Finasteride induces apoptosis via Bcl-2, Bcl-x_L, Bax and caspase-3 proteins in LNCaP human prostate cancer cell line

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Received October 15, 2007; Accepted December 28, 2007

Abstract. We investigated the effects of finasteride, a 5α-reductase inhibitor, on cell death machinery through the induction of apoptosis in an *in vitro* model for prostate cancer. Finasteride treatment of the LNCaP hormone-dependent human prostate cancer cell line caused the loss of cell viability and accelerated apoptosis in a concentration-dependent manner. The contents of immunoreactive procaspase-3 were examined by immunoblot analysis and the results suggest that the apoptosis induced by finasteride involves the increase of caspase-3 activity. Early cell changes that occur during apoptosis are associated with mitochondrial changes mediated by members of the Bcl-2 family of proteins. Therefore, Bcl-2, Bcl-x_L and Bax were evaluated by the Western blot analysis. The immunoreactivity for pro-apoptotic Bax was markedly increased whereas antiapoptotic Bcl-2 and Bcl-x_L expression was significantly reduced after incubation of cells with finasteride. These findings suggest that finasteride induces apoptosis in LNCaP cells via proteins of the Bcl-2 and caspase family.

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer in American and Western European males (1,2). However, it is still a major clinical problem due to late diagnosis, lack of solid prognostic parameters, and limited efficacy of available therapies (3). Prostate growth in normal and neoplastic conditions requires the intraprostatic conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase isozymes (4,5). The 4-azasteroid finasteride [17ß-(N-t-butylcarbamoyl)-4-aza-5α-androst-1-en-3-one] is a 5α-reductase inhibitor to be clinically approved for use in men for the treatment of human benign prostatic hyperplasia (BPH) and prostate cancer (PC) (6-9). These clinical applications are based on the ability of finasteride to inhibit the type II isoform of the 5α-reductase enzyme, which is the predominant form in human prostate, although type I isoform is also expressed (10,11). PC responds initially to finasteride therapy by inhibiting cellular proliferation and inducing apoptosis (12-14).

Apoptosis is the physiologically relevant mode of programmed cell death that counterbalances cell proliferation and tissue homeostasis in the normal prostate gland and it is maintained by the quantitative relationship between the rate of cell proliferation and the rate of apoptotic cell death (15). The central components of apoptotic pathways are the proteases of the caspase family. Caspase-3 is a potent effector of apoptosis triggered via several different pathways in a variety of mammalian cell types, and it is one of the most important caspases activated downstream of cytochrome c in the cytochrome c-dependent apoptosis pathways (16). Regulation of apoptosis is a complex process which involves a number of cellular genes including Bcl-2 and Bcl-2 related family members such as Bcl-x_L, Bad and Bax (17). The Bcl-2 gene product protects cells against apoptosis in a variety of experimental systems. Over-expression of Bcl-2 suppressed the initiation of apoptosis in responses to a number of stimuli, including anticancer drugs (18,19). Thus, cancer cells may primarily depend on Bcl-2 or related family members to prevent cell death.

The present study was undertaken to investigate the induction of apoptosis by finasteride in order to elucidate cell death mechanisms to implement effective therapeutic strategies for prostate cancer. For this purpose, we have employed several methods including measurement of cell viability, flow cytometry and fluorescence microscopy. Also, caspase-3, Bcl-2, Bax and Bcl-x_L proteins were assessed in order to determine the apoptotic machinery. In this study, we employ the LNCaP human prostate cell line (20) because it is known to be androgen dependent (21) and because both type I and type II isoforms of the 5α-reductase are present in this prostate carcinoma cell line (14).

