Camptothecin sensitizes androgen-independent prostate cancer cells to anti-Fas-induced apoptosis

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Summary Despite expressing both Fas and Fas ligand, DU145 and LNCaP prostate cancer cells were resistant to anti-Fas-induced cell death. Resistance to Fas-mediated cytotoxicity could be overcome in DU145, but not in LNCaP, cells by pretreating cells with sublethal doses of cytotoxic drugs, such as camptothecin. Activated caspases were shown to be required for this cytotoxicity. Indeed, poly(ADP-Ribose) polymerase was shown to be proteolytically cleaved in cells treated with camptothecin plus anti-Fas, but not in cells treated with anti-Fas only. Moreover, pretreatment of cells with ZVAD completely blocked camptothecin-mediated Fas-induced apoptosis. Sensitization of cells to Fas-induced cell death did not involve up-regulation of Fas or FasL, and it was independent of alterations in the cell cycle. Reactive oxygen intermediates (ROI) have been shown to be important mediators of drug-induced apoptosis. Here, we demonstrate that treatment of DU145 cells with camptothecin, anti-Fas, or both, did not alter the intracellular levels of peroxide or superoxide anion.

Keywords: apoptosis; prostate cancer; Fas; caspases; reactive oxygen intermediates

The prostate in ageing males is prone to benign and malignant proliferative changes (Carter and Coffey, 1990). Indeed, cancer of the prostate is the most prevalent malignancy and the second-highest fatal neoplasm among Western males (Chung, 1995). The mechanism of prostatic carcinogenesis and tumour development is likely to involve a multistep progression from premalignant cells to cancerous cells which proliferate and metastasize (Huggins and Hodges, 1941). Initially, the growth and development of prostate cancer appears to be androgen-dependent. Nearly all men with metastatic prostate cancer, treated with surgical or chemical castration, have an initial beneficial response to androgen withdrawal, which induces tumour regression by apoptosis. Although this initial approach has significant palliative value, nearly all patients relapse with a hormonally independent disease (Denmeade et al, 1996). This hormonally independent disease also becomes resistant to additional therapies, such as chemotherapy (Yagoda and Petrylak, 1992), and displays growth autonomy (Figueroa et al, 1995). In the normal prostatic epithelium, cell proliferation is balanced by an equal rate of apoptotic cell death. In prostate cancer, however, this fine balance is lost, causing increased cellular mass and tumour progression (Denmeade et al, 1996). Hence, an effective therapy for prostate cancer should redress the imbalance in programmed cell death, which seems to be important for disease progression.

The Fas (APO-1/CD95) receptor belongs to the nerve growth factor/tumour necrosis factor (NGF/TNF) receptor family and mediates apoptosis in a variety of cell types (Yonehara et al, 1989; Oehm et al, 1992). Although Fas seems to be a key regulator of apoptosis, the pathways through which this molecule exerts its actions and the mechanisms which determine whether any given cell is sensitive or resistant to Fas remain to be fully elucidated. It seems that there is not a strict correlation between Fas and Fas ligand (FasL) expression and sensitivity to Fas-mediated cytotoxicity. In fact, activation of the Fas signalling pathway(s) in a variety of tumour cell lines and in activated lymphocytes does not always trigger apoptosis (Owen-Schaub et al, 1994). Caspases are thought to play a central role in apoptosis and de-regulation of some caspases may contribute to the development of some cancers. Furthermore, there is evidence that caspase-dependent cleavage of specific proteins may abrogate important survival pathways (Widmann et al, 1998). Importantly, commitment to Fas-induced apoptosis was shown to require caspase activity (Longthorne et al, 1997). Following Fas trimerization there seems to be sequential activation of caspase-1 (ICE)- and caspase-3 (CPP32)-like proteases (Enari et al, 1996). The activated caspases can then cleave various substrates, which include Poly(ADP-Ribose) polymerase (PARP), Lamin, rho-GDI, Actin, Ras GTPase-activating protein, Raf-1 and Akt-1, eventually resulting in morphological changes to the cells and nuclei (Nagata, 1997; Widmann et al, 1998).

Previous work from our laboratory has shown the importance of reactive oxygen intermediates (ROI) during apoptosis in HL-60 and U937 leukaemic cell lines (McGowan et al, 1994). In particular, peroxide production has been reported in response to a variety of inducers of apoptosis (Gorman et al, 1997b; McGowan et al, 1998). The production of peroxide after cytotoxic insult and the ability of antioxidants to inhibit PARP cleavage and apoptosis, suggest that early activation of caspases may act as a common response to cytotoxic insult and that ROI may serve as common mediators of cell death in leukaemic cell lines (McGowan et al, 1996). In contrast to a well-established role in chemotherapeutic drug-induced apoptosis, the involvement of ROI in Fas-mediated signal transduction pathway(s) is still controversial. Schulze-Osthoff and colleagues (1994) have shown that neither inhibitors of the mitochondrial respiratory chain nor antioxidants protected murine fibrosarcoma cells from Fas-mediated cell death. In contrast, ROI have been reported to be involved in Fas-mediated

signal transduction pathway and to be major mediators of apoptosis in neutrophils (Kasahara et al, 1997). In addition, Dobbelsteen and co-workers (1996) have shown that Fas ligation results in a rapid and specific efflux of glutathione (GSH). Severe depletion of GSH will lower the reducing capacity of a cell, enhancing oxidative stress independent of any increase in the production of ROI. Furthermore, an oxidative stress due to elevated intracellular superoxide levels has been shown to block Fas-mediated apoptosis in cells that were constitutively Fas-sensitive (Clément and Stamenkovic, 1996). These studies suggest that an imbalance in ROI levels may play a role in Fas-mediated apoptosis.

