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The mechanism of epristeride against benign prostatic hyperplasia

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Abstract

Epristeride, a 5α-reductase inhibitor, decreases prostate size and improves symptoms in men with benign prostatic hyperplasia. However, little is known about the histopathology of the prostate after treatment with epristeride. To study the relationship between apoptosis and the mechanism of epristeride in the treatment of benign prostatic hyperplasia, the induction of apoptosis by epristeride was detected and measured in vitro by: (a) observing morphological changes in cells by light microscopy; (b) comparing the relative content of dihydrotestosterone in the rat prostate epithelial cells untreated and treated with epristeride by microspectrophotometry; (c) estimating changes in cell size and DNA integrity by flow cytometry; and (d) monitoring nucleosomal DNA fragmentation by agarose gel electrophoresis. The cells treated with epristeride showed a reduction in cell size, an increase in the cytoplasm/nuclear ratio, which is indicative of the condensation of nuclear chromatin, a significant decrease in optical density at 580 nm ($OD_{580\ nm}$), and an oligonucleosomal ladder and a subdiploid peak of DNA characteristic of apoptosis. Therefore, the mechanism of epristeride in the treatment of benign prostatic hyperplasia might be apoptosis stimulated by decreasing dihydrotestosterone level.

Keywords: Benign prostatic hyperplasia; Epristeride; Apoptosis; Cell culture; Finasteride; (Rat)

1. Introduction

Benign prostatic hyperplasia is a disease of older men, and over 50% of men older than 50 years have been found, at autopsy, to have histologic evidence of prostatic enlargement (The Finasteride Study Group, 1993; Hirosumi et al., 1995.). With advancing age, there is a progressive increase in the incidence of the disease (Paulson, 1984). Due to the circumjacent relationship of the prostate to the urethra, glandular enlargement could result in compromise urinary function requiring medical treatment. Historically, therapy most often has involved surgical procedures, which remain the most effective treatment for benign prostatic hyperplasia. Until recently, transurethral resection of the prostate represented the only recognized treatment for benign prostatic hyperplasia. The medical therapy was purported to improve the symptoms of benign prostatic hyperplasia with minimal morbidity and at a substantial cost-saving relative to transurethral resection of the prostate. These are legitimate and desirable outcomes, because the morbidity and cost of prostatectomy were significant. Combined analysis of perioperative mortality has shown a mean rate of 2.4% after open surgery and 1.5% after transurethral resection of the prostate (Geller et al., 1995). During the last few years, advances have been made in alternative approaches toward manipulation of the underlying factors contributing to prostatic growth and progression of the disease, such as selective hormonal manipulation (Levy et al., 1994). Pharmacological agents designed to relax prostatic smooth muscle (α-adrenoceptor blockade) and prostatic size (androgen suppression) have recently been reported to be a safe and effective treatment for benign prostatic hyperplasia. The medical therapies include female hormone, α-adrenoceptor blockade, 5α-reductase inhibitor, etc., although they have different side effects. 5α-Reductase inhibitors include finasteride, epristeride and others. The inhibitors of 5α-reductase, the enzyme that converts testosterone to dihydrotestosterone, have been shown to retard the growth of hyperplastic prostate glands (Robinson et al., 1997). The common side effects of finasteride reported so far are decreased libido

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and impotence. Less than 1% of patients report pelvic or testicular pain, dizziness, headache, asthenia, abdominal pain, diarrhea, flatulence, nausea or rash after treatment with finasteride (Haan et al., 1997). Epristeride (SK and F 105657), a novel 5α-reductase inhibitor, is an interesting drug in the treatment of benign prostatic hyperplasia. It inhibits the rat prostatic 5α-reductase with a K_i value of 11 nM (Lamb et al., 1992; Yasuda et al., 1997) and an IC_{50} of 60 or 58 nM in vitro (Salle et al., 1995). It also reduces the prostatic growth by inhibiting the conversion of testosterone to dihydrotestosterone in the prostate. In this study, the mechanism of epristeride in the treatment of benign prostatic hyperplasia was investigated.

2. Materials and methods

2.1. Drugs and reagents

Epristeride and finasteride were kindly donated by professor Liao, and a stock solution was prepared in ethanol. RNase A, ABC kit and diaminobenzidine were purchased from Sigma. Anti-dihydrotestosterone was purchased from the Shanghai Institute of Endocrinology. DNA molecular markers were from Sino-American Biotechnology. All other chemicals were from Shanghai No 1 Reagent.

