Fas Ligand Is Constitutively Secreted by Prostate Cancer Cells in Vitro

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ABSTRACT

LNCaP, DU145, and PC3 prostate carcinoma cells secrete the 27-kDa soluble Fas ligand (sFasL) into their local environment. sFasL arises from the 40-kDa membrane-bound form (mFasL), which can be found on the cell surface in the LNCaP line, as demonstrated by monoclonal antibody staining. mFasL was found in extracts of all three cell lines, as demonstrated by Western blotting. FasL mRNA was detected not only in the cell lines, but in the normal prostate as well. sFasL protein could also be detected immunohistochemically in prostate secretions and in human semen. Cleavage of mFasL to sFasL could be inhibited by several matrix metalloproteinase inhibitors without a change in the cellular levels of FasL. Prostate-derived sFasL is biologically active, as demonstrated by its induction of apoptosis in Fas-positive Ramos cells, which was detected by terminal deoxynucleotidyl transferase-mediated nick end labeling assay.

Mitoxantrone induces cellular apoptosis in all three prostate cancer cell lines. Mitoxantrone treatment and doxorubicin treatment also cause up-regulation of Fas, the cell surface receptor for FasL, in LNCaP cells, but not in DU145 or PC3 cells. Furthermore, the up-regulation of Fas expression by mitoxantrone at a high concentration was potentiated by hydrocortisone. When FasL interacts with its Fas, the Fas-bearing cell undergoes apoptosis. When LNCaP cells were treated with mitoxantrone and incubated with an anti-FasL monoclonal antibody, apoptosis was partially blocked. This not only further suggests that the sFasL is biologically active, but that the up-regulation of Fas in the presence of sFasL accounts, in part, for the cytotoxicity of mitoxantrone.

INTRODUCTION

Fas (APO-1/CD95) is a type I membrane protein that is a member of the tumor necrosis factor/nerve growth factor receptor superfamily (1) that also includes CD40, CD27, CD30, and OX40 (2). It is expressed in activated T and B cells, in malignant T and B cells, and in several other tissues, including the thymus, liver, heart, and ovary (2-5). Recent data (6) indicate that cell surface Fas can be up-regulated after treatment of HepG2 hepatoma cells with either bleomycin, methotrexate, or cisplatin.

FasL,3 similar to tumor necrosis factor α, is synthesized as a membrane-bound 40-kDa protein (mFasL) that can be cleaved to the soluble 27-kDa species (sFasL) by matrix metalloproteinases (7). When FasL or agonist anti-Fas Abs bind to Fas, cellular apoptosis is induced. Analysis of the Fas/FasL system has indicated that it is involved in the clonal deletion of peripheral T cells and in the general down-regulation of the immune response, including a diminution in cytotoxic T-cell-mediated cytotoxicity (8, 9).

The involvement of Fas and FasL as a possible mechanism whereby tumors escape the immune system has recently been proposed. Hahne et al. (10) detected sFasL in the serum of 18 of 35 patients with malignant melanoma and detected mFasL in several types of melanoma cells, but not in normal melanocytes. Furthermore, melanoma cells seemed to be capable of killing Fas-bearing immune effector cells. Tanaka et al. (11) detected sFasL in the sera of patients with both large granular lymphocytic leukemia and normal killer cell lymphoma. High levels of sFasL were also observed in a patient with an aggressive nasal lymphoma who succumbed to hepatic failure, perhaps due to the induction of apoptosis in Fas-expressing hepatocytes. FasL has also been proposed to have a protective role in immune-privileged sites, including the stromal cells of the retina (11, 12), inner ear, testis (Sertoli cells; Refs. 11 and 12), cornea (13), and brain (14, 15), and seems to be involved in the destruction of thyrocytes that occurs in Hashimoto’s thyroiditis (16). O’Connell et al. (17) have proposed that SW620 colon cancer cells can avoid immune surveillance by expressing high levels of FasL. Interestingly, these cells also express cell surface Fas, perhaps in an inactive form. These data are highly suggestive of the idea that the Fas/FasL system plays a general and highly complex role in the life and death cycle of human solid tumors.

