Ultrastructural validation of an improved culture system for boar efferent duct epithelium

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A tissue culture system in which cells retain defined ultrastructural and functional characteristics was established to provide a basis for functional investigations of the efferent ductules in boars. A currently used culture protocol for rat epididymal epithelium was used as a starting point and was subsequently modified because of unsatisfactory results. Epithelial plaques were isolated by mechanical uncoiling of the ductules and two sequential enzymatic digestion steps. Plaques were seeded onto extracellular matrix-coated permeable membranes and maintained in a two-chamber system. Samples taken before seeding and after 7 days in culture were processed for transmission and scanning electron microscopy. Perfusion-fixed material from earlier studies served as a reference to assess ultrastructural preservation. In addition, endocytotic activity was investigated by adding cationized ferritin to the culture medium on day 8 before fixation. At the end of the disaggregation procedure, cells were cuboidal, while cilia, microvilli and cell organelles were well preserved. After 7 days in culture, three types of cell formation were observed: cysts, pseudotubules and epithelial sheets. Cell sheets were made up of closely juxtaposed cells bearing motile kinocilia and exhibiting well-developed polar differentiation, as judged from the localization of cell junctions and organelles. Although it did not return to the values of native material, cell height was greater than that of cells grown according to the pre-existing protocol. Furthermore, preferential uptake of ferritin by principal cells after 7 days in culture was demonstrated. Preservation of these fundamental characteristics of the *in vivo* state corroborates that our *in vitro* system will furnish reliable information.

Introduction

Cell culture is a powerful tool for the study of cell functions, metabolic activities and their regulation (Freshney, 1987, 1992). Consequently, important insights into the process of sperm maturation have come from *in vitro* studies conducted on human and rat epididymal tissue (Tézon and Blaquier, 1981; Brown and Amann, 1984; Cooper *et al.*, 1989; Klinefelter *et al.*, 1992; Moore *et al.*, 1992; Wong *et al.*, 1992; Huang *et al.*, 1994). Moreover, cell culture systems may be the only possible approach in animals too large for specific *in vivo* experiments, such as autoradiographic studies. Yet few reports on cell culture of epididymal epithelia from domestic mammals are available (Wagley *et al.*, 1984; Joshi, 1985). This prompted us to develop a culture system for boar efferent duct epithelial fragments in view of intended studies on functional aspects of sperm maturation in this species.

The most recent protocols for the culture of epididymal tissue of laboratory rodents and humans have used epithelial cell aggregates generally obtained from the epididymal duct (Cooper *et al.*, 1989; Byers *et al.*, 1992; Klinefelter *et al.*, 1992; Moore *et al.*, 1992) and, in a few instances, from the efferent

ductules (Byers *et al.*, 1985a; Raczek *et al.*, 1992). Epithelial fragments are prepared by microdissection and enzymatic digestion of tubule segments. These epithelial plaques are seeded onto the extracellular matrix-coated permeable membrane of commercially available inserts. Immersion of these inner chambers in larger wells yields a two chamber system with basically separate basal and luminal compartments. This is the best approach so far to maintain some degree of histotypic organization and polar differentiation of epithelial cells in culture. However, all tissue culture systems have inherent limitations, and extrapolation of results from *in vitro* studies to the *in vivo* state requires careful assessment of structural and functional integrity. To validate our *in vitro* system, we examined its ability to preserve native ultrastructural characteristics. In addition, we wanted to procure evidence of a known vectorial function. Therefore, ultrastructure of cells before seeding and after 7 days in culture was systematically compared with perfusion-fixed tissue (Stoffel *et al.*, 1991; Stoffel and Friess, 1994) and endocytotic activity was corroborated by means of ferritin uptake.

Protocols currently used for rat epididymal epithelium (Klinefelter *et al.*, 1992) yielded unsatisfactory results with respect to morphological preservation of boar efferent duct cells *in vitro*. Therefore, we carefully optimized the disaggregation steps, composition of both culture media and

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extracellular matrix, and plating density until our ultrastructural and functional demands were fulfilled. The protocol presented here provides close approximation of native structural and functional properties after 7 days in culture and thus is suited to yield relevant and reliable information.

Materials and Methods

Animals

Epididymides from mature large white boars, *Sus scrofa* f. dom. (approximately 110 kg body mass), were obtained at castration.

Culture media

Modified Hank's balanced salt solution (HBSS; 100 ml; Sigma, St Louis, MO) was supplemented as follows: 0.20 g BSA (Sigma); 0.42 g Hepes (Sigma); 0.09 g D-glucose (Sigma); 2.5 mg soybean trypsin inhibitor (STI; Sigma); 1.0 ml MEM-sodium pyruvate 100 mmol l⁻¹ (Gibco, New York, NY), pH 7.4.

