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Induction of 5α-reductase type II mRNA transcription in primary cultured prostate epithelial cells by a soluble factor produced by primary cultured prostate fibroblast cells

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Abstract

The role of DHT in the development of BPH has resulted in the formulation of several drugs, which have been designed to inhibit the formation of DHT by the 5α-reductase enzymes (5α-reductase type I (5α-RI) & 5α-reductase type II (5α-RII)). Although the function of these enzymes is well understood, the biochemical stimulus for initiation of 5α-RI and II gene expression has not been described. Study of a co-culture model indicated the presence of a diffusible factor secreted by prostatic fibroblast cells, which is responsible for the transcription of 5α-II mRNA in primary prostatic epithelial cells. In this study, we describe the partial characterisation of a fibroblast-secreted, soluble factor which we believe induces the transcription of 5α-RII mRNA in long-term primary cultures of prostate epithelial cells which can no longer transcribe 5α-RII mRNA.

Keywords: Prostate; 5α-Reductase; Primary culture; Cell interactions

1. Introduction

The development of Benign Prostatic Hyperplasia (BPH) with age in the human male is poorly understood. There appear to be several factors, which are each in part responsible for the onset and advancement of the disease. Some of these factors have been defined while the aetiological significance of others remains uncertain. Of the known factors, the 5α-reductase isoenzymes, 5α-RI and 5α-RII have proved to be important. These enzymes are responsible for the conversion of testosterone (T) to dihydrotestosterone (DHT), a more active androgen which has been implicated in the development of BPH [1]. The observation that men with 5α-reductase (5α-R) deficiency do not develop BPH [2] implies that DHT is a major contributing factor to the development of the disease. This has resulted in the formulation of several drugs, which have been designed to inhibit the action of these enzymes.

While the biological functions of these isoenzymes are recognised and understood, the mechanisms that control gene expression have yet to be elucidated. Several theories have been proposed for the control of 5α-R in the human prostate [3]. In particular, the role of stromal epithelial interactions in the development and growth of the prostate has become increasingly evident in recent years [4,5]. With the realisation that cellular interactions play a major role in the differentiation and function of different cell types, we originally developed a co-culture model of BPH [6] to better mimic the *in-vivo* system. Study of this model suggested the presence/involvement of a diffusible factor secreted by prostate fibroblast cells, which was responsible for the initiation of transcription and expression of 5α-RII mRNA in primary prostate epithelial cells.

Here, we describe the partial characterisation of a factor secreted by primary cultured prostate fibroblast cells which induces the transcription of 5α-RII mRNA in primary cultured prostate epithelial cells that no longer express 5α-RII mRNA after a 7-day period of culturing.

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2. Materials and methods

2.1. Primary cultured cells

Primary cultured prostate fibroblast and epithelial cells were prepared as previously described in Ref. [7]. In brief, prostate chips obtained from transurethral resection of the prostate (TURP) were digested with collagenase and separated by centrifugation. Epithelial cells were grown in WAJC 404 medium supplemented with 2% fetal calf serum (FCS), penicillin/streptomycin (P/S), insulin and epidermal growth factor (EGF). Fibroblast cells were grown in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% (FCS). Cells were grown at 37 °C in 5% CO_2. The purity of the separate cultures was confirmed by immunohistochemistry and phase contrast microscopy as previously described in Ref. [7].

Primary cultured fibroblasts from breast and skin were obtained from the Molecular Research Council (MRC) unit and were grown in RPMI 1640 supplemented with 10% FCS, L-glutamine and P/S.

2.2. Preparation of conditioned medium

Conditioned medium (CM) was prepared from prostate, skin and breast fibroblast cells by changing the medium for FCS-free RPMI 1640 supplemented with penicillin/streptomycin and L-glutamine. Cells were maintained in serum-free conditions for a period of 3 days prior to the collection of the medium.

Cells and insoluble particulates were removed from the total of ten litres CM by membrane filtration (0.22 μm) prior to lyophilisation. The resulting material was re-dissolved in 50 ml of distilled water (DW) and desalted by dialysis against excess DW using a 1 kDa MWCO dialysis membrane. The dialysed CM was lyophilised a second time and re-dissolved in 10 ml DW. Soluble proteins in the resulting solution were precipitated in a stepwise manner by the addition of ammonium sulphate in accordance with the method of Scopes [8]. Proteins that precipitated at 40, 60 and 80% saturation were collected by centrifugation (300 000*g* for 10 min at +4 °C) and re-dissolved in 10 ml of DW. Samples were stored at +4 °C until required.

