Interleukin-8 signaling attenuates TRAIL- and chemotherapy-induced apoptosis through transcriptional regulation of c-FLIP in prostate cancer cells

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Abstract
Chemotherapy-induced interleukin-8 (IL-8) signaling reduces the sensitivity of prostate cancer cells to undergo apoptosis. In this study, we investigated how endogenous and drug-induced IL-8 signaling altered the extrinsic apoptosis pathway by determining the sensitivity of LNCaP and PC3 cells to administration of the death receptor agonist tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL induced concentration-dependent decreases in LNCaP and PC3 cell viability, coincident with increased levels of apoptosis and the potentiation of IL-8 secretion. Administration of recombinant human IL-8 was shown to increase the mRNA transcript levels and expression of c-FLIPL and c-FLIPS, two isoforms of the endogenous caspase-8 inhibitor. Pretreatment with the CXCR2 antagonist AZ10397767 significantly attenuated IL-8–induced c-FLIP mRNA up-regulation whereas inhibition of androgen receptor – and/or nuclear factor-κB–mediated transcription attenuated IL-8–induced c-FLIP expression in LNCaP and PC3 cells, respectively. Inhibition of c-FLIP expression was shown to induce spontaneous apoptosis in both cell lines and to sensitize these prostate cancer cells to treatment with TRAIL, oxaliplatin, and docetaxel. Coadministration of AZ10397767 also increased the sensitivity of PC3 cells to the apoptosis-inducing effects of recombinant TRAIL, most likely due to the ability of this antagonist to block TRAIL- and IL-8–induced up-regulation of c-FLIP in these cells. We conclude that endogenous and TRAIL-induced IL-8 signaling can modulate the extrinsic apoptosis pathway in prostate cancer cells through direct transcriptional regulation of c-FLIP. Therefore, targeted inhibition of IL-8 signaling or c-FLIP expression in prostate cancer may be an attractive therapeutic strategy to sensitize this stage of disease to chemotherapy. [Mol Cancer Ther 2008;7(9):2649–61]

Introduction
Prostate cancer is the second leading cause of cancer-related deaths in men in the western world. Advanced prostate cancer is poorly sensitive to chemotherapy. The combination of docetaxel with prednisone remains the only chemotherapy strategy to show even a modest survival benefit in this disease over palliative care (1). Significantly, there is no obvious second-line chemotherapy for prostate cancer patients should they fail to respond to docetaxel. Therefore, there is an urgent need to identify alternative chemotherapeutic strategies that may be more effective or, alternatively, identify additional interventions that may increase the effectiveness of current agents including taxanes.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) gene superfamily, induces apoptosis through binding to and activating death receptors (DR4 and DR5) that are coupled to the initiation of intracellular caspase-8 activation (2, 3). TRAIL may also bind to several decoy receptors that lack the death effector domain and thus are unable to promote apoptosis-inducing signaling on binding of TRAIL. Interestingly, extensive methylation of the decoy receptor genes leading to suppression of their transcription has been detected in ~60% of prostate cancers (4). The increase in DR4/DR5 expression relative to that of the decoy receptors on prostate cancer cells provides the desired targeting selectivity and justifies clinical development strategies using TRAIL to treat this disease. In vitro studies have confirmed that TRAIL induces apoptosis with varying sensitivity in prostate cancer cell lines (5–18). Increasingly, studies in prostate cancer cells have focused on using TRAIL in combination with other chemotherapy drugs (6–12), ionizing radiation (13, 14), or strategies that deplete antiapoptotic protein expression (15–18).

Elevated expression of the proinflammatory CXC chemo-kine interleukin-8 (IL-8) has been detected in the serum of prostate cancer patients (19, 20) and in prostate cancer biopsy tissue using colorimetric in situ hybridization, where elevated IL-8 mRNA transcript levels correlated with an increased Gleason score and pathologic stage (21).
Furthermore, using immunohistochemistry, we have shown elevated expression of both IL-8 and the two IL-8 receptors, CXCR1 and CXCR2, in tumor cells of prostate cancer biopsy tissue (22), suggesting that prostate cancer cells are subject to a continuous IL-8 stimulus, the magnitude of which increases with stage of disease. The effect of IL-8 signaling on prostate cancer cell function is consistent with progression of the disease, underpinning cell proliferation (22–24), cell invasion (23–25), and cell survival responses (24, 26, 27). Animal models further associate overexpression of IL-8 with the angiogenesis, tumorigenicity, and lymph node metastasis of androgen-independent prostate cancer in athymic nude mice (28, 29).

Several studies have shown that IL-8 expression is induced following administration of chemotherapy agents and that the resultant signaling is associated with chemoresistance (30–33). In prostate cancer models, we have observed that the administration of the DNA-damaging agent oxaliplatin increases the expression of IL-8 and IL-8 receptors in the androgen-independent prostate cancer cell line PC3, potentiating an IL-8 signaling pathway that is constitutively active in these cells. We have subsequently shown that IL-8 signaling reinforces nuclear factor-κB (NF-κB) transcriptional activity, resulting in elevated expression of the antiapoptotic genes Bcl-2 and survivin, known mediators of chemoresistance. Inhibition of the drug-induced IL-8 signaling resulted in the sensitization of PC3 and DU145 cells to oxaliplatin.1 TRAIL has also recently been shown to induce IL-8 expression in a variety of cancer cell lines (33, 34). In addition, the TRAIL-induced increase in IL-8 signaling has been shown to antagonize TRAIL-induced cell death in ovarian cancer cells as a consequence of decreasing DR4 expression and diminishing caspase-8 activation (33).

