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Testosterone Metabolism in Primary Cultures of Human Prostate Epithelial Cells and Fibroblasts

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We compare testosterone (T) metabolism in primary cultures of epithelial cells and fibroblasts separated from benign prostate hypertrophy (BPH) and prostate cancer tissues. In all cultures, androstenedione (Δ4) formed by oxidation of T by 17β-hydroxysteroid dehydrogenase (17β-HSD) represented 80% of the metabolites recovered. The amounts of 5α-dihydrotestosterone (DHT), formed by reduction of T by 5α-reductase (5α-R), were small: 5 and 2% (BPH) and 8 and 15% (adenocarcinoma) for epithelial cells and fibroblasts, respectively. Northern blot analysis of total RNA from epithelial cells (BPH or adenocarcinoma) attributed the reductive activity to the 5α-reductase type 1 isozyme and oxidative activity to the 17β-HSD type 2. In cancer fibroblasts, only little 17β-HSD type 2 mRNA was detected. The 5α-reductase inhibitors, 4-MA (17β-(N,N-diethyl)carbamoyl-4-methyl-4-aza-5α-androstan-3-one) and finasteride, inhibited DHT formation with a preferential action of 4-MA on epithelial cells (BPH or adenocarcinoma) and of finasteride on fibroblasts from adenocarcinoma. Neither inhibitor acted on Δ4 formation. On the other hand, the lipido-sterol extract of *Serenoa repens* (LSESr, Permixon®) inhibited the formation of all the T metabolites studied [IC_{50}s = 40 and 200 μg/ml (BPH) and 90 and 70 μg/ml (adenocarcinoma) in epithelial cells and fibroblasts, respectively]. These results have important therapeutic implications when selecting appropriate treatment options for BPH.

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INTRODUCTION

In attempts to understand the aetiology, development, and androgen responsiveness of the human prostate, in particular of hyperplastic and malignant prostatic tissue, and the mode of action of certain enzyme inhibitors, increasing attention has recently been focused on the metabolism of testosterone (T) within these tissues and in cell-lines [1–4]. In the prostate cell, T is converted by the 5α-reductase (5α-R) into 5α-dihydrotestosterone (DHT) which is further metabolized into 5α-androstane-3β,17β-diol and 5α-androstane-3α, 17β-diol [5]. In addition, there is an oxidative pathway mediated by the 17β-hydroxysteroid dehydrogenase (17β-HSD) which converts T into androstenedione (Δ4) [6] and DHT into 5α-androstane-3, 17-dione (5α-A). Prostatic 17β-HSD is a two-way enzyme exerting both keto steroid reductase and hydroxysteroid oxidase activities (Fig. 1).

Both 5α-R and 17β-HSD exist in the form of at least two isoenzymes designated in each case type 1 and type 2 [7–11]. In the human prostate, the 5α-R type 1 isozyme is expressed at a relatively low level and exhibits a neutral–basic pH optimum [7] whereas the type 2 isozyme is expressed at much higher levels and has an acidic pH optimum [8, 9]. In human placenta, type 1 17β-HSD is cytosolic and converts oestrone into

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Abbreviations: 5α-R, steroid 5α-reductase (3-oxo-5α-steroid: $NADP^+$ 4-en-oxido-reductase, EC 1.3.22); 17β-HSD, 17β-hydroxysteroid dehydrogenase (EC 1.1.1.62); T, testosterone (17β-hydroxy-4-androsten-3-one); DHT, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); Δ4, androst-4-ene-3,17-dione; 5α-A, 5α-androstanedione; 4-MA, 17β-(N,N,diethyl)carbamoyl-4-aza-5α-androstan-3-one; finasteride, N-(2-methyl(2-propyl)-3-oxo-4-aza-5α-androst-1-ene-17β carboxamide; LSESr, lipid/sterol extract from *Serenoa repens* seed.

oestradiol [12] whereas type 2 17β-HSD is microsomal and uses both oestrogens and androgens as substrates [13]. There are reports of a third 17β-HSD, present principally in testis and implicated in the development of male pseudohermaphroditism [14].

The above comments on enzyme pathways, isoforms and subcellular locations emphasize the complexity of T metabolism. Within this overall scheme, it is also necessary to consider the relationships between the rates of steroid production and removal from the prostate gland and the ability of some androgen metabolites to act as competitive substrates at several points within the pathways. Most importantly, however, special attention needs to be focused on the stroma/epithelium ratio within the prostate tissue [15–17] since it is known that these cells exhibit different metabolic activities [15] and also interact closely in eliciting prostate enlargement [18–20] in both the healthy and diseased state.

