

Original Article

Maturation of Renal Collecting Duct Cells In Vivo and Under Perifusion Culture

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Abstract. The embryonic collecting duct epithelium of neonatal kidney undergoes profound functional changes during maturation. In its initial state as inductor epithelium it appears homogeneous, but differentiates into a heterogeneously composed collecting duct epithelium consisting of principal and intercalated cells. The mechanism of this terminal differentiation process is unknown.

We used morphological and immunohistochemical methods to investigate the maturation of the collecting duct system in neonatal rabbit kidney and under organotypic culture conditions. The new perifusion culturing method allowed us to follow the differentiation of the ampullary collecting duct epithelium under conditions as close as possible to the situation within the organ. With this technique we were able to induce a differentiation process similar to that in the *in situ* situation. This process led to the appearance of a mixed cell population consisting of principal and intercalated-like cells, respectively. A continuous perifusion of the medium made it possible to stabilize the micro-environment under culture conditions and thus to maintain the heterogeneous composed collecting duct epithelium in a differentiated status over long periods of time.

Keywords: Collecting duct; Development; Intercalated cells; Kidney; Morphology; Perifusion culture

Introduction

The adult renal collecting duct regulates the Na^+/K^+ balance, the acid/base state and the water content of the organism (Koeppen et al. 1983; O'Neil and Hayhurst 1985; Star et al. 1985; Koeppen 1987; Stokes 1993). These complex physiological functions are maintained by the light principal (P) cells and at least two types of dark intercalated (α - and β -IC cells) (Kaissling and Kriz 1979; Madsen et al. 1988; Schuster 1993). The cellular heterogeneity of the collecting duct epithelium is a peculiar feature within the kidney. Each of the other tubular epithelia of the nephron consists of a homogeneous cell population with distinct transitions from one tubular segment to the other.

Within the neonatal rabbit kidney the embryonic or ampullary renal collecting duct plays an entirely different functional role than in the adult kidney. During the first steps of kidney development and until the late neonatal period the ampullary collecting duct epithelium performs the function of an embryonic inductor generating all the nephrons. In this way the collecting duct determines the future architecture of the whole kidney (Potter 1965; Saxén 1987). During the embryonic and neonatal growth of the organ this inductor capability is sustained in the ampullary tip portion, while the light P and dark IC cells gradually develop beyond the ampullary neck after the cortico-medullary elongation of the collecting duct (Neiss 1982). Six different kinds of adult collecting duct cells have been described according to the morphological appearance of their cell surfaces (Table 1; Le Furgy and Tisher 1979; Evan et al. 1991). In addition, Narbaitz et al. (1991) found that in the embryonic rat kidney differentiated α -type IC cells had already developed, in contrast to the

Table 1. Cell types in adult collecting duct of rabbit kidney according to Le Furgey and Tisher (1979)

Cell types	Luminal cell differentiation	
P-cell pattern 1	One single cilium	Short, widely-spaced microvilli
P-cell pattern 2	Once single cilium	Short, closely-packed microvilli
IC-cell pattern 1	Without a cilium	Abundant short microvilli
IC-cell pattern 2	Without a cilium	Both long and short microvilli
IC-cell pattern 3	With microplacae*	
IC-cell pattern 4	With microplacae	Microvilli

*described as IC- α type according to Narbaitz et al. (1991)

β -type IC cells, which could not be detected until three weeks after birth.

Little information is available about the origin of the IC cells. While some authors assume that different phenotypes of IC cells arise from a single precursor cell which acquires its functional polarity in response to acid/base differences (Schwartz et al. 1985; Fejes-Tóth and Náray-Fejes-Tóth 1992; Schuster 1993), other authors argue that the different IC-cell types develop from different precursor cell lineages (Madsen and Tisher 1985). Brière and Magny (1993a) describe morphological evidence that indicates the transformation of β -IC cells into α -IC cells in human fetal kidneys, supporting the findings on cultured collecting-duct β -IC cells (Fejes-Tóth and Náray-Fejes-Tóth 1992, 1993).

Although it is evident that the transition from the embryonic inductor epithelium of the neonatal rabbit kidney into the highly specialized collecting duct epithelium of the adult kidney is a process involving postnatal proliferation and differentiation steps (Evan et al. 1991), it is unknown which kind of mechanisms influence this development. Recent experiments showed that defined media (Taub and Sato 1980), growth factors, such as EGF and TGF α , insulin, transferrin, hydrocortisone or retinoic acid (Ekblom et al. 1981; Ekblom and Thesleff 1985; Segal and Fine 1989; Brière et al. 1991; Chailler and Brière 1991; Chailler et al. 1991; Weller et al. 1991; Brière and Chailler 1992; Brière et al. 1992; Humes and Cieslinski 1992; Avner et al. 1993; Brière and Magny 1993b; Ferretti et al. 1993; Nouwen et al. 1993), and even aldosterone (Minuth et al. 1988; Minuth et al. 1993) may induce the differentiation process.

