Regulation of Prostate Growth in Culture with the Pollen Extract, Cernilton T-60, and the Impact of the Drug on the EGF Tissue Profiles

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Introduction

A major difference between the prostate and other accessory reproductive glands is the susceptibility of the prostate to hyperplasia in again men. Indeed, benign hyperplasia of the prostate (BPH) affects most males over 60 years of age and causes enlargement of the inner gland. When the urethra becomes constricted, treatment is required to relieve the kidney and circulation system of the damaging effects of back pressure.

Surgery in the form of transurethral resection still remains the “gold standard” for the treatment of outflow tract obstruction (1) but recently attention has also focused on alternative forms of therapy, namely hormonal (2, 16), 5α-reeducates inhibitors (11), and α-adrenergic blockers (5). However, the long-term prognosis for medical treatment has been poor and many of the endocrine and pharmacological agents presently in use have side effects (4). This has prompted the medical and scientific community to consider new lines of treatment of BPH. One recent development was sudden and unexpected interest in phytotherapy, which was in part instigated by the encouraging results and the undoubted beneficial effects of the pollen extract, Cernilton, in the symptomatic relief of BPH (1).

The mechanism by which the pollen induces its effect on the hyperplastic prostate is not yet clear even though extensive experimentation has been undertaken by many workers (8, 9, 10). Notably however, the bulk of the earlier research was focussed on experiements with animal tissue, which constitutes an unsatisfactory model for the human gland. Additionally, the few studies on the human prostate were carried out either on whole organ homogenates or on prostate epithelical cell lines (8), both of which ignore the potential heterogeneity of the cellular activity within the gland and the importance of stroma / epithelial interactions. Futhermore, the immortal cell lines represent a highly selective cell population which might have undergone phenotypic changes and may therefore be distinctive from the cells of orgin.

In attempt to overcome these earlier limitations, efforts in our laboratory have been directed towards developing primary culture of the human prostate and the serial culture of epithelial and fibroblast cells from BPH employing defined media. Initially, progress was slow and attempts to find the optimal concentration of ingredient to permit the growth of the cells and increase their plating efficieny were repeatedly frustrated. However, thanks to our collaboration with Dr. D. *Chaproniere,* to whom much of the credit for the earlier work goes (3), combined with the perseverance of the chief tissue culturist, Mrs. *Margaret Ross,* we managed to overcome many of the initial obstacles and finally establish a reliable technique for the serial culture of both prostate stroma and epithelial cells in serum-free medium (manuscript in preparation). This model was subsequently adapted to our Cernilton studies in which the experiements were confirned to the water-soluble Cernitin T-60 fraction; this fraction accounts for approximately 60% of the pollen extract. Detail of the procedures followed and summary of our findings on the characterization of the cultured cells along with the impact of the Cernitin T-60 are described within.

This chapter also includes some preliminary data on growth factor profiles in prostate tissue specimen and in expressed prostate secretions (EPS) obtained from a group of BPH patients

receiving the pollen extract. The relevance of growth factors peptides and particularly epidermal growth factor (EGF) to the prostate stems from their ability to maintain and regulate prostat growth either by acting in tandem with androgens or possbiliy even by by-passing the steroid hormomes and imprinting their own characteristics on the gland (7, 12). Recent reports on the preferential accumulation of EGF in BPH when compared to normal prostate tissue (6, 14) supports the belief that this peptide might be implicated in the pathogenesis of this condition. Since the action of Cernilton in the prostate has been found not to be mediated via the androgen delivery system of the cell (8), we are now looking at the possibility of an association between the expression of some of these growth factors and their response to Cernilton in patients receiving the drug.