Due to the complexity of prostatic cancer, successful treatment of the disease is likely to require a multimodality therapy involving cytokines (immunotherapy), cytotoxic drugs (chemotherapy) and/or hormonal ablation. The present study was designed to examine possible ways of bypassing the resistance of prostate cancer cell lines to Fas-mediated apoptosis and to further elucidate the signalling pathways involved in Fas signal transduction.

MATERIALS AND METHODS

Reagents and cell lines

Prostate cancer cell lines LNCaP and DU145 were obtained from American Type Culture Collection (Rockville, MD, USA). Primary antibodies used in this study were anti-Fas IgM, clone Ch-11 (Upstate Biotechnology, Waltham, MA, USA); anti-Fas IgG, clone SM1/1 (Bender MedSystems, Heidelberg, Germany); anti-FasL, N-20 (Santa Cruz, CA, USA); anti-PARP, 06-557-MN (Upstate Biotechnology) and anti-β-actin (Sigma, Poole, UK). The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD) was purchased from Enzyme System Products (Dublin, CA, USA). The fluorescent probes 2′,7′-dichloro-fluorescin diacetate (DCFH-DA), dihydroethidium (DHE) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) were obtained from Molecular Probes (Leiden, The Netherlands). Cell culture reagents were purchased from Gibco/BRL (Paisley, UK), protein gel electrophoresis reagents from BioRad (Hertfordshire, UK), cytotoxic drugs, propidium iodide (PI) and RNAase were from Sigma, and all other reagents were obtained from BDH (Cork, Ireland).

Cell culture

Prostate cancer cells were cultured in RPMI-1640 medium containing 5% (v/v) fetal calf serum, 2 mM L-glutamine, supplemented with penicillin–streptomycin (10 IU ml⁻¹, 10 µg ml⁻¹) and fungizone amphotericin B (2.5 µg ml⁻¹). Cells were grown at 37°C in a humidified 5% carbon dioxide atmosphere and were routinely subcultured every 4 days. Cell density and viability were assessed by trypan blue exclusion, using a haemocytometer.

Expression of Fas and FasL by flow cytometry

For FasL expression assessment, cells were fixed with PBS/p-formaldehyde (1%, w/v, 30 min, room temperature) and permeabilized with cold ethanol (70%, v/v, overnight, −20°C). Cells were incubated with a rabbit anti-FasL (2 µg ml⁻¹, 1 h, 4°C), followed by the secondary antibody (fluorescence isothiocyanate-conjugated, 30 min, 4°C). Cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with Cell Quest
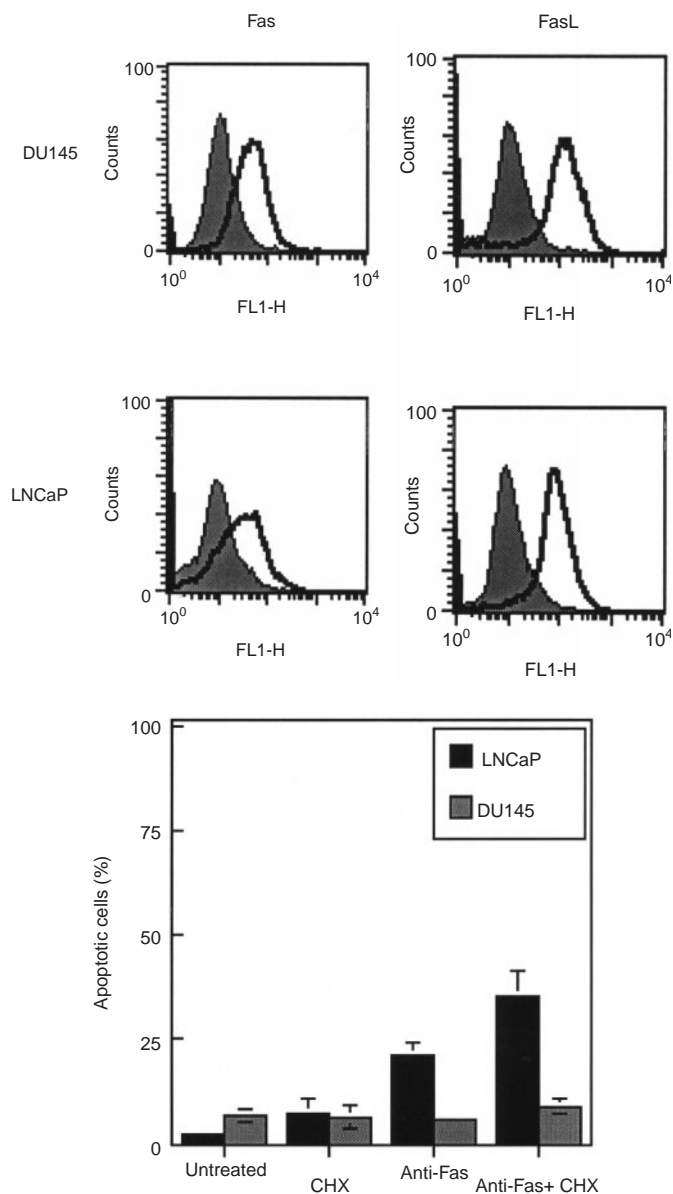
Figure 1 (**A**) Flow cytometric analysis of Fas and FasL in prostate cancer cell lines. LNCaP and DU145 cells were stained with a FITC-conjugated secondary antibody alone (intrinsic fluorescence control), (●); or with anti-Fas or anti-FasL, followed by the relevant secondary FITC-conjugated antibody, (○). Data shown is representative of at least three independent experiments. (**B**) Fas-Induced cell death in prostate cancer cell lines. Cells were incubated with cyclohexamide (CHX, 0.25 µg ml⁻¹), anti-Fas IgM (200 ng ml⁻¹), or both for 24 h, and apoptosis levels were assessed by morphological analysis as described in Materials and Methods. The data (from three independent microscopic fields) represents mean apoptosis±standard error of the mean and results shown are representative of at least three independent experiments

software (Becton Dickinson). Fixation and permeabilization steps were omitted when analysing cells for Fas expression (mouse anti-Fas IgG used at 2 µg ml⁻¹).