Cellular Fas/FasL–associated death domain protein-like inhibitory protein (c-FLIP) is a potent inhibitor of death receptor mediated apoptosis, blocking caspase-8 activation (2, 3, 35). Down-regulation of c-FLIP expression using siRNA sensitizes colorectal cancer cell lines to apoptosis induced by administration of recombinant TRAIL (rTRAIL), the Fas agonist CH-11, and chemotherapy agents [5-fluorouracil and trans-1,3-diaminocyclohexane oxalatoplat- tinum (L-OHP); ref. 36]. c-FLIP expression is elevated in prostate cancer tissue relative to normal tissue (37). Furthermore, the transcription of c-FLIP is under the control of the androgen receptor in the LNCaP cell line, and expression of c-FLIP promotes androgen-independent growth of LNCaP tumors in nude mice, suggesting an important role for c-FLIP in prostate cancer progression (36). Consistent with a critical function in regulating death receptor signaling, isoform-specific siRNA–mediated down-regulation of c-FLIP has been shown to enhance TRAIL-induced apoptosis in the androgen-independent PC3 cell line (16).

The aim of this study was to elucidate the mechanism through which chemotherapy-induced IL-8 signaling may mediate the sensitivity of prostate cancer cells to undergo chemotherapy-induced apoptosis. Our experiments focus on showing the effect of IL-8 signaling on the regulation of activation of the death receptor apoptotic pathway following TRAIL administration. We report on the role of IL-8 signaling in regulating the transcription of c-FLIP and illustrate the effect that inhibition of IL-8 signaling or c-FLIP expression has on the sensitivity of prostate cancer cells to TRAIL and the chemotherapy agents oxaliplatin and docetaxel.

Materials and Methods

Cell Culture

PC3 cells were obtained from American Type Culture Collection (European Collection of Cell Cultures, CAMR) and cultured in RPMI 1640 containing 10% (v/v) FCS and 1% (v/v) L-glutamine (Invitrogen Life Technologies). Human LNCaP cells (Cancer Research UK) were grown in RPMI 1640 supplemented with 10% (v/v) FCS, 1% (v/v) L-glutamine, and 1 mmol/L HEPES. Both cell lines were maintained at 37°C in a humidified 5% CO2 atmosphere and grown to 70% confluency before experimentation.

Materials

Chemicals were sourced from Sigma Chemical Company unless otherwise stated. Isotype control, anti-DR4, and anti-DR5 phycocerythrin-conjugated mouse IgG1 monoclonal antibodies were obtained from ebiosciences. Isotype control FITC-conjugated mouse monoclonal (DakoCytomation), anti-CXCR1, and anti-CXCR2 FITC-conjugated mouse monoclonal antibodies were from R&D Systems. Docetaxel and oxaliplatin were obtained from the Pharmacy Department, Bridgewater Chemotherapy Suite, Belfast City Hospital, and rTRAIL was purchased from Calbiochem. c-FLIP-targeted (FT) siRNA was designed to down-regulate the expression of both splice variant forms of c-FLIP as previously described (36). AZ10397767 was kindly provided by Drs. Simon Barry and David Blakey (AstraZeneca, Alderley Park, United Kingdom). Cells were stimulated with 3 mmol/L of recombinant human IL-8 (rh-IL-8; PeproTech), a concentration chosen on past demonstrations that induces robust signaling and cellular responses in prostate cancer cells (22, 27).

Immunoblotting

Protein was prepared, resolved, and blotted as previously described (22). Membranes were probed with monoclonal antibodies to c-FLIP (NF6; 1:1,000 dilution; Alexis Biochemicals), caspase-8 (1:2,000; Oncogene Research Products), and poly(ADP-ribose) polymerase (PARP; 1:1,000 dilution; ebiosciences) overnight at 4°C. Following washing in TBS/0.1% Tween, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Life Sciences). Specific staining was detected by chemiluminescence with SuperSignal (Pierce) or enhanced chemiluminescence plus (Amersham). Equal loading was assessed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal primary antibody (AbD Serotec).

1 Wilson and Waugh, unpublished observations.
ELISA
Cells (1 × 10^5 per 24-well plate) were incubated overnight at 37°C in a humidified 5% CO₂ atmosphere and replenished in serum-free RPMI 1640 before treatment with rTRAIL. Cell medium was collected at indicated times and subjected to ELISA as previously described (Pelikine Compact IL-8 Elisa Kit, Sanquin Reagents; ref. 22).

