Localization of androgen receptors in ram epididymal principal cells*

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Summary. Androgen receptor was immunolocalized in the epididymal epithelium of rams and in isolated cells using an antibody against a synthetic polypeptide representing a portion of the androgen receptor. Immunostaining was predominant in the epithelium in tissue sections. Concentrations of androgen receptor were determined in cells from the central caput, distal caput, and central corpus epididymidis enzymically dissociated and elutriated to provide two fractions. On the average ($n = 18$), Fraction I contained 8% principal cells while Fraction II contained 71% principal cells; the stromal cells in each fraction were primarily smooth muscle and fibroblasts. For each sample, the number of DHT receptors (fmol) per 10^6 total cells was greater in Fraction II than in Fraction I. Few cells in Fraction I were immunostained for androgen receptor, whereas most cells in Fraction II were intensely stained. The numbers of DHT receptors per cell, or per principal cell, were similar for the central caput and distal caput, but lower in the central corpus epididymidis. The results support our hypothesis that most epididymal DHT receptors are localized in principal cells and confirm that the region between the central caput and proximal corpus of the ram epididymis is most dependent on androgen stimulation.

Keywords: epididymis; androgen receptor; principal cells; ram

Introduction

Functions of the epididymal epithelium, and especially sperm maturation, are androgen-dependent (Amann, 1987; Robaire & Hermo, 1988), possibly through local action of dihydrotestosterone (DHT). The enzyme that converts testosterone to DHT, 5 α -reductase, apparently is localized in principal cells (Klinefelter & Amann, 1980). In rams (Amann, 1987; Amann *et al.*, 1987), in contrast to rats (Robaire & Hermo, 1988), the activity of 5 α -reductase is negligible in the initial segment, high in the central caput through to the proximal corpus, and lower in the central corpus epididymidis. Androgen receptor in ram epididymal tissue follows a similar distribution, although the concentration of oestradiol receptor in whole epididymal tissue is greater in the cauda than in the caput epididymidis (Tekpetey & Amann, 1988a). Androgen receptor is present in rat and rabbit epididymal tissue (Ritzen *et al.*, 1971; Hansson *et al.*, 1973; Danzo *et al.*, 1973; Danzo & Eller, 1975), but its regional distribution has not been quantified. At least in ram epididymal tissue, the androgen receptor is highly specific for DHT (Tekpetey & Amann, 1988a), so hereafter it will be referred to interchangeably as androgen or DHT receptor.

Androgen-dependent mRNAs are present in rat epididymal tissue (Brooks *et al.*, 1986; Brooks, 1987), and principal cells secrete proteins into the milieu around spermatozoa within the epididymal duct, which presumably mediate androgen-dependence of sperm maturation (Amann,

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1987, 1988; Robaire & Hermo, 1988). It is possible that the DHT receptor is localized in principal cells. This concept is supported by autoradiographic evaluations of epididymal tissue from mice injected with [3 H]DHT (Schleicher *et al.*, 1984). Principal cells in the caput and cauda epididymidis of mice were heavily labelled with [3 H]DHT, compared to smooth muscle and connective tissue associated with the duct.

Based on the regional distribution of receptors for DHT in the ram epididymis (Tekpetey & Amann, 1988a), we proposed that principal cells from the caput or corpus epididymidis would have a high number of DHT receptors relative to stromal cells (i.e. smooth muscle or fibroblasts). Therefore, we measured the numbers of DHT receptors in two populations of epididymal cells that contained relatively high or low percentages of principal cells. We also studied localization of androgen receptor in epididymal tissue and elutriated cells using an antibody against the androgen receptor.

Materials and Methods

Preparation of tissue and cellular populations. Tissues from the efferent ducts, caput, corpus and cauda epididymidis (Fig. 1), and liver were collected from 3 adult Western range rams. Tissues were fixed in Bouin's solution for 24 h, immersed in 70% ethanol for 48 h, dehydrated in graded ethanols and embedded in paraffin wax. Sections (6 μ m) were cut and serially collected on glass slides coated with Histostik (Accurate Chemical Corporation, Westbury, NY, USA) for immunostaining.

Epididymal tissue was obtained from 6 other adult Western range rams by castration, under local anaesthesia, in October and November. Tissue representing the central caput, distal caput and central corpus (Fig. 1) was taken from each epididymis and 2–3 g of epididymal duct were teased from the saccus epididymidis, weighed and processed for isolation of fractions enriched for, or relatively devoid of, principal cells (Wagley *et al.*, 1984). These regions were selected because they have the highest concentration of androgen receptor in total tissue (Tekpetey & Amann, 1988a). The procedures for processing tissue fragments to produce a suspension of individual cells, and subsequent fractionation of this cellular suspension by elutriation, were essentially as described by Wagley *et al.* (1984) and Amann *et al.* (1987).

