The paper is dedicated to Professor Włodzimierz Ostrowski

The effect of dihydrotestosterone on transcription of prostatic acid phosphatase mRNA in human hyperplastic gland*

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The effect of 5α-dihydrotestosterone (DHT) on the level of human prostatic acid phosphatase (hPAP) mRNA was studied using tissue slices from various benign prostatic hyperplastic glands.

The absence of DHT in the incubation medium led to a gradual, significant decrease of the hPAP mRNA level. Addition of the hormone induced hPAP mRNA in a time- and dose-dependent manner. The maximal 2–4-fold induction by 10^{-9} M DHT was observed after 3–5 h of incubation, and then the hPAP mRNA level was 6–20-fold higher than that in a parallel sample incubated without DHT. The results suggest that DHT is necessary to sustain the expression of hPAP in hyperplastic prostates.

It is commonly accepted that expression of prostatic acid phosphatase (PAP) is under androgen regulation [1, 2]. Since steroid hormones are used in prostate cancer therapy, intensive studies have been focused on their effect on metabolism of two prostate tissue specific proteins, PAP) and prostate specific antigen. Recent studies on androgen regulation of rat PAP transcripts, analysed in the ventral prostate of animals after castration and testosterone replacement therapy, showed the presence of three transcripts, two of them the medium (2.3 kb) and the shortest (1.5 kb) sized were up-regulated while the synthesis of the longest transcript (4.9 kb) was not influenced by testosterone [3]. Structures of rat and human PAP genes, including steroid response elements, are similar [4]. Bearing that in mind one could expect androgens to have a similar effect on expression

Abbreviations: BPH, benign prostatic hyperplasia; DEP, diethylpyrocarbonate; DHT, 5α-dihydrotestosterone; FCS, fetal calf serum; FCS-SF, steroid free fetal calf serum; hPAP, human prostatic acid phosphatase; PAP, prostatic acid phosphatase

and/or level of hPAP mRNA in these two species. However, there are only a few studies on hormonal regulation of PAP protein in healthy men and their results are contradictory [5, 6]. Chu *et al.* [7] reported on a significant level of PAP in circulation of patients treated for advanced prostate cancer with androgen deprivation, suggesting that, at least at this state of the disease, expression of PAP gene may not require androgen stimulation. Most of more detailed studies on the effect of 5α-dihydrotestosterone (DHT) — a main androgen regulating prostate metabolism [8–10], on expression of PAP in men, were carried out almost exclusively on cultured metastatic cell line LNCaP, and brought somewhat divergent results. Lin *et al.* [2] reported that the expression and secretion of hPAP in these cells was up-regulated by DHT although the cells did not show, an absolute requirement for DHT for enzyme synthesis. On the other hand, Henttu & Vihko [11] demonstrated that DHT down-regulated the level of hPAP synthesised by the same cell line. Later, Lin & Garcia-Arenas [12] explained the observed differences by demonstrating that DHT regulation of the PAP mRNA level depended on density of the cultured cells, and that is why the earlier results were incomparable.

Hormonal regulation of human prostate and metabolism of prostate specific antigen were recently successfully studied in organ culture [13]. Therefore we have decided to study the effect of DHT on transcription of human PAP mRNA using tissue slices as a simple model. This model allows to maintain throughout the course of the experiment a natural cellular surrounding absent in a culture of a single type cell line, e.g. LNCaP. In this work, we have applied tissue slices to study the short-term effect of DHT on hPAP mRNA level in benign hyperplastic prostate tissue.

MATERIALS AND METHODS

Synthesis of oligonucleotide probes and probe labelling. Oligonucleotide probes of 29–31 deoxyribonucleotides were synthesized basing on human PAP cDNA [14], using an automatic oligonucleotide synthesiser (Pharmacia LKB Biotechnology) as described by Matthes *et al.* [15]. Crude preparations of the probes were further purified to homogeneity by electrophoresis in 20% polyacrylamide gel (acrylamide-bisacrylamide, 19:1 w/w) in 8 M urea and chromatography on Sephadex G-25 (Pharmacia NAP-10 column) [15]. The purity of probes was verified electrophoretically on 10% polyacrylamide gel. Only non-degraded intact probes showing the absorbance ratios close to 2:1 were used to detect mRNA for the enzyme.

