Chemosensitization of Human Prostate Carcinoma Cell Lines to Anti-Fas-mediated Cytotoxicity and Apoptosis

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ABSTRACT

Androgen ablation has been an effective treatment in patients with advanced prostate cancer. However, most treated patients develop hormonally resistant disease and do not respond to conventional chemotherapy. Immunotherapy against prostate cancer is an alternative approach in overcoming hormonal/drug-resistant prostate cancer. Cytotoxic immune lymphocytes kill target cells via the perforin/granzyme and the Fas-ligand (Fas-L) pathways. We hypothesize that tumor cells respond poorly to immunotherapy by developing resistance to killing by the Fas-L mechanism. This study investigated whether prostate tumor cells are sensitive to Fas-mediated killing. The human prostate carcinoma cell lines DU145, PC-3, and LnCAP were examined for their sensitivity to killing and apoptosis by the Fas-L agonist anti-Fas antibody and CTLs. All three lines moderately expressed the Fas antigen on the cell surface; however, all three lines were relatively resistant to cytotoxicity mediated by anti-Fas (CH-11) antibody. Pretreatment of DU145 and PC-3 with subtoxic concentrations of drugs followed by anti-Fas antibody resulted in synergistic cytotoxicity and apoptosis, whereas only an additive effect was obtained with LnCAP. Chemosensitization with drugs and anti-Fas was completely blocked by the addition of neutralizing anti-Fas antibody. The murine CTL hybridoma, PMMI, which kills only via the Fas-L pathway, was able to kill chemosensitized PC-3 and DU145 but not LnCAP cells. Furthermore, this cytotoxicity was blocked by anti-Fas neutralizing antibody. Chemosensitization of PC-3 and DU145 prostate tumor cells was not due to up-regulation of Fas-neutralizing antibody. Treatment of tumor cells with cisplatin did not down-regulate the antiapoptotic genes bel-2, FAP-1, and c-myc. Further, there was no induction by cisplatin of Fas-L on the tumor cells, thus ruling out Fas/Fas-L-mediated autologous killing. These findings demonstrate that pretreatment of drug-resistant/CTL-resistant prostate DU145 and PC-3 tumor cells with subtoxic concentrations of certain chemotherapeutic drugs sensitizes the tumor cells to Fas-mediated cytotoxicity. These findings suggest that chemosensitization of tumor cells should optimize the response to immunotherapeutic interventions in the treatment of hormone-resistant/drug-resistant prostate cancer.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in men of all ages with an estimated 41,000 Americans dying from prostate cancer annually (1). Hormonal ablation is the main treatment for disseminated prostate cancer (2). However, after a median of 12–18 months, prostate cancer commonly recurs, and the patients relapse with hormonally independent tumors. Furthermore, hormonally independent tumors also become resistant to a wide range of cytotoxic drugs. Currently, there is no therapy that has been shown to prolong survival of these patients (3).

CDDP, ADR, and VP-16 are the most commonly used chemotherapeutic agents in the treatment of metastatic prostate cancer. However, the overall response rate induced by these drugs is very poor, and the duration of response is very short with limited impact on survival (4, 5). A new drug, suramin, has been introduced with a promising therapeutic activity against prostate cancer (6). Unfortunately, suramin can provoke severe adverse toxic effects, such as polyradiculopathy, coagulopathy, and adrenal insufficiency (7). Therefore, new therapeutic approaches need to be developed with the objective of overcoming tumor cell resistance and reducing drug-mediated toxicity.

One recent therapeutic approach for treating drug-resistant tumor cells is to use one agent to sensitize cells to a second cytotoxic agent (8). For instance, we have recently reported in vitro studies that treatment with combined modalities such as TNF-α/toxins and cytotoxic drugs results in potentiation, and often synergy, in cytotoxic activity in a variety of sensitive and...
drug-resistant human tumor cell lines (9–12). The combination treatment resulted in overcoming tumor cell resistance to both agents used in the combination treatments. Furthermore, the concentrations of both agents used in the combination were severalfold (>5-fold) less than the optimal concentrations of a single agent.