2.3. Fractionation of ammonium sulphate-precipitated proteins

All chromatographic separation steps were performed on a System Gold high performance liquid chromatographic (HPLC) apparatus (Beckman Instruments, USA) using a constant flow-rate of 0.5 ml/min and measuring the ultraviolet (UV) absorbance at 280 nm. Cation-exchange HPLC: the protein fraction of CM that precipitated between 40 and 60% saturation was dialysed against 25 mM (2-[*N*-Morpholino] ethanesulphonic acid) pH 6.0 (MES). Cation-exchange chromatography was performed using a MA75 (5.0×0.75 cm) column (Bio-Rad Laboratories, UK) equilibrated with MES. The entire sample was applied to the column in a series of 1ml injections allowing sufficient time for non-absorbed material in the final injection to pass through the column and the baseline to stabilise. All non-absorbed material was collected and retained for bio-assay. Absorbed material was eluted from the cation exchange matrix by the application of a linear salt gradient (0–1 M NaCl) over a period of 30 min. Fractions of 1 ml were collected and stored at +4 °C prior to use.

Anion-exchange HPLC: following the bio-assay, the non-absorbed material from the previous cation-exchange separation was dialysed against excess 25 mM Tris/HCl pH 7.5. Anion-exchange chromatography was performed using a TSK-DEAE-5PW (7.5×0.8 cm) column (Tosohass, USA) equilibrated with 25 mM Tris/HCl pH 7.5. Application of the sample to the column and elution/collection of bound proteins were performed exactly as described above for the cation-exchange chromatography. Fractions of 1 ml were collected and stored at +4 °C until required.

2.4. Size-fractionation of proteins

Protein components of anion-exchange fractions with demonstrable bio-activity (fractions 8–10) were size fractionated by passing the samples through a series of Centricon spin-columns (1 ml) (Amicon Ltd, UK) in order of descending MWCO value (50, 30, 10 and 3 kDa). Columns were spun in a micro-centrifuge in accordance with the manufacturer's recommendations and samples stored at +4 °C until required.

2.5. RNA extraction and RT-PCR

Total RNA was extracted by the method of Chomczynski and Saachi [9]. The presence of mRNA encoding 5α-RI and 5α-RII was then determined by reverse transcriptase-polymerase chain reaction (RT-PCR). 1 μg of total RNA was reverse transcribed using a commercial AMV Reverse Transcription kit (Promega, Southampton, UK) following the manufacturer's protocol. 20 μl of the RT reaction was then used for the PCR analysis in a reaction volume of 100 μl containing 10 μl 10× assay buffer (pH 9.0; Promega), 0.2 μl Taq polymerase (5000 U/ml), 2 μl deoxynucleotide triphosphate (dNTP) mix (1.25 mM) and 10 μl of intron-spanning primers (50 μg/ml). Primers used for 5α-RII were as previously published in Ref. [10] 5′CCTTGTACGTCGCGAAGC (forward) and 5′CCACCCATCAGGGTATTCAG (reverse) which amplify a 350 bp fragment between nucleotides 98 and 447. PCR was carried out in a Hybaid thermocycler. Conditions for the 5α-RII amplification

comprised 35 cycles of 96 °C, 1.5 min; 56 °C, 1 min; 72 °C, 1.5 min. Amplification products were separated on a 1.5% agarose gel and visualised by ethidium bromide staining under UV transillumination. Reactions in which cDNA was replaced with ultrapure water were included in each set of PCR as negative controls.

2.6. Effect of trypsin and heat on the ability of fibroblast-derived soluble factor to induce 5α-reductase type II expression in primary cultured prostate epithelial cells

Bio-active fractions of the CM were treated either with 1× trypsin (Gibco-Brl) or by heating in a boiling water bath for 5 min. Trypsin-treated samples were incubated in 1× trypsin for a period of 5 min at 37 °C. Subsequently, an equal volume of FCS was added to inhibit the action of the trypsin.

