Induction of Apoptosis by Diethylstilbestrol in Hormone-Insensitive Prostate Cancer Cells

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Background: Diethylstilbestrol (DES) and diethylstilbestrol diphosphate (DESdP) are effective agents for the treatment of advanced prostate cancers. Tumor-inhibiting effects of DES and DESdP are presumed secondary to suppression of androgen production in vivo. Little is known, however, about the direct cellular mechanisms of the tumor inhibition. Estrogens have been reported not only to stimulate growth but also to disrupt microtubule formation in prostate cancer cells. Purpose: The study was designed to examine and compare mechanisms of in vitro growth inhibition of DES and DESdP in human androgen-insensitive prostate cancer cells (DU145, 1-LN, and PC-3) and human androgen-sensitive prostate cancer cells (LNCaP) and to examine estrogen receptor modulation of such effects. Methods: The cytotoxic effects of DES and DESdP were examined in vitro by use of a standard microculture tetrazolium assay to quantitate numbers of viable cells. Immunofluorescence microscopy, DNA fragmentation analysis, and fluorescence flow cytometry were used to investigate microtubules, the induction of apoptosis, and changes in cell cycle distribution. The degree of estrogen receptor positivity of untreated and treated cells was determined by immunohistochemistry and quantitative image analysis. Results: LD₅₀ levels (the dose at which 50% of cells are no longer viable) in the concentration range of 19-25 µM were observed for both DES and DESdP in all cell lines examined. DESdP-induced growth inhibition was found to be dependent on heat-labile phosphatases present in fetal calf serum. DES-induced cytotoxicity was not affected by the presence of 17β-estradiol, and it was not dependent on the presence of estrogen receptor. Estrogen receptor-positive cells and estrogen receptor-negative cells were equally responsive to DES. PC-3 cells stained with fluorescent anti-tubulin, phalloidin (actin stain), and 4',6-diamidino-2-phenylindole (DNA stain) showed no inhibition of microtubules or actin filaments but revealed the presence of apoptotic bodies in the nuclei. Fluorescence flow cytometry of nuclear DNA content of propidium iodide-stained nuclei from androgen-insensitive prostate cancer cells treated with 15 or 30 µM DES or DESdP revealed an increase in relative numbers of hypodiploid (apoptotic) nuclei, a depletion of G₁- and S-phase cells, and an accumulation of cells in G₂/M phase. Conversely, androgen-sensitive cells contained a lower percentage of hypodiploid nuclei but no accumulation of cells in G₂/M phase. Conclusions: Direct cytotoxic effects of DES in prostate cancer cells are estrogen receptor independent and do not involve disruption of microtubule architecture but do involve the promotion of cell cycle arrest and apoptosis. These are the first data confirming direct cytotoxic effects of DES and DESdP in prostate cancer cells via an apoptotic mechanism. Implications: These results suggest that DES and DESdP have potential value as agents against androgen-insensitive prostate neoplasms through induction of an apoptotic cascade. [J Natl Cancer Inst 1996;88:908-17]
of primary cultures derived from benign hyperplasia and prostate carcinoma samples (10) and in prostate cancer cell lines (8). Intracellular localization studies of DES inhibition have targeted mitochondrial adenosine triphosphate synthase (11), respiratory chain enzymes (8), and microtubules (12,13) as sites of action. Hartley-Asp et al. (13) reported that DES induced metaphase arrest in DU145 prostate cancer cells. Other investigators (14,15) have found that DES increased aneuploidy in mammalian cells. It was reported that DES inhibited vitro polymerization of tubulin into microtubules, although the exact location of DES binding to tubulins or microtubule-associated proteins was not determined (16). Studies with estramustine, a structural analogue of 17β-estradiol, have shown that the 17β-estradiol carbamate linkage to nitrogen mustard may be important for binding to microtubule-associated proteins. Clinical trials of estramustine and vinblastine, both microtubule inhibitors, in the treatment of hormone-refractory prostate cancer patients have indicated that these drugs show promise for inducing antitumor responses (17). Similar antimiotic effects have been ascribed to tamoxifen (18), a compound structurally similar to DES. Estrogen receptors are present in some prostate cancer cells (19). It is unclear whether their presence is an important factor in prostate cancer response to either estrogens or antiestrogens.