The Fas antigen (APO-1, CD95) is a cell surface protein that is expressed in a variety of normal tissues and tumor cell lines (13). Fas is a member of the TNF/nerve growth factor receptor family, and anti-Fas antibody can induce apoptosis in Fas-expressing cells both in vitro (14, 15) and in vivo (16), suggesting that Fas may play a critical role in the regulation of cell death (17). Mutations that inactivate Fas have been shown to be associated with the lymphoproliferative disorder in lpr/lpr mice (18). A recent study identified a natural Fas ligand in a CTL cell line, and the Fas ligand was shown to be a member of the TNF family (19). Also, Fas and Fas ligand have recently been implicated in the mechanism of CTL and NK-mediated cell death (20, 21).

The objective of this study was to determine whether: (a) drug-resistant prostate cancer cells are sensitive to Fas-mediated killing; and (b) the prostate carcinoma cells can be sensitized to Fas-mediated cytotoxicity. This study examined the cytotoxic effect of anti-Fas antibody and CTL when used alone or in combination with drugs (CDDP, ADR, VP-16, and suramin) against the human prostatic carcinoma cell lines DU145, PC-3, and LnCAP.

MATERIALS AND METHODS

Tumor Cells

The human hormone-independent prostatic carcinoma lines, DU145 and PC-3, and hormone-dependent prostatic carcinoma cell line, LnCAP, were obtained from Dr. Belldegrun (UCLA, Los Angeles). These tumor cell lines were maintained in culture as adherent cells and cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) plus 10% heat-inactivated FCS (Atlanta Biologicals, Norcross, GA) added to 1% pyruvate (Life Technologies, Inc.), 1% nonessential amino acids (Life Technologies, Inc.), and 1% Fungi-bact solution (Irvine Scientific, Santa Ana, CA) containing 10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 25 μg/ml Fungizone. All cell lines were grown in a humidified atmosphere at 37°C in 5% CO2. When the tumor cell lines were used as target cells, they were treated with trypsin-EDTA (ICN Biomedical, Inc., Irvine, CA), washed, and resuspended in complete medium.

Reagents

Cisplatin, Adriamycin, etoposide, RNase, PI, and MTI were purchased from Sigma Chemical Co. (St. Louis, MO). Suramin was kindly supplied by Dr. Belldegrun. The cytotoxic anti-Fas mAb (IgM; clone CH-11), the neutralizing mAb (IgG1; clone ZB4), and the anti-Fas mAb (IgG1; clone UB2) for surface staining were purchased from Kamiya Biomedica Co. (Thousand Oaks, CA). Normal mouse IgM and mouse IgG1 were purchased from Amac (Westbrook, ME). Phycoprobe phycoerythrin-conjugated goat antimouse IgG was used as secondary antibody and was purchased from Biomedica Co. (Foster City, CA). Stock solutions of the reagents were routinely prepared in PBS, medium, or DMSO as appropriate.

Cytotoxicity Assay

The MTT assay was used to determine antibody-mediated cytotoxicity as described previously (10). Briefly, target tumor cells were resuspended in medium at 1 x 10^5 cells/ml after verifying cell viability by the trypan blue dye (Sigma) exclusion assay. One hundred μl of cell suspension were distributed into each well of a 96-well flat-bottomed microtiter plate (Costar, Cambridge, MA), and each plate was incubated for 24 h at 37°C and 5% CO2 atmosphere. Following the incubations, 100 μl of reagent solutions or media at the desired concentrations were distributed into each well. Treatments were performed in triplicate. Two hundred μl of the medium alone without cells and reagent were used as negative control. The microtiter plate was incubated for the desired periods of time. Thereafter, 20 μl of the MTT dye (5 mg/ml) were added into each well. The unreactive supernatants in the wells were carefully aspirated and replaced with 100 μl of isopropanol (Sigma) supplemented with 0.05 N HCl to solubilize the reactive dye. The absorbance (A) values of each well at 540 nm were read using an automatic multwell spectrophotometer (Titertek Multiscan MCC/340). The negative control well was used for zeroing the absorbance. The percentage of cytotoxicity was calculated using the background-corrected absorbance as follows:

\[
\% \text{ cytotoxicity} = \frac{(1 - A \text{ of experimental well})}{A \text{ of positive control well}} \times 100
\]

Experiments were performed at least three times with representative data presented.

CTL-mediated Cytotoxicity

PMMI is a murine CTL hybridoma derived from BALB/c PEL specific for the H-2d thymoma EL-4 (22). PMMI cells were activated in the presence of 10 ng/ml phorbol myristate acetate and 2 μg/ml ionomycin and incubated for 3 h at 37°C. At the end of the incubation period, the cells were washed once in PBS and resuspended at a final concentration of 1 x 10^6 cells/ml and used immediately in the cytotoxicity assay.