Fig. 1. Sites where samples of epididymal tissue were taken for immunohistochemistry and/or enzymic dissociation and elutriation. Regions are designated as efferent ducts, ED; initial segment, IS; central caput, CCAP; distal caput, DCAP; central corpus, CCOR; and cauda epididymidis, CAUDA.

A Beckman JE-6B elutriator rotor, fitted with a Sanderson chamber, was used to prepare two fractions. Fraction I consisted of cells eluted in the first 200 ml after loading cells into the elutriation chamber at 1600 rev./min using a flow rate of 15.5 ml/min for the caput or 16.0 ml/min for the corpus epididymidis. Fraction II was cells in the next 100 ml collected at 30 ml/min as rotor speed dropped to 0 rev./min (Wagley *et al.*, 1984). Cells in each fraction were concentrated by centrifugation and resuspended in 5–10 ml elutriation buffer. Fraction I contained relatively few principal cells, and the predominant cell types included smooth muscle cells, fibroblasts, basal cells and lymphocytes. Fraction II was enriched for principal cells, although contaminating cells were present. Thus, Fractions I and II were reciprocally enriched.

Concentration of cells (excluding spermatozoa) in aliquants of each suspension was determined with a haemocytometer and a phase-contrast microscope. Spermatozoa were excluded since they do not contain steroid receptors (Amann & Hammerstedt, 1976). Aliquants of each cellular suspension were air-dried on microscope slides and held at room temperature until differentially stained or held at -70°C until immunostained.

Fig. 2. Standard curve obtained by plotting counts of [^3H]DHT specifically bound to receptor vs fmol DHT receptor in the standard extract. ●—●; $y = -22\,893 + 11\,833x$ when values designated by ○ were excluded, x was expressed as fmol DHT receptor and y was expressed as c.p.m. [^3H]DHT; $r^2 = 0.97$. The insert (from Tekpetey & Amann, 1988b) shows a Scatchard analysis of receptor content in the standard extract.

Differential and immunostaining. Air-dried smears of cellular suspensions were stained by the Papanicolaou method. At least 200 cells/sample were classified as a principal cell or other cells. Criteria for differentiating principal cells from other cells included cell size and shape, nuclear size and shape and differential staining pattern (Wagley *et al.*, 1984).

Tissue sections and smears of cellular suspensions were utilized for immunolocalization of androgen receptor. The primary antiserum (AR1-52 donated by Dr F. S. French) was produced in rabbits against a synthetic 15-amino acid peptide sequence (Asp-His-Val-Leu-Pro-Ile-Asp-Tyr-Tyr-Phe-Pro-Pro-Gln-Lys-Thr) representing the deduced sequence of the rat androgen receptor (Tan *et al.*, 1988) and was used with the biotin-streptavidin peroxidase procedure (Stravigen Immunohistology Kit, BioGenex Laboratories, San Ramon, CA, USA). Frozen cell smears were thawed, fixed in 4% paraformaldehyde (0.1 M-sodium phosphate and 0.146 M-sucrose at pH 7.4) for 5 min at 22°C, and washed in 0.01 M-potassium phosphate-buffered saline (PBS; 0.145 M-NaCl, pH 7.4) for 10 min. Tissue sections were deparaffinized in xylene, passed through absolute ethanol and 95% ethanol for 10 min each, and rehydrated in PBS for 10 min. Cell smears and tissue sections were treated with 3% hydrogen peroxide for 10 min at 35°C to reduce endogenous peroxidase activity. Slides were washed twice in PBS for 5 min each at 22°C, and similarly washed after each subsequent step (except after incubation with normal goat serum). Cells and tissues were incubated sequentially in normal goat serum (10 min at 35°C); rabbit anti-androgen receptor serum (5 μg protein/ml PBS); preimmune rabbit serum, or primary antiserum preadsorbed with the synthetic sequence (30 min at 35°C); biotinylated goat anti-rabbit immunoglobulin (10 min at 35°C); horseradish peroxidase-labelled streptavidin (10 min at 35°C) and freshly prepared substrate reagent containing hydrogen peroxide and 3-amino-9-ethylcarbazole (AEC) or diaminobenzidine tetrahydrochloride (DAB). Peroxidase indirectly bound to androgen receptor oxidized the AEC or DAB forming a brownish-red or brown precipitate. In some cases, slides were counterstained with Mayer's haematoxylin for 5 min, dipped in ammonia water for 10 sec, and rinsed in running water. Coverslips were mounted using glycerol-gelatin. Slides were examined and photographed using a Nikon Optiphot microscope. Cells and tissues treated with preimmune rabbit serum or antiserum preadsorbed with immunogen, instead of primary antiserum, served as controls.

