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Testosterone metabolism in primary cultures of epithelial cells and stroma from benign prostatic hyperplasia

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Abstract We studied the metabolism of testosterone in primary cultures of prostate epithelial cells and fibroblasts obtained from patients with benign prostatic hyperplasia (BPH). The conversion of ^{3}H-testosterone in both cell cultures was predominantly to the oxidative pathway, with the formation of ^{3}H-androstenedione increasing with cell number and time of incubation. Although we also detected some 5α-reductase activity in these cells, the activity in the stroma component (0.00688 pmol/mg protein/min) was nonetheless insignificant when compared to the 5α-reductase activity in the tissue of origin (0.0616 pmol/mg protein/min) and well below the 17β-hydroxysteroid dehydrogenase activity of the same cells (0.0518 pmol/mg protein/min). The aromatase activity in our cells was also measured by two separate techniques, but neither the deuterium procedure nor the production of oestrone from androgen precursors yielded any positive results, suggesting that under these experimental conditions there was no aromatase activity within the cells. The shift from the reductive to the oxidative pathways in these primary cell cultures was reminiscent of the androgen-metabolizing enzyme profiles seen in poorly differentiated prostate cancer. Whether this transition is an obligatory step in the development of hormone refractiveness remains to be elucidated.

Key words Benign prostatic hyperplasia · Primary culture · Epithelial cells · Fibroblasts · Androstenedione 5α-dihydrotestosterone

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Introduction

The human prostate requires the presence of androgens for its normal development and maturation [17] and benign prostatic hyperplasia (BPH) does not occur in subjects who have been castrated before puberty [25]. A number of investigators have studied the metabolism of androgens in prostatic tissue [5], in epithelial cells and fibroblasts which were separated by a variety of techniques [3, 7, 12, 16, 19, 27] and in organ cultures [20, 23]. In most of these studies testosterone was reported to be mainly metabolized to 5α-dihydrotestosterone (DHT), with the bulk of the 5α-reductase activity found in the fibroblasts [10]. In this report, we investigated the metabolism of testosterone in primary cultures of both epithelial cells and fibroblasts derived from BPH tissue and compared these metabolic activities with those in the intact tissue. Unlike previous reports on the prostate, we found that the 17β-hydroxysteroid dehydrogenase oxidative activity was the predominant pathway in these primary cultures.

Materials and methods

Radiochemicals and steroids, [1,2,6,7-^{3}H]-testosterone (specifc activity 91 kCi/mmol), [4-^{14}C]-androstenedione (specific activity 59 kCi/mol) and [4-^{14}C]-DHT (specific activity 47 kCi/mol), were obtained from Amersham International, Amersham, UK. Non-radioactive steroids were purchased from Sigma, Poole, Dorset, UK. The purity of the radioactive steroids was checked every 2 months by thin layer chromatography (TLC).

Cell culture of prostatic stromal and epithelial cells

BPH specimens obtained from 11 patients with BPH by transurethral resection were transported under sterile conditions to the laboratory in RPMI 1640 (Gibco, Paisley, Strathclyde, UK) containing 5% fetal calf serum (FCS, Gibco) and stored in this medium for periods of up to 4 days at 4 °C. Stroma and epithelium were separated by a modification of a technique previously described [6, 33]. Basically, prostate specimens were minced into small cubes and incubated overnight (20 h, 37 °C) in RPMI 1640 containing 5% FCS and 600 IU/ml Worthington collagenase type I (Lorne Laboratories, Reading, UK). After incubation, the mixture was decanted into a centrifuge tube and centrifuged at 775 *g* for 10 min to sediment the cells. The deposits were resuspended in a 20-mM hydroxyethylpiperazine ethanesulphonic acid (HEPES)-based washing solution containing NaCl (7.07 g/l), KCl (0.15 g/l), glucose (0.72 g/l) and Na_2HPO_4 (0.136 g/l) at pH 7.6 to wash out the collagenase. The cells were sedimented by centrifugation and the process was repeated twice, following which the cell pellet was resuspended in 5 ml RPMI medium and the suspension was finally centrifuged at 135 *g* for 20 s. This resulted in deposits of acini, whereas the small aggregates of fibroblasts and single cells were dispersed in the supernatant. The acini deposits were placed in 75-cm^2 tissue culture flasks (Corning, Stone, Staffs, UK) containing 15 ml of the primary epithelial growth medium containing WAJC 404 medium (11.04 g/l; Kyokuto Pharmaceuticals, Tokyo, Japan), insulin (0.5 µg/l), dexamethasone (0.392 mg/l), epithelial growth factor (EGF) (10 µg/l), HEPES (6.7 g/l), $NaHCO_3$ (1.2 g/l), penicillin (100 IU/ml; Gibco) and streptomycin (100 mg/l; Gibco). The cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C and the media were changed on day 3. After approximately 5 days the cells showed a good spread and on reaching confluence subculturing of the cells was undertaken. The WAJC medium was removed and 5 ml solution containing 200 U/ml collagenase was added to detach the cells. Following the removal of the collagenase with the washing solution, the cells were resuspended in the WAJC medium and split into 24-well plates for subsequent experiments.