Minimum Essential Medium (MEM) Earles Salts D-Val (500 ml; Gibco) was supplemented as follows: 5.0 ml MEM-sodium pyruvate 100 mmol l⁻¹ (Gibco); 5.0 ml MEM-non-essential-amino-acids 100 × (Gibco); 292 mg L-glutamin 100 × (Gibco); 0.5 g BSA; 0.1 g L-carnitine (Sigma); 2.5 ml gentamycin sulfate (Sigma); 2.5 mg transferrin (Sigma); 2.5 mg insulin (Sigma); 0.18 mg hydrocortisone (Sigma); 0.14 mg testosterone (Fluka, Buchs); 0.15 mg dihydrotestosterone (Fluka); 50 µg retinol acetate (Sigma); 2.503 g Hepes (Sigma). Ten per cent (v/v) fetal calf serum (FCS; Biological Industries, Bet Haemek, Israel) was added and the medium was adjusted to pH 7.4.

All media were filter sterilized (Stericups GV5 from Millipore, Bedford, MA).

Extracellular matrix, cell substrate and culture plates

Inserts, 12 mm in diameter (Millipore Millicell CM PICM01250) were used as inner chambers. Before use, the permeable membranes were coated with 50 µl cm⁻² of a commercially available extracellular matrix (Basement Membrane Matrigel[®] from Becton Dickinson, Bedford, MA) diluted 1:7 with supplemented MEM (Byers *et al.*, 1992). Coated inserts were placed in 12-well cell culture clusters (Costar, Cambridge, MA) and dried for 30 min in an incubator at 34°C.

Disaggregation and culture of epithelial fragments

The following disaggregation and culture procedures were modified from protocols published by Byers *et al.* (1985b) and Klinefelter *et al.* (1992).

Immediately after castration, the head of each epididymis was transferred to sterile HBSS and the efferent ducts were carefully uncoiled with microsurgical instruments. A stereomicroscope was used for visual control to avoid damage to the

tubules and the HBSS was changed several times. Uncoiled ductules were cut into fragments of 5–10 mm, transferred to supplemented HBSS containing 300 U collagenase ml⁻¹ (Worthington, Freehold, NJ), and incubated in a water bath at 34°C with a shaker set at 120 cycles min⁻¹. Digestion was discontinued as soon as fragments appeared as fine, transparent tubules, after 60–75 min. After vigorous pipetting of the sediment, tubule fragments were allowed to settle. The supernatant was discarded and the pellet was resuspended and pipetted with supplemented MEM containing 150 U collagenase ml⁻¹. After 30 min, the size of the cell plaques was assessed every 10 min under a microscope and disaggregation was stopped as soon as fragments had reached 200–400 µm in diameter. Before seeding, a semi-quantitative approach (Raczek *et al.*, 1992) was used to calibrate the number of cell plaques per insert. A pellet volume of 0.0125 ml, diluted in 0.5 ml supplemented MEM, was used per insert and this resulted in optimal cell density. Fluid level was brought to 5 mm by adding 1.9 ml supplemented MEM to the outer chamber. Medium was changed after the first 24 h and then every 48 h. Ciliary beat, and attachment and spreading of the epithelial sheets were surveyed daily by light microscopy. The extent of the surface covered by viable cells was determined by performing a vital staining with 0.1% (w/v) acridine orange in supplemented HBSS on day 8. Epifluorescence (excitation: pass band filter 450–490 nm; emission: long wave pass band filter 520 nm) and transillumination micrographs (Zeiss Axiophot) of identical areas were compared and estimates of covered and uncovered surface areas were determined by use of an overlay grid.

Ferritin uptake

After 7 days in culture, media were removed from both chambers. After emptying the outer chamber, medium from the inner chamber was allowed to drain through the permeable membrane for 30 min. If required, remaining medium was drawn off carefully. Grown cells were gently washed with supplemented HBSS. Supplemented HBSS containing 5 mg cationized ferritin ml⁻¹ (Sigma) was then added. After incubation for 10 min at 34°C, inserts were again washed as above and cells were fixed immediately (see below).

Fixation and processing for electron microscopy

Cells were fixed by immersion in diluted Karnovsky's fixative (1% (w/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde in 0.1 mol cacodylate buffer l⁻¹ with 2 mmol CaCl₂ l⁻¹; pH 7.4; 4°C; 1 h). Tissue was then postfixed with 1% (w/v) OsO₄ in cacodylate buffer for 1 h at 4°C, dehydrated through an ascending ethanol series, and embedded for transmission electron microscopy (TEM) in an Epon-Araldite mixture according to standard protocols. Sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109. After dehydration, samples selected for scanning electron microscopy (SEM) were critical point dried (Balzers CPD 030), mounted on stubs with conducting carbon, sputter-coated with 20 nm of gold (Balzers SCD 004) and examined in a Philips PSEM 500 scanning electron microscope.