Since only limited data are available concerning the appearance of differentiated structural features during collecting duct maturation within the neonatal rabbit kidney, we developed a model which allowed us to investigate the *in vitro* cytodifferentiation within the ampullary epithelium by means of morphological and histochemical methods (Minuth 1987; Minuth et al. 1993). These *in vitro* data were compared with data obtained from the maturing tissue of the outer cortical region of the neonatal rabbit kidney.

Materials and Methods

Histology

The rabbit kidneys used for the following investigations were obtained on post-partum day 1 or 2. The renal tissue or the cultured epithelia were fixed in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL Life Technologies, Eggenstein, Germany) containing 3% glutaraldehyde (Merck, Darmstadt, Germany). Then the tissue and the epithelia were postfixed with 1% osmium tetroxide in phosphate buffered saline (PBS), pH 7.4, and dehydrated in a series of alcohols, passed through propylene oxide and embedded in Epon (Serva, Heidelberg, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate. The specimens were examined with a Zeiss electron microscope (EM 902).

For conventional scanning electron microscopy (SEM) and fixed specimens were dehydrated in a graded series of ethanols, critical point dried with CO₂ and sputter-coated with gold. Examination was carried out in a scanning electron microscope DSM 940 A (Zeiss, Oberkochen, Germany).

The binding of peanut agglutinin (PNA) on the luminal surface of cultured epithelia was visualized by a backscattered electron detector (Herter et al. 1993). Cultured epithelia were fixed for 30 min with 2% paraformaldehyde and 0.02% glutaraldehyde in PBS. The labelling was carried out with biotinylated PNA (Sigma, Deisenhofen, Germany) diluted 1:30 in PBS and a 6-nm colloidal gold-coupled antibody against biotin (Aurion-Biotrend, Köln, Germany) diluted 1:30 in PBS. Incubation with the marker and the gold label was followed by postfixation with 2% glutaraldehyde in PBS and a 5-min treatment with silver enhancement (Janssen, Olen, Belgium). The specimens were dehydrated in an ascending series of ethanols and then critical point dried. The labelled epithelia were sputter-coated with a 10-nm carbon layer using an electron-beam gun (Balzers, Liechtenstein). The distribution of PNA was visualized with an annular single crystal backscattered electron detector at an accelerating voltage of 10 kV. The working distance was 10 nm.

Fluorescence Microscopy

Fluorescence labelling was performed on cryosectioned renal tissue which was frozen in liquid nitrogen. The tissue was orientated in such a way that vertical cross sections could be obtained. 5- μ m cryosections (Cryostat HM 500, Microm, Heidelberg, Germany) were first fixed in ice-cold ethanol, then washed several times with PBS and incubated with blocking solution (PBS, pH 7.4, 10% horse serum, 1% bovine serum albumin; Sigma) for 1h. In order to detect prematured and differentiated collecting duct (CD) cells we used the monoclonal antibodies CD 7 (Kloth et al. 1993) and one against Na⁺-K⁺-ATPase (Minuth et al. 1987) according

to procedures published earlier. Biochemical characterization of the CD-antigen (PCD 7) revealed two distinct immunoreactive bands in Western blots at 37 and 57 kDa.

For the detection of peanut lectin- (PNA-) positive cells the sections were incubated for 40 min with a PNA-rhodamine conjugate (Vector, Burlingame, USA) diluted 1:2000 in PBS. The sections were embedded in FITC-guard (Testoc, Chicago, USA) and examined using a Zeiss Axiovert 35 microscope (Zeiss).

Perfusion Culture

Thin cortical explants from the kidneys of newborn New Zealand rabbits were mounted on sterile cell holder sets (Minuth et al. 1993) and placed in 24-well tissue culture plates (Becton Dickinson, Heidelberg, Germany). The explants consisted of a piece of capsula fibrosa with adherent collecting duct ampulla, S-shaped bodies and nephrogenic blastema. During the culture of these explants in Iscove's modified Dulbecco's medium (IMDM/HEPES; Gibco-BRL Life Technologies, Eggenstein, Germany) containing 10% fetal calf serum we observed an outgrowth of cells from the collecting duct ampulla. Within 24h the surface of the explant was completely covered by a single-layered collecting duct epithelium. Culture was carried out in a Heraeus tissue incubator (Hanau, Germany) at 37°C in a humidified atmosphere containing 5% CO₂/95% air for 24h. To mimic a situation comparable with that in the organ, the cell cultures were kept under permanent superfusion with fresh medium in a recently developed system (Minuth et al. 1992). The superfusion of cultures with a constant medium flow rate of 1 ml/h in the sterile system was started 24h after preparing the cultures. Total culture time was 14 days (1 day of preculture, 13 days of perfusion culture). The parts for the perfusion culture are available through MINUCELLS and MINU-TISSUE (Bad Abbach, FRG).