This study was designed to investigate the inhibitory action of DES and DESdP on cultured prostate cancer cells and to examine estrogen receptor modulation of such effects.

Materials and Methods

Cell Lines and Culture Media

The androgen-insensitive prostate cancer cell lines 1-LN, DU145, and PC-3 (20-22) were maintained in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Life Technologies, Inc. [GIBCO BRL, Gaithersburg, MD) and were replated at lower cell density with 0.25% trypsin-EDTA (Life Technologies, Inc.) twice weekly. LNCaP, an androgen-sensitive prostate cancer cell line (23), was maintained in the same growth medium enriched with 10% FBS and 5 mL of ITS+ (i.e., insulin, transferrin, and selenium; Collaborative Research, Inc., Bedford, MA). LNCaP cells were passaged by gentle agitation with phosphate-buffered saline (PBS) (Life Technologies, Inc.).

Preparation of Compounds

DES was obtained from Sigma Chemical Co. DESdP was donated as a liquid (Stilphostrol; Miles Pharmaceuticals, West Haven, CT). DESdP was diluted to appropriate concentrations with either culture medium containing heat-inactivated serum or culture medium containing non-heat-inactivated serum. 17β-Estradiol was acquired from Upjohn Co. (Kalamazoo, MI). DES and 17β-estradiol were solubilized in dimethyl sulfoxide (DMSO) (Fisher Scientific Co.). Colchicine was obtained from Sigma Chemical Co. and solubilized in dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide in PBS] was added to each well. The plates were reincubated at 37 °C for 4 hours in the dark. Supernatant was aspirated, and formazan crystals were solubilized in 150 mL DMSO at 37 °C for 10 minutes with gentle agitation. The absorbance per well was measured at 540 nm by use of an EL311 Microplate Reader (Biotek Instruments Inc., Winskis, VT) linked to a computer (Macintosh LC 475). Each assay was performed at least three times with a matrix of three to six replicate wells per drug concentration.

Statistics and LD50 Calculations

Statistical analyses were performed using the Statview II program (Abacus Concepts, Inc., Berkeley, CA). Cell viability curves were plotted from absorbance readings as a function of drug concentration by use of CA-CricketGraph III (v.1.5) software (Computer Associates International, Islandia, NY). LD50 values (the dose at which 50% of cells are no longer viable) were derived from best fit curves. Statistical analyses of the differences between mean LD50 values were determined by use of Student’s t test (two-tailed) to obtain P values.

Analysis of Estrogen Receptor Levels

Semi-quantitative analyses of estrogen receptor levels were performed by the Cell Imaging Laboratory at Duke University Medical Center by the method of Layfield et al. (Layfield LJ, Saria E, Conlon D, Kerns BJ: manuscript submitted for publication). Briefly, cells were grown to confluence in 75-cm2 flasks and washed with PBS before fixation in 10% neutral buffered formalin (Baxter, Deerfield, IL) for 30 minutes with gentle rocking. Cells were scraped off the flasks in 10% neutral buffered formalin, pelleted at 6000g for 4 minutes at 25 °C, and washed three times with cold PBS. Cell pellets were embedded in paraffin, and sections of 5 μm in thickness were cut on a microtome and mounted on clean glass slides. The slides were baked overnight at 65 °C. The following morning, they were cooled for 30 minutes at room temperature. They were then deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. The slides were heated in a pressure cooker with 1500 mL of 10 mM citrate buffer (pH 6.0) (Ventana Medical Systems Inc., Tucson, AZ) for 30 minutes in a 700-W microwave and then cooled for 30 minutes. The slides were washed in Ventana Buffer Solution and loaded into the Ventana 320 Automated Immunostainer. The estrogen receptor primary antibody was supplied in a predilution form by Ventana Medical Systems Inc. The detection system was a universal anti-rabbit secondary immunoglobulin G (IgG)-biotinylated antibody cocktail followed by an avidin–biotin conjugate (Ventana Medical Systems Inc.). Staining was visualized by diaminobenzidine chromogen with a copper sulfate enhancement. The slides were counterstained with 1% methyl green (Sigma Chemical Co.) in sodium acetate buffer (pH 5.2). Quantitative analyses were performed by use of a CAS200 Image Analyzer by use of the quantitative ER/PR Software Package Version 2.51 (Becton Dickinson/Cell Analysis Systems, Chicago, IL). The CAS200 Image Analyzer was calibrated with an estrogen receptor-positive breast tissue sample with a predetermined quantity of estrogen receptors.