For the primary culture of the fibroblasts, the supernatant containing the fibroblasts was centrifuged at 775 *g* for 10 min and the deposits were inoculated into a 75-cm^2 tissue culture flask containing 15 ml of the fibroblast growth medium of RPMI 1640 medium supplemented with FCS (10%), L-glutamine (1%; Gibco), penicillin (100 IU/ml; Gibco) and streptomycin (100 µg/ml; Gibco). The culture medium was changed on days 3 and 10. The fibroblasts spread on the bottom of culture flask 10 days later. On reaching confluence, the fibroblast growth medium was removed and the cells were passaged by detaching from the flask with a 5-ml solution of trypsin (0.05%) and ethylenediaminetetraacetate (EDTA) (0.01%) and placing the pure fibroblasts in 24-well plates for subsequent experimentation.

Characterization of fibroblasts and epithelial cells

Verification of the purity of fibroblasts and epithelial cells was established by immunohistochemical staining employing a variety of primary monoclonal antibodies including: mouse monoclonal anti-human prostatic acid phosphatase (Sigma), anti-human prostatic specific antigen (Sigma), anti-human EGF-receptor (Dako, High Wycombe, Bucks., UK), anti-human cytokeratin (Dako), anti-cow vimentin (Dako), anti-human desmin (Dako), anti-human actin (Dako) and anti-human milk fat globulin (Oxoid Unipath, Basingstoke, Hants., UK). Briefly, monolayers of prostate epithelial and fibroblast growth in 24-well plates were fixed with acetone for 20 min and allowed to dry. Primary antibodies at the appropriate dilutions and as detailed in "Results" were applied to the cells and these were allowed to incubate for 30 min before they were gently washed in TRIS saline for 10 min. A peroxidase conjugate of rabbit anti-mouse immunoglobulin was then applied to the cells for 30 min at room temperature. The peroxidase enzyme was then visualized by immersion in 0.05% (V/V) diaminobenzedine with hydrogen peroxide, after which the sections were washed in water and counterstained with haematoxylin. For each staining experiment, a negative control section was included with the primary antibody omitted from the staining procedure.

Studies on the metabolism of testosterone

Fibroblasts (5×10^5 cells/well) and epithelial cells (1.15×10^4 cells/well) were plated in 24-well plates and allowed to grow for 2 days until they reached approximately 80% confluence. At this stage, culture medium was replaced with fresh medium containing radiolabelled testosterone substrate (50 nM) and cells were incubated at 37 °C for periods of up to 3 days. An additional three wells containing the medium and substrate but no cells were also included to account for the background. At the end of the incubation, the medium was collected and transferred to extraction tubes followed by the addition of 50 µl ethanol containing approximately 500 cpm [C^{14}]-androstenedione [C^{14}] DHT and [C^{14}]3α(β)-androstanediol and 20 µg each of unlabelled 3α-androstanediol, 3β-androstanediol, testosterone, DHT and androstenedione. Each tube was vortexed for 40 s and this was followed by the addition of 1 ml diethylether, a second vortex was introduced and the phases were separated by brief centrifugation. The ether was transferred to a second tube and evaporated to dryness in a vacuum oven. Repeated extraction of the reaction mixture with ether did not increase the overall recovery of either [^{3}H]-DHT or [^{3}H]-androstenedione.