Results

Ontogenesis of Single Cell Types Within the Collecting Duct

The maturation of the renal collecting duct epithelium can be perfectly investigated on slices of clearly orientated cortico-medullary tissue through the neonatal rabbit kidney (Fig. 1; Minuth et al. 1989; Evan et al. 1991). In the outer zone of the cortex embryonic structures such as ampulla and S-shaped bodies occur (Fig. 1a), while towards the mid-cortical region semi- and fully differentiated portions are present (Fig. 1b, Fig. 2). The developmental gradient originates beneath the capsula fibrosa, where the embryonic ampulla of the collecting duct epithelium can be seen in the shape of a glove finger (Fig. 1a). The ampullary collecting duct epithelium is surrounded at the tip of the finger by

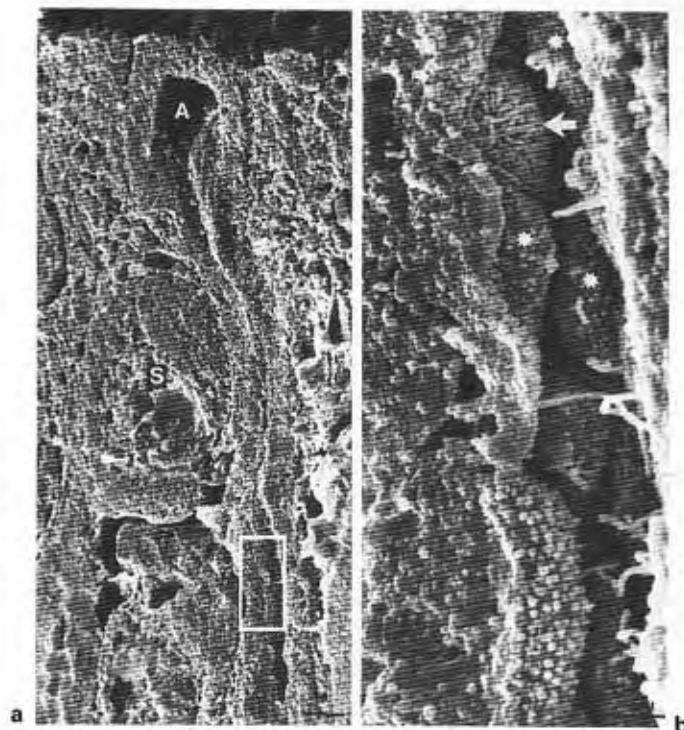


Fig. 1a,b. SEM image of the outer cortex of neonatal rabbit kidney. a SEM view into a blind ending ampullary collecting duct epithelium (A) beyond the capsula fibrosa. At the side an S-shaped body (S) is visible. b A high power magnification of the boxed area of Fig. 1a, showing a detailed view of the maturing collecting duct beyond the neck area. Note the α -type IC cell (arrow), which is surrounded by cells showing P cell characteristics (asterisks). Bar: a 15 μ m; b 5 μ m.

nephrogenic blastema and at the sides by the embryonic stages of the nephron, the comma- and S-shaped bodies.

The collecting duct of neonatal rabbit kidney reveals a remarkable zonal segmentation in its cortical-medullary course. When viewed under the transmission electron microscope the ampulla appears to be a homogeneous epithelium consisting of one cell type only (Dorup and Maunsbach 1982). In contrast, the sudden coexistence of principal (P) and intercalated (IC) cell types beyond the ampullar neck region is a characteristic feature of the more highly developed zone and illustrates the proceeding terminal differentiation process of the collecting duct epithelium in the cortico-medullary course. A flowing transition from the ampullar embryonic cell type to the adult forms of P and IC cells could not be observed in this area (Kloth et al. 1993).

