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Improved Differentiation of Renal Tubular Epithelium in vitro: Potential for Tissue Engineering

Key Words
Tissue engineering · Differentiation · Dedifferentiation · Culture · Collecting duct · Kidney

Why Epithelial Tissue Engineering?

Cells and tissues in culture often lose their typical morphological, physiological and biochemical characteristics within a matter of hours [1]. This process of dedifferentiation begins as soon as the cells are isolated from the organ and continues for various reasons in the static environment of the culture plate. Coating the cell support with extracellular matrix proteins improves differentiation, but is neither able to prevent the harmful accumulation of metabolites and growth factors nor to meet the special nutritional needs of individual cells. With this in mind we attempted to improve the quality of renal cell and tissue cultures by creating an environment like that in the organ. The new technique is based on flat cell holder sets, the so-called Minusheets®. Any support material desired can be put in the holder to optimize cell attachment and differentiation. The cell holders are placed in a culture container for constant perfusion with fresh medium over a period of weeks or months. For example, the renal collecting duct epithelium is cultured in gradient containers using capsula fibrosa as the support material. Because the cells can be bathed with a different medium on each side, the culture conditions mimic those in the organ. The result is a degree of cell differentiation never before achieved with other methods. Completely new possibilities are opened up for tissue engineering and studies on the chronic intoxication of cells and tissues in long-range culture experiments.

From the Complex Organ to a Functional Epithelial Cell Culture

The kidney is the central organ for regulating the water and electrolyte content and the acid-base balance [2]. These diverse functions are made possible by the complex histological architecture of the nephron and the collecting duct system (fig. 1a). The cell populations are homogeneous in each of the various nephron segments. In contrast, at least three different cell types can be detected in the collecting duct [3, 4]. The sodium and water content of the urine is regulated by the light principal (P) cells, while the dark α- and β-type intercalated (IC) cells are responsible for maintaining the acid-base balance. Because these functions are controlled by hormones, studies on the biochemical and physiological effects of the steroid hormone aldosterone [5] or the peptide hormone vasopressin [6] are of experimental interest. Although many electrophysiological and immunohistochemical studies have been carried out on individual cells of the collecting duct in the adult kidney and a large body of knowledge has been accumulated [7], very few data are available about the development of the collecting duct system with its various cell types [8, 9]. Because of the complexity of the organ we decided to investigate the development of the renal collecting duct using cell cultures.

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The Difficulties Begin with Isolating the Cells

Collecting duct cells can be isolated either in embryonic or in adult form [10–12]. Frequently, epithelial cells are isolated from the rest of the tissue with the help of proteases and a Ca^{2+}- and Mg^{2+}-free buffer solution. When the cells are isolated, they are separated from the natural support of their basement membrane. In addition, the tight junctions joining cell to cell are broken. What were originally geometrically shaped cells become round in the isolation process. As a result, the transport systems of the membrane, which were originally located either on the apical or the basolateral sides, are unnaturally spread over the entire cell surface [13]. If the isolated cells are then placed in a polystyrene culture plate, they soon attach themselves to the bottom of the plate and begin to divide. Many of the cells fail to regain their original isoprismatic form and consequently also fail to regain their functional polarization (fig. 1c). While the collecting duct cells in the kidney had an isoprismatic shape (fig. 1a), those on the bottom of the culture plate (fig. 1c) look more like fried eggs whose raised egg yolks are the nuclei. In order for the typical morphological differentiation of epithelial cells to take place a support material must be used which is as close as possible to the natural basement membrane with its extracellular matrix proteins [14]. In our experiments capsula fibrosa, the thin collagenic membrane enclosing the kidney, proved to be particularly suitable as a cell-support material (fig. 1b) [10].