2.2. Primary culture of prostatic epithelial cell

Sprague–Dawley rats were used. The ventral prostate lobes of five male rats (110 ± 7.5 g) were aseptically removed, freed of connective tissue, and minced into fragments of approximately 1.0 mm^3 with scissors. Tissue pieces were placed in 10 ml/g wet tissue weight of a solution containing 0.1% collagenase II, 1.75 mM HEPES, 100 u/ml penicillin, 100 μg/ml streptomycin. The tissue suspension was aspirated 3 times through a 14-gauge cannula and then incubated for 30 min at 35°C with constant shaking. After cooling to 4°C, the suspension was aspirated 3 more times to dissociate the tissue. The cell suspension was then passed through a coarse screen (wire mesh, 1 mm) to remove debris and cell aggregates. Cells were collected by centrifugation at $1000 \times g$ for 5 min at 4°C. After resuspension in 5.0 ml of the solution/g original tissue, the cell suspension was passed successively through nylon screen filters with mesh sizes of 253, 160, 100 and 41 μm, respectively, to obtain a suspension of predominately single cells. Cell suspensions containing the number of cells indicated in the test were plated out into 35-mm plastic tissue culture Petri dishes, on the bottom of which were cover-slips of 20-mm diameter. The dishes contained 2 ml of a medium consisting of McCoy's 50A (Sigma) supplemented with 10% fetal calf serum, 2 mM glutamine, 1.75 mM HEPES, 100 μ/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml insulin, 10 ng/ml epidermal growth factor (EGF), 10 ng/ml cholera toxin, 5 μg/ml transferrin. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C. The culture medium was changed on the days 3, 5, 7 and 9 of culture unless otherwise indicated (Zhang et al., 1994; Freshney, 1995). The medium was replaced with serum-free medium on the 15th day. Exponentially growing cells were not harvested, but were washed with phosphate buffer solution (PBS) three times, counted under a light microscope (5.0×10^5 cells/ml), and finally exposed to various concentrations of epristeride (180 and 360 nM) and finasteride (360 nM) for 4 days. On the seventeenth day, epristeride and finasteride were added to the Petri dishes at the same concentration when the medium was changed. Each concentration group included ten Petri dishes. One of the dishes was used for morphologic observation, and the rest were equally divided into three groups for dihydrotestosterone detection, DNA gel electrophoresis and flow cytometric analysis.

2.3. Morphology of prostate epithelial cells

After 4 days of drug exposure, on the 19th day, prostate epithelial cells on cover-slips were harvested, washed with PBS, fixed in methanol for 15 min at room temperature, and stained with hematoxylin and eosin for the purpose of observation and photograph under a microscope.

2.4. Immunohistochemical staining for dihydrotestosterone

The prostate epithelial cells on cover-slips were fixed in formalin–acetic acid–alcohol (FAA fixation) (Hattori, 1992). The fixed cells were stained for the presence of dihydrotestosterone with the double-antibody ABC immunodetection method (Bauer et al., 1992). The cells were first treated with antibodies and the antigen–antibody complexes were visualized with the indirect peroxidase technique. The first antibody was followed by incubation with the second antibody. After repeated washing with PBS and exposure to diaminobenzidine, the specimens were dehydrated, and put upside down onto a slide prior to microscopic evaluation. The optical density (O.D.) value, representative of the dihydrotestosterone level, was measured with a microspectrophotometer (MPV-SP, Leica Wetzlar) at the wavelength of 580 nm.

2.5. DNA gel electrophoresis

DNA gel electrophoresis was performed as described by Yi-He Ling (Kamesaki et al., 1993; Gorczyc et al., 1993). Untreated or drug-treated prostatic epithelial cells were harvested, collected by centrifugation, and washed with PBS buffer (without Ca^{2+} and Mg^{2+}). Negative control and drug treated cells (1×10^6) were lysed with 0.5 ml lysis buffer containing 0.2% Triton X-100 at room temper-