The steroids were taken up in 20 µl ethanol and applied to an instant thin-layer chromatography plate (ITLC) (Gelman Instrument Co., Ann Arbor, MI, USA). The plates were developed once in dichloromethane ether, 9:1 (V/V). Using this solvent system, DHT ($Rf = 0.60$) is well separated from testosterone ($Rf = 0.38$) and androstenedione ($Rf = 0.74$). The steroids were located by spraying the plates with methanol containing 0.5 mg/ml 3,5,7,2′,4′-pentahydroxyflavone and viewing under UV light. The DHT and androstenedione spots were then cut from the plate, placed in scintillation counter vials and counted for ^{3}H and C^{14} as described previously [15].

Assay for 5α-reductase activity in tissue and fibroblasts proliferations

To assess the difference in 5α-reductase activity between the cultured cells and the tissues from which these cells were derived, prostate specimens obtained by retropubic prostatectomy were chopped into small cubes and divided into two equal aliquots. One aliquot was snap frozen in liquid nitrogen and stored at −70 °C until use whereas the other aliquot was processed for primary culture.

The prostate tissue was thawed, blotted dry and weighed. The remainder of the procedure was carried out at 4 °C. A quantity of 0.2–0.3 g finely chopped tissue and 8.8×10^6 fibroblasts was homogenized initially in a Dounce glass homogenizer and this was followed by further homogenization with a "Rotary Homogenizer" (Ystral GmbH, Dottingen, Germany). This was carried out in separate tubes in 5 vols TRIS-HCl, pH 7.4, buffer. The homogenates were filtered through glass wool to remove cell debris and centrifuged at 800 *g* for 10 min to provide a postnuclear supernatant (cytosol). The 5α-reductase activity in these cytosol specimens was assayed at 37 °C by following the conversion of [^{3}H]-testosterone to [^{3}H]-DHT. Each tube contained, in a final volume of 1 ml, 100 mM TRIS-HCl pH 7.4, 5 mM glucose-6-phosphate, 0.5 mM NADP+, 0.25 U glucose-6-phosphate dehydrogenase and 50 nM [^{3}H]-testosterone. The reactions were allowed to proceed for up to 60 min and stopped by the addition of 1 ml diethylether. Details of the remaining procedures for the extraction of the steroids, their separation on

ITLC plates and the corrections for procedure losses were described in Houston et al. [15]. Protein levels were determined by the method of Bradford et al. [4] and the activity in the fibroblast and tissue was expressed as a function of the protein concentration. All measurements were carried out in triplicate on three separate tissues and results expressed as the mean of the metabolites formed ±SD. No 5α-reductase activity measurements were carried out on epithelial cells because of the difficulty of obtaining a sufficient number of cells to measure the activity.

Studies on the aromatization of androstenedione

The aromatization activity in the primary cultures of fibroblasts and epithelial cells obtained from six BPH patients was investigated by two separate techniques: (a) the deuterium method: epithelial cells (1×10^4 cells/well) and fibroblasts (1×10^5 cells/well) were cultured in the presence of 1β-^{3}H-androstenedione (175 nM) for periods of up to 72 h. The medium (0.8 ml) was subsequently transferred to a fresh tube containing 2 ml chloroform, and the contents were vortexed prior to centrifugation. The resultant supernatant was decanted into another tube and 0.4 ml of an aqueous suspension of 5% charcoal and 0.5% dextran was added. The tubes were then vortexed, allowed to settle for 30 min and centrifuged once again. The activity of ^3H_2O released into the supernatant was assessed by counting in a liquid scintillation counter. Blank reactions were run simultaneously employing the same conditions but without the cells. Finally the cells were scraped from the bottom of the plates, homogenized and the activity of ^3H_2O released was assessed as described above. The details of the procedure have been described previously [1, 32]. (b) Aromatization was also determined by quantifying the conversion of androstenedione to oestrone employing the procedure previously described from this laboratory [30]. Briefly, 130 nM [4-^{14}C]-androstenedione was added to epithelial cells (1×10^4 cells/well) and these were grown in 24-well plates for periods of up to 72 h at 37 °C. Under these incubation conditions the entire androstenedione was available for aromatization since insignificant amounts of the precursor are converted to testosterone [30]. A two-step chromatographic system was developed to isolate oestrone and oestradiol from androstenedione, testosterone and other metabolites as described previously [30]. After chromatography the steroids were localized, eluted and counted in a liquid scintillation counter.