In the present study, in order to address the issue of cell specificity, we have examined T metabolism in primary cultures of stromal and epithelial cells derived from benign prostate hypertrophy (BPH) and prostate cancer tissues employing, as in a previous study on DU145 cells [1], a reverse-phase high performance liquid chromatography method with on-line radioactive detection [21] which measures the time-courses of several enzyme activities simultaneously. The isozymes responsible for metabolism in these tissues have been identified by Northern blot analysis.

MATERIALS AND METHODS

Chemicals

[1,2,6,7-^{3}H]T (97 Ci/mmol) was purchased from Amersham (U.K.). Diethyl ether (Prolabo, France) was of analytical grade. Methanol and tetrahydrofuran (Prolabo, France) were glass distilled in our laboratory. Deionized water was triple glass distilled. Unlabeled steroids were purchased from Sigma (France). 4-MA and finasteride were synthesized at the Research Laboratories of Farmitalia Carlo Erba (Milan, Italy). LSESr (lipid/sterol extract from *Serenoa repens* seed, batch* 614) was from Pierre Fabre Médicament (Castres, France).

Cell culture medium

Epithelial cells were routinely cultured in F12-DMEM culture medium without calcium supplemented with 3% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco, Middlesex, England), 1 μg/ml cortisol, 1 μg/ml insulin, 10^{-11} M retinoic acid, 0.1 μg/ml bovine pituitary extract, 50 ng/ml phosphoethanolamine, 20 ng/ml choleratoxin, 10^{-9} M DHT (all from Sigma, France), 10^{-7} M epithelial growth factor (Euromedex, Frankfurt) and 2% HEPES buffer (Biowhittaker, Maryland). Fibroblast cells were cultured in F12-DMEM culture medium with 15% fetal calf serum, 4 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Primary cultures of epithelial cells and fibroblasts

BPH and prostate adenocarcinoma specimens obtained by prostatectomy or transurethral resection were transported under sterile conditions in F12-DMEM culture medium supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 5 μg/ml amphotericin B and 0.5 mg/ml gentamycin (all from Gibco, Middlesex, England) to our laboratory where they were dissected into 1–3 mm diameter pieces. These fragments were digested with 150 U/ml collagenase and 150 U/ml hyaluronidase for 18–20 h at 37°C in DMEM medium containing 5% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine, as previously described [22]. Organoids and cells were isolated on a ficoll gradient (lymphocyte separation medium, $d = 1.077$, from Eurobio, France) which was centrifuged at 1000 *g* for 20 min. The interface was suspended in F12-DMEM medium and centrifuged at 1000 *g* for 20 min to obtain a pellet that was treated as follows: suspension in F12-DMEM and centrifugation at 1000 *g* for 20 min (repeated twice), suspension in fibroblast culture medium and centrifugation at 1000 *g* for 6 s. The fibroblasts in the final supernatant were grown in 25 cm^2 culture flasks (Corning, U.S.A.). The epithelial cells in the pellet were suspended in epithelium culture medium and grown in 75 cm^2 culture flasks.

The presence of epithelial cells and/or fibroblasts was confirmed by immunocytochemical staining using different antibodies against vimentin (Dako A/S, Denmark), desmin (Immunotech, France), prostatic

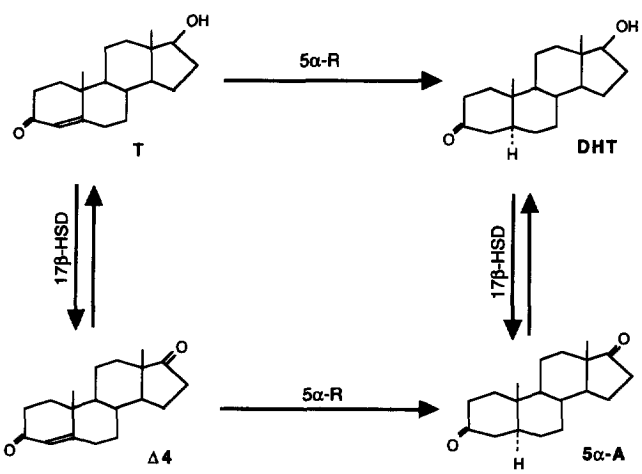

Fig. 1. Diagrammatic pathways of androgen metabolism in the prostate. T, testosterone; DHT, 17β-hydroxy-5α-androstan-3-one, dihydrotestosterone; Δ4, androst-4-ene-3,17-dione; 5α-A, 5α-androstane-3,17-dione; 5α-R, 5α-reductase; 17β-HSD, 17β-hydroxysteroid-oxido-reductase.

acid phosphatase (PAP; Amersham, UK), prostatic specific antigen (PSA; Amersham, UK) and cytokeratin (KL1 and KL4; Immunotech, France). The antigens were identified by immunoperoxidase staining.