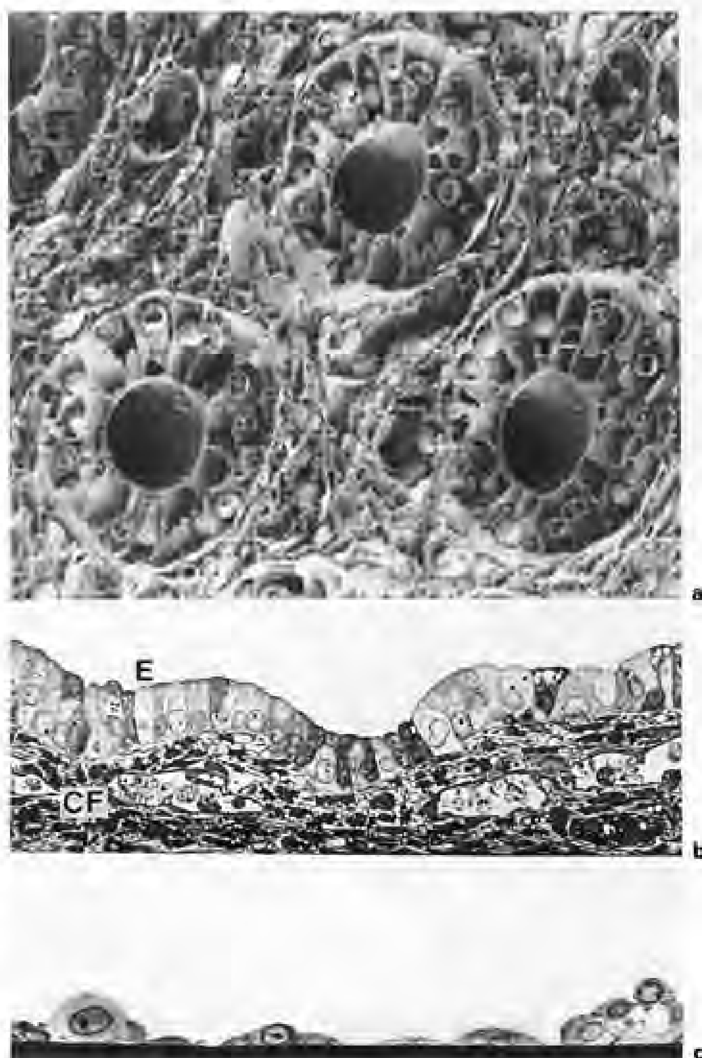

Fig. 1a–c. Morphological changes in cultured collecting duct cells. Microscopic view of collecting ducts from the rabbit kidney (**a**), of cultured collecting duct epithelium (E) grown on a renal capsula fibrosa support (CF) (**b**), and of collecting duct cells grown on the bottom of a culture plate (**c**). **a** The uriniferous lumen is clearly visible in the three collecting ducts shown in cross section under the scanning electron microscope. **b** The collecting duct epithelium cultured on a capsula fibrosa support clearly displays the same polar differentiation as in the collecting duct cells of the kidney. **c** In contrast, the polar differentiation is lost to a great degree when the cells are grown on the bottom of a plastic culture plate. **a** × 600, **b** × 900, **c** × 1,200.

The Culture Is Only as Good as the Physiological Barrier

Polar differentiation is a prerequisite for a functioning epithelium. The basal side of the collecting duct cells in the kidney adheres to the basement membrane (fig. 1a). The apical side of the epithelial cells forms the boundary to the urine compartment. The collecting duct cells have the special task of forming a functional barrier which serves to filter out urinary constituents into the urine compartment. At the same time, it ensures that metabolically harmful molecules such as urea do not reenter the interstitium. In addition, sodium and water are reabsorbed as needed. This process is regulated by the hormones vasopressin and aldosterone [15, 16]. Amiloride-sensitive sodium channels [17] and aquaporins as water channel proteins [18] are integrated into the apical plasma membrane and transport pumps such as Na/K-ATPase [19] are built into the basolateral plasma membrane. This ensures that only certain molecules like sodium or water can pass through the cells, while other substances are locked out. A seal, the tight junction (fig. 2a) is located at the border between the apical and lateral sides of the cell and forms a belt-like band encircling the cell. The tight-

