to FADD and interrupt complete caspase-8/10 processing and activation. However, the exact roles of different c-FLIP isoforms remain controversial (Peter, 2004). As an example, most published reports involving ectopic expression of c-FLIP(L) suggest that c-FLIP(L) or its caspase-cleaved 43-kDa form has an anti-apoptotic role. Moreover, c-FLIP^{-/-} mouse embryonic fibroblasts have been shown to be more sensitive to FasL-induced apoptosis (Yeh *et al.*, 2000b), which strongly suggests that c-FLIP(L) has an anti-apoptotic function. However, two reports have proposed that c-FLIP(L) may have a dual function, a pro-apoptotic function at low physiologic concentrations and an anti-apoptotic function at high cellular concentrations (Chang *et al.*, 2002; Micheau *et al.*, 2002).

3. EXPRESSION OF c-FLIP

c-FLIP is predominantly expressed in the heart, skeletal muscle, and peripheral blood leukocytes (Irmeler *et al.*, 1997). Surprisingly, some viruses encode the homolog of c-FLIP, which also controls sensitivity toward death-receptor-mediated apoptosis (Thome *et al.*, 1997). Moreover, c-FLIP-deficient mice do not survive past day 10.5 of embryogenesis and exhibit impaired heart development (Yeh *et al.*, 2000a). Hence, c-FLIP is thought to be involved in the regulation of the immune system and development. The expression of c-FLIP proteins and their role in tumor progression are still under investigation. Two recent reports showed that c-FLIP in Hodgkin's lymphomas could protect lymphoma cells from autonomous FasL-mediated cell death while preserving their ability to evade immunosurveillance (Dutton *et al.*, 2004; Mathas *et al.*, 2004), which indicates that c-FLIP has a crucial role in regulation of cell death, a potential role in malignant transformation, proliferation, and metastasis, and the levels of intracellular c-FLIP, therefore, may determine the sensitivity of cancer cells to apoptotic triggers. We collected microarray data from Oncomine

Table 20.1 c-FLIP expression in normal and cancerous tissues

	Normal vs. Normal		Cancer vs. Normal		Cancer vs. Cancer		Tumor Grade
Appendix	1						
Blood	2						
Head-Neck	1						
Heart	1						
Lung	1		1		1	1	
Lymph Node	1						
Muscle	1						
Others	1						
Ovarian	1	1					
Placenta	1						
Prostate	1						
Spinal Cord	1	1					
Thymus	2						
Tongue	1						
Tonsil	1						
Trachea	1						
Umbilical	1						
Brain	3		1		1	1	1
CellLine					1		
Lymphoma					3	4	
Multi-cancer					1	1	
Renal					1	2	
Breast							
Bladder							
Leukemia			1		1		
Normal							
Myeloma							
Sarcoma					1		

P-Value Threshold: 1E-6; Outlier Rank Threshold: 50; Numbers stands for the number of studies
■ High level; ■ Medium level; ■ Low level; ■ High level; ■ Medium level; ■ Low level

(www.oncomine.org) and analyzed the expression of c-FLIP in different normal and tumor tissues. We found that c-FLIP expression was relatively higher in blood, head-neck, lung, lymph node, muscle, tonsil, trachea, and umbilical tissues, whereas c-FLIP levels were lower in brain tissue (Table 20.1). Therefore, microarray gene data suggest that expression of c-FLIP may vary in different organs. Moreover, the expression of c-FLIP may be increased in some malignancies compared with normal tissue, whereas its expression may be decreased in other malignancies compared with normal tissues (Fig. 20.1). Immunohistochemical analyses in bladder (Korkolopoulou *et al.*, 2004) and prostate cancer (Dr. A. P. Kumar,