Preparation of cellular extracts. The fractions obtained by elutriation were centrifuged at 325 *g* for 10 min and the pelleted cells were homogenized, on ice, in low-salt TEDG buffer [10 mM-Tris-HCl; 1.5 mM-EDTA; 1.0 mM each of dithiothreitol, phenylmethyl sulphonyl fluoride and sodium molybdate; 10% (v/v) glycerol; pH 7.4], using a glass homogenizer (11 mm diam) for 20 strokes. The homogenate was centrifuged at 30 000 *g* for 30 min and the supernatant containing cytosolic receptor was saved. The pellet was resuspended in high-salt buffer (identical to low-salt TEDG buffer, except that it also contained 400 mM-KCl), held on ice and vortexed every 15 min for 1 h to extract nuclear receptors which were resistant to low-salt homogenization (Tekpetey & Amann, 1988a). The second homogenate was centrifuged (as above) and the supernatant was saved. The two supernatants were pooled into a final volume of 3–4 ml, brought to 5% (v/v) dimethylsulphoxide, snap-frozen in liquid nitrogen, and stored at -70°C until the receptors were assayed. Before quantification of DHT receptors, endogenous steroids were removed from the extracts using a suspension of dextran-coated charcoal (Tekpetey & Amann, 1988a).

Quantification of DHT receptor. The concentration of DHT receptor in each cellular extract was determined using a standard curve assay (Tekpetey & Amann, 1988b). Briefly, a 7-point standard curve was established using a standard extract of ram epididymal tissue (the same as used by Tekpetey & Amann, 1988b) that contained 6.0 fmol DHT receptor per 1.0 mg wet weight tissue and had a binding affinity of $3.5 \times 10^8 \text{ M}^{-1}$. Increasing quantities (25–400 μl , equivalent to 5–80 mg wet tissue) of the standard extract, or a sample (200 or 300 μl) of the extract from a cellular fraction, were incubated (in duplicate) with 1.5 nM-[1,2,4,5,6,7- ^3H]DHT (sp. act. 128 Ci/mmol). Parallel tubes contained a 100-fold excess of non-radioactive DHT to enable calculation of non-specific binding. After 30 h incubation at 4°C , free steroid was separated from receptor-bound steroid by adding 500 μl dextran-coated charcoal [0.6% (w/v) charcoal and 0.06% (w/v) dextran in TEDG buffer]. After incubation for 10 min at 4°C , followed by centrifugation at 5000 *g* for 10 min, a 600- μl sample of supernatant was added to 4 ml scintillation fluid. All samples were counted for 5 min.

Values for duplicate tubes were averaged and specific binding (primary value minus value for non-specific binding) was calculated for each volume of standard extract or extract of a cellular fraction. From the known concentration of DHT receptor in the standard extract, the number of receptors (fmol) for each volume of standard extract was calculated; these values were plotted to provide a standard curve (Fig. 2). The amount of [^3H]DHT specifically bound to a known volume of an extract from a cellular fraction was compared to the standard curve and the concentration of DHT receptor in the sample extract was calculated. Values for all samples were within the linear portion of the standard curve, between 100 and 250 fmol DHT receptor per assay tube. All cellular extracts were analysed for DHT receptor in one assay. Intra-assay variation, based on values for the duplicate tubes for each sample, was 2.7%.

The amount of DHT receptor in each extract was expressed on the basis of 10^6 total cells and for Fraction II on the basis of 10^6 principal cells, assuming that all receptor was in principal cells. The number of principal cells in each extract was calculated from counts of total cells and the percentage that were principal cells, as determined by evaluation of PAP-stained smears, for the corresponding cellular fraction. To estimate the amount of DHT receptor that might be associated with 'other' cells, data for cell numbers and percentage of principal cells in Fraction I, and estimates of the amount of receptor per 10^6 principal cells in Fraction II, were used to calculate principal cell-associated DHT receptor in Fraction I. This value was subtracted from the measured amount to estimate that associated with 'other' cells; the resulting value was expressed per 10^6 'other' cells.