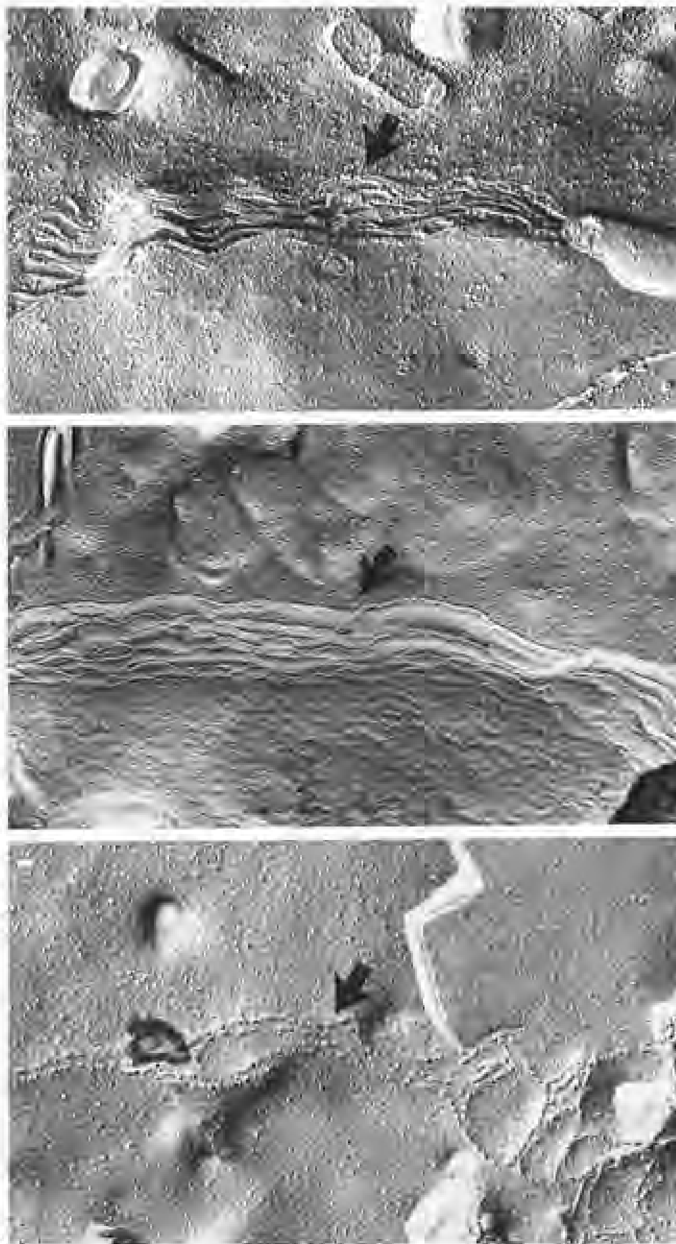

Fig. 2a–c. Qualitative differences between the tight junctions. Freeze-fracture replicas of tight junctions (arrow) from the collecting duct cells of the adult rabbit kidney (**a**) and from collecting duct cells grown on capsula fibrosa (**b**) and the bottom of a culture place (**c**). To create a functional seal in the epithelium, 5–7 anastomosing strands are formed in the zonula occludens of the tight junction both in the kidney (**a**) and in cells cultured on capsula fibrosa (**b**). Cells cultured on plastic (**c**), on the other hand, display only 2–3 strands. A tight junction with only 2 or 3 strands cannot form a satisfactory seal. × 40,000.

ness of an epithelium can be determined electrophysiologically. Perfectly sealed, 'tight' epithelia have a transepithelial resistance of at least 0.5 kΩ cm^{-2} [20]. Values smaller than this signalize a leaky condition with a paracellular and consequently uncontrolled exchange of substances which is not typical for the collecting duct epithelium of the kidney.