Statistical analysis

We expressed all data as means ± standard deviation of the mean. Data were analyzed using the SAS statistical package. Statistical analysis was performed using the unpaired Student's *t*-test. $P < 0.05$ was accepted as statistically significant.

Results

Characterization of stromal and epithelial cells

Verification of the culture as prostatic stroma and epithelial cells was determined by immunocytochemical staining employing a variety of antibodies as detailed in Table 1. Assessment of the staining patterns and their intensities was undertaken by an independent pathologist. Epithelial cells stained positively and uniformly for cytokeratin and human milk fat globulin. Furthermore, the bulk of the epithelial cells appeared to be of a secretory nature since prostatic acid phosphatase (PAP) and prostatic specific antigen (PSA) are strongly expressed (Table 1). Epithelial cells also recognized the antibody for the EGF receptor. This confirms our earlier findings on the localization of EGF receptors in epithelial cells of human prostate tissue [21].

Table 1 Immunocytochemical staining of stroma and epithelial cells in culture (intensity of staining: ++ strongly positive, + moderately positive, −/+ patchy, − negative)

Markers used	Stromal cells	Epithelial cells
Prostatic acid phosphatase	−	++
Prostatic specific antigen	−	++
Epidermal growth factor receptors	−/+	++
Cytokeratin	−/+	++
Vimentin	++	−/+
Desmin	+	−
Human milk fat globulin	−/+	++
Actin	++	−

In addition to the immunostaining, the cells were also examined by phase contrast microscopy (Figs. 1a, b). Analysis of the photomicrographs suggested that the resultant epithelial monolayers have very little or no contaminants – any residual fibroblast will be totally destroyed by the epithelium growth medium.

In contrast, the fibroblasts fail to stain for PAP and PSA but were positively labelled by antibodies for vimentin and desmin, thus confirming the purity of both cell components. However, since desmin does not differentiate between smooth and striated muscle, we undertook some staining with an antibody against actin. This also showed specific and intense labelling of smooth muscle in the fibroblast/stroma cell cultures.

Androgen metabolism in stroma and epithelium

Whilst small amounts of DHT were produced by the stromal and epithelial cells, androstenedione was the major metabolite formed, the concentrations of which measured in the media were proportional to the cell numbers (results not shown) and also to the time of incubation (Fig. 2a, b).

We also compared the 5α-reductase activities in the cytosol fractions obtained from a number of prostate specimens and from primary cultures of stromal components derived from the same specimens. As demonstrated in Fig. 3, the 5α-reductase activity in the cytosol from the stroma was significantly lower than the activity measured in the whole tissue cytosol, whereas the capacity to produce androstenedione was, on the other hand, markedly enhanced. It is evident that in the process of culturing the fibroblasts these cells have lost some of their capacity to metabolize testosterone to DHT.

