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DISTRIBUTION OF OESTROGEN AND ANDROGEN RECEPTORS BETWEEN THE STROMA AND EPITHELIUM OF THE GUINEA-PIG PROSTATE

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Summary—A procedure is described for separating guinea-pig prostatic tissue into viable epithelial and stromal fractions. Epithelial cells were isolated using 0.1% protease, but this method resulted in significant damage to the stromal cells. However, using a mechanical tissue fractionation technique, a viable stromal matrix consisting predominantly of confluent sheets of smooth muscle cells and intervening collagen fibres was obtained. Although this method selectively spilled-out the epithelial cells, the majority were non-viable and therefore not suitable for receptor studies. Electron microscopy confirmed that cell architecture and organelle morphology were well preserved in both the enzymatic epithelial preparation and the mechanically prepared stroma. Saturation analysis studies indicated that the concentration of high affinity ($K_d \sim 0.15$ nM) oestrogen receptors was approx. 10-times greater in the separated stroma than in the epithelial fraction. In contrast, the concentration of androgen receptors ($K_d \sim 2.2$ nM) was almost 2-fold greater in the epithelial than in the stromal fraction. These findings suggest that oestrogen, either independently or in association with androgen, may play a role in the growth and development of the stromal component of the guinea-pig prostate.

INTRODUCTION

In the past, studies on prostatic morphogenesis, growth and function have focused almost exclusively upon the androgenic regulation of the epithelial cells. The findings of several recent studies, however, indicate that at least some responses of prostatic epithelial cells to hormonal stimuli are not invoked directly within these cells, but instead may be the consequence of growth factors, morphogens or inductors produced in neighbouring stromal cells [1, 2]. Cunha and co-workers [2–4] have demonstrated that urogenital sinus mesenchyme directs prostatic epithelial development in embryonic and neonatal rodents. Furthermore, the maintenance of epithelial differentiation and morphogenesis in the adult prostate may be dependent on similar stromal–epithelial interactions to those observed in the developing prostate [4]. This has led to the suggestion that stromal–epithelial interactions are involved in abnormal epithelial differentiation in the prostate. McNeal[5] has proposed that benign prostatic hyperplasia in man results from reactivation of embryonic growth factors in the prostatic stroma. This concept is supported by the morphological observations of Rohr and Bartsch[6], which suggest that BPH in man is a stromal disease.

In view of the potential importance of stromal–epithelial interactions during prostatic development, it would appear that investigation of the hormonal regulation of the stromal component may lead to a better understanding of prostatic disease and may provide an insight into how some of these abnormal growth processes may be modified by hormonal means. The present study was undertaken to develop a technique which would allow separation of the guinea-pig prostate into relatively pure, viable stromal and epithelial fractions. This would enable us to examine the distribution of the oestrogen receptor in the prostate. Localization of this receptor in the prostatic stroma would suggest that stromal cells could be affected by oestrogen, and this may in turn have important implications for stromal–epithelial cell interactions.

EXPERIMENTAL

Chemicals

[2,4,6,7-^{3}H]Oestradiol-17β (Sp. act. > 85 Ci/mmol) and 5α-dihydro[1,2,4,5,6,7-^{3}H]testosterone ([^{3}H]DHT; Sp. act. > 100 Ci/mmol) were purchased from the Radiochemical Centre, Amersham (U.K.). Unlabelled steroids, protease type XIV (pronase E), bovine serum albumin, calf thymus DNA, dithiothreitol (DTT), phenylmethylsulphonylfluoride (PMSF) and benzamidine HCl, were obtained from Sigma Chemical Co. (U.S.A.). Hoechst dye 33258 was obtained from Calbiochem (Aust). Activated charcoal was purchased from Ajax Chemicals (Aust), Dextran-T70 from Pharmacia (South Seas), sodium molybdate from BDH Chemicals (Aust), and PCS scintillation fluid was from Amersham–Searle (Aust). Collagenase (from *Clostridium histolyticum*) was purchased from Boehringer Mannheim (West Germany). Hank's Balanced Salts Solution (HBSS) and foetal calf serum (FCS) were from GIBCO (U.S.A.).

