Polarized functions and permeability properties of rat epididymal epithelial cells *in vitro*

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Summary. Cultured rat caput and cauda epididymal epithelial cells are shown to exhibit polarized properties characteristic of functioning epithelia. When grown on plastic substrates coated with reconstituted basement membrane, confluent monolayers of cells from both regions formed domes characteristic of other transporting epithelia. Immunocytochemical localization of three proteins characteristically associated with epithelial junctional complexes revealed that uvomorulin, zonula occludens 1 and cingulin were present in cultured epididymal epithelial cells and that their distribution was similar to that in the epididymal epithelium *in vivo*. These three molecules were not found in epididymal stromal cells. Cells from both regions growing in two compartment chambers developed an electrical resistance across the monolayer with a magnitude characteristic of high resistance epithelia. The optimal plating density of cells was 0.75×10^6 cells cm^{-2} . The presence of reconstituted basement membrane on the filters did not affect the resistance of the cells. Inulin passage from basal to apical chambers was less than 2% over 24 h. These results show that several polarized functions of epididymal epithelial cells can be maintained in culture and that this type of culture system is useful for studying the function of the epididymis *in vitro*.

Keywords: epididymis; cell culture; permeability; rat; uvomorulin; zonula occludens 1; cingulin

Introduction

Immature spermatozoa leaving the testis acquire the ability to become motile and to fertilize eggs during transit through the epididymis (Bedford, 1975; Cooper, 1986). Although the epididymal epithelium is probably essential for sperm maturation, its precise role is largely unknown. Proteins are secreted into the epididymal lumen by the epithelial cells (Lea *et al.*, 1978; Brooks & Higgins, 1980; Jones *et al.*, 1980; Klinefelter & Hamilton, 1985), which also take up other proteins from the lumen (Attramadal *et al.*, 1981; Pelliniemi *et al.*, 1981; Djakiew *et al.*, 1984, 1985). Some molecules from the systemic circulation are excluded by the epithelium, whereas others are taken up and even concentrated (Hinton *et al.*, 1979; Hinton & Howards, 1981, 1982; Hoffer & Hinton, 1984). To learn more about the functions of the epididymis, a number of research groups have isolated and cultured these cells (Kierszenbaum *et al.*, 1981; Klinefelter *et al.*, 1982; Olson *et al.*, 1983; Byers *et al.*, 1985).

A common criticism of experiments carried out with isolated and cultured cells is that the results obtained may not reflect the function of these cells *in vivo*. In epithelial cells in general, and epididymal epithelial cells in particular, maintenance of the distinct polarity of the cell is certainly required for fully differentiated function. *In vivo*, the epididymal epithelial cells are highly polarized

(Hamilton, 1975). Tight junctions near the apical surface divide the membrane into two distinct regions. The apical plasma membrane communicates directly with the luminal environment and is presumably intimately involved in both secretion and uptake of specific proteins and other molecules directly involved in sperm maturation. The basolateral membrane is exposed to the systemic circulation (albeit modified by peritubular cells and extracellular matrix) and is probably involved largely in the uptake of nutrients. A third and particularly important function of the epithelium is to protect the maturing spermatozoa from the immune system.

To become polarized *in vitro*, epithelial cells must assemble and maintain junctional complexes. Molecules associated with the zonula occludens and zonula adherens junctions characteristic of epithelial cells *in vivo* must therefore be expressed *in vitro*. Uvomorulin, a member of the cadherin class of cell adhesion molecules (Gumbiner *et al.*, 1988) is responsible, at least in part, for the initiation and maintenance of adhesive interactions among epithelial cells. It is also thought that uvomorulin-mediated cell adhesion is a prerequisite for the assembly of tight junctional complexes (Gumbiner *et al.*, 1988). Zonula occludens 1 and cingulin are peripheral membrane proteins associated with the cytoplasmic surfaces of occluding junctions in epithelial cells (Stevenson *et al.*, 1986; Citi *et al.*, 1988; Byers *et al.*, 1991). These three molecules must not only be expressed *in vitro* but also be organized appropriately for junctional complex assembly and the resulting polarization of the epithelial cells. In other fluid-transporting epithelial cells that have successfully assembled junctional complexes, functional polarity *in vitro* is expressed as an accumulation of fluid beneath the monolayer and the formation of hemicysts or domes (Lever, 1985).

