Etoposide sensitivity of human prostatic cancer cell lines PC-3, DU 145 and LNCaP

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Summary. Metastatic prostatic cancer is typically refractory to androgen ablation therapy due to the presence of androgen-independent clones in the neoplasia. A therapeutical approach which could effectively control androgen-dependent and independent cells is, thus, needed. Maybe the failure of certain cancer cells to engage in apoptosis could explain the inherent drug resistance of many tumors. Anyway, these cells can retain the ability to undergo apoptosis in response to an adequate stimulus. We tested whether etoposide, a topoisomerase II inhibitor, could induce apoptosis in androgen-dependent (LNCaP) as well as independent (PC-3 and DU 145) human prostate cancer cell lines.

Morphological examination was performed, as it is regarded as one of the most reliable parameters for the detection of apoptotic changes. Complementarily, biochemical and flow cytometric studies were also used.

Characteristical changes of apoptosis were demonstrated in PC-3, Du 145, and LNCaP cancer cells after treatment with etoposide. These cells, thus, retain the ability to undergo apoptosis under adequate conditions, in a promising approach to hormone refractory prostate cancer therapy.

Key words: Apoptosis, Induction, Prostate cancer, Etoposide

Introduction

Metastatic prostatic cancer which is refractory to hormone therapy remains an incurable disease for which there is no effective therapy. Nearly all men with metastatic prostate cancer treated with surgically or chemically-induced castration have an initial, often dramatical, beneficial response to such androgen withdrawal therapy. While this initial response is of substantial palliative value, essentially all treated patients eventually relapse into an androgen insensitive state and succumb to the progression of their cancer. Several studies have demonstrated that the reason for this universal relapse of metastatic prostate cancer to androgen ablation is that prostate cancer within an individual patient is heterogeneously composed of clones of both androgen-dependent and independent cancer cells even before hormone therapy has begun (Kiprianou et al., 1990; Isaacs et al., 1992; Berges et al., 1993; Denmeade and Isaacs, 1996). While the concept of early combinational chemohormonal therapy for prostate cancer is valid, for such an approach to be therapeutically effective in humans, a chemotherapeutic agent which can effectively control the growth of the preexisting androgen-independent prostatic cancer cells must be available.

Observation that many types of cells undergo apoptotic cell death, an active process, have received renewed attention. An understanding that tumor cell population dynamics depends upon changes in the balance of cell loss and gain has raised the possibility of pharmacological intervention to increase cell loss by apoptosis. Many anticancer drugs initiate apoptosis and it is possible that the failure of certain cancer cells to engage in apoptosis may explain the inherent drug resistance of many tumors. (Oberhammer et al., 1993a; Schwartz et al., 1993; Schwartz and Osborne, 1993; Schwartzman and Cidlowski, 1993).

Combined hormone ablation and chemotherapy have been attempted in clinical studies with a broad spectrum of cytotoxic drugs. Attention has recently focused on the use of oral etoposide for treatment of prostate cancer. (Crawford et al., 1992; Pienta and Lehr, 1993; Pienta et al., 1994; Scherr and Fossa, 1995; Colleoni et al., 1997).

In the present work, we tested whether etoposide, a topoisomerase II inhibitor that acts at the level of nuclear matrix could induce apoptosis in androgen-dependent (LNCaP) and independent (PC-3 and DU 145) human prostatic cancer cell lines. In that case, we would be able to demonstrate that these human metastatic prostatic cancer cells still retain the ability to undergo apoptosis so that adequate chemotherapeutical approaches could improve hormone refractory prostate cancer patient development. Morphological changes of apoptosis as well as biochemical and cell cycle parameters were assessed.
Apoptosis induced in prostate cancer cells

Materials and methods

Cell lines

Three prostatic cancer cell lines were used: LNCaP, an androgen-dependent prostatic cell line derived from a lymph metastasis, supplied by American Type Culture Collection (ATCC), and two androgen-independent cell lines: PC-3, a p53 deficient prostate cell line derived from a bone metastasis (Nuclear Iberica); and Du 145, (ATCC), derived from a brain metastasis of a prostatic carcinoma.

Androgen-independent cells were grown in Dulbecco Modified Essential Medium (DMEM, ICN Flow) supplemented with 10% Foetal Bovine Serum (FBS, Serva), 4% penicillin-streptomycin (Biochrom) and 0.4% gentamycin (Gibco) under standard conditions, in a water saturated atmosphere of 5% CO₂ until the experience was started. Androgen-dependent cells were grown in RPMI 1640 (ICN Flow) under the same conditions.