The homogeneous oligonucleotide (100 ng each) was labelled at its 5′ terminus using T4 polynucleotide kinase (Amersham) with 50 μCi of [γ-^{32}P]-ATP (5000 Ci/mmole, Amersham) according to Maniatis [16]. Excess of label was removed on DEAE-cellulose 52 column equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 5 mM EDTA, followed by elution with 0.2 M and 0.5 M sodium chloride.

Clinical samples. Samples of prostate tissues from patients with diagnosed and histopathologically confirmed hyperplasia (age 72 ± 10) supplied by Dr. S. Mrozicki (Urology Department; Military Hospital, Cracow) and Dr. Gałka (Urology Department, G. Narutowicz Hospital, Cracow) were collected at the time of surgery. Immediately after resection of a gland, a part of tissue was immersed in a sterile phosphate buffered saline, pH 7.2 containing antibiotics (streptomycin and penicillin), and kept on ice until used for further studies (not later than 30 min). The other part of tissue was snap-frozen in liquid nitrogen and stored at −70°C for reference studies.

Preparation and treatment of tissues slices. The entire preparation of tissue slices was performed under sterile conditions until otherwise stated. A piece of prostate tissue brought from the surgery unit was cut with a razor blade into small slices (1 mm × 1 mm × 2mm) and continuously washed during the procedure with RPMI 1640 medium (Sigma) to remove blood and cell debris from the cut surfaces.

The weighed tissue slices (0.1–0.2 g) were incubated at 37°C in an incubator (5% CO_2

and 95% air) for 1, 3, 5 h or overnight in the RPMI 1640 medium containing 1% L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) and additionally supplemented with 10% fetal calf serum (FCS; Gibko, Great Britain) or 10% steroid free serum, FCS-SF (charcoal-stripped fetal calf serum) [17] in the absence or presence of DHT (10^{-7}–10^{-9} M). The analysis of total RNA isolated from prostate slices showed that the amount of hPAP mRNA depended on the conditions of the experiment. After 24 h incubation in the presence of FCS-SF instead of FCS in the RPMI 1640 medium, the level of hPAP was significantly lower. Thus to avoid any interference of FCS with expression of the PAP gene in benign hyperplastic prostate, a steroid free serum (FCS-SF) was used in the experiments with various amounts of DHT.

Isolation of RNA. Total RNA was isolated from human prostate tissue samples using a guanidinium isothiocyanate procedure, followed by centrifugation in a cesium chloride gradient [18] or by the Chomczyński method [19] based on commercial Trizol reagent (Gibco), according to the instructions of the manufacturer. Usually 0.2 mg of RNA was obtained from 1 g of prostate tissue.

Poly(A)$^+$RNA was obtained from total RNA using affinity oligo(dT) cellulose chromatography [16].

The quality of RNA samples was always verified by spectrophotometric (A_{260}/A_{280}) and electrophoretic (1.2% agarose gel) analysis [16].

Northern blot analyses. An aliquot of each RNA sample was electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, with a running buffer (20 mM morpholino-propane sulfonic acid, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). Prior to electrophoresis 25 µg of each RNA sample was denatured by heating at 65°C for 15 min in a solution containing the running buffer, 50% formamide and 6% formaldehyde. Intactness of RNA in each sample was ascertained after electrophoresis by staining with ethidium bromide [16], then RNAs were transferred to a nylon membrane (Zeta-Probe Blotting Membranes BioRad) by capillary blotting in 10 × SSC (1 × SSC = 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7) and covalently bound to the membrane. The filter was placed between two sheets of 3MM paper, and baked at 80°C for 1.5 h in a vacuum oven.