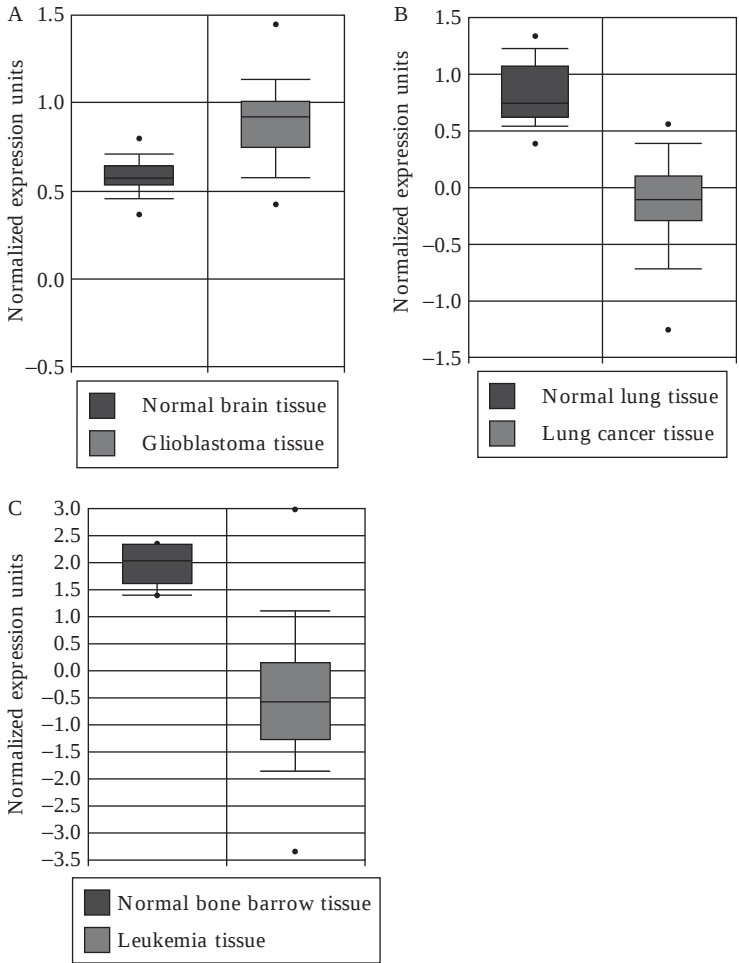


Figure 20.1 The differential expression of c-FLIP between normal and cancerous tissues. (A) c-FLIP level in glioblastoma is higher than that of normal brain tissues. (B) c-FLIP level in lung cancer is lower than that of normal lung tissues. (C) c-FLIP level in leukemia is lower than that of normal bone marrow tissues.

University of Texas, personal communication) suggest that c-FLIP's expression may correlate with more advanced tumors.

4. REGULATION OF c-FLIP EXPRESSION

Intracellular c-FLIP(L) can be regulated at the transcriptional, translational, or posttranslational levels (Kim, 2002; Zhang *et al.*, 2004). We have shown in the past that persistent expression of c-FLIP(L) is necessary and

sufficient to maintain resistance to TRAIL-induced apoptosis. Expression of c-FLIP(L) has been shown to be modulated by NF- κ B (Benoit *et al.*, 2004; Okamoto *et al.*, 2006), Akt (Nemet *et al.*, 2003; Skurk *et al.*, 2004), c-Myc (Ricci *et al.*, 2004), p53 (Fukazawa *et al.*, 2001), and E3-ubiquitin ligase (Chang *et al.*, 2006).