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Key words: finasteride, apoptosis, Bcl-2, Bcl-x_L, Bax, caspase-3, LNCaP cells, prostate cancer

Materials and methods

Chemicals. The prostate cancer cell line LNCaP was purchased from the American Tissue Type Culture Collection (Rockville, MD). Tablets of Proscar® were kindly supplied by Merk Sharp and Dohme España, S. A. Media, antibiotics and sera were from Gibco (Grand Island, NY, USA). Cell Proliferation Kit I was from Roche (Mannheim, Germany). Propidium iodine, 4',6-diamino-2-phenylindole (DAPI), dimethylsulfoxide (DMSO), NP-40, sodium dodecyl sulphate (SDS) and aqueous mounting medium were from Sigma (St. Louis, MO, USA). Anti-actin, anti-procaspase-3, anti-Bcl-2, anti-Bcl-x_L, anti-Bax and horseradish peroxidase conjugated anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL Chemiluminescence detection kit was from Pharmacia Biotech Europe (Freiburg, Germany). Prestained SDS-PAGE standards and dye reagent for protein assay were from Bio-Rad (Munich, Germany). All other chemicals were of the highest grade available from commercial sources.

Extraction of finasteride. Finasteride was extracted as previously described (22) with small modifications. Briefly, tablets of Proscar®, each containing 5 mg of drug, were used. The excipients were hydrous lactose, microcrystalline cellulose, pregelatinized starch, sodium starch glycolate, hydroxipropyl cellulose, titanium dioxide, magnesium stearate, talc, docusate sodium, FD&C Blue 2 aluminium lake and yellow iron oxide. Twenty-eight tablets were powdered in a mortar. The powder was transferred to a B-29 tube and extracted three times with 30 ml of ethyl acetate. The combinated extracts were filtered and evaporated. The residue was separated by thin layer chromatography on Silica gel G plates developed in ethyl acetate. The zones on the silica gel corresponding to finasteride were scraped off the plate, and the drug was extracted with ethyl acetate. The evaporation of the solvent yielded the final product.

LNCaP cell cultures. LNCaP cells (passages 5-15) were routinely grown in RPMI-1640 medium supplemented with 5% of foetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 0.125 μg/ml fungizone, seeded at a density of $3x10^4$ cell/cm^2 on 75-cm^2 culture flasks or different plates, and placed in a 37˚C-incubator with a humidified 5% CO_2/95% air environment. Medium was changed every 2-3 days.

Finasteride treatments. Medium of LNCaP cell cultures was exchanged for RPMI-1640 medium containing various concentrations (0.1, 1, 10 and 50 μM) of finasteride. The cultures were left in the incubator for different time periods (0-10 days). In all cases, finasteride was added dissolved in DMSO. Samples with DMSO without finasteride were used as controls (final concentration of DMSO in the incubation 0.1%).

Cellular viability assay. After the finasteride treatments in P24 plates, cellular viability was evaluated by measuring the formation of a dark blue formazan product by the reduction of the tetrazolium ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Briefly, 50 μl of MTT (5 mg/ml) was added to each well. The microplate was incubated for 4 h. Then, 500 μl of the solubilization solution (10% SDS in 0.1% HCl) was added and the plate stood overnight in the incubator. Thereafter, the colored solution was measured in an ELISA reader at 570 and 630 nm. MTT reduction determined in control cells represented 100% cellular viability.

Detection of apoptosis. Apoptotic cells were identified by the appearance of piknotic nuclei. To identify these features, LNCaP cells were grown on glass coverslips into P24 plates. After 4 days of treatment with various finasteride concentrations, the glass coverslips were washed with PBS and the cells were fixed for 5 min at room temperature with 3.7% paraformaldehyde in PBS. Following fixation, cells were permeabilized for 5 min with 0.05% Triton X-100 in PBS. After washing again with PBS, DAPI was added to cells at a concentration of 1 μg/ml in PBS and allowed to incubate for 20 min at room temperature. Cells were washed again with PBS, mounted with aqueous mounting medium and visualized under a fluorescence microscope.

