Fas-mediated killing of primary prostate cancer cells is increased by mitoxantrone and docetaxel

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Abstract
Therapies for prostate cancer based on Fas (CD95) modulation have been under active development at the preclinical stage using immortalized cell lines. To address clinical applicability, the potential of 11 cultures of primary prostate cancer cells to be killed by Fas-mediated apoptosis was investigated. In addition, the effect of the chemotherapeutic agents mitoxantrone and docetaxel on this killing was determined. Apoptosis was induced in patient-derived, primary prostate cancer cells using effector cells engineered by recombinant lentivirus infection to express Fas ligand (FasL) and measured by 51Cr release assays. All cultured prostate cells were found to undergo Fas-mediated killing; cytotoxicity ranged from 12% to 87% after 6 h. These cells were significantly more sensitive to FasL-mediated killing than PC-3 cells. The basal expression of Fas or the expression of five inhibitors of apoptosis (c-FLIP, survivin, cellular inhibitors of apoptosis protein 1 and 2, and bcl-2) was not found to correlate with susceptibility to Fas-mediated killing. Both mitoxantrone and docetaxel were able to induce Fas receptor expression on primary prostate cancer cells, which translated into a 1.5- to 3-fold enhancement of apoptosis mediated by FasL. Whereas mitoxantrone increased the Fas-induced apoptotic response of all cultured prostate cells tested, docetaxel pretreatment was found to preferentially enhance the killing of bcl-2-expressing cells. These findings show that cultured primary prostate cancer cells are sensitive to Fas-mediated apoptosis. Furthermore, the incidence of apoptosis was found to be improved by combining Fas-mediated therapy with standard chemotherapeutic agents. These findings may have significant implications for prostate cancer therapy. [Mol Cancer Ther 2008;7(9):3018–28]

Introduction
Since the discovery of Fas-mediated apoptosis, there has been sustained interest in exploiting this pathway in therapeutic strategies against prostate cancer (1–7). Apoptotic signaling is induced in cells on binding of Fas ligand (FasL) to its receptor, Fas. A caspase cascade is initiated, leading to death of the Fas-expressing cell. Approaches manipulating this pathway are growing more sophisticated and functionally efficacious while at the same time limiting off-target toxicity mediated by this ligand/receptor axis (8–11).

Although these advances are bringing FasL-based therapy closer to clinical reality, the effectiveness of such therapies against prostate cancer has not been thoroughly investigated. Functional studies conducted to date make use of immortalized prostate cancer cell lines as models, which may not accurately reflect the potential clinical response of prostate tumors to FasL-mediated killing. Indeed, the majority of these cell lines, including PC-3, LNCaP, and DU145 cells, were derived from metastases or were artificially created by oncogene overexpression and are likely intrinsically resistant to apoptosis. In addition to immortalization, extensive culture periods may have further altered the phenotype of these commonly used models. Primary prostate cancer cells are thus a more representative model to study the response of prostate cancer to Fas-mediated killing in a preclinical setting.

Immunohistochemical studies of primary human prostate cancer tissues have shown expression of key apoptotic pathway proteins, including Fas and caspases (12, 13). On the other hand, abundant expression of inhibitors of apoptosis, such as survivin and cellular inhibitors of apoptosis protein 1 and 2 (cIAP1 and cIAP2, respectively), have also been documented (14, 15). Without functional studies, it is not known whether the Fas pathway is operational in primary malignant prostate cells. In fact, based on expression profiling of such apoptotic mediators, it has been predicted that apoptotic signaling may be dramatically interrupted in prostate cancer tissues (12). To date, no functional studies have been conducted looking at Fas-mediated killing of patient-derived primary prostate tumor cells.

Various chemotherapeutic drugs, such as camptothecin compounds, have been shown to enhance the sensitivity of prostate tumor cell lines to Fas-mediated apoptosis (16, 17). Certain other anticancer agents, including mitoxantrone, are known to increase cellular Fas expression in LNCaP but not PC-3 or DU145 cells (18). Reports suggest that
increasing Fas receptor expression in this way can lead to heightened susceptibility of cells to apoptosis in some (19, 20) but not all (21) cell types. This must be examined on a case-by-case basis.

In this study, we investigated the susceptibility of cultured primary prostate cells to Fas-mediated apoptosis. Primary prostate cells were maintained in short-term cultures (22), which may more accurately reflect the characteristics of prostate cancer cells in situ. The effect of mitoxantrone and docetaxel, chemotherapeutics currently used against prostate cancer, on tumor cell killing by FasL was also explored. Successful combination of novel FasL-based treatments with existing anti-prostate cancer chemotherapeutics that results in increased tumor cell apoptosis would be an important advance for therapy of prostate cancer.