Prostate cancer is now the second leading cause of cancer...
deaths in adult males, claiming over 41,000 lives in the United States annually (18). Whereas primary treatment for metastatic disease consisting predominately of the administration of lev- 

tinizing hormone-releasing hormone antagonists in combination with a nonsteroidal antiandrogen (e.g., flutamide) will palliate as many as three-fourths of patients, the tumor will invariably cease to respond to this therapy within a median time of 3.5 years (19). Until recently, treatment of metastatic HRPC has been relatively ineffective. How- 

ever, recent studies have demonstrated clinical activity by a combination of a taxane plus estramustine (20, 21). Furthermore, a study by Tannock et al. (22) has suggested that the combination of mitoxantrone plus prednisone may significantly improve quality of life in as many as 38% of patients with HRPC.

The mechanisms of prostate cancer cell apoptosis are com- 

plicated and are not well understood. Approximately 40% of 

advanced clinical prostate cancers overexpress bcl-2 (23). Taxol-induced changes in the phosphorylation pattern of bcl-2 seem to correlate with the induction of cellular apoptosis, at least in cell lines (24, 25). Furthermore, Taxol-induced apopto- 

sis, as demonstrated by the formation of ladders and DAPI staining, was associated with the down-regulation of both bcl-xL mRNA and protein. No changes were observed in the cellular levels of bax, bcl-xL, or Fas mRNA or protein (26). Neverthe- 

less, Fas has also recently been implicated in cellular apoptosis in two of six prostate cancer cell lines (27).

Here we present evidence that not only do all three prostate cancer cell lines constitutively secrete biologically active FasL into their local environment, but that FasL mRNA can be found in the normal prostate, and FasL protein can be found in normal prostate secretions. We also show that whereas Fas is minimally expressed on the surface of these prostate cancer cell lines, treatment of LNCaP cells but not DU145 or PC3 cells with mitoxantrone plus hydrocortisone dramatically up-regulates its expression. In this context, the anthracycline-induced up-regu- 

lation of Fas in LNCaP cells may possibly then create a SF loop, which seems to be one of the mechanisms by which androqui- 

rones act to kill susceptible cells.

MATERIALS AND METHODS

Cells. LNCaP, DU145, and PC3 prostate cancer cells were purchased from American Type Culture Collection (Rock- 

ville, MD) and cultured in RPMI 1640 supplemented with 10% 

FCS in 95% air:5% CO2. Cells in complete media were treated with different concentrations of mitoxantrone (Immunex, Seat- 

tle, WA) or doxorubicin (Gensia, Irvine, CA) for the times 

indicated in “Results.” The cells were harvested, and the pro- 

teins and total RNA were extracted as described below.

Isolation of Apoptotic DNA Fragments. DNA fragment- 

ation assays were performed as described previously, with 

modification (28). Briefly, 2 106 LNCaP cells treated with 

0.2–1 μm mitoxantrone for 24 h were lysed in 1% NP40, 20 mm 

EDTA, and 50 mm Tris-HCl (pH 7.5; 100 μl106 cells). After 

centrifugation for 5 min at 15,000 9 g, the supernatant was 

collected, and the extraction was repeated with additional lysis 

buffer (50 μl106 cells). The supernatants were brought to a 1% 

SDS concentration and treated with RNase A (final concentra- 

tion, 200 μg/ml) at 56°C for 1 h, followed by digestion with 

proteinase K (final concentration, 2.5 mg/ml) at 56°C for 2 h. 

The DNA was then precipitated with 2.5 volumes of ethanol and 
dissolved in Tris-EDTA (ethylenediaminetetraacetate) buffer 
(pH 7.4). Equal amounts of DNA as measured by UV absorb- 
ance at 260 nm were electrophoresed on 1.2% agarose gels 
containing 0.5 mg/ml ethidium bromide and visualized by UV 
transillumination.

