The localisation and expression of 5α-reductase Types I and II mRNAs in human hyperplastic prostate and in prostate primary cultures

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Abstract

The expression and localisation of mRNAs for 5α reductase Type I (5α R-I) and Type II (5α R-II) isoenzymes in human benign prostatic hyperplasia (BPH) were investigated by RT-PCR and by in situ hybridisation (ISH) using digoxigenin labelled riboprobes. In addition, we also examined the isoenzymes mRNA expression in primary BPH cultures of separated stroma/fibroblast and epithelial cells to determine whether primary cultures are appropriate models in which to investigate 5α R activity and regulation. The results demonstrated conclusively the presence of mRNA encoding both isoenzymes in all specimens so far examined. Additionally, the presence of a functional 5α R-I and -II activity in BPH was confirmed by enzyme assays. ISH studies localised the mRNA expression to both the fibroblast/stromal component as well as the epithelial cells of the hyperplastic tissue. In the glandular regions the expression for both isoenzymes was particularly strong in the basal layers of the epithelium whereas mRNA expression in the secretory cells was less pronounced. Expression of 5α R-I and -II mRNAs in fibroblast was on the other hand variable with high expression in some areas and little in others. These findings were supported by our primary culture experiments which demonstrated that both the fibroblast and epithelial cells maintain a capacity to express both isoenzymes in vitro. In the case of the fibroblast, the capacity to express the isoenzymes was maintained following the sequential passage of the cells up to passage 6, after which the cells no longer expressed either isoenzyme.

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Introduction

The development of benign prostatic hyperplasia (BPH) is a process dependent on a number of factors including testicular androgen and aging. The active androgen regulating the growth and function of the prostate is 5α dihydrotestosterone (5α DHT) which, in vitro, is formed by the irreversible reduction of testosterone by the enzyme 5α reductase (5α R) (cholesterol 5α-reductase, 3-oxo-5α-steroid:NADP+ 4-ene-oxidoreductase, EC1·3·1·22). 5α R activity can therefore play an important role in the development of the prostate gland in health and disease (Anderson & Liao 1968, Bruchovsky et al. 1981, Hudson 1981, Houston et al. 1985b, Enderle–Schmitt et al. 1986). However, these conflicting messages have now been resolved, due mainly to the recent successes in cloning the genes responsible for the two steroid 5α R isoenzymes, designated Types I and II, which have been characterised in a variety of tissues from man and rat (Anderson et al. 1989, 1991, Andersson & Russell 1990, Jenkins et al. 1992). The Type I enzyme (5α R-I), which in man maps to chromosome 5, is expressed in large amounts in skin and liver. The Type II enzyme (5α R-II), which shows 50% nucleotide identity with Type I, is located on chromosome 2 and is expressed mainly in male reproductive tissues: 5α R-II mRNA and protein have been demonstrated in...
normal adult prostate, as well as in BPH. Mutations in 5α R–II result in male pseudohermaphroditism and lack of prostate formation (Andersson et al. 1991) suggesting a central role for the 5α R–II isoenzyme in the normal development of the prostate. However the possible presence of 5α R–I in human prostate remains controversial. Whilst some studies reported the absence of 5α R–I mRNA and protein in human prostate (Thigpen et al. 1993, Silver et al. 1994), other investigations demonstrated 5α R–I expressed in primary prostate tissue by Northern analysis (Bonnet et al. 1993) and in cultures of epithelial cells (Hirsch et al. 1993). However neither study addressed the important issue of whether the two isoenzymes were expressed in intact BPH tissue or established their cellular distribution within the gland. To resolve this matter we have investigated the presence of mRNAs encoding the two isoenzymes in freshly obtained BPH specimens, measured the functional activities of the enzymes by following the conversion of [3H]testosterone to [3H]DHT at basic and acidic pH and investigated their cellular distribution within the hyperplastic prostate. In addition, we have explored the mRNA expression of 5α R–I and –II in primary cultures of prostate stromal/fibroblast and epithelial cells at different stages of passaging to demonstrate whether normal cellular expression/distribution of the isoenzymes was retained in culture.

