

Upregulation of estrogen and androgen receptors modulate expression of FGF-2 and FGF-7 in human, cultured, prostatic stromal cells exposed to high concentrations of estradiol

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This study was performed to develop an experimental model in which expression of estrogen receptors (ER) by human prostatic stromal cells could be reproducibly enhanced relative to similar cells with low ER expression. The second aim was to characterise changes in expression of ER, androgen receptor (AR), FGF-2 and FGF-7 in stromal cells exposed to high and low concentrations of estradiol and testosterone mimicking the different sex hormone levels between young and elderly men. Five strains of human prostatic stromal cells, isolated from BPH resections, were grown in steroid-free medium plus 1 μmol 17 β -estradiol. After 10 days, cells were passaged and grown in the same medium without estradiol until confluent. In a second study four cell strains were exposed to high and low concentrations of 17 β -estradiol and testosterone for 10 days. Cells were labelled with fluorescent antibodies to ER α , AR, FGF-2 and FGF-7 and the fluorescence intensity measured by flow cytometry. Following exposure to 1 μmol estradiol, stromal cells showed reduced expression of AR and ER α but after passage without estradiol they showed a 25% increase in both receptors over controls. Different combinations of sex hormones induced inconsistent changes with respect to expression of ER, AR and FGFs in the various cell-strains. However, there was a highly significant correlation between AR, ER and FGF-2 and FGF-7, which was cell strain-specific. Thus, changes in sex hormone balance *per se* may not be solely responsible for the observed increases in prostatic ER levels in BPH. Since expression of ER is correlated with synthesis of FGF-2 and FGF-7, it is likely that increases in stromal ER may mediate the synthesis of stromally-derived growth factors which contribute to the aetiopathogenesis of benign prostatic hyperplasia.

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Introduction

Benign prostatic hyperplasia (BPH) occurs in approximately 70% of men over 70 y old and develops at a time

when levels of testosterone are falling and estrogens are rising, thus resulting in an increase in the ratio of estrogen: androgen.^{1–3} This age-related shift in hormone balance has long been suspected to be implicated in the aetiology of BPH particularly following the discovery that 17 β -estradiol acts synergistically with testosterone in experimentally induced BPH in the dog.^{3,4} For estrogen to be directly involved in the genesis of human BPH, the prostate should contain estrogen receptors (ER)^{1–4} which should be upregulated as in the canine model.^{1–5} Recent

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studies have demonstrated high levels of ER in human hyperplastic prostates which are located predominantly within the cells of the stroma,^{6–10} where the tissue concentration of 17 β -estradiol is highest.^{1,2}

In addition to their complement of androgen receptors (AR) and ER, prostatic stromal cells also synthesise several peptide growth factors,^{11–13} production of which is controlled by the binding of androgens to stromal AR. They are likely to be important second messengers in the genesis of BPH through an autocrine interaction with the stroma or a paracrine effect on the epithelium. However, the influence of estrogens and ER on the production of these growth factors in the prostate has not been explored. Such studies would be facilitated by an *in vitro* model in which cultured human prostatic stromal cells are induced to express levels of ER that are consistently elevated with respect to similar cells in which ER expression is low. Therefore, the first part of this paper tests the hypothesis that growth of human, isolated, prostatic stromal cells in an environment of high 17 β -estradiol concentration, followed by passage and growth in the absence of steroid hormones, leads to increased expression of ER. This possibility was originally suggested by data obtained during recent studies.¹⁴

This investigation then examines the interaction of different combinations of testosterone and 17 β -estradiol on the immunorexpression of both AR and ER as well as fibroblast growth factors – 2 and – 7. FGF-2 (bFGF) has been shown by immunohistochemistry to be present in stromal cells^{15,16} and is increased in BPH.^{16,17} This peptide appears to stimulate growth of stromal cells in an autocrine manner.^{17,18} FGF-7 (KGF), is produced exclusively by stromal cells.^{11,19–21} Immunorexpression of both growth factors and that of sex hormone receptors was quantified by flow cytometry. The anti-ER antibody used in all experiments was the alpha subtype since this is the classical form of ER which is predominantly localised to the prostatic stroma,^{8,9,22} unlike the more recently discovered subtype, ER β which is found mainly in the prostatic epithelium.^{23,24}

Materials and methods

Prostatic tissues were obtained from six patients who had undergone transurethral prostatic resection for urinary

retention. Thorough histopathological examination of these tissues excluded prostatic cancer and confirmed the diagnosis of BPH. Stromal cells were isolated from the tissues by collagenase digestion using a method which we have described previously.²⁵ The cells were serially passaged two–five times before being used in the experiments.