Most of the collecting duct cultures grown on the bottom of a culture plate were unable to form a completely closed epithelium (fig. 1c). Although the cultured cells did not have the typical cobblestone shape of those in the kidney, but were uncharacteristically flat, we found that most of them did indeed have tight junctions. In order to evaluate the seal formed by these tight junctions, the cells were broken apart at the cell-cell connection. The junctional band was examined under the electron microscope using the freeze-fracture method (fig. 2) and evaluated by comparing it morphometrically with cells taken from a kidney. The tight junction in collecting duct cells from the rabbit kidney consists of 5–7 anastomosing strands (fig. 2a). Physiological studies have shown that precisely this number of strands is needed to maintain a functional seal, which can be demonstrated electrophysiologically in a resistance of at least 0.5 kΩ cm^{-2} [20]. Cultured cells were also able to form such tight junctions with 5–7 strands, like those in the collecting duct cells of the adult kidney. Such impermeable tight junctions, however, were only found in cells which had been cultured on capsula fibrosa (fig. 2b). If the cells were grown on the bottom of an untreated culture plate, on the other hand, tight junctions were formed which consisted of only 2 or 3 strands (fig. 2c). Similar conditions are found in embryonic epithelia [21, 22]. A tight junction with 2–3 strands is not sufficient to form a functional barrier. Our experiments clearly show that the cell support material by itself can already determine whether or not a functional barrier will be formed.

The Development of Special Transport Porperties

Another important criterion for the functionality of the collecting duct epithelium cells is whether the transport functions found in the organ are retained under culture conditions, or if crucial functions are lost. We were able to show in several papers that the collecting duct epithelia we cultured are well suited for studying the physiological and biochemical effects of aldosterone [23, 24]. However, the prerequisite is that the collecting duct cells are cultured on a support of capsula fibrosa from the kidney

Table 1. Collecting duct-specific proteins

Protein	MW kD	mAb	Collecting duct cells in the kidney	Primary CD cultures on capsula fibrosa	Primary CD cultures on plastic	Ref. No.
$GP_{CD}1$	150	pk	+	+	+	30
$GP_{CD}2$	85	pk	+	+	–	30
$P_{CD}1$	190	mk	+	–	–	27
$P_{CD}2$	210	mk	+	+	–	27
$P_{CD}3$	45	mk	+	+	–	27
$P_{CD}4$	40	mk	+	+	–	31
$P_{CD}6$	–	mk	+	+	o	32
$P_{CD}9$	32/39	mk	+	+	o	33

The expression of collecting duct-specific proteins can be demonstrated immunohistochemically with special antibodies. It can be seen that cells cultured on the bottom of a culture plate do not produce specific CD proteins. If the cells are cultured on the organ-specific capsula fibrosa, however, most of these CD proteins are produced. These results underscore the importance of the right cell support material for the development of specific functions.

+ = Present; – = not present; o = not tested; pk = polyclonal; mk = monoclonal.

(fig. 1b, 2b). Aldosterone plays a decisive role in the formation of the functional seal of the eqithelium [24]. In comparison to controls, aldosterone raises the transepithelial resistance in the cultured epithelium to more than 0.5 $k\Omega\ cm^{-2}$ and thus induces a selective seal. A similar value can be demonstrated in the collecting duct epithelium of the kidney and other tissues [20]. The effect of aldosterone began much earlier than had been assumed until then [25], because a noticeable increase in sodium transport could already be seen about 15 min after the application of aldosterone, even though aldosterone-induced proteins were not expressed until approximately 6 h later [26]. An important finding turned out to be that increased sodium transport was associated with the simultaneous expression of collecting-duct-specific proteins ($P_{CD}2,3$) in the apical plasma membrane of the cultured collecting duct epithelia. These proteins are found in the collecting duct epithelium of the adult kidney and on epithelium cultured on capsula fibrosa, but not in the embryonic collecting duct ampullas of the maturing kidney [24].

In contrast, water transport experiments with cultured collecting duct cells have failed to yield the expected results [27]. The water reabsorbed from the collecting duct lumen passes through special water channels or aquaporins into the cell interior and is pumped out again on the basolateral side of the cell [18]. Under the electron microscope such water entry sites can be seen on the apical side of principal cells in the kidney. These water channel structures can also be seen on cells cultured on capsula fibrosa, but not on collecting duct cells grown on the bottom of culture plates. Further, a regulatory subunit of adenylate cyclase which stimulates water transport is not sufficiently expressed in the epithelial cells or is not functionally connected with the receptor, preventing vasopressin from developing its stimulatory effect.