Fas-induced cell death

Cells were plated in 24-well plates and treatment with anti-Fas IgM (200 ng/ml) was carried out for 24 h. Whenever used, cytotoxic

drugs (100 ng ml^{-1} actinomycin D, 100 ng ml^{-1} camptothecin, 1.25 μg ml^{-1} etoposide, 1 μg ml^{-1} *cis*-platinum(II)diaminedichloride, 0.25 μg ml^{-1} cyclohexamide), were added 5 min prior to anti-Fas IgM. When required, ZVAD (50 μM) was added 5 min prior the addition of cytotoxics.

Morphological analysis

Briefly, supernatants were transferred to labelled test tubes and cells were washed once in HBSS. Cells in each well were then trypsinized using Trypsin/EDTA (TE) for 10 min at 37°C. TE was neutralized with complete media and cells were transferred to the appropriate test tube. Cells were then cytospun at 100 *g* (500 rpm) for 2 min. Slides were stained using the Rapi-Diff II kit (Langan Back Services, Ireland) and cell morphology was assessed by light microscopy, using previously defined criteria (Wyllie et al, 1980).

Cell cycle analysis

Freshly harvested cells (2.5 × 10^5 ml^{-1}) were fixed in ice-cold ethanol (70%, v/v), on ice for 15 min. Ethanol was removed by centrifugation (200 g × 5 min) and cells were resuspended in 1 ml of a PBS/PI (50 μg ml^{-1}) solution containing RNAase (0.5 mg ml^{-1}). Following a 30 min incubation on ice, in the dark, cells were analysed by flow cytometry in FL2-H, on a FACScan equipped with Cell Quest software.

Western blot

Whole cell lysates were prepared by solubilizing ~10^6 cells in 20 μl of lysing buffer (10 mM Hepes/NaOH, pH 8.0; 150 mM sodium chloride; 500 mM sucrose; 1 mM EDTA; 0.15 mM spermine; 1.0% v/v Triton X-100; 0.2 mM phenylmethylsulphonyl fluoride; 1.0 μg ml^{-1} antipain; 1.0 μg ml^{-1} aprotinin; 1.0 μg ml^{-1} chymostatin; 0.1 μg ml^{-1} leupeptin; 4.0 μg ml^{-1} pepstatin) and incubating cells on ice for 1 h. Prior to electrophoresis, cells were diluted in 2× sodium dodecyl sulphate (SDS) gel-loading buffer (100 mM Tris–HCl, pH 6.8; 200 mM dithiothreitol; 4.0% w/v SDS; 0.2% w/v bromophenol blue; 20% v/v glycerol) and boiled for 15 min. Proteins were separated in a 10% (w/v) polyacrylamide gel and blotted onto Trans-Blot nitrocellulose membrane. After blocking, the membrane was incubated with anti-human PARP (1/500 dilution, 4°C, overnight), followed by an anti-rabbit-HRP-conjugated antibody (1/1000, 1 h, room temperature). The membrane was probed with ECL (Amersham) and exposed to Kodak X-OMAT LS film (Sigma).

Detection of intracellular ROI and measurement of mitochondrial transmembrane potential (Δψm)

Mitochondrial membrane depolarization, peroxide and superoxide levels were measured as previously described (Gorman et al, 1997a; Costa-Pereira and Cotter, 1999). Briefly, cells (2.5 × 10^5) were harvested and incubated with 5 μg ml^{-1} JC-1 (Δψm measurement), for 15 min; or with 5 μM DCFH-DA (peroxide level) for 1 h at 37°C; or with 5 μM DHE (superoxide anion) for 30 min at 37°C. Fluorescence due to JC-1 and oxidized DCFH-DA was measured on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with Cell Quest software (Becton Dickinson), in FL-1,

whereas the fluorescence due to oxidation of DHE was measured in FL-2. Cells were treated with the apoptosis-inducing agents either before or during the incubation period, depending on the time point at which ROI or Δψm were to be studied.

