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

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in a variety of tumorigenic and transformed cell lines but not in many normal cells. Hence, TRAIL has the potential to be an ideal cancer therapeutic agent with minimal cytotoxicity. FLICE inhibitory protein (c-FLIP) is an important regulator of TRAIL-induced apoptosis. Here, we show that persistent expression of c-FLIP(Long) [c-FLIP(L)] is inversely correlated with the ability of TRAIL to induce apoptosis in prostate cancer cells. In contrast to TRAIL-sensitive cells, TRAIL-resistant LNCaP and PC3-TR (a TRAIL-resistant subpopulation of PC3) cells showed increased c-FLIP(L) mRNA levels and maintained steady protein expression of c-FLIP(L) after treatment with TRAIL. Ectopic expression of c-FLIP(L) in TRAIL-sensitive PC3 cells changed their phenotype from TRAIL sensitive to TRAIL resistant. Conversely, silencing of c-FLIP(L) expression by small interfering RNA in PC3-TR cells reversed their phenotype from TRAIL resistant to TRAIL sensitive. Therefore, persistent expression of c-FLIP(L) is necessary and sufficient to regulate sensitivity to TRAIL-mediated apoptosis in prostate cancer cells.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in the United States, accounting for an estimated 220,900 of newly diagnosed cancers in 2003 (1). Localized prostate cancer can be effectively managed with surgery and radiation therapy in a majority of cases or by watchful waiting in select cases. However, advanced prostate cancer results in 28,900 deaths annually (1), and newer treatments are needed to reduce the mortality from advanced prostate cancer. Progression of prostate cancer often is associated with misregulation of many apoptotic related genes. Therefore, targeting apoptotic signaling pathways for therapy can be useful for management of prostate cancer.

The cytotoxic effects on normal cells frequently limit systemic therapies. A relatively new proapoptotic agent, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, also known as Apo2L; refs. 2–4), has been used effectively in systemic animal trials and has been shown to be more sensitive to FasL-induced apoptosis (30), which strongly suggests that c-FLIP(L) has an antiapoptotic function. Furthermore, two recent reports have proposed that c-FLIP(L) may have a dual function, a proapoptotic function at low physiologic concentrations and an antiapoptotic function at high cellular concentrations (31, 32).

Although many cancers undergo TRAIL-induced apoptosis, some develop resistance, making TRAIL ineffective as an anticancer agent. Expression of certain apoptotic mediating genes has been suggested to regulate sensitivity of cancer cells to TRAIL-mediated apoptosis, including nuclear factor κB (NFκB; refs. 33, 34), Akt (35–37), Bcl-2 (38), Bax (39), and c-FLIP (35, 37, 40). Considering that there are numerous ways to regulate TRAIL-mediated apoptosis, this study focused on the role of c-FLIP(L) in mediating resistance to TRAIL-induced apoptosis. Although expression of c-FLIP(L) has been correlated with TRAIL resistance in select cancer models, the direct functional role of c-FLIP(L) in TRAIL-mediated apoptosis in prostate cancer has not been well studied. In this study, we show that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to apoptotic pathways induced by TRAIL, whereas silencing c-FLIP(L) expression converts TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype. Therefore, regulation of c-FLIP(L) is sufficient to overcome the necessary threshold to modulate TRAIL-mediated apoptosis in prostate cancer.

MATERIALS AND METHODS

Antibodies to DR4 and DR5, lamin A/C, and horseradish peroxidase-conjugated secondary antibodies, goat-antimouse, goat-antrabbit, and goat-antirabbit antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human TRAIL/TNFFSF10 was obtained from R&D Systems Inc. (Minneapolis, MN). Monoclonal anti-FasL antibodies (Dava II)
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 × 10^5 LNCap cells were seeded in 96-well plates and cultured for 24 hours before treatment. Cells were then treated with various concentrations of TRAIL for 24 hours. MTT was added, followed by solubilization buffer 4 hours later. Absorbance was measured at 590 nm (630 nm was the reference wavelength) according to the manufacturer’s protocol (Roche Diagnostics, Indianapolis, IN) with BrdUrd and propidium iodide (PI; 50 μg/mL) staining. All of the results were from at least triplicate experiments.