Materials and Methods

Source of prostate tissue

Hyperplastic prostate specimens were obtained from 15 patients undergoing transurethral resection to relieve bladder obstruction. These specimens were either snap frozen in liquid nitrogen immediately following removal for mRNA analysis or placed in ice cold R.P.M.I 1640 medium (Gibco, Paisley, Strathclyde, UK) containing 5% foetal calf serum (FCS, Gibco) and transported to the laboratory for primary culture experiments. Random selected chippings from each specimen were sent to pathology for histological examination. In addition, archival paraffin-embedded BPH material was obtained from the Department of Pathology for use in the in situ hybridisation studies. Serial 5 μm sections were cut and alternate sections were stained by haematoxylin and eosin (H and E) to confirm morphology.

Primary cultures of prostate fibroblast and epithelial cells

Human prostatic fibroblast and epithelial cells were cultured from prostate chips as previously described (Habib 1992, Tsugaya et al. 1996). Verification of the purity of the fibroblast and epithelial cells was established by immunohistochemical staining employing a variety of primary monoclonal antibodies and by phase contrast microscopy as detailed in our earlier reports (Habib 1992, Tsugaya et al. 1996).

Measurement of 5α R activity

Primary culture of prostate fibroblast and epithelial cells Fibroblast (4.0 × 10⁴ cells/well) and epithelial cells (4.0 × 10⁴ 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 radio-labelled testosterone substrate (1,2,6,7-[3H]testosterone; specific activity 91 Ci/mmol; Amersham International, Amersham, Bucks, UK) and cells were incubated at 37 °C for periods of up to 5 days. In addition, three wells containing the medium and substrate but no cells were also included, to account for breakdown of [3H]testosterone. At the end of the incubation, the medium was collected and transferred to extraction tubes, followed by the addition of approximately 1000 c.p.m. [14C]labelled steroids for each of the androgens to be assayed to calculate losses. Subsequent steps, including extraction and separation of steroids, development of thin layer chromatography plates and the recovery of steroids, have all been described previously (Houston et al. 1985a, Tsugaya et al. 1996).

Tissue homogenates Tissue homogenates were prepared by pulverising frozen tissue (2–5 g) and homogenising in approximately five volumes of 40 mM sodium phosphate buffer containing 0.32 M sucrose and 1 mM dithiothreitol (DTT) at either pH 7.5 for 5α R–I or pH 5.0 for 5α R–II. The 5α-reductase assays were set up in tubes containing an NADPH generating system as described by Tsugaya et al. (1996) and the reactions proceeded following the addition of 200 μl homogenate along with [3H]testosterone solution (20 nM; 1 μCi). The tubes were incubated in a shaking water bath at 37 °C for 30 min and the reactions were stopped by the addition of two volumes of diethyl ether containing 500 c.p.m. [14C]DHT and 25 μg each of unlabelled steroids. Details of the remaining procedures for the extraction of steroids, their separation on ILTC plates and the correction for procedure losses are described in Houston et al. (1985).

Protein levels were determined using a Bio-Rad kit (Bio-Rad Laboratories Ltd, Hemel Hampstead, Herts, UK) and enzyme activity was expressed as a function of the protein concentration. The activities of 5α R–I (pH 7.5) and 5α R–II (pH 5.0) were also measured in the presence of the 5α R–II inhibitor (Moore et al. 1995, Habib et al. 1997), Finasteride, at a concentration of 270 nM.

Preparation of RNA

Total cellular RNA was extracted using the acid–guanidium–phenol–chloroform (AGPC) method of
Reverse transcription-polymerase chain reaction (RT-PCR)

One microgram total RNA or 0·1 µg poly(A)^+ RNA was reverse transcribed in 10 × reaction buffer (Promega) containing 2 µl 10 mM dNTP mix (Pharmacia, Milton Keynes, Bucks, UK), 20 U RNAsin (Promega), 500 ng oligo(dT)_{15} primer (Promega), 5 mM MgCl_{2} and 15 U avian myeloblastosis virus reverse transcriptase (Promega). Reactions were incubated at 42 °C for 60 min then 20 µl reaction mix were used for PCR amplification. PCRs were performed in a volume of 100 µl containing 0·2 mM dNTPs (Promega), 40 mM KCl, 8 mM Tris HCl pH 9, 0·08% Triton X-100, 2 mM MgCl_{2}, 1·5 U Taq DNA polymerase (Promega) and 75 pmol/primer. Human 5α R–I and –II cDNAs were amplified using two sets of intron-spanning primers. Primers for the 5α R–I were: 5′ TGC TGA CTG GGT AGC AGC 3′ and 5′ GCTCGAGATGTGATGAAG3′* (Andersson et al. 1989). Primers for 5α R–II were: 5′ CCT TGT TAC GTC CGA AGC 3′ and 5′ GCC CCC ATC AGG GTA TTC AG 3′, amplifying nucleotides 98–447 (Andersson et al. 1989).