Conventional cell culture systems do not permit investigations of the polarized functions of epithelial cells. We have shown that polarized monolayers of epididymal epithelial cells and Sertoli cells can be maintained in dual environment culture chambers (Byers *et al.*, 1986). This report shows that, as well as having a polarized morphology, epididymal epithelial cells *in vitro* express and appropriately organize uvomorulin, zonula occludens 1 and cingulin, and form hemicysts. We also show that cells growing in dual compartment chambers establish a permeability barrier and an electrical resistance characteristic of high resistance epithelia.

Materials and Methods

Cell isolation and culture

Epididymal epithelial cells were isolated from either the caput-corpus or cauda epididymidis of 40–50-day old rats. The epididymides were divided at the isthmus of the corpus epididymidis. After a single collagenase (GIBCO) digestion for 60–90 min (Byers *et al.*, 1985), free tubule fragments were incubated with 0.2% pronase (Sigma, P5147) in Hank's balanced salt solution free of calcium and magnesium to release sheets of epithelial cells (Olson *et al.*, 1983). This isolation procedure differs from that described earlier (Byers *et al.*, 1985) as it was necessary to reduce the size of the fragments of epithelium plated to get a more even monolayer than could be obtained using two collagenase digestions. Epididymal stromal cells were isolated as described previously (Byers *et al.*, 1985). Cells were plated at a density of $0.5\text{--}2.0 \times 10^6$ cells cm^{-2} on Millicell HA culture chambers (Millipore Corporation, Bedford, MA, USA) impregnated with Matrigel (Collaborative Research, Lexington, MA, USA). The Matrigel was first diluted 1:7 in sterile distilled water and air dried in the Millicells to coat the filters. This resulted in a thinner ($1\text{ }\mu\text{m}$) more even layer of basement membrane than could be obtained using the undiluted material. The initial volume of medium in the upper chamber was 2 ml and that in the lower chamber 1.5 ml when large chambers (30 mm) were used (inulin permeability experiments). For electrical resistance measurements, cells were grown in 12 mm chambers with initial apical and basal volumes of 0.6 ml. Monolayers were used for inulin permeability and electrical resistance studies after 4–6 days. Cells were grown in serum-free defined medium (Byers *et al.*, 1985). Some cells were also plated on Matrigel-coated plastic petri dishes, uncoated plastic dishes and uncoated Millicells.

Immunocytochemistry

Antibodies to uvomorulin, zonula occludens 1 and cingulin were used for immunocytochemistry on methanol-fixed $4\text{ }\mu\text{m}$ frozen sections of epididymis and on cells growing on Matrigel-coated glass coverslips (Byers *et al.*, 1988). Affinity-purified rabbit polyclonal antibody to zonula occludens 1 fusion protein (Anderson *et al.*, 1989) was used at a

dilution of 1:100. Rabbit polyclonal antiserum to the extracellular domain of uvomorulin (Schuh *et al.*, 1986) was used at a dilution of 1:200 and rabbit polyclonal antiserum to whole cingulin was used at a dilution of 1:500 (Citi *et al.*, 1988).

Inulin permeability and electrical resistance measurements

After 6 days in culture, four 30 mm Matrigel-coated chambers from each region were incubated in [^3H]methoxyinulin ($1\ \mu\text{Ci ml}^{-1}$ in the basal chamber). After 1, 3, 6 and 24 h, aliquots of medium from both compartments were collected and the radioactivity was measured. Results were expressed as a percentage of the original radioactivity added to the basal chamber present in the apical medium. This experiment was also carried out with Matrigel-coated chambers in the absence of cells. Electrical resistance across monolayers of cells was measured using a transepithelial resistance device (Millipore Corporation, Bedford, MA, USA). Care was taken to ensure that fluid volumes in the apical chambers were at the same level for each chamber measured. Small differences in the fluid levels made small (20%) but measurable differences in the resistance measurements, owing to variations in the length of the electrode immersed. This was most important at the early time points before a complete monolayer was established.

Results

General comments on the cell cultures

Epithelial cells were isolated from the epididymides of six pubertal animals which were usually 40–50 days old. At this time spermatozoa were beginning to enter the tubules, but were not present in large numbers. When older animals were used, it was more difficult to achieve consistent confluent monolayer formation, as the cultures were more likely to be contaminated with nonepithelial cell types. Yields of cells were remarkably consistent among cultures, with a range of $5.0\text{--}8.2 \times 10^6$ cells per rat for the caput-corporis, and $3.0\text{--}5.4 \times 10^6$ cells per rat for the corpus-cauda epididymidis. Cell yields were much lower when long-term castrated rats were used (1.66×10^6 cells per rat for both caput and cauda). Cell counts were carried out originally by digesting an aliquot of the final cell preparation to single cells and counting with a haemocytometer. In later experiments the number of cells was estimated by measuring the volume of the final cell pellet after centrifugation in a 15 ml calibrated centrifuge tube (80 *g* for 5 min), 50 μl of cell pellet being equivalent to 15×10^6 cells.