2.7. In-situ hybridisation of 5α-reductase type I and II mRNA in metastatic prostate cancer

Digoxygenin-labelled RNA probes were prepared using a DIG RNA labelling kit (Roche) in accordance with the manufacturer's instructions and as previously described in Ref. [10]. Archival sections of metastatic prostate cancer in lymph and bone were obtained and subjected to digestion with Proteinase K (Sigma) at 37 °C for 15 min following de-waxing and washing in 40% paraformaldehyde. Sections were re-hydrated and allowed to air dry. Hybridisation was carried out overnight in the dark at 50 °C. Sections were then washed with decreasing concentrations of SSC buffer to remove any non-specifically bound probe. Sections were visualised by incubation with alkaline phosphatase-labelled anti-DIG antibody (Roche) as per the manufacturer's instructions using nitroblue tetrazolium salt as the colour substrate. Colour was allowed to develop overnight at room temperature in a light-tight box. Sections were then mounted with aqueous mount (Shandon Immumount) prior to viewing using a microscope.

3. Results

3.1. Transcription of 5α-RII mRNA in primary cultured epithelial cells

Epithelial cells which had been grown in primary culture for a period of 14 days demonstrated no 5α-RII transcripts (Fig. 1a) (prior to 10 days of incubation, 5αRII transcripts can be detected by RT-PCR). This is in contrast to primary cultured epithelial cells, which had been treated with CM obtained from prostate fibroblast cells. These cells clearly demonstrated the expression of 5α-reductase type II mRNA when analysed by RT-PCR (Fig. 1b).

CM prepared from human breast and skin fibroblast cells in an identical manner to that from prostate fibroblast cells was also tested for its ability to induce the transcription of 5α-RII mRNA in primary cultured prostate epithelial cells. The breast and skin fibroblast CM produced no demonstrable upregulation of 5α-RII mRNA transcription in the prostate epithelial cells (Fig. 2a). All RT-PCR experiments were performed in triplicate.

In order to determine if the effect seen was due to differentiation of the cells, samples of epithelial cells were subjected to immunocytochemistry employing anticytokeratin 14 antibody (DAKO). These results

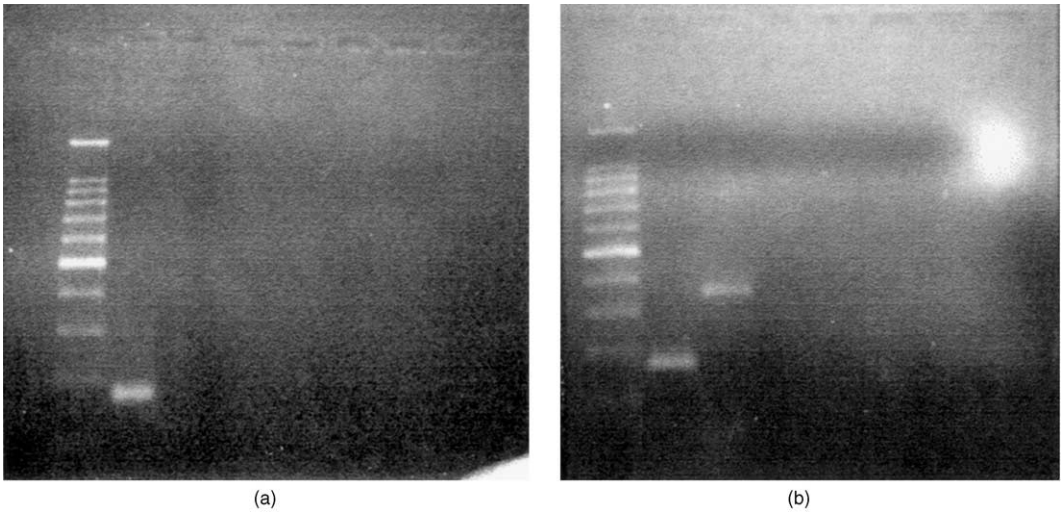

Fig. 1. (a) Reverse transcriptase-polymerase chain reaction (RT-PCR) of 5α-RI and II in primary cultured epithelial cells. Lane 1, 100 bp markers; lane 2, 5α-RI; lane 3, 5α-RII; lane 4, 5α-RI RT-negative; lane 5, 5α-RI PCR-negative; lane 6, 5α-RI RT-negative; lane 7, 5α-RII PCR-negative. (b) RT-PCR of 5α-RII transcription in primary cultured prostate epithelial cells incubated in high performance liquid chromatographic (HPLC) fractionated Conditioned Medium (CM). Lane 1, 100 bp markers; lane 2, 5α-RI; lane 3, 5α-RII; lane 4, 5α-RI RT-negative; lane 5, 5α-RI PCR-negative; lane 6, 5α-RII RT-negative; lane 7, 5α-RII PCR-negative.