ature for 30 min. The supernatant fractions were collected by centrifugation at 12,000 × *g* for 30 min. The DNA in these fractions was precipitated overnight with 100 μl 5 M sodium chloride and 0.5 ml 2-isopropanol at −20°C. The DNA was dissolved in 20 μl of 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA buffer. Ten units RNase were added to the samples to remove RNA before incubation at 60°C for 60 min. DNA content was determined spectrophotometrically at 260 and 280 nm; purity requirement was set at $A_{260}/A_{280} > 2$. After the addition of an equal volume of loading buffer, the DNA samples were obtained. DNA samples extracted from control and drug-treated groups were electrophoretically separated on 1.8% agarose gels in TBE (Tris 45 mM borate buffer, edetic acid 1 mM, pH 8.0), and electrophoresed at 40 V for 5 h. A group ladders of 64, 89, 124, 184, 267, 289, 328, 434 and 657 base pairs was used as a marker. The DNA in gels was visualized under UV light after staining with ethidium bromide 5 mg/l.

2.6. DNA flow cytometry

Flow cytometric analysis to determine the ratio of apoptotic cells was accomplished as described below. Nearly 5000 prostatic epithelial cells were examined by flow cytometry for each sample, using a Becton Dickinson FACSCalibur, and the cells were excited using a 488-nm argon laser. Prostatic nuclei were prepared for flow cytometric analysis, using propidium iodide as the fluorescence dye instead of diamidino-2-phenylindole. The method was developed in the FACS Lab, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were scraped from the Petri dishes and aspirated three times with PBS buffer, then passed through a nylon screen filter of mesh size 41 μm. After being washed with PBS buffer for 1 h, the cells were fixed with citrate buffer for 1 h and collected by centrifugation at 100 × *g* for 5 min; the supernatant was abandoned. The cells were digested with trypase, and then 100 mg/l RNase was added for 5 min to remove RNA. In studies with propidium iodide, the cells after the final harvest were resuspended in 1 ml of PBS buffer containing propidium iodide and were then put on ice. The cell debris was gated out on the basis of propidium iodide (50 mg/l) threshold. Cells were counted and the concentration was adjusted to 1×10^6/ml after filtration through the nylon screen filter. Cells were then analyzed with a flow cytometer (FACSCalibur, Becton Dickinson).

2.7. Statistical analysis

The results are expressed as percentages and statistical comparisons were made with X^2 ($R \times C$) test, with a *P*-value < 0.05 considered significant.

3. Results

3.1. Morphology of prostate epithelial cells

To visualize morphological changes indicative of programmed cell death, slides for subsequent microscopic examination were prepared by placing cover-slips in the bottom of Petri dishes before the cells and medium were added. The cover-slips to which the cells attached, were harvested, fixed and stained as described in Section 2. Apoptosis was considered to have been demonstrated when at least two of the following three criteria were met: (a) > 30% decrease in cell size, as determined by visual observation; (b) chromatin condensation, as evidenced by the presence of dark, heavily stained nuclei; and (c) fragmentation, as indicated by the appearance of at least three discrete masses of heavily stained nuclear material (Roboz et al., 1997). The prostatic epithelial cells treated with finasteride (360 nM, B in Fig. 1) and epristeride (180 nM, C in Fig. 1; 360 nM, D in Fig. 1) showed a reduction in cell volume (> 30% decrease) and an increase in the cytoplasmic/nuclear ratio, which is indicative of condensation of nuclear chromatin. On the 17th day, the density of cells in control, 180 nM epristeride, 360 nM epristeride and 360 finasteride dishes was approximately 2×10^6, 1×10^6, 1×10^6 and 1×10^6 cells/ml. On the 19th day, the density decreased to approximately 1×10^7, 5×10^5, 1×10^5 and 1×10^5 cells/ml, respectively.

3.2. Immunohistochemical staining for dihydrotestosterone

In preliminary test in vitro, various concentrations (45, 90, 180, 360, 720, 1800, 3600 and 7200 nM) of epristeride were added into Petri dishes containing prostatic epithelial cells. The rate of apoptosis was approximately 6, 9, 15, 45, 73, 86, 90 and 93%, respectively. Therefore, we chose 180 and 360 nM (IC_{50} = 390 nM) of epristeride in this experiment.