Animals and tissues

Male guinea-pigs (IMVS coloured; outbred strain) with body weights ranging from 380 to 450 g were maintained on a standard laboratory diet and lighting regime (0700–1900 h). Animals were killed by intraperitoneal injection of sodium pentobarbitone (80 mg/kg). The prostate gland was rapidly excised, dissected clear of connective tissue, weighed and immediately rinsed in chilled calcium and magnesium free HBSS supplemented with 20 mM sodium molybdate and 5% FCS, pH 7.4 (medium A) using a tissue:medium ratio of 1:20. Individual prostates were used to evaluate the tissue fractionation procedures. However, to examine both the oestrogen and androgen receptor distribution in the separated components, it was necessary to pool prostate glands obtained from animals with similar body weights.

Tissue fractionation

Prostatic stromal and epithelial fractions were prepared using mechanical and enzymatic tissue fractionation procedures (Fig 1). The viability and purity of the different preparations were assessed using both light and electron microscopy. This evaluation enabled the optimum methods for preparing the stromal and epithelial fractions for receptor distribution studies to be determined.

The prostate glands were transferred into a petri-dish containing 5 ml of fresh medium A and teased into 2–4 mm fragments using forceps. All manipulative procedures were conducted on ice unless otherwise stated. Several tissue fragments were removed to enable estimation of oestrogen and androgen receptor concentrations in the whole tissue. These fragments were washed, resuspended in TEDGM buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol and 20 mM sodium molybdate, pH 7.4) containing 1 mM PMSF and 1 mM benzamidine–HCl and maintained on ice until homogenized.

Mechanical fractionation. The mechanical procedure developed to prepare prostatic stromal and epithelial fractions relied on the selective release of epithelial cells when the gland was sliced. Approximately 200–250 mg of the teased fragments were transferred into a petri-dish containing medium A supplemented with the protease inhibitors PMSF and benzamidine–HCl at a final concentration of 1 mM (medium B) and then sliced into small (1–2 mm) pieces with a scalpel. To facilitate further release of epithelial cells, the flat surface of a syringe plunger was employed to apply gentle pressure to the chopped tissue.

After buttering the tissue with the plunger in a gentle rubbing action for 90 s, the epithelial cells released into the medium were separated using a 250 μm stainless steel sieve. The fragments retained on the sieve were resuspended in medium B and the buttering process was repeated twice. Less than 5% of the total epithelial cells released during the mechanical procedure were recovered in the medium following the third buttering process. The residual tissue retained on the sieve at this point was designated the stromal fraction. A sample of the stromal fraction was routinely fixed in phosphate-buffered formalin, pH 7.4 and embedded in paraffin for histological analysis. The fixed tissue was sectioned and stained with haematoxylin and eosin. The remaining stromal fragments were washed in medium B and maintained on ice in TEDGM buffer containing protease inhibitors in preparation for receptor analysis. The epithelial cells released during the buttering process were washed twice by centrifugation in medium B. The cell concentration was determined using a haemocytometer and viability was assessed by trypan blue (0.25%) exclusion. A sample of the epithelial cells was stained with haematoxylin and eosin for cytological examination.

Enzymatic fractionation. Approximately 300–400 mg of teased fragments were washed in calcium and magnesium free HBSS containing 20 mM sodium molybdate, resuspended in 20 ml of the same medium containing 0.1% protease and incubated for 30 min at 25°C on a rotating gyro-shaker (Adams Nutator). After this preincubation, the tissue fragments were allowed to settle for 2–3 min and the supernatant, which contained mainly red blood cells and some damaged stromal and epithelial cells, was aspirated and discarded. The preincubation tissue fragments were resuspended in an additional 20 ml aliquot of protease medium and digested for 60 min at 37°C. The prostatic fragments were maintained in suspension by intermittent agitation. To obtain a maximum yield of viable epithelial cells, the cells released from the tissue fragments were collected by sieving at 15 min intervals. The tissue retained on the sieve was resuspended in fresh medium containing enzyme, while the separated cells were retrieved by centrifugation (300 *g* for 10 min) and resuspended in medium B. After 2 washings, the cell fractions were pooled to form the enzymatic epithelial fraction. The residual tissue fragments, which constituted the enzymatic stromal fraction, were also washed twice with medium B. The yield, viability and cytology of the enzymatically prepared epithelial and stromal fractions were assessed in the same manner described for the mechanically prepared fractions.

Electron microscopy

Preparations of stromal and epithelial fractions obtained using both the mechanical and enzymatic fractionation procedures were fixed in 2.5% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanols and embedded in araldite. Ultrathin (70 nm) sections were stained with uranyl acetate and lead citrate and examined with a Philips 201 electron microscope.

