

Gene expression in the dog epididymis: a model for human epididymal function

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Summary

The physiology of the epididymis is an integral part of the maturation process by which human spermatozoa acquire the ability to reach and fertilize an oocyte. Because of the high degree of species specificity exhibited by the epididymal proteins involved in sperm maturation, we have assessed tissue from several alternative species for their suitability as a model for human epididymal physiology. Of these, the dog appears to offer an appropriate system. Northern hybridization using cDNA probes specific for human epididymal genes established that, irrespective of dog breed, the canine equivalents of the epididymis-specific HE1, HE4 and HE5 mRNAs were expressed highly in the canine epididymis. cDNA cloning and sequencing confirmed that the canine gene products, CE1, CE4 and CE5 were indeed true structural homologues of their human counterparts. Finally, tissue culture conditions were established wherein all three specific canine genes remained up-regulated after 5 days of culture. Thus, the prerequisite criteria for the development of a system which models human epididymal physiology are to a large degree fulfilled by this canine culture system.

Keywords: epididymis, canine, sperm maturation, cDNA cloning, secretory glycopeptides

Introduction

The ability of spermatozoa to reach and fuse with an oocyte in the female tract, and to give rise to a healthy embryo, is a property which has not been attained fully when spermatozoa leave the testis. Further maturation occurs while the spermatozoa pass through the different regions of the epididymis (Bedford, 1975; Cooper, 1986; Schoysman & Bedford, 1986; Blaquier *et al.*, 1989; Silber, 1989). Spermatozoa traversing the epididymis interact with the luminal milieu created by the epididymal epithelium, and in particular with proteins secreted from these cells, which may have hormonal, enzymatic or other surface-modifying properties. Knowledge of these maturation processes, and the potential impact of an epididymal pathol-

ogy on human fertility, is hampered by the limited research undertaken on this organ, but more particularly by the high degree of species diversity evident between different mammals. This diversity is evident in the low degree of homology between species of epididymal secretory proteins or their respective gene transcripts (Brooks *et al.*, 1986a,b; Kirchhoff *et al.*, 1990). In order to determine the role played by the epididymis in human fertility, we have begun recently to develop tools and assess systems appropriate for studying human epididymal physiology. This has included the cloning and analysis of the gene transcripts corresponding to the major epididymal-specific secretory proteins in the human (Kirchhoff *et al.*, 1990, 1991, 1993, 1994; Osterhoff *et al.*, 1994).

In order to study the mechanisms of human sperm maturation *in vitro*, a cell culture system is required wherein the sperm-epididymal interactions can be investigated under

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controlled conditions. The use of primary human epididymal cells is handicapped greatly by the limited availability of tissues, mostly from elderly patients undergoing orchidectomy as a treatment for prostatic carcinoma. Although these tissues can be maintained in culture for quite long periods (Cooper *et al.*, 1990; Moore *et al.*, 1992), they appear with time to lose their ability to express the genes for specific secretory proteins (McArdle, 1991). Cell-lines with properties of the mature, differentiated epididymal epithelium are lacking. Therefore, we have assessed the use of tissues from alternative mammalian species for primary cell culture in order to develop a model system which has relevance for the human.

The following criteria were applied in the selection of a model system. Tissue should be available conveniently in moderate quantities and should involve the ethical use of laboratory or domestic animal species. The epididymides should express to a high level, marker genes which are the counterparts of genes expressed tissue-specifically in the human epididymis. Primary cultures of epididymal epithelial cells should be stable over periods of several days and maintain expression of the tissue-specific phenotype *in vivo*, i.e. expression of marker genes.

Analysis of epididymal secretory proteins and their encoding mRNAs from both rats and mice have revealed little homology between rodents and humans in the protein components involved in epididymal sperm maturation. Of the five major human epididymally expressed genes studied by us so far, only HE1 and HE5 appear to have rodent homologues (Kirchhoff *et al.*, 1990; Uhlenbruck *et al.*, 1993; Kirchhoff, 1994). Furthermore, of the major rodent epididymally expressed genes no homologues have been identified within the human epididymis (Brooks *et al.*, 1986a, 1986b; Kasahara *et al.*, 1989). Bovine tissues, although available from commercial slaughterhouses in large quantities, only appear to express HE1 and HE4 (Kirchhoff *et al.*, 1990; Uhlenbruck *et al.*, 1993). Porcine epididymides appear to offer a good model with four of the human genes being expressed, namely, HE1, HE3, HE4 and HE5 (Uhlenbruck *et al.*, 1993; Kirchhoff *et al.*, 1994). However, since the overwhelming majority of commercially slaughtered pigs are castrated before they reach sexual maturity, a ready supply of adult tissue is not available. Amongst primates, only the marmoset has been assessed to date, but despite close homology to the human, it was eliminated as a frequent source of tissue for ethical and practical reasons. Amongst the carnivores, the cat was shown to express the counterpart of only one human gene, HE1 (Kirchhoff *et al.*, 1990).