DU145 and PC-3 adherent cells were trypsinized for 5 min, collected, and washed once in PBS. The cells were incubated in 100 μCi of Na_2^51CrO_4 for 1 h and then washed three times in medium; 10^6 cells were added to flat-bottomed 96-well cell culture plates in the presence or absence of 10 μg/ml CDDP. The plates were allowed to incubate for 18 h at 37°C and 5% CO_2. Two h prior to addition of activated PMMI effector cells, the supernatant was removed, and 100 μl of fresh medium plus or minus 1 μg/ml of neutralizing antibody (clone ZB4) were added to the cells. Then, 100 μl of PMMI effector cells were added to each well at the indicated E:T ratio. The plates were centrifuged and incubated for 7 h at 37°C and 5% CO2. Following the incubation, 100 μl of supernatant were harvested from each well and counted in a Beckman Gamma 4000 gamma counter. Total 51Cr release was determined by lysing target cells with 50 μl of 10% SDS buffer and collecting 150 μl for count.
The percentage of specific $^{51}$Cr release was determined as follows:

$$\text{% }^{51}\text{Cr release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

All values are presented as the mean ± SD of triplicate samples.

**DNA Staining**

**PI Method.** Samples containing $2 \times 10^5$ cells/well were washed twice with $1 \times$ PBS and incubated for 30 min in 70% ethanol on ice. The cells were washed twice with $1 \times$ PBS, and 70 μl of RNase (1 mg/ml) and 140 μl of PI (10 μg/ml) were added in each sample. After 1 h of incubation in the dark, DNA analyses were determined using an Epic C flow cytometer, and DNA fragmentation (apoptosis) was determined by DNA hypoploidy as described previously (23).

**TUNEL Assay.** For staining by the TUNEL method, the tumor cells were added in microtiter wells, fixed with 1% formaldehyde for 15 min on ice, and washed twice to remove the formaldehyde. The cells were suspended in 70% cold ethanol for 1 h at 20°C and washed twice. The cells were then treated with the terminal deoxyribonucleotidyl transferase reaction mixture (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C according to the manufacturer’s instructions. Controls consisted of treating the samples as above except that terminal deoxynucleotidyl transferase was absent. The various treated samples were then washed twice with PBS and analyzed by flow cytometry (24) and as reported by us recently (25).

**Flow Cytometric Analysis**

The expression of Fas antigen on tumor cells was determined by flow cytometry (26). Staining was performed in 96-well U-bottomed plates (Costar, Cambridge, PA). Dilutions and washings were performed in $1 \times$ PBS containing 2% heat-inactivated FCS and 0.1% sodium azide. Prior to staining, the cells ($2 \times 10^5$) were pretreated with human AB serum (Gemini Bioproducts, Calabasas, CA) for 1 h, washed twice, and resuspended in 50 μl of PBS. The cells were incubated 1 h with 10 μg/ml of anti-Fas mAb (IgG1) or 10 μg/ml of normal mouse IgG1, then washed twice and resuspended in 50 μl of $1 \times$ PBS containing goat antimouse phycoerythrin-conjugating IgG antibody for an additional hour. The cells were then washed twice and fixed in 2% paraformaldehyde solution (Sigma), and flow cytometry was performed using an Epic C flow cytometer.

**Detection of Gene Expression by Reverse Transcription-PCR**

Total RNA was extracted and purified from $1 \times 10^6$ cells for each different condition by a single-step guanidinium thiocyanate-chloroform method with STAT 60™ reagent (Tel-Test “B.” Inc., Friendswood, TX). One μg of total RNA (approximately 10–20 ng of mRNA) was reverse transcribed to first-stranded cDNA for 1 h at 42°C with SuperScript™ II reverse transcriptase (200 units) and random hexamer primers (20 μM; Life Technologies, Bethesda, MD). Amplification of one-tenth of these cDNA by PCR was performed using the following gene-specific primers: c-myc sense, 5’-GTGGCACCTTGTAGAGCA-3’; c-myc antisense, 5’-TG-GTGC TTCAATAGAGA-3’ (195-bp expected product); bcl-2 sense, 5’-CGACTCTCAGCGAAGAT GTCCAGCAG-3’, bcl-2 antisense, 5’-ACTTTGGCTCAGATAGGACCCG-3’ (389-bp expected product); FAP-1 sense, 5’-GAAATACGGTTCGACATGG-3’, FAP-1 antisense, 5’-AGGTCTGCAGAGAAGCAA-3’; Fas ligand sense, 5’-CCTCAGTCCATCCTCTG-3’, Fas ligand antisense, 5’-TAGCTGAACCTGTCGACCTTG-3’, Fas receptor sense, 5’-ATGCCTGGGACCATGTCACC-3’; and Fas receptor antisense, 5’-GCCATGCTCCCTCATTACACAA-3’ (338-bp expected product).