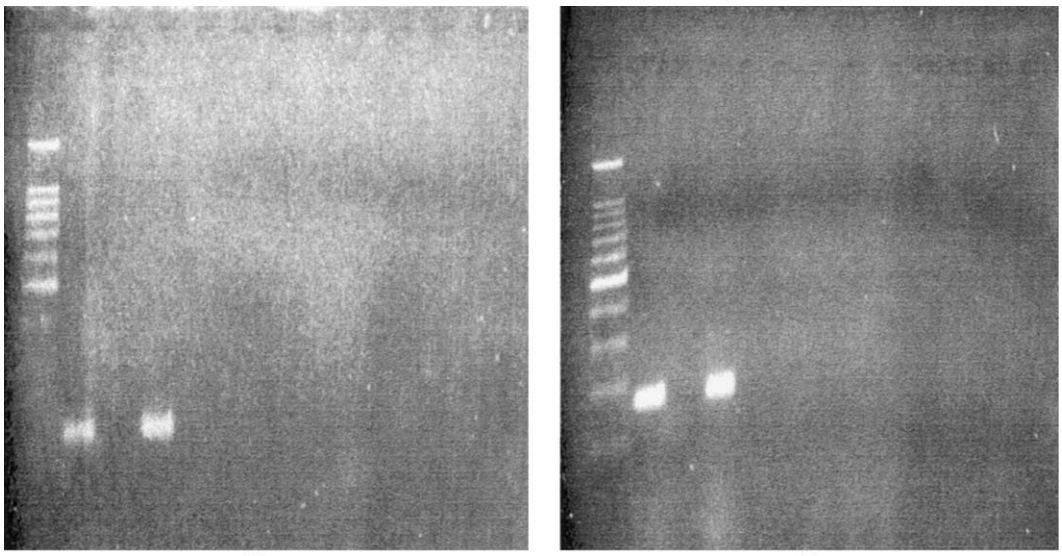

Fig. 2. (a) RT-PCR for 5α-RI and RII transcripts in primary cultured epithelial cells treated with breast and skin fibroblast CM. Lane 1, 100 bp markers; lane 2, 5α-RI breast fibroblast CM; lane 3, 5α-RII breast fibroblast CM; lane 4, 5α-RI skin fibroblast CM; lane 5, 5α-RII skin fibroblast CM; lane 6, 5α-RI breast fibroblast CM RT-negative; lane 7, 5α-RI breast fibroblast CM PCR-negative; lane 8, 5α-RII breast fibroblast CM RT-negative; lane 9, 5α-RII breast fibroblast CM PCR-negative; lane 10, 5α-RI skin fibroblast CM RT-negative; lane 11, 5α-RI skin fibroblast CM PCR-negative; lane 12, 5α-RII skin fibroblast CM RT-negative; lane 13, 5α-RII skin fibroblast CM PCR-negative. (b) RT-PCR for 5α-RI and RII transcripts in prostate epithelial cells incubated with heat (lane 1, 100 bp ladder; lane 2, 5α-RI; lane 3, 5α-RII) and trypsin-treated (lane 4, 5α-RI; lane 5, 5α-RII) CM-active fraction. Lane 6, 5α-RI heat-treated CM RT-negative; lane 7, 5α-RI heat-treated CM PCR-negative; lane 8, 5α-RII heat-treated CM RT-negative; lane 9, 5α-RII heat-treated CM PCR-negative; lane 10, 5α-RI trypsin-treated CM RT-negative; lane 11, 5α-RI trypsin-treated CM PCR-negative; lane 12, 5α-RII trypsin-treated CM RT-negative; lane 13, 5α-RII trypsin-treated CM PCR-negative.

indicated that there was no change in differentiation of the cells during the culture period or following incubation with CM (data not shown).

