

Relationship between upregulated oestrogen receptors and expression of growth factors in cultured, human, prostatic stromal cells exposed to estradiol or dihydrotestosterone

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This study investigated the hypothesis that, in benign prostatic hyperplasia (BPH), upregulated oestrogen receptors (ER) and the action of androgens differentially regulate expression of stromal growth factors. Eight human prostatic stromal cell strains were subjected to a procedure to upregulate their ER by exposing them to 1 μ mol 17 β -estradiol for 10 days followed by passage and growth in the absence of steroids. Four of the cell strains instead received 100 nmol dihydrotestosterone for 48 h. Immunoprecipitation of ER α , AR and six growth factors was quantified by flow cytometry in each case. Expression of ER α was significantly increased in six of eight cell strains. Expressions of six growth factors (FGF-2, FGF-7, IGF-1, TGF- β ₁, NGF and e NOS) were elevated but only for FGF-7 was it significant. There was a significant positive correlation between the change in ER α and the change in FGF-2 and FGF-7, but not the other growth factors. Exposure to dihydrotestosterone reduced expression of ER α and all six growth factors, compared with oestrogen-treated cells but not significantly. It is concluded that upregulated ER α in prostatic stroma may have a greater modulating influence on synthesis of certain growth factors than the direct action of androgens and, by enhancing synthesis of FGF-2 and FGF-7, could play a significant role in the development of BPH.

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Introduction

Benign prostatic hyperplasia (BPH) develops at a time when levels of testosterone are falling and oestrogens are rising, resulting in an increase in the oestrogen: androgen ratio.^{1,2} This age-related shift in sex hormone balance has long been suspected to be implicated in the aetiology of BPH involving activation of oestrogen receptors in the prostate. Recent studies have demonstrated high levels of oestrogen receptors (ER) in human hyperplastic prostates which are located predominantly within cells

of the stroma.^{3–5} These stromal ER are of the alpha subtype,^{3,4,6} the beta subtype being not detected immunohistochemically or flow cytometrically (as confirmed by preliminary studies for this investigation). In addition to their complement of androgen receptors (AR) and ER α , stromal cells also synthesise growth factors^{7,8} by a process controlled by binding of androgens to AR. It has been hypothesised that these growth factors are important in the genesis of BPH by an autocrine action on the stroma and a paracrine influence on the epithelium.⁸ It is possible that synthesis of these growth factors is at least partly regulated by binding of oestrogen to ER; however, the relationship between oestrogen, ER and growth factor production by the prostatic stroma has received relatively little attention. Such studies would be greatly facilitated if stromal ER could be modulated *in vitro* to mimic the situation in the

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intact hyperplastic prostate. Recently, we described a simple experimental model in which expression of ER α protein is reliably upregulated in human prostatic stromal cells by exposing them to a high concentration of estradiol for 10 days followed by steroid deprivation.⁹ In the current paper, this technique is utilised to determine the effect of ER α upregulation on the expression of six growth factors and hence gain some insight into the importance of ER α in the pathogenesis of BPH.

Two of these factors, fibroblast growth factor 2 (FGF-2) and keratinocyte growth factor (FGF-7), have both been localised to prostatic stromal cells and are increased in BPH.^{7,10–16} Insulin-like growth factor (IGF) isoforms have also been demonstrated within stromal cells^{7,17} and are increased in elderly men¹⁷ and in cases of BPH.⁷ Transforming growth factor beta (TGF- β) isoforms are synthesised by the stroma and their receptors expressed by the epithelium.^{18,19} TGF- β ₁, the isoform used in the present study, inhibits growth of the epithelium but is stimulatory on the stroma.^{19–21} The remaining two growth factors studied, nerve growth factor (NGF) and endothelial nitric oxide synthase (e NOS or NOS 3), have a more speculative role in stromal cell function.

The purpose of the present study was to test the hypothesis that, in a new stromal cell model in which expression of ER protein is upregulated, dihydrotestosterone and estradiol differentially regulate the expression of particular growth factors, thus defining some of the events that occur during development of BPH.

Materials and methods

Prostatic tissues were obtained from eight patients who had undergone transurethral prostatic resection for urinary retention. Thorough histopathological examination of these tissues excluded prostatic neoplasia or malignancy and confirmed the diagnosis of BPH. Stromal cells were isolated by collagenase digestion²² to produce eight different cell strains, which were passaged three to five times before being used in the experiments.