Hybridisation. The nylon membranes were always pre-hybridised for 3 h at 42°C in 10 ml of hybridisation cocktail containing 5 × SSC, 50% formamide, 20 µg/ml salmon sperm DNA, 0.5% SDS, 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidine, 0.02% Ficoll) in Ziploc storage bags (Gripper Zipper). The labelled oligonucleotide probes (5×10^{7}–1×10^{8} c.p.m.) were then added and hybridisation was performed at 42°C for 18 h in a water bath with constant rocking (Heidolph Polymox 1040) in 10 ml of hybridisation solution. After hybridisation, the filters were washed very thoroughly twice for 15 min each in 5 × SSC, 0.1% SDS solution at room temperature and subsequently twice for 30 min in 1 × SSC, 0.1% SDS solution at 65°C. The filters were dried in air and exposed to Medical X-ray film Blue AGFA at –70°C, for 4–15 days [16]. After exposure the nylon filters were cut into small fragments, soaked with scintillation fluid (Bio Care) and counted in Pacard Tri-carb Liquid Scintilation Spectrometer C 2425.

The amount of mRNA present in every analysed sample was expressed in percent of counts referring to the non-incubated sample.

All reagents were of analytical grade.

RESULTS

To study the effect of DHT on hPAP mRNA level in benign hyperplastic prostate tissue four deoxyribonucleotide probes corresponding to cDNA of PAP were used. Two of them, the 29-mer 5'-TGGGACTTCG GTCTCC-ATGC CGAAACACC-3' and 31-mer 5'-GGGATCACAG GGCCAACCAG CTCAG-CAAACC -3', were identical to the fragments of sense strand of hPAP cDNA between positions 143–171 and 1087–1117, respectively [14]. The probes were purified to homogeneity as proved by polyacrylamide gel electrophoresis.
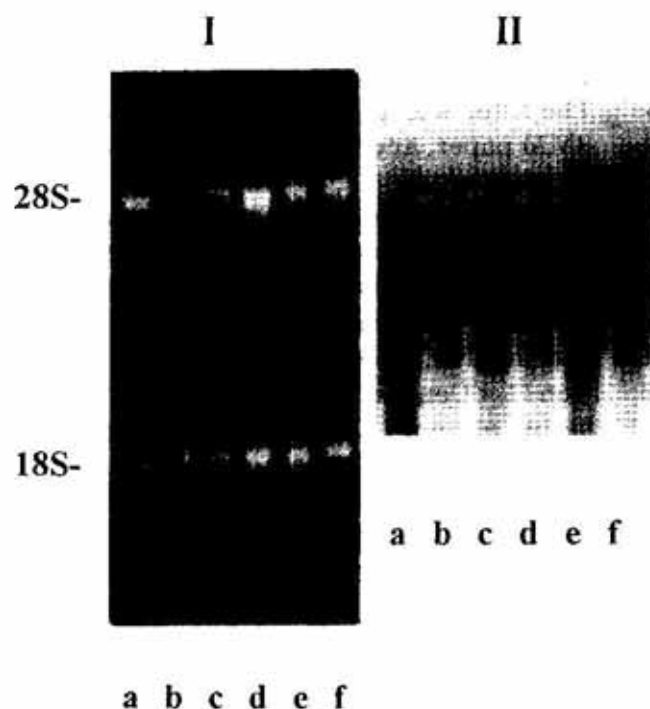
I   II

28S-

18S-

a b c d e f

Figure 1. Northern blot analysis of hPAP mRNA in total RNA isolated from human prostate tissues.

Total RNA (25 µg) from different benign hyperplastic prostate tissues (lanes a, b and d–f) and poly(A)$^+$ RNA (8 µg) (lane c) fractionated by electrophoresis and visualized with ethidium bromide (I) was hybridized to ^{32}P-labelled oligonucleotide probes (II).

a b c d e f

Figure 1 depicts autoradiographs of total RNA samples hybridised with ^{32}P-labelled probes. The results pointed to the presence, in total RNA, of a 3.3 ± 0.1 kb fraction specifically reacting with the probes regardless of the origin of the samples. This fraction was observed both in RNA from prostate tissue snap-frozen after surgery in liquid nitrogen, and in RNA from the slices of acinar part of the gland cultured as described in Materials and Methods.