Upregulation of estrogen receptors

To test the effects of estradiol on ER in growing cells, one strain of stromal cells, was plated onto four 9 cm Petri dishes at a density of 1.5×10^3 cells/cm² and fed with RPM1 1640 containing 2 mmol l-glutamine, antibiotics, 1% (v/v) of an insulin, transferrin and selenium supplement (ITS; Gibco), and activated charcoal-stripped 5% (v/v) fetal calf serum (FCS). To two dishes, designated EST 1 was added 17 β -estradiol (Sigma Chemical Co.) at a concentration of 1 μ mol. Two dishes designated CON 1 were left untreated (Table 1). Medium and estradiol were replaced thrice weekly for a total of 10 days when one dish from each group (EST 1 and CON 1) was trypsinised until a monodisperse suspension of cells was obtained which were fixed in 1 ml of 70% (v/v) chilled methanol for subsequent flow cytometry. The remaining cells were passaged each into two dishes at a density of 1.5×10^3 cells/cm². Both pairs of dishes (EST 2 and CON 2) received identical medium but without estradiol and fed thrice weekly until confluent (11 days). One dish from each pair was then fixed in methanol as above, the other two dishes (EST 3 and CON 3) subcultured and treated as above.

The second experiment was designed to examine the effect of estrogen on oestrogen receptors in confluent, quiescent stromal cells. Five stromal cell strains were each plated onto four Petri dishes and grown in medium containing 10% complete FCS until confluent. The medium was then changed to that containing 5% activated charcoal-stripped FCS and the cells allowed to become quiescent for 48 h. Thereafter, two dishes of each cell strain received 1 μ mol 17 β -estradiol (EST 1) and two dishes were left as untreated controls (CON 1). Medium and estradiol were replaced thrice weekly for 10 days after which the cells from one dish of each pair were harvested and fixed in methanol. The remaining two dishes were subcultured at a split ratio of 1:4 into

Table 1 Effects of estrogen on the expression of estrogen and androgen receptor in proliferating stromal cells

Treatment	Antibody	Immunofluorescence units		Percentage of control
		Control cells	Estrogenised cells	
First passage:	Anti estrogen receptor alpha	CON 1	EST 1	87
EST 1 cells given 1 μ mol estradiol		4.38	3.80	
Second passage:		CON 2	EST 2	130
No estradiol		4.23	5.49	
Third passage:		CON 3	EST 3	114
No estradiol		4.38	5.00	
First passage:	Anti androgen receptor	CON 1	EST 1	81
EST 1 cells given 1 μ mol estradiol		4.19	3.40	
Second passage:		CON 2	EST 2	143
No estradiol		3.27	4.68	
Third passage:		CON 3	EST 3	113
No estradiol		3.86	4.36	

medium plus 5% stripped FCS, grown until confluent and then fixed for flow cytometry (EST 2).

Flow cytometry

Methanol-fixed cells from each sample were counted and 0.5×10^6 cells transferred to microcentrifuge tubes. The cells were further permeabilised in acetone for 5 min and hydrated in phosphate buffered saline (PBS). After blocking in 1% bovine serum albumin (w/v) in PBS, the cells were incubated with 100 μ l of monoclonal mouse antibodies to either ER alpha (Dako) or AR (Dako) diluted 1:50 in blocking buffer with 0.5% Triton x-100 at 37°C for 75 min. Untreated cells of the same strain were incubated with isotype-specific non immune IgG of equivalent concentration. After washing with PBS, supernatants were replaced with 100 μ l of secondary antibody comprising sheep anti-(mouse IgG) conjugated to fluorescein isothiocyanate diluted 1:100 in blocking buffer. Incubation was for a further hour at 37°C after which supernatants were replaced with 200 μ l of PBS.