Quantitative Real-time PCR Analysis
RNA was harvested from cultured cells using RNAsStat60 (Biogenesis Ltd.) according to the manufacturer's instructions and cDNA was synthesized from 2-μg total RNA by priming with random hexamers and reverse transcribing with Moloney murine leukemia virus reverse transcriptase (Invitrogen) as per manufacturer's protocol. Quantitative real-time PCR analysis was done on the DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research, Bio-Rad Laboratories). The forward and reverse primers used were, for c-FLIP L, CCTAggAAATCTgCCTgATTAATCGA (forward) and TgCgATATACCAgATCAGG (reverse); c-FLIPL, gcAgCAATCCAAAAgAgTCTCA (forward) and ATTTCCAAgAATTTTCAgATCAGG (reverse); and 18S, CATTgTATTgCCgCTAT (forward) and CgACgTATgATgTC (reverse). All primers were supplied by MWG Biotech AG. The quantitative real-time PCR reaction consisted of 1-μL undiluted cDNA, 0.225 μmol/L final concentration of forward and reverse primers, and 2× SYBR Green 1 master mix (Finnzymes). Standard cycling procedures were used with an annealing temperature of 55°C for all primer pairs tested. Specific amplicon formation with each primer pair was confirmed by melt curve analysis. Gene expression was quantified using a standard curve and normalized to the 18S housekeeping gene.

siRNA Transfections
Cells were seeded at 5 × 10^5 per P90 plate in Optimem 1 (Invitrogen)-10% (v/v) FCS medium and allowed to grow to 50% confluency. siRNA transfections were done as previously described (36) and incubated in unsupplemented Optimem 1 medium using Oligofectamine reagent (Invitrogen) for 4 h at 37°C. Cells were replenished in 20% (v/v) FCS-enriched medium for 2 h and then treated with rTRAIL, docetaxel, or AZ10397767.

Cell Viability Assay
Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay. Cells were seeded at 2,000 per well in a 96-well plate. After 24 h, the cells were either transfected as described above or treated with increasing concentrations of rTRAIL. After 48 h, 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL) were added to each well and cells were incubated at 37°C for 4 h. The formazan crystals were reabsorbed in 200-μL DMSO and absorbance was read at 570 nm using a microplate reader (Molecular Devices) as previously described (36).

Flow Cytometry
For cell cycle analysis, cells (1 × 10^5) were seeded in a six-well tissue culture plate, allowed to adhere overnight, and then replenished in fresh RPMI 1640. Cells were treated with varying concentrations of rTRAIL, docetaxel, oxaliplatin, or FT siRNA oligonucleotides and incubated before fixation in 100% ethanol. Cellular DNA profile was evaluated by propidium iodide staining of cells using an EPICS XL Flow Cytometer (Beckman Coulter). In some experiments, rTRAIL, docetaxel, or FT was given in the presence of 20 nmol/L AZ10397767. For receptor protein analysis, cells (5 × 10^4) were seeded and cultured overnight, replenished in RPMI 1640, and treated with rTRAIL or replenished with serum-free medium and treated with rh-IL-8. The cell-surface expression of CXCR1, CXCR2, DR4, and DR5 were examined in conjunction with their appropriate isotype control antibodies. Cells were collected and washed twice in PBS/0.2% (v/v) bovine serum albumin, then incubated with their specific antibody, at a dilution of 2:1 (anti-CXCR1/anti-CXCR2) or 1:20 (anti-DR4/anti-DR5), at 4°C for 1 h. The cells were washed twice in PBS/0.2% (v/v) bovine serum albumin, washed once in PBS, resuspended in PBS, and analyzed immediately on an EPICS XL Flow Cytometer. Each flow cytometry analysis was done in three separate experiments and data are represented as an overall average of experiments.

Statistical Analysis
Differences observed in apoptosis levels in control and drug-treated groups were analyzed for statistical significance using two-tailed Student's t test. The effect of combining agents on apoptosis induction relative to their individual effects was analyzed using a two-way ANOVA test. Two-tailed Student's t test was used to test for statistical significance between gene transcript levels detected in rh-IL-8- or AZ10397767-treated cells. All statistical calculations were conducted using GraphPad Prism 3.0 software.

Results
IL-8 Induces the Expression of c-FLIP in Both Androgen-Dependent and Androgen-Independent Prostate Cancer Cells
Ligand-induced activation of the death receptor or extrinsic apoptosis pathway is dependent on the relative expression levels of transmembrane death receptors (DR), decoy receptors, caspase-8, and the endogenous inhibitor of caspase-8, c-FLIP. Initial experiments were undertaken to characterize the expression levels of each of these proteins in the androgen-dependent LNCaP and androgen-independent PC3 cell lines. Flow cytometry analysis confirmed cell-surface expression of the death receptors DR4 and DR5 in LNCaP and PC3 cells (Fig. 1A). We also observed a differential expression of the decoy receptors DcR1 and DcR2 in these cells; DcR1 expression was only detected in LNCaP cells whereas neither decoy receptor was detected in PC3 cells (Fig. 1A).

IL-8 signaling has previously been reported to regulate DR expression in ovarian carcinoma cells (33). Flow cytometry confirmed the expression of the two IL-8 receptors, CXCR1 and CXCR2, on the cell surface of LNCaP cells (Fig. 1B), consistent with our prior characterization of their...
expression in PC3 cells (22), indicating that each cell line is capable of responding to exogenous administration of rh-IL-8. However, we observed that the constitutive levels of membrane-associated DR4 and DR5 expression were not altered in either cell line following a 24-hour stimulation with rh-IL-8 (Fig. 1C). Furthermore, immunoblotting showed that rh-IL-8 also had no effect on procaspase-8 expression in PC3 cells over a 24-hour time course. However, immunoblotting suggested that IL-8 signaling may act to increase procaspase-8 expression in LNCaP cells with densitometry, indicating increases of 1.8- and 1.6-fold in caspase-8 expression observed between the 2- and 6-hour time points (Fig. 1D).