Testosterone metabolism in epithelial cells and fibroblasts

Cells (10^5 cells/well) were plated in 12-well culture plates (Corning, U.S.A.) in their appropriate culture medium. At cell confluence, the medium was replaced by 1 ml fresh medium containing 0.1 μM T, 0.6 10^6 cpm [^{3}H]T, and various concentrations of inhibitors. The cells were then incubated for 2 (epithelial cells) or 24 h (fibroblasts) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 after which time the medium was removed and stored at −20°C. Cells were harvested by trypsinization and the cell number was determined manually in at least six dishes incubated in parallel with the inhibitors. T metabolism kinetic was realized by incubating confluent cells with 0.1 μM T and 0.6 × 10^6 cpm [^{3}H]T for different times (15–1440 min).

Radioactive T and metabolites were extracted from the incubation medium twice with 3 ml diethyl ether. The solvent was evaporated and the residue taken up in 200 μl of methanol/tetrahydrofuran/water (40/13/47, v/v/v). A 100 μl sample was injected into an automated system combining an HPLC apparatus with an on-line radioactivity detector (Flo-one, Radiomatic Instruments, La Queue-Lez-Yvelines, France) [21].

RNA extraction and Northern blot analysis

Total RNA from human tissues (placenta, meningioma, BPH or prostate adenocarcinoma) and confluent prostatic cells in primary culture were isolated by the acid guanidinium thiocyanate–phenol–chloroform method [23], analyzed (25 μg) on denaturing formaldehyde–1% agarose gels, and capillary-transferred to nylon Hybond-N filters (Amersham, U.K.). Human placenta 17β-HSD 1 and 2 probes as well as human prostatic 5α-R 1 and 2 probes were labeled with [α-^{32}P]dCTP using the random priming method [24]. Prehybridization, hybridization and washing conditions were performed as previously described [25]. Briefly, the membranes were prehybridized at 42°C for 2 h, in a solution containing 250 μg/ml of denatured salmon sperm DNA, 50% formamide, 5 × Denhardt's, 0.1% SDS, 5 × SSC, 50 mM Na_2HPO4. Hybridization was carried out in the same solution containing 5% dextran sulfate and one of the ^{32}P-labeled probes at 42°C for 16 h. The membranes were washed sequentially in 2 × SSC containing 0.1% SDS at 55°C for 1 h, in 0.5 × SSC–0.1% SDS at 60°C for 30 min and in 0.1 × SSC–0.1% SDS at 65°C for 30 min, and exposed for a week to X-ray films at −80°C (Hyperfilm-MP, Amersham, U.K.).

Table 1. Immunocytochemical staining of epithelial cells and fibroblasts in primary culture

	BPH and cancer cells	
	Epithelial cells	Fibroblasts
Prostatic specific antigen	+	−
Prostatic acid phosphatase	+	−
Cytokeratin		
KL1 (56 kDa)	+	+/−
KL4 (50–67 kDa)	+	−
Vimentin	+	+
Desmin	−	−

Intensity of staining: (+) positive; (−) negative; (+/−) patchy.

RESULTS

Characteristics of the cell cultures

Using the technique of collagenase digestion, it was possible to establish and serially culture pure populations of both human prostate cells in well-defined media. The type of cell, epithelial cell or fibroblast, was checked by immunocytochemical staining. A typical staining pattern indicating efficient cell separation is given in Table 1.

Epithelial cells in culture reached confluency in approx. 7 days. Cells examined by light microscopy were characteristically polygonal in shape and closely associated with each other. Fibroblasts were confluent after about 10 days. Confluency was generally reached at a lower cell population density than for epithelial cells. Cultured fibroblasts were characteristically elongated, spindle-shaped, and loosely associated with each other.