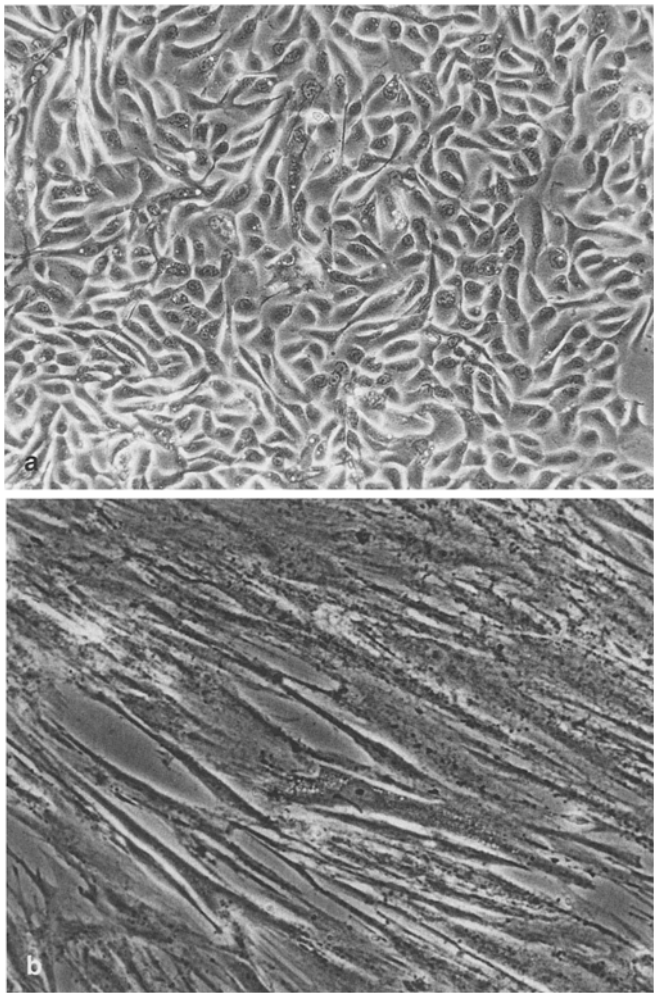

Fig. 1a, b Phase-contrast micrograph of a primary culture of epithelium (**a**, × 100) and a serial culture of stromal cell (**b**, × 200) from a patient with benign prostatic hyperplasia

We also examined the capacity of the primary cultures to aromatize androstenedione, employing two different techniques. Our results demonstrated the absence of tracer amounts of oestrogen and/or ^3H_2O in the primary cultures (results not shown), suggesting that under the present experimental conditions the primary culture of epithelial cells and stroma were not involved in the conversion of androstenedione to oestrone. The possibility that later intermediates in the aromatization pathway, namely the 19-hydroxy and 19-oxo derivatives, may be converted into oestrogen via the primary cultures has not, however, been excluded.

Discussion

There have been marked improvements over the last few years in the primary culture of prostate cells [6, 26, 33]. The development of such in vitro models has become an important step in the understanding of the events leading to benign and malignant prostate disease. In this study we have successfully established the growth of both epithelial cells and stroma derived from BPH and investigated the metabolism of testosterone in these cells. The predominant conversion of testosterone into androstenedione confirmed that 17β-hydroxysteroid dehydrogenase is the main metabolic pathway in our cultured stroma and epithelial cells. Although there was some 5α-reductase activity in these cells, the reductive pathways were insignificant when compared to the oxidative activity of the two cell types, resulting in the formation of large concentrations of androstenedione. It is not clear why there is this shift to the oxidative pathway, but removing the cells from their in vivo setting induces the dedifferentiation of the stroma and epithelial cells. This, in turn, might be responsible for the shift in the metabolic pathways – a situation not totally dissimilar from the physiological changes reported in the human prostate gland immediately following the onset of the cancer [13]. In an earlier study on prostate-derived fibroblasts, high 17β-hydroxysteroid dehydrogenase activity was also reported [27] but this was limited to a 1-h period and throughout this period there was also significant 5α-reductase activity. The high 17β-hydroxysteroid dehydrogenase activity in stroma from human adult prostate was also confirmed by Boudou et al. [3], who also reported that immortalized epithelial cells preferred the reductive 5α-dihydrotestosterone pathway [3]. In a separate study (paper in press) we have also shown that the supplementation of the media with androgens neither alters the metabolic pathways described in this document nor increases the growth of the cells [9]. These cells have become refractive to androgens as a result of the dedifferentiation process [9].

At a time when two isoenzymes have been characterized for both the 5α-reductase [31] and the 17β-hydroxysteroid dehydrogenase [2, 22], it is difficult, at this stage, to apportion the reductive and oxidative activities measured in these cells to any particular isoenzyme. This is also complicated by the fact that the isoenzyme distribution for 5α-reductase and 17β-hydroxysteroid dehydrogenase is different in stroma and epithelium [14] and this may well be reflected in the distinctive patterns of androgen metabolic pathways seen in the prostate.