The prostate epithelial cells were divided into four groups: untreated cells (served as negative control), 180 nM epristeride-treated cells, 360 nM epristeride-treated cells and 360 nM finasteride-treated cells (positive control). The $OD_{580\ nm}$ value of 100 cells in each group was measured. The $OD_{580\ nm}$ values of normal cells and of the cells treated with 180 nM epristeride, 360 nM epristeride and 360 nM finasteride were 0.32 ± 0.04, 0.12, 0.08 ± 0.02 and 0.06 ± 0.01, respectively). The decrease in $OD_{580\ nm}$ value in the drug-treated groups was significant ($P < 0.01$ vs. negative control).

3.3. DNA flow cytometry

Flow cytometry was accomplished using a Becton Dickinson FACSCalibur. Nearly 5000 cells were analyzed by

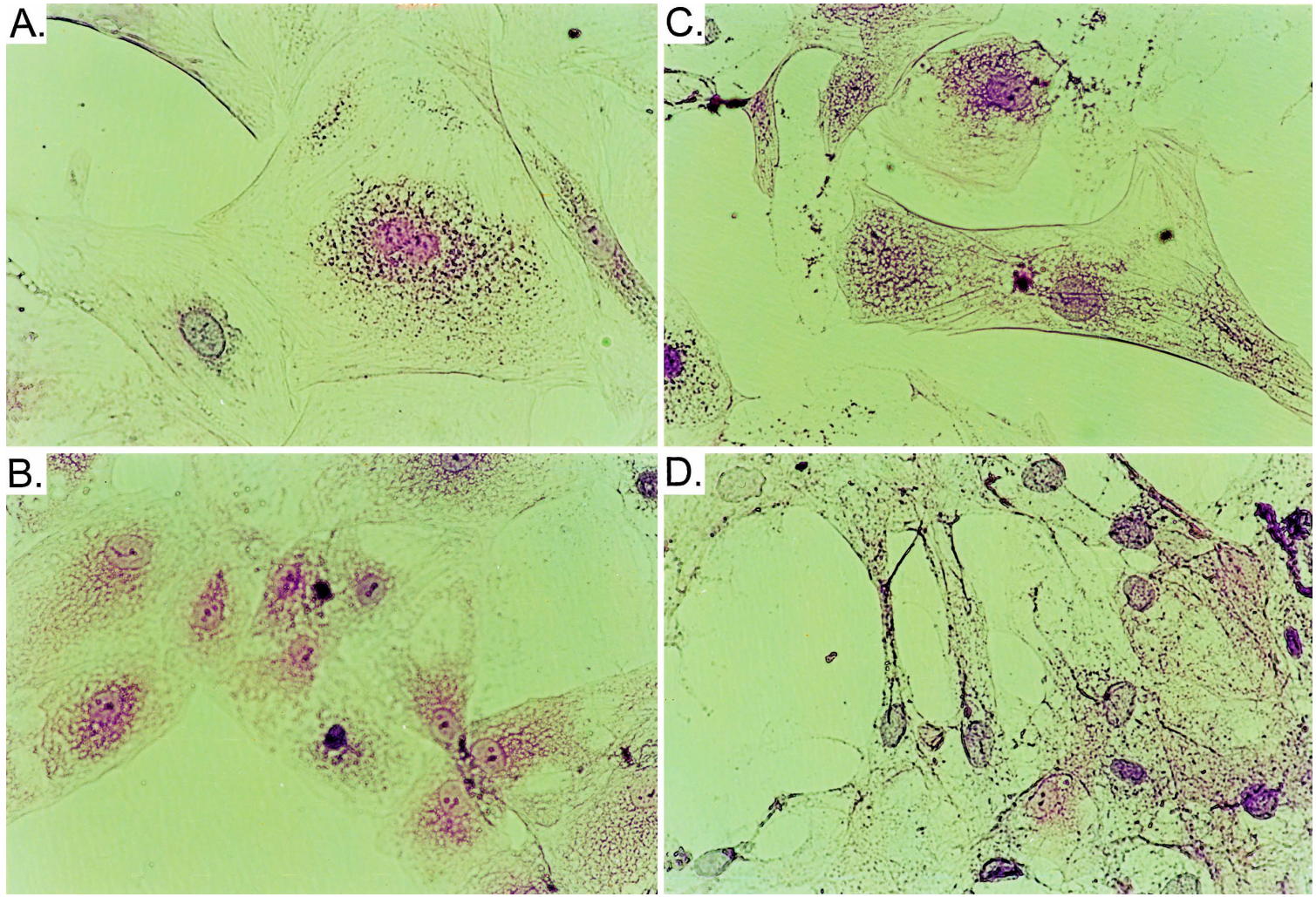

Fig. 1. Light microscopic examination of prostatic epithelial cells untreated and treated with finasteride and epristeride. Exponentially growing cells were treated with finasteride and epristeride. Following a 4-day incubation period, cells on cover-slips were fixed with methanol, stained with hematoxylin and eosin dye, and