T metabolism in cultured epithelial cells and fibroblasts

In the following experiments, neither the composition of the culture medium, nor the number of cultured cell passages influenced T metabolism (data not shown). In BPH and prostate cancer epithelial cells, T was entirely transformed within 4 h (Fig. 2). Δ4-androstenedione was the first and main (80%) metabolite formed. 5α-androstanedione was more abundant in cancer cells than in BPH cells (35 and 15% of the metabolites, respectively). Under these experimental conditions, DHT represented only 5% of the metabolites formed. In BPH fibroblasts, a similar metabolic profile was observed only after 6 h and metabolite levels remained lower than in epithelial cells. In cancer fibroblasts, T biotransformation was very slow. Metabolites began to be detected 4–6 h after incubation. After 24 h, 70% of T was still detectable. The results are summarized in the top part of Table 2.

Enzymes responsible for T metabolism

Total RNA content in confluent cultured cells and prostate tissue was examined in Northern blots

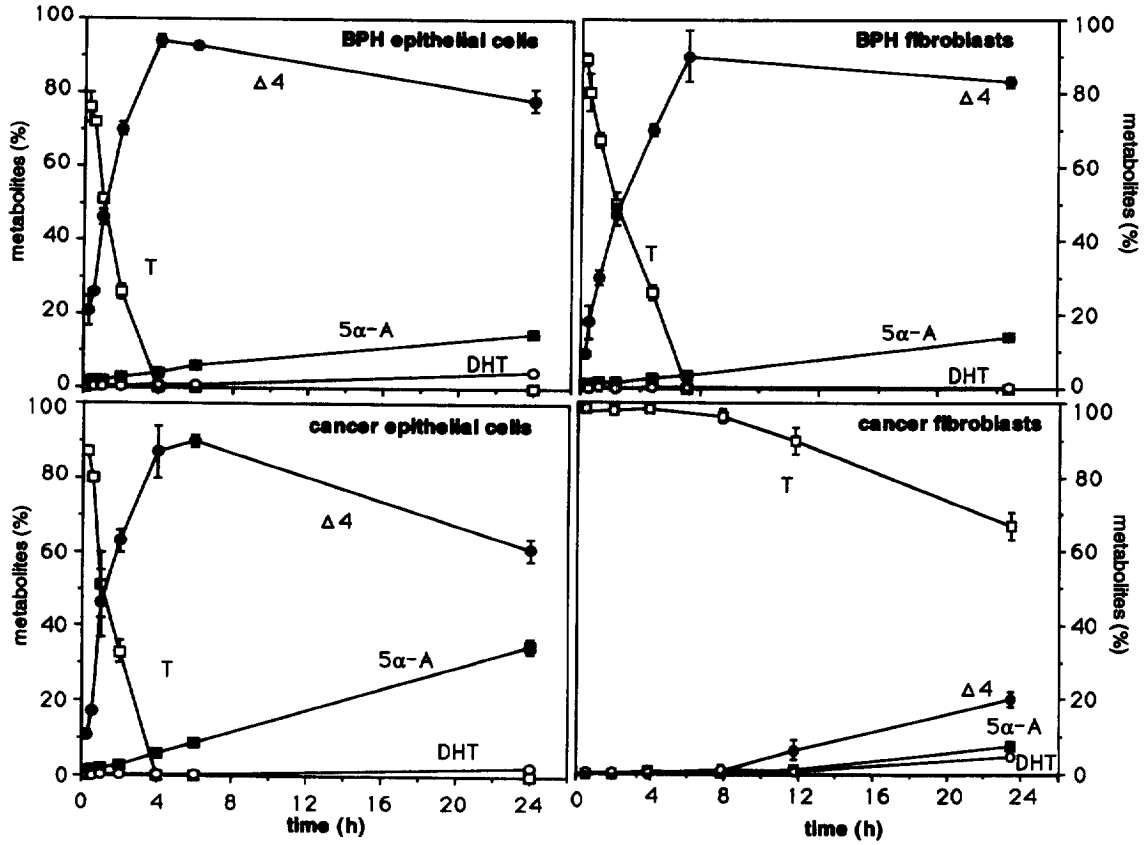

Fig. 2. Time course of T metabolism in human prostate epithelial cells and fibroblasts in primary culture. Confluent BPH and cancer cells were incubated with 0.1 μM T and 0.6 × 10^6 cpm [^{3}H]T for 15–1440 min. Results are expressed as percent of produced steroids. Each point is the mean of three experiments.

(Figs 3 and 4). 5α-R type 1 mRNA and 17β-HSD type 2 mRNA were found in BPH and adenocarcinoma epithelial cells. However, no 5α-R type 1 mRNA was detected in the corresponding fibroblasts whereas some 17β-HSD type 2 mRNA (weak band of 1.5 kb) was observed in the cancer fibroblasts. Neither type 2 5α-R nor type 1 17β-HSD were detected in any culture cells. Experiments on BPH and cancer tissues revealed the presence of the 5α-R type 2 mRNA only.