To determine whether alterations in transcription can affect TRAIL-induced apoptosis, TRAIL-resistant (PC3-TR and LNCaP) and TRAIL-sensitive (PC3) cells (Zhang *et al.*, 2004; Zhang *et al.*, 2007b) were treated with TRAIL/Apo-2L (100 ng/ml) in the presence or absence of actinomycin D (Fig. 20.2A), a general inhibitor of transcription. We found that actinomycin D had little effect on the cell viability of the TRAIL/Apo-2L-sensitive cells, yet a combination of actinomycin D and TRAIL/Apo-2L enhanced apoptosis in TRAIL-resistant PC3-TR and LNCaP, suggesting that inhibition of cellular transcription can enhance TRAIL-induced apoptosis (Fig. 20.2B). Microarray analysis of differentially expressed genes after TRAIL treatment suggested that expression of c-Fos was significantly upregulated in the TRAIL-sensitive PC3 cells. In contrast, expression of c-Fos was significantly down regulated in the TRAIL-resistant PC3-TR and LNCaP cells (Table 20.2).

Because c-FLIP(L) is partially regulated transcriptionally (Gao *et al.*, 2005; Li *et al.*, 2007; Roue *et al.*, 2007), its putative promoter region contains multiple c-Fos/AP-1 binding sites (Fig. 20.2C), and c-Fos is differentially expressed in TRAIL-sensitive and TRAIL-resistant cells, we wished to examine whether c-Fos regulates expression of c-FLIP(L). We hypothesized that AP-1 family of proteins may be an important regulator of c-FLIP(L) and, as a result, play a key role in mediating a cell's response to TRAIL-induced apoptosis. We examined the potential AP-1 binding sites in the putative c-FLIP(L) regulatory region (17,000 base pairs upstream of c-FLIP(L)'s ATG start codon [Fig. 20.2C]). We identified and examined binding of c-Fos to 14 AP-1 binding sites in the putative c-FLIP(L) regulatory region by means of chromatin immunoprecipitation (ChIP) assays, which included six AP-1 binding sites upstream of exon 1 (designated sites "a" through "f" in Fig. 20.2C) and eight within intron 1-2. We only detected binding of c-Fos protein to c-FLIP(L)'s AP-1(f) site (Fig. 20.2D). ChIP assays demonstrated that binding of c-Fos to the c-FLIP(L) AP-1(f) site increased in the TRAIL-sensitive PC3 cells, whereas c-Fos binding to the c-FLIP(L) AP-1(f) site was reduced in the TRAIL-resistant PC3-TR and LNCaP cells after treatment with TRAIL/Apo-2L. To confirm the importance of c-Fos/AP-1 binding AP-1(f) site on regulating c-FLIP(L) expression, we deleted this AP-1(f) site in our c-FLIP(L) promoter luciferase reporter. We found that deletion of c-FLIP(L)'s AP-1(f) site abolished the ability of c-Fos to suppress c-FLIP(L) expression (Fig. 20.2D). More detailed studies have shown that TRAIL treatment in TRAIL-sensitive cancer cells promote c-Fos to translocate from the cytoplasm to the nucleus and upregulate AP-1 activity. We have found that