Dome formation

Confluent monolayers of cells formed after 3–5 days in culture. At this time, if cells were growing on Matrigel-coated plastic, many areas of the cultures from both regions of the epididymis had formed hemicysts or domes (Fig. 1). This phenomenon has been described in a number of epithelial cell lines (Lever, 1985). The domes were formed by an accumulation of fluid beneath the epithelial cells, a result of vectorial transport. Dome formation was not observed when cells were growing on permeable supports or on impermeable supports in the absence of Matrigel. Incubation of cultures for 1 h at 4°C resulted in the collapse of most of the domes (data not shown).

After 10 days in culture, the epithelial cell monolayers became patchy (Fig. 2). This was not due to a simple detachment of cells from the substrate but to a folding of the epithelium. At this time, many tubule-like structures were observed floating freely in the medium. It was, therefore, important to carry out studies of the polarized function of these cells before this time.

Immunocytochemical localization of uvomorulin, zonula occludens 1 and cingulin

In sections of epididymis, uvomorulin was distributed in a pattern consistent with a concentration at sites of apical junctional complexes (Fig. 3a). This was observed as a bright honeycomb of staining at the apical part of the epithelium, which presumably corresponds to the zonula adherens or belt desmosome, which lies basal to the tight junction. Uvomorulin was also localized over the rest of the basolateral plasma membrane but the staining intensity was reduced. No

Fig. 1. Paired phase contrast micrographs of rat epididymal epithelial cell monolayers growing on Matrigel-coated plastic after 4 days in culture. Dome formation (arrows) is shown in monolayers isolated from the caput epididymidis ($\times 75$). (a) The monolayer is in the plane of focus and (b) the domes are in focus.

Fig. 2. (a) Micrograph of rat cells isolated from the cauda epididymis after 9 days in culture ($\times 75$). The cell layer is patchy. Many tubule-like structures were observed floating in the medium above the cells. (b) Higher power micrograph ($\times 300$) of rat cells after 9 days in culture. The edges of this region were folding in to form a cord or tubule.

specific staining of the epididymal interstitium was observed. In cultured cells from both regions of the epididymis, uvomorulin staining was concentrated at the cell periphery at regions of cell-cell contact (Fig. 3b–d). In areas of the monolayers that had formed hemicysts, uvomorulin was present on cells that formed the hemicysts as well as on the surrounding epithelial cells (Fig. 3b,c). In cells at subconfluent densities, uvomorulin staining was more concentrated on parts of the epithelial cells in contact with other cells and was considerably less intense on the free borders (Fig. 3c). In co-cultures of stromal cells and epididymal epithelial cells, stromal cells were completely negative (Fig. 3e).

In sections of the epididymis, both cingulin and zonula occludens 1 were distributed as discrete rings of staining at the apical part of the epithelial cells (Figs 4a, 5a). No staining of the rest of the basolateral membrane was observed. Tight junctions of endothelial cells were also stained but no other cells in the epididymal interstitium exhibited specific staining. The zonula occludens 1 antibody also stained spermatozoa in the lumen of the epididymis. This particular antibody preparation was purified immunoglobulin G (IgG), not serum. At similar concentrations, purified rabbit IgG also stained spermatozoa but did not give the characteristic zonula occludens 1 ring-like staining (not shown). Since it has been shown that spermatozoa do have receptors for the constant region of IgG (Sethi & Brandis, 1980), we interpret this staining of spermatozoa as an interaction of the purified IgG with these Fc receptors. The other antibodies were whole sera preparations in which the IgG concentration at the effective dilution for immunostaining was considerably less than that required for staining for zonula occludens 1.

Cultured epithelial cells showed a similar pattern of staining. Cingulin and zonula occludens 1 were localized as a fine ring of staining around the borders of epithelial cells that were in contact with one another (Figs 4b, 5b). As with uvomorulin, no staining of the free borders of epithelial cells or stromal cells was observed. Controls, including normal rabbit serum or purified IgG instead of primary antibody, and omission of one or more steps of the immunocytochemical procedure, did not show staining.

Inulin permeability and electrical resistance

Complete monolayers of cells from proximal and distal regions were remarkably impermeable to inulin passage from basal to apical compartments with a rate of $0.1\% \text{ h}^{-1}$ for the proximal and $0.05\% \text{ h}^{-1}$ for the distal epididymidis, respectively (Fig. 6).