Meningioma and placenta tissues were used as control tissues. As expected [10, 25], the mRNAs of

Table 2. Testosterone metabolism and inhibition in primary cultures of epithelial cells and fibroblasts*

	Epithelial cells			Fibroblast cells		
	Δ4	5α-A	DHT	Δ4	5α-A	DHT
Testosterone metabolism (pmol/10^6 cell/24 h)						
BPH (n = 3)	65 ± 3	12.5 ± 1	3.7 ± 0.4	53 ± 25	8 ± 4	0.8 ± 0.4
Cancer (n = 3)	40 ± 3	23 ± 2	5 ± 2	53 ± 3	3.5 ± 1	2.4 ± 1
Inhibition IC_{50}						
BPH (n = 3)						
4-MA (nM)	Not active	10	20	Not active	25	50
Finasteride (nM)	Not active	500	400	Not active	30	30
LSESr (μg/ml)	40	60	30	200	30	10
Cancer (n = 3)						
4-MA (nM)	Not active	3	7	Not active	40	15
Finasteride (nM)	Not active	>100	>100	Not active	40	20
LSESr (μg/ml)	60	70	70	70	70	<10

*Confluent cells were incubated (2 h for epithelial cells and 24 h for fibroblasts) with 0.1 μM testosterone, 0.6 × 10^6 cpm [^{3}H]T, with or without inhibitor. Radioactive steroids were extracted from incubation medium and analyzed by HPLC as detailed in Materials and Methods.

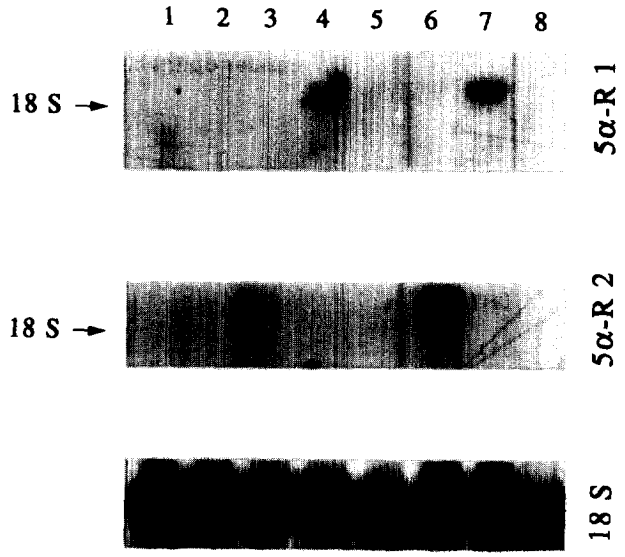

Fig. 3. Northern blot analysis using 5α-reductase type 1 and type 2 cDNA probes. Total RNA (25 μg) from the sources indicated below were processed as described under Materials and Methods. 18S RNAs are indicated on the left. Lane 1, human placenta; lane 2, human meningioma; lane 3, BPH tissue; lane 4, BPH epithelial cells; lane 5, BPH fibroblasts; lane 6, human prostate adenocarcinoma tissue; lane 7, human prostate adenocarcinoma epithelial cells; lane 8, human prostate adenocarcinoma fibroblasts.

both 17β-HSD were detected in the placenta whereas the type 2 mRNA was observed in meningioma.

Effect of 5α-reductase inhibitors on T metabolism

In both BPH and cancer epithelial cells (Fig. 5), 5 nM 4-MA inhibited DHT formation by 30% and 5α-A formation by 40% whereas 100 nM finasteride was required to obtain this level of inhibition. In BPH and cancer fibroblasts (Fig. 6), 4-MA and finasteride inhibited DHT and 5α-A formation with comparable IC_{50}s (between 15 and 50 nM), equivalent to the IC_{50} of 4-MA in BPH epithelial cells (10–20 nM). Neither inhibitor had any effect on Δ4 formation. The LSESr inhibited the formation of all metabolites. These results are summarized in the bottom of Table 2.