Upregulation of oestrogen receptors

Expression of oestrogen receptor alpha (ER α) was increased in the stromal cultures using the protocol devised previously.⁹ Stock cultures of stromal cells were seeded onto four, 9 cm diameter culture dishes (Nunc, Roskilde, Denmark), and grown in RPMI 1640 medium supplemented with 2 mmol L-glutamine, antibiotics and

10% fetal calf serum (FCS; Gibco, Invitrogen, Paisley, Scotland). When the cells had just reached confluence, the medium in two dishes was changed to that containing only 5% activated charcoal-stripped FCS to remove all steroid hormones. To these dishes was added 17 β -estradiol (Sigma Chemical Co., Gillingham, Dorset, UK) (hereafter referred to as estradiol) to a final concentration of 1 μ mol. The medium and estradiol were changed on alternate days for a total of 10 days. The other two dishes acted as controls and continued to be fed with the medium plus 10% complete FCS. The cells in both pairs of dishes were then passaged into eight dishes and grown in a medium containing 5% activated charcoal-stripped FCS together with insulin, transferrin and selenium supplement (ITS; Gibco). No estradiol was added at this stage. When the cultures were fully confluent (at 10–12 days), four dishes of control cells (C1) and four of test cells (E1) were trypsinised until a monodisperse suspension of cells was obtained, which was fixed in 2 ml of 70% (v/v) chilled methanol for subsequent flow cytometry. To the remaining four dishes of each group was added 1 nmol estradiol for 48 h to determine whether binding of the upregulated ER to their ligand was necessary to activate synthesis of growth factors. These cells (C2 and E2) were harvested and fixed as above.

Addition of dihydrotestosterone

Stromal cells from four cell strains showing the greatest elevation of ER α expression were each plated onto four culture dishes. They were treated in the same way as the controls except that for the last 48 h before harvesting, all four dishes of each strain were given a solution of dihydrotestosterone (Sigma) at a final concentration of 100 nmol.

Flow cytometry

Methanol-fixed cells from each cell strain were counted and aliquots of 0.25×10^6 cells transferred to eight microcentrifuge tubes. Cells were rehydrated in phosphate-buffered saline (PBS) and then left overnight in blocking buffer consisting of 1% bovine serum albumin (w/v) in PBS. They were then labelled, separately, with 100 μ l monoclonal antibodies to ER α and AR and with rabbit polyclonal antibodies to six growth factors comprising FGF-2, FGF-7, TGF- β ₁, e NOS, IGF-1 and NGF (Table 1). All antibodies (Santa Cruz Biotechnology, CA, USA) were diluted 1:50 in blocking buffer

Table 1 Mean values of fluorescence intensity expressed as a percentage of control for eight stromal cell strains exposed to 1 μ mol estradiol

Treatment designation	Fluorescent antibodies against							
	ER α	AR	FGF-2	FGF-7	TGF β ₁	IGF-1	e NOS	NGF
C2	104.7 (12.3)	98.5 (7.8)	100.7 (14.9)	103.1 (9.3)	99.2 (4.0)	101.4 (7.4)	102.3 (3.3)	99.0 (6.8)
E1	115.2 (17.1)	103.3 (20.3)	116.0 (30.4)	116.9 (21.4)	106.4 (20.1)	106.8 (22.8)	106.2 (19.6)	102.2 (28.6)
E2	109.5 (15.5)	99.0 (18.8)	115.1 (30.4)	108.8 (14.9)	102.4 (16.1)	110.4 (18.9)	109.5 (16.8)	106.7 (28.8)

C2: control cells exposed to 1 nmol estradiol for the last 48 h only; E1: test cells exposed to 1 μ mol estradiol followed by growth in the absence of steroid hormones; E2 = test cells exposed to 1 μ mol estradiol followed by growth in the absence of steroid hormones plus exposure to 1 nmol estradiol for the last 48 h only. Figures in parentheses denote ± 1 standard deviation from the mean.

containing 0.5% Triton X-100 and the cells incubated at 37°C for 75 min. Untreated cells of the same strain were incubated with non-immune IgG of equivalent concentration. After washing with PBS, the supernatants were replaced with 100 µl of secondary reagent. For the monoclonal primary antisera, the secondary antiserum was goat anti-(mouse IgG, Fc specific) FITC conjugate (flow cytometry grade, Sigma Chemical Co.) diluted 1:100 in PBS. For polyclonal primary antisera, the secondary antiserum was goat anti-(rabbit IgG) FITC (Sigma) also diluted 1:100 in PBS.