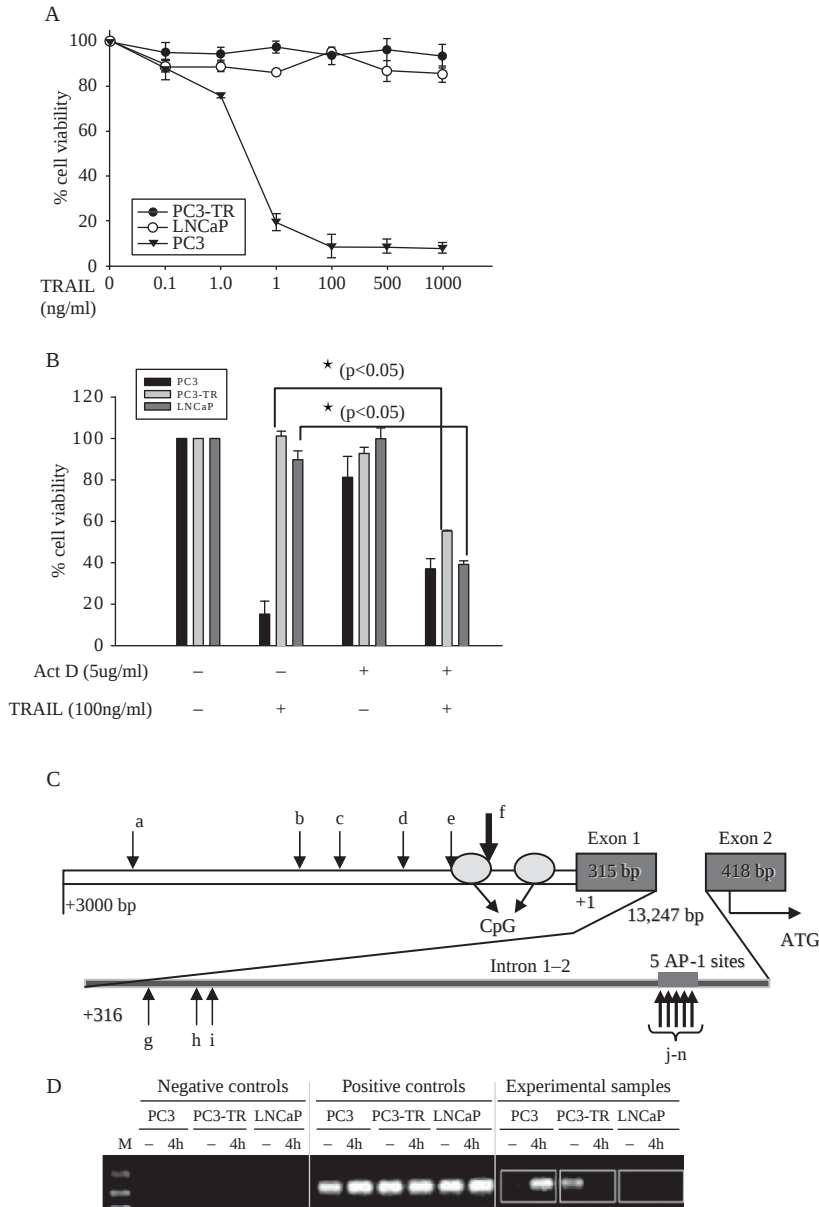


Figure 20.2 c-FLIP in prostate cancer cells can be regulated at the transcriptional level. (A) Cell viability of prostate cancer cells measured by MTT assay. (B) Cell viability was assessed in prostate cancer cells that were pretreated with RNA synthesis inhibitor, Actinomycin D (Act D), for 1 h followed by treatment with TRAIL (100 ng/ml) for another 24 h. (C) Potential AP-1 binding sites in the cFLIP(L) promoter and regulatory region. (D) AP-1(f) binding to c-FLIP(L) promoter analyzed by CHIP assay. Error bars indicate SD of three replicate experiments. “★” Represents significant differences between controls and experimental samples. (C and D were reproduced with permission from *Cancer Res.* 67, 9425; 2007.)

Table 20.2 Differentially expressed genes in TRAIL-sensitive and TRAIL-resistant cells

Genes	PC3 U	PC3 T	LCB	PC3-TR U	PC3-TR T	LCB	LNCaP U	LNCaP T	LCB
SOCS box-containing WD protein SWiP-1	91.61	155.32	1.54	210.78	102.14	−1.85	307.6	103.79	−2.67
Notch homolog 3 (Drosophila)	95.16	231.15	2.22	90.97	37.69	−1.63	126.91	49.19	−2.2
v-fos FBJ murine osteosarcoma viral oncogene homolog	−0.07	53.02	2.57	93.08	−3.34	−4.55	61.81	7.13	−2.2
collagen, type VI, alpha 1	718.18	1790.2	2.16	235.85	136.6	−1.55	401.67	240.52	−1.51
myosin VIIB	33.06	92.81	2.53	32.59	17.05	−1.52	52.44	20.92	−1.92
hypothetical protein FLJ14360	14.36	66.69	3.79	27.88	5.79	−2.26	25.29	9.5	−1.53

upregulation and cytoplasmic to nuclear translocation of c-Fos is necessary, but insufficient, for cancer cells to be sensitive to TRAIL. We postulate that one of the mechanisms that c-Fos/AP-1 primes cancer cells to TRAIL is through direct binding of c-Fos protein to the c-FLIP(L) putative promoter region and repressing the expression of c-FLIP(L) (Zhang *et al.*, 2007b).