**Statistical Analysis and Synergy**

All assays were set up in triplicate, and the results were expressed as the mean ± SD. Statistical analysis was determined by the Student’s t test. For synergy, isobologram analysis was performed according to Berenbaum (27). The isobole method has been used to evaluate the presence of synergism or antagonism in many fields. It requires experimental data for agents used alone and in different dose combinations at equieffective levels. These data are plotted on isoeffective graphs with the axis representing the doses of each agent. If two agents do not interact, the line forming the point corresponding to the combination with those on the axis representing doses isoeffective with the combination will be a straight line. When agents in combination are more effective than what might be expected from their dose-response curves (synergy), smaller amounts will be needed to produce the effect under consideration, and a concave-up isobole results. On the other hand, when agents in combination are less effective than expected (antagonism), greater doses than expected will be needed to produce the same effect, and a concave-down isobole is generated. Fraction of inhibitory concentrations were calculated as the percentage of each treatment to effect a fixed level of cytotoxicity.

**RESULTS**

**Sensitivity of Human Prostate Carcinoma Cell Lines to Treatment with Either Anti-Fas Antibody or Combination CDDP and Anti-Fas Antibody**

The expression of the surface receptor Fas antigen on tumor cells has been considered by some investigators as a target for cytotoxic therapy by anti-Fas antibody or by Fas ligand-bearing cytotoxic lymphocytes. Accordingly, we examined whether prostate carcinoma cell lines express the Fas receptor on the cell surface. Three prostate carcinoma cell lines, DU145, PC-3, and LnCAP, were examined. All three lines express Fas antigen, although the intensity of Fas antigen expression was moderate when
Immunosensitization of Prostate Carcinoma to Fas Signaling

Control

CDDP 1\mu g/ml

CDDP 10\mu g/ml

AD10

DU145

PC-3

LnCAP

Fig. 1  Surface Fas expression on AD10, DU145, PC-3, and LnCAP cells following treatment with different concentrations of CDDP. The cells were treated with various concentrations of CDDP and incubated at 37°C for 18 h, washed, and stained with normal IgG, or anti-Fas IgG antibody. Upper right corner of panels. percentage of stained cells and mean fluorescence.

Fig. 2  Cytotoxicity and isobolograph analysis of treatment with combination of CDDP and anti-Fas (CH-11) antibody. Cytotoxicity was determined by the MTT assay in a 48-h culture. The results are expressed as the means of three different experiments; bars, SD. Normal mouse IgM was used as an isotype control for anti-Fas IgG antibody. Upper right corner of panels. percentage of stained cells and mean fluorescence.

Mechanism of Tumor Cell Sensitivity to Cytotoxicity and Synergy by CDDP and Anti-Fas Antibody

Effect of Sequential Treatment. The previous findings demonstrated that treatment of tumor cells with CDDP and anti-Fas (CH-11) antibody resulted in synergy. We examined the effect of sequential treatment of DU145 and PC-3 tumor cells with either CDDP or anti-Fas antibody (CH-11) and sub-
expect the abolition of synergy in the presence of neutralizing antibody. The previous findings indicated clearly that CDDP Antibody. Indeed, when the tumor cells were sensitized by CDDP for 24 h, washed, and then treated with neutralizing anti-Fas antibody (ZB4) for 2 h and then treated with cytotoxic anti-Fas antibody (CH-11), synergy was not observed. The extent of blocking was a function of the concentration of the anti-Fas neutralizing antibody (ZB4) used. At high concentrations of neutralizing anti-Fas antibody (ZB4), complete inhibition of synergy was achieved (Table 2). These findings demonstrate that the augmented cytotoxicity seen in synergy by CDDP and anti-Fas (CH-11) was due exclusively to Fas-mediated cytotoxicity.