On the other hand, LNCaP cells were incubated for 4 days with different finasteride concentrations. Then, cells were dissociated with trypsin (0.25%) plus EDTA (0.05%) and pelleted by centrifugation (400 x g, 5 min). Cells were washed twice with PBS, resuspended in 475 μl PBS containing 1% NP-40 and 0.5 mg/ml RNAse and incubated 30 min at room temperature. Afterwards, the cells were stained with 0.5 mg/ml propidium iodine and the cellular suspensions were analyzed for cell cycle distribution in a FAScalibur flow cytometer (BD, San Jose, CA, USA) equipped with a 488 nm Ar-laser. The fraction of apoptotic cells were quantified by analysis of sub G1 peak (sub-diploid) cells with Win MDI 2.8 (Joe Trotter, free disposition software).

Immunoblot analysis. After treatment with finasteride for 4 days, cells were scraped in ice-cold sonication buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.1% Triton X-100, 2 mM EDTA, 2 mM EGTA and the protease inhibitors: 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml phenylmethylsulfonyl fluoride. The cell lysates were sonicated (3x5 sec at 4˚C) and centrifuged at 27,500 x g for 10 min at 4˚C. Protein content in the supernatant was determined by the Bio-Rad protein assay with BSA as a standard. Aliquots containing 50 μg of protein were added to SDS sample buffer and separated by SDS-PAGE (23). Standard proteins were also routinely electrophoresed. After electrophoresis, the proteins were transferred to nitrocellulose membranes for Immunoblot analysis by the method of Towbin *et al* (24).

Membranes were incubated at room temperature for 3 h in blocking buffer containing 5% not-fat dry milk and 0.1% of Tween-20, and then incubated at 4˚C overnight with anti-procaspase-3 (1:200 dilution), anti-Bcl-x_L (1:200 dilution), anti-Bcl-2 (1:200 dilution) or anti-Bax (1:200 dilution) and anti-ß-actin (1:2,000). After being washed three times with the blocking solution, the membranes were incubated for 90 min at room temperature with the secondary antibody (1:2,000 dilution) conjugated with horseradish peroxidase. The unbound antibody was washed out five times with Tris-

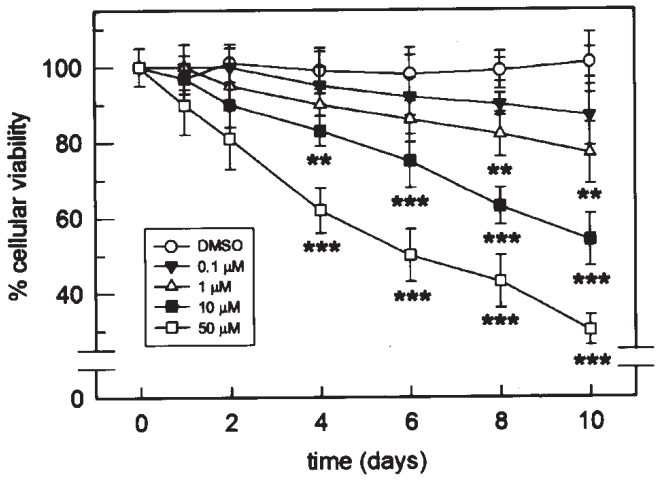

Figure 1. Effect of finasteride treatment upon the cellular viability of LNCaP cell cultures estimated by MTT reduction. Cells were incubated in absence (control and DMSO) or presence of several finasteride concentrations (0.1, 1, 10 and 50 μM) for different time periods (0-10 days). Then, MTT reduction was expressed as a percentage of cellular viability (control cells represented 100% cellular viability). Results are expressed as the mean ± SE of 6 independent determinations. Asterisks indicate significant differences from control values (**P<0.01; ***P<0.001).