Materials and Methods

Culture of Cells
Prostate tissue was obtained from needle biopsies from patients undergoing radical prostatectomy. Tissue from palpable tumors were collected and cells were processed and cultured as described (23). Briefly, tissue was finely minced, digested 12 to 18 h in 100 units/mL collagenase I in PrEGM medium (Lonza), and pipetted to disperse clumps. Cells were washed in PBS and cultured on collagen-coated (PureCol; Inamed) tissue-culture plates (BD Biosciences) in PrEGM medium. This protocol favors the growth of epithelial cells. Cell lines were named T1 to T11 and used within 3 weeks of culture. Characterization studies were done on the resulting cell lines (see below). All tissues were collected under an approved University Health Network ethics protocol from patients granting informed consent. PrEC cells were obtained from Clonetics and cultured in PrEGM medium. PC-3 cells were obtained from the American Type Culture Collection and cultured in F-12 Ham’s medium (Sigma) supplemented with 10% FCS, 2 mmol/L l-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma).

Immunofluorescence of Primary Cell Cultures
T3, T7, and T8 cells were cultured on chamber slides (BD Falcon), fixed with acetone/methanol (1:1) for 10 min at -20°C, and rehydrated in PBS. Nonspecific binding was blocked by incubating cells with 1% goat serum in PBS + 1% bovine serum albumin for 30 min. Mouse anti-human cytokeratin 8 + 18 and/or anti-cytokeratin 5 + 14 antibodies (Abcam) were used at 1:50 or 1:100, respectively, in 3% bovine serum albumin in PBS + 0.1% Tween 20 for 1 h. Alexa 488 goat anti-mouse IgG1 or Alexa 568 goat anti-mouse IgG2a (Invitrogen) were added at 1:500 in PBS + 0.1% Tween 20 + 1% bovine serum albumin for 1 h. When slides were stained for cytokeratins 5 + 14 and 8 + 18, staining was done sequentially and an additional blocking step with 5% mouse serum was done following addition of the first secondary antibody. Nuclei were visualized with 1 μg/mL 4,6-diamidino-2-phenylindole (Sigma). Slides were mounted with Fluoromount-G (Southern Biotech) and analyzed on a Zeiss Axioskop 2 fluorescent microscope. Mismatched combinations of primary and secondary antibodies were used as a negative staining control, and MCF-7 cells were used as a positive control.

MTT Growth Assay
The growth rates of all prostate tumor cultures were compared using a MTT assay. Here, 4,000 cells per well were plated in a 96-well plate and allowed to adhere overnight. Ten microliters of 5 mg/mL MTT reagent (Sigma) were added followed 4 h later by 100 μM solubilization solution (10% SDS in 0.01 mol/L HCl). In some samples, recombinant FasL (0.01-10 ng/mL; SuperFasL; Alexis) was added for 24 to 72 h to assess Fas-induced proliferation. Plates were analyzed at 570 nm on a v Max Kinetic microplate reader (MDS Analytical Technologies).

Flow Cytometric Analysis for Fas and FasL Expression
Cells were harvested and blocked with 10% mouse serum. Cells were labeled with PE-anti-FasL antibody clone NOK-1 (eBioscience) or APC- or FITC-anti-Fas antibody clone DX2 (BD Biosciences) and 7-AAD. Cells were analyzed on a Cell-Quest Pro software. Increases in Fas expression were assessed following 4, 24, 48, or 72 h treatment with irradiation (2, 4, or 8 Gy), mitoxantrone (10, 100 or 1,000 nmol/L), or docetaxel (100 or 1,000 nmol/L). Mean fluorescence intensity (MFI) of Fas staining was recorded. To correlate MFI with the number of Fas receptors on the cell surface, a series of Quantum Simply Cellular anti-mouse IgG microspheres were labeled with anti-Fas antibody for 30 min and a calibration curve was established using QuickCal software (Bangs Laboratories). MFI readings for the treated and untreated cells were converted to antibody-binding capacity (ABC) values based on a standard curve.