To verify the integrity of the RNA, control RT-PCRs were performed using primers designed to amplify human cDNA encoding the housekeeping protein hypoxanthine–guanine–phosphoribosyl transferase (HGPRT): 5′ CCA CCC ATC AGG GTA TTC AG 3′ and 5′ GTCTGCAGTTACGTATTC3′* (Andersson et al. 1989), amplifying nucleotides 453–624 (Andersson et al. 1989). Primers for 5α R–I and –II cDNAs were amplified using two sets of intron-spanning primers. Primers for the 5α R–I were: 5′ TGC TGA CTG GGT AGC AGC 3′ and 5′ GCTCGAGATGTGATGAAG3′* (Andersson et al. 1989). Primers for 5α R–II were: 5′ CCT TGT TAC GTC CGA AGC 3′ and 5′ GCC CCC ATC AGG GTA TTC AG 3′, amplifying nucleotides 98–447 (Andersson et al. 1991).

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In situ hybridisation

Paraffin wax embedded BPH blocks were cut into 5 µm thick sections and mounted on aminopropyltriethoxysilane (TESPA)-coated glass slides. Prehybridisation was carried out by baking the section for 15 min at 60 °C followed by dewaxing in xylene, refixing and permeabilisation by covering slides with 100 µl 50 mM Tris–HCl pH 7·6 containing 20 µg Proteinase K (Sigma) and 5 mM EDTA for 7·5 min at room temperature. Hybridisation to digoxigenin (DIG–UTP) labelled RNA probes (1·6 µg/80 µl) was carried out in a hybridisation mixture containing 40 µl formamide, 9·6 µl 5 M NaCl, 1·6 µl 50 × Denhardt’s solution, 0·8 µl 1 M Tris–HCl pH 7·4, 0·4 µl 200 mM EDTA, 0·8 µl 1 M DTT, 1·6 µl tRNA (10 µg/µl) and 16 µl 50% dextran sulphate and proceeded overnight at 50 °C in a moist chamber under sealed silane-treated coverslips. Post hybridisation washes (30 min each) included 2 × SSC (300 mM sodium chloride and 30 mM sodium citrate) at 37 °C followed by a single wash in 2 × SSC at room temperature before RNase treatment (30 min at 37 °C in 2 × SSC containing 10 µg/ml RNase-A) then washed in 2 × SSC for 20 min, 2 × SSC containing 50% formamide at 65 °C for 20 min, 2 × SSC at 65 °C for 20 min, 2 × SSC at room temperature.

Figure 1 Elimination of the expression of 5α R-I pseudogene from RNA preparations derived from BPH tissue following RT-PCR. One microgram total RNA or 0·1 µg poly(A)^+ RNA was reverse transcribed and the cDNA sequences for 5α R-I were amplified by PCR (35 cycles) employing primers for 5α R-I and Taq DNA polymerase. The PCR products and 100 bp markers were size fractionated on 2% agarose gel, stained with ethidium bromide and visualised under UV light. Lane 1: total RNA; Lane 2: total RNA (no RT); Lane 3: poly(A)^+ RNA; Lane 4: poly(A)^+ RNA (no RT); Lane 5: no DNA control; Lane 6: 100 bp ladder.
temperature for 10 min and finally in 0.1 × SSC at 42 °C for 20 min. The hybridised riboprobes were subsequently visualised by immersion in the DIG–alkaline phosphatase conjugated antibody (Boehringer Mannheim, Lewes, Sussex, UK) at a dilution of 1:2500 for 30 min at room temperature after which the sections were washed in 0.1 M Tris pH 9.5 containing 0.1 M NaCl and 0.05 M MgCl₂ for 30 min at room temperature and developed by immersion overnight in a Boehringer Mannheim developing reagent.