The intensity of signal visualised on film after hybridisation with PAP specific probes depended on the amount of total RNA applied on agarose gel indicating that the method can be used for quantitation of hPAP mRNA in total RNA (Fig. 2).

The effect of 5×10^{-9} M DHT on hPAP mRNA level in the samples of benign hyperplastic prostate tissue from different donors was time-dependent. The mRNA level increased significantly during the first few hours of incubation, reaching the highest level after 3–5 h (Fig. 3). The time-dose relation between concentration of DHT in the incubation medium and PAP mRNA level showed that the most efficient response — 2–4-fold induction of hPAP mRNA was that to 10^{-9} M DHT on 5 h incubation (Fig. 4).

In the tissues from different donors the dose-dependence on 5 h incubation confirmed that the highest induction of hPAP mRNA was at 10^{-9} M concentration of DHT. The hPAP mRNA level was 6–20-fold higher than the level in the same tissue incubated in parallel without DHT. At higher concentrations the expression of PAP gene was lowered (Fig. 5A). Prolonged incubation for 24 h resulted in a marked decrease of hPAP mRNA level irrespective of tissue donor, although at
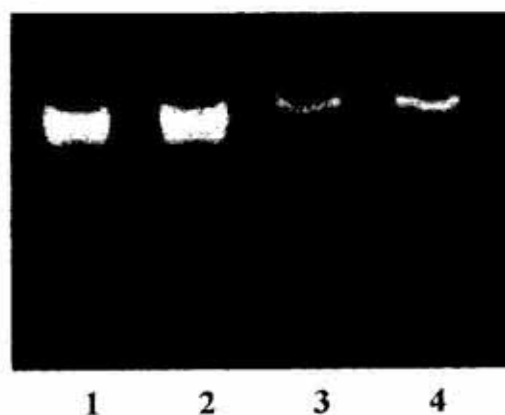
1   2   3   4

Figure 2. Quantitation of hPAP mRNA in total RNA isolated from hyperplastic human prostate.

Lane 1, 20 µg; lane 2, 15 µg; lane 3, 10 µg. Positions of 28S and 18S rRNAs are indicated.

DHT

-28S

-18S

1   2   3   4   5
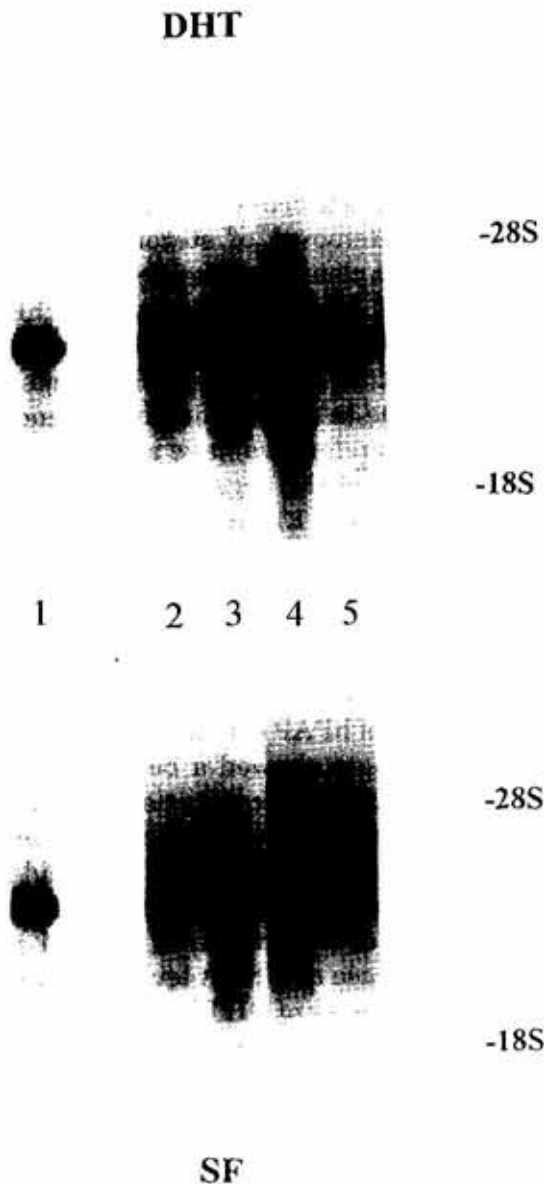

-28S

-18S

SF

Figure 3. Autoradiogram of human prostatic RNA blot analysis.

