Evaluation of the Cytotoxic Activity of Diethylstilbestrol and Its Mono- and Diphosphate towards Prostatic Carcinoma Cells

Peter Schulz, Hartwig W. Bauer, Wolfgang P. Brade, Alois Keller, and Friedrich Fittler


ABSTRACT

To evaluate a possible direct cytotoxic effect of diethylstilbestrol diphosphate (DESDP) in the treatment of prostate cancer we exposed three prostatic carcinoma cell lines (LNCaP, DU 145, and PC-3), 2 nonprostatic neoplastic cell lines (KB and EJ), and one nontransformed cell line (MRC-5) to diethylstilbestrol (DES), diethylstilbestrol monophosphate, and DESDP at levels occurring in patients' sera during p.o. DES therapy (2 to 5 ng/ml) or DESDP infusions (1 to 20 µg/ml), respectively. With 5 ng/ml of DES no effect was seen in LNCaP cells, even after 14 days of exposure. In contrast, drug levels attained during DESDP infusions showed marked, dose-dependent cytotoxicity towards all cell lines under study. Prostatic cells were not exceptionally sensitive. High-dose DES slightly stimulated the synthesis of prostatic acid phosphatase in LNCaP cells. Formation of foci of polygonal cells was induced by 1/2 fold amount of DESDP compared to DES results in equimolar solution. With 5 ng/ml of DES no effect was seen in LNCaP cells, even after 14 days of exposure. In contrast, drug levels attained during DESDP infusions showed marked, dose-dependent cytotoxicity towards all cell lines under study. Prostatic cells were not exceptionally sensitive. High-dose DES slightly stimulated the synthesis of prostatic acid phosphatase in LNCaP cells. Formation of foci of polygonal cells was induced by 1/2 fold amount of DESDP compared to DES results in equimolar solution.

INTRODUCTION

The infusion of high doses of DESDP has shown effective symptomatic relief in patients relapsing on conventional hormone therapy (3 to 5 mg of DES per day p.o.) of carcinoma of the prostate (1, 2). In view of the cardiovascular complications associated with high doses of synthetic estrogens (3) a more detailed understanding of the mode of action of DESDP at the cellular level could contribute to the development of more efficient therapeutic schedules.

Some authors have proposed that, in addition to the inhibition of the pituitary testicular axis leading to suppression of gonadal androgen production (4), high doses of DESDP also exert cytotoxic effects on prostatic tumor cells (5). Different experimental approaches have been used to evaluate direct cytotoxicity. The effects of DES on the androgen response mechanism of benign hyperplastic prostate cells were investigated using short-term cultures of surgically removed tissue. No significant suppression of nuclear androgen uptake, change in testosterone/dihydrotestosterone ratios in nuclei, or 5α-reductase activity could be detected at reasonably low DES concentrations (6). Specific enrichment of DES in prostatic tissue concentrations (6). Specific enrichment of DES in prostatic tissue was reported to occur 1 h after infusion of 1 g or more DESDP into patients suffering from benign hypertrophy or carcinoma of the prostate (7). Yet direct effects of synthetic estrogens on prostatic carcinoma cells remained hypothetical.

With the successful cultivation of hormone-responsive hu-

mancarcinoma cells straightforward investigations of direct drug effects have become possible. In human MCF-7 breast cancer cells the cytotoxicity of DES has been shown to be independent of the presence of estradiol receptor (8, 9), although it specifically binds to this receptor. In the work presented we compare the inhibition of cell proliferation caused by DES, DESMP, and DESDP at concentrations attained during therapeutic infusions (10) in cell lines derived from three different carcinomas of the prostate (LNCaP, PC-3, and DU 145), one bladder carcinoma (EJ), one epidermoid carcinoma (KB), and in one nontransformed human embryonic fibroblast cell line (MRC-5) with limited life span.

We could demonstrate significant suppression of cell growth in all cell lines tested using DES, DESMP, and DESDP as antiproliferative agents. The fibroblast line MRC-5 in addition exhibited morphological changes upon exposure to the drugs. These results suggest that DES and its phosphorylated derivatives act upon a regulatory or metabolic mechanism common to many if not all human cells.

MATERIALS AND METHODS

Cell Lines, Culture Media, and Drugs. All lines used in this work are of human origin. The prostate carcinoma lines LNCaP, PC-3, and DU 145 were from the Human Tumor Cell Laboratory, Sloan Kettering Institute for Cancer Research, Rye, NY. The remaining lines were obtained from the following sources: EJ (bladder carcinoma), K. Wil- 

lecke, Essen, Federal Republic of Germany; KB (epidermoid carci-

noma), American Type Culture Collection, CCL 17; and MRC-5 (em-

bryonal lung, nontransformed with limited lifetime), Flow Laborato-

ries. The culture medium RPMI 1640, DMEM, and fetal calf serum were from Boehringer Mannheim. DES, DESMP, and DESDP were from Asa-Werke, Degussa Pharma Gruppe, D-4800 Bielefeld, Federal Republic of Germany.