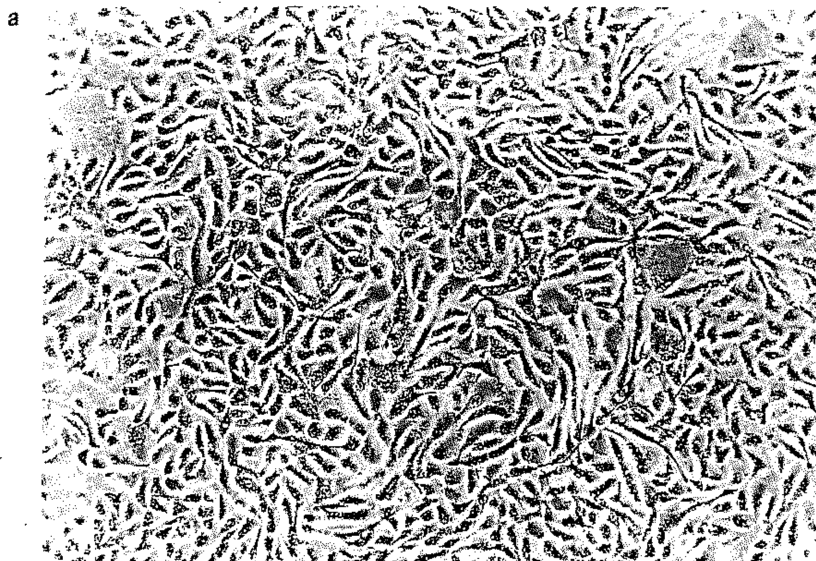
Serial Culture of Prostate Epithelial and Fibroblast Cells

BPH specimens obtained by transurethral resection were transported under sterile conditions to the laboratory in transport medium. Acini and fibroblast cells were released from prostate tissue by collagenase digestion and primary and sub-cultures were grown by plating onto plastic culture flasks and incubating at 37°C in a 95% air-5% CO_2 humidified atmosphere. By using this sytem it was possible to establish and serially culture pure populations of both epithelial (Fig **1a**) and fibroblasts (Fib **1b**) cells in well-defined media. For epithelial cells the WAJC404 medium (3) was serum free and was supplemented with insuling (2.5µg / ml), EGF (10 ng / ml), dexamethasone (1µM), and choleratoxin (10µg / ml); this medium selects against the growth of the fibroblast cells. Four days after inculation of the epithelial cells onton T-75 flasks, the acini demonstrated good spread, and confluence was usually reached by day five. Fibroblast cells were maintained in RPMI1640 supplemented with fetal calf serum (10%) and penicillin and streptomycin (10µg / ml each). Fibroblast cells were initially slow in growing and confluence was reach usually after ten days.

Verification of the culture as prostatic fibroblast and epithelial cells is accomplished by immunocytochemical staining employing a variety of antiobodies including those for vimentin, desmin, prostatic-specific antigen (PSA), prostatic acid phosphatase (PAP), and cytokeratin. Assessment of the staining patterns and their intensities was always undertaken by an independent pathologist. A typical pattern of the staining profiles obtained is illustrated in Table **1**.

In addition to the immunostaining (Table **1**), the cells were also examined by phase contrast microscopy. Analysis of the photomicrographs (Figs. **1a** and **1b**) suggest that the resultant epithelial monolayers contain very little or no contaminants – any residual fibroblasts will be totally destroying by the epithelial growth medium. Futhermore, the bulk of the epithelial cells appear to be of a secretory nature since PAP and PSA are strongly expressed (Table **1**). The epithelial cells also stain uniformly for cytokeratin and recognize the antibody for the epidermal growth factor receptor. This confirms our earlier findings on the presence of EGF-receptors in epithelial cells of human prostate tissue (14).

In contrast, the fibroblast cells failed to stain for PAP and PSA but were positively labeled by antibodies for vimentin and desmin. Somewhat surpisingly, the fibroblast cells were also
outlined by the antibodies for cytokeratin and for Human Milk Fat Globulin (HMFG), which are exclusively epithelial in nature. This raises the possilbity that the fibroblast cells might contain small contaminats of a secondary cell. Closer examination of those fibroblastic cells by microscopy highlights the presence of small numbers of epithelial-like cells amongst the stromal monolayers. The secondary cells could be either non-secretory epithelial or endothelial cells which maintain an ,,epithelioid”-like appearance, but this needs to be confirmed. The presence of the fibroblast contaminants was also confirmed by flow cytometry and we are at present attempting to segregate the two cell populations employing a cell sorter. Interestingly, however, the “epithelioid”-like material appears not to multiply but remains constant throughout each passage and might merely act as a supportive matrix for the fibroblast.