Total RNA was isolated from slices of benign hyperplastic prostate gland incubated for various time intervals in RPMI 1640 medium with 10% FCS-SF containing 5×10^{-9} M DHT (DHT). As a control, RNA was incubated in 10% FCS-SF without DHT (SF). Lane 1, reference sample (0 h incubation); Lanes 2–5, incubation for 1, 3, 5 h and overnight, respectively.

DHT concentration of 10^{-9} M this level exceeded the initial reference level (Fig. 5B).

DISCUSSION

Oligonucleotide probes used in examination of the effect of 5α-dihydrotestosterone on expression of PAP gene enabled detection
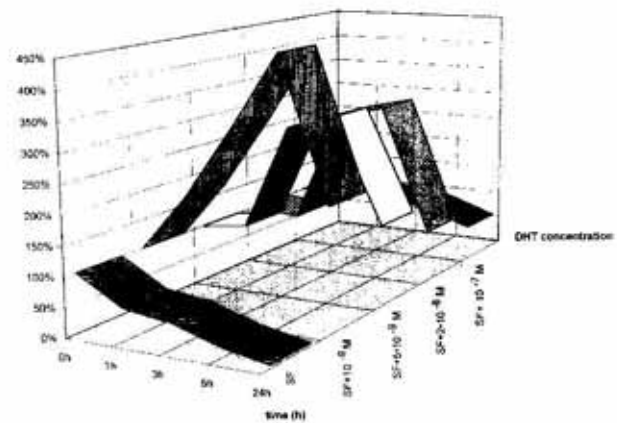

Figure 4. The time-dose dependence of hPAP mRNA level on DHT concentration.

Tissue slices were cultured for the time indicated in RPMI 1640 medium with FCS-SF (SF) or FCS-SF supplemented with DHT (SF + 10^{-9}–SF + 10^{-7} M). The hPAP mRNA level was expressed in percent of the level in non-incubated samples.

and quantitation of one RNA transcript in total RNA from human benign hyperplasia prostates tissue. The size of the transcript, 3.3 ± 0.1 kb, was in agreement with the value previously reported for hPAP mRNA [2, 20–22].

For examination of the effect of androgens on PAP synthesis tissue slices seem to be more useful than a metastatic cell line. The lack of intercellular, stromal-epithelial interactions in cell culture might lead to misleading conclusions. Several lines of evidence suggest that complex interactions between epithelium, extracellular matrix and stroma play an important role in maintaining normal function of prostate epithelial cells [23, 24]. Guenette & Tenniswood [25] hypothesized that disruption of the growth factor mediated cell-cell communications and destruction of basement membrane leads to apoptosis of prostatic secretory epithelial cells. The results of Krieg *et al.* [9, 10] on distribution of androgens and the activities of androgen metabolising enzymes in various parts of prostate tissue also support the view that the effect of these hormones on metabolism of this gland involves different types of cells. Thus, the reported discrepancies and uncertainties with respect to androgen regu-
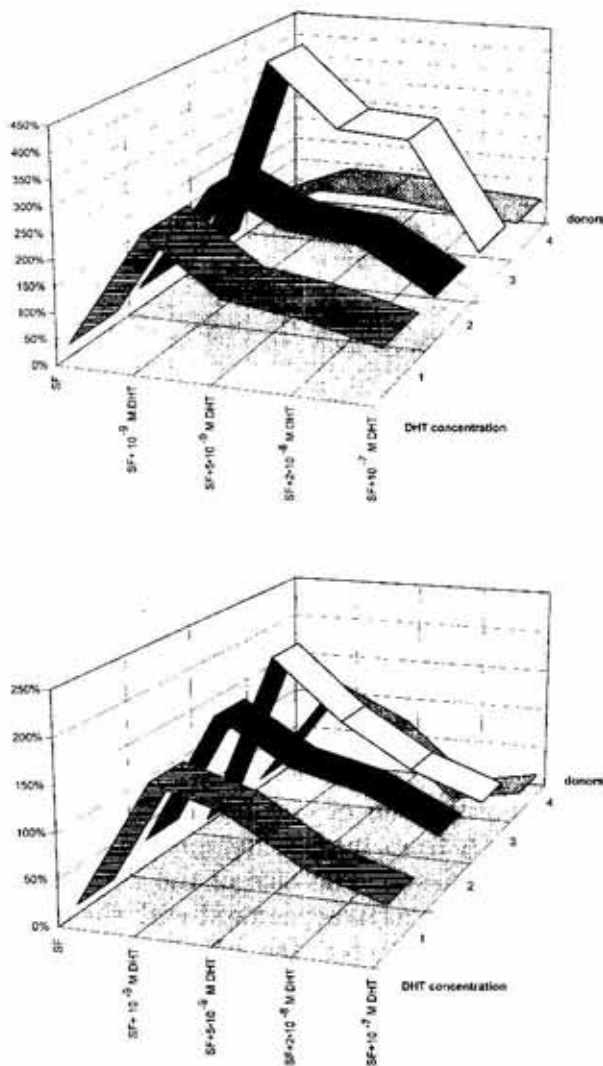
potent androgenic hormone, on the level of hPAP mRNA in tissue slices from prostate tissue with benign hyperplasia.