Treatment protocols

All experiments were started with unsynchronized exponentially growing cultures. Cells were seeded in microplates (Nunclon) at a density of 10,000 cells/ml in each well and culture media was supplemented with 5% FBS. Then 4% penicillin and 0.4% gentamycin were added and 48 hours later shifted to medium with etoposide (Sigma) added, from 2mM stock solution in DMSO, at doses of 80 μg/ml, 100 μg/ml, and 150 μg/ml, to assess induction of apoptosis. The cells were examined at 0, 24, 48, and 72 hours of treatment.

In the androgen-dependent cell line, a second protocol including hormonal deprivation was used. Androgen withdrawal was carried out by withdrawing from RPMI containing 5% FBS after 2 days, to RPMI containing 5% Steroid Free Serum (SFS, Biogenesis) for 2 more days. The cultures were then treated as described previously adding a group treated with dihydrotestosterone (DHT, Sigma), 1nM. The same protocol used in androgen-independent cells offered no positive results, so was abandoned.

In conclusion we obtained for each cell line a control group only with culture media supplemented with 5% FBS and a group with each dose of etoposide. In androgen-dependent cell line, the same number of groups were made for the deprivation protocol, and we added a control group with 5% SFS supplemented culture medium, and a group treated with DHT.

Cell parameter analysis

Direct examination by phase contrast microscopy

With a Nikon Diaphot phase contrast microscope adapted to a photographic system, we could observe morphological changes such as cell surface alterations, blebbing, detachment and round up of treated cells.

Growth kinetics and cell viability

Determined by trypan blue exclusion, with trypan blue in culture media (0.5%). After incubation of cells with trypan blue v/v, non stained cells were regarded as viable cells, and blue cells were considered non viable, when observed in the hematocytometer.

Percentages of viable cells: Number of non stained cells/ (total cell number) x 100.

Determination of apoptotic process

For microscopical quantification of apoptotic cells, we used cytosin preparations obtained from in vitro cell cultures. Apoptotic cells round up and detach from the substrate. The sample was taken by collecting the supernatant, containing the floating apoptotic cells, followed by trypanization of the rest of the monolayer, containing healthy cells. Both fractions were added together to reconstitute the total population and then centrifuged at 1,000 rpm for 5 minutes to get the pellet. Cells were then washed twice in PBS and cytosin by means of cytoclones, at 1,500 rpm for 5 minutes. Samples were air dried and afterwards stained for their observation with light and fluorescence microscopy.

Haematoxylin-eosin stain

Air dried slides were fixed in 10% formaldehyde and stained with haematoxylin and counterstained with eosin

Apoptosis staining: (fluorescent DAPI)

Air dried slides were fixed in methanol at -20 °C for 20 minutes, air dried and stained with 4',6'-diamino-2-phenilindol DAPI (Serva) at room temperature and in darkness for 20 minutes, and mounted with antifading media O-phenilendiamine (Sigma) in glycerol (Merck) and preserved in darkness at -20 °C until examination. Fluorescence range: 300-400nm.

Percentages of apoptotic cells

After microscopical examination they were established as (number of apoptotic cells/total cell number) x 100.

Flow cytometry

Cells (10⁵) were centrifuged at 1,000 rpm for 5 min, and washed three times in PBS. The pellet was resuspended in 425 μl PBS and 25 μl propidium iodide, and 50 μl NP40 in 1% PBS was added, prior to cytometric analysis (Epics XL Coulter).

DNA fragmentation

DNA fragmentation was monitored by a gel electrophoresis method. Briefly, samples of 10⁶ were washed in PBS and resuspended in 50 μl Tris Borate
Apoptosis induced in prostate cancer cells

EDTA, pH 8 (Merck), and 2.4 μl Nonidet P40 (Sigma). Then 2 μl RNAsa (1/100), 1mg/ml, (Sigma) were added to each sample prior to incubation at 37 °C for 2 hours. 10 μl proteinase K (Boehringer Manheim) was added and incubation at 37 °C continued overnight. Samples were heated to 65 °C, and 20 μl agarose was mixed with each sample before loading them into the dry wells of a 2% agarose gel in TAE 1x (Merck). Molecular weight marker was loaded with 4 μl marker (Amresco E-261), 8 μl water and 0.25 bromophenol blue in 10%, agarose 1% (Pronadisa). The gels were run at 70 V until the marker dye had migrated 3-4 cm, and then at 15 V overnight. DNA was visualized by staining in ethidium bromide and destaining in water.