Statistical tests. The cellular fractionation and receptor data were subjected to analysis of variance (Statistical Analysis System, 1982), using a model that included region of the epididymis (fixed), cellular fraction (fixed), replicate (ram; random) and the interactions of epididymal region and cellular fraction. Differences between means were tested using the Student-Neuman-Keuls multiple comparison. Data with non-homogeneous variance, as indicated by Bartlett's test, were transformed logarithmically before analysis.

Fig. 3. Immunolocalization of androgen receptor in the ovine epididymis (a–e; $\times 125$) and elutriated cells of central caput epididymidis (f–i; $\times 400$). Androgen receptor was sparse in the efferent ducts (ED), but immunostaining was very conspicuous in epithelium of the initial segment (IS) of the epididymis (a). Immunostaining also was predominant in epithelium of the central caput (b), central corpus (c), and cauda (d) epididymidis. At distal sites, some immunostaining of smooth muscle associated with the duct was evident. Immunostaining was not observed when preimmune serum (e) or antiserum preadsorbed with synthetic peptide was substituted for primary antiserum. Only a few cells were immunostained in Fraction I that was enriched for stromal cells (f), whereas a large proportion of cells was conspicuously stained in Fraction II that was enriched for principal cells (g). Immunostaining was not observed when preimmune serum (h) or antiserum preadsorbed with the synthetic peptide (i) was substituted for primary antiserum.

Results

In tissue sections, immunostaining for androgen receptor was predominant in the epithelium of all regions of the epididymis (Figs 3a–3d). Staining in the initial segment was conspicuous and more intense than in any other region of the epididymis, but staining in efferent ducts was faint (Fig. 3a). In the corpus and cauda epididymidis, some staining also was evident in smooth muscle associated with the duct. Staining was not observed in liver or when preimmune serum (Fig. 3e) or antiserum preadsorbed with the immunogen was substituted for primary antiserum.

The results for individual elutriations of tissue from each of the 3 regions for the 6 rams (Table 1) show that Fractions I and II were quite different, and that Fraction II was enriched for principal cells. In general, Fraction I contained more cells per unit volume than did Fraction II. Excluding data for the distal caput from Ram 128, for which distinct cellular fractions were not produced, the percentages of principal cells in samples from the central caput, distal caput and central corpus epididymidis were similar ($P > 0.05$) among regions.

For each of the 18 samples, the number of DHT receptors per 10^6 cells was greater ($P < 0.01$) in Fraction II than in Fraction I (Table 1). When number of DHT receptors in Fraction II was expressed on a basis of 10^6 principal cells, assuming that all DHT receptor was associated with principal cells, values for the central caput and distal caput were higher ($P < 0.05$) than those for the central corpus epididymidis (Table 2). Interactions of fraction by region of the epididymis were not significant.

Immunostaining of elutriated cells revealed only a few cells that were distinctly stained in cellular Fraction I, while some others stained faintly (Fig. 3f). For Fraction II, however, most cells were conspicuously stained (Fig. 3g). This staining pattern was similar in all 3 regions studied.

Table 1. Cellular yield, percentage of principal cells, and concentration of DHT receptor in cellular fractions from three regions of the epididymis of the ram

Region of epididymis	Ram	Total cells (10^6 /ml)		Principal cells (%)		DHT receptor (fmol/ 10^6 cells)	
		Fraction I	Fraction II	Fraction I	Fraction II	Fraction I	Fraction II
Central caput	128	32	9	8	70	9.3	37
	129	52	7	12	76	5.7	47
	130	74	18	6	60	5.2	29
	131	28	7	10	63	10.6	43
	132	52	18	12	55	5.7	25
	133	63	24	10	57	5.1	14
	Mean	50	14	10	63	6.9	32
Distal caput	128*	62	52	38	45	ND	ND
	129	26	6	7	68	11.3	50
	130	71	6	7	60	6.7	50
	131	74	12	8	64	5.6	34
	132	87	10	6	77	4.3	47
	133	42	13	6	76	8.5	27
	Mean	60	9	7	69	7.3	42
Central corpus	128	42	15	5	69	8.5	23
	129	85	30	10	77	5.1	13
	130	136	24	9	73	4.0	16
	131	72	16	6	63	5.7	19
	132	111	35	14	81	3.9	15
	133	131	25	5	71	3.5	16
	Mean	96	24	8	72	5.1	17

*Values for this sample were excluded from mean values, because of failure to isolate distinct fractions; ND=not determined.