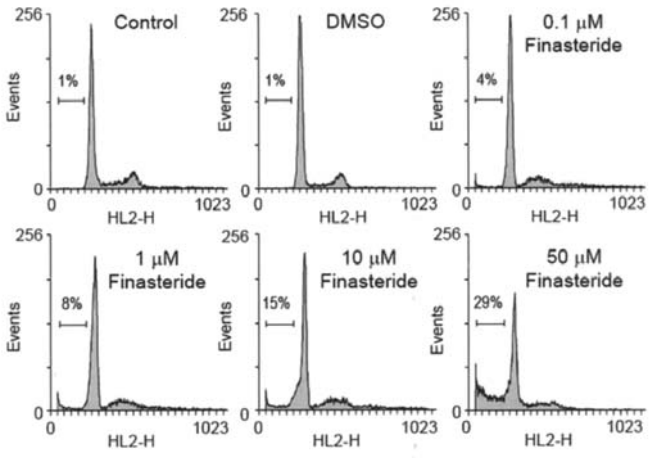

Figure 2. Effect of finasteride treatment on apoptosis of LNCaP cell cultures determined by flow cytometry. Cells were incubated in absence (control and DMSO) or presence of different finasteride concentrations (0.1, 1, 10 and 50 μM) for 4 days. Hypodiploid DNA content was evaluated by propidium iodine staining and flow cytometry as described in Materials and methods. The percentage of apoptotic cells is indicated above each hypodiploid (sub-G1) peak. A representative experiment is shown.

buffered saline. Immunoreactive bands were visualized by chemiluminescence using luminol and autoradiography, according to the instructions in the ECL Amersham kit. The autoradiograms were scanned and the level of procaspase-3, Bcl-2, Bcl-x_L and Bax expression was quantified by densitometry using image analysis software (MIP4, Digital Image Systems, Spain).

Statistical treatment of the results. Results are expressed as mean ± SE of six independent determinations. One-way analysis of variance (ANOVA) was used to determine statistically significant differences from untreated controls. Two-way analysis of variance (ANOVA) was performed to determine statistically significance of difference for multiple comparisons between treatments. Mean contrast tests were performed using Tukey-Kramer's t-test. Comparisons of two means were made using Student's t-test (GraphPad Instat). In all cases, P<0.05 was considered the statistically significant level.

Results

LNCaP cell damage caused by finasteride exposure was quantified using the MTT reduction assay. No significant differences in cellular viability from 0 to 10 days of exposure time were noted between untreated cultures and cultures incubated with 0.1% DMSO, used as vehicle for finasteride, with the techniques employed. When 0.1 μM finasteride was added to LNCaP cell cultures, no significant changes in the metabolic reduction of MTT by active cells were observed from 0 to 10 days of exposure time (Fig. 1). After 8 and 10 days of exposure with 1 μM finasteride, significant decreases of cell viability were noted with respect to the cultures incubated with 0.1% DMSO (P<0.01). The cellular viability decreased after 4 days of exposure at the highest finasteride concentrations utilized (Fig. 1, 10 μM, 4 days P<0.01, 6-10 days P<0.001 and 50 μM, 4-10 days P<0.001).

To elucidate whether finasteride induced LNCaP cell death through an apoptotic mechanism, we evaluated DNA fragmentation by flow cytometry after the propidium iodine labeling of nuclei. Results illustrated in Fig. 2 revealed that LNCaP cells treated with several finasteride concentrations (0.1, 1, 10 and 50 μM) for 4 days increase in a concentration-dependent manner the percentage of apoptotic nuclei in the subdiploid (hypodiploid) region. The first large spike in each plot represents the G1 phase (2n) of the cell cycle. The plots for finasteride treated cells show a large sub-G1 population of cells that appears as a very large spike (peak) on the left of the G1 spike (subdiploid region). This population represents the apoptotic particles (i.e., membrane-enclosed particles that contain <2n amount of DNA). After 4 days of treatment with finasteride, 4% (0.1 μM), 8% (1 μM) 15% (10 μM) and 29% (50 μM) of the cells became apoptotic in the subdiploid region. The evidence of apoptosis was also examined by fluorescence microscopy after stained with DAPI, which binds to DNA. Results in Fig. 3 show piknotic nuclei and condensed balls of chromatin in LNCaP cells treated with finasteride during 4 days.