A proper choice of conditions for experimentation on hormonal regulation in prostatic tissue cultures is decisive for arriving at right conclusions. Removal of steroids from the culture to get a clear, univocal response of the gland to DHT leads to a decrease of hPAP mRNA to an undetectable level. On the other hand, involutive morphological changes of prostate tissue take place in the absence of DHT [13]. Therefore we have applied a simplified version of the organ culture used by Nevalainen *et al.* [13] and we have not extended our observations on the DHT effect beyond 24 h. Spectro- and electrophoretical analysis of total RNA isolated from the tissues incubated for 24 h pointed to integrity and intactness of RNA (not shown).

Comparison of hPAP mRNA levels in tissue of various donors showed a bell-shaped dependence on DHT concentration regardless of the time of incubation. The concentration of DHT in the medium above 10^{-8} M did not significantly influence the level of PAP mRNA. DHT concentration of 10^{-7} M prevented a decrease of mRNA level which took place in the absence of DHT but did not cause an increase in hPAP expression. The observed decrease of hPAP mRNA level at DHT concentration above 10^{-8} M confirmed the previous reports on a bell-shaped dependence of prostatic cell proliferation in culture on androgen concentration [27]. Growth of LNCaP cells exposed to higher DHT concentrations was significantly inhibited [17, 28]. Although a characteristic shape of this dose-response curve remains poorly understood, one possible and presently accepted explanation of such a relation indicates down-regulation of androgen receptor and lack of its stabilisation at high hormone concentrations [29, 30]. However, one should not exclude other factors, not only the direct hormonal impact, as probably much more complicated mechanisms are involved in regulation of hPAP gene expression [3].

Figure 5. The dose dependence on DHT concentration of hPAP mRNA level following 5 h (A) and overnight (B) incubation.

Tissue slices obtained from four benign prostatic hyperplasia donors (1–4) were incubated for 5 h or overnight in RPMI 1640 medium with FCS-SF (SF) or FCS-SF supplemented with DHT at the concentrations indicated. The results expressed in percent of the level in the non-incubated samples.

lation of hPAP synthesis [2, 5–7, 11] justify the use of tissue slices.

Recently organ culture was found to be suitable for evaluation of hormone regulation in human prostate [13], and Wheeler & Lebovitz [26] suggested that the prostate tissue obtained after prostatectomy might, and perhaps should, be stored in a medium containing androgens for even a week at 4°C before harvesting of RNA. Therefore we have decided to study the effect of DHT, the most

sity, Cracow) for histopathological verification of the tissues studied.

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