Preparation of cytosol fractions

All tissue fractions were resuspended in TEDGM

buffer containing PMSF (1 mM) and benzamidine–HCl (1 mM) at 4°C. The undissociated tissue samples and stromal fractions were disrupted with an Ultra Turrax homogenizer. The epithelial cell fractions were homogenised using a Teflon-glass homogenizer (Potter–Elvehjem; 102–105 m clearance). The final cytosol fractions were obtained by centrifugation of the homogenates at 105,000 *g* for 60 min at 4°C.

Oestrogen and androgen receptor assays

Oestrogen receptor levels were measured using a dextran-charcoal saturation analysis method as previously described [7]. Briefly, cytosol fractions were incubated in microtiter plates for 20 h at 4°C with [^{3}H]oestradiol (0.05–1.2 nM), both alone and in the presence of a 100-fold excess of radioinert diethylstilboestrol to determine total and nonspecific binding, respectively. Bound and free hormones were separated by incubating with dextran-coated charcoal (0.5% activated charcoal and 0.05% Dextran T-70 in TEGM buffer). The binding data was analysed by the method of Scatchard[8].

Androgen receptor levels were quantitated using a protamine sulphate method similar to that reported by Mobbs *et al.*[9]. Cytosol fractions were incubated with [^{3}H]DHT (0.1–10.0 nM) for 20 h at 4°C. Nonspecific binding was estimated in a parallel series of incubations containing [^{3}H]DHT and a 100-fold excess of unlabelled DHT. The cytosol incubations were terminated by addition of an equal volume of chilled protamine sulphate (1.0 mg/ml) in TEGM buffer. After a further 15 min incubation at 0°C, the samples were centrifuged at 2000 *g* for 10 min. The supernatant was discarded and the precipitates washed twice with TEGM buffer prior to extracting the receptor bound [^{3}H]DHT with 2 × 1 ml aliquots of ethanol for 30 min at room temperature. The ethanol extracts were counted for 2 min in 10 ml of a toluene based scintillation fluid (0.4% PPO and 0.005% POPOP).

Protein and DNA measurements

Protein determinations were performed according to the method of Lowry *et al.*[10] using bovine serum albumin as a standard. Absorbance was read at 750 nm using a Gilford 250 spectrophotometer.

Quantitation of DNA in homogenates was based on the method of Labarca and Paigen[11]. Fluorescence of Hoechst 33258 was measured using a Perkin–Elmer Spectrofluorimeter (model 3000) with excitation and emission wavelengths set at 356 and 460 nm, respectively. Calf thymus DNA was used as a standard.

RESULTS

Tissue fractionation

(*a*) *Mechanical preparations.* Light microscopy revealed that mechanical fractionation of the prostate denuded the acinar structures of epithelial cells leaving an intact stromal matrix (Fig. 2). Electron microscopy confirmed that the mechanically prepared stroma consisted of confluent sheets of smooth muscle cells and small numbers of intervening collagen fibres (Fig. 3).

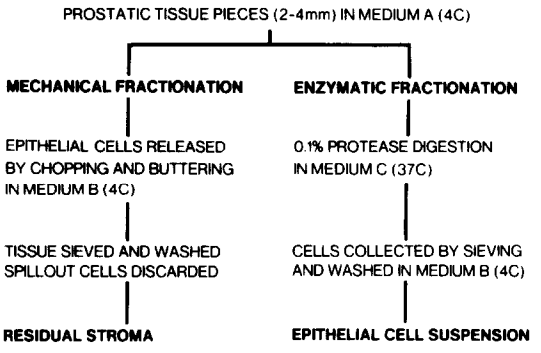

Fig. 1. Schematic outline for the preparation of prostatic stromal and epithelial fractions.

Longitudinally arranged microfilaments with associated dense bodies were clearly maintained. The endoplasmic reticulum displayed mild to moderate cisternal dilatation; some mitochondria were swollen, with lucent matrices, while others appeared condensed. Contaminating epithelial cells were not observed.