5. TARGETING c-FLIP TO ENHANCE APOPTOSIS IN CANCER CELLS

Because c-FLIP is an important modulator of apoptosis, and its expression and activity can be regulated at multiple levels, targeting c-FLIP(L) as a cancer therapeutic agent can be attractive. For example, the combination of TRAIL with the chemotherapeutic agent, doxorubicin, has been shown to effectively decrease the expression of c-FLIP(L), thus sensitizing prostate cancer cells to TRAIL-induced apoptosis (Kelly *et al.*, 2002). Others have shown that CDDO (a novel triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) compounds may sensitize cells to pro-apoptotic agents by downregulation of c-FLIP(L) or upregulation of the cell surface TRAIL receptors, TRAIL-R1 and TRAIL-R2 (Hyer *et al.*, 2005). Previously, we had also found that c-Fos/AP-1 functions as a pro-apoptotic molecule by directly repressing the anti-apoptotic gene, c-FLIP(L). Other groups have shown that expression of c-FLIP(L) could be modulated by NF- κ B (Benoit *et al.*, 2004; Okamoto *et al.*, 2006). These findings suggest that strategies to potentiate c-Fos/AP-1 activation and/or inhibit NF- κ B may repress the expression of c-FLIP(L) and enhance the efficacy of apoptotic inducers, such as TRAIL, for treatment of various malignancies.

12-O-Tetradecanoylphorbol-13-acetate (TPA) is a strong inducer of c-Fos/AP-1. We have demonstrated that TRAIL or a TRAIL-R2 agonist antibody combined with low-dose TPA upregulates AP-1 proteins and its activity, reduces c-FLIP(L) levels, and potentiates apoptosis in TRAIL-resistant LNCaP cells in *in vitro* and *in vivo* experiments (Zhang *et al.*, 2007a). Therefore, TPA, when combined with the pro-apoptotic agent TRAIL, is effective in changing the phenotype of some TRAIL-resistant prostate cancers to a TRAIL-sensitive phenotype.

We have also used the proteasome inhibitor, MG-132, to inhibit NF- κ B. We have found that MG-132 not only inhibited NF- κ B activity (Fig. 20.3A), but it also increased AP-1 activity by promoting nuclear translocation of c-Fos and c-Jun and their heterodimerization (Fig. 20.3B) (Li *et al.*, 2007). Treatment of the cells with MG-132 alone did not affect the level of c-FLIP(L) protein levels, however, when combined with TRAIL, c-FLIP(L) level decreased at both the mRNA and protein level (Fig. 20.3C, D). Therefore, MG-132 significantly increases sensitivity of PC3-TR cells by concomitant