observed under a light microscope (40× objective lens). (A) untreated prostatic epithelial cells; (B) prostatic epithelial cells treated with 180 nM epristeride; (C) prostatic epithelial cells treated with 360 nM epristeride; (D) prostatic epithelial cells treated with 360 nM finasteride. (A) does not show apoptotic cells, but (B), (C) and (D) do.

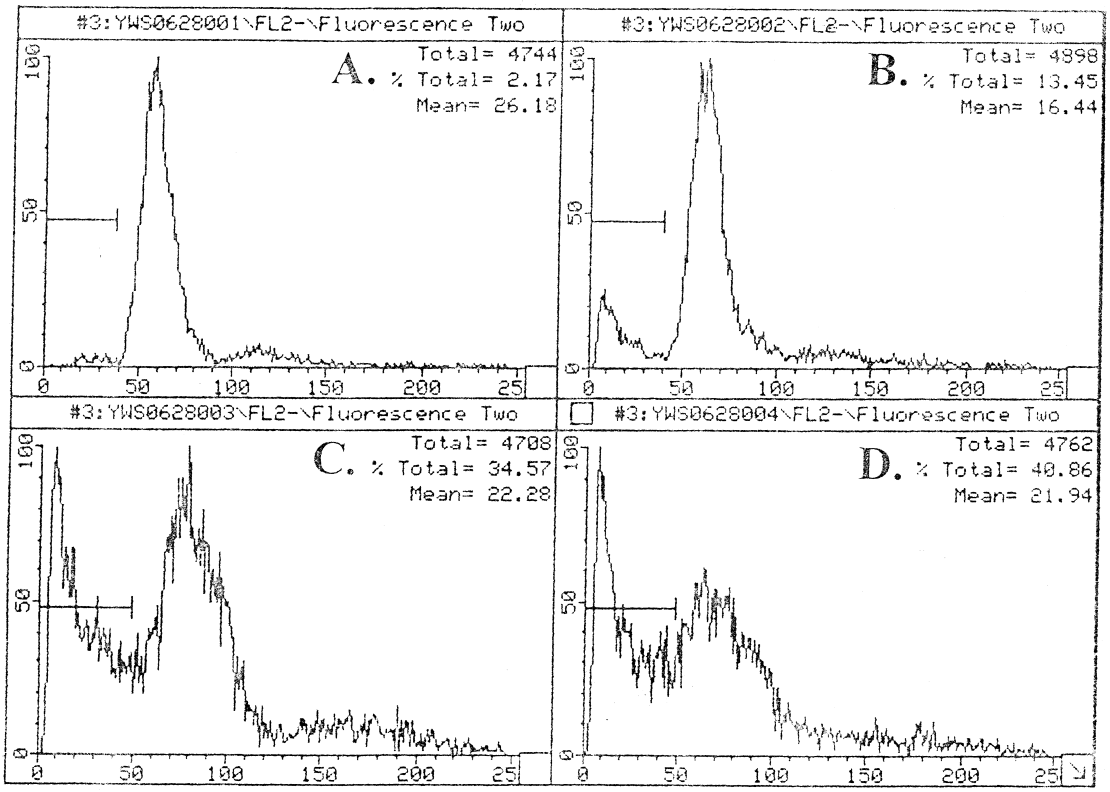

Fig. 2. Ratio of apoptotic prostatic cells in cultures treated with episteride and finasteride. (A) prostatic epithelial cells were fixed with citrate and stained with propidium iodide, and 4744 cells were examined by flow cytometry: 2.17% apoptotic cells were counted. (B) the cells were treated with 180 nM episteride, and 4898 cells were detected by flow cytometry: 13.45% of the cells were apoptotic. (C) the cells were treated with 360nM episteride and 4708 cells were counted by flow cytometry: 34.57% of the cells were apoptotic. (D) the cells were treated with 360 nM finasteride and 4762 cells were counted: 40.86% or the cells were apoptotic. See Table 1 for quantitation of the G_1, S, G_2-M and apoptotic peaks.