We were interested in the effect of cell density on the development of electrical resistance. We found that densities of $>1.0 \times 10^6$ per chamber ($1.6 \times 10^6 \text{ cm}^{-2}$) actually delayed and in some instances prevented, the development of significant resistance (Fig. 7). The deleterious effects of high initial plating density were probably the result of many cells failing to attach under these conditions. Some chambers did eventually develop resistance, probably a result of the surviving cells dividing and forming a monolayer. The appropriate initial plating density for both regions was $0.75 \times 10^6 \text{ cells cm}^{-2}$. Even at these ideal densities, 30% of the chambers did not develop a high resistance (>200 ohms). The large standard deviations at certain times reflected the inclusion of values for chambers that did not establish high resistance. Many chambers eventually established resistances exceeding the limits of the detector (>2000 ohms) and these values were included in the results as 2000 ohms. Despite these variations, it was clear that cells from both regions formed high-resistance, impermeable monolayers *in vitro*. Epithelial cells in those chambers that did not develop a resistance may have been contaminated with nonepithelial cells. Our demonstration that uvomorulin, zonula occludens 1 and cingulin were specifically expressed in epithelial cells indicates that these markers could also be used to establish the purity of cell culture preparations. Indeed, we found that an initial contamination of $>10\%$ of nonepithelial cells was sufficient to prevent the development of high-resistance monolayers (authors' unpublished observations).

For experiments of polarized protein secretion, inulin permeability and transport studies, chambers with an electrical resistance of more than 500 ohms were selected. At an initial cell

Fig. 3. (a) Uvomorulin immunostaining of the rat epididymis. Specific staining was concentrated as a ring at the apical surface of the cells (arrows), but was also present on the rest of the basolateral plasma membrane. lum: lumen; int: interstitium. (b) Uvomorulin immunostaining of rat epididymal cells in culture. A dome was seen in the centre of the field. The cells within the dome were out of focus and the staining of the cells surrounding this was concentrated at the cell borders (arrows). (c) Uvomorulin staining of the cells in the dome. The borders of the cells were specifically stained (arrows). (d) A colony of rat epididymal epithelial cells stained for uvomorulin. Note that the staining was concentrated at the cell borders where adjacent cells were in contact (small arrows) but was less intense at the free borders (large arrowheads). (e) Stromal cells in co-culture with epithelial cells were negative for uvomorulin. n: nucleus; large arrowheads indicate epithelial cells at the edge of the field. ($\times 280$.)

Fig. 4. (a) Cingulin staining of rat epididymis. Note that staining was restricted to a honeycomb (arrows, tight junctions) at the apical part of the cells. lum: lumen; int: interstitium. (b) Cingulin staining of epididymal epithelial cells in culture. Arrows indicate the staining restricted to the cell borders.

density of 1.0×10^6 cells per chamber, there was no difference in the resistances of cells growing on uncoated or Matrigel-coated filters (Fig. 8). It was clear from these experiments that not all chambers could be used to investigate vectorial functions. The routine measurement of electrical resistance was essential before selecting chambers for use in such studies.

Discussion

We have shown that epididymal epithelial cells and Sertoli cells can be maintained as morphologically polarized monolayers on permeable supports coated with extracellular matrix (Byers *et al.*, 1986) and demonstrated the transferrin-mediated transcytosis of iron and the polarized secretion of androgen-binding protein and transferrin by Sertoli cells in culture (Djakiew *et al.*, 1986; Hadley

Fig. 5. (a) Zonula occludens 1 staining of the epididymis. Staining was restricted to a honeycomb (arrows, tight junctions) at the apical part of the cells. lum: lumen; int: interstitium. (b) Zonula occludens 1 staining of epididymal epithelial cells in culture. Arrows indicate the staining restricted to the cell borders.

et al., 1987). The present experiments show that cultured epididymal epithelial cells express adhesive and junctional proteins and form a permeability barrier when grown in a two-chamber culture system.

Dome formation in epithelial cell cultures is acceptable presumptive evidence that a number of polarized functions are being expressed (see Lever, 1985 for review). Lifting of the epithelial cell layer, owing to active transport of ions and water across the cell monolayer in an apical to basolateral direction, traps a bubble of fluid between the cell layer and the culture dish resulting in

Fig. 6. [^3H]inulin permeability of monolayers from the proximal (●) and distal (○) regions of the epididymis. (△) Permeability of the matrix coated filter alone. % diffusion is the percentage of the original radioactivity added to the basal chamber present in the apical chamber at the times indicated. Data are means $\pm$ SEM.