Detection of Apoptotic Nuclei by DAPI Staining. Mor- 

phological changes characteristic of apoptosis were determined by staining cell nuclei with DAPI. Briefly, cells were plated onto poly-d-lysine-coated tissue culture chamber slides (Nunc, Inc., Naperville, IL) and incubated overnight. The cells were 
treated with mitoxantrone (0.2 and 0.6 μm) for 24 h and then 
washed once with PBS. The cells were then fixed with 90% 
ethanol and 5% acetic acid for 1 h at room temperature, washed 
twice with PBS, and treated with a 1.5 mg/ml solution of DAPI in 
PBS for 30 min at room temperature. The slides were washed 
twice with PBS, mounted, and photographed using a Nikon 
phase-fluorescence microscope.

RT-PCR. The first strand cDNA was synthesized from 2 
μg of total RNA isolated from LNCaP, DU145, or PC3 cells in a 
20-μl reaction mixture containing 4 μl of 5× RT reaction buffer, 10 units of RNasin (Promega, Madison, WI), 200 μm 
deoxynucleotide triphosphate, 40 pm oligodeoxythymidylic acid 
primer, and 20 units of Moloney murine reverse transcriptase (Promega). The mixture was incubated at 42°C for 1 h and then 
incubated at 53°C for 30 min. The unhybridized RNA was then 
digested with 10 units of RNase H at 37°C for 10 min. The RT 
products were diluted to 200 μl with Tris-EDTA (ethylenedi- 
aminetetraacetate) buffer, and 4 μl of the diluted RT products 
were subjected to PCR amplification using Fas- and FasL- 
specific primers. The primer sequences were as follows: Fas 
upstream, 5′-ATGCCCAAGTGACTGACATC-3′; Fas downstream, 
5′-GTCAATTCGATCATCCTATG-3′ (29): FasL upstream, 
5′-TCAACTCAGGTTCTCATGCC-3′; and FasL downstream, 
5′-CAGAGAGGCTCAGATGT-3′ (16). Thirty-five cycles of amplification were performed in a thermocycler at 94°C (1 min), 57°C (1 min), and 72°C (2 min). The RT-PCR products (725- and 342-bp fragments predicted for Fas and FasL, respectively) were analyzed on 1.2% agarose gels. 

The amount of template RNA was normalized by using 
human GAPDH primers as internal controls. The primer se- 
quences for GAPDH were 5′-GTCACAGGATTTGCTCTG- 
TAAT-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′, and 
the cycling conditions were 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. To check for artifacts based on the possible contami- 
nation of RNA by genomic DNA during RT-PCR, several PCR 
reactions were performed using the same amount of mRNA as 
template under identical conditions, but with no RT step (i.e., 
no added reverse transcriptase).

Western Blot Analysis and Immunoprecipitation. Cells (106) were lysed in 100 μl of ice-cold radioimmuno- 
precipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM 
NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate] 
with 0.1 mg/ml freshly added phenylmethylsulfonyl fluoride, 1 
m sodium orthovandate, and 30 mg/ml aprotonin; mixed 
gently with a pipette; and incubated on ice for 30 min. Cell 
debris was removed by centrifugation. Protein concentrations
were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell extracts containing 20 μg of protein were resolved by 10% SDS-PAGE and transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL). Filters were incubated at 25°C for 1 h in Blotto A (5% nonfat milk powder in TBS [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl] plus 0.05% Tween 20) and then incubated at 25°C for 1 h in Blotto A containing a 1:200 dilution of either rabbit anti-Fas or anti-FasL polyclonal Abs (all from Santa Cruz Biotechnology, Santa Cruz, CA). After washing in TBS + 0.05% Tween 20 buffer, filters were incubated for 45 min at 25°C in TBS + 0.05% Tween 20 buffer containing a 1:10,000 dilution of peroxidase-conjugated secondary Ab (Amersham Life Sciences, Arlington Heights, IL). After washing, ECL was performed using the procedure recommended by the manufacturer (Amersham). The filters were exposed to the X-ray film for times ranging from 10–120 s. For immunoprecipitations, complete media (fresh and conditioned) were cleared with irrelevant mAb and protein A-Sepharose CL-4B (Sigma, St. Louis, MO). Anti-FasL mAb (PharMingen, San Diego, CA) was then added (2 μg/ml) and incubated at 4°C for 1 h. Protein A-Sepharose beads were then added, and the incubation was continued at 4°C overnight. The beads were then collected by centrifugation, washed three times with PBS, and resuspended in 2X SDS-PAGE loading buffer, boiled for 5 min, and electrophoresed on a 10% SDS-containing gel. Subsequently, the proteins were transferred to Hybond ECL filters as described above and probed with the anti-FasL mAb.