Results

Characterization of the apoptotic process

The morphology of treated and control cells analyzed in cell extensions revealed that control nuclei were round and had a blue fluorescent staining with one or two nucleoli when examined by fluorescence microscopy. When examined with light microscopy cells were round with well preserved cytoplasm and plasma membrane, and nonuniform stained nuclei with preserved nucleolus (Fig. 1).

Apoptotic cells passed through a series of morphologically identifiable stages in their pathways to death. In the initial phase, the cell losses contacted with their neighbors. The nucleus was the locus of much of the drama in apoptosis, and although the exact pattern was different and asynchronous for each cell type, in general it shrinks and its chromatin became very dense, collapsing into patches, then into crescent caps in tight aposition to the nuclear envelope (Fig. 2). The cell shrank due to a loss of cytoplasmic volume. Cells detached from their neighbors and from culture substrata, and adopted a smooth contour and the cell membrane did not become permeable to vital dyes at this stage. In a following phase, the plasma membrane became ruffled and blebbed, "zeiosis", (Figs. 3, 4) with crenation of the nuclear outline. As might be predicted from this agonized appearance, both nucleus and cytoplasm split into fragments of various sizes and the cell became a cluster of round, smooth membrane-bound apoptotic bodies, some containing nuclear fragments, others without (Fig. 5). There was no spilling of intracellular content. These bodies could be shed into the culture medium and be phagocytosed by neighboring cells or degenerate within a couple of hours. Neighboring cell phagocytosed the fragments and completely degraded them into phagosomes. In the third phase, there was progressive degeneration of residual nuclear and cytoplasmic structures. In culture cells, this was manifested as membrane rupture, producing permeability to vital dyes.

Flow cytometric study of cell cycle distribution showed an increase of fluorescence in the sub G1 region and also an accumulation of cells in the G1 area.
especially in DU 145 cells, and in G2-M for PC-3 cells as described previously for etoposide-induced apoptosis (Fig. 6). Although cells detached and exhibited classical apoptotic morphology, no subsequent DNA internucleosomal cleavage was observed in DU 145 cells, a cell line that typically exhibits resistance to the production of internucleosomal ladders (Fig. 7).

**Percentages of apoptotic cells**

Androgen-independent cell line PC-3

Maximal induction of apoptosis, 61.7%, was achieved after 48 hours of treatment with the 100µg/ml dose. Control group had 7.1% apoptotic cells, and 17%

**Fig. 2.** a-d. Apoptotic nuclei of etoposide-treated Du 145 prostate cancer cells. Chromatin collapses into patches in an asynchronous way, and cell becomes detached from its neighbors. Haematoxylin/eosin technique. ×1,000

**Fig. 3.** Androgen-dependent LNCaP cell line after treatment with etoposide. Apoptotic cells (arrow) detach from culture substrata and round up. Plasma membrane becomes ruffled and blebbled. Phase contrast microscopy. ×400
of apoptosis was found in the 80μg/ml etoposide-treated group (Fig. 8).

Androgen-independent cell line DU-145

Etoposide-treated cells (80 μg/ml dose) experimented maximal induction of apoptosis, 62%, after 48 hours of treatment. Control group reached 8.75% apoptotic cells, and 56% of apoptosis was found in the 100μg/ml etoposide-treated group during 48 hours. The latter presented higher cell damage and necrosis (Fig. 8).

Androgen-dependent cell line

In the androgen-deprived cell line LNCaP, maximal induction of apoptosis was achieved after 48 hours of treatment in the androgen-deprived group that also received 100μg/ml etoposide, with 60% of apoptotic cells. The deprived control group presented 9% apoptotic cells. In those groups supplemented with foetal bovine serum and treated with etoposide at the same dose for the same time, the number of apoptotic cells was markedly decreased (3.4%), almost matching control cells at 3.5%. 15% of apoptotic cells were counted in the DHT group (Fig. 9)

Growth kinetics and cell viability

All etoposide-treated groups presented worse viability than control groups. In some cases the
percentage of stained cells did not exactly correlate with the apoptosis percentage, due to a higher number of cells in the initial stages with non permeable membrane. (Figs. 10, 11).