Maintenance of the Cell Lines and Assays for Inhibition of Cell Proliferation. The cell line LNCaP was maintained in RPMI medium, and the lines PC-3, DU 145, KB, EJ, and MRC-5, in DMEM. All lines were grown in the presence of 10% fetal calf serum and phenol red. The serum was not heat inactivated. The doubling times, splitting ratios, and splitting frequencies of the individual cell lines were as follows: LNCaP (42 h, 1:4 to 1:5, once a week); PC-3 (40 h, 1:3, twice a week); KB (42 h, 1:4, once a week); and MRC-5 (90 h, 1:2 to 1:3, once every 10 days). For LNCaP, DU 145, KB, and MRC-5 the culture medium was renewed once between splittings. All cell lines reached near or complete confluence before being split, except PC-3, which was kept at lower density. For the preparation of seed stocks, cells were split as indicated above and grown to 50 to 75% confluency before use. The period between splitting and seeding for growth inhibition assays of the individual cell lines was as follows: LNCaP (3 to 4 days); PC-3 (2 to 3 days); DU 145 (3 to 4 days); EJ (2 days); KB (3 to 4 days); and MRC-5 (6 to 7 days). To assay the growth inhibition cells were seeded into 60-mm Petri dishes in 8 ml of culture medium. The inoculum sizes were held constant within cell lines and were as follows: LNCaP (7 × 10^4); PC-3 (5 × 10^4); DU 145 (7 × 10^4); EJ (5 × 10^4); KB (5 × 10^4); and MRC-5 (7 × 10^4). The drugs were added 16 to 24 h after seeding as 20-µl ethanol solutions to give the final concentrations indicated in the figures. The addition of the 1.5-fold amount of DESMP or the 2-fold amount of DESDP compared to DES results in equimolar solu-

Received 7/1/87; revised 12/21/87; accepted 1/28/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Asa-Werke AG, Degussa Pharma Gruppe, Bielefeld (Federal Republic of Germany), Deutsche Forschungsgemeinschaft, and Fonds der Chemischen Industrie.

2 The abbreviations used are: DESDP, diethylstilbestrol diphosphate, tetradsodium salt; DES, diethylstilbestrol; DESMP, diethylstilbestrol monophosphate, disodium salt; PAP, prostate-specific acid phosphatase (EC 3.1.3.2); DMEM, Dulbecco’s modified Eagle’s medium.

2867

Downloaded from cancerres.aacrjournals.org on July 24, 2017. © 1988 American Association for Cancer Research.
tions. Controls received ethanol only. After 96 h of incubation (192 h for MRC-5 cells) at 37°C, the medium containing nonadherent cells was aspirated, and the cells remaining on the bottom of the dish were detached by trypsin treatment, suspended in phosphate-buffered saline (138 mM NaCl, 3.9 mM KCl, 1.5 mM KH2PO4, 7.2 mM Na2HPO4, pH 7.5), and counted in a Neubauer counting chamber. In each experiment assays in two or three dishes were run in parallel.

Prostatic acid phosphatase assays were carried out using a commercially available test kit (Enzygnost) from Behringwerke (D-3550 Marburg, Federal Republic of Germany) according to the recommendations of the supplier.

To determine the phosphatase activity in culture fluids, RPMI and DMEM media without phenol red were supplemented with 10% fetal calf serum and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.2), incubated at 37°C with 29 µg/ml of p-nitrophenylphosphate. The molarity of the p-phosphate monoesters in p-nitrophenylphosphate was equal to the phosphate ester concentration in 20 µg/ml of DESDP. After various times of incubation, 1.9-ml aliquots were taken, 0.5 ml of 1.5 M NaOH was added, and the absorbance at 405 nm was measured. The molar absorptivity of nitrophenol used for calculations was 18.5 x 10^3 M⁻¹ cm⁻¹.

**RESULTS**

**DES Does Not Inhibit Growth of LNCaP Cells at Concentrations Attained during p.o. Therapy.** We tested the response of LNCaP cells to 3, 5, 10, and 100 ng/ml of DES. No difference in appearance of the cells and in cell numbers could be detected in comparison to untreated controls (Fig. 1).