Studios on normal human seminal fluid were performed on samples purchased from Cryogenics Laboratories (Minneapolis, MN). Western blotting was performed as described above.

**Immunohistochemistry for Fas and Fasl.** Prostatic tissue was acquired from radical prostatectomy specimens with institutional review board approval. Tumor samples were snap-frozen and stored at −80°C. After endogenous peroxidase activity had been quenched with 1.5% H2O2 in PBS and nonspecific binding of the Abs had been blocked by 10% goat serum in PBS, sections were incubated in PBS with 5% goat serum for 1 h at room temperature with primary rabbit polyclonal Abs (both diluted 1:50). The anti-FasL and anti-Fas Abs were from Santa Cruz Biotechnology. Sections were then rinsed three times for 5 min each in PBS and incubated with a secondary biotinylated goat antirabbit Ab (1:100 in PBS) for 30 min, rinsed again with PBS, and incubated with avidin-peroxidase complex (1:100; Vector Laboratories, Burlingame, CA) for 30 min. Diaminobenzidine (250 μg/ml in PBS) was used as a substrate for peroxidase to detect the presence of target antigen by deposition of a brown reaction product. Sections were counterstained with Mayer’s hematoxylin, dehydrated, cleared with xylene, and coverslipped.

**Determination of Cell Surface Fas and Fasl. Expression by Flow Cytometry.** Prostate cancer cells were detached from the bottom of dishes in PBS containing 2.5 mM EDTA. The cells were centrifuged, washed twice with PBS, and then incubated at 4°C for 60 min with 10 μg/ml rabbit anti-Fas polyclonal Ab (IgG1; Santa Cruz Biotechnology) in PBS containing 1% FCS (PBS/BSA) and 0.5 mM EDTA. As controls, staining was performed with both the secondary Ab only and a nonspecific isotype-matched (anti-cyclin A) polyclonal Ab (Santa Cruz Biotechnology). The cells were then washed twice with PBS and incubated for 30 min on ice with 10 μg/ml affinity-purified FITC-conjugated goat antirabbit IgG1 (Jackson ImmunoResearch Laboratories, San Diego, CA) in the same buffer. Detection of mFasL was accomplished by the use of an antihuman Fasl mouse IgG1 (clone G247-4; PharMingen), with a FITC-labeled goat antimonouse IgG used as a secondary Ab (Life Technologies, Inc., Grand Island, NY). Controls were as described above, except that the irrelevant Ab was an antihuman bcl-2 IgG1 mAb (Santa Cruz Biotechnology). Cells were washed twice with PBS and then resuspended in PBS/BSA. Cell aggregates were removed by filtering through a 60-μm mesh. The mean fluorescence intensity of a population of 5000 cells was determined on a Becton Dickinson FACScalibur in the

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**Fig. 1.** A, detection of sFasL in conditioned media. Aliquots of fresh media (Lane 1), LNCaP cell-conditioned media (Lane 2), LNCaP cells treated with 1 μM mitoxantrone for 2 days (Lane 3) and with 4 μM mitoxantrone plus 10 μM hydrocortisone for 2 days (Lane 4), PC3 cells (Lane 5), and DU145 cells (Lane 6) were subjected to SDS-PAGE and immunoblot analysis using an anti-FasL polyclonal Ab. B, immunoprecipitation of sFas-L from conditioned media. Lane 1, LNCaP-conditioned media were immunoprecipitated with a specific anti-Fasl mAb as described in the text; Lane 2, immunoprecipitation of LNCaP-conditioned media with nonspecific control anti-bcl 2 mAb; Lane 3, immunoprecipitation of fresh media with anti-Fasl mAb.
Prostate Cancer Cells Secrete FasL in Vitro