Western Blotting
Protein lysates were prepared from 80% confluent plates of cells. Protein (30 μg) was loaded onto 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked using 10% nonfat dry milk in PBS + 0.1% Tween 20. Anti-bcl-2 (Cell Signaling Technologies), anti-chromogranin A (Abcam), anti-smooth muscle actin (Abcam), anti-cIAP1 (R&D Systems), anti-cIAP2 (R&D Systems), anti-survivin (Novus Biologicals), anti-c-FLIP (Kamiya Biomedical), anti-p53 (Cell Signaling Technologies), anti-actin (Chemicon International), horseradish peroxidase–linked anti-rabbit (Santa Cruz Biotechnology), or horseradish peroxidase–linked anti-mouse IgG (GE Healthcare) antibodies were applied in PBS + 0.1% Tween 20 with 5% nonfat dry milk. Immunoreactive bands were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer). 293T cells overexpressing bcl-2 were used as a positive control for the bcl-2 Western blot (provided by E. Zacksenhaus; University Health Network).
RNA Extraction and Quantitative Reverse Transcription-PCR Analyses of Prostate Tumor Markers and Characteristic Apoptosis Pathway Proteins

RNA was extracted from cells using the Trizol reagent (Invitrogen). cDNA was synthesized from 5 μg total RNA using a SuperScript II Reverse Transcription kit (Invitrogen). Quantitative real-time reverse-transcription PCR was done on a Rotor-Gene 3000 thermal cycler (Corbett Life Science) using the following primer sets (Invitrogen): cIAP1 forward 5′-TTTTGTCACCTCAGATACCCTG-3′ and reverse 5′-CAGCTGAGTAGATCTCTGGAAGG-3′, cIAP2 forward 5′-GGAGCCATTGTCATCCGTCAGT-3′ and reverse 5′-TGGATAATTTGACTCTGCATTTTTC-3′, survivin forward 5′-CCCCATAGAGCAACTAAAAGGATTTCC-3′ and reverse 5′-TCATCTAAAATCCACCAAGGTTAATTCT-3′, c-FLIP forward 5′-CCTAGGAAATCTGCCTGATAATCGA-3′ and reverse 5′-TGGGATTATACCATGCATACTGAG-3′, and Bcl-xL forward 5′-CGGCTACCACATCCAAGGAA-3′ and reverse 5′-GCTGGAATTACCGCGGCT-3′. RNA was extracted from cells using the Trizol reagent (Invitrogen). cDNA was synthesized from 5 μg total RNA using a SuperScript II Reverse Transcription kit (Invitrogen). 

Mitochondrial Membrane Depolarization Assay

Tetramethylrhodamine ethyl ester (TMRE) dye (Invitrogen) was used to measure mitochondrial outer membrane integrity of prostate cancer cells after induction of Fas-mediated apoptosis (26). Target cells (2.5 × 10⁶-3 × 10⁶) were co-incubated for 6 h at a 20:1 effector-to-target cell ratio with K562-enGFP or K562-ncFasL cells that had been labeled with 5 μM carboxytexasin succinimidyl ester (Invitrogen). Following incubation, cells were harvested and incubated with 100 nmol/L TMRE in culture medium for 30 min at 37°C. The percentage of carboxytexasin succinimidyl ester–negative cells retaining TMRE dye was assessed by flow cytometry. Z-VAD-FMK (BD PharMingen), a general caspase inhibitor, was included at 10 μM/L in a subset of cocultures of target cells with K562-ncFasL cells as a negative control.

Incubation of Primary Prostate Cancer Cells with Mitoxantrone and Docetaxel

Mitoxantrone (Sigma) was reconstituted in water and stored at room temperature; docetaxel (Fluka) was prepared in DMSO and stored at −20°C. The IC₅₀ for docetaxel was determined to be 1,000 nmol/L after 48 h exposure using T4 and T9 cells and analysis by MTT assays (data not shown). For Fas up-regulation and cytotoxicity assays, 10 or 100 nmol/L mitoxantrone or 100 or 1,000 nmol/L docetaxel was added to plates of 70% confluent primary prostate cells 24, 48, or 72 h before ⁵¹Cr release or flow cytometry assays.

p53 Inhibition Assay

Cells were cultured with 30 μmol/L pifithrin α (Sigma) for 24 h before the addition of 100 nmol/L mitoxantrone or 1,000 nmol/L docetaxel and refreshed every 24 h. Forty-eight hours after mitoxantrone and docetaxel addition, cells were collected for use in ⁵¹Cr release assays as before. Cell lysates were probed for p53 expression by Western blotting.

Statistical Analysis

Data are presented as mean ± SD. Sample means were compared using two-tailed unpaired Student’s t tests, with a significance level of P < 0.05. Correlation was measured using Spearman rank-order correlation on SigmaStat software with P < 0.05.