We next examined whether IL-8 signaling regulated the expression of the native inhibitor of death-inducing signaling complex–induced caspase-8 activation, c-FLIP. Quantitative real-time PCR analysis revealed a time-dependent increase in mRNA transcript levels encoding IL-8 Regulates c-FLIP Expression
Figure 2. Characterization of IL-8–promoted increases in c-FLIP in prostate cancer cells. A, histograms illustrating the mRNA transcript levels detected over a 24-h time course for c-FLIP L and c-FLIP S in PC3 (left) and LNCaP (right) cells following stimulation with rh-IL-8 (3 nmol/L). B, histograms illustrating the effect of adding the CXCR2 receptor antagonist AZ10397767 (20 nmol/L) on the rh-IL-8–induced increase in c-FLIP L and c-FLIP S mRNA expression detected after 24 h in PC3 (left) and LNCaP (right) cell lines. *, P < 0.05; **, P < 0.01. C, histograms illustrating the effect of inhibiting NF-κB transcriptional activity using 5 μmol/L BAY-11-7082 in PC3 (left) and LNCaP (middle) cells and the effect of inhibiting androgen receptor transcriptional activity in LNCaP cells (right) using 1 μmol/L bicalutamide on the rh-IL-8–induced increase in c-FLIP L and c-FLIP S mRNA expression detected after 24 h. Values for all quantitative real-time PCR data shown above were normalized to 18S mRNA expression and are the mean of three independent experiments; bars, SE. D, immunoblots showing basal expression of c-FLIP isoforms in prostate cancer cells (left) and a time-dependent increase in c-FLIP L and c-FLIP S protein in PC3 (middle) and LNCaP (right) cells following stimulation with 3 nmol/L rh-IL-8; GAPDH expression was assessed as a loading control.
c-FLIP in both PC3 and LNCaP cells, in response to rh-IL-8 (Fig. 2A). Using primers that selectively detect the two predominant splice forms of c-FLIP, we observed a differential regulation of c-FLIP_L and c-FLIP_S between the two cell lines. In PC3 cells, rh-IL-8 induced increases in both c-FLIP_L and c-FLIP_S mRNA transcript levels, although fold changes were markedly higher for c-FLIP_L. Maximal induction of the c-FLIP_S transcript was observed within 1 hour of IL-8 stimulation, whereas c-FLIP_L transcript levels peaked 8 hours after stimulation in PC3 cells. Our data also confirmed a sustained up-regulation of both slice variants out to 24 hours after rh-IL-8 stimulation (Fig. 2A, left). In contrast, rh-IL-8 induced a marked increase in the mRNA transcript encoding for c-FLIP_S with a minimal increase in
c-FLIP\(_L\) transcript levels (<2-fold at 24 hours) in LNCaP cells. The increase in c-FLIP\(_L\) transcript levels also showed sustained up-regulation in the LNCaP cell line, with peak induction of c-FLIP\(_S\) observed at time points >8 hours after rh-IL-8 administration (Fig. 2A, right).

We have previously shown that coadministration of a selective CXCR2 receptor antagonist, AZ10397767, attenuates rh-IL-8-induced NF-kB activation and the downstream transcriptional regulation of the Bcl-2 and survivin genes.\(^1\) Using quantitative real-time PCR, we observed that pretreatment with AZ10397767 attenuated the rh-IL-8–promoted increase in c-FLIP\(_L\) and c-FLIP\(_S\) mRNA transcript levels in PC3 cells (\(P < 0.05\) and \(P < 0.05\), respectively; Fig. 2B, left). In LNCaP cells, addition of AZ10397767 alone decreased endogenous c-FLIP\(_L\) (\(P < 0.01\)) and c-FLIP\(_S\) mRNA transcript levels (Fig. 2B, right). The IL-8–promoted increase in c-FLIP\(_S\) mRNA expression was also reversed to below basal levels in the presence of AZ10397767 (\(P < 0.001\)). Consistent with our prior observations, IL-8 signaling had a minimal effect in modulating c-FLIP\(_L\) mRNA transcript levels in LNCaP cells (Fig. 2B, right).

Transcription of c-FLIP has been reported to be regulated through NF-κB in breast cancer cells (38) and via the androgen receptor in prostate cancer cells (37). PC3 cells are devoid of androgen receptor expression but exhibit constitutive activation of NF-κB. Both of these transcription factors are expressed in LNCaP cells, although constitutive NF-κB activity is lower relative to that in PC3 cells. We have confirmed that IL-8 signaling is coupled to the activation of both these transcription factors in either PC3 or LNCaP cells (39).\(^1\) To determine the mechanism underpinning the IL-8–promoted increase in the transcript levels of c-FLIP isoforms in these cells, PC3 or LNCaP cells were treated with the NF-κB inhibitor BAY-11-7082 (5 μmol/L) or the androgen receptor antagonist bicalutamide (1 μmol/L). In these experiments, prostate cancer cells were stimulated with 3 nmol/L rh-IL-8 for 24 hours. Inhibition of NF-κB activity was shown to abrogate the IL-8–induced increases in c-FLIP\(_L\) and c-FLIP\(_S\) transcript levels in PC3 and LNCaP cells (Fig. 2C, left and middle). Administration of bicalutamide also abrogated the rh-IL-8–induced increase in c-FLIP\(_S\) and attenuated the increase in c-FLIP\(_L\) transcript levels in LNCaP cells (Fig. 2C, right).