DISCUSSION

Our experimental design enabled us to compare epithelial cells with fibroblasts and hyperplastic with

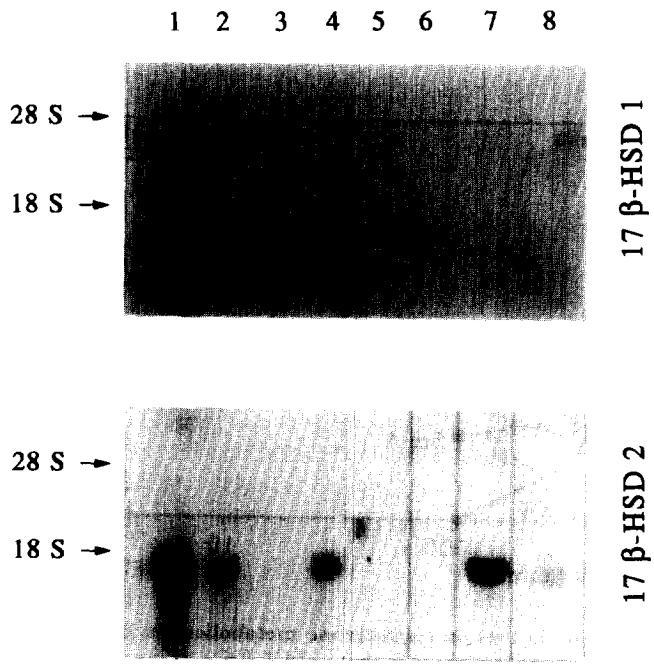

Fig. 4. Northern blot analysis using 17β-HSD type 1 and type 2 cDNA probes. 28S and 18S RNAs are indicated on the left. For details, see legend to Fig. 3 and Materials and Methods.