3.2. Effect of trypsin and heat on activity of CM

HPLC-purified CM fractions, which had demonstrated activity prior to treatment with either trypsin or heat, were subsequently found not to induce 5α-RII mRNA transcription in primary cultured prostate epithelial cells (Fig. 2b) following either treatment. This procedure was carried out on three separate samples to confirm those effects.

3.3. Effect of fractionation of CM on ability to induce 5α-reductase type II mRNA transcription in primary cultured epithelial cells

Primary cultured epithelial cells, which had been treated with the various fractions of the CM, were analysed for the presence of 5α-reductase type II mRNA transcripts by RT-PCR. The 60% ammonium sulphate fraction was found to contain the active fraction. Following cation-exchange chromatography, flow-through from the cation-exchange column was found to contain the

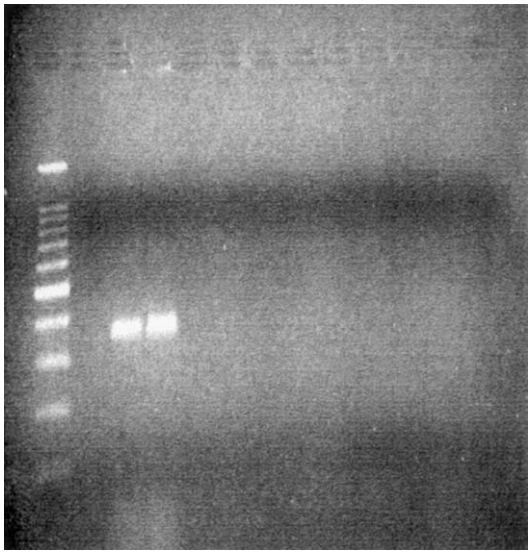

Fig. 3. RT-PCR of RNA obtained from primary cultured prostate epithelial cells treated with size-fractionated CM. Lane 1, 100 bp markers; lane 2, <3 kD; lane 3, >3 kd <10 kD; lane 4, >10 kD <30 kD; lane 5, >30 kD; lane 6, <3 kD RT-negative; lane 7, <3 kD PCR-negative; lane 8, >3 kD <10 kD RT-negative; lane 9, >3 kD <10 kD PCR-negative; lane 10, >10 kD <30 kD RT-negative; lane 11, >10 kD <30 kD PCR-negative; lane 12, >30 kD RT-negative; lane 13, >30 kD PCR-negative.

bio-active component. Subsequent fractionation of this material by anion-exchange chromatography isolated the active component to two collected fractions corresponding to a retention time of 19.96 min on the column after commencing with the application of the salt gradient. Material contained in these samples was fractionated further by passing it sequentially through a descending series of MWCO spin columns. Cells which had been treated with anion-exchange fractions obtained from the <10 kD and $<10>3$ kD MWCO columns demonstrated