Immunofluorescence and UV Microscopy

PC-3 cells were plated on sterile coverslips in six-well tissue culture dishes at 1 x 10^4 cells per well. Cells were allowed to reattach overnight; the next day, they were exposed to DES (0-50 μM) or colchicine (0.01-1.0 μM) for 24, 48, and 72 hours. They were then rinsed with PBS buffer (50 mM PIPES [pH 6.9], 10 mM EGTA, and 6 mM MgSO4) (Sigma Chemical Co.) and made permeable for 1 minute at 37 °C with 0.5% Triton X-100 in PBS. Following a 15-minute fixation with 4% paraformaldehyde, cells were rinsed three times with PBS and incubated for 1 hour at room temperature with 0.5% preimmune goat serum in Hank’s balanced salt solution (Life Technologies, Inc.). Cells were then incubated for 1 hour with monoclonal anti-β-tubulin antibody (clone TUB 2.1, diluted 1:1000 in PBS) (Sigma Chemical Co.). After incubation, cells were rinsed three times with PBS and incubated with either (a) fluorescein isothiocyanate-labeled goat anti-mouse IgG (TAGO Inc., Burlingame, CA), (b) tetra-methylrhodamine–isothiocyanate-labeled phallolidin (Sigma Chemical Co.) to stain filamentous actin, or (c) 4',6-diamidino-2-phenylindole (Sigma Chemical Co.) to stain DNA. Cytospin slides were prepared for comparison. Briefly, PC-3 cells were plated in 100-mm dishes at 5 x 10^5 cells per dish and allowed to

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recovered overnight. Cells were then exposed to either DES (0-30 M) or DESdP (0-30 M) for 72 hours. Adherent and nonadherent cells were collected and centrifuged at 400g for 10 minutes at 25°C (i.e., cytopsin) on glass slides. Slides were air-dried overnight and frozen at -20°C. Cytospin preparations were stained in the same manner as that used for cells grown on coverslips. Stained cells were rinsed with PEM, mounted in 5-10 μL of antifade, and sealed with clear nail polish. Both coverslip or cytospin preparations were visualized by use of a Zeiss Axioskop fluorescence photomicroscope and were photographed on black-and-white film (35 mm, TMAX, 400 ASA, Kodak) or visualized with a Color CCD monochrome video camera. Video images were captured digitally by use of a Scion Leica 6.3 digital capture board and Macintosh-based software (Scion Image, Adobe Photoshop, and Canavas, Dense Head Software, Miami, FL). Video images were processed with a Phaser II-SDX dye sublimation printer (Tectronics Inc., Beaverton, OR).