Fig. 7. Electrical resistance of cell monolayers from the (a) proximal and (b) distal regions of the rat epididymis growing on Matrigel-coated filters. Cells were plated at 2.0×10^6 cells per chamber (○), 1.0×10^6 cells per chamber (●) and 0.5×10^6 cells per chamber (△). Error bars indicate the SD of results from three chambers.

the formation of domes which can be viewed by microscope. For this to occur, three fundamental processes must take place: (i) cell-cell adhesion and the formation of adherens and occluding junctions, (ii) plasma membrane polarization resulting in the restriction of ion pumps to the appropriate membrane domain and (iii) vectorial transepithelial ion transport. In the present study, we demonstrated the uvomorulin, zonula occludens 1 and cingulin molecules associated with adherens and occluding junctions (Stevenson *et al.*, 1986; Citi *et al.*, 1988; Gumbiner *et al.*, 1988), are present and appropriately organized in cultures of epididymal epithelial cells. In earlier studies, we observed the presence of adherens and occluding junctions at the electron microscope level (Byers *et al.*, 1985, 1986). We recently localized $\text{Na}^+ - \text{K}^+$ ATPase to the basolateral plasma membrane of the epididymal epithelium *in vivo* (Byers & Graham, 1990). Isolated epididymal

Fig. 8. Cells from the proximal (○) and distal (●) regions of the rat epididymis were grown at 1.0×10^6 cells per chamber on uncoated filters. Electrical resistance was measured daily. Error bars indicate the SD of results from six chambers.

epithelial cells growing in Boyden chambers also carry out vectorial ion transport (Wong, 1988). These observations and the ability of epididymal epithelial cells to form domes in culture provide evidence that these cells are functionally polarized *in vitro*. The presence of domes was more obvious in cultures growing on Matrigel, possibly indicating that extracellular-matrix-cell interactions are needed for polarized functions in this system. Alternatively, the Matrigel-plastic interface may be more easily displaced by accumulating fluid than the cell-plastic interface. Matrigel is not required for epididymal epithelial cells growing in culture chambers to establish high resistance monolayers. We have not noticed dome formation in confluent and apparently polarized monolayers of Sertoli cells, perhaps because these cells are involved in fluid secretion from their apical, rather than basolateral, surface.

The relative impermeability to inulin of the epididymal epithelium *in vitro* in the present study differs from that reported previously (Byers *et al.*, 1986; Cooper *et al.*, 1989). There may be several explanations for this. First, the cells in the earlier studies may have undergone the folding and tubule formation described here, with a concomitant increase in inulin permeability. Second, at the time of our previous study, chamber construction was not always consistent; we now use commercially available Millicells. Third, we now use younger animals and achieve more reproducible results. The inulin permeability of epididymal cells *in vitro* in our study compares favourably with that described for high resistance epithelia (Simons & Fuller, 1985); indeed, our electrical resistance measurements indicate that the epididymal epithelium *in vitro* falls into this category. Other cells in this category include the high resistance strain of the Madin Darby Canine Kidney (MDCK) cell line and the A6 toad bladder epithelial line (see Simons & Fuller, 1985 for review). Confluent monolayers of Sertoli cells in culture do not approach this resistance or inulin impermeability *in vitro*, despite the presence of extensive tight junctional complexes (Hadley *et al.*, 1985; Byers *et al.*, 1986; Djakiew *et al.*, 1986; Janecki & Steinberger, 1986; Ailenberg & Fritz, 1988; Onoda *et al.*, 1990). The role of the various structural components of occluding junctions in epithelial resistance is not well defined. Neither the number of junctional strands nor the relative amounts of the tight-junction associated protein zonula occludens 1 correlate with electrical resistance in low and high resistance strains of MDCK cells (Stevenson *et al.*, 1988). The molecular and structural basis underlying differences in electrical resistance among the various types of epithelia in the reproductive system, and elsewhere, is therefore not clear.

In this paper we have described several factors that are important in the isolation and culture of polarized monolayers of epididymal epithelial cells: (i) cells must be plated at the correct cell

density, (ii) routine electrical resistance measurements must be made and (iii) great care must be taken to ensure that the isolated cells are at least 90% epithelial. We showed that several of the polarized functions of the epididymal epithelium can be preserved *in vitro*. This, with the effective separation of apical from basal compartments possible in dual-compartment chambers, should permit more meaningful *in vitro* studies of the role of the epididymis in sperm maturation.

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