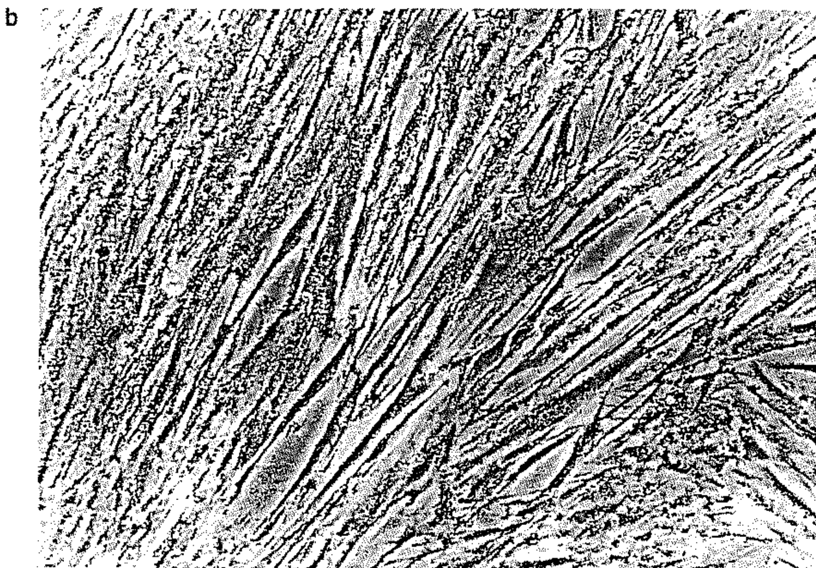


Fig. 1 Phase-contrast micrograph of a primary culture of epithelium (1 a; ×100) and a serial culture of fibroblast (1 b; × 200) from a patient with benign prostatic hyperplasia.

Tab. 1 Immunocytochemical Staining of Epithelial and Fibroblast Cells in Culture.

Markers used	Fibroblast cells	Epithelial cells
Prostatic acid phosphatase	–	++
Prostatic specific antigen	–	++
Epidermal growth factor receptor	–/+	++
Cytokeratin	–/+	++
Vimentin	++	–/+
Desmin	+	–
HMFG (Human Milk Fat Globulin)	–/+	++

Intensity of staining: (++) strongly positive; (+) moderately positive; (–/+) patchy; (–) negative.

The Effect of T-60 on Epithelial and Stromal Cell Growth in vitro

Dose response curves of Cernitin T-60 treatment were determined using the following method: triplicate determinations for each treatment were performed in 96 well culture plates; each well was seeded with 2.5 x 10^4 cells and incubated overnight at 37°C in the medium under defined incubation conditions. The following day, the Cernitin T-60 stock solution was serially diluted in the defined medium to yield a concentration varying from 0.05-1mg / ml. Controlled cultures received culture medium alone. For the dose response curve studies, the cells were exposed to Cernitin T-60 for a total period up to 4 days with changes of freshly diluted T-60 in medium every 2 days. For the time course study, cells were treated in the presence and absence of T-60 a total period of 7 days. After the incubation periods, the cells were pulse-labelled with radiolabelled thymidine whilst remaining in the defined medium for a further 24 hours.

For the determination of the rate of the DNA synthesis the cells were trypsinized and 10% ice cold tricholoacetic acid was added for 2 hours. The cells were subsequently harvested onto filter mats, dried at 60°C for 30 minutes and each disc of filter paper containing the precipitable material was then counted in scintillation fluid. The results illustrated in Fig **2** (fibroblast cells) and Fig. **3** (epithelial cells) are expressed as the percentage of ^{3}H-thymidine incorporated relative to the untreated control. These demonstrate that the effect of Cernitin T-60 on stroma and epithelial cells is biphasic: initially and at the low concentrations of T-60 (up to approximately 0.1 mg / ml) we detect significant stimulation, particularly in the fibroblast cells which show after 2 days of exposure an increase of appromately 75% in DNA synthesis. However, exposure to higher concentrations of the T-60 inhibits the uptake of thymidine and after 3-4 days exposure we do find that the concentrations of T-60 ($P > 0.25$ mg / ml) almost totally inhibit the fibroblast growth.