DNA Gel Electrophoresis

PC-3 cells were harvested while in log-phase growth by trypsin treatment. They were then plated at a density of 5 × 10^5 cells per 100-mm dish. Cells were allowed to recover for 24 hours, and DES was added at a concentration of 15 or 30 M. Dishes with or without drug were incubated for 24, 48, or 72 hours. Adherent and nonadherent cells were harvested in PBS, centrifuged at 600g for 10 minutes at 25°C, and frozen at -20°C until time of assay. DNA was then extracted by the method of Botermann et al. (25). Cells were centrifuged at 600g for 5 minutes at 25°C, and the resultant pellets were lysed in 10 mM Tris-Cl (pH 7.4), 25 mM EDTA, and 0.1% Nonidet P-40. The samples were exposed to 300000 μg/mL proteinase K for 2 hours at 55°C. The lysate was centrifuged at 300000 g for 2 hours at 4°C in an Optima TLX ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was treated with 100 μg/mL ribonuclease (RNase) (Sigma Chemical Co.) and incubated for 18 hours at 37°C. DNA was precipitated in 2 volumes of 100% ethanol for 20 minutes at 4°C and harvested as maximal speed in a microfuge. DNA pellets were resuspended in distilled water and loaded in a 1.0% agarose gel. DNA was electrophoresed for 1.5 hours at 10 V/cm. Gels were stained with 30-40 μL ethidium bromide (10 mg/mL) (Sigma Chemical Co.) and incubated for 15 minutes at 25°C and frozen at -20°C until time of assay. DNA was then excised, resuspended in distilled water containing 30-40 μL ethidium bromide (10 mg/mL) (Sigma Chemical Co.) and photographed under UV light.

Fluorescence Flow Cytometry

Cells were harvested at log phase by trypsin treatment and plated at a density of 5 × 10^5 cells per 100-mm tissue culture dish. Cells were allowed to recover for 24 hours before DES or DESdP was added at various concentrations bracketing the LD$_{50}$ values (15-30 M). Cells with and without drug were incubated for 72 hours, and then adherent and nonadherent cells were harvested. Approximately 1 × 10^6 cells were centrifuged at 600g for 5 minutes at 25°C, and the supernatant was removed. The pelleted cells were resuspended in 1 mL of nuclear isolation medium (i.e., 9 mM CaCl$_2$, 3 mM KCl, 1 mM KH$_2$PO$_4$, 5 mM MgCl$_2$, 137 mM NaCl, 8 mM Na$_2$HPO$_4$, 0.5% bovine serum albumin [Sigma Chemical Co.], 0.2% nonionic detergent, and Nonidet P-40 [Sigma Chemical Co.]). RNA was digested by the addition of 10 μL (10 mg/mL) deoxyribonuclease-free RNase (Sigma Chemical Co.) to the nuclear preparation. The isolated nuclei were stained by the addition of 10 μL of propidium iodide (5 mg/mL) (Sigma Chemical Co.). The stained nuclei were kept in the dark at 4°C for 24 hours before analysis on a FACScan II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Fluorescence DNA histograms were generated from DNA signal width versus DNA signal area to identify the G$_0$/G$_1$, G$_2$/M populations on the basis of the relative propidium iodide (DNA) fluorescence. The G$_0$/G$_1$ peak was placed at about one third the propidium iodide fluorescence scale, and the lower discriminator was set at about channel 10 to capture the hypodiploid region of fluorescence (just below the G$_0$/G$_1$ peak) but to exclude the signals present in the lowest channels. The lower cutoff was set and kept exactly at the same position for the analysis of the DES- or DESdP-treated cells. The upper discriminator was set to exclude cell doublets or clumps but to include nuclei with aneuploid or polyploid DNA content.

Results

Heat Inactivation of FBS

DESdP inhibited the growth of PC-3 cells grown in medium supplemented with non-heat-inactivated FBS. There was an eightfold reduction in PC-3 viability with a 50% cytotoxicity (LD$_{50}$) at approximately 20 M DESdP. In contrast, DESdP did not reduce the viability of PC-3 cells when grown medium was supplemented with heat-inactivated FBS (Fig. 1). All subsequent assays for DESdP were therefore carried out with non-heat-inactivated serum-supplemented media.

Comparison of DESdP-Induced and DES-Induced Cytotoxicity

As summarized in Table 1, the LD$_{50}$ values (means ± standard error) for DESdP were in the range of 20.6 μM ± 1.9 M to 25.0 μM ± 3.4 μM, with a group LD$_{50}$ mean of 23.0 μM ± 1.0 M. DESdP reduced the viability of each of the cell lines by 50% at concentrations of approximately 20 μM. The LD$_{50}$ values (means ± standard error) for DES were within a range of 19.6 μM ± 0.6 μM to 21.7 μM ± 4.0 μM, with a group LD$_{50}$ mean of 20.9 μM ± 0.5 μM DES. The differences between group LD$_{50}$ means for DES and DESdP were not statistically significant (Table 1). Representative trypan blue dye exclusion hemocytometer counts verified observed cell number reductions measured by the MITT assays.