Surprisingly, the dog appears to express three homologues of the human epididymally expressed genes, HE1, HE4 and HE5 (Kirchhoff *et al.*, 1990, 1991, 1993). Since canine tissue is conveniently available as the product of routine veterinary castration for non-endocrine related disturbances, this source has been assessed in greater depth for its suitability as a model for human epididymal function.

Materials and methods

Tissues

Canine tissues were obtained from local veterinary practices where dogs were being castrated for non-endocrine, mostly behavioural disturbances. Tissues from sexually mature animals were removed rapidly, the epididymides cleaned of irrelevant tissue and snap-frozen in liquid nitrogen either entire or separated according to gross morphology into caput, corpus and cauda regions. Since the domestic dog shows a wide range of size between breeds, as well as positive organ allometry (Woodall & Johnstone, 1988), preliminary studies involved collecting tissues from a wide variety of breeds and mongrels. Table 1 indicates the weights of the individual epididymides from single animals used.

Table 1. Fresh weights of individual epididymides from different dog breeds

Breed	Epididymal fresh weight (g)			
	Caput	Corpus	Cauda	Total
Chihuahua	0.07	0.12	0.10	0.29
Poodle	ND	ND	ND	0.85
Small mongrel	0.42	0.52	0.88	1.82
Chow Chow	0.55	0.90	1.39	2.84
Beagle	0.70	0.95	1.76	3.41
Alsation halfbred	ND	ND	ND	4.20
Alsation	1.23	1.79	1.54	4.56
Hovaward	1.29	2.51	2.48	6.28
Deutsche Dogge	ND	ND	ND	8.50

Weights refer to individual epididymides from single animals after careful removal of extraneous fat and connective tissue. ND, not determined.

Where tissues were to be used for cell culture, organs were transferred to culture medium (DMEM plus antibiotics only; see below) and transported quickly to the laboratory where they were processed without delay.

RNA preparation and analysis

RNA was extracted into 10–12 volumes of guanidinium isothiocyanate solution as described previously (Chirgwin *et al.*, 1979; Kirchhoff *et al.*, 1990). The high volume : weight ratio was necessary because of the large proportion of DNA within this tissue, in the form of stored spermatozoa, which tends to disturb the subsequent ultracentrifugation step. The RNA extracts were then purified by ultracentrifugation through a 5.7M CsCl cushion (Chirgwin *et al.*, 1979). Then 20 µg of the total RNA thus obtained was electrophoresed in formaldehyde-buffered agarose gels, transferred to a nylon membrane (Hybond N, Amersham-Buchler, Braunschweig,

Germany) and hybridized as described previously (Kirchhoff *et al.*, 1990) to various radioactively labelled probes for the epididymis-specific human genes HE1 through HE5 (Kirchhoff *et al.*, 1990, 1991, 1993; Osterhoff *et al.*, 1994). Even loading was confirmed by UV fluorescence of the ethidium bromide stained ribosomal RNA bands. Probes corresponding either to the full-length human cDNA clones (HE1, HE2, HE4), or to a 500 bp 3' fragment (HE3), or to a 270 bp 5' fragment (HE5), were used as excised plasmid inserts, and labelled to high specific activity ($>5 \times 10^8$ cpm/ μ g) by random primer extension (Feinberg & Vogelstein, 1983) in the presence of [α - ^{32} P]dCTP (Amersham-Buchler).

Construction and analysis of a canine epididymal cDNA library

Pooled RNA extracted from the epididymides of two dogs (a half-bred Alsatian mongrel and a Deutsche Dogge) was enriched in poly(A)-containing mRNA by oligo(dT) affinity chromatography (Aviv & Leder, 1972), and 5 μ g of this mRNA was used to programme cDNA synthesis from an oligo(dT) primer. Double-stranded cDNA was prepared by a modification of the Gubler & Hofmann (1983) procedure, using a commercially available kit (ZAP-cDNA Synthesis Kit, Stratagene, La Jolla, C.A., U.S.A.). This cDNA was then directionally cloned into the bacteriophage Lambda UniZAP-XR (Stratagene), which were used to infect the bacterial strains *E. coli* 'SURE' or XL1-Blue (Stratagene). The library was screened and analysed using standard bacteriophage procedures (Sambrook *et al.*, 1989) and the same ^{32} P-labelled human epididymal cDNA probes (HE1, HE4 and HE5) described above.

Positive plaques were purified by successive dilution, and the inserted cDNA isolated by rescue of the specially encoded phagemid sequence by in-vivo excision (Short *et al.*, 1988). The resulting recombinant plasmid DNA was purified according to standard procedures (Sambrook *et al.*, 1989) and subjected to DNA sequence analysis using the dideoxy termination procedure as modified for double-stranded plasmid DNA (Chen & Seeburg, 1985).