In contrast, the mechanically prepared epithelial cells were poorly preserved. The majority of cells were present as dissociated single cells (Fig. 4) and more than 90% were non-viable according to their ability to exclude trypan blue. The majority of cells showed extensive vesiculation of the cytocavitary network, with disruption of the cytoplasm. Scattered nuclei almost entirely devoid of surrounding cyto-

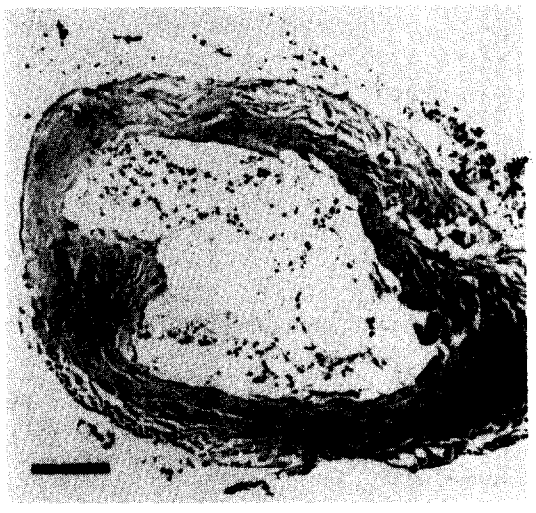

Fig. 2. Light micrograph of mechanically prepared prostatic stroma. The acinar structure has been denuded of epithelial cells leaving an intact stromal matrix. Stained with haematoxylin and eosin. Bar = 50 μm.

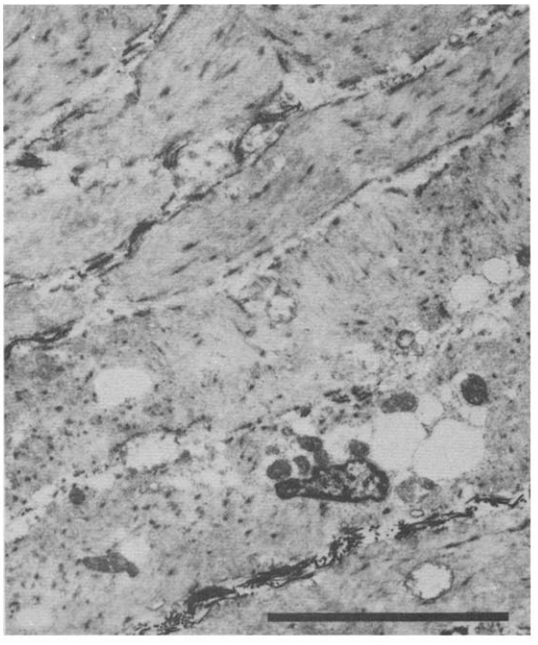

Fig. 3. Electron micrograph (EM) of mechanically prepared prostatic stroma. Microfilaments and dense bodies are preserved, but vesiculation of the endoplasmic reticulum and mitochondrial ballooning are evident. Bar = 5 μm.

plasm were also seen. Few recognizable contaminant smooth muscle cells could be found.

(*b*) *Enzymatic preparations.* The epithelial cells prepared using 0.1% protease were present as both dissociated cells and cohesive cellular clusters. Viability, assessed by trypan blue exclusion, was

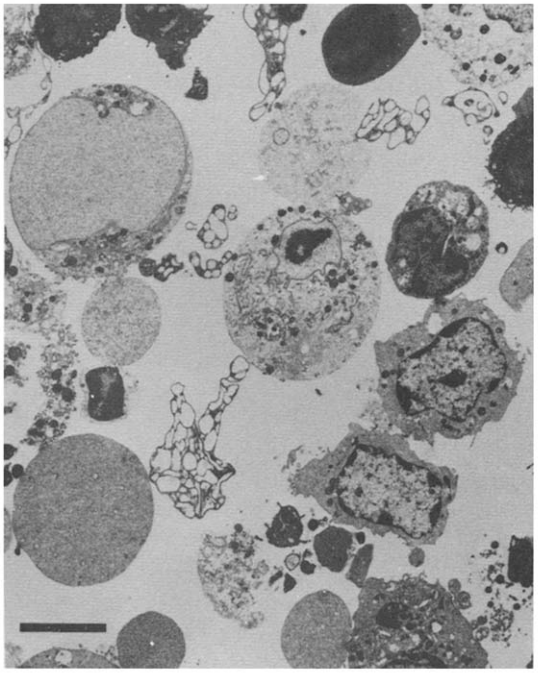

Fig. 4. Mechanically prepared prostatic epithelium. (EM). Isolated cells showing varying degrees of degenerative changes are depicted. Bar = 5 μm.