Although the epithelial cells do also show an inhibition in cell growth which is time-and concentration-dependent, it appears that the epithelial cells are slightly more resistant to the pollen extract than the fibroblast cells. Though there is initially a minute stimulation in the DNA synthesis of up to 25% after 2 days of exposure (results not shown), this is rapidly reserved, and inhibition is observed at approximately the same concentrations of T-60 as those required to induce the same effect with the fibroblast but following longer periods of response to the Cernitin T-60 (Fig. **3**).

EGF Concentrations in Prostate Tissue and Prostate Secretions following Cernitin Treatment

Prostate tissue was obtained at the time of transurethral resection from 19 patients with the BPH; the patients had been enterend into a Cernilton double-blind placebo-controlled study over a six-month period. The tissue was transported immediately to the laboratory in iced saline, dry blotted, snap-frozen in liquid nitrogen and stored at -70°C until analysis. Matching expressed prostatic secretions (EPS) were collected by transrectal massage before the commencement of the trial and at approximatelty three-month intervals with the last specimen obtained immediately prior to transurethral resection whilst the patient was under either regional or general anesthesia. The fluid was collected into 1-ml insulin syringes, frozen without delay, and stored at -70°C until needed.

Studies on EPS Specimens

Pre- and post-treatment samples of EPS were obtained from 8 patients in the Cernilton treatment group and 5 patients in the placebo group; the mean length of treatment with Cernilton was 147 ± 42 days. A comparison of EGF concentrations in both group before commencement of treatment revealed to significant difference ($P > 0.5$; Fig. **4**). Similarly, comparison of the EGF concentrations in samples before and after treatment also showed no significant difference ($P > 0.5$); these data are illustrated in Fig. **4**. In addition we have also examined the changes in EGF concentrations of consecutive samples of EPS from individual participants in the double-blind placebo-controlled study; the patterns obtained are illustrated in Fig. **5**. Clearly, there are no consistent patterns of change which could be of use for monitoring response to treatment.

Studies on Prostate Tissue

In addition to the measurements undertaken on EPS, we have also measured the ECG

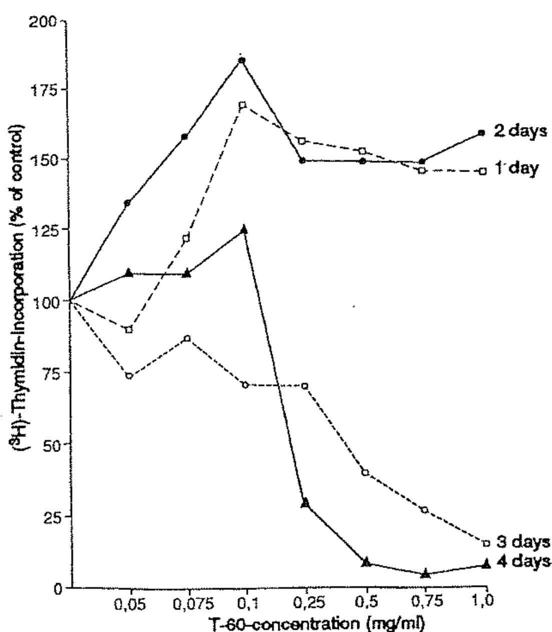


Fig. 2 Effect of T-60 concentration on fibroblast cell survival. Fibroblast cells (2.5×10^4 cells/well) were plated overnight in 96 well plates. Increasing concentrations of T-60 were added for varying times. (^{3}H)-thymidine was then added for 24 hours and the cells were trypsinized in 10 % TCA. The cells were then harvested onto filter mats, dried and counted in scintillation fluid. The data is normalized relative to the untreated control (100 %).