RESULTS

## Prostate cancer cells are resistant to Fas-mediated cytotoxicity

Fas and FasL expression were assessed in LNCaP and in DU145 prostate cancer cells by flow cytometry (Figure 1A). Although both cell lines expressed the ligand and the receptor, treatment of cells with anti-Fas IgM (200 ng ml^{-1}; 24 h) induced only moderate levels of apoptosis in the androgen-responsive cell line, LNCaP, having no significant cytotoxic effects on the androgen-independent cell line, DU145 (Figure 1B). LNCaP (but not DU145) cells were more susceptible to Fas-mediated apoptosis when treated concomitantly with anti-Fas and cyclohexamide (CHX, 0.25 μg ml^{-1}), suggesting the presence of an endogenous protein inhibitor of the Fas signalling pathway in these cells. These results are in contrast to an article by Rokhlin et al (1997). However, their DU145 were only susceptible to treatment with 1 μg ml^{-1} anti-Fas at high concentrations of CHX (2.5–25 μg ml^{-1}), and the resistance displayed by their LNCaP cells is uncharacteristic of our cell lines. The sensitization of LNCaP cells mediated by CHX was not due to up-regulation of Fas, as assessed by flow cytometry (results not shown). Dose–response curves for both LNCaP and DU145 cell lines treated with anti-Fas IgM showed that, even at high anti-Fas IgM concentrations (1.5 μg ml^{-1}), DU145 cells were resistant to anti-Fas-induced apoptosis, indicating that there is a bona fide blockage in the Fas apoptotic pathway(s) in these cells. LNCaP cells were more susceptible to anti-Fas and there was, to a certain extent, a dependency on the dose of antibody used. The apoptosis levels, however, never surpassed those observed when LNCaP cells were treated with 0.25 μg ml^{-1} CHX (data not shown).
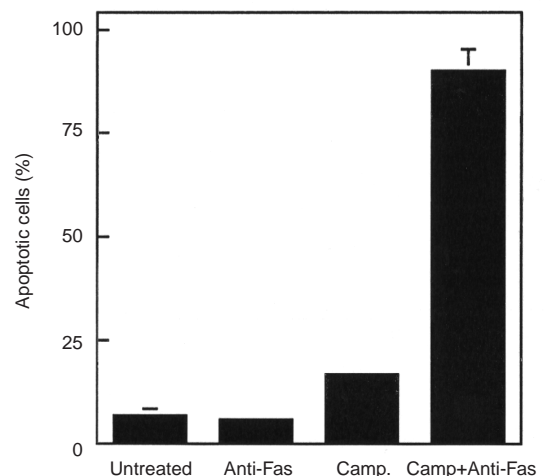
Figure 2 Chemosensitization of androgen-independent prostate cancer cells to Fas-induced cell death. DU145 cells were treated with subtoxic doses of camptothecin (100 ng ml^{-1}) and concomitantly incubated with anti-Fas IgM (200 ng ml^{-1}), for 24 h. Apoptosis was assessed as described in Figure 1B

Fas
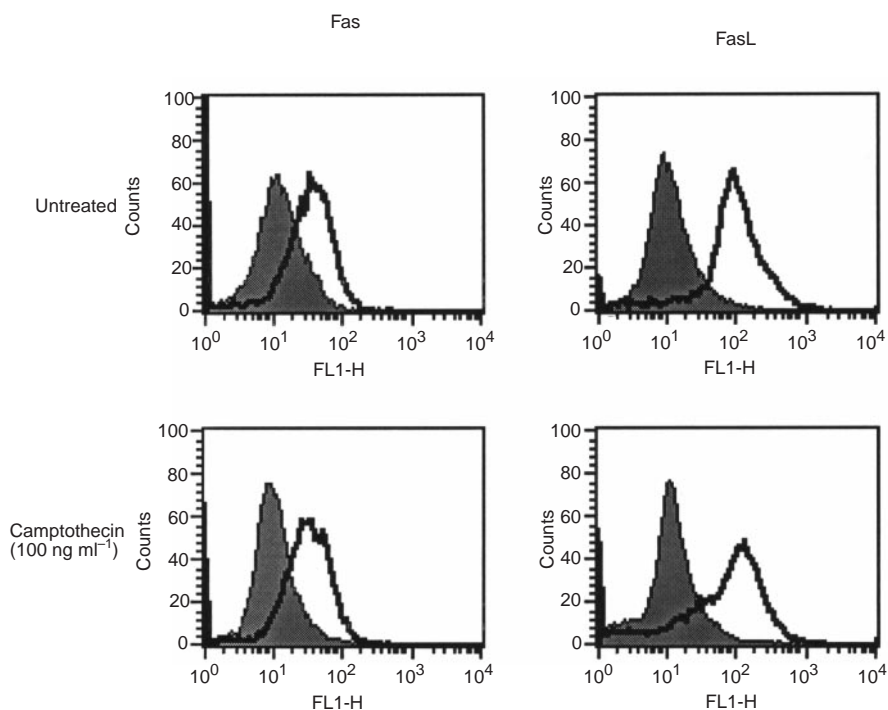
FasL

Figure 3 Fas and FasL expression in untreated DU145 cells and in cells treated with camptothecin (100 ng ml^{-1}). DU145 cells were stained with a FITC-conjugated secondary antibody alone (intrinsic fluorescence control), ($\bullet$); or with anti-Fas or anti-FasL, followed by the relevant secondary FITC-conjugated antibody, ($\circ$). Data are for the 24 h time point and are similar to those seen for the 4 and 8 h points
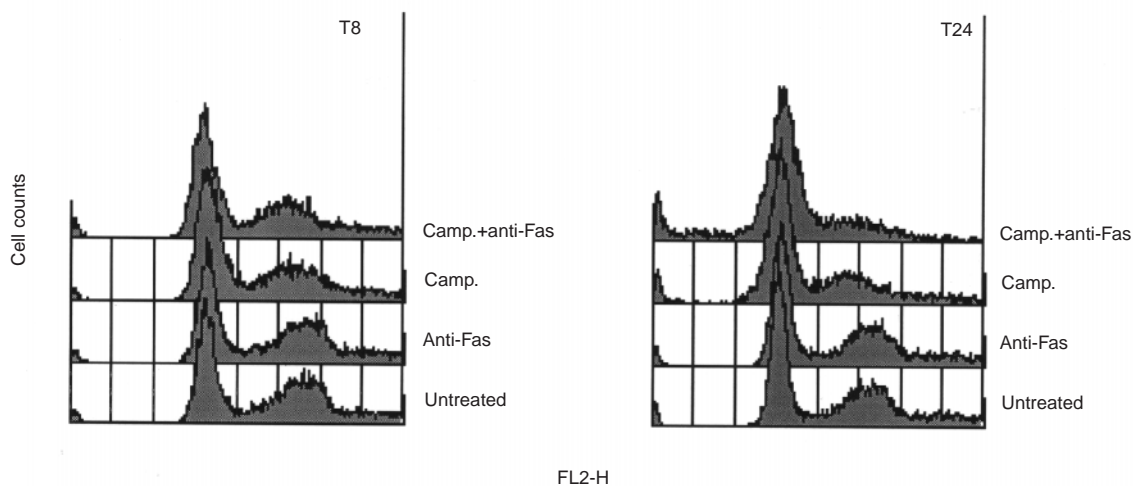