Immunoblotting was conducted to analyze isoform-specific expression of c-FLIP in PC3 and LNCaP cells. PC3 cells were observed to preferentially express c-FLIP\(_L\) whereas LNCaP cells were shown to have equivalent expression of both c-FLIP isoforms (Fig. 2D, left). The effect of IL-8 signaling on c-FLIP expression in either cell line was studied over a 24-hour time course by immunoblotting. c-FLIP\(_L\) and c-FLIP\(_S\) expression was increased within 1 hour of the addition of rh-IL-8, remaining elevated for an initial period of ~6 hours (Fig. 2D, middle). In addition, we observed a secondary increase in c-FLIP\(_L\) and c-FLIP\(_S\) expression in PC3 cells between 12 and 16 hours and between 8 and 16 hours posttreatment, respectively. Similarly, IL-8 signaling increased c-FLIP\(_L\) and c-FLIP\(_S\) expression in LNCaP cells (Fig. 2D, right).

Interestingly, increased expression of c-FLIP\(_L\) and c-FLIP\(_S\) was observed at early time points where increases in transcript levels had previously not been detected. Therefore, these data suggest that IL-8 signaling may regulate c-FLIP isoform expression through an additional posttranscriptional mechanism.

Effect of rTRAIL on the Induction of Apoptosis in Prostate Cancer Cell Lines

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done to determine the sensitivity of PC3 and LNCaP cells to rTRAIL. We observed a concentration-dependent decrease in cell viability in response to rTRAIL in each cell line, with a more pronounced cytotoxic effect being observed in PC3 cells (Fig. 3A). However, even at the highest dose used (50 ng/mL), an IC\(_{50}\) cytotoxic level was not achieved, showing that both these cell lines are poorly sensitive to rTRAIL. This was further confirmed at a molecular level by immunoblotting, where we only observed the emergence of significant PARP cleavage at high concentrations of rTRAIL (25–50 ng/mL) in both cell lines (Fig. 3B). Similarly, caspase-8 processing was observed between 25 and 50 ng/mL in the PC3 cell line (Fig. 3B, left). In LNCaP cells, we observed loss of procaspase-8 in response to 25 to 50 ng/mL of rTRAIL (Fig. 3B, right), which is indicative of caspase-8 activation, although no caspase-8 cleavage products could be detected. Interestingly, we were able to detect that administration of rTRAIL up-regulated c-FLIP\(_L\) and c-FLIP\(_S\) protein expression in a concentration-dependent manner in both PC3 and LNCaP cells.

Effect of TRAIL on IL-8 Secretion and IL-8 Receptor Expression

Administration of 10 ng/mL (data not shown) or 25 ng/mL of TRAIL was shown to increase the secretion of IL-8 by PC3 and LNCaP cells (Fig. 3C). The induction of IL-8 secretion following administration of rTRAIL was significantly more pronounced in LNCaP cells than in PC3 cells (403% versus 24% increase, respectively). This may be due to the much higher constitutive secretion of this chemokine from PC3 cells under unstimulated conditions. Flow cytometry analysis also confirmed that the relative cell-surface expression of CXCR1 and CXCR2 was not altered on exposure to either 10 ng/mL (data not shown) or 25 ng/mL of rTRAIL for 24 hours in PC3 cells (Fig. 3D, left). In contrast, administration of 10 ng/mL rTRAIL reduced the cell-surface expression of CXCR1 but increased CXCR2 distribution in the plasma membrane of LNCaP cells (Fig. 3D, right). This altered expression profile of the receptors may reflect the effect of TRAIL-induced increases on IL-8 secretion in altering the dynamics of receptor internalization and recycling in LNCaP cells.

Targeting c-FLIP Induces Spontaneous Apoptosis and Sensitizes Prostate Cancer Cells to TRAIL-Induced Cell Death

To establish the significance of c-FLIP expression on prostate cancer cell function, we used a siRNA strategy that specifically targets the down-regulation of both splice forms c-FLIP\(_L\) and c-FLIP\(_S\) (designated FT), as previously...
described (36). Treatment with increasing concentrations of FT siRNA (range, 0.1–10 nmol/L) resulted in a decrease in the viability of PC3 and LNCaP cells as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 4A, left). To confirm that the decrease in cell viability of both cell lines was due to an apoptotic event, we conducted flow cytometry analysis, which showed a concentration-dependent increase in apoptotic cells in the