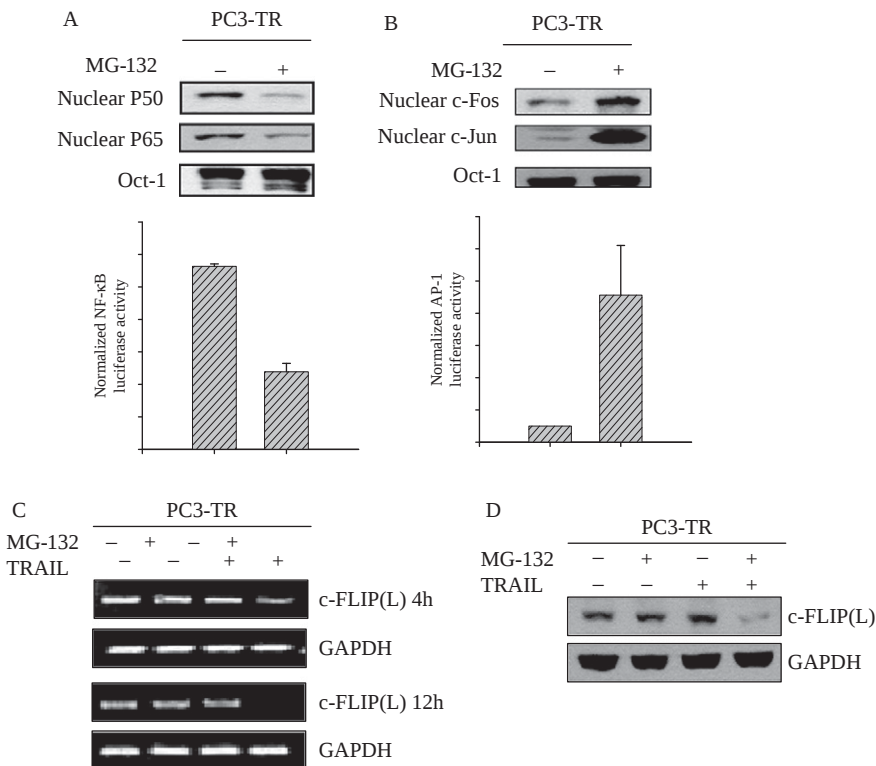


Figure 20.3 MG-132 sensitizes resistant prostate cancer cells to TRAIL. (A) MG-132 decreased NF- κ B-related proteins (p50 and p65) (*top panel*) and inhibited NF- κ B's activity (*bottom panel*). (B) MG-132 upregulated nuclear protein levels of AP-1 family members, c-Fos and c-Jun, (*top panel*) and activated AP-1 activity (*bottom panel*). TRAIL combined with MG-132 represses c-FLIP(L) as demonstrated in the semiquantitative reverse transcription-PCR (C) and Western blot (D) analyses (C and D were reproduced with permission from *Cancer Res.* 67,2247;2007).

activation of AP-1 and repression of NF- κ B to prime cancer cells to undergo TRAIL-induced apoptosis.

6. METHODS AND MATERIALS

6.1. Cell culture and production of PC3-TR

All cell culture materials were obtained from Cellgro (Herndon, VA) and plasticware was from Becton Dickinson Labware (Bedford, MA). PC3, DU145, and LNCaP prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PC3-TR was

a TRAIL-resistant subline established from parental PC3 cells by TRAIL selection. PC3 cells were treated with TRAIL (100 ng/ml). After 24 h, viable cells were rescued by removing TRAIL and replenishing the cells with full medium. When the plates reached 80% confluency, the cells were again treated with TRAIL (100 ng/ml) for 24 h. The cycle was repeated, and PC3-TR cells were generated after 2 months and maintained in medium with TRAIL. PC3-TR cells were released from TRAIL at least one passage before use. All cells were cultured in RPMI-1640 tissue culture medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin (each at 50 μ g/ml) at 37 °C with 5% CO₂.

6.2. Cell viability assays

Cell viability was determined by MTT method in accordance with the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). In brief, 5×10^4 PC3, DU145 cells and 7.5×10^4 LNCaP cells were seeded in 96-well plates and cultured for 24 h before treatment. Cells were then treated with various concentrations of TRAIL for 24 h. MTT was added followed by solubilization buffer 4 h later. Absorbance was measured at 590 nm (630 nm was the reference wavelength) by use of a microtiter plate reader. Viability of untreated cells was set at 100%, and absorbance of wells without cells was set at zero. All results were from at least triplicate experiments.