Figure 4 Cell cycle analysis of untreated DU145 cells and cells treated with anti-Fas (200 ng ml^{-1}), camptothecin (100 ng ml^{-1}), or both, at the indicated times. The first peak represents G0/G1 phase, the second peak cells in G2/M

Sensitization of androgen-independent prostate cancer cells to anti-Fas-induced cell death

To address the possible mechanism(s) involved in the resistance displayed by prostate cancer cells to Fas-induced apoptosis, we have used a number of cytotoxic drugs known to have distinct intracellular targets. The chemotherapeutic drugs used were: actinomycin D (RNA transcription inhibitor, which can also induce topoisomerase I and II cleavable complexes), camptothecin (topoisomerase I inhibitor), *cis*-platinum(II)diamminedichloride

(CPDD) (non-classical alkylating agent, which forms intrastrands linkages with DNA) and etoposide (topoisomerase II inhibitor). DU145 were sensitized by ~20-fold (average of five independent experiments yielding similar results) when treated with subtoxic concentrations of camptothecin (100 ng ml^{-1}) and subsequently treated with anti-Fas IgM (200 ng ml^{-1}) (Figure 2). The sensitization does not seem to be particular to topoisomerase I inhibitors, as actinomycin D (100 ng ml^{-1}), CPDD (1.0 µg ml^{-1}) and etoposide (1.25 µg ml^{-1}) also sensitized DU145, although to a lesser extent

A

B
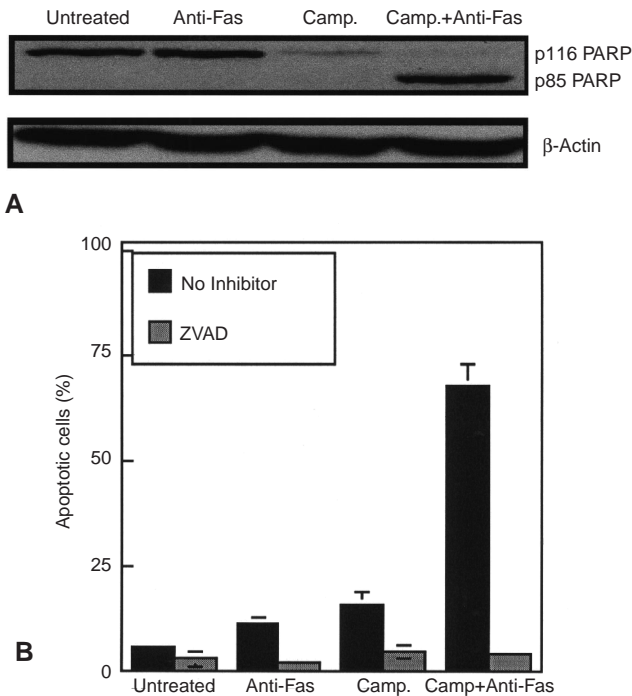
Figure 5 (**A**) Caspases are involved in camptothecin-mediated anti-Fas-induced apoptosis of DU145 cells. Western blot analysis of PARP cleavage in untreated (lane 1) DU145 cells, or treated with anti-Fas IgM, 200 ng ml⁻¹ (lane 2); Camp, 100 ng ml⁻¹ (lane 3); Camp. and anti-Fas (lane 4). The membrane was also probed with anti-β-actin to ensure equal protein loading (30 µg protein/lane). (**B**) Inhibition of Fas-induced apoptosis in DU145 cells pretreated with camptothecin by a caspase inhibitor, ZVAD. Cells (5 × 10⁴ ml⁻¹) were seeded in 24-well plates and cultured for 48 h. Cells were incubated for 5 min with 50 µM of ZVAD, followed by treatment with camptothecin (100 ng ml⁻¹), anti-Fas IgM (200 ng ml⁻¹), or both, for 24 h. Apoptosis levels were assessed as described in Figure 1B

(data not shown). Identical concentrations of actinomycin D, camptothecin, or etoposide failed to sensitize the androgen-responsive cells, LNCaP (not shown).

Camptothecin-mediated sensitization of DU145 cells to anti-Fas-induced cell death does not involve up-regulation of Fas or FasL proteins

Recent reports (Friesen et al, 1996; Herr et al, 1997) suggested that apoptosis caused by certain anticancer drugs was mediated by the Fas/FasL system. In order to determine whether camptothecin was sensitizing DU145 cells to Fas-mediated apoptosis by up-regulating Fas and/or FasL, cells were treated with camptothecin and flow cytometric analysis of Fas and FasL expression was assessed at different time points (4 and 24 h post-treatment). Results show that there was no change, at the protein level, of Fas or FasL (Figure 3), suggesting that sensitization was not due to upregulation of Fas and/or FasL.

