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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.

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-κB (NF-κB), 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

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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 pro-apoptotic 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-^{32}P–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).

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-IP11-536I18 (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′-AGATCTGAGGCAAAGAAACCGAAAGC-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′-GGCTGGTCTCCAACTGCTGGT-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 ^{32}P-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′-AT*CA*CTTGAGGAT*CA*CTTGAGGAT*CA*CTTGAGGAT*CA*CTTGAGG-3′ (wild-type) and 5′-AT*TG* CTTGAGGAT*TG* CTTGAGGAT*TG*CTTGAG-AGGAT*TG*CTTGAGG-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. 4*A* are 5′-CCTGTGATCCCAG-CACTTTG-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 deoxyribonucleotide 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-phenyl-indole (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 defied 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. 1*A*). 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 promotor and regulatory regions of *c-FLIP(L)* by using the Alibaba 2.1 software (17). We 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. 1*B*). We identified potential binding sites for NF-κB (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-FILP(L)*, we also found multiple potential AP-1 binding sites in the *c-FLIP(L)* regulatory region (Fig. 1*B*). 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. 1*C*). 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. 1*C*; 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. 1*D*, *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. 1*D*, *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 deceased in LNCaP cells (Fig. 1*D*, *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. 1*D* and data not shown). Therefore, the change in

nuclear c-Fos protein levels (Fig. 1*D*) correlated with AP-1 activity levels (Fig. 1*C*) 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. 2*A*) and changed PC3 cells from TRAIL-sensitive to a more TRAIL-resistant phenotype (Fig. 2*B*). 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. 2*C*), and converted the PC3 cells from a TRAIL-sensitive to a TRAIL-resistant phenotype (Fig. 2*D*). 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.

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. 1*C* 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. 1*B*), 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. 3*A*). 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. 3*B*). 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. 3*C*). Moreover, protein levels of c-FLIP(L) were maintained when c-Fos expression was either reduced by siRNA (Figs. 2*A* and 3*D*) or its AP-1 activity was suppressed by a AP-1 dominant-negative A-Fos (Figs. 2*C* and 3*D*).

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. 1*B*]. 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. 1*B*) 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. 1*B* and 4*A*). 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. 4*B*). 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. 4*C*). 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. 5*A*), AP-1 activity was increased (Fig. 5*B*), and cell viability was decreased (Fig. 5*C*). 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. 5*C*). 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 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), 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 proteosome-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 proteosome 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 anti apoptotic 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-κB (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. 4*A*), 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. 1*B*); 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. 1*B*), 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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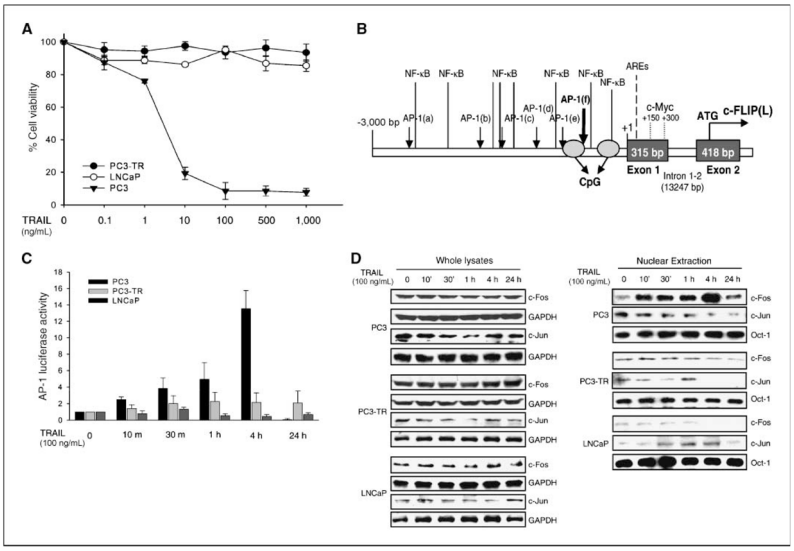

Figure 1.