Figure 4. siRNA targeting of c-FLIP induces spontaneous apoptosis and sensitizes prostate cancer cells to TRAIL. A, left, histogram showing the effect on cell viability 48 h posttransfection with increasing concentrations of FT siRNA in PC3 (black columns) and LNCaP (white columns) cells. Columns, mean of three independent experiments; bars, SE. Right, histogram illustrating the percentage of the sub-G0/G1 population (apoptotic cells) detected by FACS analysis of PC3 (black columns) and LNCaP (white columns) cells 48 h posttransfection with FT siRNA. Columns, mean of three independent experiments; bars, SE. B, panels of immunoblots showing PARP cleavage and suppression of c-FLIP expression in response to increasing concentrations of FT siRNA in PC3 (left) and LNCaP (right) cells. Protein samples were extracted from cells 48 h posttransfection. GAPDH expression was assessed as a loading control. C, histogram illustrating the percentage of the sub-G0/G1 population (apoptotic cells) detected by FACS analysis in PC3 (left) and LNCaP (right) cells following depletion of c-FLIP expression in the absence and presence of indicated concentrations of rTRAIL. Apoptosis in PC3 cells was determined 48 h posttransfection with 1 nmol/L FT siRNA whereas FACS analysis was conducted 24 h posttransfection with 0.5 nmol/L siRNA. Equivalent concentrations of the scrambled oligonucleotide (SC) were used as parallel controls. *, P < 0.05; **, P < 0.01; ***, P < 0.001. D, panels of immunoblots showing PARP cleavage and caspase-8 cleavage in response to the combination of TRAIL with FT siRNA in PC3 cells (left) and LNCaP cells (right) using the experimental conditions described above. Equal protein loading was confirmed by reprobing the membrane for GAPDH.
sub-G0/G1 population on transfection of FT siRNA in LNCaP and PC3 cells (Fig. 4A, right). LNCaP cells were observed to be more sensitive to apoptosis following the inhibition of c-FLIP relative to the response observed in PC3 cells. Immunoblotting also showed an enhanced PARP cleavage in a concentration-dependent manner in response to FT siRNA in both cell lines (Fig. 4B). Consistent with past reports, FT siRNA abrogated the expression of both splice forms of c-FLIP at concentrations as low as 0.5 nmol/L in the PC3 cell line (Fig. 4B, left) and 1 nmol/L in the LNCaP cell line (Fig. 4B, right).

We next assessed whether suppressing c-FLIP expression would sensitize the prostate cancer cell lines to rTRAIL. FT siRNA was effective in potentiating rTRAIL-induced apoptosis in both cell lines (Fig. 4C). In PC3 cells, transfection with FT (1 nmol/L) increased rTRAIL-induced apoptosis at both 5 ng/mL (sub-G0/G1, 19.5 ± 3.0%; P < 0.0001) and 10 ng/mL (sub-G0/G1, 26.1 ± 4.1%; P < 0.0001) when compared with the effect of rTRAIL at equivalent concentrations in nonsilencing control–transfected cells (sub-G0/G1, 8.1 ± 1.5% and 12.5 ± 3.4%, respectively; Fig. 4C, left). In addition, there was a significant increase in the degree of apoptosis induced on FT siRNA in combination with rTRAIL (10 ng/mL) compared with FT alone (sub-G0/G1, 17.7 ± 2.9%; P < 0.001; Fig. 4C, left). Immunoblotting also showed an enhanced cleavage of PARP and caspase-8 in PC3 cells on administration of rTRAIL (10 ng/mL) in FT siRNA (1 nmol/L)–transfected cells compared with FT siRNA (1 nmol/L) alone (Fig. 4D, left). Similarly, in LNCaP cells, we observed a significant increase in apoptosis induced on treatment of FT siRNA (0.5 nmol/L)–transfected cells with rTRAIL (5 ng/mL) relative to levels detected in TRAIL- and non–TRAIL-treated nonsilencing control siRNA (0.5 nmol/L)–treated cells (P < 0.001; Fig. 4C, right). The combination of FT siRNA (0.5 nmol/L) and rTRAIL (5 ng/mL) was also shown to potentiate the effect observed with FT siRNA alone (sub-G0/G1, 19.1 ± 2.7%; P < 0.001). This increase in apoptosis was further confirmed by the detection of enhanced cleavage of PARP and caspase-8 in LNCaP cells following the combination of rTRAIL and FT siRNA (Fig. 4D, right).

IL-8 Signaling Modulates the Sensitivity of Prostate Cancer Cells to rTRAIL

Previously, we observed that rTRAIL potentiated IL-8 signaling (Fig. 3C) and up-regulated c-FLIP expression in PC3 and LNCaP cells (Fig. 3B). To show the link between these two observations, we first determined whether addition of exogenous rh-IL-8 could antagonize TRAIL-induced apoptosis. Immunoblots revealed that prestimulation of both PC3 and LNCaP cells with 3 nmol/L rh-IL-8 for 8 hours increased the expression of PARP in these cells. In further experiments, we investigated whether rTRAIL-induced,
cell-derived IL-8 contributed to the TRAIL-induced increase in c-FLIP expression. Coadministration of AZ10397767 to PC3 cells attenuated the up-regulation of c-FLIP induced by the addition of either 10 or 50 ng/mL rTRAIL (Fig. 5B). In addition, coadministration of AZ10397767 increased rTRAIL-induced apoptosis in PC3 cells (Fig. 5C, left). Administration of 10 ng/mL rTRAIL alone for 48 hours increased the apoptotic cell fraction from 3.0 ± 0.5% in untreated cells to 18.2 ± 2.1% (n = 3; P < 0.001). Whereas AZ10397767 had no effect on apoptosis by itself (sub-G0/G1, 3.6 ± 0.9%), coadministration of this antagonist with 10 ng/mL rTRAIL further increased the apoptotic cell fraction to 23.4 ± 2.9% (P < 0.01, relative to rTRAIL alone). Immunoblotting also confirmed an enhanced cleavage of PARP on coadministration of AZ10397767 with rTRAIL, compared with the effect of rTRAIL alone (Fig. 5C, right). Although rTRAIL induced apoptosis in LNCaP cells (sub-G0/G1 = 20.7 ± 0.5%; P < 0.001, compared with 9.3 ± 0.8% in untreated cells), coadministration with AZ10397767 did not potentiate the level of apoptosis (sub-G0/G1 = 20.6 ± 1.0) detected by either fluorescence-activated cell sorting (FACS) analysis or immunoblotting to analyze PARP cleavage (data not shown).
siRNA Targeting of c-FLIP Sensitizes Prostate Cancer Cells to Docetaxel and L-OHP