Cell culture

Essentially following previous protocols elaborated for human and rodent epididymal tissue (Klinefelter *et al.*, 1982; Byers *et al.*, 1985; McArdle, 1991), freshly obtained canine epididymides were cleaned carefully of extraneous tissue and macerated with scissors in Dulbecco's Modified Eagle's Medium (DMEM) containing 50 U/ml penicillin and 50 μ g/ml streptomycin (Seromed, Biochrom, Berlin, Germany) only. The resulting tissue fragments were then digested three times for 50 min at 37°C with 0.2% collagenase (Type II) and 20mg/ml DNaseI (both from Sigma, Deisenhofen, Germany). After brief centrifugation the pellet was dispersed in culture medium (DMEM supplemented with 2 mM L-glutamine, 100 ng/ml vitamin A, 200 ng/ml

vitamin E, 5ng/ml selenium dioxide, 5 μ g/ml transferrin, 0.1 μ M testosterone (all from Sigma), 10% fetal calf serum (FCS; Gibco-BRL, Berlin, Germany) and antibiotics (as above). Tissue fragments were plated out in T25 culture flasks and incubated at 33°C in 5% CO₂ / 95% air for the times indicated in the Results section. After culture for 24 h, medium was changed and the FCS replaced by 1 mg/ml bovine serum albumin (BSA, fraction V, cell culture grade; Sigma).

For RNA preparation, medium was removed carefully and cells lysed in 1 ml per culture flask of the chaotropic solution RNA-Clean™ (AGS GmbH, Heidelberg, Germany). Pure RNA was isolated following the protocol supplied with the reagent, which is essentially a modification of the procedure described by Chomczynski & Sacchi (1987), and submitted to northern blot analysis as described above.

In-situ transcript hybridization of primary cultures

Tissue fragments were prepared as above and plated out onto Lab-Tek chamber slides (Nunc, Naperville, I.L., U.S.A.). For the in-situ hybridization, culture medium was removed, cells were washed briefly in PBS, then fixed for 30 min in 4% paraformaldehyde in PBS, followed by a further brief wash in PBS. Cells were then dehydrated in ascending ethanol, followed by 2 min in the xylol-replacement Rotihistol (Roth, Karlsruhe, Germany), and then rehydrated in descending ethanol, post-fixed in 4% paraformaldehyde in PBS and washed briefly in PBS. The slides were then treated for 5 min in 0.1M triethanolamine, pH 8.0, followed by 10 min in 0.5% acetic anhydride in 0.1M triethanolamine, washed in 2 $\times$ SSC for 5 min and finally dried for 30 min at 52°C. Prehybridization, hybridization, stringency washing and autoradiography were exactly as described in Krull *et al.* (1993). As hybridization probes, sense and antisense ^{35} S-labelled cRNA transcripts were prepared by in-vitro transcription from an 800 bp 5' EcoRI-ApaI restriction fragment of the CE1 cDNA subcloned into the pBS-SK transcription vector.

Results

Northern hybridization analysis

The domestic dog is a very heterogeneous species, with a large divergence in size between the smallest and largest breeds (Table 1). Additionally, there is evidence for positive allometry of some organs of the male reproductive system; organ weight being overproportionate to body weight (Woodall & Johnstone, 1988). Since size reflects the balance of growth factors during development of a given breed, and it is possible that growth factors may influence epididymal function, it was important to check whether the breed of dog and size of organ influenced specific epididymal gene expression. This was assessed by measuring the levels of the canine counterparts hybridizing to the human epididymal-

specific gene transcripts HE1, HE4 and HE5 (Fig.1). A constant amount of total RNA (20µg) was loaded for