### Effect of CDDP on Surface Fas Antigen Expression

We examined if CDDP-mediated sensitization of tumor cells to anti-Fas cytotoxicity is due to up-regulation of surface Fas antigen expression. CDDP had a minimal effect on Fas expression in the prostate carcinoma cell lines (Fig. 1). These findings indicate that CDDP-mediated sensitization of DU145 and PC-3 to anti-Fas antibody (CH-11) cytotoxicity is not due to up-regulation of surface Fas expression.

### Effect on Apoptosis

CDDP and anti-Fas can mediate programmed cell death or apoptosis. We examined whether anti-Fas antibody and CDDP can also induce apoptosis in a synergistic manner. Apoptosis was assessed by two methods: P1 fragmentation. In the P1 method, CDDP was slightly cytotoxic and anti-Fas antibody and CDDP can also induce apoptosis in a

### Table 1

<table>
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<tr>
<th>Target cells</th>
<th>1st treatment a (24 h)</th>
<th>2nd treatment b (24 h)</th>
<th>% cytotoxicity b (mean ± SD)</th>
<th>1st treatment a (24 h)</th>
<th>2nd treatment b (24 h)</th>
<th>% cytotoxicity b (mean ± SD)</th>
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<td>DU145</td>
<td>Medium only</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>4.6 ± 2.2</td>
<td>Medium only</td>
<td>CDDP (10 μg/ml)</td>
<td>13.7 ± 3.5</td>
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<td>CDDP (10 μg/ml)</td>
<td>Medium only</td>
<td>17.8 ± 4.7</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>Medium only</td>
<td>2.0 ± 1.3</td>
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<td>CDDP (10 μg/ml)</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>51.2 ± 4.0</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>CDDP (5 μg/ml)</td>
<td>10.6 ± 2.2</td>
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<td>CDDP (10 μg/ml)</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>60.6 ± 4.6</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>CDDP (10 μg/ml)</td>
<td>15.5 ± 2.4</td>
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<td>PC-3</td>
<td>Medium only</td>
<td>Anti-Fas (1000 ng/ml)</td>
<td>16.3 ± 3.3</td>
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<td>19.3 ± 4.6</td>
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<td>Anti-Fas (1000 ng/ml)</td>
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<td>CDDP (5 μg/ml)</td>
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<td>Anti-Fas (1000 ng/ml)</td>
<td>66.3 ± 3.0</td>
<td>Anti-Fas (1000 ng/ml)</td>
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### Table 2

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<th>Target cells</th>
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<th>2nd treatment b</th>
<th>% cytotoxicity b (mean ± SD) a</th>
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</thead>
<tbody>
<tr>
<td>DU145</td>
<td>Medium only</td>
<td>Medium only</td>
<td>0.1 ± 1.9</td>
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<td></td>
<td>CDDP (10 μg/ml)</td>
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<td>24.0 ± 4.9</td>
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<td></td>
<td>Medium</td>
<td>Medium</td>
<td>0.4 ± 2.9</td>
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<td>CDDP (10 μg/ml)</td>
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<td>7.0 ± 7.0</td>
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<td>Medium</td>
<td>Medium</td>
<td>0.4 ± 1.6</td>
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<tr>
<td>PC-3</td>
<td>Medium only</td>
<td>Medium only</td>
<td>32.8 ± 2.7</td>
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<tr>
<td></td>
<td>CDDP (10 μg/ml)</td>
<td>Medium</td>
<td>41.8 ± 4.1</td>
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### Notes

1. The results are expressed as the mean ± SD of three different experiments.
2. Normal mouse IgM was used as an isotype control and showed no effect on the cells, and the data were comparable to cells incubated in medium only.
3. The cells were pretreated with medium or CDDP for 24 h (1st treatment). Thereafter, the medium was aspirated, and the tumor cells were washed twice with RPMI and subsequently incubated with either medium only or anti-Fas (IgM) antibody for another 24 h (2nd treatment). The plates were processed to measure cytotoxicity by the MTT assay.
for DU145 (Fig. 3A) and mediated some apoptosis (Fig. 3B), whereas anti-Fas antibody (1000 ng/ml), which was not cytotoxic, resulted in no detectable apoptosis (Fig. 3C). However, when CDDP and anti-Fas antibody were used in combination, synergy in apoptosis was observed (Fig. 3D). Likewise, in the TUNEL method, significant DNA fragmentation was observed in tumor cells treated with CDDP and anti-Fas (CH-11) antibody (Fig. 4). Apoptosis was inhibited by the addition of neutralizing anti-Fas IgG (ZB4) antibody (Fig. 4). These results indicate that there was a good correlation between cytotoxicity and synergy, as measured by the PI and TUNEL assays.