**Western Blot Analysis.** Cell lysates were prepared in RIPA buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 1% NP40, and 0.1% SDS] supplemented with a protease inhibitor mixture stock solution (Roche Molecular Biochemicals, Mannheim, Germany). After sonication for 10 seconds, cell debris was removed by centrifugation at 12,000 × g for 10 minutes at 4°C, and the protein concentration was determined by BCA protein assay reagent (Pierce, Rockford, IL). Equivalent amounts of proteins, as verified by Porcine S staining and by immunoblot analysis against α-tubulin, were resolved on 10% SDS-PAGE gels, and the bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

**Real-Time Quantitative Reverse Transcription-PCR.** Total RNA was isolated with the RNaseasy Mini Kit (Qiagen, Valencia, CA). The yield and quality of RNA was evaluated by measuring its absorbance at A260/A280 and gel electrophoresis. A total of 0.3 μg of each sample was isolated in a 50-μL reaction containing 25 μL of 2× Taqman universal PCR master mix, 1.25 μL of 40× Multiscribe Reverse Transcriptase/RNase inhibitor mix (Applied Biosystems), 0.25 μL of forward primer, 0.25 μL of reverse primer, 9.75 μL of 2× Taqman master mix, 0.75 μL of RNase-free water, and 0.25 μL of cDNA. Thermal cycling conditions were 30 minutes at 48°C, a 10-minute initial denaturation step at 95°C, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Thermal cycling was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems). The sequence of the custom primers was c-FLIP(L) forward primer, 5′-TCT CAC AGC TCA CCA TCC CTG-3′; reverse primer, 5′-CAG GAG TGG GCG TTT TTC TCT-3′. Each sample was run in duplicate for c-FLIP(L), negative control of c-FLIP(L), and glyceraldehyde-3-phosphate dehydrogenase–positive and –negative controls. Results were from at least three independent experiments and normalized to the glyceraldehyde-3-phosphate dehydrogenase control amplification.

**RESULTS**

**Sensitivity of Prostate Cancer Cells to Recombinant Human TRAIL.** To determine whether prostate cancer cells have variable sensitivity to TRAIL-induced apoptosis, LNCaP, DU145, and PC3 cells were treated with increasing doses of TRAIL (Fig. IA). Cell viability assays showed that LNCaP cells were resistant to TRAIL, DU145 cells were moderately sensitive to TRAIL, and PC3 cells were sensitive to TRAIL (Fig. 1A), a finding consistent with others (36). In accordance with the cell viability results (Fig. 1A), phase microscopy (Fig. 1B) showed that LNCaP cells were viable when treated with TRAIL (100 ng/mL), whereas the majority of TRAIL-sensitive PC3 cells had undergone cell shrinkage and pyknosis, morphologic findings consistent with cell death. Although the results shown herein represent cells that had been treated with TRAIL for 24 hours, numerous apoptotic-appearing PC3 cells could be found even after 4 hours of TRAIL treatment (data not shown). To ensure that the TRAIL-sensitive cells had undergone apoptosis, we further confirmed our cell viability (Fig. 1A) and morphologic findings (Fig. 1B) by assessing the sub-G1, apoptotic cell population of LNCaP, DU145, and PC3 cells (Fig. 1C). Prostate cancer cells were treated with TRAIL (100 ng/mL) for 4 hours, and the sub-G1 DNA content was assessed by flow cytometry. Nearly 50% of PC3 cells were apoptotic, whereas the LNCaP and DU145 cells showed much lower percentages of cell death after treatment with TRAIL (Fig. 1C).