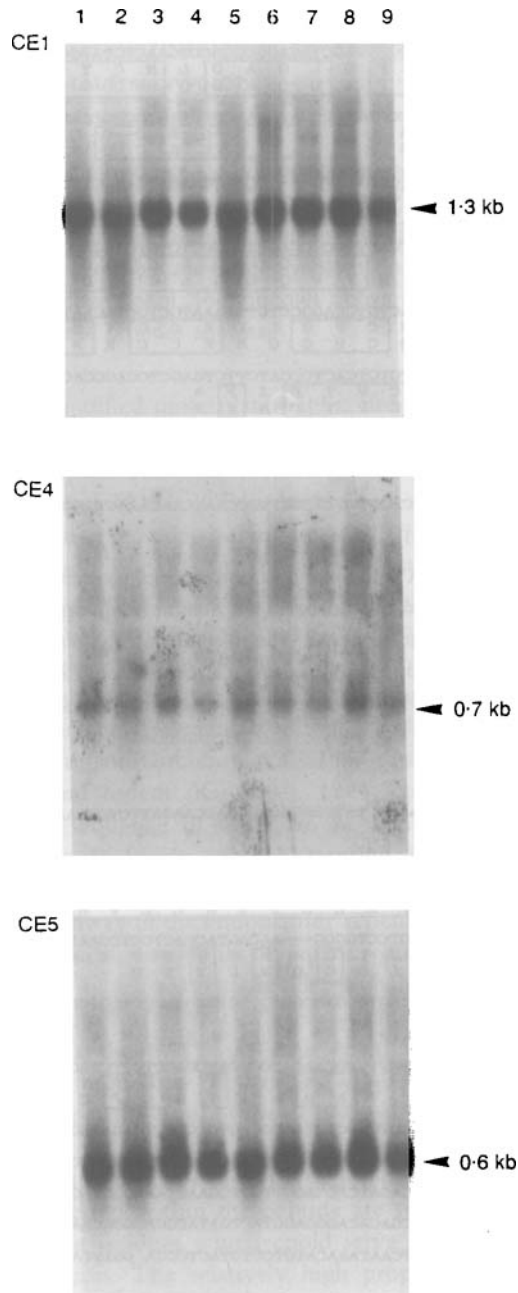


Figure 1. Northern hybridization of total RNA extracted from individual epididymides of different dog breeds. The lane numbers refer to the dogs listed in Table 1. (1) Alsatian halfbred, (2) Deutsche Dogge, (3) Chow Chow, (4) small mongrel, (5) Hovaward, (6) Alsatian, (7) Chihuahua, (8) Beagle, (9) Poodle. All lanes are equally loaded with 20 µg total RNA, excepting lane 9 (10 µg). Blots hybridized as indicated against the human gene probes for HE1, HE4 and HE5 (see Materials and methods), corresponding to the canine mRNAs CE1, CE4 and CE5 respectively, were exposed to autoradiographic film overnight with one intensifying screen.

each individual dog assessed (except for lane 9, 10µg), irrespective of breed and size of epididymis. The specific hybridization signals obtained were very constant for all mRNA species and all breeds of dog. Since the total amount of RNA extracted per epididymis varied according to organ size, this implies that the proportions of the three different gene transcripts, and presumably the cell types expressing them remain constant, independently of how big the organ is. This important finding meant that epididymal tissue can probably be taken from any breed of dog with the expectation of comparable results. Nevertheless, for subsequent studies preference was given to medium-sized, outbred (mongrel) dogs.

The northern hybridization data confirmed further that only for HE1, HE4 and HE5 could homologous gene transcripts be detected. For HE2 and HE3 no positive hybridization was obtained (not shown). In analogy to the human system we refer to the canine homologues of HE1, HE4 and HE5 as CE (Canine Epididymal) 1, 4 and 5, respectively. The sizes of the canine transcripts agreed approximately with those for the corresponding human mRNAs, with CE1 mRNA at 1.3kb (HE1 mRNA is 1.0 kb), CE4 mRNA at 0.7 kb (HE4 mRNA is 0.7 kb) and CE5 mRNA at 0.6 kb (HE5 mRNA is also 0.6 kb).

cDNA cloning and sequence analysis

Although there appears to be a high degree of homology between the canine and human epididymal gene transcripts, hybridization alone is not a sufficient criterion. The possibility remains that the canine homologues may not represent functional transcripts, or may encode proteins with divergent amino acid sequence and hence possibly different function. Therefore, a cDNA library was constructed from the pooled mRNA of two canine epididymides. The complexity of the unamplified library was 2.3 million independent clones. Blue/white plaque selection for functional, i.e. non-recombinant β -galactosidase activity showed that recombinant bacteriophage, with cDNA inserts, represented more than 97% of all clones. Screening of this library for the canine homologues of HE1, HE4 and HE5 yielded a high proportion of positive clones (Table 2). Of several subjected to preliminary restriction analysis, one full-length clone for each gene was selected and sequenced. The full sequences and their alignments with the human homologues

Table 2. Frequency of specific CE and HE epididymal cDNA clones in relation to the total library size

CE1: 3.3%	HE1: 0.5%
CE4: 0.7%	HE4: 0.05%
CE5: 2.6%	HE5: 0.2%

Data obtained by rescreening the unamplified libraries with the specific cDNA probes.