However, scanning electron microscopy revealed the first steps of cytodifferentiation between P and IC cells, which can be distinguished by the differences in their cell surface organization (Table 1; Le Furgy and Tisher 1979; Evan et al. 1991). A clear distinction between the apical surfaces of the light and dark cell types within the maturing collecting duct is first confirmed in the lower neck region (Fig. 1b). A single IC- α -type cell according to Narbaitz et al. (1993) (Fig. 1b, Fig. 2a) with microplacae was found to be surrounded by cells bearing a

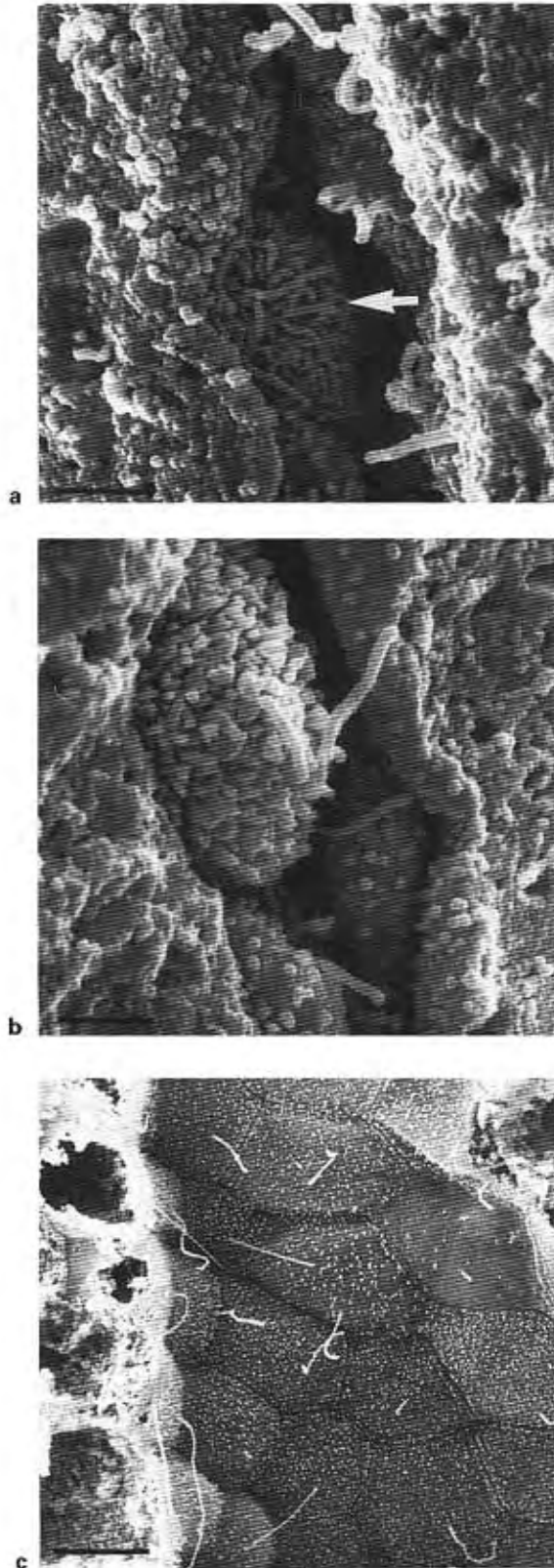


Fig. 2a-c. SEM of the neonatal collecting duct. a SEM reveals an α -type IC cell (arrow) surrounded by P cells. b Differentiating IC-like cells with numerous long microvilli and a cilium beside cells expressing P-cell-like features. c Differentiated collecting duct cells in the inner cortical region with microvilli in various densities and a cilium. Bar: 5 μ m.

cilium with short, stubby microvilli, in other words, cells resembling maturing P cells. In contrast with this result, Evan et al. (1991) noted no intercalated cells in the outer cortex of 2-day-old rabbit renal collecting ducts.

A new cell type with a cilium and extremely long microvilli could be observed in the neighbourhood of the primary occurrence of the α -IC cell (Fig. 2b). In the rabbit collecting duct such a cell type had not yet been reported, but it is known from guinea-pig collecting duct epithelium (Kim et al. 1992). This cell probably differentiates into a β -type IC cell possessing densely packed, long microvilli and may lose the cilium during maturation. The other surrounding cells generally showed P cell like characteristics with short and stubby microvilli, each cell with an individual cilium. In the inner cortical region (Fig. 2c) typical P cells with various densities of microvilli were found resembling those of the adult collecting-duct epithelium (Le Furgy and Tisher 1979).

In Vitro Development of Renal Collecting Duct Cell Features

In a series of experiments we investigated the process of cytodifferentiation by culturing the ampullary collecting duct epithelium. The ampullary collecting duct epithelium was isolated by stripping off the capsula fibrosa of neonatal kidneys, put in culture and subjected to permanent superfusion with fresh medium over 13 days in a recently developed cell culture contained (Fig. 3; Minuth et al. 1993). After continuous superfusion a morphological and histochemical examination of the cultured epithelia revealed a polarized epithelium (Fig. 4a). The luminal side of the epithelium was in contact with the medium and the basal face rested on the supporting capsula fibrosa. Immunostaining with the antibody against Na^+/K^+ -ATPase showed that all of the epithelial cells were labelled on the basolateral side (Fig. 4b) as is known for collecting duct cells (Minuth et al. 1987). PCD 7 antibody labelling, a marker we used