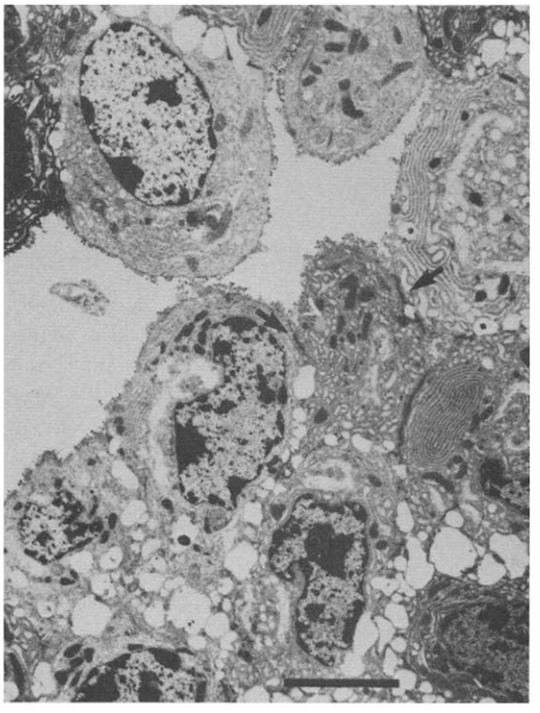

Fig. 5. Enzymatically prepared prostatic epithelium. (EM). These cells form cohesive clusters, with maintenance of junctional complexes (arrows), but there are extensive vesicular expansions of the intercellular space. Mitochondrial morphology is maintained and targetoid formations of RER can be seen. Bar = 5 μm.

85–98% and the yield of viable cells was approx. 0.5×10^7 cells per 100 mg of tissue. Electron microscopy demonstrated that surface microvilli, junctional complexes and other intercellular junctions were maintained in the clusters, but there were prominent focal expansions of the intercellular space (Fig. 5). Rough endoplasmic reticulum (RER) was a major feature of these cells, forming both parallel and complex targetoid patterns. Mitochondria were generally condensed. The nuclei often contained marginated heterochromatin. Contaminant stromal elements were not seen. Replacement of pronase by 0.1% collagenase, resulted in a decreased yield of viable epithelial cells and an increase in the percentage of contaminating stromal cells present in the final cell suspension.

Preservation of the enzymatically prepared stroma was visibly inferior to the mechanical preparation. The myocytes appeared less cohesive, although intercellular junctions were discernible in some areas (Fig. 6). The cells showed variable electron-density and in many the sarcolemma formed frequent irregular folds and undulations. The appearance of these cells resembled a partially deflated balloon, and was similar to that of atropic striated muscle cells [12]. Longitudinally oriented microfilaments averaging 5 nm in diameter were evident within the myocytes, but in some cells their associated dense bodies were not as obvious as in the mechanically prepared stroma. In

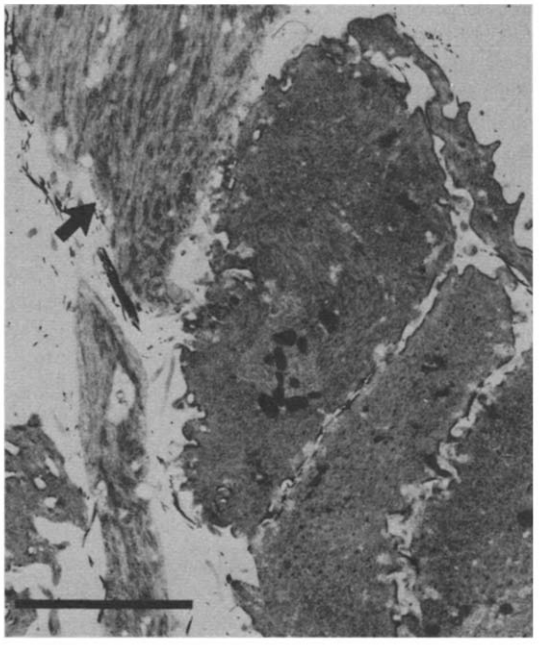

Fig. 6. Enzymatically prepared prostatic stroma. (EM). Myocytes appear separated with irregularly convoluted plasmalemmal regions. Thick myofilaments inhabit one of the myocytes (arrow). Bar = 5 μm.

addition, the myocytes often contained elongated thick myofilaments. Clusters of contaminating epithelial cells were also present, their morphology being similar to that of the enzymatically prepared epithelial fraction.