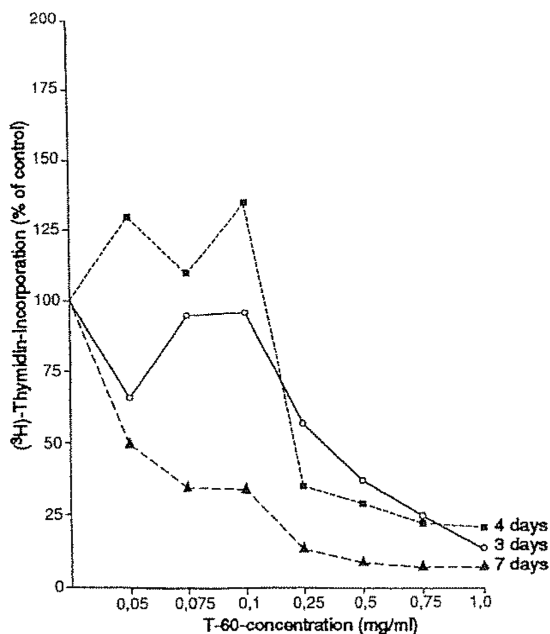


Fig. 3 Effect of T-60 concentration on epithelial cell survival. Details were identical to those followed for the fibroblast cells as detailed in legend to Fig. **2**.

concentrations in prostate tissue obtained from 15 BPH patients undergoing prostatectomy. These were compared to the concentrations found in a parallel group of 7 patients who were taking Cernilton as part of the double-blind placebo-controlled study. The data was expressed as ng EGF / mg protein in the tissue and the results obtained for the individual patients are outlined in Table **2**. Although the levels of EGF in the treated group appear to be considerably lower than those measured in the controlled group, the difference is not statistically different. However, it should be noted that the population receiving the Cernilton tablet is comparatively small and the results obtained might have been slightly biased by the fact that 2 out of 7 patients showed relatively high concentrations of EGF whereas the remainder of the population had levels considerably lower than those measured in any of the other individuals in the controlled group. We are at present extending this study to incorporate a further 20 patients on the drug in the hope that this might show some light no the mechanism of action of Cernilton and whether the differences between the control and test groups are genuine and reflect actual changes in the metabolic pathways of the gland following treatment with the pollen extract.

Conclusion

The precise mode of action of Cernilton in BPH is not clearly understood even though many studies have been undertaken to elucidate the mechanism by with this pollen extract promotes

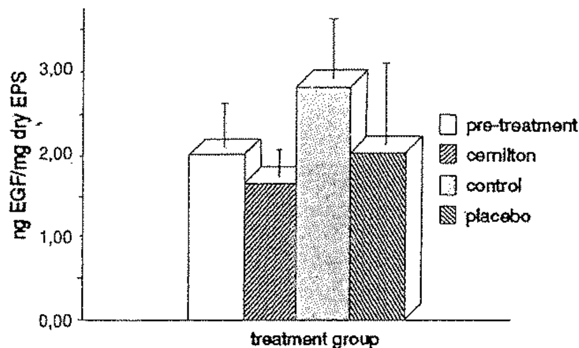


Fig. 4 EGF concentrations in expressed prostate fluid (EPS). Aliquots of EPS were taken from a group of patients who had entered the double-blind placebo trial of Cernilton, and EGF was measured in samples taken at the start and towards the end of the trial. The concentrations in the treated group were compared to those on placebo. Results are expressed as mean ± SEM for 8 patients in the treated group and 5 patients on placebo. Bars show SEM.

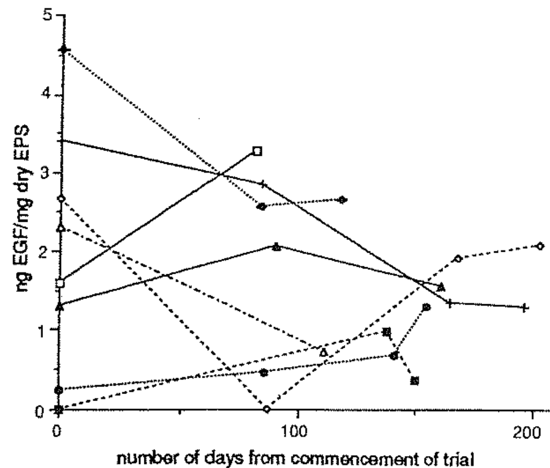


Fig. 5 EGF concentrations in consecutive EPS specimens taken from patients receiving Cernilton. For each patient EPS specimens were obtained before commencement of treatment and then at approximately 3 and 6 months into the trial.