17β-Estradiol Receptor Analysis

To test the responsiveness of these cell lines to a natural estrogen, we added 17β-estradiol to growth media at concentrations ranging from 0 to 50 μM. 17β-Estradiol stimulated growth of LNCaP cells but did not affect the growth of 1-LN, PC-3, and DU145 cells at the concentrations tested. The presence of...
estrogen receptors was also analyzed in these cell lines. 1-LN, DU145, and PC-3 cells were negative for estrogen receptor (i.e., <5 fmol/pg DNA), whereas LNCaP cells were positive for estrogen receptor (>10 fmol/pg DNA). Table 1 summarizes these results and gives previously reported results relating to the androgen sensitivity of these cell lines (8,23,26). There was no apparent association between estrogen receptor positivity or androgen sensitivity and the cytotoxic effects of either DES or DESdP in these prostate cancer cell lines (Table 1). However, the growth of LNCaP cells, positive for estrogen receptors, was stimulated by the addition of exogenous 17β-estradiol, as shown by the increase in MTT optical density or control cells in Fig. 2. A number of apoptotic nuclei were also observed in control cells at the 72-hour time point (arrows in Fig. 4, C and E). Cytospin preparations were also analyzed by staining with 4′,6-diamidino-2-phenylindole to determine whether DES or DESdP increased the proportion of “mitotic” nuclei within the G2/M population. Nuclei with condensed chromatin or “chromosomes,” as in metaphase, anaphase, and telophase, were infrequently seen in cytospin or coverslip preparations of control or DES- or DESdP-treated cells (arrowheads in Fig. 4, A, B, C, E).

In agreement with the flow cytometry data, this observation suggests that a small number of mitotic cells are represented in the G2/M subpopulation.

### Failure of 17β-Estradiol to Reverse DES-Induced Cytotoxicity

17β-Estradiol rescue of DES-treated cells was not observed in triplicate assays combining both agents in four separate cell lines. Cells were pretreated with DES (0-50 μM) for 24 hours, and 17β-estradiol was added at a concentration of 50 μM for an additional 48 hours, after which cell viability was measured by the MTT assay. There was no apparent difference in loss of viability for prostate cancer cells exposed to DES in the absence or presence of 50 μM 17β-estradiol. Fig. 2 shows the results of such an experiment with LNCaP cells.

### Immunofluorescence and UV Microscopy

It had been previously suggested that DES inhibits microtubule formation (13). To examine this issue, we studied changes in microtubule architecture in control versus DES-treated PC-3 cells. Colchicine, a known microtubule inhibitor, was used as the positive control (27,28). Compared with control (untreated) cells (Fig. 3, A), microtubules in PC-3 cells treated for 72 hours with 0.1 μM colchicine were completely disrupted (Fig. 3, B). However, microtubules were not altered by a 72-hour exposure to 15 μM DES (Fig. 3, C) or 30 μM DES (Fig. 3, D). Actin filaments in these cells were also unaffected by DES at the same concentrations (data not shown).

UV light fluorescence microscopy of cells grown on coverslips or of cytosin preparations stained with the DNA fluorochrome 4′,6-diamidino-2-phenylindole revealed the presence of apoptotic nuclei in PC-3 cells treated with DES or DESdP (Fig. 4). Cells treated with DES for 72 hours had smaller nuclei often associated with a scalloped outline (Fig. 4, B; arrows). A small number of apoptotic nuclei were also observed in control cells at the 72-hour time point (arrows in Fig. 4, C and E). Cytospin preparations were also analyzed by staining with 4′,6-diamidino-2-phenylindole to determine whether DES or DESdP increased the proportion of “mitotic” nuclei within the G2/M population. Nuclei with condensed chromatin or “chromosomes,” as in metaphase, anaphase, and telophase, were infrequently seen in cytospin or coverslip preparations of control or DES- or DESdP-treated cells (arrowheads in Fig. 4, A, B, C, E).