excitation of the dyes, and emission was examined at 488 nm. The number of cells examined in the negative control group, the 180 nM episteride group, the 360 nM episteride group and the 360 nM finasteride was 4744, 4898, 4708 and 4762, respectively (Fig. 2 Table 1). Examination of negative control prostatic epithelial cells by flow cytometry revealed essentially a population of cells that had a normal rate of apoptosis (2.17%). When episteride and finasteride activated apoptosis, a subdiploid peak of DNA characteristic of apoptosis was observed. The percentage of apoptotic cells increased when the inhibitors were added, and the ratio of apoptotic cells in the 180 nM episteride cells, the 360 nM episteride cells and the finasteride cells was 13.45, 34.57 and 40.86%, respectively.

3.4. DNA gel electrophoresis

To study the apoptotic changes, prostatic epithelial cells were analyzed by DNA fragmentation analysis. The cells showed dramatic changes in DNA fragmentation after incubation with inhibitors for 4 days, whereas the negative control cells did not exhibit DNA fragmentation over the same period. The 180–200 base pair DNA fragments and the multiples of them that were observed in this study

Table 1
Effect in vitro of episteride and finasteride on cell cycle distribution of prostatic epithelia cell

Treatment	Cell cycle distribution (% of cycling cells)			Apoptosis (%)
	G_1	S	G_2-M	
Negative control	84.00	14.40	1.60	2.17
Episteride (180 nM)	79.20[a]	20.20[a]	0.60[a]	13.45[a]
Episteride (360 nM)	69.50[a]	27.40[a]	3.10[a]	34.57[a]
Finasteride (360 nM)	65.50[a]	24.90[a]	9.60[a]	40.86[a]

[a] Indicates a significant change ($P < 0.05$) as compared to the ratio recorded in negative control cells.
Flow cytometric analysis to determine the ratio of apoptotic cells and the cell cycle distribution of unaffected cells was accomplished as described in Section 2.

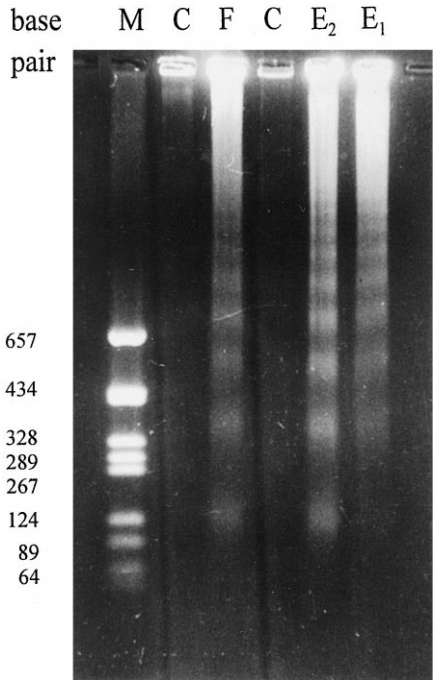

Fig. 3. Agarose gel electrophoresis of DNA from prostatic epithelial cells treated with episteride and finasteride. A typical 'ladder' indicative of DNA equivalent to the size of single and oligo nucleosomes is characteristic of apoptosis. The lane marked 'M' represents the DNA markers. The size of each band is, from bottom to top 64, 89, 124, 184, 267, 289, 328, 434 and 657 bp. The untreated cells served as control (C). E_1 and E_2, 180 nM and 360 nM episteride-treated cells, respectively. F, 360 nM finasteride-treated cells. The prostatic epithelial cells were treated with episteride and finasteride for 4 days. The cells were harvested and the fragmented DNA was prepared and electrophoresed as described in Section 2.

represent a characteristic fragmentation pattern attributed to apoptotic phenomena (Shao et al., 1997). The identification of apoptosis by observation of these DNA fragmentation patterns was carried out using procedures suggested previously (Hotz et al., 1992). The appearance of an oligonucleosomal ladder in drug-treated cells indicates the breakage of double-stranded DNA at the linker region between nucleosomes. The DNA ladder was not observed in negative control cells (see Fig. 3). Thus, apoptosis was activated by episteride. There was also a significant decrease in the dihydrotestosterone level in the episteride-treated cells.