**DES and Its Phosphorylated Derivatives Are Cytotoxic to Prostatic Carcinoma as well as to Neoplastic Cells of Diverse Origin at Concentrations Attained during DESDP infusions.** Drug levels in patients' sera during therapeutic DESDP infusions have been precisely measured. Infusion of 1500 mg of DESDP yielded peak concentrations of 15 to 18 ng/ml of DESDP, 14 to 17 ng/ml of DESMP, and 2 to 4 µg/ml of DES (10).

We found LNCaP cells to be sensitive to DES at a concentration as low as 1 µg/ml (Fig. 3). The antiproliferative effect of DES is clearly dose dependent. At 10 µg/ml of DES the cell number after 4 days of exposure to the drug was equal to or lower than the cell number seeded. The proportion of severely damaged and dead cells increases with time (Fig. 2). These findings suggest that, in addition to a possible antimitotic action, DES clearly exerts a cytotoxic effect at high concentrations.

The weaker effect of the phosphorylated compounds is consistent with the assumption that cleavage of the phosphate esters yields the active substance DES. A direct effect of DESMP and DESDP cannot be excluded.

![Fig. 1. Low-dose DES does not affect the proliferation of LNCaP cells.](image)

**Fig. 1.** Low-dose DES does not affect the proliferation of LNCaP cells. r, ratio (average cell number in drug-containing dishes)/average cell number in control dishes. The r value of controls (rC) is defined as 1.00 (=100% or unity). Bars, SD of three independent experiments with at least two replicate dishes.

The prostatic carcinoma cell lines DU 145 and PC-3 have been characterized as true prostatic carcinoma cell lines (11), but do not produce PAP. Hence they provide the opportunity to test to what extent the cytotoxicity of DESMP and DESDP depends on the presence of high phosphatase concentrations on the target cell.

The results (Fig. 3) show that the cytotoxic effect of the phosphorylated compounds towards phosphatase-negative cells is equal compared to LNCaP cells. Phosphatase production by the target cell is not a strict precondition for the cytotoxic action of DESMP and DESDP. The phosphatase activity in the culture medium due to enzymes contained in the fetal calf serum was determined to be sufficient to remove 54 nmol/ml of P from p-nitrophenylphosphate within 24 h. Assuming a comparable activity towards phosphorylated DES, 70% of the phosphate moieties are hydrolyzed. In conditioned medium (pH 7.2) from LNCaP cells containing 4 ng/ml of PAP according to immunological assays no significant enhancement of phosphatase activity was observed. Of course, this finding does not exclude high local phosphatase activities in tumor tissues in vivo.

Since DES, DESMP, and DESDP proved cytotoxic to all prostatic cell lines tested, we asked if this finding was due to some peculiarity of cells derived from steroid hormone-responsive tissues. For a comparative study we chose two neoplastic human cell lines of nonprostatic origin.

Although there is some variation in the magnitude of the
Cytotoxicity of DESDP towards prostatic carcinoma cells

Fig. 3. High-dose DES, DESMP, and DESDP are cytotoxic to various cell types. r, ratio (average cell number in drug-containing dishes)/(average cell number in control dishes). The r value of controls (re) is defined as 1.00 (=100% or unity). Bars, SD of three independent experiments with at least two replicate dishes. For LNCaP cells, more than 10 independent experiments have been carried out.

response among the different transformed cell lines (Fig. 3), it could be demonstrated that the cytotoxicity of the drugs under investigation is not restricted to prostatic carcinoma cells. The bladder (EJ) and epidermoid (KB) carcinoma cells responded in a similar dose-dependent fashion to high concentrations of DES and its phosphorylated derivatives as prostatic carcinoma cells.

The Nontransformed Cell Line MRC-5 Is Responsive to DES and Its Phosphorylated Derivatives. High-dose DES inhibits the growth of all malignant cell lines tested. In order to evaluate its specificity as an antitumor agent we conducted control experiments using a nontransformed embryonal fibroblast cell line. These cells exhibited obvious morphological changes (Fig. 4) and a reduced growth rate (Fig. 3) upon exposure to the drugs. Untreated controls showed the typical appearance of fibroblasts, parallel arrangements of long spindle-shaped cells. After 10 days of incubation with 5 μg/ml of DES, dense foci of polygonal cells became visible. They were still surrounded by spindle-formed fibroblasts.

DES Slightly Stimulates PAP Synthesis in LNCaP Cells. In previous work we showed that estradiol stimulates PAP synthesis in LNCaP cells (12). DES is known to be a highly efficient inducer of the transcription of estrogen-regulated genes, e.g., of egg white protein genes in chicken oviduct (13). Since PAP levels in patients’ sera are used to monitor the response of a prostatic tumor to therapeutic measures, PAP induction by DES could lead to overestimation of tumor mass under DESDP therapy judging from PAP levels only. The results shown in Fig. 5 indicate that high-dose DES has a slight stimulatory effect on the PAP synthesis per cell. However, DES is a weaker inducer than estradiol (12). We ascribe the large variation in PAP synthesis to the cytotoxicity of high-dose DES which may counteract specific hormonal activities.