Discussion

Hormone refractory prostate cancer is characterized by a low response rate following second line therapy (Colleoni et al., 1997). The development of hormone resistant tumor cells may be explained either by a multiclonal theory, by mutation of previously sensitive tumor cell clones or, most likely, by both mechanisms. Thus, vigorous efforts are needed to develop non-endocrine treatment approaches (Crawford et al., 1992; Denmeade and Isaacs, 1996). Apoptosis is a death process which involves a series of well organized events which require active cell participation and is primarily caused by physiological stimuli. Cell death induced by therapeutical agents may take the form of either apoptosis or necrosis (Lennon et al., 1991; Cohen, 1993). The primary importance of the apoptosis concept for oncology lies in its being a regulated phenomenon subject to stimulation and inhibition. It seems reasonable to suggest that greater understanding of the processes involved might lead to the development of improved treatment regimens (Kerr et al., 1993; Denmeade and Isaacs, 1996; Raghavan et al., 1997).

A wide variety of cytotoxic agents with different intracellular targets can induce the uniform phenotype of apoptosis and the propensity of cells to undergo apoptosis has been proposed to be a determinant for chemotherapy sensitivity. This implies that the cytotoxic activity of anticancer drugs is not solely dependent on

Fig. 6. Cell cycle distribution in PC-3 control cells (cc) and etoposide-treated cells (E). *: corresponding to sub G1 area.

Fig. 7. DNA internucleosomal ladder in PC-3 cells after treatment with etoposide, wells 3 and 4. Agarose gel electrophoresis. Wells 1, 2 corresponding to controls. Wells 5, 6: Du 145 cells (5: control cells; 5: etoposide treated cells).
specific drug-target interactions but also on the activity of an apoptotic machinery (Borner et al., 1995). It has been suggested that failure of the damaged cells to successfully transit cell cycle checkpoints such as the G2/M boundary may lead to apoptosis. Cells with damaged genetic material may have abortive passages through these checkpoints as a strategy for cell death. (Fesus et al., 1991; Barbiero et al., 1995; Sinha et al., 1995; Siegmund et al., 1997). Etoposide is a topoisomerase II inhibitor which selectively inhibits new DNA synthesis on the nuclear matrix and causes accumulation of cells in the G2/M phase (Barbiero et al., 1995; Pienta et al., 1996). Human autopsy tissue distribution of etoposide reveals high concentrations of etoposide on prostate tissue (Stewart et al., 1993) and several Phase I and II clinical trials with oral etoposide have been developed. Single administration of drug reveals minimal activity in hormone refractory patients (Hussain et al., 1994) and preliminary studies on combined therapy with estramustine, CDDP, pirarubicin, cis platinum, cyclophosphamide, adriamycin and metotrexate sometimes associated to radiation or hormone ablation therapy, show variable results, some promising even in stages C or D, and in metastasis of soft tissues. Slowly growing cancers seem to show a better response to chemotherapy than do rapidly proliferating ones (Amato et al., 1992; Moore et al., 1992; Akimoto et al., 1994; Debras et al., 1994; Pienta et al., 1994; Naito et al., 1995; Maulard Durdoux et al., 1996; Pienta et al., 1996; Fuse et al., 1996; Coleoni et al., 1997).

Results from experiments using cytotoxic agents in human hormone-independent prostate cancer are scarce, raising the question as to whether the relative drug insensitivity of advanced prostate cancer is due to the loss of the ability to undergo apoptosis (Kiprianou et al.,

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**Fig. 8.** Percentages of apoptosis in androgen-independent prostate cancer cell lines after 48 hours treatment with etoposide and in control cells.

**Fig. 9.** Percentages of apoptosis in androgen dependent prostate cancer cell line LNCaP after 48 hours treatment with etoposide and in control cells.

**Fig. 10.** Androgen-independent prostate cancer cell lines. Percentages of viability assessed by means of trypan blue exclusion after 48 hours treatment with etoposide respect to control cells.

**Fig. 11.** Androgen-dependent prostate cancer cell lines. Percentages of viability after 48 hours treatment with etoposide with respect to control cells.
Apoptosis induced in prostate cancer cells

1990; Oberhammer et al., 1993a,b; Schwartzmann and Cidlowski, 1993; Borner et al., 1995).