Results

Acquisition and Characterization of Prostate Tissue

Cell cultures were established from needle biopsy cores of tissue from palpable prostate tumors from 11 men undergoing radical prostatectomy. Ten of these cultures were derived from Gleason score 7 tumors, whereas one culture (T9) was derived from a Gleason score 9 (4, 5) tumor from a patient who had been receiving luteinizing hormone-releasing hormone therapy. To characterize the type of cells populating the resulting cultures, immunofluorescence analysis was done to measure levels of basal mediated FasL activity was blocked by the addition of 25 μg/mL anti-FasL NOK-1 or isotype control antibody (BD Biosciences) to K562-enGFP or K562-ncFasL cells 30 min before use in ⁵¹Cr release assay.

Retroviral Transduction and Selection of K562 Cells

K562 cells, a human erythroleukemia cell line, were cultured in RPMI (Sigma) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma). Cells were transduced with lentiviral particles produced from pHRC-pCFP-ENGF-W-SIN (24) or pHRC-pCFP-ncFasL-W-SIN vectors to engineer expression of enGFP or noncleavable Fasl (ncFasL). ncFasL was created by removing the proteolytic cleavage site at amino acids 103 to 136, rendering FasL–membrane-bound for increased expression and potency (8). The pHRC-pCFP-ncFasL-W-SIN vector was constructed by replacing the enGFP cDNA with the ncFasL cDNA. Viral particles were produced by transient transfection of 293T cells as described previously (25). K562 cells were transduced in the presence of 8 μg/mL protamine sulfate for 16 h. Transgene expression was assayed by flow cytometry 72 h post-transduction. Clonal populations of enGFP-expressing (K562-enGFP) and ncFasL-expressing (K562-ncFasL) cells were isolated by limiting dilution.

⁵¹Cr Release Assays

Primary prostate cells (3 × 10⁵) were labeled with 40 μCi ⁵¹Cr-sodium chromate (GE Healthcare) and plated in triplicate in round-bottomed 96-well plates at 10⁵ cells per well. K562-ncFasL or control K562-enGFP cells were added at effector-to-target cell ratios of 1:1, 10:1, or 25:1. Background control wells contained only labeled prostate cells, whereas maximum release control wells additionally contained 1% Triton X-100. After 6 h co-incubation, counts/min from 100 μL medium from each well was determined on a gamma counter (1277 GammaMaster; Perkin Elmer). Percent cytotoxicity was calculated as (experimental counts/min - background release counts/min) / (maximum release counts/min - background release counts/min) × 100. To verify that cytotoxicity was directly Fas

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epithelial cell markers (cytokeratins 5 + 14) and luminal epithelial cell markers (cytokeratins 8 + 18). Cell lines T3, T7, and T8 were analyzed as representative samples. All cells were found to express both cytokeratins 5 + 14 and 8 + 18 (Fig. 1A). Although 100% of the cells from the tested groups co-expressed these cytokeratins, a small number of cells were found to express cytokeratins 5 + 14 more strongly than cytokeratins 8 + 18. To exclude the presence of neuroendocrine or stromal cells in the primary prostate cancer cell cultures, chromogranin A, a neuroendocrine cell marker, and smooth muscle actin, a stromal cell marker, levels were measured by Western blot. T3, T7, and T8 cells were found to have undetectable levels of chromogranin A and very low levels of α smooth muscle actin (Fig. 1B). These data, combined with the immunofluorescence data, suggest that the cultures were composed almost exclusively of epithelial cells with an insignificant presence of stromal cells in the cultures. Based on this expression profiling, the cultures consisted of cells that resemble an intermediate transitional epithelial type (27).

During carcinogenesis, activation of pathways that drive proliferation can render cells resistant to apoptosis (28). To determine whether the rate of proliferation of primary prostate cells might influence the incidence of apoptosis in these cell lines, their relative rates of growth were measured by MTT assay. As expected, growth rates varied somewhat between cell lines (Fig. 1C).