The interplay between androgens and oestrogens is critical to the development of BPH and over the years oestrogens have consistently been implicated in the pathogenesis of this condition. Oestrogen formation resulting from the aromatization of androgens occurs at several sites, but reports concerning the presence of aromatase activity in the prostate are conflicting [18, 28, 30]. However, we have recently demonstrated the expression of aromatase mRNA in BPH and prostate cancer [34]. Aromatase mRNA was identified in all specimens by Southern blot analysis of the reverse

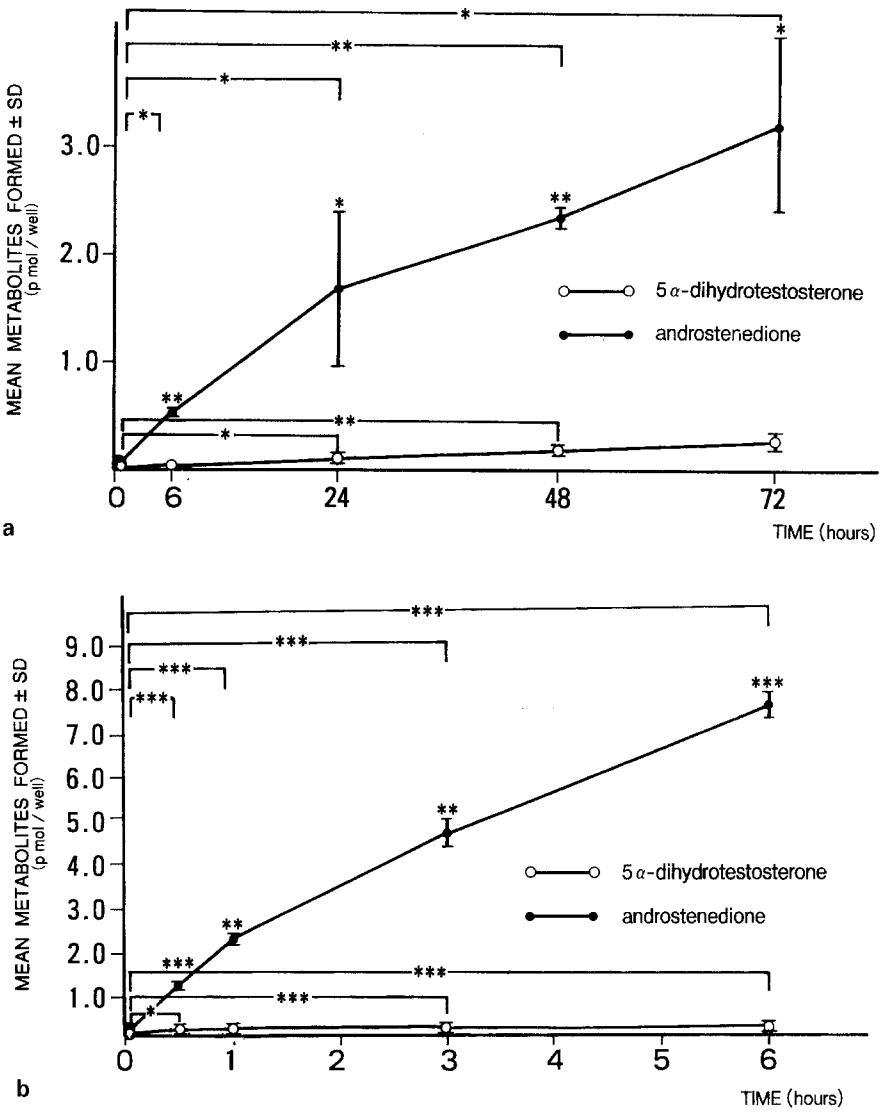

Fig. 2a, b The formation of [^{3}H]-androstenedione mainly increased in epithelial cells (**a**) and fibroblasts (**b**) derived from BPH depending on the time of incubation. The incubation of epithelial cells (1.15×10^4/well in 24-well plates) and fibroblasts (6.0×10^5/well in 24-well plates) was carried out up to 72 h. Each point represents the mean of three different specimens. Each run is in triplicate and values are expressed as pmol metabolite formed/mg protein ± SD ○–○ 5α-dihydrotestosterone, ●–● androstenedione; means ± SD). Significantly different from control. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