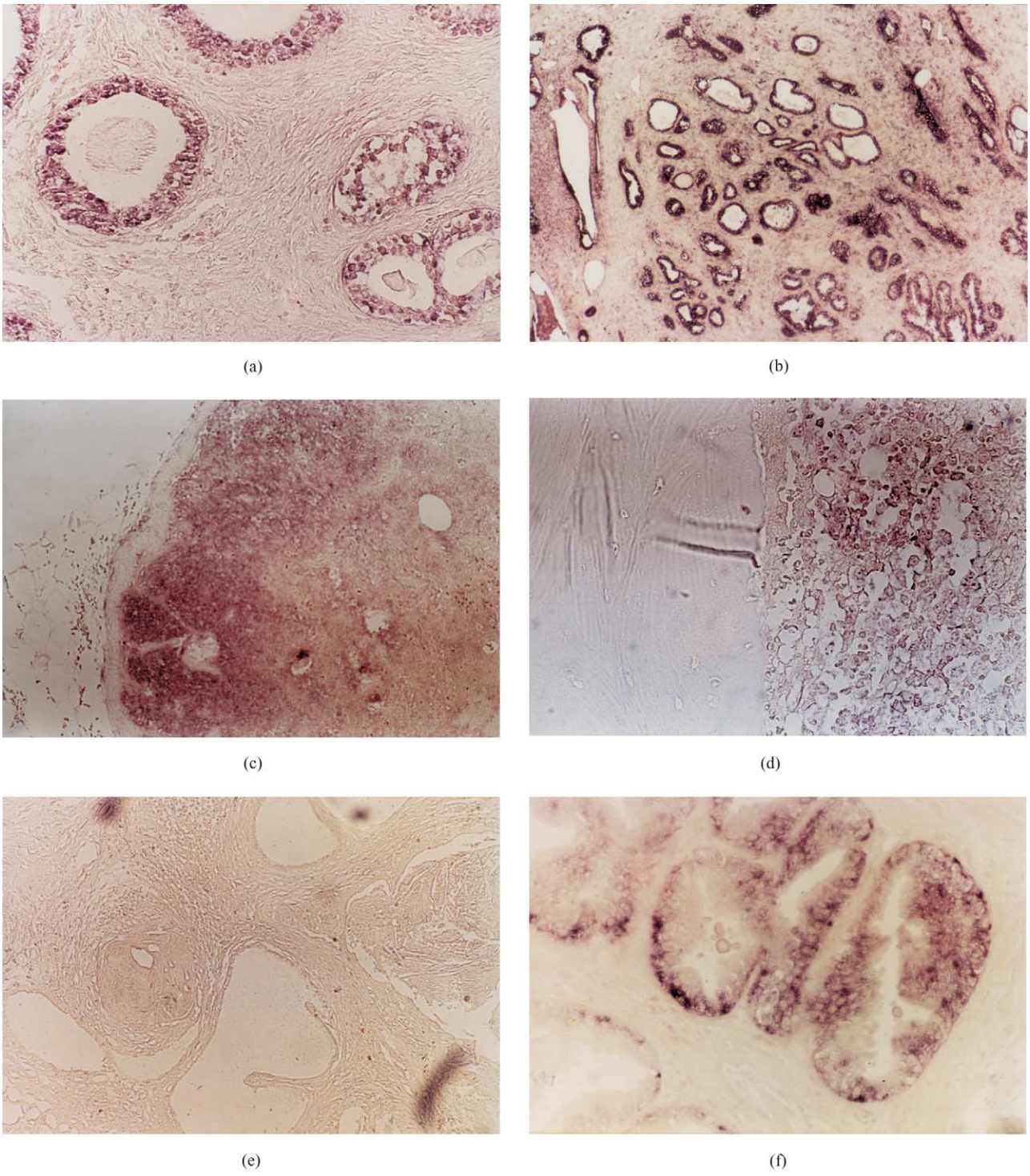

Fig. 4. *In-situ* hybridisation in metastatic prostate samples (a) 5α-RI BPH, (b) 5α-RI CaP, (c) 5α-RI metastatic CaP in lymph node, (d) 5α-RI metastatic CaP in bone, (e) 5α-RI-negative (BPH), (f) 5α-RII BPH, (g) 5α-RII CaP, (h) 5α-RII metastatic CaP in lymph node (i) 5α-RII metastatic CaP in bone, (j) 5α-RII-negative (BPH).