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 *cFLIP(L)* promoter and regulatory region. Putative AP-1, NF- κB, 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.

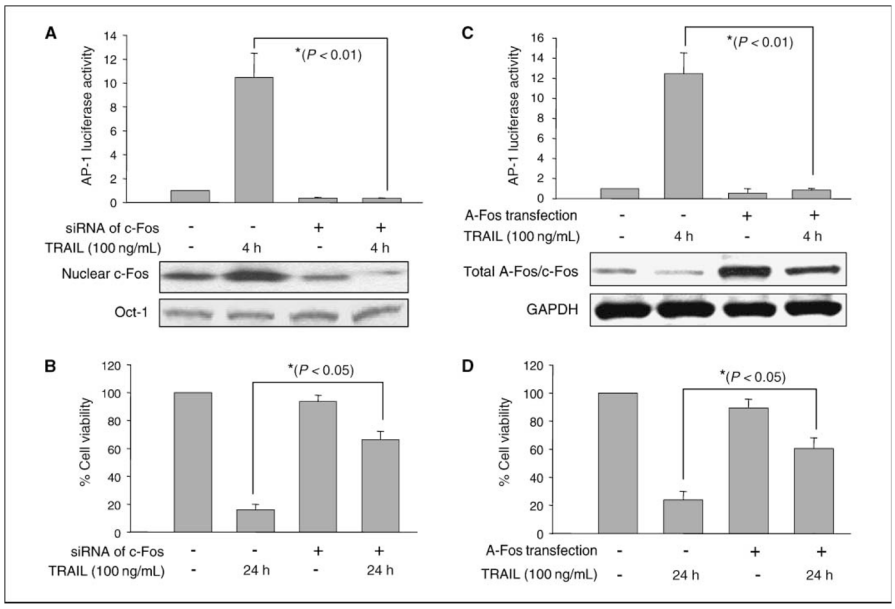

Figure 2.

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.

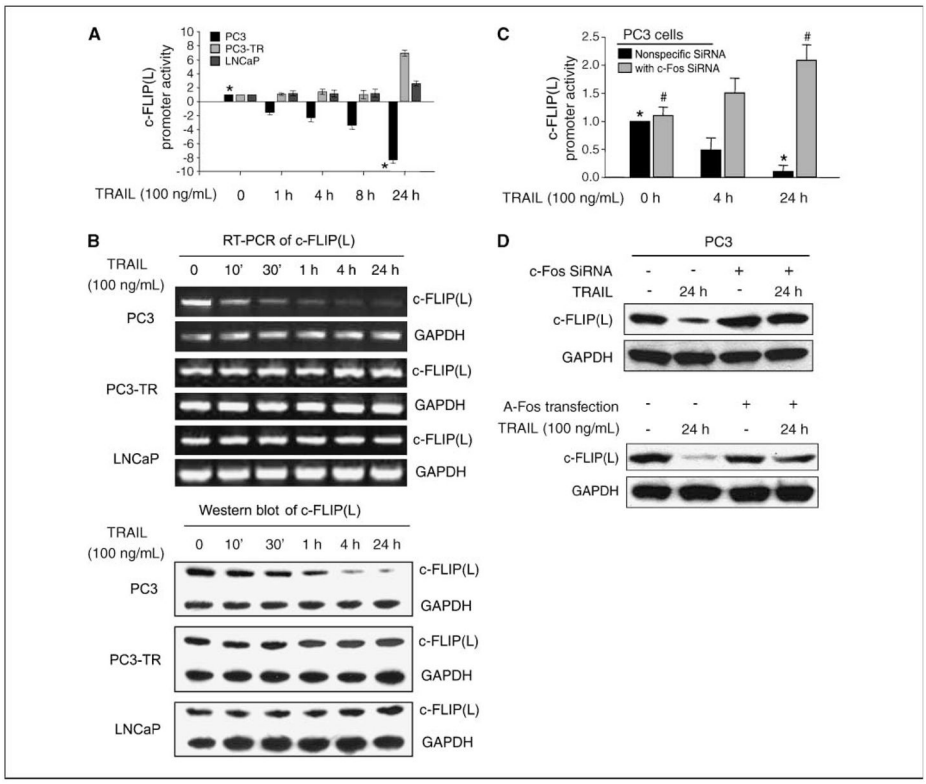

Figure 3.