In agreement with the flow cytometry data, this observation suggests that a small number of mitotic cells are represented in the G2/M subpopulation.

### Table 1. LD₅₀ values* in prostate cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Androgen sensitivity†</th>
<th>Estrogen receptor</th>
<th>DES§</th>
<th>DESdP§</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-LN</td>
<td>-</td>
<td>-</td>
<td>19.6 ± 0.6</td>
<td>25.0 ± 3.4</td>
<td>&lt;.29</td>
</tr>
<tr>
<td>DU145</td>
<td>-</td>
<td>-</td>
<td>21.6 ± 1.7</td>
<td>24.3 ± 3.5</td>
<td>&lt;.54</td>
</tr>
<tr>
<td>PC-3</td>
<td>-</td>
<td>&lt;.79</td>
<td>20.6 ± 4.8</td>
<td>20.6 ± 1.9</td>
<td>&lt;.95</td>
</tr>
<tr>
<td>LNCaP</td>
<td>+</td>
<td>+</td>
<td>21.7 ± 4.0</td>
<td>22.1 ± 2.1</td>
<td>&lt;.95</td>
</tr>
<tr>
<td>Group LD₅₀</td>
<td></td>
<td></td>
<td>20.9 ± 0.5</td>
<td>23.0 ± 1.0</td>
<td>&lt;.19</td>
</tr>
</tbody>
</table>

*LD₅₀ = the dose at which 50% of cells are no longer viable.
†Previously reported (8,23,26).
§Diethylstilbestrol.
§Diethylstilbestrol diphosphate.
Fig. 3. Immunofluorescence microscopy of fluorescein isothiocyanate-stained microtubules of PC-3 cells treated with colchicine or diethylstilbestrol (DES) for 72 hours. A) Control cells (original magnification x100). Cells exposed to one of the following: B) 0.1 μM colchicine (original magnification x100), C) 15 μM DES (original magnification x100), and D) 30 μM DES (original magnification x100).

Observation of Apoptosis by Electrophoresis

PC-3 cells treated with DES were examined by DNA extraction and electrophoresis on a 1.0% agarose gel (Fig. 5). A DNA pattern suggestive but not diagnostic of apoptosis was noted at the 48- and 72-hour time points for 15 and 30 μM DES exposures. There was no low-molecular-weight DNA in the control lanes, and there was a clear dose- and time-dependent response to DNA fragmentation with DES (Fig. 5). A small amount of DNA fragmentation was observed within the 24-hour time limit with 30 μM DES. The induction of apoptosis was dependent on time and concentration.

Flow Cytometric Analysis

DES and DESdP have been shown to induce metaphase arrest and to inhibit growth of a variety of mammalian cells (13). It was therefore of interest to determine whether DES and DESdP could also disrupt cell cycle progression in cultured prostate cancer cells. A disruption or blockage of the cell cycle is expressed in the DNA profile of cells in the various stages of the cell cycle. Such a profile is readily obtained by means of propidium iodide-fluorescence flow cytometry. Fluorescence discriminators were lowered to detect the incidence of hypodiploid bodies at propidium iodide-fluorescence levels below the 2N DNA level. The appearance of propidium iodide-stained nuclei with aneuploid chromosome complement (i.e., DNA content <2N) was therefore detectable. Propidium iodide-fluorescence (DNA) profiles were obtained for the four cell lines exposed to DES and DESdP at concentrations bracketing the LD₅₀ values (15 and 30 μM).