**Protein Expression of c-FLIP(L) Correlated with TRAIL Sensitivity in Prostate Cancer Cells.** TRAIL mediates apoptosis via the DR4/DR5 receptors, and FADD is essential during this process. Therefore, we examined the DR4, DR5, and FADD protein expression. As assessed by immunoblot, we observed no significant changes in the expression of DR4, DR5, or FADD before or after TRAIL treatment in prostate cancer cells (data not shown).

c-FLIP(L) has been shown to be an important mediator of cell death signaling downstream of the DR4/DR5 receptors and FADD complexes. Therefore, we postulated that expression of c-FLIP(L) might play a critical role in regulating TRAIL sensitivity in prostate cancer. Basal c-FLIP(L) expression was equivalent in DU145, PC3, and LNCaP cells. However, treatment with TRAIL led to decreased c-FLIP(L) protein expression in PC3 cells, a moderate reduction of c-FLIP(L) in DU145 cells, and no change in c-FLIP(L) expression in LNCaP cells (Fig. 2A). As for c-FLIP(s), it could not be detected in LNCaP or PC3 cells. In contrast, DU145 cells expressed a relatively high level of c-FLIP(s), which was decreased after TRAIL treatment (data not shown). These results show that persistent expression of
c-FLIP(L) correlates with TRAIL resistance, whereas expression of c-FLIP(s) does not correlate with TRAIL sensitivity. To better define the correlation between c-FLIP(L) expression and TRAIL treatment, we measured c-FLIP(L) protein expression as a function of variable TRAIL treatment in time- and dose-dependent studies (Fig. 2B). Because the cell viability results (Fig. 1A) showed that ~80% of PC3 cells died when treated with 10 ng/mL of TRAIL for 24 hours, we chose the dose of 10 ng/mL for the time course experiments. LNCaP cells showed no significant changes in c-FLIP(L) protein expression, and DU145 cells showed a moderate reduction in the level of c-FLIP(L). In contrast, PC3 cells exhibited a gradual reduction in the level of c-FLIP(L) following TRAIL treatment. These results correlated with the degree of TRAIL sensitivity in LNCaP, DU145, and PC3 prostate cancer cell lines (Fig. 1). Interestingly, PC3 cells treated with 10 ng/mL of TRAIL for 24 hours reexpressed c-FLIP(L) protein. Reexpression of c-FLIP(L) occurred when PC3 cells were treated at low concentrations of TRAIL, whereas higher TRAIL concentrations (50 or 100 ng/mL) for 24 hours did not lead to c-FLIP(L) reexpression. This finding may be associated with the survival of a subpopulation of PC3 cells that are resistant to TRAIL-mediated apoptosis (see below).

Because protein expression of c-FLIP(L) seems to be correlated
with resistance to TRAIL-mediated apoptosis, we wished to determine whether differential expression of c-FLIP(L) after TRAIL treatment is regulated at the transcriptional level. Therefore, we assessed the relative mRNA levels of c-FLIP(L) in prostate cancer cells after treatment with TRAIL (Fig. 2C). c-FLIP(L) mRNA levels were reduced in all of the prostate cancer cell lines following 4 hours of treatment with TRAIL. However, after 24 hours c-FLIP(L) transcription was increased to threefold above baseline in the TRAIL-resistant LNCaP cells. In contrast, the c-FLIP(L) mRNA levels were not altered significantly in the DU145 and PC3 cells following 24 hours of TRAIL treatment (Fig. 2C). Collectively, these data suggest that expression of c-FLIP(L) is correlated with resistance to TRAIL-mediated apoptosis, and steady expression of c-FLIP(L) protein in LNCaP cells (Fig. 2A) is correlated with increased c-FLIP(L) mRNA levels 24 hours after TRAIL treatment (Fig. 2C).

Ectopic Expression of c-FLIP(L) Increased TRAIL Resistance in PC3 Cells. Because expression of c-FLIP(L) correlated with TRAIL resistance in prostate cancer cells, we wished to determine whether c-FLIP(L) is directly responsible for inducing resistance to TRAIL-mediated apoptosis. c-FLIP(L) was overexpressed in TRAIL-sensitive PC3 cells using a FLAG-tagged vector (Fig. 3A). Transient overexpression of c-FLIP(L) changed the phenotype of PC3 cells from TRAIL sensitive to TRAIL resistant (Fig. 3B). Therefore, these data indicate that c-FLIP(L) expression is sufficient to cultivate resistance to TRAIL-mediated apoptosis (Figs. 2 and 3B).