Since caspase-3 activation is an early marker and an irreversible point in the development of apoptosis (25), we have determined the effects of finasteride on the procaspase-3 content. Caspases are expressed as inactive proenzymes and are proteolytically activated to form active enzymes (26). Therefore, caspase-3 precursor protein (procaspase-3) was evaluated by Western blot analysis. Fig. 4 presents representative immunoblots, and Fig. 5 shows the mean distribution of immunoreactive procaspase-3 from all the experiments. A significant decrease of procaspase-3 content was observed after 4 days of exposure to 0.1, 1, 10 and 50 μM finasteride (P<0.001).

Early cell changes that occur during apoptosis are associated with mitochondrial changes mediated by members

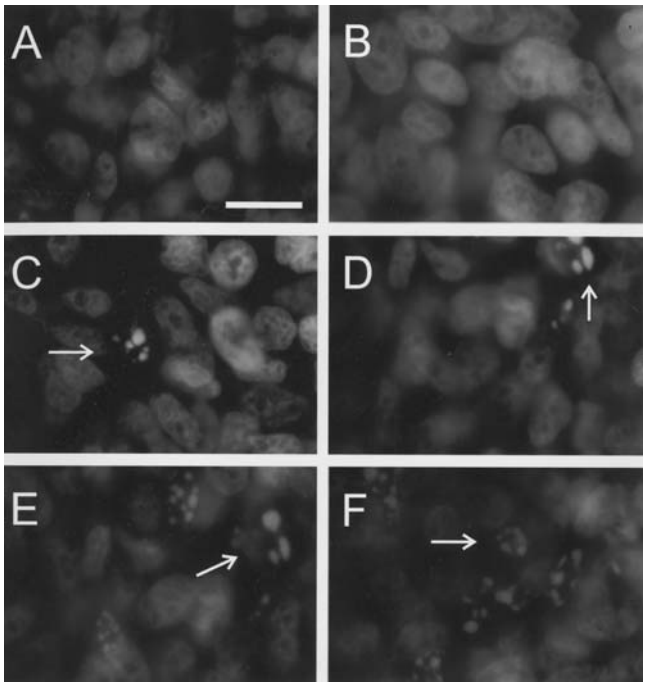

Figure 3. Nuclear morphological changes characteristic of apoptosis in LNCaP cell cultures induced by finasteride. Cells were incubated in absence [(A) control; (B) DMSO] or presence of several finasteride concentrations [(C) 0.1; (D) 1; (E) 10; (F) 50 μM] for 4 days. Following fixation and staining with DAPI, cultured were examined and photographed using a fluorescence microscope as described in Materials and methods. Some of the condensed and fragmented nuclei are indicated by arrows. Bar, 10 μm. A representative experiment is shown.

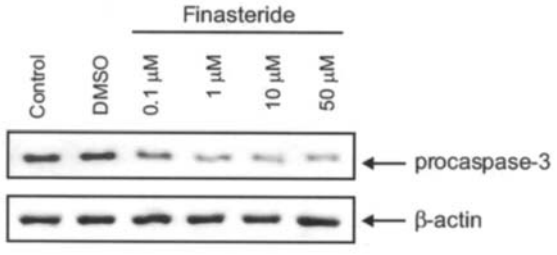

Figure 4. Effect of finasteride upon the immunoreactive procaspase-3 protein in LNCaP cell cultures. Cells were incubated in absence (control and DMSO) or presence of different finasteride concentrations (0.1, 1, 10 and 50 μM) for 4 days. Lysates containing 50 μg of protein were subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-procaspase-3 and detected using the ECL Amersham kit. ß-actin was used as a lane loading control. A representative autoradiograph is shown.