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.

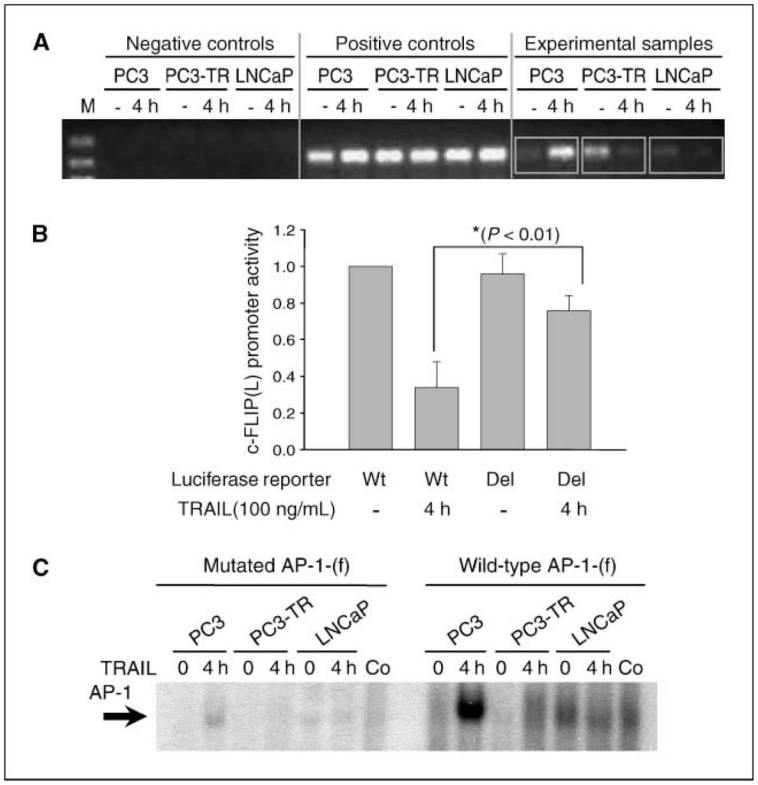

Figure 4.

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.