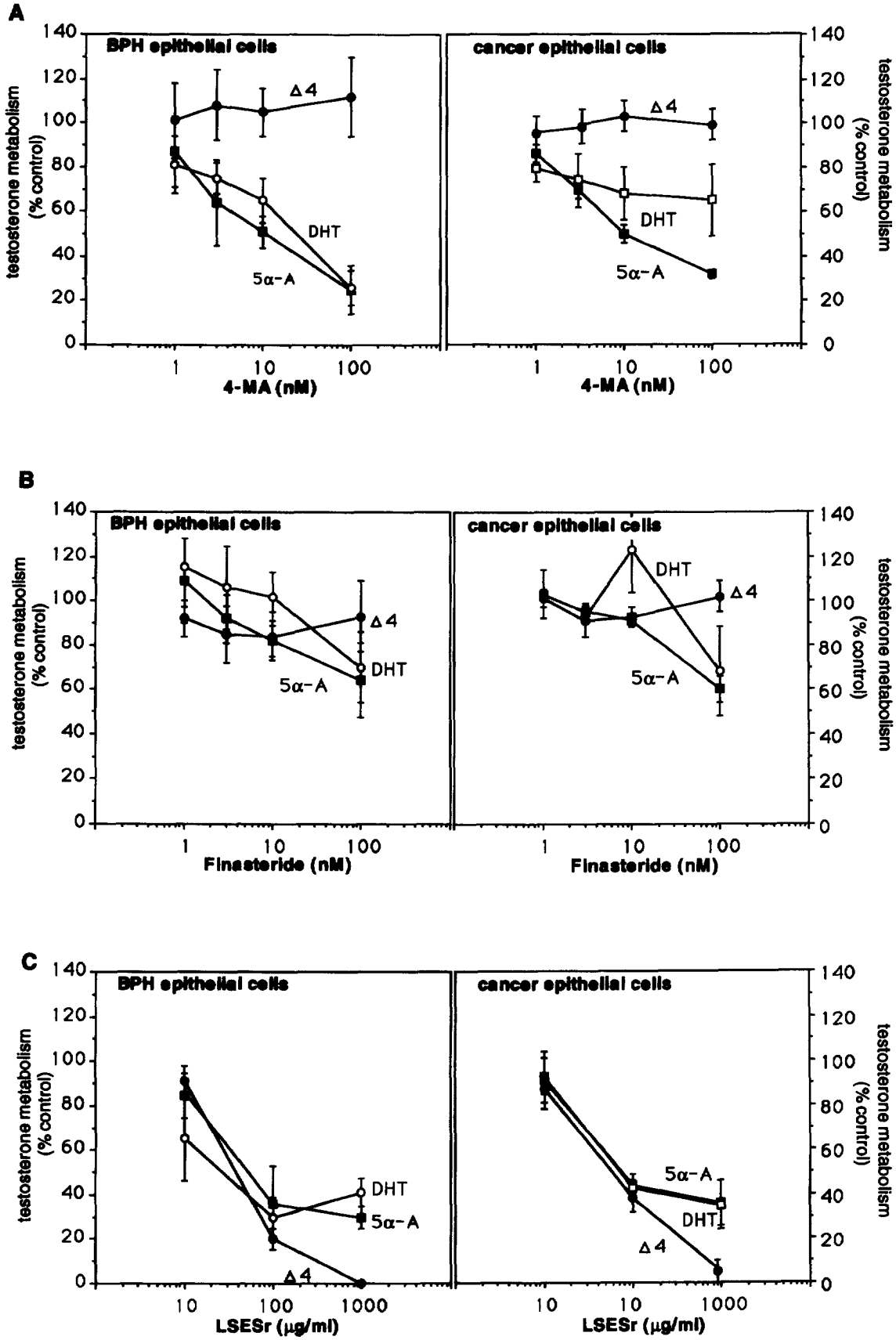

Fig. 5. Effects of 5α-reductase inhibitors on testosterone metabolism in BPH and cancer epithelial cells in primary culture. Confluent cells were incubated with 0.1 μM T, 0.6 × 10^6 cpm [^{3}H]T and various concentrations of 4-MA (A), finasteride (B), and LSESr (C). Results are expressed as percent control (without inhibitor). Each point is the mean of six experiments.

malignant prostate tissue. In all instances, T conversion into Δ4 by 17β-HSD was the predominant activity. Epithelial cells converted T into high levels of Δ4 within 4 h regardless of whether the cells were of hyperplastic or malignant origin. Fibroblasts operated this conversion slightly slower (6 h) when derived from BPH tissue and very much slower (>24 h) when derived from an adenocarcinoma. In fact, the metabolic profile of BPH fibroblasts was highly analogous to that of epithelial cells. These results support observations