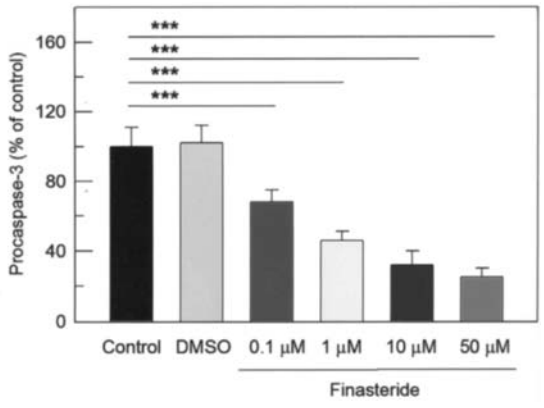

Figure 5. Quantitative analysis of the immunoreactive procaspase-3 protein in LNCaP cell cultures treated with different finasteride concentrations (0, 0.1, 1, 10 and 50 μM) for 4 days. Lysates for SDS-PAGE were prepared and Western immunoblot was performed as described in Materials and methods. The autoradiographs were scanned and the level of procaspase-3 expression was quantified by densitometry. Results are expressed as optical density presents in control cells. Each value is the mean ± SE of 6 independent determinations. Asterisks indicate significant differences from control values (***P<0.001).

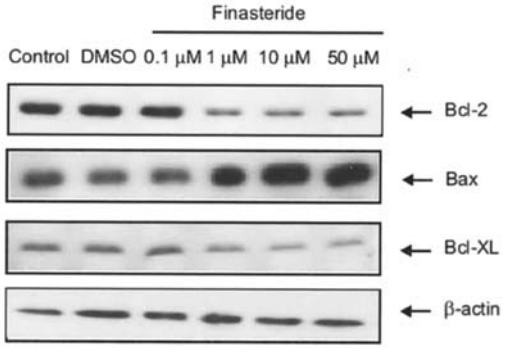

Figure 6. Effect of finasteride upon the Bcl-2, Bax and Bcl-x_L expression in LNCaP cell cultures. Cells were incubated in absence (control and DMSO) of presence of different finasteride concentrations (0.1, 1, 10 and 50 μM) for 4 days. Lysates containing 50 μg of protein were subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-Bcl-2, anti-Bax, and anti-Bcl-x_L and detected using the ECL Amersham kit. ß-actin was used as a lane loading control. A representative autoradiograph is shown.

of the Bcl-2 family of proteins, including anti-apoptotic Bcl-2, Bcl-x_L and proapoptotic Bax proteins. Therefore, Bcl-2, Bcl-x_L and Bax were evaluated by the Western blot analysis in order to elucidate the cell death machinery induced in LNCaP cell cultures by finasteride. After finasteride treatment at doses of 0.1, 1, 10 and 50 μM for 4 days the cells were collected to perform the Western blot analysis. We observed the altered expression of Bcl-2, Bax and Bcl-x_L in treated cultures comparing with the controls (Fig. 6). The immunoreactivity for Bax was markedly increased whereas Bcl-2 and Bcl-x_L expression was significantly reduced after incubation of cells with finasteride. Therefore, the results indicate a concentration-dependent decrease of the Bcl-2/Bax and Bcl-x_L/Bax ratios at all the concentrations of finasteride assayed (Figs. 7 and 8 respectively, P<0.001).