Fig. 2 Determination of cell surface FasL expression by flow cytometry. Shown are histograms of LNCaP cells in which the mean channel number of fluorescence, as determined on a FACScalibur flow cytometer, is plotted against arbitrary fluorescence units (counts). Each histogram represents the counting of 5000 cells, as described in the text. A, control fluorescence, secondary Ab only. B, staining with anti-FasL mAb + secondary Ab, as described in the text.

presence or absence of mAb staining. Mean fluorescence channel intensities were expressed as the average of these measurements (n = 3).

Induction of Apoptosis by LNCaP Supernatants. A total of 5 × 10^5 Fas-positive Ramos 296 cells or control Fas-negative 293/CD8 cells were washed and incubated overnight in either complete media alone or complete media plus either OKT4 control Ab (10 μg/ml), anti-Fas Ab (PharMingen; 10 μg/ml), or culture supernatants of LNCaP cells obtained from a dense culture of LNCaP cells grown for approximately 1 week. Cells were then assayed for apoptosis via terminal deoxynucleotidyl transferase-mediated nick end labeling assay and flow cytometry using the Boehringer Mannheim (Indianapolis, IN) in situ cell death fluorescence detection kit.

MTT Assay. Cells (7 × 10^3) were plated in 96-well plates and incubated for 24 h in 100 μl of complete media. Various concentrations of mitoxantrone and the anti-FasL mAb G247-4 (0.5 μg/ml; PharMingen) were added, and the cells were incubated for an additional 48 h. Then, 10 μl of MTT/PBS (5 mg/ml MTT in PBS; Sigma) were added to each well and incubated for 2.5 h at 37°C, and 0.04 M HC1 (100 μl) in isopropanol was added with vigorous mixing. The absorption was determined in a microtiter plate reader at 540 nm.

RESULTS
Prostate Cancer Cells Secrete Fas Ligand. Aliquots of LNCaP-, DU145-, or PC3-conditioned media (1 or 2 days) were electrophoresed in polyacrylamide gels under the conditions given in “Materials and Methods.” As shown in Fig. 1, a band migration with an apparent molecular mass of 27 kDa that corresponds to the sFasL (10) was observed. The 40-kDa mFasL was not detected in samples of conditioned media. The 27-kDa sFasL could also be immunoprecipitated from conditioned media (2 days) by the use of the anti-FasL mAb described in “Materials and Methods” (Fig. 1). No sFasL was immunoprecipitated with this mAb in fresh media. Furthermore, the secretion of sFasL into the conditioned media (2 days) was not significantly diminished in the continuous presence of mitoxantrone (1–5 μM) with or without hydrocortisone (10 μM).

The 27-kDa sFasL represents the cleaved fragment of the 40-kDa mFasL. To demonstrate that the presence of this fragment in the media was not only the result of prolonged cell culture and concomitant cell death and release of cellular components, we examined the cell surface expression of mFasL on LNCaP cells using mAb staining and flow cytometry. As shown in Fig. 2, mFasL could easily be detected on the cell surface of LNCaP cells. Furthermore, as shown in Fig. 3, the approximately 40-kDa mFasL was readily detected in whole-cell extracts of all three prostate cancer cell lines by Western blotting. The cleavage of mFasL to sFasL could be blocked by the MMIs CGS 27023A (M, 448) and 3390A (M, 547; Novartis, Basel, Switzerland). These compounds are highly specific inhibitors of matrix metalloproteinases but do not distinguish between them. Thus, CGS 27023A inhibits collagenase (MMP-1), gelatinase B (MMP-9), gelatinase A (MMP-2), and stromelysin with IC_{50} of 33, 8, 11, and 50 nm, respectively. The blockade of cleavage was demonstrated by a >80% decline in the level of sFasL in LNCaP 2-day culture supernatants. The IC_{50} of cleavage in both cases was <1 μM. No cellular cytotoxicity was observed, as determined by the MTT assay. Moreover, as determined by Western blotting, no change in the level of FasL in whole-cell extracts was observed in these cells after MMI treatment.