Oestrogen and androgen receptor distribution

Cytosolic oestrogen and androgen receptor concentrations were determined in the mechanically prepared stromal and enzymatically prepared epithelial fractions and in the whole tissue from which these fractions were derived. Examples of Scatchard plot analyses for the binding of [^{3}H]oestradiol to cytosol

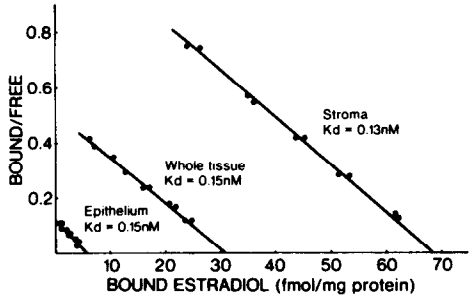

Fig. 7. Example Scatchard plot analyses of the binding of [^{3}H]oestradiol to cytosol fractions obtained from separated prostatic stromal and epithelial fractions, and from the whole tissue. Cytosol fractions were incubated with 0.05–1.2 nM [^{3}H]oestradiol for 20 h at 4°C prior to dextran charcoal adsorption of unbound ligand. Nonspecific binding was determined using a parallel series of incubations containing a 100-fold excess of DES. The protein concentration of the cytosol fractions was adjusted to 2.0–2.5 mg/ml prior to commencing the binding studies.

Table 1. Oestrogen and androgen receptor distribution relative to cytosolic protein

	Receptor concentration (fmol/mg protein)*		
Receptor	Whole prostate	Stroma	Epithelium
Oestrogen	31.7 (±4.6)	70.7 (±9.1)	5.8 (±3.7)
Androgen	15.6 (±4.3)	14.6 (±4.1)	23.3 (±4.4)

*Values are mean (±SD) for 10 tissue fractionations.

fractions derived from each tissue component are shown in Fig. 7. Expressed relative to cytosolic protein, the stromal oestrogen receptor concentration was approximately twice the value observed in non-dissociated tissue and more than 10-times the epithelial concentration (Table 1). In contrast, a higher androgen receptor concentration was measured in the epithelial fraction than in either the stromal fraction or the non-dissociated prostate. A similar distribution of oestrogen and androgen receptors was observed when receptor concentrations were expressed relative to cellular DNA (Table 2).

Omission of a protease substrate (FCS) and protease inhibitors from the wash medium, combined with homogenization in the absence of protease inhibitors, resulted in a reduction in epithelial androgen receptor concentration from 23.3(±4.4) to 8.2(±5.4) fmol/mg protein. Similarly, if proteolytic activity was not inhibited, the epithelial oestrogen receptor concentration decreased from 5.8(±3.7) to 2.6(±2.3) fmol/mg protein. Omission of the protease inhibitors did not affect the measurable levels of oestrogen and androgen receptors in the mechanically prepared stromal fractions. Neither receptor was detected in the mechanically prepared epithelial fraction, while low levels of oestrogen (20.1 ± 9.3 fmol/mg protein) and androgen (2.7 ± 2.2 fmol/mg protein) receptors were measured in the residual enzymatic stromal fraction.

Dissociation constants

The equilibrium dissociation constants for the [^{3}H]oestradiol- and [^{3}H]dihydrotestosterone–receptor interactions are shown in Table 3. The K_d for oestradiol binding in the separated fractions did not

Table 2. Oestrogen and androgen receptor distribution relative to DNA

	Receptor concentration (fmol/μg DNA)*		
Receptor	Whole prostate	Stroma	Epithelium
Oestrogen	0.84 (±0.15)	1.64 (±0.21)	0.15 (±0.03)
Androgen	0.62 (±0.11)	0.51 (±0.09)	1.11 (±0.14)

*Values are mean (±SD) for 10 tissue fractionations.

Table 3. Dissociation constants for oestrogen and androgen receptors in cytosols from whole prostate and separated stromal and epithelial fractions

	Dissociation constant (nM)*		
Receptor	Whole prostate	Stroma	Epithelium
Oestrogen	0.14 (±0.08)	0.15 (±0.11)	0.17 (±0.09)
Androgen	2.29 (±0.94)	2.23 (±0.64)	1.96 (±0.37)

*Values are mean (±SD) for 10 tissue fractionations.

differ from that in the whole prostate and was characteristic of binding to high affinity oestrogen receptors. Similarly, there was no difference in the K_d for the androgen receptor.