**Effect of CDDP on Regulation of Genes Associated with Apoptosis**

The potential mechanism of chemosensitization of PC-3 tumor cells by CDDP to Fas-mediated cytotoxicity and apoptosis was examined. Both bcl-2 (29) and FAP-1 (30) have been shown to influence the Fas signaling apoptotic pathway. We examined if CDDP inhibits these genes and therefore renders the cells sensitive to Fas. The findings in Fig. 5 demonstrate that the levels of both bcl-2 and FAP-1 mRNA are not affected by CDDP. We then examined if CDDP modulates c-myc expression because c-myc has also been associated with apoptosis (31). No effect was observed (Fig. 5).

It has been reported that certain drugs induce the expression of Fas-L on tumor cells, thus inducing autologous killing (32). We examined whether CDDP induces Fas-L on PC-3 that enhances killing by Fas/Fas-L interaction. PC-3 did not express a basal level of Fas-L, and Fas-L was not inducible by CDDP.

**Sensitization of DU145 and PC-3 Prostate Carcinoma Cell Lines to Anti-Fas Cytotoxicity by Adriamycin and VP-16 but not by Suramin**

The previous findings demonstrated that CDDP sensitized DU145 and PC-3 prostate carcinoma cell lines, but not LnCAP, to anti-Fas (CH-11) antibody mediated cytotoxicity. We examined if other cytotoxic drugs like ADR, VP-16, and suramin can also sensitize the tumor cells to cytotoxicity by anti-Fas. Both ADR and VP-16, but not suramin, sensitized DU145 and PC-3 to anti-Fas (CH-11) cytotoxicity, and the extent of sensitization was a function of both the drug and antibody concentrations used (Figs. 6, A and B). However, like CDDP, neither ADR nor VP-16 sensitized LnCAP to anti-Fas cytotoxicity (Fig. 6C).

Suramin has been used recently in the treatment of prostate cancer. We examined whether suramin can also sensitize the cells to anti-Fas cytotoxicity. No synergy was observed in all three lines tested, demonstrating that suramin is not a sensitizing agent to anti-Fas (CH-11) cytotoxic activity (Fig. 6). These findings indicate that CDDP is not a unique sensitizing agent, but other drugs like ADR and VP-16 are also effective in chemosensitization of tumor cells to Fas killing.

**Sensitization of DU145 and PC-3 to CTL-mediated Cytotoxicity**

The above findings demonstrated that sensitization was achieved with the Fas-L agonist anti-Fas antibody. We examined whether sensitization to Fas-L cytotoxicity is also achieved. The CTL hybridoma PMMI mediates its cytotoxic function exclusively by the Fas/Fas-L pathway (22). Furthermore, this CTL hybridoma kills both murine and human Fas-bearing target cells. Both DU145 and PC-3 were resistant to PMMI-mediated
DUI45 and PC-3 with combinations of anti-Fas antibody or Fas-L bearing CTL and either CDDP, ADR, or VP-16 results in a synergistic cytotoxic activity and apoptosis. The synergy observed was the result of chemosensitization of the tumor cells to Fas/Fas-L cytotoxic activity because synergy was completely inhibited by the addition of neutralizing anti-Fas antibody. Synergy in apoptosis was confirmed by DNA fragmentation experiments. These findings demonstrate that chemotherapeutic drugs, used at subtoxic concentrations, can sensitize certain drug/CTL-resistant prostate tumor cells to cytotoxicity and apoptosis by Fas-mediated cytotoxic mechanisms.

Fas is a receptor for Fas-L, and when Fas-L binds to Fas, the targeted cell undergoes apoptosis. Human Fas is a type I membrane protein with a signal sequence at the NH₂ terminus and a membrane spanning region in the middle of the molecule. Anti-Fas antibody works as an agonist and mediates killing of Fas⁺ cells. The cytoplasmic domain is sufficient to transduce apoptosis and has a sequence called "the death domain." In humans, Fas is weakly expressed on thymocytes but is expressed on activated mature lymphocytes, although many tumor cells express Fas (13). Whereas all three prostate carcinoma cell lines expressed surface Fas, they were relatively resistant to cytotoxic anti-Fas antibody. Thus, the mere expression of Fas is not sufficient to trigger cells for apoptosis. Indeed, unlike DUI45 and PC-3, LnCAP was not killed by anti-Fas after treatment with the drugs. It is possible that LnCAP may express potent antiapoptotic gene products that inhibit apoptosis by anti-Fas antibody (33).