Camptothecin's effect is independent of alterations in the cell cycle

Camptothecin has been reported to cause a cell cycle arrest in S/G2 (Hsiang et al, 1989). Thus it was considered that the sensitization to Fas-mediated apoptosis could be a consequence of cell cycle arrest. Flow cytometric analysis of untreated cells, or cells

treated with camptothecin (100 ng ml⁻¹), anti-Fas IgM (200 ng ml⁻¹), or camptothecin plus anti-Fas, for 8 h, showed no significant cell cycle abnormalities in the cell cycle profile of each sample. After a 24 h treatment with camptothecin plus anti-Fas IgM, only sub-G0/G1 population was slightly increased indicative of apoptosis (Figure 4).

Activation of caspases is required for camptothecin-mediated sensitization of DU145 cells to anti-Fas-induced apoptosis

There have been several reports showing that caspases play a pivotal role in Fas-induced apoptosis (Enari et al, 1996; Longthorne and Williams, 1997). Western blot analysis demonstrated that poly(ADP-ribose) polymerase was proteolytically cleaved in camptothecin-treated cells, but not in cells treated with anti-Fas only (Figure 5A). A down-regulation of PARP, in the absence of cleavage product, was noted in cells treated with camptothecin only. This may be due to an inhibition of transcription. Apoptosis in cells treated with camptothecin plus anti-Fas could be completely blocked by pretreatment with ZVAD (50 µM) (Figure 5B). Thus, camptothecin sensitization involves the activation of caspases analogous to apoptosis in Fas-sensitive cells.

Mitochondrial depolarization is a late and non-specific event in DU145 cells undergoing camptothecin-mediated anti-Fas-induced apoptosis

Emerging evidence suggests a key role for mitochondria during apoptosis induced by a diverse range of stimuli. Discontinuity of the outer mitochondrial membrane results, in some instances, in redistribution of cytochrome *c* from the intermembrane space to the cytosol, followed by subsequent inner mitochondrial membrane depolarization (Kroemer, 1997). Disruption of the transmembrane potential (Δψm) and generation of reactive oxygen intermediates (ROI) have been reported as early events in apoptosis (Lennon et al, 1991; Kroemer, 1997). Δψm depolarization was only significant (but not complete) at 24 h, when > 80% of cells were dead (Figure 6). Δψm disruption was evidently a late event in our system. Taken together, results suggest that, although degradation of Δψm might contribute to the apoptosis of DU145 cells, it is unlikely that camptothecin causes sensitization to anti-Fas through this mechanism.

Camptothecin opens the Fas apoptotic pathway(s) in DU145 cells through an ROI-independent mechanism

Apoptotic cells are believed to be subject to some form of oxidative stress, as they accumulate oxidized proteins and lipids (Buttke and Sandstrom, 1994) and, in a number of cell lines, apoptosis induced by chemotherapeutic drugs is accompanied by rapid production of ROI (Lennon et al, 1991; McGowan et al, 1996; Gorman et al, 1997b). Furthermore, Fas-mediated cell death has been shown to be blocked by superoxide anion (Clément and Stamenkovic, 1996), suggesting that an imbalance in ROI levels may have a role to play in Fas-mediated cell death. The existence of a short-lived inhibitor protein of Fas signalling transduction pathway(s) has been widely suggested. However, since the protein synthesis inhibitor cyclo-hexamide did not sensitize DU145 cells to anti-Fas-mediated apoptosis, we sought to investigate alternative mechanism(s) which did not directly involve proteins. We hypothesized that camptothecin could be opening the Fas pathway in DU145 cells by either
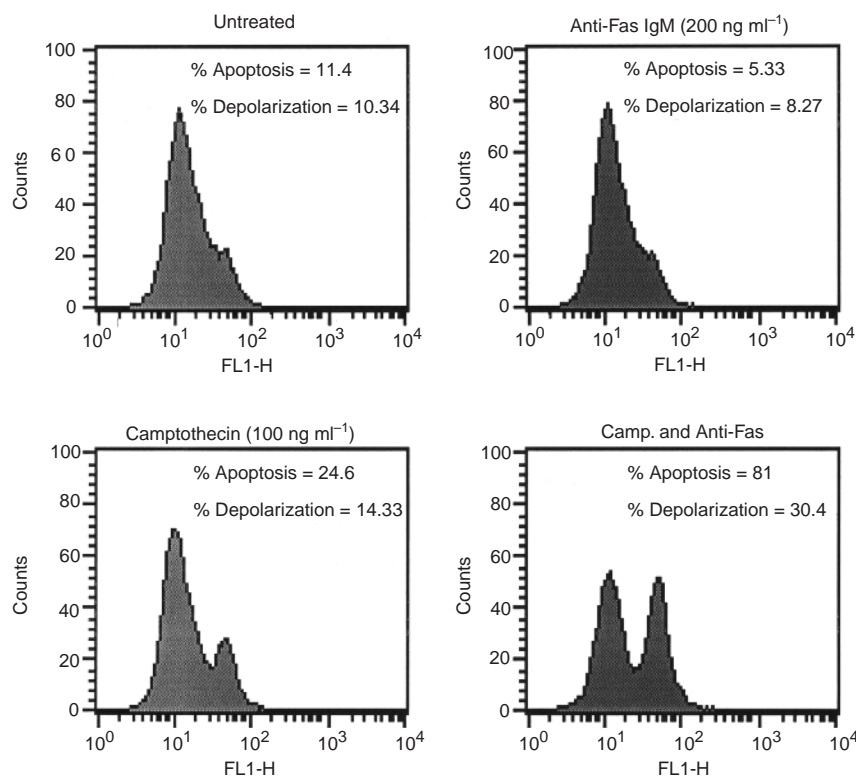
Figure 6 Mitochondria transmembrane potential disruption in DU145 cells. In cells treated with camptothecin (100 ng ml^{-1}), anti-Fas IgM (200 ng ml^{-1}), or both, $\Delta\psi m$ depolarization is a late event and does not correlate with apoptosis levels. FL-2H fluorescence of cells treated with camptothecin, anti-Fas, or both remained unchanged and was identical to the FL-2H of untreated cells. $\Delta\psi m$ was measured by flow cytometry as described in Materials and Methods. Data are for the 24 h time point and are representative of three independent experiments