The fluorescence intensity of each sample of cells was measured in either a Becton Dickinson FACSort (San Jose, CA, USA) or a Beckman Coulter EPICS XL flow cytometer (High Wycombe, UK). Sensitivity of each instrument was adjusted against the non-immune control so that 95% of events fell within the first log decade. A total of 20 000 events were counted with subcellular debris excluded by setting a threshold on forward light scatter. Data were recorded as frequency distribution histograms and mean fluorescence intensity units. To facilitate comparison of data, all cells derived from a particular cell strain were labelled with a specific antibody and passed through the flow cytometer on the same single occasion.

The results were analysed as raw data generated by the flow cytometer, or as normalised data where values were expressed as percentages of control. Some parameters had skewed distributions or widely divergent variances. Consequently, all data were analysed by the Wilcoxon's signed-rank test for nonparametric, related sets of data.

Results

Raw data

Six of the eight cell strains treated with estradiol (E1) showed upregulated ER relative to control. This difference was significant ($P=0.035$). However, exposure of these cells to 1 nmol estradiol for the last 48 h (E2) induced no further significant effect. Fluorescences of cells labelled for FGF-7 were significantly higher than control ($P=0.018$), but there were no significant differences between test and control for the remaining antibodies.

Normalised data

Data were also expressed as a percentage of control values to provide an indication of the relative changes in immunoeexpression of the proteins induced by oestrogen treatment. Mean values of the eight cell strains are shown in Table 1 and, in each case, cells with upregulated ER (group E1) were in excess of 100% of control values indicating that expressions of ER α , AR and the six growth factors were increased. However, none was significantly greater than the control cells that were cultured in medium containing 1 nmol estradiol for 48 h (C2). Regression analysis of immunofluorescence of cells with upregulated ER (E1) expressed as a percentage of control revealed statistically significant positive correlations between ER α and FGF-2 ($r=0.8028$, $P<0.05$) and

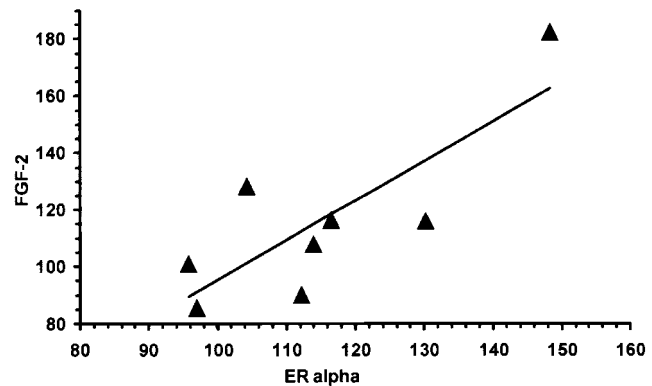


Figure 1 Plot of immunofluorescence of cells labelled for ER α vs FGF-2 expressed as a percentage of control from eight stromal cell strains subjected to the protocol to upregulate their ER. The regression line is drawn and there is a significant correlation between the two parameters ($P<0.05$).

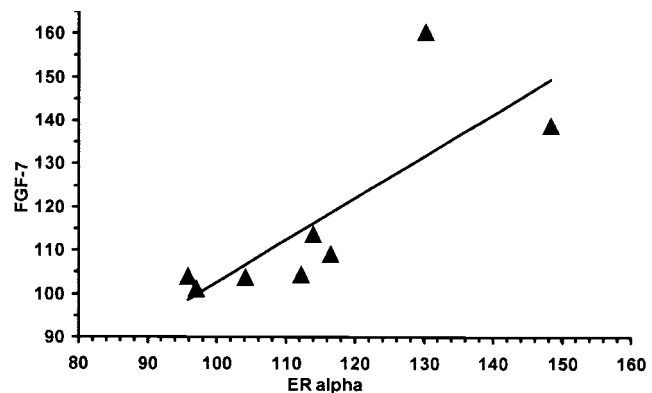


Figure 2 Plot of immunofluorescence of cells labelled for ER α vs fibroblast growth factor-7 (FGF-7 or KGF) expressed as a percentage of control from eight stromal cell strains subjected to the protocol to upregulate their ER. The correlation is significant ($P<0.05$).

between ER α and FGF-7 ($r=0.7968$, $P<0.05$). These are illustrated graphically in Figures 1 and 2. ER α was not significantly correlated with the percentage immunofluorescence of any of the remaining four growth factors.