We then evaluated the presence of FasL mRNA by RT-PCR. As shown in Fig. 3, the predicted 342-bp fragment can be observed in all three prostate cancer cell lines and in the total RNA obtained from normal human prostate (Clontech, Palo Alto, CA) as well. The identity of the 342-bp fragment was confirmed by dideoxy sequencing.

We then examined radical prostatectomy specimens to
mFasL was not seen on either normal or neoplastic prostate cells. This was not surprising because of its cleavage to sFasL, which would be washed out during specimen processing. However, strong sFasL staining was observed in the intraluminal secretions from benign prostate glands (Fig. 4A). No staining of intraluminal secretions was observed with an irrelevant isotypematched Ab. Strong mFasL staining was also observed on peripheral nerves in the prostate stroma (Fig. 4C). We then examined specimens of normal human seminal fluid and were able to detect sFasL in six of eight specimens by Western blotting, implying that the normal prostate gland secretes sFasL into the seminal fluid. However, it is possible that the sFasL in seminal fluid may be produced by other organs in addition to the prostate, e.g., testis.

sFasL secreted by prostate cancer cells is biologically active. Fas-positive Ramos 296 B cells were treated overnight with 1-week culture supernatants from LNCaP cells. DNA nicking, as assessed via a flow cytometric terminal deoxynucleotidyl transferase-mediated nick end labeling assay, was dramatically increased in treated cells versus Ramos 296 cells treated with the anti-Fas Ab (10 μg/ml) alone (43 versus 18% apoptotic; 12% of untreated cells were apoptotic (average of two separate determinations; mean difference, <15%).) Fas-negative 293 control cells were only minimally affected by the culture supernatants. In addition, mAb Mike-1 (IgG rat monoclonal antihuman FasL, extracellular domain), but not an irrelevant isotypematched mAb, almost completely blocked the induction of apoptosis by the LNCaP supernatants.

**Mitoxantrone Causes Apoptosis and Up-Regulation of Cell Surface Fas in LNCaP Cells.** To determine whether mitoxantrone-induced LNCaP death demonstrates the characteristic DNA laddering of apoptosis, we examined genomic DNA after treatment with 0.2 and 1.0 μM mitoxantrone. As shown in Fig. 5A, internucleosomal fragmentation of the DNA was observed in the cells after treatment for 24 h. The extent of the laddering was drug dose dependent. To further demonstrate that mitoxantrone induced cellular apoptosis, we stained the cells with the nuclear stain DAPI. As shown in Fig. 5B, characteristic cellular changes of apoptosis, including chromatin condensation and nuclear fragmentation, were observed in at least 50% of the LNCaP cells after 24 h of treatment with 0.6 μM mitoxantrone.

Furthermore, the treatment of LNCaP cells with mitoxantrone caused a dramatic up-regulation in the amount of total cellular Fas expression, as determined by Western blotting (Fig. 6A). However, FasL expression was not affected (Fig. 6B). Cellular Fas expression, as detected by Western blotting, was also equally dramatically up-regulated by doxorubicin (0.2 and 1.0 μM; 1 or 2 days; data not shown). No up-regulation of cellular Fas expression could be detected in either DU145 or PC3 cells in the presence of mitoxantrone.

Cell surface expression of Fas, as evaluated by flow cytometry (Fig. 7, left), was also dramatically increased after treatment of LNCaP cells with 1 μM mitoxantrone for 24 h (mean channel number of control, 6 ± 0.15; + anti-Fas mAb, 182 ± 2). The addition of hydrocortisone (10 μM) to mitoxantrone under identical conditions did not further increase up-regulation of cell surface Fas (Fig. 7, right; mean channel number, 127 ± 1.5; control, 11 ± 0.3) However, at higher mitoxantrone concentrations, hydrocortisone (10 μM) potenti-