Special Antibodies Signalize the Difference between Embryonic and Functional Cells

We began to look for specific marker proteins which could be used to positively identify the differential stage of collecting duct cells (table 1) [27, 28]. Since such marker proteins were unavailable at the time, we developed our own monoclonal antibodies reacting with the collecting duct cells of the adult and maturing rabbit kidney [29]. However, the embryonic cells of the collecting duct ampulla do not express the proteins recognized by the antibodies [24]. This makes it possible to readily distinguish between mature and still embryonic cells. For example, the protein $P_{CD}2$, could be demonstrated in the plasma membrane of adult collecting duct cells in the kidney [24]. The antibody also reacts with single epithelial cells cultured on capsula fibrosa. If the epithelial cells are grown in the presence of aldosterone, however, the protein is no longer found in isolated cells, but is produced by all of them. At the same time, a transepithelial resistance of

Fig. 3a–c. Kidney-like culture conditions. **a** The cells are grown on a special holder set with individually exchangeable supports. **b, c** In the newly developed cell culture containers the cultures are continuously supplied with fresh medium. In the perfusion container (**b**) the cell holder sets are bathed with the same culture medium on both the top and the bottom. In the gradient perfusion container (**c**), however, the cells are supplied with completely different media from above and below, just like in the actual kidney.

more than 0.5 kΩ cm^{-2} and an amiloride-sensitive sodium transport can be demonstrated in the cultured epithelium, just like in the kidney [23, 24].

Differentiation and Dedifferentiation of Collecting Duct Cells in Culture

We ran additional experiments to clarify whether collecting duct cells cultured on the bottom of culture plates (fig. 1c, 2c) express typical collecting-duct proteins in the same way as cells grown on a capsula fibrosa support (fig. 1b, 2b). In only one case did collecting duct cells grown on culture plates show a weak immunohistochemical reaction to the antibodies used (table 1; $GP_{CD}1$) [30]. Thus, immunological identification criteria [27, 28, 30–33] showed that cells grown on the bottom of culture plates bear absolutely no resemblance to collecting duct epithelium cells grown on capsula fibrosa or isolated from the adult or maturing kidney. In contrast, the immunohistochemical reaction pattern of cells grown on capsula fibrosa was typical for the organ (fig. 1b, 2b) with the exception of $P_{CD}1$ (table 1) [27, 30–33]. In the collecting duct cells grown on the bottom of culture plates we were no longer able to recognize the typical morphology (fig. 1c), nor did we find completely developed tight junctions (fig. 2c) or typical water channels. The downregulation of typical collecting-duct proteins is therefore not an isolated phenomenon, but is closely connected with a loss of epithelium-specific properties.

Simulating the Microenvironment in the Kidney

In the experiments described until now the collecting duct epithelium cells were kept in a static environment for days. Although it was possible to culture principal cells as a functioning epithelium in these experiments [23], we were unable to observe any intercalated cells under static culture conditions, despite using capsula fibrosa as a support. Our goal for further experiments was to create culture conditions as closely as possible the conditions in the kidney [34, 35]: (1) the cells should be grown on a support material as organ-specific as possible (fig. 3a); (2) the culture medium should be constantly exchanged (fig. 3b); (3) it should be possible to apply different media on the luminal and the basal sides of the epithelium to create a natural gradient under experimental conditions (fig. 3c).