Discussion

The fundamental biological reason of the hormonal therapy of prostate cancer resides in the androgen sensitivity of the prostate, both normal and neoplastic. Human prostate carcinoma cell lines represent a good experimental model to assess hormonal therapies since most of these cell lines are derived from metastatic foci of prostate carcinoma and represent therefore a highly advanced phase growth (20). In short, the demonstration of a possible effect of finasteride on the androgen-dependent LNCaP cell line (equivalent to a less undifferentiated epithelial prostate cell line) could represent an important achievement for the proposed use

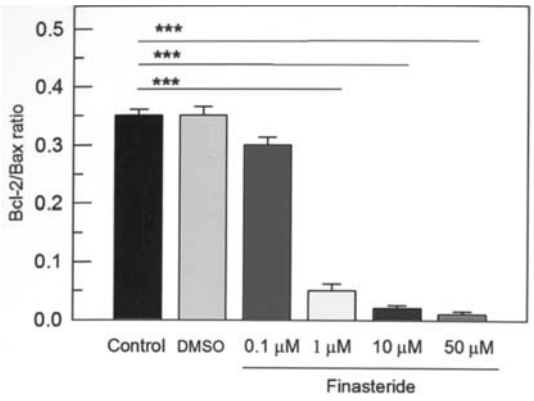

Figure 7. Quantitative analysis of the immunoreactive Bcl-2 and Bax proteins in LNCaP cell cultures treated with different finasteride concentrations (0, 0.1, 1, 10 and 50 μM) for 4 days. Lysates for SDS-PAGE were prepared and Western immunoblot was performed as described in Materials and methods. The autoradiographs were scanned and the level of Bcl-2 and Bax expression was quantified by densitometry. Results are expressed as optical density presents in Bcl-2 vs. Bax band. Each value is the mean ± SE of 6 independent determinations. Asterisks indicate significant differences from control values (***P<0.001).

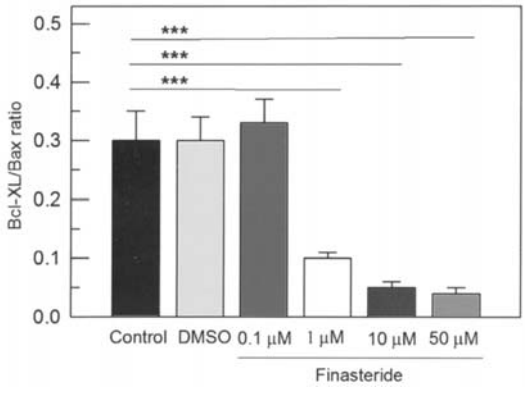

Figure 8. Quantitative analysis of the immunoreactive Bcl-x_L and Bax proteins in LNCaP cell cultures treated with different finasteride concentrations (0.1, 1, 10 and 50 μM) for 4 days. Lysates for SDS-PAGE were prepared and Western immunoblot was performed as described in Materials and methods. The autoradiographs were scanned and the level of Bcl-x_L and Bax expression was quantified by densitometry. Results are expressed as optical density presents in Bcl-X_L vs. Bax band. Each value is the mean ± SE of 6 independent determinations. Asterisks indicate significant differences from control values (***P<0.001).

of this antiandrogen in the management of early prostate cancer and perhaps also in the prevention of development of more advanced forms of this neoplasm.

This study demonstrates that finasteride significantly induces a loss of cell viability in LNCaP cell cultures in a concentration- and time-dependent manner. Under our experimental conditions, the loss of cell viability originated by the treatment of LNCaP cell cultures with finasteride is due mainly to the induction of apoptosis. However, other results (27) suggest that finasteride has no apoptotic effects in the LNCaP prostate cancer cell line, even at a high concentration. Methodological aspects may account for the differences. Thus, the discrepancy between results could be due to the different sensitivity of the apoptosis determination methods. Moreover, the content of steroid in the culture medium when carrying out the treatment with finasteride can be decisive in the study of apoptotic effect of this compound. On the other hand, and in agreement with our data, several authors have established that finasteride induces apoptotic cell death in prostate tissues of rat (13,28) and men (29,30).