The PC3-TR Subpopulation Persistently Express c-FLIP(L) after TRAIL Treatment. Investigation of the molecular differences between TRAIL-resistant and TRAIL-sensitive prostate cancer cell lines, as assessed in LNCaP, DU145, and PC3 cells, may introduce a bias because of the distinct genetic backgrounds of the cells. Therefore, to have a more homogeneous basis in the TRAIL-sensitive and TRAIL-resistant prostate cancer cells, we used the TRAIL-sensitive parental PC3 population to develop a TRAIL-resistant subpopulation named PC3-TR (Fig. 4A and B; see Materials and Methods for details). Evaluating the genetic components that have been altered between PC3 and PC3-TR cells should serve as a better model to study TRAIL sensitivity. After exposure to TRAIL, PC3-TR cells had...
normal-appearing morphology (Fig. 4B) and underwent apoptosis at low rates compared with the parental PC3 cells (Fig. 4C). The relative mRNA levels of c-FLIP(L) in PC3 cells were reduced after treatment with TRAIL, whereas PC3-TR cells showed equivalent (4-hour treatment) or increased (24-hour treatment) mRNA levels following treatment with TRAIL (Fig. 4D). In accordance with our earlier findings, protein expression of c-FLIP(L) was reduced after treatment with TRAIL in PC3 cells, whereas TRAIL-resistant PC3-TR cells had a higher baseline level of c-FLIP(L) expression and maintained a steady expression of c-FLIP(L) protein after TRAIL treatment (Fig. 4E). Maintained protein expression of c-FLIP(L) after treatment with TRAIL can be partially explained by the increased transcription of c-FLIP(L) mRNA in PC3-TR cells (Fig. 4D). These results are consistent with our findings in the TRAIL-resistant LNCaP cells, which exhibited increased c-FLIP(L) mRNA levels 24 hours after TRAIL treatment and concurrent steady c-FLIP(L) protein expression (Fig. 2B and C).

Silencing of c-FLIP(L) Reverses the TRAIL-Resistant Phenotype in Prostate Cancer Cells. c-FLIP(L) protein expression may be directly related to TRAIL resistance in prostate cancer cells, and we postulated that inhibition of c-FLIP(L) protein might reverse the phenotype of TRAIL-resistant cells such that they become TRAIL sensitive. Cycloheximide (10 μmol/L) is a nonspecific protein synthesis inhibitor that when used in combination with TRAIL (100 ng/ml) instigated PC3-TR cells to become TRAIL sensitive. Treatment of PC3-TR cells with cycloheximide alone did not lead to significant cell death (data not shown). To directly examine the role of c-FLIP(L) in mediating resistance in TRAIL-induced apoptosis in prostate cancer cells, we inhibited the expression of the c-FLIP(L) protein by using siRNA in PC3-TR cells (Fig. 5A). As shown in Fig. 5A, c-FLIP(L) protein was knocked down after siRNA transfection. Silencing the c-FLIP(L) isoform, but not the c-FLIP(s) isoform, followed by treatment with TRAIL led to a significant reduction in cell viability and apoptosis in PC3-TR cells (Fig. 5B). c-FLIP(s) siRNA served as a negative control for the aforementioned experiments and successfully reduced the expression of c-FLIP(s) protein in DU145 cells, which was the only prostate cancer cell line that expressed c-FLIP(s) in our studies (data not shown). These results suggest that silencing c-FLIP(L) protein can convert TRAIL-resistant prostate cancer cells to become TRAIL sensitive, whereas maintained expression of c-FLIP(L) is necessary and sufficient to render prostate cancer cells resistant to TRAIL-mediated apoptosis.

DISCUSSION

TRAIL is a type II transmembrane protein that is a member of the TNF family (2–4). TRAIL has been shown to selectively induce apoptosis in tumors and transformed cells but not in normal cells (5–7). Moreover, the combinations of TRAIL and certain DNA-damaging drugs (5, 41) or radiotherapy (42, 43) may exert synergistic antitumor activity. Thus, TRAIL is potentially a powerful inducer of apoptosis. Therefore, it is essential to identify the precise mechanisms by which select cancers develop resistance to TRAIL. In this article, we showed that persistent protein expression of c-FLIP(L) after treatment with TRAIL was necessary and sufficient to render cells resistant to TRAIL-mediated apoptosis in prostate cancer cells.