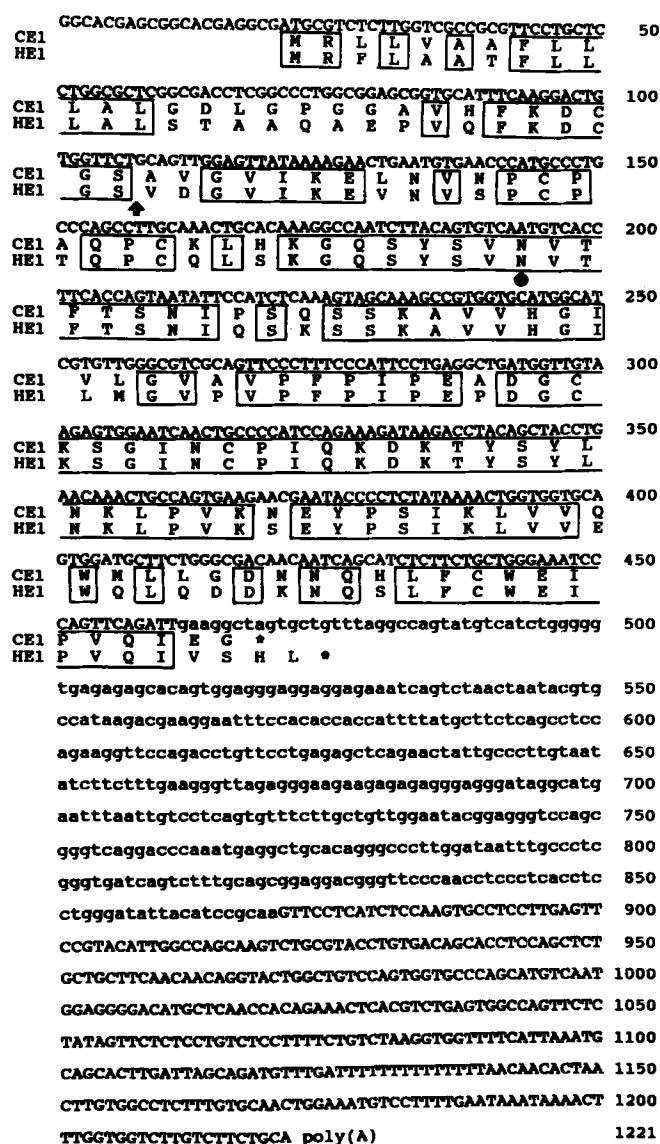


Figure 2. cDNA sequence and corresponding amino acid sequence for CE1 compared to the human homologue HE1. Homologous regions of the amino acid sequences are enclosed in boxes. The putative cleavage site for the removal of the N-terminal signal peptide, following the consensus rules of Von Heijne (1983), is indicated by the arrow. A putative site for N-glycosylation is indicated by the rhombus. The lower case lettering in the 3' non-coding region of the canine cDNA sequence represents a region without an homologous equivalent in the human cDNA sequence.

are shown in Figs 2, 3 and 4 for CE1, CE4 and CE5, respectively.

All of the predicted canine epididymal proteins were furnished with typical N-terminal signal sequences, implying that, like their human homologues, these represent proteins secreted from the epididymal epithelium. Like the HE1 product, the CE1 protein represents a small glycopeptide

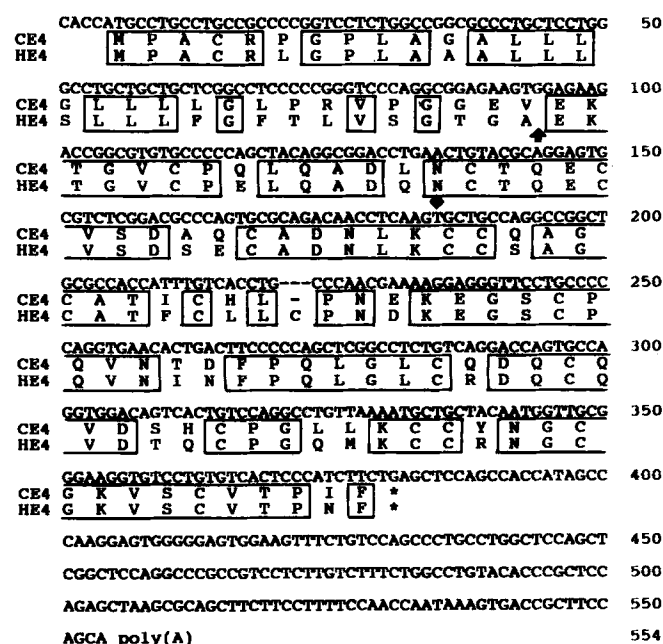


Figure 3. cDNA sequence and corresponding amino acid sequence for CE4 compared to the human homologue HE4. For details see Legend to Fig. 2. The mismatched cysteine in the HE4 sequence corresponding to nucleotides 221–223, though verified at the cDNA level, may represent a polymorphism, and is being checked currently at the genomic level.



Figure 4. cDNA sequence and corresponding amino acid sequence for CE5 compared to the human homologue HE5. For details see Legend to Fig. 2. A second putative cleavage signal to remove the C-terminal signal peptide involved in formation of the GPI-anchor is also indicated by an arrow. The location of this site is based on the homology to the human sequence for which the cleavage site is known. A dashed line has been introduced in the CE5 cDNA sequence at nucleotide 120 to accommodate a supernumerary amino acid in the homologous human sequence. Similarly, dashes have been introduced into the human amino acid sequence to optimize alignment of the sequences.

