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In vitro development and preservation of specific features of collecting duct epithelial cells from embryonic rabbit kidney are regulated by the electrolyte environment

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Abstract During kidney development the embryonic collecting duct (CD) epithelium changes its function. The capability for nephron induction is lost and the epithelium develops into functional principal (P) and intercalated (IC) cells. Aldosterone is able to modulate this differentiation. Consequently we investigated whether increased concentrations of extracellular NaCl or Na gluconate may also have an influence on the development of individual CD cell features. Embryonic CD epithelia were isolated from neonatal rabbit kidneys, placed on tissue carriers and cultured in gradient containers, which were constantly perfused with medium for 13 days. Isotonic culture conditions could be mimicked, when on both the luminal and basal side standard Iscove's Modified Dulbecco's Medium (IMDM) was used. In another set of experiments, gradient culture was performed. Standard IMDM was applied on the basal side and IMDM supplemented with 12 mM NaCl and 17 mM Na gluconate on the luminal side. This adaptation of IMDM led to the same Na concentrations as found in the serum of neonatal rabbits. The development of CD cell features was monitored by cellular markers such as the monoclonal antibodies (Mabs) 703 and 503 recognizing P and IC cell features respectively. Epithelia cultured under isotonic conditions showed less than 5% Mab 703- and 503-immunopositive cells. In contrast, epithelia cultured in a luminal-basal medium gradient revealed more than 80% positive cells. Immunoreactivity started to develop after a long lag period of 4 days, then increased continuously during the following 5 days and reached a maximum at day 14. When the medium gradient was then changed to an isotonic environment for another 5 days immunoreactivity for Mab 703 remained stable, while the number of Mab 503-positive cells was found to be decreased to 10%. Thus, the extra-

cellular electrolyte environment not only induces but also preserves individual cell features.

Introduction

The development from an embryonic into a functional nephron epithelium starts when the tubular portion has elongated to its final length. The cells downregulate the mitotic activity, attach closely to the basement membrane, upregulate integrin synthesis, polarize and develop intercellular junctions [3, 6, 24, 31, 32, 37, 39]. The maturation is completed, when functional proteins such as channels, pumps and cotransporters have been synthesized, so that the tissue can start to work as it is known from the adult kidney [10, 14, 28, 35, 36].

Each of the individual segments of secretory nephrons consists of a homogenous cell population, which derives from cells within the S-shaped body [29, 32]. In contrast, the collecting duct (CD) epithelium originates from a highly specialized tissue within the CD ampulla. As an embryonic inducer it first generates all of the nephron anlagen [29]. Then after the induction process the ampulla elongates and develops into a heterogeneously composed epithelium consisting of principal (P) and different kinds of intercalated (IC) cells [1, 2, 13, 27].

Little knowledge is available about the mechanism by which different cell types arise in the renal collecting duct system. Some data is available from tissue culture experiments on embryonic CD epithelia. Aldosterone is known to stimulate the expression of peanut lectin binding molecules, a typical feature of β -type IC cells [20]. Amiloride was shown to influence the development of a P cell feature [21]. Thus, it appeared obvious that the extracellular Na metabolism in general is involved in the differentiation of P and IC cells. Further experiments showed that addition of 12 mM NaCl and 17 mM Na gluconate to standard Iscove's Modified Dulbecco's Medium (IMDM) led to a drastic increase of antibody binding cells signaling expression of individual P and IC cell features in the embryonic CD epithelium [23].

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The extracellular environment influences mitosis, differentiation and function of kidney cells [4, 8, 9, 11, 17, 25, 26, 30, 34, 38, 40], especially in the collecting duct [9, 11, 26]. It is unknown whether the extracellular environment triggers only the primary appearance of CD cell features in the embryonic epithelium or also controls the preservation over a period of time. Consequently, we treated embryonic collecting duct epithelia with standard IMDM and IMDM containing additional Na. Then after 14 days the medium was switched back to standard IMDM. By immunohistochemical methods we analyzed the primary appearance, preservation and loss of individual CD cell features.