DIG–UTP labelled riboprobes were prepared as follows using the DIG–RNA labelling kit from Boehringer Mannheim. DIG-labelled RNA complementary to 5α R-I mRNA (sense and antisense probes) was synthesised from a 780 bp fragment cDNA probe of human 5α R-I cDNA (Andersson et al. 1989), subcloned into pBSII SK and linearised either with KpnI using T7 RNA polymerases for the antisense probe or with NsiI using T3 RNA polymerase for the sense probe, as detailed in the manufacturer’s protocol. The DIG-labelled riboprobes for the 5α R-II isoenzyme were synthesised in a similar fashion except that a 365 bp cDNA probe (5α R-II; Andersson et al. 1991) complementary to the 5α R-II mRNA was used. The specificity of the 5α R-I and -II antisense probes was checked by in situ hybridisation of CHO cells which had been transfected with either the 5α R-I or -II gene. The results showed no cross-reactivity between the probes (Fig. 2A–D). For negative control, hybridisation with sense-strand riboprobes was also carried out on BPH sections.

Results

5α R-I and 5α R-II gene expression in BPH specimens

The mRNA expression for 5α R-I and -II was investigated in nine BPH specimens. Figure 3 demonstrates the PCR products corresponding to the expected sizes for 5α R-I (170 bp) and 5α R-II (350 bp) mRNA isolated from BPH tissue. Identity of the two PCR products was confirmed by restriction enzyme digest using HinfI for 5α R-I and NciI for 5α R-II. HinfI is predicted to cleave the
170 bp 5α R-I product once, producing restriction fragments of 72 bp and 98 bp, whilst NciI should cleave the 350 bp 5α R-II product, producing two fragments of 137 and 213 bp (Fig. 3). Analyses carried out on RNA isolated from nine patients is detailed in Table 1. In each case, a single PCR product was seen for each of 5α R-I and -II and no competition between Type I and II fragments under the conditions used (Fig. 3).

Localisation of the 5α R-I and 5α R-II mRNA in BPH

In situ hybridisation was carried out on wax-embedded BPH sections obtained from four different patients. DIG-UTP labelled antisense riboprobes complementary to 5α R-I and -II RNAs demonstrated strong mRNA expression for both the 5α R-I and -II in the glandular areas of all BPH specimens, but weaker staining in the stromal/fibroblast component (Figs 4 and 5). We also noted that (5α R-I and -II) labelling in the glandular areas was stronger and more pronounced in the basal cells whereas the secretory cells demonstrated a much lower expression. Furthermore, labelling of epithelial cells was uniform throughout the four specimens examined but there was considerable variability in the labelling patterns of the stromal/fibroblast component. Hybridisation experiments employing the sense probes for both the 5α R-I and -II enzymes yielded no specific signal (Figs 4 and 5).

mRNA expression of 5α R isoenzymes in primary cultures of prostate fibroblast and epithelial cells

In order to determine whether primary cultures are appropriate models in which to investigate 5α R activity and regulation, we have used a qualitative RT-PCR to examine the mRNA expression of 5α R-I and -II in epithelial cells (Fig. 6) and fibroblasts (Fig. 7) cultured separately from six freshly obtained BPH specimens. As summarised in Table 1, both fibroblast and epithelial cells contained mRNAs encoding isoenzymes as demonstrated by PCR products of 170 bp for 5α R-I (Figs 6 and 7) and 350 bp for 5α R-II (Figs 6 and 7). Restriction digestion of PCR products with HinfI (5α R-I) and NciI (5α R-II) confirmed the identity of the PCR products thus demonstrating that both the fibroblast and epithelial cells expressed mRNA for both isoenzymes. Expression of mRNA encoding both isoenzymes persisted over several passages of the fibroblast cells (Table 1; Fig. 7). RT-PCR demonstrated the presence of 5α R-I and -II mRNA in cells up to passage 6; at later passages, no 5α R-I or -II

Table 1 The expression of mRNA transcripts for both 5α R-I and -II in BPH and in primary cultures of stromal/fibroblast and epithelial cells

<table>
<thead>
<tr>
<th>5α reductase isoenzymes</th>
<th>BPH (9)*</th>
<th>Epithelial cells (6)</th>
<th>Stromal/fibroblast cells (6)</th>
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<tr>
<td></td>
<td>Type I</td>
<td>Type II</td>
<td><strong>p-2</strong></td>
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<td>RT-PCR</td>
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*Number of patients; **Passage number.
mRNA was detectable by RT-PCR (Table 1; Fig. 7). Because of the difficulties in passaging epithelial cells, we were unable to investigate the impact of serial passaging of these cells on the mRNA expression of the isoenzymes.