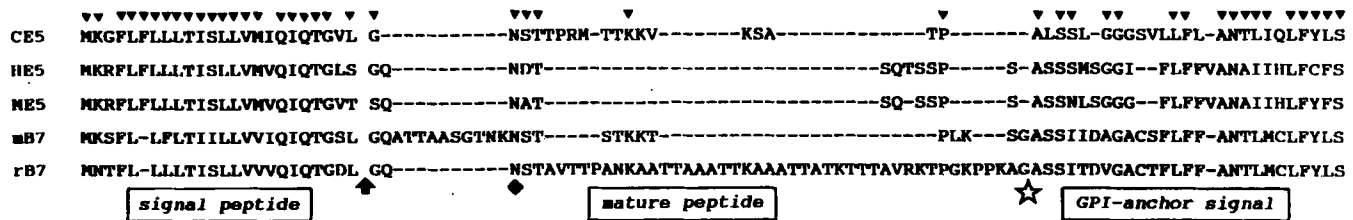


Figure 5. Comparison of the primary amino acid sequences for the precursor polypeptides corresponding to CE5 and its homologues in different species. CE5, canine; HE5, human; ME5, macaque monkey; mB7, mouse; rB7, rat. Dashed lines have been introduced to optimize the alignment of the sequences. The arrow indicates the putative cleavage site for the N-terminal signal sequence. The rhombus marks the probable site for N-glycosylation. The star marks the region of attachment of the putative GPI-anchor after removal of the C-terminal anchor signal. The triangles above the CE5 sequence highlight highly conserved amino acids which appear in equivalent positions in three or more species.

with an unmodified molecular weight, after removal of the signal peptide, of approximately 12 kDa. Thus, *in vivo*, this would be expected to give rise to a glycosylated protein of between 15 and 20 kDa. CE4 is highly homologous to HE4 (for the mature peptide, homology is 81.9% at the amino acid level) and has the same multiple cysteine structure, divided into two repeated WAP (whey acidic protein) domains, which places these molecules amongst the family of extracellular protease inhibitors (Kirchhoff *et al.*, 1991). Taking account of the glycosylation, this product also might exhibit a molecular weight of about 15–20 kDa *in vivo*.

Like its human (Kirchhoff *et al.*, 1993), monkey (Perry *et al.*, 1992) and rodent (Kirchhoff, 1994) counterparts, the amino acid sequence of CE5 can be divided into three domains (Fig. 5). Firstly, at the N-terminus is a highly conserved sequence suitable as a signal peptide for entering the secretory pathway of the endoplasmic reticulum. This is followed by a small, but highly divergent region, representing the sequence of the mature peptide, probably expressed on the cell surface (Xia *et al.*, 1993). However, there is very little similarity at the level of the primary amino acid sequence (Fig. 5). The homology between the different species rather appears to be at the level of the post-translational modifications. There is in all species a typical site for N-glycosylation (Asn-X-Thr) near the N-terminal region of the mature peptide. Interspersed within the peptide are several threonine and/or serine residues which could serve as substrates for O-glycosylation. The relatively high proportion of these residues suggests that the CE5 molecule, like its counterparts in the other species, might present an extended, highly glycosylated, mucin-like structure projecting from the membrane as part of the glycocalyx. Binding to the cell surface appears to be the function of the third, conserved domain at the C-terminus. In all species this has the characteristics of a signal peptide for the appendage of a hydrophobic glycosylphosphatidyl-inositol (GPI)-anchor to the C-terminus of the mature peptide region. The precise point of cleavage is only known with certainty for the human lymphocyte form of HE5 (CDw52) (Xia *et al.*, 1991, 1993). In the human, rat and mouse, it has been suggested that the homologues use

this GPI-anchor to become inserted into the sperm membrane (Hale *et al.*, 1993; Kirchhoff, 1994).

Epididymal cell culture

In the presence of testosterone, canine epididymal cells appeared to survive well in primary culture. Microscopic examination showed that starting from small clumps of cells a relatively homogeneous monolayer grew out from these tissue fragments (Fig. 6), as has been described previously for the rat (Klinefelter *et al.*, 1982), and with medium change every 1–2 days, can survive in culture up to 21 days. Within the monolayer, a few cells appeared to retain some ciliary activity.

Using the cDNA clones for CE1, CE4 and CE5 as indicators of specific epididymal function, primary cultures of canine epididymal cells were assessed for the maintenance of cell-type specific gene expression. Northern hybridization of RNA collected at day 5 of culture showed that all three gene transcripts, for CE1, CE4 and CE5 were still being expressed at high levels (Fig. 7). The sizes of these mRNAs extracted from the cultured cells were identical to those from intact tissue. In order to assess which cells might be responsible for the specific hybridization signals obtained with the northern blots, *in-situ* transcript hybridization of the cultured cells was performed using sense and anti-sense cRNA probes specific for CE1. Both monolayers and source tissue clumps gave clear and confluent positive signals with the antisense strand but not with the control sense strand (Fig. 8).