The fluorescence intensity of each sample of cells was measured in a Becton Dickinson FACsort flow cytometer (San Jose, CA, USA) in which the sensitivity was adjusted against the isotype control so that 95% fell within the first log decade. A total of 20 000 events was counted with electronic noise and subcellular debris excluded by setting a threshold on forward light scatter. Data were recorded as frequency distribution histograms and mean fluorescence intensity units.

Different combinations of estrogen and androgen

In this experiment the effects of high and low concentrations of estradiol and testosterone in various combinations were tested on the subsequent expression of sex hormone receptors and FGF-2 and FGF-7. Concentrations of estradiol and testosterone were chosen from published measurements on patients. Thus, the high dose of testosterone was 2000 nmol and the high dose of 17 β -estradiol 500 nmol, both being within the range of prostatic tissue concentrations (1500–2800 nmol and 200–650 nmol, respectively).²⁶ The low concentration of 17 β -estradiol was set at 0.075 nmol since this is the plasma level in a normal young man.²⁶ The low concentration of testosterone was 1 nmol, this being within the range of plasma levels in a castrated man (0–2 nmol).²⁷ The purpose of this was to simulate, in an exaggerated form, the hormonal balance in patients with BPH (high estrogen; low testosterone) and in healthy young men (low estrogen; high testosterone). Equal levels of both extremes were also included so that all four combinations were represented.

Four strains of stromal cells isolated from four BPH patients were each plated onto eight culture dishes and grown in complete medium until the cells were confluent. Thereafter, the medium was changed to that containing 5% charcoal-stripped FCS. Estradiol and testosterone were added to pairs of dishes in all four combinations of concentration; there was also one set of untreated control dishes. Medium and estradiol were replaced thrice weekly for 10 days and the cells were then trypsinised and fixed in 70% methanol.

Flow cytometry was performed as described above using monoclonal antibodies to ER α (D12, Santa Cruz Biotechnologies), AR (441, Santa Cruz Biotechnologies), and rabbit polyclonal antibodies to FGF-2 and FGF-7 (147 and H-73 respectively, Santa Cruz Biotechnologies) all diluted 1:100. The secondary antibody for monoclonal primary antisera was goat anti-(mouse IgG, Fc specific) FITC conjugate (flow cytometry grade, Sigma Chemical Co.) diluted 1:100 with blocking buffer, and for polyclonal primary antisera, goat anti-(rabbit IgG) FITC (Sigma) diluted 1:100.

Results

Upregulation of estrogen receptors

Mean immunofluorescence units obtained from flow cytometry of cell strain 1 exposed to estradiol whilst proliferating are shown in Table 1. Expression of ER α following exposure to 1 μ mol 17 β -estradiol for 10 days was reduced to 87% of the control value. Following subsequent passage and growth in the absence of sex hormones, ER expression was increased to 130% whereas control cells were largely unchanged. Even after a further, third passage the descendants of the estrogenised cells retained an elevated expression of ER although this had fallen to 114% of control. The expression of AR followed a similar trend. Table 2 shows the results of flow cytometric analysis of five stromal cell strains exposed to estradiol when confluent and non-proliferating. These cells too showed a reduction in expression of ER α after incubation with estradiol (EST 1) followed by average increases to 125% of control on subsequent second passage and growth without estradiol (EST 2). Analysis of variance of these data demonstrated a significant difference ($P < 0.025$) between the three treatments, ie control, 1 μ mol estradiol, and estradiol with subsequent passage in the absence of sex hormones. Mean values for EST 1 and EST 2 were significantly different by t -test ($P < 0.02$).