**5α R activity in primary culture of fibroblast and epithelial cells and in human BPH homogenates**

Primary cultures were tested for 5α R activity by measuring the metabolites formed following incubation of the cells with radiolabelled testosterone. The results obtained from eight separate incubations demonstrated that the metabolism of testosterone and the formation of DHT in the epithelial cells (means ± s.e.m.: 4.4 × 10⁻⁸ ± 0.12 pmol/cell/h) was slightly greater than the activity measured in the fibroblasts (2.6 × 10⁻⁸ ± 0.21 pmol/cell/h). However the live cell assay does not differentiate between the activities of the Type I and II isoenzymes and in all probability the values obtained represent a combination of the two activities. With repeated passaging of the fibroblast cells, 5α R activity gradually diminished and became undectable by passage 3 (data not shown).

We also examined the metabolism of [³H]testosterone to [³H] DHT by homogenates from four separate BPH tissues each run in triplicate. Both 5α R-I and -II activities were detected but the mean ± s.e.m. 5α R-II activity (0.426 ± 0.058 pmol/mg protein/h) was higher than that for 5α R-I (0.128 ± 0.032 pmol/mg protein/h). Furthermore, our studies confirmed Finasteride as a potent inhibitor for 5α R-II inducing a 75% decrease in the activity of this enzyme (0.109 ± 0.018 pmol/mg protein/h). However the inhibitor had little effect on the activity of 5α R-I which was very slightly depleted (0.114 ± 0.016 pmol/mg protein/h).
Discussion

The results described in this report demonstrated conclusively that mRNAs encoding both \( 5\alpha \) R-I and -II isoenzymes are expressed in human BPH tissue. Using RT-PCR, we have shown mRNA expression for both isoenzymes in nine out of nine BPH tested. In situ hybridisation further demonstrated the cellular distribution of these isoenzymes in the epithelial as well as in the fibroblast components of the prostate. This was subsequently confirmed by primary cultures which demonstrated expression of mRNA encoding \( 5\alpha \) R-I and -II in both fibroblast and epithelial cells. The presence of a functional \( 5\alpha \) R-I and -II in BPH was also established by enzyme assay. The activity for both isoenzymes was demonstrated in the tissue, with the Type II exhibiting approximately three times as much activity as that measured for \( 5\alpha \) R-I. The presence of an activity for either isoenzyme in BPH was also confirmed by the use of Finasteride, which selectively inhibited \( 5\alpha \) R-II but was a poor inhibitor of \( 5\alpha \) R-I.

There is general consensus that \( 5\alpha \) R-II is expressed in both the normal prostate and in BPH (Silver et al. 1994, Levine et al. 1996); however the presence of the \( 5\alpha \) R-I enzyme and its encoding mRNA have been controversial. Bonnet et al. (1993) demonstrated, using Northern analysis, that \( 5\alpha \) R-I and -II were expressed in human prostate and suggested that both enzymes were located predominantly in epithelial cells. However, Thigpen et al. (1993) were unable to detect either \( 5\alpha \) R-I mRNA or protein using Northern and Western analysis respectively. Here we conclusively demonstrate the presence of \( 5\alpha \) R-I mRNA in human BPH and show that the enzyme is functionally active in the hyperplastic gland.

Reports as to the cellular location of \( 5\alpha \) R are also controversial: in rat, \( 5\alpha \) R-I is in basal epithelial cells and the \( 5\alpha \) R-II in stroma (Berman & Russell 1993). Silver et al. (1994) have reported that human \( 5\alpha \) R-II in some BPH specimens is predominantly in the basal epithelial cells whereas in other specimens it is in both stroma and basal epithelial cells. They were, however, unable to detect \( 5\alpha \) R-I in prostate by employing specific antibodies. In their studies, \( 5\alpha \) R-II was highly expressed in stromal cells for BPH and CaP. In contrast, we found a similar distribution for both mRNAs in BPH, with both isoenzymes expressed in basal epithelial and fibroblast cells. Expression in fibroblasts was variable, with high expression in some areas and little in others. These findings are