Processing and signal transductions associated with TNF may involve two sequential signaling complexes: a cell survival promoting complex I and a cell death promoting complex II (44). It has been postulated that complex I, the initial plasma membrane bound complex, rapidly activates NFκB and promotes cell survival. In contrast, complex II promotes cell death through a mechanism involving TNF-R1-associated death domain and RIP1, which associate with FADD and caspase-8 and -10. The first complex activates NFκB, which stimulates its target genes, including c-FLIP(L) (45, 46). c-FLIP(L), in turn, becomes incorporated into complex II and inhibits its function, thus promoting cell survival. In the absence of c-FLIP(L), the second complex is activated and leads to programmed cell death (44). It is presently unknown whether TRAIL-induced apoptosis involves formation of complex I and complex II in a manner similar to TNF (44) or whether the initial TRAIL-induced apoptotic events are solely mediated through the death-inducing signaling complex plasma membrane complex (8–15). Nonetheless, our results regarding TRAIL treatment are consistent with the findings of Micheau and Tschopp (44) because we have shown that persistent c-FLIP(L) protein expression in prostate cancer cells leads to cell survival in correlative and functional studies.

The exact function of c-FLIP(L) in the regulation of apoptosis mechanisms remains controversial. Although many studies have shown that c-FLIP(L) has an antiapoptotic function (17, 19–22), others have shown c-FLIP(L) functions as a proapoptotic molecule at low concentrations and an antiapoptotic molecule only at high concentrations (31). Our data indicated that c-FLIP(L) transcript and protein levels were higher in TRAIL-resistant LNCaP and PC3-TR cells than in TRAIL-sensitive cells 24 hours after treatment with TRAIL. Moreover, c-FLIP(L) protein and mRNA levels were decreased in TRAIL-sensitive PC3 cells after TRAIL treatment, thus showing a strong correlation between expression of c-FLIP(L) and TRAIL sensitivity (Figs. 2 and 4). In direct experiments focusing on c-FLIP(L) function, we showed that ectopic expression of c-FLIP(L) in TRAIL-sensitive PC3 cells and silencing of c-FLIP(L) in TRAIL-resistant PC3-TR cells resulted in conversion of TRAIL sensitivity (Figs. 3 and 5). These results strongly indicate that c-FLIP(L) is necessary and sufficient to promote TRAIL resistance in prostate cancer cells and that c-FLIP(L) expression may be partially regulated at the transcriptional level.

Persistent expression of c-FLIP(L) also may play a critical role in mediating the activation of cytoplasmic and mitochondrial caspase-mediated apoptotic pathways (47, 48). In additional studies, we have found that persistent expression of c-FLIP(L) inversely correlates with the activation of the apical cytoplasmic caspases (caspase-8 and -10),...
which mediate TRAIL-induced apoptosis, whereas secondary activation of mitochondrial-mediated caspase-9 was less crucial. We are actively pursuing these findings in greater depth.

The antiapoptotic role of c-FLIP(L) in TRAIL-induced apoptosis has been supported in an erythroid model system, wherein c-FLIP(L) was down-regulated during differentiation (49). Further cytoplasmic apoptotic pathways also may regulate the activity of caspase-mediated pathways, thus affecting TRAIL sensitivity and potentially the expression and function of c-FLIP(L) as well. For example, Akt protects LNCaP cells from TRAIL-induced apoptosis, and suppression of Akt causes LNCaP cells to become sensitive to TRAIL (36, 50). Expression of c-FLIP(L) seems to depend on the activity of the phosphatidylinositol 3'-kinase/Akt pathway (37). Akt may inhibit apoptosis in multiple other ways, including modulation of the proapoptotic proteins BAD and caspase-9, activation of NFκB pathway, inhibition of FOXO transcription factors, and inhibition of BID protein (51). In conclusion, although there seems to be multiple mechanisms by which to regulate TRAIL sensitivity, regulation of c-FLIP(L) was sufficient to overcome the necessary threshold to modulate TRAIL-mediated apoptosis in prostate cancer.

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