Fig. 1. Cell plaques prior to seeding: (a) scanning electron micrograph of the apical side, exhibiting well preserved microvilli (MV) and kinocilia (KC); (b) scanning electron micrograph of the basal side, showing that epithelial cells are freed of peritubular elements; (c) transmission electron micrograph showing principal cells (PC) and ciliated cells (CC) as well as indentations of nuclear membranes (N). Scale bars represent 5 μ m.

Results

Morphology of cell plaques

The disaggregation protocol yielded epithelial plaques of about 200–400 μ m in diameter and some single cells. Breakdown of efferent ductules was complete since tubular structures were never observed in samples collected before seeding. Examination of cell plaques fixed at the end of the disaggregation protocol (Fig. 1) revealed a substantial decrease

in cell height to approximately 25% of that of perfusion-fixed epithelium. Both principal and ciliated cells were cuboidal but still contained the normal complement of organelles. However, nuclear shape seemed less regular than in native tissue owing to indentations of the nuclear envelope. The apical plasma membrane was typical, the principal cells forming a cobblestone-like pattern which was interrupted by tufts of kinocilia. Examination of the basal side of the cell plaques revealed a complete absence of peritubular elements.

Fig. 2. Spreading of epithelial fragments: (a) semithin section through epithelial sheet (ES) with attached cyst (CY); (b) epifluorescence image of cultured tissue adhering to filter membrane on day 8, vital staining with 0.1% acridine orange: fluorescence clearly reveals epithelial sheets (ES), three-dimensional formations (arrows), and a few bare filter membrane areas (asterisks); (c) transillumination micrograph of identical area as (b): though less distinctly, epithelial sheets (ES) and three-dimensional formations (arrows) can nonetheless be discerned. Scale bars represent 100 μm .

Attachment and spreading of cell plaques

Attachment of cell plaques to the extracellular matrix was complete after 24 h in culture. It was followed by outgrowth from the borders of the original fragments and by local three-dimensional reorganization of the cell aggregates (see

below). About 65% of the surface area was occupied by epithelial sheets, while three-dimensional formations took up some 25% (Fig. 2). By day 8, filter membranes were, therefore, covered to approximately 90%, that is, confluence was not complete. No evidence of mitosis was detected by either light or transmission electron microscopy.

Fig. 3. Transmission electron micrographs revealing spatial organization of epithelial cells after 7 days in culture: (a) cyst: the large lumen (LU) is delimited by the basal side of the cells, while the apical side, bearing microvilli (MV), faces outwards; (b) pseudotubule: the lumen (LU) is bounded by the apical membrane of the cell, while the basal plasmalemma rests on the matrix-coated permeable support (M); (c) epithelial sheet made up of polarized principal cells (PC) and ciliated cells (CC) which rest on the matrix-coated permeable membrane (M). Scale bars represent 5 μ m.

Types of cell aggregate

After 7 days in culture, three types of cell aggregate could be distinguished (Figs 2a and 3). Three-dimensional formations were designated as cysts when the lumen enclosed was bounded by the basal plasmalemma (Figs 2a and 3a), and as pseudotubules when the lumen was delimited by the apical cell membranes (Fig. 3b). Hemicysts and tubule-like structures became apparent after 3 days in culture. Between cysts and pseudotubules, large areas were covered by polarized epithelial sheets resting on the extracellular matrix with their basal plasmalemma. While these sheets typically were made of monolayers, overlapping of epithelial cells in two or three layers was also encountered in some areas. Lateral plasma

membranes were closely apposed over wide areas, whereas the apical plasmalemma bore microvilli and kinocilia, respectively. Cells were cuboidal and reached about 25–33% of the epithelial height of native tissue (Fig. 3c). They contained a typical complement of cell organelles. No ultrastructural evidence of other cell types (fibroblasts, smooth muscle cells) was found.

Principal cells and ciliated cells in epithelial sheets

Principal cells and ciliated cells are shown (Fig. 4). Plasma-lemma was continuous in both cell types. Principal cells showed apical pits between well preserved microvilli, while

Fig. 4. Transmission electron micrographs of epithelial sheets after 7 days in culture: (a) principal cell (PC) containing numerous vesicles in apical region and ciliated cell (CC) with typical complement of organelles; (b) desmosomes connecting a principal (PC) and a ciliated cell (CC); (c) supranuclear region of adjacent principal cells showing a desmosome (D), well-preserved mitochondria (M), Golgi apparatus (G), endoplasmic reticulum (ER), and a large vesicle (V); (d) unstained section of a principal cell with membrane-bound and endocytosed ferritin. Scale bars represent 0.5 μ m.

ciliated cells exhibited microvilli and kinocilia. Lateral plasma membranes displayed elaborate interdigitations and were connected to neighbouring cells by desmosomes and tight junctions. Nuclei were oval with prominent nucleoli. Both outer and inner membranes of cristae-type mitochondria were intact. Cisternae of smooth and rough endoplasmic reticulum

were distributed over the cytoplasm, while Golgi complexes were found in their typical supranuclear location. As in native tissue, Golgi complexes were conspicuous in principal cells but small in ciliated cells. Vesicles and lysosomes were particularly numerous in the apical cytoplasm of principal cells. Cytoskeletal elements were also detected.