Prompted by our prior studies (36), we investigated whether FT siRNA could potentiate oxaliplatin (L-OHP)-induced apoptosis in cells. PC3 and LNCaP cells were poorly sensitive to L-OHP. However, suppression of c-FLIP using FT siRNA was able to sensitize both PC3 and LNCaP cells to undergo oxaliplatin-induced apoptosis (Fig. 6A, left and right). Interestingly, the combination of FT siRNA with oxaliplatin was more potent in inducing apoptosis relative to the combination of AZ10397767 with oxaliplatin in both PC3 and LNCaP cells. These observations were confirmed by immunoblotting analysis of PARP cleavage and/or the detection of caspase-8 activation in PC3 and LNCaP cells, illustrating that oxaliplatin-induced apoptosis was enhanced particularly by the suppression of c-FLIP (Fig. 6B). No further increase in apoptosis induction was observed using a combination of oxaliplatin, FT siRNA, and AZ10397767 in either cell line (data not shown).

Docetaxel is a chemotherapeutic agent used in the treatment of advanced metastatic prostate cancer (1). Therefore, we sought to determine whether FT siRNA could sensitize prostate cancer cell lines to docetaxel therapy. As observed before, FT siRNA was more effective in inducing apoptosis in PC3 and LNCaP cells than administration of 1 nmol/L docetaxel alone (Fig. 6C). The combination of FT siRNA (1 nmol/L) with 1 nmol/L docetaxel also induced a further increase in apoptosis relative to the effect of the taxane alone in both PC3 and LNCaP cells ($P < 0.05$ and $P < 0.001$, respectively). Furthermore, the combination of docetaxel (1 nmol/L) with FT siRNA was more effective in inducing apoptosis than that induced by suppressing c-FLIP expression alone in PC3 cells ($P < 0.01$) and LNCaP cells ($P < 0.05$). In probing for PARP cleavage and/or caspase-8 activation, silencing of c-FLIP expression was shown to be especially effective in potentiating docetaxel-induced apoptosis in these cells (Fig. 6D).

Discussion

c-FLIP is a key regulator of Fas and DR4/DR5 signaling at the death-inducing signaling complex formed by these receptors (2, 3, 35). Overexpression of c-FLIP in cell lines decreases the activation of caspase-8, inhibiting the proapoptotic signaling stimulus resulting from ligand-induced activation of DRs (40). c-FLIP expression is elevated in prostate cancer tissue relative to normal tissue and contributes to the androgen-independent growth of prostate cancer cells in vivo (37). In this study, we have confirmed endogenous expression of the short and long isoforms of c-FLIP in representative cell-based models of androgen-dependent (LNCaP) and androgen-independent (PC3) prostate cancer. Consistent with past observations, we have shown that down-regulation of c-FLIP expression sensitizes prostate cancer cells to TRAIL (16). We have also observed for the first time that down-regulating c-FLIP expression increases the apoptosis of prostate cancer cells induced by chemotherapeutic agents, oxaliplatin and docetaxel. Interestingly, down-regulation of c-FLIP alone using siRNA induced spontaneous apoptosis in both LNCaP and PC3 cells. Our current observations in prostate cancer cells add to the expanding number of cell-based model systems in which depleting c-FLIP expression results in a spontaneous induction of apoptosis, including colorectal, breast, and non–small-cell lung cancer cells (36, 41–43). This current study also adds to the growing literature reporting the importance of c-FLIP as a key regulator of cell survival, disease progression, and the intrinsic resistance of prostate cancer cells to therapeutic intervention.