Addition of dihydrotestosterone

The four stromal cell strains given 100 nmol dihydrotestosterone for 48 h were compared with the same cell strains that had been treated with 1 µmol estradiol. The results are shown graphically in Figure 3. The protocol for upregulating ER induced a mean increase in ER α expression of 20% in these four stromal cell strains. Percentage immunofluorescences of all six growth factors were also increased by 14–29% above control but AR levels were increased by only 5%. In contrast, cells that were not given estradiol but dihydrotestosterone instead all showed a lower immunofluorescence in comparison to their estradiol-treated counterparts, which in several instances was below control levels. However, the differences between estradiol- and DHT-treated cells were not significant.

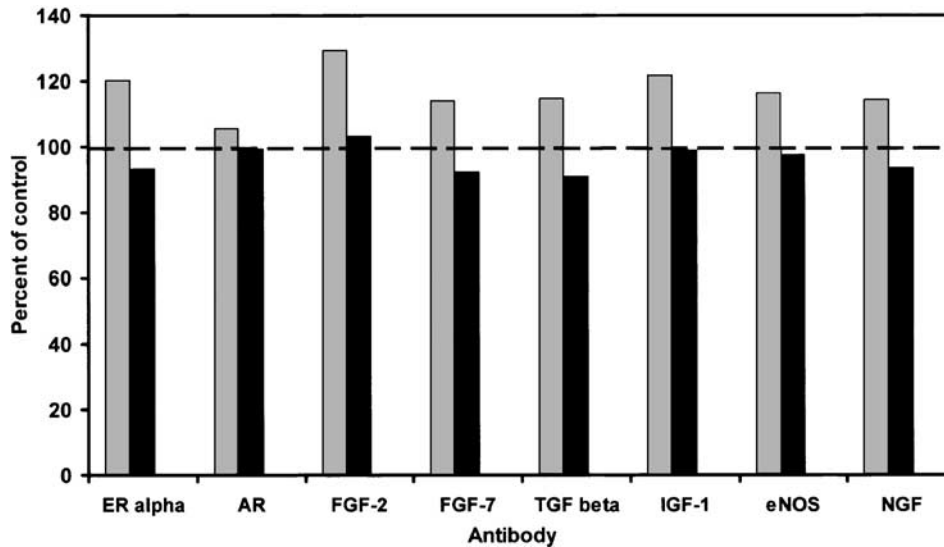


Figure 3 Bar chart to compare the percentage immunofluorescence of four stromal cell strains with upregulated ER (shaded bars), with similar cells without ER upregulation but exposed to DHT for 48 h instead (black bars). The horizontal dotted line denotes control levels. The mean values of ER α , AR and six growth factors are shown, expressed as percentages of control. For each antibody, mean values for DHT are lower than those for ER upregulated cells but not statistically significantly so.

Discussion

This investigation confirms and extends our previous study⁹ that showed that exposure of prostatic stromal cells to 1 μ mol estradiol for 10 days followed by passage and growth in the absence of steroid hormones enhanced expression of ER. In the previous study, all stromal cell strains exhibited upregulation of ER to varying degrees. In the present experiments, six of eight cell strains responded in this manner. The average increase in ER expression (15% above control levels) was significant.

The observed increases in ER α expression recorded by the flow cytometer were not simply due to increases in size of the cells stimulated by estradiol. The forward light scatter recorded by the instrument, an index of cell diameter, was closely similar between oestrogen-treated and control cells. Thus, with the exception of two unresponsive cell strains, this protocol can be used as an experimental model to induce moderate upregulation of stromal ER.