6.3. cDNA microarray assays

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Chatsworth, CA). The RNA yield and purity were evaluated by measuring A_{260}/A_{280} and agarose gel electrophoresis. cDNA Microarray was performed by use of the human gene arrays GeneChip (Affymetrix, Santa Clara, CA) and 21,625 genes were analyzed according to the manufacturer's instructions. Arrays were scanned by use of an Affymetrix confocal scanner and analyzed by the Microarray software (Affymetrix). Intensity values were scaled so that the overall fluorescence intensity of each chip of the same type was equivalent. If the 90% lower confidence bound (LCB) of the fold change (FC) between the experiment and the baseline was >1.5 , the corresponding genes were considered to be differentially expressed. In our analysis, LCB readings were considered to be more reliable than FC readings for analysis of differential gene expression (Ramalho-Santos *et al.*, 2002).

6.4. Luciferase assays

Cells were seeded into 24-well plates. When the cells were 80% confluent, both AP-1 luciferase reporter (25 ng/well) and Ranilla reporter (5 ng/well) from Stratagene (La Jolla, CA) or NF- κ B reporter and Ranilla reporter from

Stratagene (La Jolla, CA) were cotransfected into cells. Here, Renilla served as an internal control for transfection efficiency. After 24 h of transfection, cells were treated with TRAIL (100 ng/ml) for 4 h, and then both attached and floating cells were collected and centrifuged at 1000 rpm for 5 min at 4 °C. Pellets were rinsed twice with phosphate-buffered saline (PBS), and the cell pellet was prepared in the presence of 1× passive lysis buffer (Dual-Luciferase Assay System Kit, Promega, Madison, WI). Samples were stored at −20 °C until detection. The activities of NF-κB and AP-1 luciferase and Renilla luciferase were determined following the dual-luciferase reporter assay protocol recommended by Promega (Madison, WI, USA). Twenty microliters of cell lysate was transferred into the luminometer tube containing 100 μl luciferase assay reagent (LAR), and firefly luciferase activity (M_1) was first measured then Renilla luciferase activity (M_2) was measured after adding 100 μl of Stop & Glo Reagent. The results were calculated and expressed as the ratio of M_1/M_2 . The experiments were carried out three times with duplicate samples. The data are presented as mean ±SD.

6.5. ChIP assays

ChIP assay was performed by the ChIP Assay Kit (Upstate Cell Signaling Solutions, Lake Placid, NY). Cells were cultured in 10-cm dishes treated with or without TRAIL for 4 h. Fixation of cross-linked DNA and proteins was carried out by adding formaldehyde for final concentration of 1% and incubated for 10 min at 37 °C. Both attached and floating cells were collected, washed, and resuspended in 200 μl of SDS lysis buffer for 10 min and then sonicated for 10 sec 10 times on ice. Selecting the appropriate time and intensity of sonication was very important for successful CHIP results. In pilot experiments, samples were prepared and sonicated for different times and intensity, and then agarose gels were run to evaluate size of the DNA fragment. The DNA gel showed DNA ladder and size of most of the DNA was between 500 bps and 1000 bps. Samples were centrifuged for 10 min at 13,000 rpm at 4 °C, and the supernatant was harvested. The concentration of each sample was quantitated by use of BCA protein assay. Positive controls were 10% of each DNA sample, which did not include the immunoprecipitation step. The remainder of the samples was equally divided into two groups. The experimental group was immunoprecipitated with specific c-Fos (D-1) antibody, whereas the negative control group was immunoprecipitated with general mouse IgG antibody. After immunoprecipitation, protein-DNA crosslinking was reversed. The isolated DNA was first purified, then amplified by PCR, by use of specific primers encompassing the c-FLIP(L) AP-1(f) binding site (GeneBank). The PCR conditions were denaturation at 94 for 50 sec, annealing at 56 °C for 50 sec, and polymerization at 72 °C for 1 min (total number of cycles = 30), final extension at 72 °C for 10 min for 35 cycles. The primers for the

experiments in Fig. 20.4A were 5'-CCT GTG ATC CCA GCA CTT TG-3' (forward primer) and 5'-CAC CAT GCC CGA CTA ATT TT-3' (reverse primer).