Endocytosis

Ferritin was preferentially bound and endocytosed by principal cells (Fig. 4d).

Discussion

Although *in vitro* systems have been widely used for the study of epididymal functions, only a limited number of reports have included detailed morphological data (Wagley *et al.*, 1984; Byers *et al.*, 1985a, b; Joshi, 1985; Cooper *et al.*, 1989; Raczek *et al.*, 1992). We consider ultrastructural validation of the culture system to be critical with respect to both the identification of the cell types present and to the status of polar differentiation, especially in highly specialized epithelia. Evidence of morphological resemblance between cultured and native cells is, therefore, a prerequisite to any conclusions inferring *in vivo* functions from *in vitro* experiments.

Vital staining of cultured cells on day 8 revealed that 10% of the filter membranes remained devoid of viable cells and that confluence, therefore, was not complete. However, beginning on day 2, cell plaques expanded on the filter membrane, thereby generating epithelial sheets which eventually covered 65% of the available surface area. Since no evidence of mitosis was ever found by light or electron microscopy, it must be assumed that spreading of epithelial sheets is due to a spatial reorganization of the cells brought into culture.

Ultrastructural observations in the study reported here show that fibrocytes were successfully precluded by careful design of the disaggregation steps and by excluding L-valine from the culture media (Gilbert and Migeon, 1975; Olson *et al.*, 1983). The reduced epithelial height of cultured cells as compared with native tissue (Stoffel *et al.*, 1991; Stoffel and Friess, 1994) remains a disturbing drawback. However, this is the first report to our knowledge that shows that epithelial plaques take on a more cuboidal appearance after disaggregation, that is before plating. Since the cells at the boundaries of the plaques were particularly low, it seems that the loss of tubular organization contributes to this alteration. The culture system itself, therefore, did not lead to this change but did fail to provide an environment in which cells would return to their original shape. Pseudotubules and cysts were also lined by cuboidal cells, casting some doubt on whether three-dimensional reorganization in culture helps in restoring columnar cell shape.

Despite this alteration in epithelial height, polar differentiation was maintained as shown by the proper orientation of the apical cell membrane as well as by the location of cellular junctions and organelles. In an earlier study (Stoffel and Friess, 1994), we interpreted interdigitations of the lateral plasma membrane within a slightly extended intercellular space as evidence of water transport through the epithelium. Adjacent cells in culture exhibit a very similar interlocking pattern of their plasmalemma. Taken together, these findings support the view that vectorial metabolic pathways are intact.

Besides morphological evidence of kinocilia, ciliary beat after 7 days in culture could still be observed by light microscopy.

Ferritin is a tracer for adsorptive endocytosis. Experiments *in vivo* revealed that ferritin binds almost exclusively to the

apical plasma membrane of principal cells and that these cells are involved in its endocytosis (Stoffel and Friess, 1994). Demonstration of preferential uptake of ferritin by principal cells *in vitro*, therefore, is an indication that normal cellular activity was maintained after 7 days in culture.

To achieve satisfactory preservation of ultrastructural and functional characteristics of boar efferent duct epithelium in culture, protocols currently used for rat epididymal epithelia (Klinefelter *et al.*, 1992) required some adjustments. These included ensuring an adequate and equal size of the plaques to be seeded (200–400 μm), achieving an optimal cell concentration per insert by means of a semi-quantitative determination of the tissue volume used in culture, reducing the thickness of the extracellular matrix to about 1 μm and modifying the composition of the culture media (major changes included lower concentrations of hydrocortisone, testosterone, and dihydrotestosterone, exclusion of L-valine, and the addition of retinoic acid).

Collectively, these results show that species and tissue differences must be taken into account when establishing an *in vitro* system and that refining culture parameters may lead to substantial improvements. Although further efforts are needed to attain the goal of full morphological congruity between native and cultured epithelium, we consider that boar efferent duct cells have adequately maintained their fundamental ultrastructural and functional characteristics in our culture system to make it trustworthy for functional investigations. We, therefore, expect this technique to be of value in the effort to unravel the sperm maturation process in this domestic species.

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