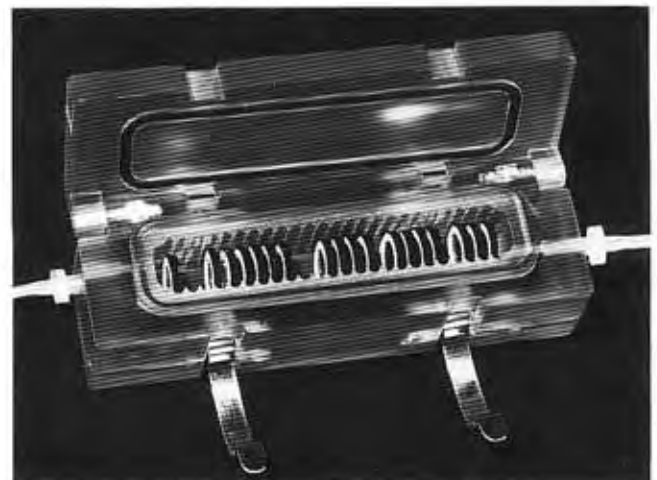


Fig. 3. Microphotograph of the culture container for perfusing the cells with fresh medium.

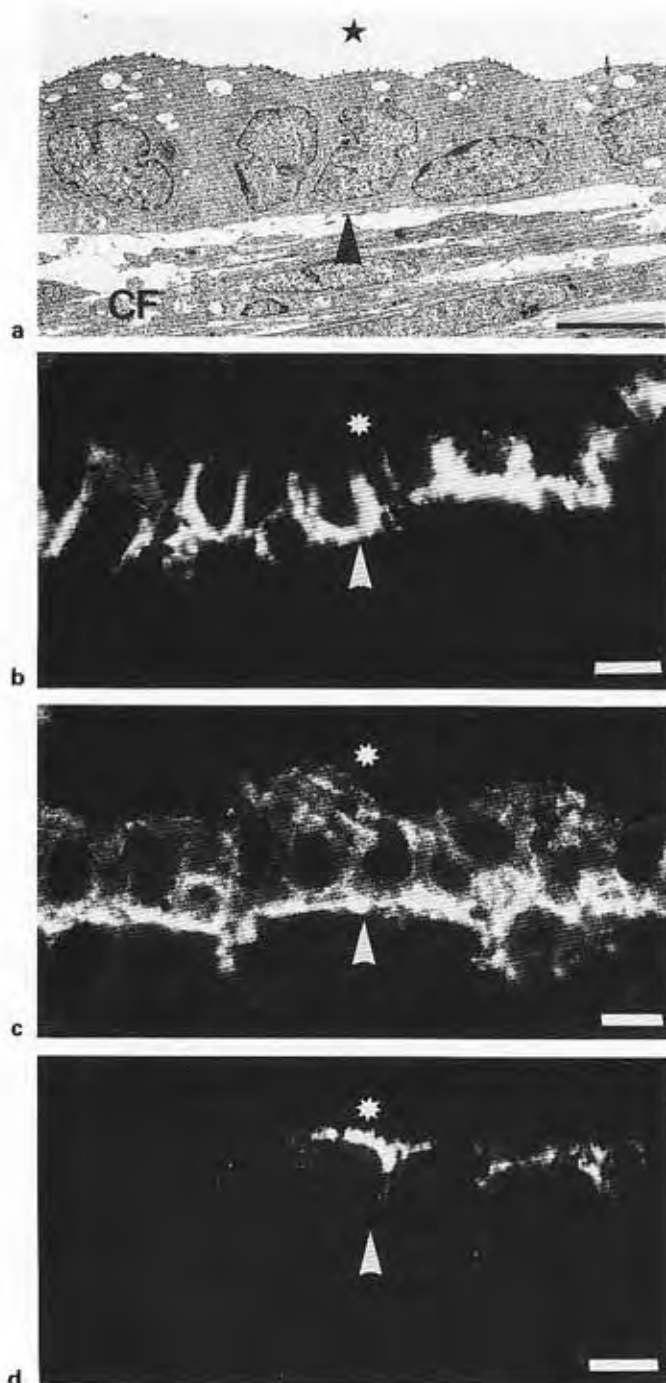


Fig. 4a-d. Ultrathin section **a** and immunohistochemistry **b-d** of cultured collecting duct epithelium. **a** The micrograph shows the polarity in the differentiation of the collecting duct epithelium (asterisks, luminal side; arrowheads, basal membrane; CF, capsula fibrosa). **b** Na^+/K^+ -ATPase is expressed in all epithelial cells. **c** The monoclonal antibody CD 7 identifies the cells as differentiated collecting duct epithelium. **d** PNA labels individual cells at the luminal cell poles. Bar: 10 μm .

for differentiated collecting duct cells in neonatal kidneys (Kloth et al. 1993) demonstrated that the detected antigen was also expressed in all cultural epithelial cells (Fig. 4c). In contrast, the β -IC-cell-specific marker PNA

was found only on single cells (Fig. 4d). All of the cultured epithelia tested so far showed comparable immunohistochemical characteristics of the collecting duct in the neonatal kidney.