DISCUSSION

Recent observations demonstrating the importance of stromal–epithelial interactions during prostatic development [1–4], have stimulated interest in the hormonal regulation of the stromal component. One approach has been to examine the distribution of oestrogen and androgen receptors in the prostatic epithelial and stromal fractions [13, 14, 15]. These studies, however, have been hindered by the lack of a single suitable method for preparing both tissue fractions. In the present study, this problem was overcome by using independent enzymatic and mechanical procedures to separate the guinea pig prostate into relatively pure, viable epithelial and stromal fractions, respectively.

Initial attempts to mechanically disrupt the prostate by mincing the tissue with razor blades or scalpels, produced a relatively pure suspension of epithelial cells, but the majority of these cells failed to exclude trypan blue. However, when enzymatic techniques were used, it was found that protease (0.1%; Sigma Type XIV) gave the best yield of viable epithelial cells. A similar observation has been reported for the isolation of epithelial cells from human prostatic tissues [16]. To maximize cell viability it was found to be important to remove separated cells from the incubation medium at regular intervals and to protect these cells from further proteolysis by resuspending them in medium containing FCS and protease inhibitors. Electron microscopy confirmed that the enzymatically prepared epithelial cells were well preserved. The ultrastructural appearance of the isolated cells closely resembled that of epithelial cells prepared from the rat seminal vesicle by trypsin-collagenase digestion [17].

In contrast, the residual stroma obtained enzymatically was not well preserved. Cell architecture and organelle morphology were not maintained. These observations suggest that protease may be more toxic to stromal cells than to epithelial cells. Such a toxic effect of protease on stromal cells released during the proteolytic digestion may in part explain why the enzymatic epithelial fraction appeared to be devoid of stromal cells.

Although mechanical disruption was unsuitable for preparing viable epithelial cells, the ability to selectively spill-out epithelial cells by alternately slicing and buttering the prostate provided a means of obtaining a relatively pure and well preserved stromal matrix. A similar approach has been reported for preparing stromal fractions from the rat ventral prostate [13]. In our study, it was found that excessive mechanical disruption resulted in the release of stromal cells from the residual tissue. Thus, it was necessary to examine the cell composition following each step of the mechanical procedure. This was successfully accomplished by light microscopy of the spilled-out cells.

Receptor measurements performed on the enzymatically prepared epithelial and mechanically prepared stromal fractions of the guinea-pig prostate, indicated that the oestrogen receptor was predominantly localized in the stroma. The stromal oestrogen receptor concentration was approx. 10-times greater than in the epithelium and twice the concentration measured in the whole prostate. The androgen receptor, however, was distributed more evenly between the stromal and epithelial fractions, with the concentration in the epithelial fractions being approximately twice the stromal value. Similar conclusions were reached irrespective of whether the receptor values were expressed relative to cytosolic protein or cellular DNA.

Assuming that approx. 25–50% of the prostate is comprised of stromal elements, then a stromal localization of the oestrogen receptor would imply that the stromal receptor concentration should be at least twice the level in the non-dissociated tissue. Apart from the present study, such a phenomenon has been demonstrated only for the rat ventral prostate [13]. In that study, prostatic stroma was prepared using a mechanical method similar to that employed in the present investigation, but the spilled-out cells were retained as the epithelial fraction. Since it appeared that many of the spilled-out cells were damaged, it was not possible in that study to conclude with any certainty that the epithelial cells did not contain substantial levels of oestrogen receptor. In another study using the canine prostate [14], the oestrogen receptor concentration was found to be considerably higher in the stroma than in the epithelium, but the concentration in the stroma did not differ significantly from that measured in the whole tissue. The apparently low oestrogen receptor level found in the enriched stromal fraction in the canine study, may again be attributed to cell damage and consequent loss of receptor during tissue fractionation. Similarly, the use of tissue homogenates containing extensively damaged cells may explain why oestrogen receptors were not consistently detected in human BPH tissue stromal fractions [15].

In the present study, the measurement of receptor levels in viable stromal and epithelial fractions in which cell architecture and organelle morphology were maintained, has clearly demonstrated the stromal localization of the oestrogen receptor in the guinea-pig prostate. This finding suggests that oestrogen may play an important role in the regulation of the prostatic stroma. Further investigations are required to elucidate the significance of a stromal localization of the oestrogen receptor in relation to the growth and development of the prostate.

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