**Primary Prostate Cell Cultures Express High Levels of Surface Fas but No FasL**

The first protein in the Fas-mediated apoptosis cascade is the Fas receptor. To verify that cultured prostate cancer cells express Fas, cells were analyzed by flow cytometry. High, uniform expression of Fas receptor was observed in all tested cells (MFI = 409.9 ± 166.1 for Fas versus 10.7 ± 3.8 for isotype control; P < 0.01; Fig. 2A). A calibration curve to translate MFI values into ABC was generated using antibody-labeled microspheres with known numbers of antibody-binding sites. ABC was then used as a surrogate measure of the number of molecules of Fas on the cell surface. The Fas expression on the prostate cancer cell lines ranged from 52,475 ABC (T11) to 208,726 ABC (T4), with an average of 96,346 ± 42,450 ABC. Of note, the Gleason score 9 tumor-derived cells (T9) had significantly reduced levels of Fas receptor compared with the overall mean of all tested samples (MFI = 311.8 for T9 versus 409.9 average; P < 0.05) as has been...
reported by others for advanced tumors (13). Surface expression of FasL was not detected by flow cytometry under these conditions (MFI = 4.0 ± 1.0 for FasL versus 5.3 ± 1.8 for isotype control; Fig. 2A).

Characterization of Expression of Inhibitors of Apoptosis

Although expression of the Fas receptor is necessary for induction of apoptosis by FasL, complex modulation of this pathway is asserted by protein inhibitors of apoptosis. Malignant cells often acquire dysfunctions in apoptotic signaling caused by mutations in pro-apoptotic proteins or by overexpression of inhibitors of apoptosis (29). To evaluate the levels of expression of several such inhibitors, Western blots for bcl-2, c-FLIP, survivin, cIAP1, and cIAP2 proteins were performed. The expression of p53 protein was detected in all primary cell lines (Fig. 2B). Bcl-2 is often up-regulated in prostate cancers (30), and robust expression of this protein was observed in T2, T4, T5, T6, T9, and T11 cells at levels comparable with that of PC-3 cells (Fig. 2B). Conversely, low or no expression of bcl-2 was seen in T1, T8, T3, T7, or T10 cells.

Expression of c-FLIP, survivin, cIAP1, and cIAP2 mRNA was measured by quantitative reverse transcription-PCR (Fig. 2C). At both protein and mRNA levels, variable expression of cIAP1, cIAP2, survivin, and c-FLIP mRNA was observed in the primary cell lines. Co-expression of cIAP1 and cIAP2 or survivin and c-FLIP protein was strongly correlated (Spearman rank-order correlation \( r_s \geq 0.6, P < 0.05 \)). Protein and mRNA expression of all tested inhibitors of apoptosis was independent of levels of Fas expression (Spearman rank-order correlation, \( P > 0.05 \)).
Prostate Cell Cultures Undergo Fas-Mediated Apoptosis

Although these cultured primary prostate cancer cells strongly express the Fas receptor, they also co-express several inhibitors of apoptosis, and as such, functional assays are required to determine whether they are sensitive to apoptosis via Fas. This was assessed in cytotoxicity assays by co-incubating primary cancer cells with FasL-overexpressing effector cells. To generate these potent effector cells, K562 cells were transduced with lent-i-ncFasL or lent-i-enGFP vectors leading to stable overexpression of ncFasL or control enGFP, respectively. Clonal populations of K562-ncFasL and K562-enGFP cells were co-incubated with each of the 11 prostate cell cultures in 6 h 51Cr release assays. After co-incubation with K562-enGFP cells at a 25:1 effector-to-target ratio, 5.0 ± 4.9% of cultured primary prostate cancer cells were killed (data not shown). When K562-ncFasL cells were used at the same ratio, the resulting cytotoxicity was 34.1 ± 20.3% above the level of nonspecific background killing induced by the K562-enGFP cells. Percent cytotoxicity ranged from 12.2 ± 2.5% (T8) to 87.1 ± 42% (T2) (Fig. 3A). In comparison, PrEC cells, a commercial nonmalignant primary prostate cell line, were shown to have a similar cytotoxic response to K562-ncFasL cells, undergoing 44% cytotoxicity under the same conditions. The sensitivity of the primary prostate cancer cell lines to Fas-mediated apoptosis far exceeded that of immortalized PC-3 cells, which underwent negligible killing by K562-ncFasL cells. The cytotoxicity induced by K562-ncFasL cells was shown to be entirely mediated by FasL, as addition of the antagonistic anti-FasL antibody NOK-1 completely prevented any killing by K562-ncFasL cells (Fig. 3B). FasL binding has been reported in some situations to induce a proliferative response in target cells (31). To rule this out in our system, T2, T8, and T11 cells were incubated with increasing doses of soluble FasL and growth rate assessed by MTT assay. No increase in growth was observed even at nontoxic low doses (data not shown).