symptomatic relief in patients with BPH. The earlier studies concentrated mainly on animal models and as reported by *Ito* et al. (9), Cernilton produced in mature Wistar rats a significant reduction in the size of the prostate as well as in PAP concentrations whilst also inducing a parallel increase in blood and tissue zinc concentrations. Additionally, Cernitin T-60 produced relaxtion of the smooth muscle of the mouse and increased the contraction of the bladder muscle in a concentration-dependent manner (10).

In view, however, of the specie differences in prostate anatomy and function, a fundamental distinction must be made between animal studies and experiements on human tissue. The attentions of this laboratory were therefore focused initially on the immortal human prostate cell lines which demonstrated an inhibitory response following treatment with Cernitin T-60 (7). Interestingly, the inhibitory effect was far more marked in the hormone unresponsice cell line when compared to the androgren-sensitive human prostate cells. Human prostate cell lines derived from non-prostatic tissue failed to exhibit a similar sensitivity to the pollen-extract (7).

Although the usage of immortal cell lines in our earlier studies was most helpful in dentifying the specificity and selectivity of the drug, their use is somewhat limited because of: (a) the cancer nature of the continuous cells whilst Cernilton is prescribed purely for BPH; (b) immortal cells are identical clones and do not therefore take account of the morphological heterogeneity of the prostate; and (c) continuous cell lines may undergo phenotypic changes and this might render them distinctive from the cells of origin. In view of these limitations we have decided to continue our work on Cerntin T-60 employing the well-established cultures of epithelial and fibroblast cells from human hyperplastic prostats (3, 15). Those studies were facilitated by our ability to establish and serially culture pure populations of epithelial and fibroblast cells in a well-defined serum-free medium. By using this system the specifc characteristics of Cernitin T-60 could be assessed in a cohesive and systematic fashion.

Clearly, the data outlined in this report indicates that Cernitin T-60 is a powerful mitogenic inhibitor of fibroblastic and epithelial

Control group	EGF concentration	Cernilton Group	EGF concentration
J. W.	1.50	W. F.	3.45
J. G.	1.35	C. F.	2.43
J. N.	2.09	C. S.	1.51
D. D.	1.61	K. B.	0.45
G. T.	2.07	A. S.	0.31
H. H.	1.20	T. S.	1.10
R. H.	2.84	R. H.	0.89
A. C.	3.98		
W. T.	3.67		
W. B.	4.38		
W. H.	1.50		
K. H.	3.40		
H. J.	2.57		
K. N.	3.00		
T. S.	1.76		
hEGF-Concentrations (µg EGF/mg Protein)			
Mean± S.D.	2.39 ± 0.85		1.45 ± 1.31

proliferation. Although the mechanism involved is not as yet understood, we have evidence derived from our earlier studies (8) to indicate that these responses are not medicated via the androgenic pathways. We have therefore decieded to look at the impact of Cernitin T-60 on the expression of growth factors which have been implicated in the growth of the prostate cells. Though the results on the prostate fluid indicate little difference in EGF concentrations between the control and test groups, the evidence derived in this report suggests that there might be some impact on the epidermal growth factor concentration of the tissue.

EGF is a well-established secretory product of the prostate and is retained in large concentrations by BPH when compared to the normal gland (6). This retention might be associated with the high concentrations of the EGF receptors found BPH which must sequester the growth factor for internal use (14). We are not too clear on the mechanism responsible for this build-up of EGF receptors and whether it is a causal factor or merely a result of the development of hyperplasia. We are also not cerntain whether there is an association between these abnormal growth factor concentrations and the dihydrotestosterone levels which have previously been linked to the growth of the gland (17). Significantly however, our most recent studies reveal no correlation between EGF recpetors and the endocrine status of the gland, suggesting that androgens do not modulate EGF-receptor expression in the prostate (13). Since the action of Cernilton on the prostate seems also to be independent of the endocrine functions of the gland, the impact of the pollen exract on the tissue EGF concentrations might be of significant importance, not only in controlling the abnormal growth of the gland but also in pinpointing new pathways relating to the pathogenesis of BPH.