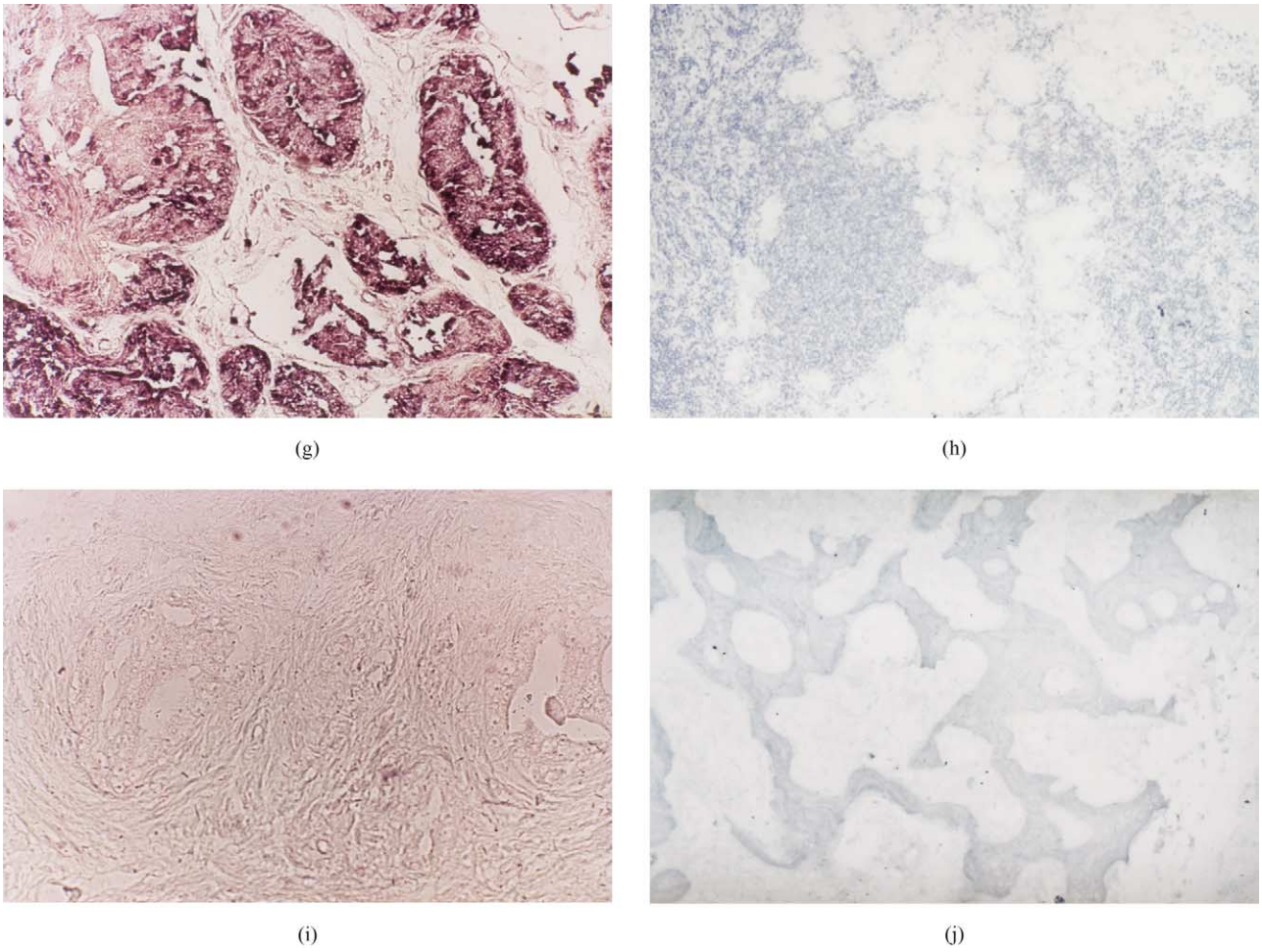

Fig. 4 (*continued*).

the ability to induce transcription of 5α-RII mRNA in prostate epithelial cells (Fig. 3). N-Terminal amino acid sequencing by Edman degradation of the clean and unambiguous fractions produced a partial sequence of I P L Q C/W T A/P D/F. A blast search of the Swissprot protein database revealed no significant matches.

3.4. In-situ *hybridisation for 5α-RII mRNA in prostate metastasis*

Archival sections of metastatic prostate cancer in lymph node and bone were analysed for the presence of 5α-RII mRNA transcripts by *in-situ* hybridisation. All metastatic CaP samples thus investigated demonstrated no transcription of 5α-RII mRNA (Fig. 4). Negative controls hybridised with sense probe were included (data not shown). All *in-situ* hybridisation was performed in triplicate.