A flow cytometric analysis for PC-3 nuclei is shown in Fig. 6. The cell cycle phases are defined in terms of propidium iodide-fluorescence units on the abscissa: Hypodiploid bodies (nuclei) = 10-50, G₁ phase = 50-75, S phase = 75-125, and G₂/M phase = 125-150. The frequency of hypodiploid nuclei and the distribution (%) of cells within each cycle phase were calculated from histograms for each cell line (Table 2). All androgen-insensitive cells had an increase in apoptotic percent, whereas androgen-sensitive LNCaP cells had an increase in apoptotic percent to a lesser degree. A pronounced decrease in the proportion of G₁ cells in each of the androgen-insensitive prostate cancer cell lines (1-LN, DU145, and PC-3) was observed in cell lines exposed to either DES or DESdP. For DES-treated cells, a decrease in S-phase cell number was noted; a similar but less pronounced decrease occurred in DESdP-treated cells. An increase in the proportion of nuclei in G₂/M phase was noted in all DES-treated, androgen-insensitive prostate cancer cell lines, but only PC-3 cells responded to DESdP with an increase in G₂/M. In contrast, androgen-sensitive LNCaP cells showed no change in the proportion of cells in G₁ or S phase and a decrease in the proportion of cells in G₂/M phase after treatment with DES or DESdP.

Discussion

In this study, DES and DESdP were equally cytotoxic to prostate adenocarcinoma cells, regardless of androgen sensitivity (Table 1) or estrogen receptor status (Fig. 2). Our experiments implicate the presence of heat-labile phosphatases in serum and support the previously published work of Schulz et al. (8).
DES is a synthetic estrogen and should exhibit similar effects as those exhibited by other estrogenic compounds, i.e., 17β-estradiol. Estrogen receptor analysis did reveal that LNCaP cells were estrogen receptor positive and that androgen-insensitive cell lines were estrogen receptor negative. Even though the growth of LNCaP cells was stimulated by 17β-estradiol (Fig. 2), a similar effect with DES was not seen (Table 1). On the contrary, we found that DES abrogated 17β-estradiol stimulation of LNCaP cells, yielding LD₅₀ values that were essentially equal to those seen with no 17β-estradiol stimulation. Our findings agree with previous results (29) that revealed a similar effect of estramustine on prostate cancer cells independent of the presence of estrogen receptors. These observations have led us to conclude that DES-induced growth inhibition is not mediated via the estrogen receptor but may involve alternate cellular mechanisms.

In previous studies, investigators (13,15) have observed DES-induced mitotic spindle disruptions in vitro. Hartley-Asp et al. (13) noted that DES-induced mitotic arrest in DU145 prostate cancer cells was accompanied by inhibition of microtubule assembly. This inhibition was reversible and required high concentrations of DES (up to 100 μM). On the basis of UV fluorescence microscopic analysis of 4',6-diamidino-2-phenylindole-stained nuclei of DES- and DESdp-treated PC-3 cells (Fig. 4), there is little evidence in our study to indicate that these cells were arrested at mitosis. The preponderance of apoptotic nuclei was most likely blocked at the G2/M interface, which is in agreement with the DNA fluorescence flow cytometry profiles shown in Fig. 6.

DES-induced microtubule inhibition has also been shown in breast cancer cell lines. Aizu-Yokota et al. (12) reported the occurrence of microtubule inhibition in breast cancer cell lines, regardless of the presence of estrogen receptor. However, microtubular inhibition was induced at DES concentrations more than twofold higher (80 μM) than those used in this study (15-30 μM). Immunofluorescence microscopy failed to demonstrate DES inhibition of microtubule formation in PC-3 prostate cancer cells (15-30 μM). Our results agree more with the work...
also proposed that the nucleosome-length DNA may represent only the end product of excessive digestion and that nucleosomal fragments may be lost or digested further. Thus, while the presence of DNA ladders is consistent with apoptosis, the absence of DNA ladders does not rule out apoptosis either.

Sawada and Ishidate (44) noted in DES-treated Chinese hamster fibroblasts histologic features consistent with the apoptotic changes described for the DES- and DESdp-treated PC-3 cells examined in this article. Expression of testosterone-repressed prostatic message 2 (TRPM-2) messenger RNA (mRNA) has been associated with the induction of apoptosis in cells of the rat ventral prostate following castration (i.e., androgen ablation) (41). Similarly, higher levels of TRPM-2 mRNA have been observed in human prostate xenograft tumor cells following castration of the host animals (42). In a study of normal rat ventral prostate (43), DES increased TRPM-2 expression for a longer time than did castration. DES-induced chromatin condensation has also been observed (44). Evaluating 25 prostate cancer patients treated with DES, Szende et al. (45) noted the occurrence of apoptosis in biopsy specimens of tumor tissue from 20 (80%) of 25 patients, along with clinical tumor regression. Histologic evidence of apoptosis included foci of pyknosis and nuclear fragmentation. Such morphologic changes are consistent with the nuclear disruption (Figs. 3 and 4) and DNA fragmentation (Fig. 5) observed in our in vitro study.