Even though all three cell lines used have been described to be relatively resistant to etoposide (Berchem et al., 1995; Borsellino et al., 1995; Chatterjee et al., 1996), we have demonstrated etoposide-induced proliferation-dependent death of androgen-dependent and independent prostate cancer cell lines PC-3, DU145 and LNCaP, based upon the temporal sequence of DNA fragmentation, morphological changes and loss of cell viability (Pienta and Lehr, 1993; Hashimoto et al., 1995; Furuya et al., 1997). Our results with androgen-independent prostatic PC-3 cell line showed that continuous exposure for 48-72 hours to a 100 μg/ml dose was required to induce apoptosis. That might be an expression for a low propensity of this cell line to undergo apoptosis. It can only be speculated that the absence of wild type p53 in these cells was a contributing factor, since this tumor suppressor gene has been shown to be an essential component of the apoptosis pathway induced by genotoxic insult (Borner et al., 1995; Denmeade and Isaacs, 1996) In Du 145 cells, a dose dependent induction of apoptosis was observed, as higher doses of cytotoxic agents tend to cause necrosis rather than apoptosis. A marked resistance of androgen-dependent LNCaP line to etoposide has been described, possibly related to growth factor interference with the apoptosis pathways that can confer resistance to cytotoxic agents. In our experiment, hormonal withdrawal was, in fact, required to achieve etoposide-induced apoptosis with a 100 μg/ml dose for 48 hours (Berchem et al., 1995; Denmeade and Isaacs, 1996).

When we routinely performed dye exclusion assays in conjunction with a cytopsin preparation stained for light or fluorescence microscopy, identification of apoptotic cells determined morphologically should precede the loss of membrane integrity determined by dye exclusion, since apoptosis is a controlled type of cell death. Since the preapoptotic cell is undergoing a "managed retreat", apoptosis must be initiated while the cell membrane is intact (Arends and Wylie, 1991; Fesus et al., 1991; Lennon et al., 1991; Kerr et al., 1993; Stewart, 1994; Earnshaw, 1995; Payne et al., 1995).

We agree with the opinion of those authors that support microscopic examination to be one of the most reliable parameters for the detection of apoptosis due to the lack of a DNA ladder formation in apoptotic models, and the difficulty to recognize apoptotic cells by flow cytometry when DNA cleavage apparently does not reach the internucleosomal level, as happened with our Du 145 cells, which exhibited resistance to the production of internucleosomal ladders. The reason for this is unclear, and could reflect a phenotypically inherent low level of an endonuclease or a topographical difference in chromatin structure such that it is inaccessible to a preexisting endonuclease. Our results suggest that the appearance of a DNA ladder is not mandatory in an apoptotic cell death defined by characteristic changes in chromatin condensation. A number of reports encompassing responses to exogenous stimuli have described the occurrence of apoptosis, assessed by morphological criteria, in the absence of DNA ladders (Arends and Wylie, 1991; Gerschenson and Rotello, 1992; Bowen, 1993; Cohen, 1993; Kerr et al., 1993; Oberhammer et al., 1993a,b; Pienta and Lehr, 1993; Schwartz and Osborne, 1993; Schwartzmann and Cidlowski, 1993; Stewart et al., 1993; Schulze Osboff et al., 1994; Stewart, 1994; Catchpoole and Stewart, 1995; Earnshaw, 1995; Payne et al., 1995; Philpott et al., 1996; Furuya et al., 1997; Sanders, 1997).

Apoptotic cells are easily identifiable by condensation of their cytoplasm, formation of surface protruberances, chromatin condensation, and nuclear fragmentation. Many of these changes appear to be directed at making the cells as "palatable" as possible to phagocytic cells and perhaps the process of zeviosis breaks the apoptotic cells up into bite-sized pieces for rapid consumption by "non-professional" phagocytes. Recognition of the cellular corpses and their removal by phagocytosis occurs without disturbance to tissue architecture or function and without initiating inflammation (Bowen, 1993; Kerr et al., 1993; Schwartz and Osborne, 1993; Schwartzmann and Cidlowski, 1993; Martin et al., 1994; Bellamy et al., 1995; Bicknell and Cohen, 1995; Evan et al., 1995; Tounetki et al., 1995).

The results obtained demonstrate that the studied androgen-dependent and independent cell lines retain the ability to undergo apoptosis induced by etoposide treatment. The implications for cancer therapy are obvious: a drug that stimulates apoptosis in the malignant cells would be therapeutically valuable and the promising results of etoposide-based chemotherapy have encouraged additional laboratory and clinical investigations to develop more effective therapy for hormone refractory disease (Cohen, 1993; Hudes, 1997).

References


Apoptosis induced in prostate cancer cells

Apoptosis induced in prostate cancer cells


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