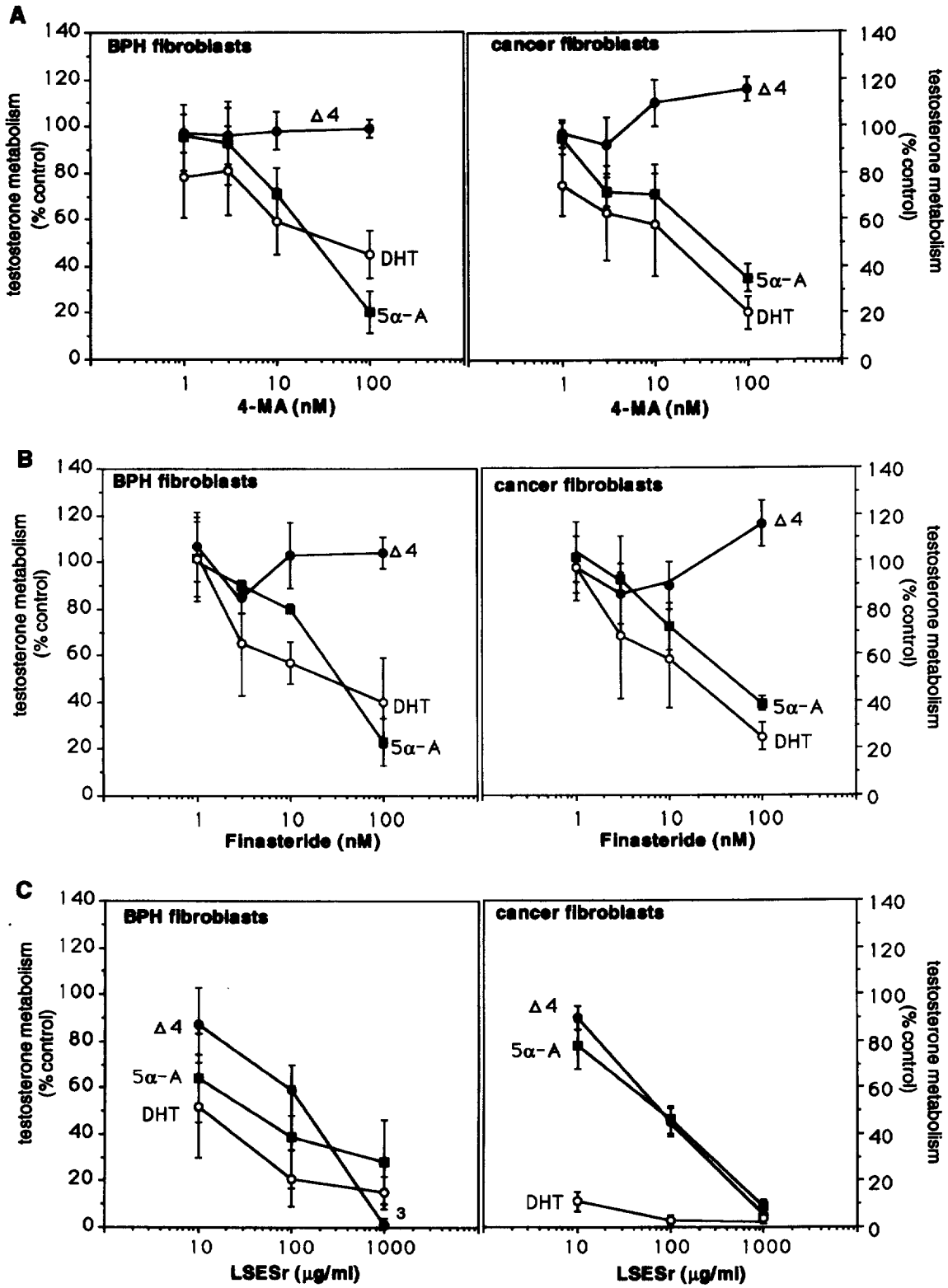

Fig. 6. Effects of 5α-reductase inhibitors on T metabolism in BPH and cancer fibroblasts in primary culture. For details see legend Fig. 5.

published for primary cultures of immature rat ventral prostate [15] and human prostate fibroblasts [16]. They also support more recent studies [2] showing Δ4 formation by cultured epithelial cells and indicating that several human prostatic carcinoma cell lines degrade T quite differently, favoring alternatively reductive or oxidative metabolic pathways.

In Northern blot analysis of total RNA, cultured confluent epithelial cells were found to contain 5α-R type 1 mRNA whereas prostate tissue displayed 5α-R type 2 as previously reported [9, 26, 27]. Neither 5α-R isozyme was detected in fibroblasts. This observation is found whatever the origin of the cells or tissue (BPH or adenocarcinoma). Recently, it has been shown that freshly isolated prostate cells exhibited 5α-R type 2, whereas cells which were obtained from BPH tissue and maintained in culture for several days exhibited 5α-R type 1 [28]. These results suggest that prostate cells are able to express both 5α-R isozymes.

That prostate can also express the 5α-R 1 isoenzyme is indicated by several lines of evidence. Cultured human prostate cells tested with an apparently selective inhibitor (LY191704) were found, by this means, to express the 5α-R 1 isozyme [28]. *In situ* mRNA hybridization and immunohistochemical analysis on regenerated rat ventral prostate have demonstrated that 5α-R type 1 mRNA expression is largely confined to the basal epithelial cells whereas type 2 mRNA expression occurs predominantly in the stromal cells [29]. DU145 cells derived from a brain metastasis of an epithelial human prostate carcinoma [30] preferentially express the 5α-R type 1 isoform [1].

By Northern blot analysis, we also detected 17β-HSD type 2 mRNA which was more abundant in epithelial cells than in fibroblasts. No 17β-HSD type 1 mRNA was found in these cells. Nor did we find any significant 17β-HSD expression in prostate tissues confirming the results by Ling Wu *et al.* for 17β-HSD type 2 mRNA [11] but in disagreement with the observations of Luu-The *et al.* [31] who detected 17β-HSD type 1 mRNA with poly(A)$^+$RNA. However, as expected, we noted 17β-HSD type 1 mRNA in control placenta tissue [25, 31] although not in control meningioma tissue.

Experiments with 5α-R inhibitors confirmed previous observations [15] but also provided new insights. DHT formation in epithelial cells, which preferentially express the type 1 isoform, was, as expected [8], inhibited more markedly by 4-MA than by finasteride [respective IC_{50}s = 20 and 400 nM (BPH); 7 and > 100 nM (adenocarcinoma)] whereas in fibroblasts the two compounds were equipotent [respective IC_{50}s = 50 and 30 nM (BPH); 15 and 20 nM (adenocarcinoma)].

Phospholipids such as phosphatidylserine and phosphatidylcholine can modulate 5α-R activity [32]. The *n*-hexane LSESr differed from the above inhibitors in that it effectively inhibited DHT and also Δ4 formation, moreover in both cell-types, suggesting that it is not only an inhibitor of 5α-R activity but also of 17β-HSD activity. This more universal enzyme inhibitory action might be due to its lipid/sterol composition which doubtlessly influences the lipid environment of membrane-associated enzymes. These new data therefore provide important information to be taken into account when selecting appropriate treatment options for BPH.

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