Table 2. Estimated concentrations of DHT receptor in principal cells or in 'other' cells from ram epididymis*

Region of epididymis	Ram	Principal cells in Fraction II (fmol/10 ⁶ cells)	'Other' cells in Fraction I (fmol/10 ⁶ cells)
Central caput	128	52	5.6
	129	63	0.0
	130	49	2.4
	131	68	4.2
	132	46	0.2
	133	24	0.1
	Mean	50	2.1
Distal caput	129	74	6.6
	130	86	0.7
	131	53	1.5
	132	61	0.7
	133	36	6.6
	Mean	62	3.2
Central corpus	128	33	7.2
	129	17	3.8
	130	23	2.1
	131	31	4.1
	132	19	1.4
	133	23	2.5
	Mean	24	3.5

*Calculated assuming that DHT receptor was found only in principal cells in Fraction II, and that DHT receptor not present in principal cells was equally distributed among all cells in the 'other' population of Fraction I regardless of cell type or cell volume.

Immunostaining of elutriated cells was not observed when preimmune serum (Fig. 3h) or antiserum preadsorbed with synthetic peptide (Fig. 3i) was substituted for primary antiserum.

Discussion

Based on Papanicolaou-stained slides, principal cells averaged 63, 69 and 72% of the total population of cells in Fraction II from elutriations for the central caput, distal caput and central corpus epididymidis. These values were considerably lower than values reported by Wagley *et al.* (1984), although the procedures used for elutriation and determination of the percentage of principal cells were essentially identical. This difference was probably due to the batch of enzyme, and the facts that in the present study more tissue was dissociated per flask and more cells were loaded into the elutriation system.

Because of the obvious enrichment of Fraction II for principal cells (Table 1), the approximately 5-fold greater number of DHT receptors per cell in Fraction II, and immunolocalization of receptor in most cells in Fraction II (Fig. 3), we conclude that most DHT receptors are located in principal cells. However, some cells in the 'other' population (primarily smooth muscle cells and fibroblasts) probably contain a low level of androgen receptor, as evidenced by immunostaining of some non-epithelial cells. If all principal cells in Fractions I and II have the same number of receptors, the 'other' cells must have contained about 2.9 fmol DHT receptor per 10⁶ cells, regardless of region (Table 2), as compared to 50, 62, or 24 fmol per 10⁶ principal cells in the central caput, distal caput, or central corpus epididymidis, respectively. Alternatively, about 5% of the 'other' cells in Fraction I have a number of DHT receptors similar to that for principal cells in the caput epididymidis. In either case, most epididymal DHT receptors must be localized in principal cells.

This conclusion is supported by immunohistochemical localization of androgen receptor, using the same antiserum, in rat epididymal tissue (Sar & Wilson, 1989).

The concentration of DHT receptor for cells in Fraction II was greater for the central caput and distal caput than the central corpus epididymidis. This probably reflects a true regional difference in receptor number per principal cell (930 in central or distal caput vs 400 in central corpus epididymidis for Fraction II), since the percentage of principal cells (of all somatic cells) in Fraction II was similar for all regions (Table 1). The current results are consistent with our previous finding that the concentration (fmol/mg protein) of DHT receptor in extracts of whole epididymal tissue, collected at a similar time of year, was higher in the central caput and proximal corpus epididymidis than in the proximal caput, distal corpus or cauda epididymidis (Tekpetey & Amann, 1988a). The apparent discrepancy between intense immunostaining for androgen receptor in the initial segment (Fig. 1) and the relatively low tissue concentration of receptor in the proximal caput (Tekpetey & Amann, 1988a) probably results from inclusion of the efferent ducts in their tissue preparation. Although our immunocytochemical observations are not quantitative, and cannot be interpreted as concentration DHT receptor per 10^6 principal cells or per milligram protein, they clearly suggest that the epithelium of the initial segment contains considerable androgen receptor. However, there probably is little DHT in the initial segment or proximal caput epididymidis since there is little 5α -reductase in these tissues (Amann, 1987), although testosterone should be available. Affinity of the androgen receptor in ram epididymal tissue for testosterone is only 10% of that for DHT (Tekpetey & Amann, 1988a). Collectively, these results support the conclusion that in rams the region between the central caput and proximal corpus epididymidis probably is most dependent on androgen stimulation, presumably for functions important in providing a milieu essential for sperm maturation.

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