Methods

Tissue isolation and generation of an embryonic collecting duct epithelium

Cortical explants from the kidneys of newborn New Zealand rabbits were isolated according to methods described earlier [18]. The explants were mounted in sterile tissue carriers (Fig. 1a; Minucells and Minutissue, Bad Abbach, Germany), which were placed in 24-well culture dishes (Greiner, Nürtingen, Germany). The explants consisted of a piece of the fibrous capsule with adherent collecting duct ampullae, S-shaped bodies and nephrogenic blastema. During the culture of these explants in Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL-Life Technologies, Eggenstein, Germany) and 10% fetal bovine serum (Boehringer, Mannheim, Germany) an outgrowth of cells from the CD ampullae was observed. Within 24 h after the initiation of culture the entire surface of the explant, 6 mm in diameter, was covered by a polarized CD epithelium. Culture for the first day was carried out in a tissue incubator (Heraeus, Hanau, Germany) at 37° C in a humidified atmosphere containing 5% CO₂/95% air.

Perfusion culture of embryonic collecting duct epithelia in a gradient container

Twenty four hours after the initiation of culture the epithelia on the tissue carriers (Fig. 1a) were transferred to a newly developed gradient culture container (Fig. 1b; Minucells and Minutissue) to create optimized culture conditions [19, 21–23]. Fresh medium was continuously superfused on the luminal and basal sides of the epithelia for the further culture period of 18 days (Fig. 1c). The container was placed on a 37° C heating plate (Medax, Kiel, Germany). IMDM [12] without serum was used as the standard medium which was continuously perfused at a rate of 1 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). Aldosterone (1×10^{-7} M; Sigma-Aldrich-Chemie, Deisenhofen, Germany) and 1% antibiotic-antimycotic solution (Gibco BRL-Life Technologies) was added to all culture media. Furthermore, 50 mM HEPES (Gibco BRL-Life Technologies) was added to the medium to maintain a constant pH of 7.4 in perfusion culture under laboratory atmosphere. Serum addition to the culture medium during perfusion culture was avoided.

Fig. 1a–c Gradient perfusion culture with a renal collecting duct (CD) epithelium. **a** The renal explant with the epithelium (E), 6 mm in diameter, is mounted in a tissue carrier. **b** Six carriers can be placed in a gradient perfusion container, which is connected to a luminal and a basal perfusion line. **c** A peristaltic pump transports the media and a thermo plate gives the right temperature. The closed gradient container allows luminal and basal perfusion with two different media. Three tissue carriers are superfused with test medium, while the other three carriers are used as controls