To determine whether basal levels of Fas expression may affect the apoptotic response of these cell lines, Fas ABC of each cell line was plotted against the observed cytotoxicity. No correlation was seen between these two variables (Fig. 3C; Spearman rank-order correlation $r_s = 0.35, P = 0.28$).

The induction of apoptosis also did not correlate with relative rates of growth or expression of the inhibitors of apoptosis survivin, cIAP1, cIAP2, or c-FLIP at the protein or mRNA level as assessed by quantitative RT-PCR or...
Western blot (Spearman rank-order correlation, \( P > 0.13 \) in all cases; data not shown).

Fas-mediated apoptosis can proceed through a mitochondrial-dependent or mitochondrial-independent pathway depending on the cell type. Loss of mitochondrial membrane potential is a hallmark of apoptotic signaling through the mitochondria. To examine the pathway followed by Fas-mediated apoptosis in these prostate cancer cells, the integrity of the mitochondrial outer membrane was measured by the ability of these cells to retain the mitochondrial dye TMRE after co-incubation with K562-ncFasL cells. Co-incubation of T10 and T11 cells with K562-ncFasL cells resulted in a significant reduction in the number of cells retaining TMRE dye, indicating a loss of membrane potential in these cells (Fig. 3D). The addition of Z-VAD-FMK to block caspase activation and thus apoptotic signaling prevented the loss of mitochondrial membrane potential.

Chemotherapeutic Drugs Mitoxantrone and Docetaxel Increase Fas Expression

Chemotherapeutic agents have been observed to induce increases in Fas receptor expression on various cancer cell lines, which in some cases translates into increased Fas-mediated apoptosis (18). The potential of the anti-prostate cancer chemotherapeutics docetaxel and mitoxantrone to enhance cell surface expression of Fas on primary prostate cancer cell lines was investigated. Cell cultures were exposed to increasing doses of mitoxantrone (1-100 nmol/L) or docetaxel (10-1,000 nmol/L) for 24, 48, or 72 h. Changes in Fas expression were measured by flow cytometry and calculated as the change in Fas MFI for treated cells over that of untreated cells. The greatest increase in Fas expression by T1 and T8 cells was seen following a 72 h exposure to 100 nmol/L mitoxantrone (3.2-fold increase in MFI; Fig. 4A and B). Similar results were found for T3 and T4 cells (data not shown). Pretreatment of prostate cells with docetaxel resulted in a 1.5-fold increase in Fas expression at both 100 and 1,000 nmol/L concentrations, with no evidence of dose-dependency at these concentrations (Fig. 4C, T8 cells). Enhancement of Fas expression in this manner significantly increased the ABC of T8 cells to 215,138 \( \text{F}_9,172 \) ABC after 48 h exposure to 100 nmol/L mitoxantrone compared to the basal expression level of 139,385 \( \text{F}_10,283 \) ABC (\( P < 0.05 \)). In accordance with published reports (32), irradiation of prostate cells was also found to lead to increased Fas expression in these cells, with a 1.4-fold increase in MFI in T1 cells 72 h after irradiation with 8 Gy in our hands (data not shown).

**Combination of Mitoxantrone and Docetaxel with Fas Therapy Leads to Increased Cell Killing**

The effect of mitoxantrone- and docetaxel-mediated Fas up-regulation on the effectiveness of Fas-induced apoptosis was tested on T3, T4, T5, T8, and T11 cells. Increases in Fas-mediated apoptosis of mitoxantrone-treated T3, T8, and T11 cells peaked following 48 h treatment with 100 nmol/L mitoxantrone, resulting in a 1.5- to 3-fold enhancement of cytotoxicity following combination treatment (Fig. 5A). When the same cells were pretreated with docetaxel before \(^{51}\text{Cr} \) release assays, no increase in Fas-mediated killing of T3 or T8 cells was seen despite similar increases in cell surface Fas expression (Fig. 5B). In fact, small but significant decreases were actually seen in Fas-mediated apoptosis following docetaxel treatment in these cells (\( P < 0.05 \)). When T11 cells were used in this assay, again no difference in apoptosis induction was seen at 24 h (Fig. 5B). However, increases in cytotoxicity of 1.4-fold were observed by 48 and 72 h after addition of 1,000 nmol/L docetaxel (Fig. 5B; \( P < 0.05 \)). One activity of docetaxel is the inhibition of bcl-2 (33). Whereas T11 prostate cancer cells
expressed high levels of bcl-2, T3 and T8 cells expressed low levels or no detectable bcl-2 (Fig. 2B). Similar increases in Fas-mediated cytotoxicity were obtained when bcl-2-expressing T4 cells were pretreated with docetaxel (data not shown). No further improvements in FasL-induced cytotoxicity of T8 or T11 cells were observed when mitoxantrone and docetaxel were added simultaneously (data not shown).