6.6. Semiquantitative reverse transcription-PCR analysis

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA yield and purity were evaluated by measuring A_{260}/A_{280} and agarose gel electrophoresis. RT-PCR was performed by use of a Superscript One-Step RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA); 0.4 μ g of the total RNA was used for RT-PCR in 25 μ l of total volume. cDNA synthesis was performed at 50 for 30 min with the following cycle temperatures and times: denaturation at 94 °C for 50 sec, annealing at 56 °C for 50 sec, and polymerization at 72 °C for 2 min (total number of cycles = 30), final extension at 72 °C for 10 min. In each reaction, the same amount of GAPDH was used as an internal control. The primers used for PCR were as follows: c-FLIP(L), 5'-GTC TGCTGA AGT CAT CCA TCAG-3' (forward) and 5'-CTT ATG TGT AGG AGA GGA TAA G-3' (reverse); c-Fos, 5'-GAA TAA GAT GGC TGC AGC CAA ATG C-3' (forward) and 5'-AAG GAA GAC GTG TAA GCA GTG CAG C-3' (reverse); GAPDH, 5'-TCC ACC ACC CTG TTG CTG TA-3' (forward); and 5'-ACC ACA GTC CAT GCC ATC AC-3' (reverse). The PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and then photographed.

6.7. Cell extracts and western blot analysis

Cells were harvested for total cell lysates with RIPA buffer (1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholate, and 0.1% SDS) containing a mixture of protease inhibitors (cocktail 1 $\times$, 1 mM PMSF, 20 mM, 40 mM NaF, and 3 mM Na_3VO_4). After sonication for 15 sec, cell debris was discarded by centrifugation at 12,000g for 10 min at 4 °C, and the protein concentration was determined by BCA protein assay reagent (Pierce, Rockford, IL). The procedure for the nuclear protein extraction was carried out according to the manufacturer's instructions (NE-PER nuclear and cytoplasmic extraction reagents Kit [Pierce Biotechnology, Rockford, IL]). The cell pellet was harvested in Eppendorf tubes, and the supernatant was carefully removed and discarded; 200 μ l of ice-cold CER I was added to the cell pellet (per 40 mg). The tube was vigorously vortexed on the highest setting for 15 sec to fully resuspend the cell pellet. The tube was incubated on ice for 10 min, then 11 μ l of ice-cold CER II was added to the tube. The mixture was mixed in the tube with vortex for 5 sec on the highest setting and then incubated on ice for 1 min. The reaction was again vortexed for 5 sec on the highest setting, then centrifuged for 5 min

at maximum speed in a microcentrifuge ($\sim 16,000g$). Immediately, the supernatant (cytoplasmic extract) fraction was transferred to a clean pre-chilled tube. This tube was placed on ice until further use or storage. The insoluble (pellet) fraction was resuspended, which contains nuclei, in 100 μ l of ice-cold NER. The nuclear fraction was vortexed on the highest setting for 15 sec and then iced for 10 min. Vortexing and icing of the nuclear fraction was repeated every 10 min five times. The nuclear fraction was centrifuged at maximum speed ($\sim 16,000g$) in a microcentrifuge for 10 min. Immediately, the supernatant (nuclear extract) was transferred to a clean prechilled tube and iced. Extracts were placed in -80°C storage until use.

The amount of nuclear protein was quantitated by immunoblot analysis, with anti-Oct-1 or GAPDH as controls. Protein extracts were resolved by 10 to 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblot analysis. Nitrocellulose blots were blocked with 5% (w/v) nonfat dry milk or 3% BSA in Tris-buffered saline/Tween buffer, and incubated with the indicated primary antibody in Tris-buffered saline/Tween containing 2% milk or 1% BSA overnight at 4°C . The blots were stained with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunostained proteins were visualized on X-ray film by use of the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

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