The new culture technique makes it possible to grow the cells in their natural extracellular matrix environ-

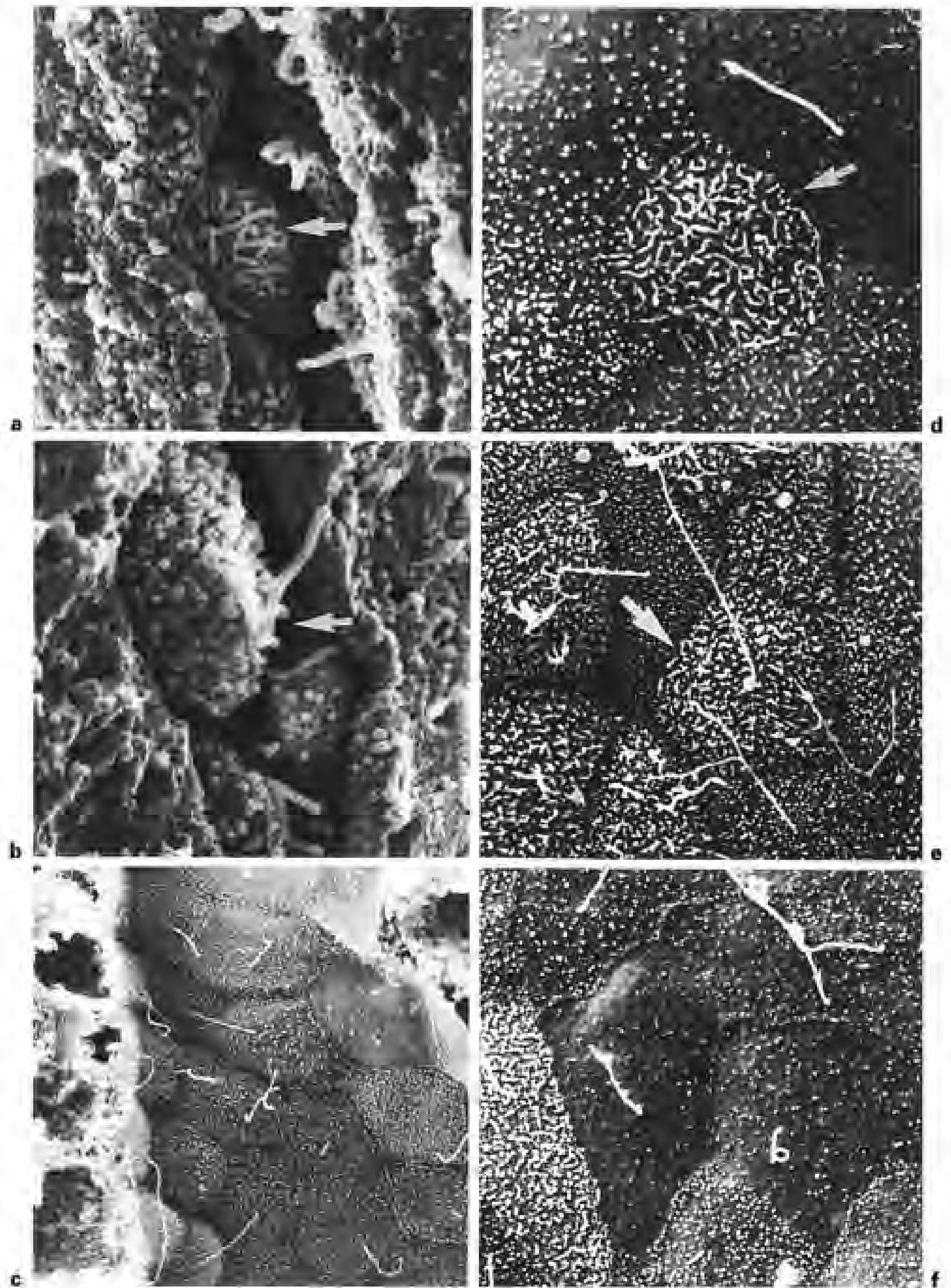

Fig. 4a–f. Morphological quality control. Scanning electron microscope view of the cell surfaces of the collecting duct in the neonatal rabbit kidney (**a–c**) and of collecting duct cells cultured on capsula fibrosa under perfusion conditons (**d–f**). In the kidney the following cell types can be recognized: **a** α-type intercalated cells (arrow). **b** β-type intercalated cells (arrow). **c** Principal cells. Under perfusion culture the following cell types can be identified on the basis of morphological features: **d** α-type intercalated cells (arrow). **e** β-type intercalated cells (arrow). **f** Principal cells. Thus, the same cell-surface structures are developed under perfusion culture conditions as in the kidney. **a, b** × 10,000, **c** × 3,000; **d–f** × 4,500.