Sensitization of DUI45 and PC-3 was not due to up-regulation of Fas expression. However, treatment of other tumor cell lines like ovarian carcinoma resulted in up-regulation of Fas and sensitization to anti-Fas antibody (28). These data suggest that up-regulation of surface Fas expression by drugs is not required for sensitization to Fas-mediated killing.

Fas mediates cell death via apoptosis (19). One mechanism by which resistant tumor cells can be sensitized to the Fas/Fas-Ligand cytotoxic pathway is by up-regulation of apoptotic genes or down-regulation of antiapoptotic genes (33). The drugs, although not cytotoxic, may modify the DNA, augmenting the Fas-mediated apoptotic signal. For example, FAP-1 is a protective factor that has been shown to block the Fas death signal (30). Down-regulation of FAP-1 by drugs would be expected to
Fig. 6  Cytotoxicity by treatment with a combination of ADR, VP-16, or suramin and anti-Fas (IgM) antibody on PC-3 (A), DU145 (B), and LnCAP (C). For details, see the legend for Fig. 2. Synergy was determined by isobologram analysis. Bars, SD.
increase sensitivity to Fas/Fas-L cytotoxicity as reported (34). However, CDDP did not inhibit FAP-1 in PC-3 cells.

Recently, Berchem et al. (35) showed that androgen can mediate resistance to VP-16-induced cytotoxicity by increasing bcl-2 expression. The bcl-2 family of gene products are mitochondria-associated proteins that have been implicated in preventing apoptosis triggered by a variety of death stimuli such as radiation, growth factor deprivation, and chemotherapy (29, 36, 37). The role of bcl-2 in Fas/Fas-L-mediated cytotoxicity is not clear. For example, Chiu et al. (38) showed that bcl-2 expression in transfected target cells had no effect on anti-Fas or Fas-L CTL-mediated cytotoxicity. In contrast, Lacronique et al. (39) showed that bcl-2 could protect human bcl-2 transgenic mice from anti-Fas-induced hepatic cell apoptosis. Previous studies have reported that drug-resistant PC-3 and DU145 cells express high levels of bcl-2 and c-myc and may contribute to drug resistance (40). In this study, CDDP-treated PC-3 cells did not modulate the expression of bcl-2 or c-myc, suggesting that chemosensitization occurs via other genes.

Recent findings demonstrate that certain chemotherapeutic drugs induce the expression of Fas-L on tumor cells, and cytotoxicity is achieved via autologous Fas/Fas-L interaction (32). This new mechanism of drug-induced cytotoxicity was tested in our system and was not observed. PC-3 did not express Fas-L or induce the expression of Fas-L. Altogether, these findings suggest that CDDP-mediated sensitization is the result of the regulation of other apoptosis-associated genes.

Previous studies have reported that CDDP can exert an immunostimulatory activity. It has been demonstrated that CDDP augments the antigen response of lymphocytes, monocytes, and NK cells (41–43). The interaction of Fas and Fas-L plays an important role in CTL- and FAP-mediated cytotoxicity (14, 15). It is noteworthy that both DU145 and PC-3 were relatively resistant to Fas-L-mediated cytotoxicity, corroborating the agonist anti-Fas antibody findings. Furthermore, our results demonstrate that sensitization of tumor cells by CDDP enhances tumor cell sensitivity to CTLs. The relative resistance of the tumor cells to Fas-L-mediated cytotoxicity has important implications in immunotherapy. Several approaches including gene therapy have been developed to generate an antitumor cytotoxic T-cell response with the objective to eradicate drug-resistant tumor cells. It is probable that even if an antitumor CTL is generated, not all tumor cells will be sensitive to killing by the CTL. For instance, this study shows that the prostate tumor lines were not sensitive to CTL-mediated killing by the Fas/Fas-L pathway. Consequently, tumor cells that are not sensitive to CTL will not be eradicated. Therefore, our findings suggest that chemosensitization of tumor cells to Fas-mediated killing is one approach to potentiate CTL-mediated immunotherapeutic approaches in the eradication of cancer.

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Chemosensitization of human prostate carcinoma cell lines to anti-fas-mediated cytotoxicity and apoptosis.

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