Discussion

DES at low concentrations (2 to 5 ng/ml in serum) (14) acts as an estradiol analogue inhibiting testicular androgen produc-
tion and causing estrogenic side effects, e.g., gynaecomastia in men. The standard DES therapy of carcinoma of the prostate (5 mg per day p.o.) seems to be based on the hormonal effects of DES only. We could not detect any inhibitory effects on growth or changes in morphology of LNCaP cells at 3 and 5 ng/ml of DES in the culture medium.

The infusion of 1500 mg per day of DESDP leads to 500- to 1000-fold higher DES levels in serum (10). At high doses DES causes a set of reactions which seem to be unrelated to its hormonal action. In a high proportion of patients relapsing on androgen-suppressing therapy for relief of bone pain, decrease of PAP serum levels and regression of the tumor and its metastases are observed (1, 2).

A specific cytotoxic activity of DES liberated from DESDP has been postulated to be the basis of its benefit to patients with advanced disease (5). It was the aim of this work to evaluate the direct inhibition of proliferation of prostatic carcinoma cells by DES and its phosphorylated derivatives in vitro. Of the three in vitro models of prostate cancer used (LNCaP, DU 145, and PC-3) the behavior of the LNCaP cell line most closely resembles that of an authentic transformed prostatic epithelial cell. Most important are its continued synthesis of PAP and its responsiveness to steroid hormones (15).

We have experimentally demonstrated that therapeutic concentrations of DESDP and its metabolic products DESMP and DES are cytotoxic to LNCaP cells. The basic postulate of the DESDP therapy has been confirmed. In order to examine the cell type specificity and the role of phosphatase synthesis by the target cells, we included 2 other prostatic carcinoma and 3 nonprostatic cell lines in this study.

We have shown that high concentrations of DES, DESMP, and DESDP are also cytotoxic to bladder and epidermoid carcinoma cell lines. Prostatic carcinoma cells do not appear to be exceptionally sensitive or resistant to synthetic estrogens, but respond in a manner similar to other neoplastic cell lines.

The nontransformed cell line MRC-5 also proved responsive to DES. A reduced proliferation rate coincided with striking morphological changes. The cytotoxic activity of DES appears not to be restricted to neoplastic cells. Our results suggest that the mechanism of action of high-dose DES involves a general regulatory or metabolic pathway.

In the context of clinical application it is of interest to know whether synthetic estrogens stimulate PAP synthesis in prostate carcinoma cells. Our experiments with cultured hormone-responsive LNCaP cells indicate that DES, at concentrations as observed in patients’ sera after infusion of high-dose DESDP, causes a slight increase of PAP production per cell. However, the stimulation is not as dramatic as observed with DES for the synthesis of the chicken egg white proteins and is not of importance when the tumor mass is estimated on the basis of PAP levels in patients’ sera.

Therapeutic efficacy is thought to be based on selective killing of tumor cells. The rationale leading to the introduction of DESDP has been an enhanced liberation of the active compound DES near the surface of phosphatase-producing cells (5). Due to the phosphatase activity in the culture medium we cannot decide whether DESDP itself is cytotoxic or not.

Phosphatase production by the target cells appears to be but one mechanism of enhancing sensitivity to phosphorylated DES derivatives.

Selective inhibition of prostatic carcinoma cells could be brought about by substantial accumulation of DES liberated from DESDP in prostatic tissue. A 100-fold enrichment of DES in prostates has been reported. The DES concentration in the prostatic tissue samples examined with a biological assay system attained 2.5 µg/g (7). According to our in vitro experiments DES should inhibit cell growth at this concentration. It would be of great interest to confirm high intraprostatic DES levels following DESDP infusion using advanced analytical equipment. Selective DES/DESDP uptake and accumulation by the prostate could explain the therapeutic value of DESDP infusions.

ACKNOWLEDGMENTS

We thank Alexandra Lepler, Irene Vetter, and Laura Chaudhouri for skillful cell culture experiments and Sylvia Dannert for PAP determinations. We are indebted to Dr. Dorothea Messerer (Institut für Medizinische Informationsverarbeitung, Statistik und Biomathematik der Universität München) for advice on the evaluation and presentation of the data.

REFERENCES

Evaluation of the Cytotoxic Activity of Diethylstilbestrol and Its Mono- and Diphosphate towards Prostatic Carcinoma Cells

Peter Schulz, Hartwig W. Bauer, Wolfgang P. Brade, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/10/2867

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.