Since apoptosis is closely involved in the initiation, progression, and metastasis of human prostate cancer (31), we have advanced to study the programmed cell death machinery. The mechanism of apoptosis is remarkably conserved across species, and is executed with a cascade of sequential activation of initiation and effector caspases. Caspase-3, which is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis (16), and is synthesized as an inactive precursor. Proteolytic processing of this zymogen of 32 kDa (procaspase-3) generates active enzyme (caspase-3), and this caspase-3 activation is an early and irreversible point in the development of apoptosis. This study demonstrates that finasteride significantly decreases procaspase-3 content as assessed by immunoblot analysis using a specific procaspase-3 antibody. Therefore, finasteride induces apoptosis in LNCaP cells by activating caspases. Other investigators have shown that DHT inhibited apoptosis in LNCaP prostate cancer cells (32,33). It was also found (34), that DHT protected LNCaP from radiation- and etoposide-induced apoptosis. In these experiments, LNCaP was pre-treated with DHT and decreased levels of several apoptosis regulators including caspase-3 were found. Keeping in mind that finasteride is a 5α-reductase inhibitor; it is possible to consider that DHT plays an important role in the apoptosis induced by finasteride via caspases in LNCaP cells. On the other hand, an earlier report (35) indicates a significant loss of caspase-3 protein expression in human prostatic tumors compared with the normal gland and a deregulated expression of caspases in prostate tumorigenesis. Therefore, the loss of expression of key caspases, including caspase-3, would confer protection against apoptosis in malignant prostate cells and finasteride could revert, at least in part, this process according to our results in LNCaP prostate cells.

The most common pathway of apoptosis, the intrinsic pathway, is activated from within the cell itself, such as in response to the stress initiated by damage-inducing agents like cytotoxic drugs. Apoptosis mediated through the intrinsic pathway involves the release of cytochrome c from mitochondria, an essential cofactor required for the activation of caspases and is regulated by the Bcl-2 family of proteins upstream of caspase activation (17). A major characteristic of these proteins is their frequent ability to form homo- as well as heterodimers suggesting a neutralizing competition between these proteins. Briefly, Bcl-2 and Bcl-x_L are apoptosis suppressing factors that heterodimerize with Bax and neutralize the effects of the latter. When Bcl-2 and Bcl-x_L are present in excess, cells are protected against apoptosis. In contrast, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to programmed death. Therefore, the ratios Bcl-2/Bax and Bcl-x_L/Bax determine the fate of a cell rather than the absolute concentration of either (36). The treatment of LNCaP cell cultures with 1, 10 or 50 μM finasteride originate a significant descent in the Bcl-2/Bax and Bcl-x_L/Bax ratios (P<0.001). Thus, the

imbalance between Bcl-2, Bcl-x_L and Bax expressions during finasteride treatment is believed to play a significant role in finasteride-induced apoptosis as observed here and in other experimental systems (28). Many of these apoptosis regulators have been associated with various human malignancies. For example, studies on human tumors (reviewed in ref. 31) have demonstrated an overall positive correlation between increased expression of Bcl-2 (or Bcl-x_L), decreased expression of Bax and uncontrolled tumor cell growth (due to suppressed apoptosis), and, in some cases, with tumor progression and poor prognosis of cancer patients.

In summary, a wide range of experimental and clinical reports have demonstrated biological effects for finasteride. Our current study supports the presence of a specific proapoptotic mechanism in LNCaP cultures, and showed that the 5α-reductase inhibitor finasteride causes the loss of cell viability and accelerated apoptosis in LNCaP cell line (which may correspond to a biologic model more similar to a non-invasive, low-grade tumor, in which differentiated prostate features are still predominant) in a dose- and time-dependent fashion. The apoptotic cell death induced by finasteride is regulated by the Bcl-2 family of proteins upstream of caspase activation. Thus, information gained in our present study provides novel aspects concerning mechanisms of apoptosis in the treatment by finasteride.

Acknowledgments

This work was supported by the Consejería de Sanidad de la Comunidad de Castilla-La Mancha (grant 03024-00).

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