As an ELR-positive CXC chemokine, IL-8 is primarily associated with the induction of angiogenesis and the promotion of metastasis in prostate cancer (28, 29). However, IL-8 signaling may also contribute to the intrinsic resistance of prostate cancer cells to undergo apoptosis in response to environmental or chemical stress. We have previously shown that the hypoxia-induced potentiation of IL-8 signaling, mediated in part via hypoxia-inducible factor-1 and NF-κB–promoted transcription of CXCR1 and CXCR2, renders hypoxic prostate cancer cells less sensitive to DNA damage–based chemotherapy (26). Similarly, we have shown that administration of oxaliplatin potentiates autocrine IL-8 signaling in prostate cancer cells, reinforcing the transcriptional activity of NF-κB and the expression of antiapoptotic genes of the Bcl-2 and IAP gene families that decreases the sensitivity of these cells to oxaliplatin.6 We have now shown that administration of...
TRAIL also potentiates IL-8 signaling in prostate cancer cells. We have presently not studied the mechanism by which TRAIL signaling regulates IL-8, CXCR1, and CXCR2 gene transcription. However, we have shown that administration of TRAIL, potentiates the secretion of IL-8 by each of the prostate cancer cell lines, with the greatest increase observed in LNCaP cells (which have a lower basal rate of IL-8 secretion relative to the PC3 cell line). Flow cytometry has also confirmed that each of the IL-8 receptors is expressed on the cell surface of both PC3 and LNCaP cells and before following TRAIL administration, indicating that the cells can respond to the increased secretion of the ligand. Therefore, our data suggest that the magnitude of IL-8 signaling is increased in prostate cancer cells following exposure to TRAIL.

The significance of TRAIL-induced IL-8 signaling in regulating the response of prostate cancer cells to TRAIL was studied in further experiments. IL-8 signaling induced a marked, time-dependent, isoform-selective increase in the transcript levels for c-FLIPL and c-FLIPS in PC3 and LNCaP cells. The IL-8–induced transcription of c-FLIP was shown to be mediated through a NF-κB– and/or androgen receptor–dependent mechanism, consistent with past observations from other laboratories reporting NF-κB–dependent transcription of c-FLIP in breast cancer cells and the role of the androgen receptor in regulating expression of this gene in LNCaP cells (37, 38). Inhibition of CXCR2 signaling attenuated IL-8–promoted c-FLIP gene transcription, consistent with the role that we have shown of this receptor in mediating IL-8–induced transcriptional activation of NF-κB and androgen receptor in PC3 and LNCaP cells, respectively (39). Thus, we have characterized c-FLIP as a further downstream transcriptional target of these IL-8–induced signaling pathways in prostate cancer cells. Furthermore, time course analysis of c-FLIP protein levels suggests that IL-8 signaling may also regulate c-FLIP expression at the posttranscriptional level. Indeed, the early increases in c-FLIP expression are consistent with the previously characterized effect of IL-8 signaling in potentiating the translation of oncopgenes in prostate cancer cells (27).

Administration of TRAIL alone was shown to increase c-FLIP expression in PC3 and LNCaP cells. Experiments conducted on PC3 cells confirmed that the TRAIL-induced increase in c-FLIP expression was attenuated when TRAIL was co-administered with the CXCR2 receptor antagonist AZ10397767. Furthermore, treatment with AZ10397767 alone also reduced endogenous c-FLIP expression in these cells, confirming the association of both endogenous and TRAIL-induced IL-8 signaling in potentiating the expression of this antiapoptotic gene. The sensitivity of PC3 cells to undergo TRAIL-induced apoptosis was also enhanced by the coadministration of AZ10397767. In contrast, coadministration of AZ10397767 did not inhibit TRAIL-induced expression of c-FLIP in LNCaP cells and failed to enhance TRAIL-induced apoptosis in this cell line. The differential effect of AZ10397767 in modulating the sensitivity of these two cell lines to TRAIL is interesting and warrants further investigation. Given that IL-8 signaling was shown to increase caspase-8 expression in LNCaP but not PC3 cells, the differential sensitivity of the two cell lines to IL-8 antagonism may reflect the relative ratio of c-FLIP/caspase-8 expression. Irrespective, these initial experiments confirm that the induction of IL-8 signaling can act to functionally antagonize the death-inducing effects of TRAIL in representative models of androgen-independent prostate cancer, potentially by up-regulating expression of the endogenous inhibitor of caspase-8, c-FLIP.

Our current observations highlight the relevance of IL-8 signaling in modulating the susceptibility of prostate cancer cells to therapeutic interventions. Previously, it had been reported that IL-8 signaling resulted in decreased DR4 expression in ovarian cancer cells (33). Here we provide evidence that IL-8 signaling can also modulate the activation of the extrinsic apoptosis pathway via transcription-mediated increases in c-FLIP expression. In either case, IL-8 signaling is coupled to a decreased sensitivity of tumor cells to undergo caspase-8–mediated, TRAIL-induced apoptosis. Our observation that the inhibition of c-FLIP sensitizes prostate cancer cells to oxaliplatin suggests that transcriptional regulation of c-FLIP, in addition to our unpublished observations about the roles of Bcl-2 and survivin, describes a mechanism by which IL-8 signaling attenuates the cytotoxic effects of this DNA damage agent in prostate cancer cells. However, of more direct clinical relevance to current treatment of metastatic prostate cancer, we have shown that the inhibition of c-FLIP increases the cytotoxicity of docetaxel in prostate cancer cells. Therefore, this study characterizes the importance of stress-induced IL-8 signaling and its association with c-FLIP in underpinning prostate cancer cell survival and its functional importance as an intrinsic mode of chemoresistance in advanced prostate cancer.

Disclosure of Potential Conflicts of Interest
D.J.J. Waugh received research support from AstraZeneca. The other authors disclosed no potential conflicts of interest.

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Interleukin-8 signaling attenuates TRAIL- and chemotherapy-induced apoptosis through transcriptional regulation of c-FLIP in prostate cancer cells

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