Figure 6 The RT-PCR expression of \( 5\alpha \) R-I and -II mRNA in primary cultures of epithelial cells from BPH. One microgram total RNA (\( 5\alpha \) R-II) or 0.1 \( \mu \)g poly(A)\(^+\) RNA (\( 5\alpha \) R-I) isolated from primary cultures of fibroblast cells derived from BPH tissue was used to prepare cDNA in an oligo-dT primed reverse transcription. cDNAs were subjected to 35 cycles PCR with either \( 5\alpha \) R-I or -II primers and the resulting products separated on a 2% agarose gel containing ethidium bromide. The PCR products were visualised under UV light. Lane 1: 100 bp ladder; Lane 2: epithelium/\( 5\alpha \) R-I; Lane 3: \( 5\alpha \) R-I negative control (no RT); Lane 4: epithelium/\( 5\alpha \) R-II; Lane 5: \( 5\alpha \) R-II negative control (no RT); Lane 6: epithelium/HGPRT; Lane 7: HGPRT negative control (no RT); Lane 8: 100 bp ladder; Lane 9: no DNA control.

Figure 7 The RT-PCR expression of \( 5\alpha \) R-I and -II mRNA in primary cultures of BPH fibroblast from BPH at different passages. One microgram total RNA (\( 5\alpha \) R-II) or 0.1 \( \mu \)g poly(A)\(^+\) RNA (\( 5\alpha \) R-I) isolated from primary cultures of fibroblast cells derived from BPH tissue was used to prepare cDNA in an oligo-dT primed reverse transcription. cDNAs were subjected to 35 cycles PCR and the resulting products for \( 5\alpha \) R-I (170 bp) and \( 5\alpha \) R-II (350 bp) were electrophoresed on 2% agarose gels containing ethidium bromide and visualised under UV light. Lane 1: 100 bp ladder; Lane 2: no DNA control for \( 5\alpha \) R-I and -II; Lane 3: RT negative for \( 5\alpha \) R-II; Lane 4: stroma passage 2/\( 5\alpha \) R-II; Lane 5: stroma passage 4/\( 5\alpha \) R-II; Lane 6: stroma passage 6/\( 5\alpha \) R-II; Lane 7: RT negative for \( 5\alpha \) R-I; Lane 8: stroma passage 2/\( 5\alpha \) R-I; Lane 9: stroma passage 4/\( 5\alpha \) R-I; Lane 10: stroma passage 6/\( 5\alpha \) R-I; Lane 11: 100 bp ladder.
supported by our primary culture experiments which demonstrate that both the fibroblast and epithelial cells maintain a capacity to express both isoenzymes in vitro. In the case of the fibroblast, this capacity was maintained following the sequential passaging of the cells up to passage 6, following which the cells no longer expressed either isoenzyme. However it has not been possible for us at this stage to identify the reason for this loss of expression with repeated passaging. Our initial data on DHT supplementation of the primary cultures (work in progress) suggest that the down-regulation in 5α R activity was not in any way linked to a depletion in DHT concentrations as previously suggested (George et al. 1991).

Because of the difficulties of passaging primary cultures of human epithelial cells, we have not been able to assess the impact of passaging of these cells on the mRNA expression of the 5α R isoenzymes. It was however interesting to note that our observations on the expression of 5α R-I mRNA in epithelial cells confirm the earlier reports of 5α R-I enzyme in primary culture demonstrating that 5α R activity in primary epithelial cultures from BPH, inferred as the 5α R activity in these cells, was far more susceptible to 5α R-I inhibitors than to 5α R-II inhibitors (Hirsch et al. 1993). Whilst it was not possible for us to quantify the relative activity of each isoenzyme in the primary cultures, the combined activities of 5α R-I and –II isoenzymes appeared to be slightly higher in the epithelial cells than in the fibroblast/stromal cultures.

The question of why the prostate has two isofoms of 5α R still remains open. The possibility that the fibroblast and epithelial cells might be expressing different isofoms has now been discounted by the results of the present study. This leaves us with another alternative and that is, whilst 5α R-II is responsible for the metabolism of testosterone to DHT, the 5α R-I isoenzyme might be associated with the reduction of androstenedione to androstanedione. To the best of our knowledge no one has so far investigated this alternative mechanism but this is a possibility which is worth exploring.

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