Two cell strains (1 and 2) were also labelled for androgen receptor, one of which showed a reduced AR expression after incubation with β -estradiol. After subsequent

Table 2 Flow cytometric analysis of the effects of estrogen on expression of estrogen receptor alpha in confluent stromal cells

Treatment	Immunofluorescence of cell strains					Mean	Percentage of control
	1	2	3	4	5		
CON 1. Untreated control	4.14	3.46	3.79	3.42	3.18	3.60	100
EST 1. 1 μ mol estradiol	2.84	3.08	3.78	3.25	2.89	3.17	88
EST 2. Passage without estradiol	5.35	5.73	4.14	3.84	3.53	4.52	125

passage, both cell strains showed increases in AR immunorexpression of 145 and 138% of control values, respectively.

Different combinations of estradiol and testosterone

The effects of high and low doses of estradiol and testosterone in different combinations produced no consistent change in the expression of ER α (data not shown). In particular, there was no consistent difference between a low testosterone/high estrogen environment and a high testosterone/low estrogen one. Immunofluorescences of AR were similarly variable.

Immunofluorescence values of cells labelled with antibodies to FGF-2 and FGF-7 were greater than those for the steroid receptors. In all but two instances the expression of FGF-2 was slightly lower than that of its respective controls, whilst expression of FGF-7 was variable both between and within cell strains. Irrespective of these inconsistent responses, when the values of AR and ER α were plotted against those for FGF-2 significant positive correlations were obtained ($r=0.7479$, $P<0.001$; and $r=0.9165$, $P\ll 0.001$, respectively). Similarly, plots of AR and ER α against FGF-7 also showed significant positive correlations ($r=0.9366$, $P\ll 0.001$; and $r=0.8404$, $P<0.001$, respectively). Expression of ER α against AR was also significantly correlated ($r=0.7983$, $P<0.001$). Two examples are shown graphically in Figures 1 and 2. They reveal a tendency for the data points corresponding to each cell strain to be clustered around a particular region of the regression line. It indicates that correlations between the sets of data are more a reflection of the properties of each cell strain than they are of differences in response to the various hormone concentrations.

Discussion

This investigation tested the hypothesis that ER can be upregulated in recently-isolated and non-immortalised

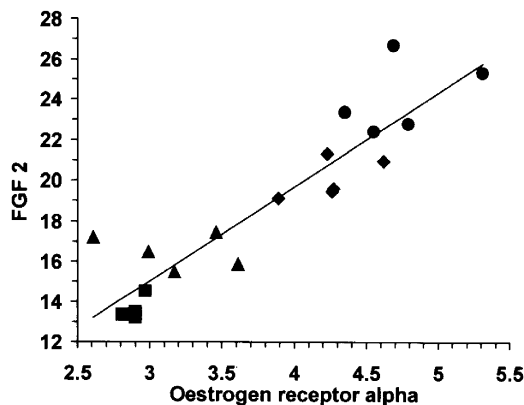


Figure 1 Plot of immunofluorescence of cells labelled for estrogen receptor vs cells labelled for fibroblast growth factor-2 (FGF-2 or bFGF) in four prostatic stromal cell strains cultured in high or low concentrations of estradiol and testosterone. In this and Figure 2 axes show the mean immunofluorescence units obtained from the flow cytometer and the regression line is drawn. Different data points represent the four cell strains such that diamonds = strain 3, circles = strain 4, triangles = strain 5 and squares = strain 6.

prostatic stromal cells by culturing them in a high concentration of estradiol followed by passage and growth in the absence of sex hormones. This elevated expression of ER α occurs irrespective of whether the cells are growing or are confluent and relatively quiescent and persists for at least two passages. We suggest that this upregulation, which represents a significant increase in receptor expression of 25%, can be used as a consistently-reproducible experimental model for studying the effects of ER on stromal cell phenotypes. The basis for the observed change in ER α expression is unclear. One can speculate that high titres of estradiol saturate the ER so that some become redundant and their synthesis declines. This would explain the fall in ER expression 10 days after exposure to 1 μ mol estradiol. Subsequent passage and withdrawal of estradiol then leads to renewed synthesis of receptors which overshoots the original level in a rebound phenomenon.