increasing peroxide levels (Figure 7A) or by directly or indirectly causing a fall in superoxide levels (Figure 7B). None of the treatment (anti-Fas, camptothecin, camptothecin plus anti-Fas), under our experimental conditions, caused a significant increase in peroxide or a decrease in superoxide anion levels, as measured by flow cytometry. Camptothecin, at higher concentrations (1–10 µg ml^{-1}), did cause a rapid and marked increase in ROI levels (results not shown), but not at the concentrations used here (100 ng ml^{-1}, 24 h treatment). While it is possible that an alternative mechanism of oxidative stress contributes to camptothecin and anti-Fas cytotoxicities, the data presented in this study suggest that ROI are not significant players in camptothecin-mediated sensitization of DU145 cells to anti-Fas-induced apoptosis.

DISCUSSION

Although prostate cancer cells expressed both Fas and FasL, they were significantly resistant to Fas-induced cell death. This observation, and the fact that the Fas/FasL system is emerging as one of the key apoptotic pathways in eukaryotic cells, prompted us to investigate why Fas-mediated apoptotic pathway(s) were blocked in these cells. Understanding the mechanisms underlying this resistance may allow the development of strategies for effectively killing prostate cancer cells, using a combination of cytotoxic and immune approaches. In this study, two prostate cancer cell lines (LNCaP, DU145) were used. It was shown that LNCaP and DU145 cells expressed both Fas and FasL. Treatment of LNCaP and DU145 with anti-Fas IgM and CHX up to 24 h induced

moderate levels of apoptosis in LNCaP, having no significant cytotoxic effects on DU145 cells. LNCaP were more susceptible to anti-Fas when pretreated with CHX, suggesting that there might be a short-lived inhibitor in these cells. The existence of a hypothetical suppressor of Fas-induced apoptosis ('Saf' – suppressor of apoptosis by Fas) has been postulated (Green, 1997). Such suppressor is likely to exist, as in many cells resistance to Fas can be overcome by treatment with cyclohexamide or actinomycin D, which are thought to eliminate such short-lived inhibitor. FLIP (for Flice inhibitory protein), a molecule recently identified by Irmler and co-workers (1997), seems to be an appropriate candidate for such a role. A number of cytotoxic drugs were shown to sensitize androgen-independent cells (DU145) to anti-Fas-induced apoptosis. Indeed, DU145 cells were sensitized by ~20-fold when treated with subtoxic doses of camptothecin and concomitantly incubated with anti-Fas. This sensitization was not particular to topoisomerase I inhibitors, as actinomycin D, CPDD and etoposide also sensitized cells to cell death. Interestingly, we were unable to chemosensitize the androgen-responsive cells (LNCaP) using the above mentioned cytotoxic drugs.