trascriptase polymerase chain reaction (RT-PCR) products. The concentrations of aromatase mRNA (means ± SD) which were measured by fluorometric quantitation in 16 of 19 patients with BPH and in 3 of 4 patients with prostate cancer were 1.81 ± 3.02 and $0.84 \pm 0.27 \times 10^{-3}$ amol/μg total RNA, respectively. BPH patients showed no relationship between any previous hormonal treatment, their age, the weight of the excised adenoma, the stromal element/epithelial plus stromal elements ratios or the aromatase mRNA levels. However, a significant correlation ($r = 0.979$; $P < 0.05$) between the stromal element/epithelial plus stromal elements ratios and the levels of aromatase mRNA in the patients with prostate cancer was observed [34]. Even so, the studies described in this report failed to demonstrate the presence of aromatase activity in either cell type in spite of the high concentrations of androstenedione present in the culture medium along with the absence of any significant 5α-reductase activity in these cells. The failure of these cells to aromatize androstenedione may be connected to some anomaly in the transcription process as a result of a deletion or point mutation in the aromatase gene. This may be induced by the primary culture of these cells, but such a hypothesis will need to be examined further.

The metabolic pathways of the prostate cell vary considerably depending on the histopathology of the gland. The major metabolites in normal and hyperplastic cells are DHT and androstanediols, whereas in prostate cancer there is a shift to the oxidative pathways, with androstenedione being the major steroid found [8, 24]. This shift is prompted by a downregulation in the 5α-reductase activity of the gland as the tumour grade progresses from a well-differentiated to a poorly differentiated state [13]. This naturally increases the availability of the testosterone precursor for the oxidative pathway, resulting in the formation of

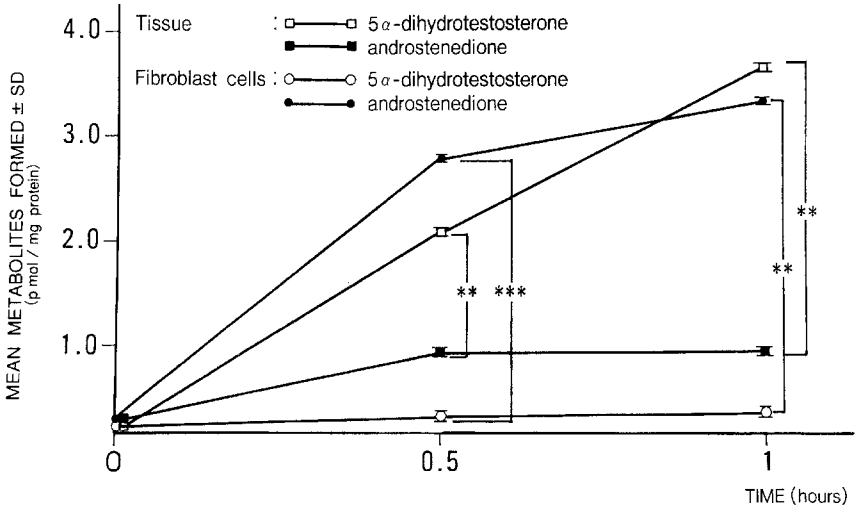

Fig. 3 Comparison of testosterone metabolism in a cytosol fraction obtained from a prostate specimen and in the primary cultures of fibroblasts derived from the same specimen. The tissue (2.173 mg) and the cells (8.82×10^6) were homogenized and cytosol fractions were obtained by centrifugation. Conversion of [^{3}H]-testosterone to radiolabelled DHT and androstenedione was measured at different times and the incubation was carried out at 37 °C. *Each point* represents the mean of three experiments and values are expressed as pmol metabolite formed/mg protein ± SD (Tissue: □–□ 5α-dihydrotestosterone, ■–■ androstenedione; fibroblast cells: ○–○ 5α-dihydrotestosterone, ●–● androstenedione; pmol/mg protein, means ± SD)

large concentrations of androstenedione. In an earlier retrospective study we attempted to correlate 5α-reductase activity in patients with prostate cancer to responsiveness to endocrine therapy and found that those patients with little or no 5α-reductase activity were less likely to respond to hormone treatment than those with high 5α-reductase activity [11]. It is interesting to note that many of the prostate cell lines derived from poorly differentiated tumours also express little 5α-reductase activity but high 17β-hydroxysteroid dehydrogenase [29], and it may be that our primary cultures are in a state of transition towards becoming ultimately hormone refractive [9].

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