As revealed by scanning electron microscopy, the cells of the cultured epithelium showed differences in surface organization. This indicated the presence of different cell types which develop under superfusion conditions (Fig. 5). Cells with a cilium and with short and few microvilli, resembling principal cells (Fig. 5a,b), were found beside cells with numerous microvilli of variable, sometimes extreme length at the luminal cell poles (Fig. 5c-f). These cells can be classified as IC-type cells (Fig. 5c), but they are not identical to the α -type of IC cells characterized by microplicae (Fig. 5d; Herter et al. 1993; Narbaitz et al. 1993). A further novel finding was that IC-cell-like characteristics such as long and dense microvilli occurred with a single cilium (Fig. 5e,f). This cell feature is also present in the outer cortex of maturing renal tissue (Fig. 2b). It probably marks a transition from the embryonic to the adult state, because the IC cells in rabbit collecting duct epithelium do not bear a cilium.

PNA labels β -type IC cells in the adult rabbit kidney (Schuster et al. 1986; Holthöfer 1988; Satlin et al. 1992; Herter et al. 1993). By backscattered scanning electron microscopy the binding of gold-labelled PNA on the apical side of cultured collecting duct epithelium was investigated (Fig. 6). It was observed that not only the typical β -type IC cells without a cilium were labelled, but various intermediate cell forms were marked, even cells bearing a cilium. The label was predominantly found at the more or less numerous occurring microvilli. The various PNA-binding intensities corresponded to the different lengths and densities of the individual microvilli population on individual cells. In conclusion, the PNA label revealed several intermediate forms between embryonic and further developed, but not yet fully differentiated collecting duct cells. The IC cells with microplicae (Fig. 5d) were not labelled. This mirrored the results found in adult renal collecting duct (Herter et al. 1993).

Discussion

The developing kidney is an organ uniquely suited for studying the differentiation of a composite epithelium and the concomitant development of specific functions (Ekblom et al. 1980; Saxén et al. 1981; Saxén et al. 1986; Aufderheide et al. 1987; Klein et al. 1988; Furuse et al. 1989; Vainio et al. 1989; Ekblom and Weller 1991; Perantoni et al. 1991; Sainio et al. 1992; Sorokin and Ekblom 1992; Vainio et al. 1992). On the one hand, of course, the complex architecture of this organ represents a major obstacle to biological investigations for a number of reasons. First, it is difficult to harvest sufficient quantities of defined embryonic nephron segments. Second, the application of drugs via the blood stream in order to influence the development within the