ment. There is a permanent and controllable exchange of liquids and gases through medium perfusion. The result is that differentiation-regulating factors are not accumulated continuously in ever greater concentrations which could be harmful to metabolism, but are maintained at a constant level [36, 37]. The creation of a medium gradient makes it easier for the epithelium to organize its polar differentiation. By removing growth factors and high serum concentrations we attempted to provide the cells with contact inhibition typical for the organ and not expose them to constant mitotic stress. In the natural cell cycle, a dividing cell cannot simultaneously perform organ-specific functions. In consequence, labeling the cultured collecting epithelia after perfusion culture with the proliferation marker Ki 67 gave no reaction and shows a postmitotic status.

The Creation of a Heterogeneous Epithelium Cell Population

In the adult kidney, intercalated cells are found along with principal cells [3, 38]. Under the scanning electron microscope it is especially easy to recognize intercalated cells because of their striking surface structures [39]. The α-type intercalated cells have long folds on the luminal side of the cell (fig. 4a), while the aciliate β-type has long, very dense microvilli (fig. 4b). The principal cells can be recognized by their single cilium and short, relatively sparse microvilli (fig. 4c). The natural cellular heterogeneity in the kidney is not found in collecting duct epithelium grown under the static conditions of a conventional culture plate (fig. 2c). Even when capsula fibrosa is used as cell support material to encourage differentiation, we do not see differentiation towards the intercalated cell type. Only when the cells are grown on special supports (fig. 3a) and continuously supplied with fresh culture medium in a specially constructed perfusion container (fig. 4b) do we see the differentiation characteristics familiar to us from the kidney [40]. Isolated α-type intercalated cells could be seen (fig. 4d). By and large, however, we find cells with long, dense microvilli typical for the beta-type intermediate cells (fig. 4e). It was striking that many of these cells had a cilium not found in this cell type in the adult kidney. However, such a cell type with one cilium was identified in the maturing neonatal kidney during a brief developmental stage (fig. 4b). We can conclude that after perfusion the cultured cells are phenotypically similar to the maturing cells of the neonatal kidney, but are still lagging behind the adult kidney cells in their development.

Intelligent Bioengineering Results in New Discoveries

Without exception all of our cell culture experiments so far have shown that we must recreate the natural conditions in the kidney as closely as possible if we wish to generate cell cultures of similar quality as in the organ. Our present research efforts with cultured collecting duct epithelium are directed towards matching the histophysiological concept of the kidney. Since epithelial cells always form a boundary surface, they are exposed to completely different environmental conditions on the apical than on the basal side. The epithelium of the kidney forms the barrier between the urine and blood compartments. These are exactly the conditions we would like to simulate in future experiments with cultured collecting duct epithelium. We will use a special gradient perfusion container (fig. 3c) to bath the epithelium with completely different media on the top and the bottom for the entire duration of the culturing process. First results with this new culturing technique indicate that the embryonic epithelial cells have a different distribution of receptors for the steroid hormone aldosterone than the adult kidney. In adult tissue, the receptors for aldosterone are described on the basolateral side of the cell [41]. When the cultured embryonic collecting duct epithelium was provided with aldosterone from the apical side, however, we were surprised to find the same properties as would have been developed if only the basolateral side had been exposed to the hormone, like in the adult kidney.

By comparing embryonic and functionally mature cells from the kidney on the one hand (fig. 4a–c) with cells grown under improved culture conditions on the other (fig. 1b, c, 4d–f), we tried to determine clear differentiation criteria for the in vitro situation. In the process we learned that cultured cells do not necessarily mature into high-quality, i.e., specially differentiated organ cells, but can only attain full development with the aid of culture techniques which mimic the organ with respect to morphology, physiology and biochemistry. It is not only the extracellular matrix [42] and the differentiation hormones [37] which have an enormous effect on the development of cell properties, but also environmental conditions such as the electrolytes present or a varying pH [43].

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