It has been postulated that chemotherapeutic drugs induced apoptosis by activating the Fas/FasL apoptotic pathway (Friesen et al, 1996; Müller et al, 1997). There is an accumulating body of evidence which suggests that this is not always the case (Eischen et al, 1997; Villunger et al, 1997; McGahon et al, 1998). It has been suggested that lack of p53 expression might account for the lack of recruitment of Fas and FasL (Müller et al, 1997). Treatment of DU145 cells with camptothecin did not alter the levels of Fas, or
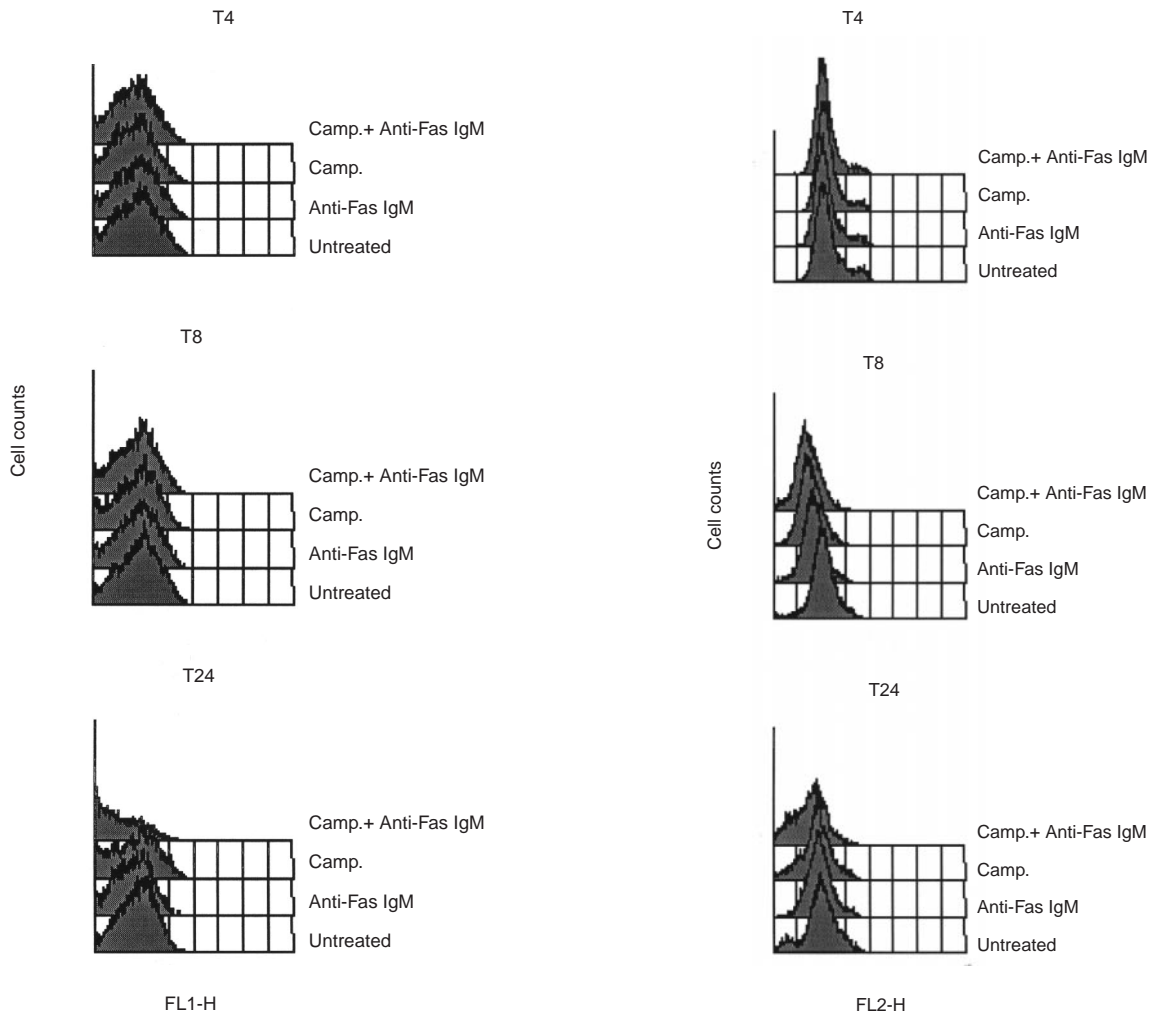
Figure 7 (**A**) Camptothecin-mediated anti-Fas-induced apoptosis is independent of production of peroxide. Cells were treated with camptothecin (100 ng ml^{-1}), anti-Fas IgM (200 ng ml^{-1}), or both, and peroxide levels were assessed, as described in Materials and Methods. Data shown are for the 4, 8 and 24 h time point and are similar to those seen for earlier time points (0.5, 1 and 2 h). Data shown is representative of three independent experiments. (**B**) Levels of superoxide anion remained unaltered in DU145 cells undergoing camptothecin-mediated anti-Fas-induced apoptosis. Cells were treated with camptothecin (100 ng ml^{-1}), anti-Fas IgM (200 ng ml^{-1}), or both, and superoxide anion was as described in Materials and Methods. Data shown are for the 4, 8 and 24 h time points and are similar to those seen for 0.5, 1 and 2 h time points. Data shown is representative of three independent experiments

FasL, as shown by flow cytometry. DU145 cells have been shown to possess a mutated p53 (Isaacs et al, 1991), which further suggests that DU145 cells might be unable to up-regulate Fas or FasL proteins. In addition, cell cycle analysis of cells treated with anti-Fas, camptothecin, or camptothecin plus anti-Fas, has shown that camptothecin was mediating Fas-induced cell death through a mechanism which was independent of cell cycle alterations. Camptothecin has been shown to induce single-strand DNA breaks in the presence of topoisomerase I, which is a major target for the anti-tumour effect (Hsiang et al, 1989). How this is translated into a cytotoxic effect is, however, currently unknown.

Camptothecin sensitization was shown to involve activation of caspases analogous to apoptosis in Fas-sensitive cells. Indeed, poly(ADP-ribose) polymerase was proteolytically cleaved in cells treated with camptothecin plus anti-Fas, but not in cells treated with anti-Fas only. Furthermore, camptothecin-mediated sensitization of DU145 cells to Fas-induced apoptosis was completely blocked by the caspase inhibitor ZVAD.

A variety of stimuli have been shown to cause an accumulation of cytochrome *c* in the cytosol, cause disruption of mitochondria transmembrane potential and an increase in ROI, leading to activation of caspases, PARP cleavage, DNA degradation and subsequent apoptosis. We hypothesized that camptothecin could be sensitizing DU145 cells to anti-Fas-induced apoptosis through an ROI-mediated mechanism. Results, however, seem to negate this hypothesis: (1) an increase in peroxide, which is thought to induce apoptosis by increasing cellular oxidative stress beyond the tolerable threshold, could not be detected; (2) no decrease in superoxide anion was observed.

In summary, our results show that a combinatory treatment of androgen-independent DU145 cells with sublethal concentrations of camptothecin and very low concentrations of anti-Fas IgM, will kill nearly all cells (75–95%) in 24 h. The mechanism of this cooperation appears to be independent of alterations in the levels ROI (peroxide and superoxide anion). Because we were able to chemosensitize the androgen-independent cells (DU145), but not

the androgen-responsive cells (LNCaP), it is tempting to speculate that the resistance of a prostate cell line to anti-Fas IgM treatment may be inversely proportional to its positive response to chemosensitization. This would have important implications for therapy since, although nearly all men with metastatic prostate cancer respond well initially to some sort of hormonal ablation therapy, prostate cancer cells invariably become androgen-independent. This is invariably fatal because no effective systemic therapy currently exists. Taken together, these results seem to indicate that a combination of chemotherapy and immunotherapy may present a novel approach for the elimination of androgen-independent cells.

ACKNOWLEDGEMENTS

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