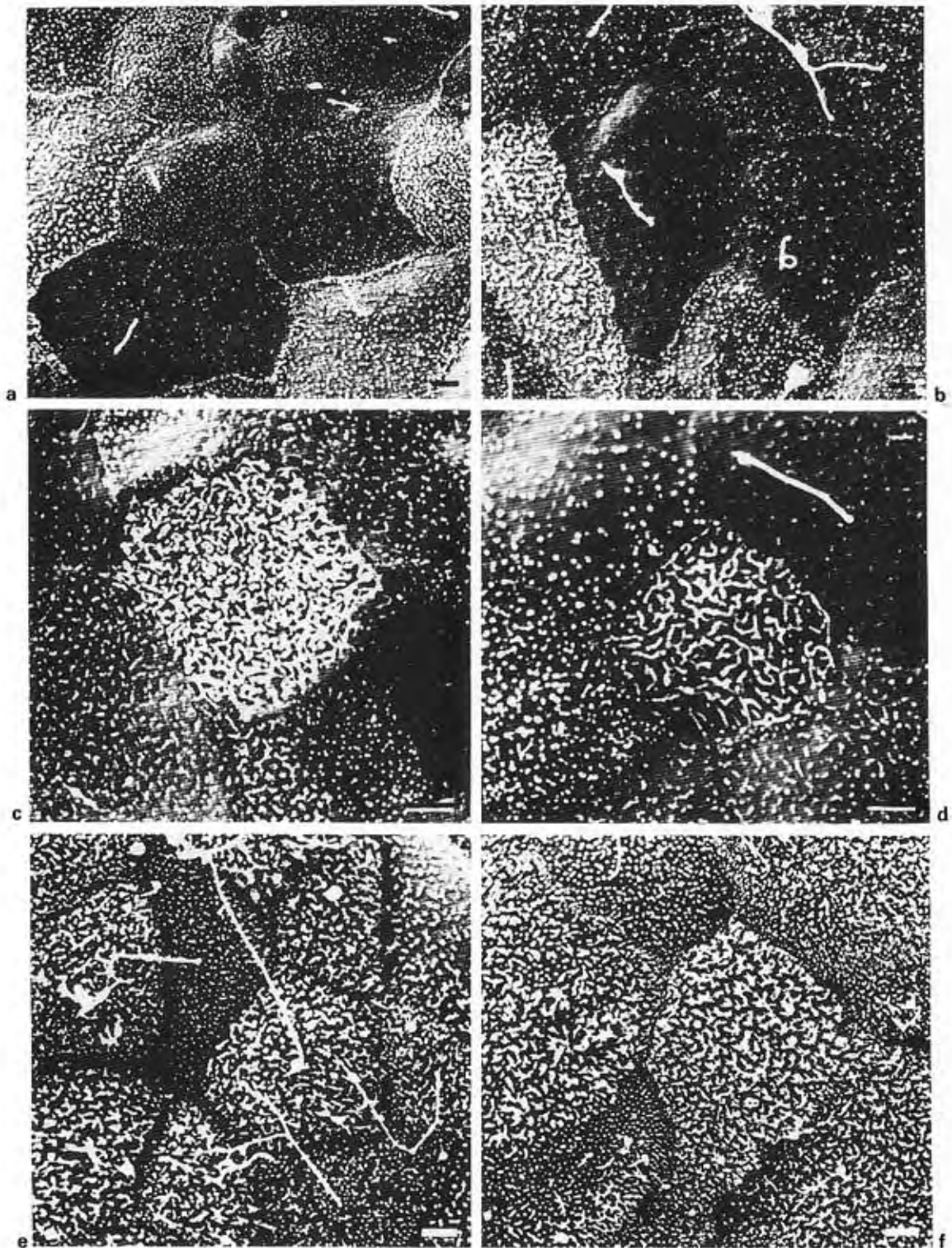


Fig. 5a-f. SEM of cultured collecting duct epithelium perfused with IMDM containing 10% fetal calf serum without additional hormonal supplements. a,b Numerous cells possess a cilium and short microvilli in various densities and resemble differentiated P cells (compare Fig. 3c), and c,d Single cells without a cilium and with long microvilli (c) or microplicae (d) resembling cells in the adult kidney (see Le Furgy and Tisher 1979). e,f IC-like cells with long and dense microvilli and an atypical cilium. This feature was also observed in the maturing collecting duct (compare Fig. 3b). Bar: 2 μ m.

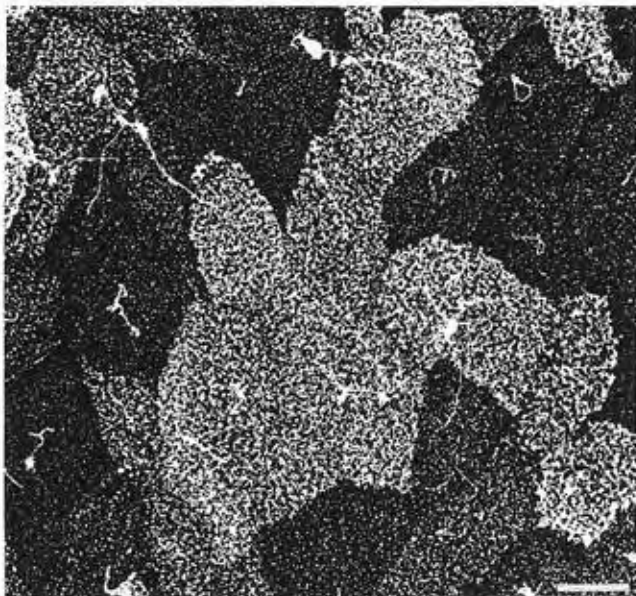


Fig. 6. PNA-labelled cultured cells represented by backscattered SEM analysis. The different labelling intensity is due to various intermediate features of the collecting duct cells, because PNA binds on the microvilli. Thus, cells with a higher density of long microvilli are labelled more than cells with shorter microvilli. Bar: 5 μ m.

outer cortical region remains a problem, because the vessel system is not completely developed. This means that substances reach the embryonic zone solely by a long diffusion pathway (Kloth et al. 1992). Third, because of the complexity of the organ, the effect of applied hormones can be ascribed directly to the target site of a specific cell type, but could also be the effect of a long cascade of reactions between neighbouring cells (Brière et al. 1991). Fourth, the metabolism pathway of cells is not the same in the embryonic stage and the different stages of maturation. Finally, the equipment of specific cell receptors in the embryonic zone is completely different from that in the adult organ (Satlin 1991; Farman 1992; Robillard et al. 1992).