Discussion

Three out of five tissue-specifically expressed human epididymal genes were expressed abundantly in the dog epididymis (Fig. 1). Based on these findings, the dog is probably amongst the best species to use as a potential model of human epididymal function. In the accompanying article (Pera *et al.*, 1994), we also show, using *in-situ* transcript hybridization, that the regional specialization of this gene expression in the dog epididymis is very similar to the

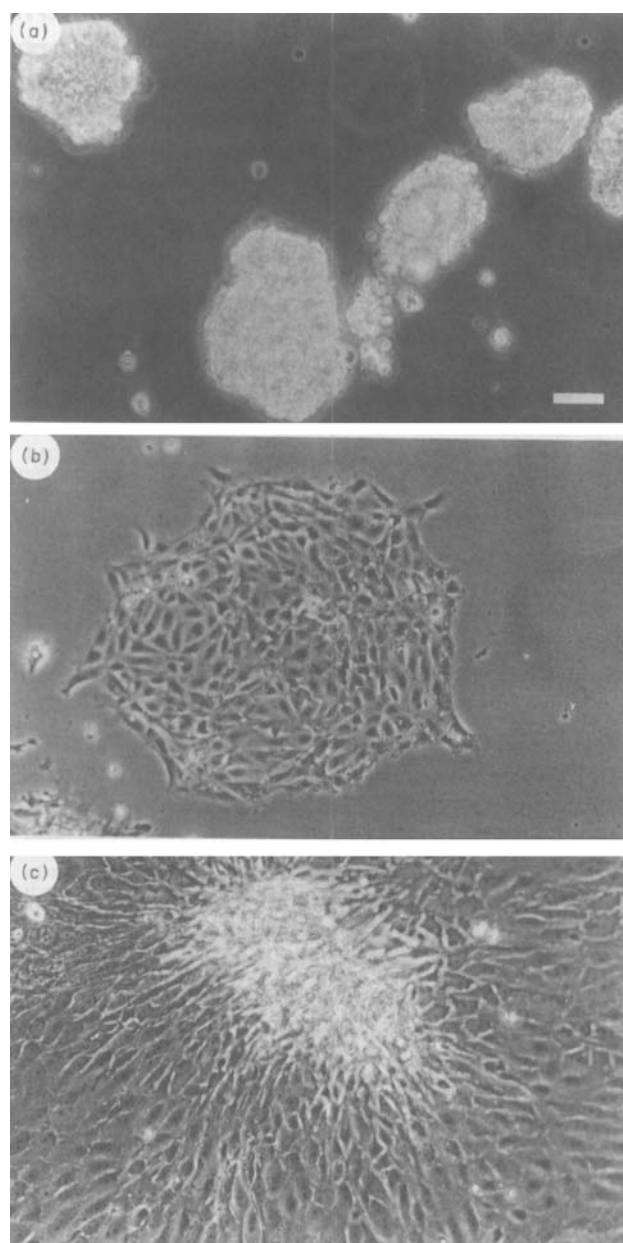


Figure 6. Development with time of canine epididymal tissue cultures. (a) Epididymal fragments, from a Boxer dog, as plated out on day 0; (b) typical monolayer established from a similar small fragment on day 2; (c) extensive monolayer of cells still linked to a larger epididymal fragment on day 5. Scale bar = 100 µm; all panels photographed at the same magnification.

pattern observed in the human. Not only are these genes expressed, but deduced from their frequency as cDNA clones, the corresponding mRNAs are expressed at relative levels almost ten-fold greater than in the human (Table 2), albeit in the same relative proportion to one another. A reason for the high level of expression may relate to the increased functional level of the canine epididymis. This

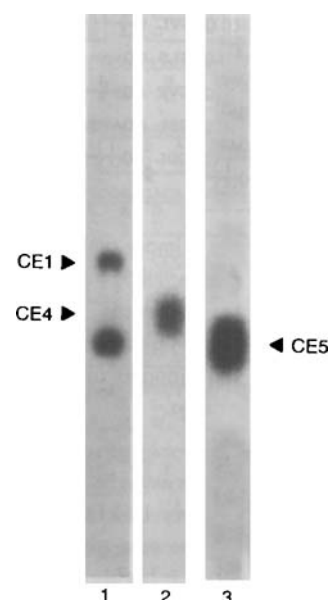


Figure 7. Northern hybridization of RNA extracted from a single T25 flask of the canine epididymal tissue culture prepared from a Poodle as described in Materials and methods and cultured for 5 days. Five micrograms of total RNA were loaded per lane. Lane 1 represents the rehybridization with a probe for CE1 of the blot used in Lane 3, without intermediate removal of the old probe (CE5). Lane 2, is an independent hybridization vs. a CE4 probe. Lane 3, is the first hybridization of the blot used in Lane 1 vs. the CE5 probe. The sizes of the transcripts in the cell cultures corresponded exactly with those for the in-vivo gene transcripts (Fig. 1).

species has no seminal vesicle, and thus a larger contribution of seminal fluid secretions has to be made by the epididymis. Also, ejaculate volumes as well as total sperm numbers are each about ten-fold higher in the dog than in the human (Setchell *et al.*, 1994). Further, it may be relevant that all data on levels of gene expression in the human come from tissues obtained from elderly patients (Kirchhoff *et al.*, 1990); younger subjects may exhibit different levels of epididymal gene expression.

A comparison between different breeds of dog showed that it did not matter greatly from which breed of dog tissue was obtained. The levels of gene expression remained the same for a given amount of RNA and hence weight of tissue. Also, the relative mRNA levels between CE1:CE4:CE5 were similar to those between HE1:HE4:HE5 (Table 2), and hence the relative functional importance of these gene products is probably similar. Taken together with the high absolute levels of gene expression observed, and the relative ease and ethical acceptability of obtaining dog tissue, this suggests that the canine epididymis could be a highly appropriate model in which to examine the role of this tissue in sperm maturation in a fashion which is relevant to the human.