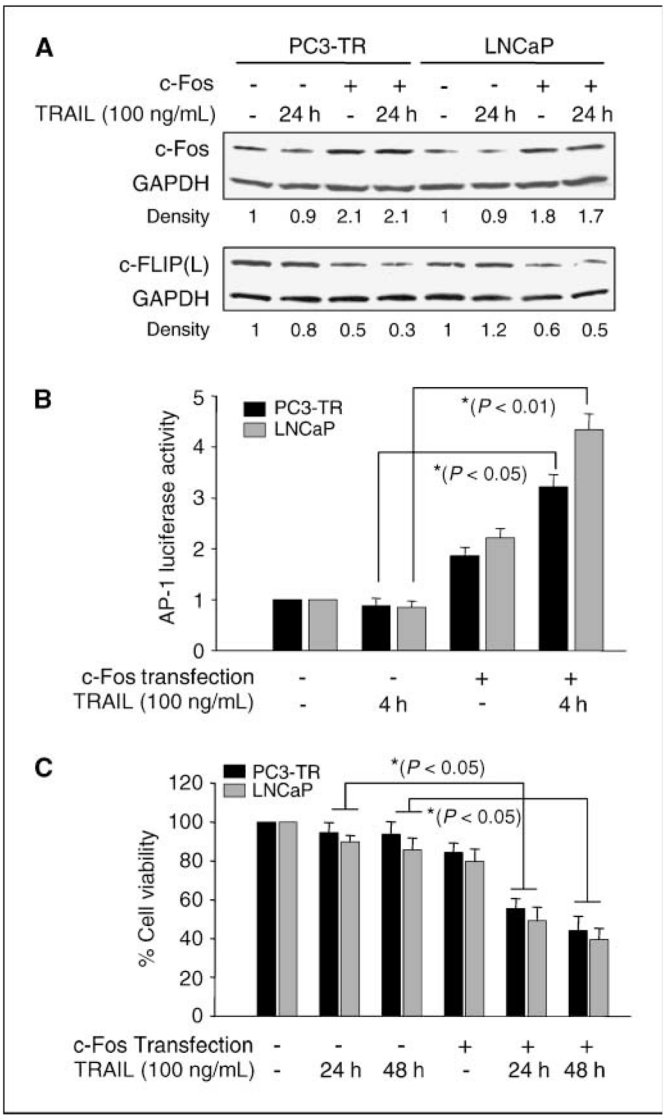

Figure 5.

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.

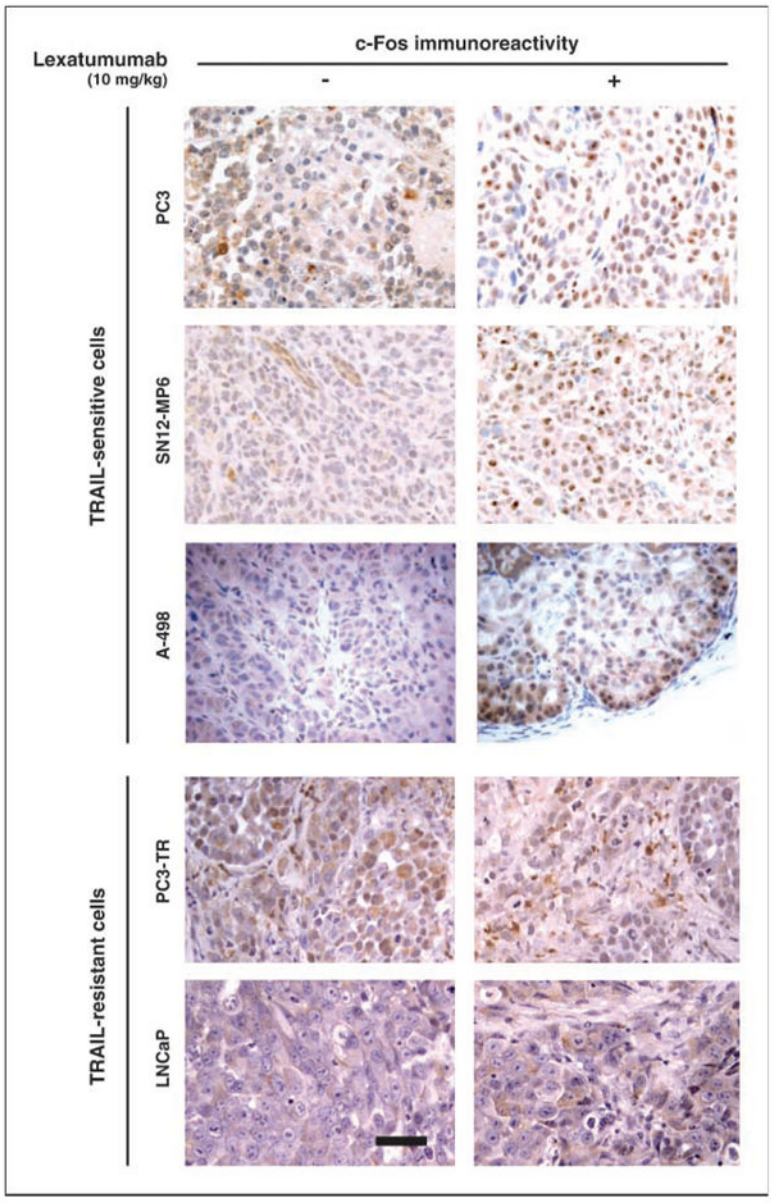

Figure 6.

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 μm.