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**Fig. 4** FasL is found in the normal prostate. Frozen sections from human prostate specimens are shown. Representative nontumoral regions are depicted. A, sFasL, is found intraluminally in the secretions from prostate epithelial cells. B, normal prostate control specimen stained with isotypematched anti-fas Ab. No staining is seen within the gland lumena. C, mFasL, is found on peripheral nerves in the prostate stroma. After treatment with either the anti-Fas or anti-FasL polyclonal Abs, specimens were treated with biotinylated goat antirabbit Ab and then with avidin-peroxidase and diamobenzidine, as described in "Materials and Methods." Sections were counterstained by Mayer's hematoxylin.
ated the up-regulation of total cellular Fas, as assessed by Western blotting. In the absence of the steroid, Fas protein was up-regulated after treatment with up to 2 μM mitoxantrone, but not at higher concentrations (up to 5 μM). In the presence of the steroid, approximately equal up-regulation was seen in LNCaP cells at all mitoxantrone concentrations tested (1–10 μM; data not shown). Up-regulation of Fas protein could also be correlated with simultaneous up-regulation of the Fas mRNA, as shown by semiquantitative RT-PCR, in which the predicted 725-bp DNA fragment could be observed (Fig. 6C). The identity of this fragment was verified by dideoxy sequencing.

Maximal up-regulation of total cellular Fas expression was dependent on the presence of hydrocortisone after mitoxantrone washout. When LNCaP cells were treated for either 1 or 2 h with 4 μM mitoxantrone, washed, and then reincubated in fresh complete media for either 1 or 2 days, Fas expression was not up-regulated (Fig. 8). In contrast, if hydrocortisone (10 μM) was added to the initial incubation, up-regulation of Fas was observed, even after as long as 2 days. Moreover, as assessed morphologically and by the MTT assay, these cells had undergone apoptosis and cell death, in sharp contrast to the LNCaP cells that were hydrocortisone naive.

These data suggested that in the presence of mitoxantrone plus hydrocortisone, a SF pathway of cell death had been established. This pathway could be established because previously secreted sFasL still present in the supernatants could now interact with cell surface Fas, whose expression had been up-regulated by the drug combination. Alternatively, it is also possible that mFasL may interact with the up-regulated cell surface Fas on the same cells or on neighboring cells. To validate this hypothesis, we treated LNCaP cells with increasing and continuous concentrations of mitoxantrone (0–10 μM) and evaluated the fraction of living cells after 2 days by using the

**Fig. 6** Mitoxantrone induces up-regulation of Fas expression in LNCaP cells. A, immunoblot analysis of total protein (20 μg protein/lane) extracted from untreated cells (Lane 1), cells treated with 0.2 μM mitoxantrone for 24 and 48 h (Lanes 2 and 3), and cells treated with 1 μM mitoxantrone for 24 and 48 h (Lanes 4 and 5). After gel electrophoresis, the blots were probed with an anti-Fas polyclonal Ab and visualized by ECL as described in the text. B, reprobing of the stripped blot in A with an anti-FasL polyclonal Ab. C, expression of Fas mRNA in LNCaP cells treated with 1 μM mitoxantrone for 6 h to 2 days. The cDNA was synthesized and amplified by PCR using primers specific for Fas. The PCR product of GAPDH amplification was used as a control. After 35 cycles, the PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. M, φX174/HaeIII markers. The 725-bp Fas cDNA fragment can be seen between the 603- and 872 bp markers.
DISCUSSION

It has previously been shown (14) that FasL mRNA can be detected in moderate levels in mouse embryo seminal vesicles and at low levels in the prostate. In this work, we have demonstrated that three commonly available human prostate cancer cell lines secrete FasL, and that FasL mRNA is found in the normal human prostate gland and in human semen as well. In clinical specimens, sFasL can be seen by immunohistochemistry in prostatic secretions, but not on the cell membrane. It is possible that matrix metalloprotease activity in the prostate (30) may contribute to the cleavage of FasL from its membrane-bound form. This activity, as shown, can be inhibited by several new MMIs. FasL present on intraprostatic nerve bundles is not cleaved, possibly because these metalloproteases are intraluminally located. In addition, the role of this peripheral nerve FasL is unknown. mFasL has previously (31) been shown to be present on the surface of human central nervous system neurons, where it is thought to contribute to local immune protection. Perhaps a similar mechanism is operative for peripheral nerves in the normal prostate.