An indication of the relative increases in the expression of ER α , AR and the six growth factors was obtained by expressing fluorescence intensities as a percentage of control. With these data, cells treated with 1 μ mol estradiol showed significant positive correlations between percentages of ER α and both FGF-2 and FGF-7. We have previously found highly significant correlations between immunofluorescence values of ER α and both FGF-2 and FGF-7 in the absence of ER α upregulation,⁹ suggesting that there is an intrinsic relationship between ER α and the two growth factors in the prostatic stroma. The present study has further demonstrated that elevation of ER α expression also tends to be associated with elevated expression of FGF. FGF-2 (bFGF) is synthesised by the prostatic stroma but is also located to a lesser extent within the epithelium, especially the basal cells.^{8,10,13,15} FGF-2 is assumed to act in an autocrine fashion on the stroma causing a dose-dependent increase in its growth.²³ Increased levels of FGF-2 have been

found in the stroma of prostates with BPH^{13,14} coincident with an increased expression of its receptor FGFR1,¹⁶ and its synthesis is considered to be under androgenic control. The correlation between percentage change in the expression of FGF-2 and ER α suggests that activation of ER is also likely to be of importance.

The same possibility also applies to FGF-7 for which immunoexpression was also correlated with the percentage change in ER α as well as being significantly greater than control. FGF-7 is produced exclusively by the stroma^{5,11,24} from which it is secreted to exert a paracrine mitogenic influence on epithelial cells via FGF receptors.^{11,12} Stromal cells from patients with BPH contain high levels of FGF-7 protein and mRNA associated with higher than normal expression of AR.²⁴

Thus, our data support the hypothesis that increased plasma estradiol and elevated stromal ER cause transactivation of genes determining the synthesis of FGF-2 and FGF-7, the former has an autocrine mitogenic effect and the latter a paracrine stimulatory effect on the epithelium. Interestingly, exposure of cultured stromal cells to a physiological concentration of estradiol (1 nmol) for the last 48 h of the experiment (E2 cells in Table 1) induced no further increase in the expression of either of the above growth factors. It is therefore possible that it is not a prerequisite for upregulated ER to bind to their ligand in order to activate synthesis of stromal growth factors.

Growth factors TGF- β , IGF-1, NGF and eNOS showed no correlation with the percentage change in ER α . Isoforms of TGF- β ₁ and IGF have been clearly demonstrated within prostatic stromal cells.^{7,18,19} IGF appears to have a stimulatory effect on those prostatic epithelial cells that possess receptors to both IGF-1 and IGF-II.⁷ Furthermore, the levels of IGF protein and mRNA in stromal cells are particularly increased in elderly men,¹⁷ and by as much as 10 times in cases of BPH,⁷ suggesting a likely role for this growth factor in the development of BPH. Conversely, TGF- β inhibits growth of prostatic epithelium but appears to have a mitogenic effect on the

stroma, also inducing these cells to adopt a more smooth muscle-like phenotype.^{18,19,21} Our current data indicate that the roles of TGF- β and IGF occur independently of the upregulation of stromal ER α reported in BPH. NGF protein has been identified, by immunohistochemistry, to be expressed by the prostatic stroma while its receptor is confined to the epithelium.^{25,26} NGF appears to have a stimulating influence on epithelial cell growth²⁵ although its expression is reduced in BPH.²⁶ All three isoforms of nitric oxide synthase have been detected in the human prostate but are not specific to the stroma.²⁷ Expression of the isoform examined in the present study (endothelial nitric oxide synthase, e NOS) is increased in prostatic carcinoma cell lines following exposure to estradiol.²⁸ Any putative role of NGF and e NOS in the regulation of prostatic growth appears to act independently of ER.

Cells from four of the stromal cell strains that were not exposed to estradiol instead received 100 nmol DHT for 48 h. The purpose of this was to determine the relative importance of upregulation of ER and the direct action of androgens on the levels of stromal growth factors. For ER α , AR and the six growth factors, DHT-treated cells exhibited a lower immunofluorescence, expressed as a percentage of control, than the cells with upregulated ER (Figure 3). Although these differences were not significantly different, it is a fact that values for DHT were lower rather than higher than those of cells with upregulated ER, which is of interest since it has been reported that DHT at a similar concentration to ours stimulates synthesis of growth factors by the stroma.²⁹ This finding suggests that enhanced ER α expression may be a more potent transducer of stromal growth factor synthesis than is the action of androgens, the levels of which are in a steady continuous decline in elderly men. We postulate that upregulated ER α in the prostatic stroma of elderly men has a greater modulating influence on the expression of FGF than the action of androgens and, also by enhancing synthesis of FGF-2 and FGF-7, could play a significant role in the aetiopathogenesis of BPH.

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