ER were not upregulated independently since AR were consistently increased by a similar amount. This occurred in the total absence of testosterone suggesting that ER and AR are in some way interlinked. This close relationship between the two classes of receptor has been demonstrated in cultured human prostatic stromal cells in which exposure to estradiol also raised the level of AR.⁶ A similar phenomenon has also been reported in cultured prostatic carcinoma cell lines,²⁸ in stromal cells of prostatic carcinoma from patients treated with androgen ablation therapy¹⁰ and in castrated dogs treated with oestrogen.⁵

Culture of stromal cells with different concentrations of estradiol and testosterone, produced no consistent pattern of ER α and AR expression between the various treatments. It is possible that the effect of different hormone levels is obscured by lack of specificity for their receptors since it has been demonstrated that AR can bind estradiol promiscuously and thus become directly activated.²⁸ Whatever the reason for their variability, the data suggest that changes in sex hormone balance *per se* may not be solely responsible for the observed increases in prostatic ER levels reported in men with BPH.

We found that the expression of ER and AR was highly significantly correlated with levels of two stromally

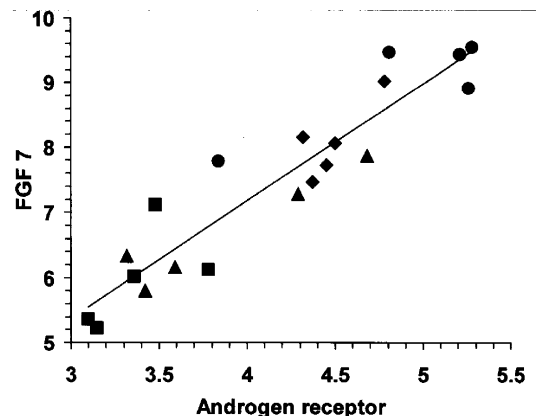


Figure 2 Plot of immunofluorescence for androgen receptor vs fibroblast growth factor-7 (FGF-7 or KGF) in four prostatic stromal cell strains cultured in high or low concentrations of estradiol and testosterone. Key to different data points is shown in legend to Figure 1.

produced growth factors, FGF-2 and FGF-7. It was noted earlier that in the graphs of ER α and AR *vs* the expression of both fibroblast growth factors there was a tendency towards clustering of data points for individual cell strains. It could be argued that this created spurious correlations brought about by differences in cell size. However, the range of forward light scatter recorded by the flow cytometer (an indication of cell diameter) was closely similar between all four cell strains. It is likely, therefore, that the obtained correlations reflect intrinsic differences in receptor levels of stromal cells derived from different patients. FGF-2 or bFGF is produced by the prostatic stroma but is also located to a lesser extent within the epithelium, particularly the basal cells.^{13,15,16} FGF-2 is not normally secreted and is thus assumed to act in an autocrine fashion on the stromal cells causing a dose-dependent increase in their growth¹⁸ which may be as high as 4.8 times that of unstimulated cells.¹⁷ Increased levels of FGF-2 have been found in the stroma of prostates with BPH.^{16,17} Its synthesis is considered by some to be under androgenic control^{13,17} but the close correlation which we found between FGF-2 and ER α suggests that activation of ER may also be involved.

The same possibility also applies to FGF-7 (KGF), the expression of which was also closely correlated to ER α as well as to AR. KGF is produced exclusively by the stroma^{11,19,20,29} from which it is secreted to have a paracrine growth promoting influence on the epithelium.^{19–21} Stromal cells from patients with BPH contain high levels of KGF associated with a higher than normal expression of AR.²⁹ This growth factor too may, therefore, be involved in the pathogenesis and/or maintenance of BPH and its secretion may, in part, be regulated by transactivation of ER as well as by AR.

Conclusions

This study has demonstrated that ER can be upregulated, in a consistent and reproducible manner, in cultured prostatic stromal cells by exposing them for several days to a high concentration of estradiol followed by passage and growth in a steroid-deficient medium. Immunoeexpression of FGF-2 and FGF-7 is closely correlated with the level of ER α as well as with AR. Thus, in BPH, a conjoint increase both in plasma estradiol and stromal ER is likely to participate in the synthesis of growth factors which stimulate modification of both stromal and epithelial cells, although in a differential manner with respect to individual prostate glands.³⁰

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