4. Discussion

Symptoms of prostatism related to benign prostatic hyperplasia result from two major causes. The first and most important of these is the growth of the prostate gland with development of a periurethral adenoma, which compresses the urethra, resulting in obstruction of the flow of urine from the bladder. The other cause is increased smooth muscle tone of the bladder neck and of the prostate, which is regulated by α_1-adrenergic receptors (Geller et al., 1995). The cellular availability of sufficient amounts of dihydrotestosterone is thought to be a prerequisite for the normal growth and function of the human prostate. Moreover, the development of human benign prostatic hyperplasia seems to be a pathobiological process that is at least in part dependent on dihydrotestosterone (Krieg et al., 1995). Dihydrotestosterone, the 5α-reduced metabolite of testosterone, is the active molecule triggering androgen action. As a consequence, the conversion of testosterone to dihydrotestosterone by 5α-reductase is a key step in this mechanism, and the target tissue concentration rather than the plasma dihydrotestosterone level is the important parameter (Mestayer et al., 1996). This transformation of testosterone to dihydrotestosterone may be blocked by castration or by 5α-reductase inhibitors, for example, finasteride and episteride. Both approaches cause a decline in the dihydrotestosterone level. In this study, the decline in the dihydrotestosterone level in drug-treated cells was measured.

Apoptosis is a form of programmed cell death through which specific cells are lost when exposed to a certain drug (Thompson, 1994). During apoptosis, the nucleus and the cytoplasm condense, and the dying cell often fragments into membrane-bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or by neighboring cells. In this way, dead cells are rapidly removed, and any leakage of their noxious and possibly dangerous content is avoided. Apoptosis is usually associated with the activation of nucleases that degrade the chromosomal DNA first into large (50 to 300 kilobases) and subsequently into very small oligonucleosomal fragments (Steller, 1995). Apoptosis is associated with the death of isolated cells, rather than with the death of contiguous patches or areas of tissue. There is no inflammatory infiltrate. Nuclear shrinkage occurs relatively early, whereas changes to the organelles and loss of membrane integrity are relatively late events. The dying cells are phagocytosed by neighboring cells, rather than by migrating phagocytes. The degraded DNA from apoptotic cells forms a characteristic ladder when analyzed by electrophoresis because endonucleases gain access to the internucleosomal regions of the DNA (Vaux, 1993).

To examine apoptosis in individual cells, Banerjee et al. (1995) used in situ labeling of fragmented DNA, followed by biochemical analysis of DNA integrity on agarose gels. The results clearly indicated that castration caused apoptosis of the prostate of the rat (Banerjee et al., 1995). It has also been reported that a progressive decrease in epithelial cell size and function occurs during the first few months in the prostates of men treated with finasteride, and that an increased rate of apoptosis occurs transiently in these prostates (Rittmaster et al., 1996; Padayatty et al., 1997). Finasteride is a competitive inhibitor of 5α-reductase, and episteride has been shown to be an uncompetitive inhibitor of both human 5α-reductase isoenzymes. The mechanisms of the inhibition of both isoenzymes involve

the formation of a ternary complex with epristeride, $NADP^+$, and enzyme. The dosage of finasteride used clinically would increase with the testosterone level in prostate, whereas epristeride would not. These results suggest that this 3-androstene-3-carboxylic acid is a specific and selective inhibitor of the human type 2 5α-reductase, and that epristeride is an attractive compound for further investigation as a safe and effective therapeutic agent for the potential treatment of disease states associated with dihydrotestosterone-induced tissue growth (Levy et al., 1994). In this study, prostatic epithelial cells treated with epristeride showed a reduction in cell volume (> 30% decrease), an increased cytoplasmic/nuclear ratio in some cells, which is indicative of condensation of nuclear chromatin, and a decreased level of dihydrotestosterone. To assess the presence of apoptosis histologically, we chose other two events that are characteristic of tissues undergoing apoptosis: DNA ladder and increased rate of apoptotic cells. The percentage of apoptotic cells increased after the inhibitors were added, and the DNA ladder appeared in the drug-treated cells but not in the control cells. These results indicate that epristeride reduces the number of prostatic epithelia cells via an apoptotic mechanism stimulated by the decline in the level of dihydrotestosterone in the cell.

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