**Mechanism of Sensitization to Fas-Mediated Apoptosis Involves p53**

Induction of p53 by DNA-damaging agents has been reported to stimulate expression of the Fas gene and to render certain cell types more sensitive to Fas-mediated apoptosis (34). To verify that the mechanism of sensitization of primary prostate cancer cells to Fas-mediated killing is dependent on p53 activity, apoptosis experiments were repeated in the presence of pifithrin α, a chemical inhibitor of p53. T11 cells were treated with pifithrin α for 24 h before the commencement of 48 h mitoxantrone or docetaxel treatment. Both the basal expression level of p53 and its induction by mitoxantrone and docetaxel were reduced by pifithrin α treatment (Fig. 6A). When 51Cr release assays were done using pifithrin α–treated cells, a general reduction in the level of cytotoxicity was observed. As well, any increase in cytotoxicity induced by docetaxel was completely prevented, whereas sensitization to Fas-mediated apoptosis by mitoxantrone treatment was limited (Fig. 6B). These data show the important role of p53 in sensitizing primary prostate cancer cells to Fas-mediated apoptosis.

**Discussion**

This study shows for the first time that primary human prostate cancer cells are sensitive to Fas-mediated killing and that this killing can be further improved by pretreatment of the cells with mitoxantrone and, in select cases, docetaxel. Fas signaling induces depolarization of the mitochondrial outer membrane, suggesting a mitochondrial-dependent mechanism behind this killing. Cytotoxicity studies revealed no difference in the ability of six of our primary tumor cell lines and PrEC normal prostate epithelial cells to undergo apoptosis in response to FasL. This suggests that malignant transformation may not necessarily lead to acquisition of resistance to Fas-mediated killing at this stage of disease. The sensitivity of all 11 primary prostate cancer cell lines to apoptosis was found to exceed that of PC-3 cells, revealing that the use of immortalized prostate cancer cells

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**Figure 5.** Pretreatment of primary human prostate cancer cells with mitoxantrone increases sensitivity to Fas-mediated killing. Bcl-2-negative T8 and T3 cells and bcl-2-expressing T11 cells were treated for 24, 48, or 72 h with (A) 10 or 100 nmol/L mitoxantrone or (B) 100 or 1,000 nmol/L docetaxel before assessment of sensitivity to FasL-mediated killing in 6 h 51Cr release assays. Drug-treated T3, T8, and T11 cells were co-incubated with K562-ncFasL or K562-enGFP cells at 1:1, 10:1, or 25:1 effector-to-target cell ratios. Percent cytotoxicity was calculated as in Fig. 3, and target cell cytotoxicity induced following incubation with K562-ncFasL cells at a 25:1 ratio is shown, normalized to cytotoxicity values for target cells not treated with drug. Triplicate samples from one of two representative experiments are shown. *, P < 0.05, Student’s t test.
in functional assays may underestimate the responsiveness of prostate cancer to Fas-mediated apoptosis. This result provides important evidence validating the suitability of Fasl-based therapies for prostate cancer and suggests that novel combinatorial approaches with chemotherapy might be most effective.

Encouragingly, we have shown that apoptosis occurs readily in Gleason score 7 prostate tumor-derived cells despite the expression of inhibitors of apoptosis in many of these primary lines. c-FLIP expression has been shown to cause resistance of prostate DU145 cells to Fas-mediated apoptosis (35) and is sufficient to induce TRAIL resistance in prostate cancer cells (36). IAP expression is also associated with resistance to apoptosis (37). In this study, simultaneous expression of a number of apoptosis inhibitors was observed; however, we did not show a correlation between expression of inhibitors and induction of Fas-mediated killing. Furthermore, the primary human prostate cell lines with the lowest response to Fas, T1 (19.9%) and T8 (12.2%), were found to be bcl-2 negative, whereas the most sensitive cell line, T2 (87.1%), expressed abundant bcl-2. These observations suggest that measurement of expression of only these inhibitors of apoptosis is not a valid predictor of apoptotic responses in these primary cells.