On the other hand, the complexity of the maturing neonatal kidney offers some considerable advantages for developmental biological studies. The developmental gradient of various nephron generations between the capsula fibrosa and the mid cortex allows us to observe embryonic, maturing and differentiated cells all in one single section. Further, it allows us to investigate whether a primary and final expression of functional proteins has occurred in different cell types during maturation. All of these morphological and histochemical observations can be performed in the natural environment of the organ. However, the method is limited because the embryonic tissue cannot be rapidly influenced due to its poorly developed vessel system (Kloth et al. 1992). As a consequence, tissue culture techniques are alternatives isolating defined regions and using them for individual and nephron-specific questions. Tissue culture experiments are limited, however, by the fact that cells will lose specific features by

dedifferentiation when they are isolated from the organ and grown in a dish (Minuth and Gilbert 1988; Koechlin et al. 1991). Thus, the separation and culture of cells are accompanied by an unknown degree of morphological, physiological and biochemical alterations.

In order to partially surmount some of the above-mentioned difficulties, we followed a strategy combining non-disaggregating tissue culture methods with an organotypic cell culture environment. Two features made this approach possible: the easy isolation of the embryonic ampullary collecting duct epithelium from the neonatal rabbit kidney (Minuth 1987) and the use of a new culture technique which allowed us to keep the epithelia under controlled conditions by a permanent exchange of medium (Minuth et al. 1992; Minuth et al. 1993). Under these conditions, the ampullary collecting duct epithelium was cultured to observe the maturation capability of these cells. The ultimate aim was to compare *in vitro* differentiation with the situation in the maturing neonatal kidney.

Development of the Renal Collecting Duct *In Vivo*

As documented by Le Furgey and Tisher (1979), Evan et al. (1991) and Herter et al. (1993), principal (P) cells bear one single cilium with a variable number of short microvilli at the cell surface. In contrast, intercalated (IC) cells with long microvilli do not bear a cilium in the collecting duct of the adult kidney. Therefore, it was something of a surprise that in the neonatal kidneys (Fig. 2b) and even in cell cultures IC-like cells were seen both with (Fig. 5e,f) and without cilia (Fig. 5c,d). This finding indicates that both P and differentiating IC cells bear a cilium.

Furthermore, all IC cells may stem from a cell type with short microvilli as seen within the ampulla (Kloth et al. 1993). During development the long microvilli or microplicae appear although the actual mechanisms of transition are unknown. Some authors assume that the α -IC cells arise from the β -IC cells depending on the physiological status of the cells (Schwartz et al. 1985; Brière and Magny 1993a). The last developmental event seems to be that most of the IC cells lose their cilium. Thus, the present morphological results speak in favour of a common precursor cell type for P and IC cells in the ampulla. Recent immunohistochemical data showed that the ampullary epithelium displayed homogeneous and heterogeneous composition depending on the markers used (Kloth et al. 1993).

The Collecting Duct Epithelium *In Vitro*

Culturing the embryonic ampullary collecting duct epithelium resulted in the development of P and IC-like cells (Figs 5 and 6). When the apical sides of both cell types were compared by backscattered scanning electron microscopy, we observed that not only were the typical luminal PNA-labelled features of adult IC cells developed, but several intermediate cell forms could be detected as well (Fig. 6; Herter et al. 1993).

The same results were obtained by SEM analysis of cultured P cells (Fig. 5a,b). Consequently, both similarities and dissimilarities between adult, neonatal and cultures are evident. However, these contrasting observations lead us to the conclusion that a collecting duct cell lineage with many indifferent cell types may exist in the neonatal kidney during development.

What is the developmental stimulus for a heterogeneously composed ampullary epithelium? In the close vicinity of the ampullary epithelium the induced comma-shaped bodies go on to develop into the S-shaped bodies (Potter 1965; Saxén 1987). During the following developmental step a connection between the ampullary epithelium and the developing connecting tubule must be established. Both epithelia are oriented in such a way that the basement membranes of the ampullary epithelium and the presumptive connecting tubule are attached to each other. To establish the junction, the process requires a solubilization of the basement membranes to combine both epithelia. This 'melting' event may be responsible for an inductive signal which targets the ampullary tip cells and radiates out to induce a new generation of nephrons. It may then also give a signal to the remaining ampullary cells to differentiate into individual P and IC cells. The heterogeneous composition of the ampullary epithelium may reflect the first signs of this differentiation step.

Acknowledgements. This investigation was supported by the Deutsche Forschungsgemeinschaft (Mi 331/2-5). The skillful photographic assistance of Mrs A. Pieringer and Mrs K. Dassler is gratefully acknowledged.

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