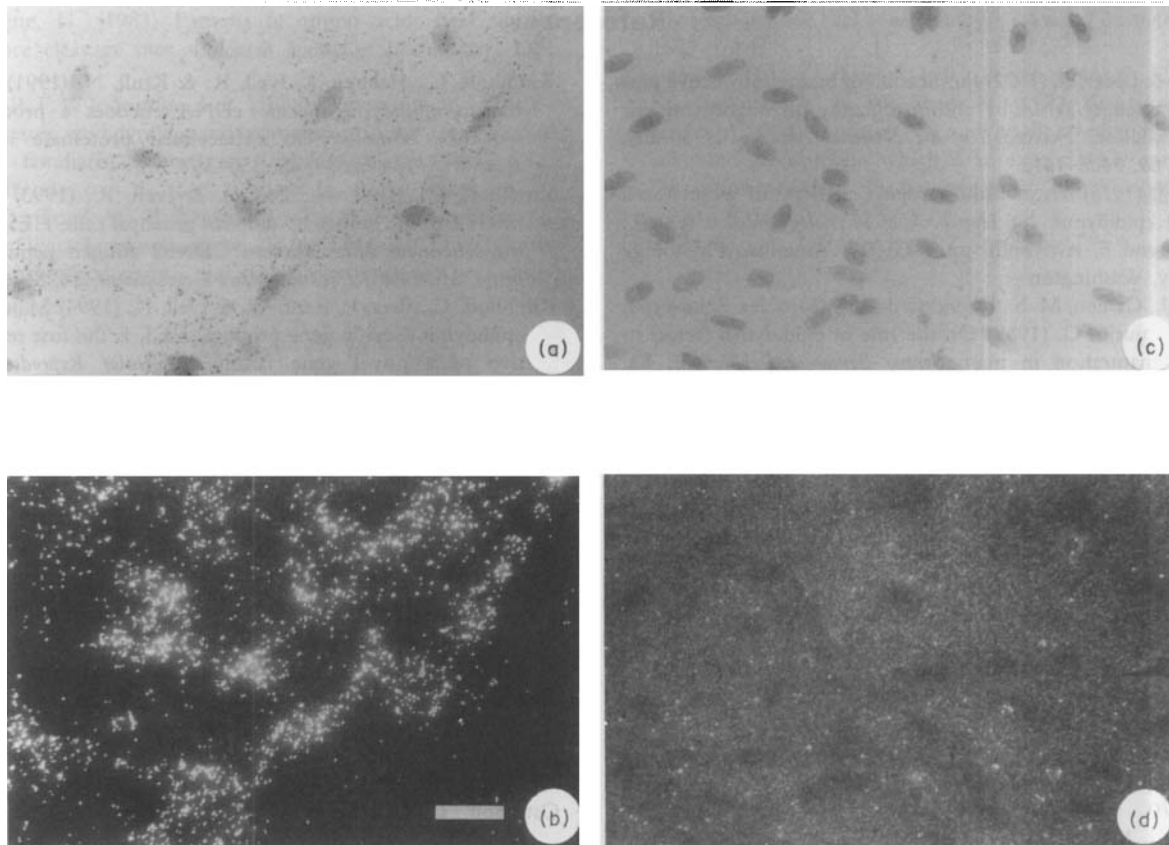


Figure 8. In-situ transcript hybridization for CE1 mRNA in a day 5 culture of canine epididymal cells, obtained from a Doberman half-bred dog. Panels A and B were hybridized with the anti-sense strand cRNA probe, panels C and D with the control sense strand cRNA. Panels A and C are viewed in brightfield transmitted light to identify the extent of the monolayer in the field of view. Panels B and D are the equivalent darkfield reflectance images to emphasize silver grains resulting from positive hybridization signals. Scale bar = 35 μ m; all panels were photographed at the same magnification. Cell nuclei were counterstained with Mayer's haemalum.

The high levels of gene expression *in vivo* appear to be maintained in cultured cells, with all three canine genes analysed still being expressed after 5 days in culture. Although the cultured cells lost the typical morphological appearance of a tall columnar epithelium, in-situ hybridization showed that the flattened cells of the new monolayer were still expressing the epididymal marker gene CE1, indicative of an epididymal, epithelial phenotype. In some experiments mRNA expression was still detectable after 21 days of culture (not shown). The culture medium chosen in these preliminary studies was sufficient for culture survival and maintenance of specific gene expression. Future, more extensive investigations will aim to determine which factors are essential or optimal for expression of the epididymal phenotype, and how this expression is regulated.

The ready availability of canine epididymal tissue from routine veterinary castration also means that a culture system is now available to assess not only the regulation of epididymal physiology, but also sperm-epididymis interactions, and

the pathological/pharmacological effects of substances with potential influence on male fertility.

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