The observed lack of correlation between apoptosis induction and inhibitor expression may be due to the limited sample size studied or may result from the complex cellular mechanisms controlling apoptosis. In addition to the inhibitors studied, overexpression of other bcl-2 family proteins, dysregulation of mitogen-activated protein kinase or phosphatidylinositol 3-kinase activity, expression of decoy receptors, or deletion/mutation of caspases have been described in cancer (38). Thus, although expression of IAP proteins or c-FLIP may limit apoptosis in some cell lines, other factors may be more influential in regulating this important cellular process. In addition, protein and mRNA expression was measured from pooled lysates and mRNA. It is possible that any cells surviving after 6 h in our assays will express these proteins at higher levels than the cells that were readily killed. Future studies will address this issue.

In our study, mitoxantrone, a topoisomerase II inhibitor and molecule that integrates into DNA, along with docetaxel, a microtubule-stabilizing agent, were both
effective at increasing Fas receptor expression on primary prostate cancer cells. However, only mitoxantrone was able to improve the susceptibility of all tested primary prostate cultures to Fas-mediated apoptosis. In the majority of the cells tested, docetaxel did not improve sensitivity to apoptosis despite also enhancing Fas expression. One explanation for this difference could be the existence of a threshold effect, as mitoxantrone was more effective at increasing Fas expression than docetaxel (3.2-fold versus 1.5-fold induction). Alternatively, mitoxantrone and docetaxel may differentially affect the expression of multiple cell survival or apoptosis proteins. Indeed, we have observed a change in expression in 110 proteins in primary prostate cancer cells on addition of mitoxantrone. This concept is backed up by the lack of correlation in this study between basal Fas expression and cytotoxicity (Fig. 3C). Furthermore, one observed mechanism of action of docetaxel is the inactivation of bcl-2 (39). In our study, docetaxel pretreatment was only found to increase apoptosis induction by FasL-based therapy in bcl-2-expressing cells (Fig. 5B). In contrast, mitoxantrone was able to sensitize both bcl-2-negative and bcl-2-expressing cells to Fas-mediated killing. It is thus possible that docetaxel-induced inactivation of bcl-2 is a more potent sensitizer to apoptosis than docetaxel-induced Fas up-regulation. Further investigation into the effect of these agents on apoptotic pathways is warranted, as the molecular profile of an individual cancer is likely to influence the success of any apoptosis-based therapeutic strategy. It is also possible that no single apoptosis-sensitizing agent will be optimal for all tumors.

Expression of p53 protein was detected in all cell lines tested by Western blot analyses (Fig. 2B). Addition of pifithrin α, an inhibitor of p53, to cultures of T8 and T11 cells limited the induction of apoptosis and the sensitization to Fas-mediated killing by docetaxel and mitoxantrone. p53 has been implicated in control of Fas expression in several cell models (20). Other studies show that combination cisplatin treatment and Fas-triggering induced apoptosis in p53-mutant cell lines (19). In the present study, apoptosis was not completely inhibited by pifithrin α, suggesting that although p53 plays a role in sensitivity to apoptosis, loss of p53 activity does not completely abrogate this process.

FasL-based therapies will require careful design to avoid off-target toxicity. This can be addressed by introducing stringent delivery, expression, and activation controls on FasL. Several such approaches have been used with success, including the development of gene therapy-based strategies engineering tissue-specific FasL expression (8), bispecific anti-Fas antibodies that aggregate and become active only in the presence of a specified tumor antigen (9, 10), and recombinant FasL prodrg constructs that are functional only at the tumor site (11). These innovations in FasL-based drug design are likely to culminate in a molecule with a more favorable toxicity profile and are bolstering enthusiasm for these types of signaling-directed anticancer approaches. In addition, as we have shown that chemotherapy is able to improve the therapeutic efficacy of FasL, controlled delivery of chemotherapeutic agents could be used to further direct the specificity of Fas-mediated toxicity. For example, nanoparticle-based drug delivery methods are under development, which, by incorporation of antigen- or receptor-targeting molecules into the particle surface, can deliver high levels of localized drug to specific target cells (40). In this way, tumor-specific targeting can be achieved, reducing toxicity to nonmalignant cells.

In conclusion, we have shown in primary prostate cells that Fas-based approaches to prostate cancer cell killing are possible. Mitoxantrone and docetaxel were shown to further improve this killing. Although neither mitoxantrone nor docetaxel is curative for prostate cancer in themselves, their use in combination with FasL-based therapies could potentially increase anticancer efficacy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
3028  FasL-Induced Apoptosis in Primary Prostate Cancer Cells


Fas-mediated killing of primary prostate cancer cells is increased by mitoxantrone and docetaxel


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