3.5. *Effect of cycloheximide on the ability of CM to induce 5α-RII mRNA transcription*

Cells treated with an active fraction and cycloheximide (10 μg/ml) demonstrated no 5α-RII mRNA

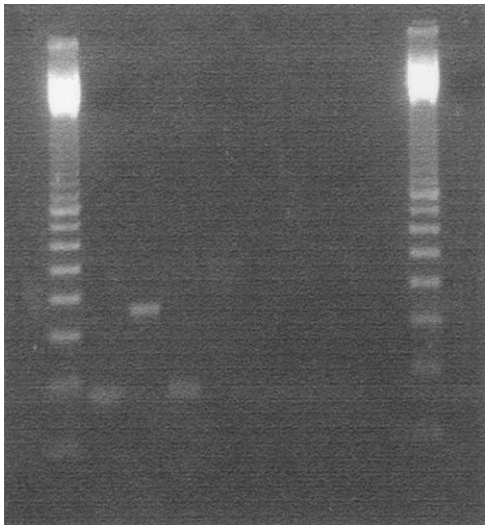

Fig. 5. RT-PCR of 5α-RII transcripts in RNA extracted from primary prostate epithelial cells treated with CM and cycloheximide (10 μg/ml). Lane 1, 100 bp ladder; lane 2, 5αRI (no cycloheximide treatment); lane 3, 5αRII (no cycloheximide treatment; lane 4, 5αRI transcription following cycloheximide treatment prior to incubation with CM fraction; lane 5, 5αRII transcription following cycloheximide treatment prior to incubation with CM fraction.

transcription when analysed by RT-PCR. Cultured epithelial cells which were incubated with active fraction but no cycloheximide, demonstrated 5α-RII mRNA transcription following analysis by RT-PCR (Fig. 5). Control cells which received neither fraction, nor cycloheximide were found not to transcribe 5α-RII mRNA. The effect of cycloheximide on RNA transcription was repeated three times to confirm the results.

4. Discussion

Transcriptional and translational regulation of genes encoding the two isoenzymes of 5α-R in the human prostate has been the subject of intense debate. Earlier evidence had demonstrated a possible link between DHT and an increase in the transcription of 5α-reductase type II mRNA [3]. Although the evidence for this association was compelling, there was no data which directly associated the increase of 5α-RII mRNA in primary cultured epithelial cells treated with DHT. However, when this data is considered along with later studies, which demonstrate cellular interactions between the stromal and epithelial components of the prostate [4,5,11], a paracrine control system becomes probable. Although the exact nature of this paracrine control system remains to be elucidated, it presents a number of opportunities for manipulation of steroid metabolism which may provide a better understanding of the processes involved in the development of BPH.

Earlier studies have shown that the epithelial compartment of the prostate has higher levels of 5α-reductase activity than the stromal compartment [12]. Correspondingly, this increased the likelihood that the site of DHT production is the epithelium. However, it has since been shown that the site of DHT activity is in-fact the stromal compartment of the prostate and that many of the actions of DHT are actually mediated through the stroma. This prompted us to examine possible influences of the fibroblast cells on the epithelial component of the gland. Our earlier work demonstrated that cell interactions were responsible for the maintenance of many *in-vivo* characteristics of the gland which were subsequently lost following primary culturing of the cells [6]. By examining the effect of fibroblast cells on the primary cultured epithelial cells, we have shown that a soluble factor produced by the fibroblast component of the human prostate can induce 5α-RII mRNA transcription.

We have also demonstrated that fibroblast cells derived from breast and skin do not appear to secrete the same soluble factor as the prostate fibroblast cells. This may be of importance when considering metastatic prostate cancer (CaP). By removing the epithelial cells from the controlling influence of the prostate fibroblast cells, the prostate epithelial cells may then de-differentiate thereby increasing the ability of the tumour to metastasise. Indeed this hypothesis has been supported by the results obtained by *in-situ* hybridisation on metastatic prostate sections which failed to provide evidence of 5α-RII transcriptional activity.

As the ability of the CM to induce the transcription of 5α-RII mRNA is inhibited by cycloheximide (a protein interaction inhibitor [13]), it is probable that the soluble factor is not acting directly with epithelial DNA, but is instead activating a signalling system resulting in an increase in the transcription of 5α-RII mRNA in the epithelial cells. If this is the case, it offers the opportunity to artificially regulate this system and thereby influence the development of BPH.

Although we have restricted this investigation to the induction of transcription of 5α-RII mRNA, we have demonstrated that prostate epithelial cells require the products of prostate fibroblast cells for normal function. It is therefore conceivable that many other 'normal' epithelial functions are under the direct or indirect control of the surrounding stromal compartment of the prostate. A better understanding of the epithelial/stromal relationship may prove invaluable in the search for an understanding and treatment for the development of BPH.

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