References

1 *Buck, A. C., Cox, R. W. M. Resse, L. Ebeling, A. John:* T Treatment of outflow tract obstruction due to benign prostatic hyperplasia with the pollen extract, Cernilton. A double-blind, placebo-controlled study. Brit. J. Urol. 66 (1990) 398-404.

2 *Caine, M., S/ Perlberg, R. Gordon:* The treatment of benign prostatic hypertrophy with flutamide (SCH12521): A placebo controlled study. J. Urol. 114 (1975) 564-568.

3 *Chaproniere, D. M., W. L. McKeehan:* Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain. Cancer Res. 46 (1986) 819-824.

4 *Chisholm, G. D.:* Benign prostatic hyperplasia: The best treatment. Brit. Med. J. 299 (1989) 215-216.

5 *Gower, R. P. Wells:* United Kingdom Indoramin Study Group. Low dose indoramin, an α-adrenorecptor antagonist, in the management of benign prostatic hypertrophy. Neurourol. Urodyn. 7 (1988) 216-217.

6 *Gregory, H., I. R. Willshire, J. P. Kavanagh, N. J. Blacklock, S. Chowderry, R. C. Richards:* Urogastrone epidermal growth factor concentrations in prostate fluide of normal individuals and patients with benign prostatic hypertrophy. Clin. Sci. 70)1986) 359-363.

7 *Habib, F. K.:* Peptide growth factors: A new frontier in prostate cancer. In *Prostate Cancer and Testicular Cancer.* EORTC Genito-Urinary Group Monograph. 7 (ed. *Newling, D. W. W.)* Alan D. Liss, Inc. New York 1990 (pp. 107 -115).

8 Habib, F. K., M. Ross, A. C. Buck, L. Ebeling, A. Lewenstein: In vitro evaluation of the pollen extract, Cernitin T-60, in the regulation of prostate cell growth. Brit. J. Urol. 66 (1990) 393-397.

9 *Ito, R, M. Ishi, S. Yamashita* et al.: Cernitin pollen-extract (Cernilton): Anti-prostatic hypertrophic actic of cernitin pollen extract. Pharmacometrics 31 (1986) 2-14.

10 *Kimura, M., I. Kimura, K. Nakase* et al.: Micturition activity of pollen extract: Contractile effects on bladder and inhibitory effects on urethral smooth muscle of mouse and pig. Planta Medica 2 (1986) 148-151.

11 *McConnell, J. D., J. D. Wilson, F. W. George* et al.: An inhibitor of 5α-reductase, MK-906 suppresses prostatic dihydrostestosterone in men with benigh prostatic hyperplasia. J. Urol. 141 (1989) 239a.

12 *MacDonald, A., G. D. Chisholm, F. K. Habib:* Production and response of a human prostatic cancer line to transforming growth factor-like molecules. Brit. J. Cancer 63 (1990) 579-584.

13 *MacDonald, A., F. K. Habib:* Divergent response to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. (Submitted for publication) (1991).

14 *Maddy, S. Q., G. D. Chisholm, R. A. Hawkin, F. K. Habib:* Localization of epidermal growth factor receptors in the human prostate by biochemical and immunocytochemical methods. J. Endocrinol. 113 (1987) 147-153.

15 *Peehl, D. M., T. A. Stamey:* Growth responses of normal, benign hyperplastic and malignant human prostatic epithelial cells *in vitro* to cholera toxin, pituitary extract and hydrocortisone. Prostate 8 (1986) 51-61.

16 *Peters, C. A., P.C. Walsh:* The effect of nafarelin acetate, luteinizing-hormone-releasing hormore agonist, on benign prostatic hyperplasia. New Engl. J. Med. 317 (1987) 599-604.

17 *Wilson, J. D.:* The pathogenesis of benign prostatic byperplasia. Amer. J. Med. 68 (1980) 745-756.