Following previously described methodology (46-48), fluorescence flow cytometry was used in this study to detect apoptotic cells. Wei et al. (47) noted that apoptosis was accompanied by a decrease in G, and S phases of the cell cycle upon tumor exposure to quercetin, a growth-inhibiting bioflavonoid. In our study, androgen-insensitive prostate cancer cells (1-LN, DU145, and PC-3) responded in a similar manner when treated with DES or DESdp: The percentage of cells in G, and S phases decreased as the percentage of G2/M and hypodiploid nuclei increased (Table 2). G2/M arrest is commonly caused by DNA-damaging agents (49,50). We found normal arrays of microtubules in DES-treated PC-3 prostate cancer cells, suggesting that disruption of microtubules was not the cause of the G2/M block. Sorenson et al. (49) described such events associated with G2-phase arrest and subsequent apoptosis in L1210 murine leukemia cells treated with cisplatin. Cells were shown to progress to G2 phase and to remain arrested for several days, followed by a slow death at the G2/M transition (49).

Barry et al. (40) proposed the following: “There are two forms of apoptosis, a rapid, and presumably more direct, activation of the endonuclease, and a delayed activation that requires passage to a specific cell cycle phase, the G2/M phase, and the activation of events normally associated with the G2/M phase transition.”

Events that occur in this phase of the cell cycle may therefore be involved in the onset of apoptosis. One of the essential proteins for passage through G2/M is cyclin B (51). Cyclin B complexes with p34/cdc2 and phosphorylates structural proteins such as the spindle apparatus (52). Histone H1, which is involved in chromatin condensation, is also phosphorylated by the cyclin B—p34/cdc2 complex (52). Preliminary immunohistochemical analyses in our laboratory revealed that cyclin B expression is decreased in DES-treated, androgen-insensitive prostate cancer cells. A link may exist between apoptosis, cyclin B—p34/cdc2 complex, and G2 arrest (50,53,54). The androgen-
Fig. 6. DNA fluorescence flow cytometry histograms of propidium iodide (PI)-stained nuclei obtained from PC-3 cells following 72-hour treatment with diethylstilbestrol (DES) or diethylstilbestrol diphosphate (DESDP) at concentrations of 15 and 30 µM. Panels A and D show profiles of nuclei from untreated control cells. Panels B and E show the histograms obtained for PC-3 nuclei treated with 15 µM DES or DESDP, respectively. Panels C and F show the histograms for PC-3 nuclei treated with 30 µM DES or DESDP, respectively. The cell cycle phases are indicated on the abscissa in PI-fluorescence units: 10-50 = hypodiploid (apoptotic) nuclei; 50-75 = G1; 75-125 = S; and 125-150 = G2/M.
sensitive prostate cancer cell line LNCaP did not show a decrease in the proportion of cells in G1 phase after treatment with DES and DESdP, but did show a decrease in the proportion of cells in the G2/M phase, which was concurrent with a slight increase in hypodiploid nuclei (Table 2). Details of the mechanism of DES-induced apoptosis and its relationship to cyclin B expression, however, are currently unknown.

Our results highlight the induction of apoptosis and cell cycle arrest upon prolonged exposure to DES or DESdP. The intensity of this phenomenon appears to depend on the androgen sensitivity of prostate cancer cells under study. Androgen-insensitive prostate cancer cells were more susceptible to DES-induced apoptosis than were androgen-sensitive prostate cancer cells. Whether high-dose DES treatment represents a window of opportunity for hormone-insensitive prostate cancers will require further laboratory and clinical studies to be warranted.

References


Notes
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