MTT assay in the presence or absence of the anti-FasL mAb. As shown in Fig. 9, the presence of the mAb increased the LD_{50} of mitoxantrone by approximately three to four times, indicating that the SF loop is one of the mechanisms by which mitoxantrone is able to kill prostate cancer cells. The addition of an isotype-matched anti-bcl-x_{L} mAb did not change the LD_{50} of mitoxantrone.
Why should sFasL be found in normal human semen? Given the ability of prostate cancer cell line-derived sFasL to induce apoptosis in Fas-positive lymphocytes and the previously demonstrated immunosuppressive properties of sFasL (32), we postulate two reasons: (a) the presence of immune effector cells and/or high local concentrations of cytokines and peroxides, such as occurs in inflammatory conditions of the prostate, can be very deleterious to spermatozoa and can lead to infertility; and (b) the presence of activated immune cells in the female genital tract. Indeed, the immunosuppressive properties of seminal fluid have been long recognized and have been ascribed to such soluble mediators as transforming growth factor β and prostaglandins (33).

It is also possible that the constitutive secretion of FasL, although possibly originating as a protection system for spermatozoa, is co-opted by at least some tumor cells as a self-protectant. In essence, this strategy is similar to the secretion of immunoinhibitory factors such as transforming growth factor β by some glioblastomas (34). In this context, it should be noted that it is very rare to find infiltrating lymphocytes in specimens of clinical prostate cancer.

Recently, Friesen et al. (35) demonstrated that treatment of CEM T cells with an anti-Fas Ab inhibited doxorubicin-induced apoptosis. This occurred because doxorubicin (and methotrexate as well) stimulated FasL expression. Our data on prostate cancer cells, on the other hand, demonstrated increased membrane Fas protein and Fas mRNA expression after anthracycline treatment, with no change in constitutive in FasL expression. Thus, our observations are similar to those of Muller et al. (6) in HepG2 hepatoma cells, which indicated that cell surface Fas can be up-regulated after treatment with either bleomycin, methotrexate, or cisplatin.

Nevertheless, as demonstrated above, it is possible to use the apoptosis-inducing properties of the Fas system as an antineoplastic strategy. Cell surface Fas was up-regulated in LNCaP cells by anthracycline treatment, and this up-regulation was, in the case of mitoxantrone, potentiated at some concentrations by hydrocortisone. This observation suggests that additional clinical therapeutic efficacy may be achieved in HRPC if the steroids are administered before as well as during the mitoxantrone infusion. The mechanism of anthraquinone induction of Fas up-regulation remains uncertain, but it is known that wild-type p53 can regulate Fas expression (6, 36). Possibly, DNA damage, which is known to be induced by anthracyclines, either by a free radical mechanism or by topoisomerase II inhibition (37), causes activation of wild-type p53, which can then mediate Fas expression. This mechanism is consistent with our observations that Fas up-regulation occurs only in LNCaP cells (wild-type p53), and not in DU145 (mutant p53) or PC3 (null p53) cells (38). However, in other cell types, the regulation of cell surface Fas expression may be p53 independent (39). In addition, the Fas system is most likely not the only proapoptotic pathway activated by anthracyclines in prostate cancer cells. Regardless, the addition of an anti-FasL Ab to cultured LNCaP cells increases the LD50 of mitoxantrone by 4-fold. This demonstrates that the Fas system is at least a contributor to the net anthracycline-induced apoptotic process. An additional demonstration of this lies in the fact that in the absence of up-regulation of Fas, as described in the mitoxantrone washout experiments, cellular apoptosis is greatly diminished. Finally, both bcl-2 and bcl-xL seem to protect Fas-overexpressing MCF-7 breast cancer cells from apoptosis induced by an anti-Fas Ab (40). This suggests that in at least some cell types, the two systems share common effector molecules. However, the LNCaP cell line, which expresses high levels of bcl-xL, is not protected against Fas/FasL-mediated apoptosis.

ACKNOWLEDGMENTS


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