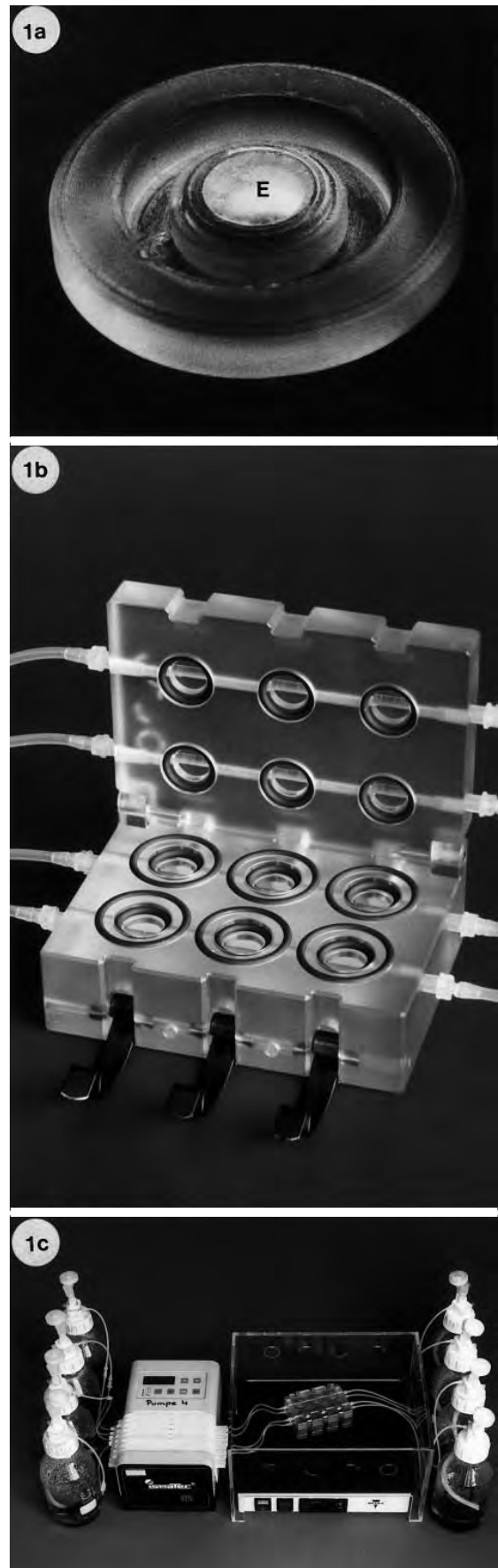


Table 1 Analytical parameters of neonatal rabbit serum, standard Iscove's modified Dulbecco's medium (IMDM), NaCl and NaCl/Na-gluconate-altered IMDM for culturing embryonic collecting duct epithelia. Mean \pm half of range is given

| mM | Serum | IMDM | IMDM+12 mM NaCl/ 17 mM Na-gluconate |
|------------------|-------------------------------|---------------------------------|--|
| Ca ²⁺ | 1.66 \pm 0.46 | 1.15 \pm 0.27 | 1.15 \pm 0.03 |
| K ⁺ | 6.04 \pm 1.7 | 4.25 \pm 0.1 | 4.25 \pm 0.04 |
| Cl ⁻ | 99 \pm 5.8 | 85.1 \pm 1.0 | 98.5 \pm 1.2 |
| Na ⁺ | 137 \pm 5.7 <i>n</i> =17 | 112.3 \pm 1.6 <i>n</i> =40 | 136.9 \pm 1.0 <i>n</i> =32 |

Determination of physiological parameters in the culture medium

Culture parameters such as pH, pCO₂, pO₂, lactate, osmolarity and electrolyte concentrations such as Na⁺, K⁺, Cl⁻, soluble and unbound Ca²⁺ of IMDM or neonatal rabbit serum specimens were determined within an undiluted 200 μ l sample by a Stat Profile 9 Plus analyzer according to the manufacturer's instructions (Nova Biomedical, Rödermark, Germany). Solutions with defined electrolyte concentrations served as controls.

Electrolyte adaptation of the culture medium

Commercially available IMDM (Gibco-BRL Life Technologies) was used as the standard medium [12]. In control series the epithelia were superfused under isotonic conditions from the luminal and basal side with standard IMDM. In experimental series the epithelia were exposed to a luminal – basal medium gradient. At the basal side of the epithelia standard IMDM was superfused. In contrast, the luminal side was superfused with altered IMDM. The content of electrolytes in IMDM and serum are different. For that reason the Na concentration of the standard IMDM was increased by the addition of 12 mM NaCl and 17 mM Na gluconate. This alteration of IMDM resulted in 137 mM Na⁺ and 99 mM Cl⁻ as measured in the serum of neonatal rabbits (Table 1). NaCl and Na gluconate were obtained from Sigma-Aldrich-Chemie.

Immunohistochemistry

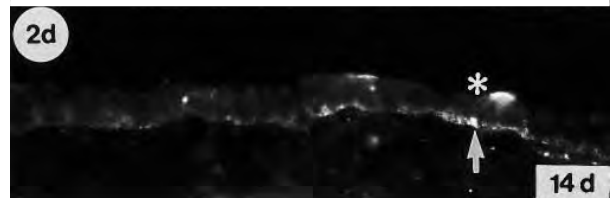
For microscopical examination of cultured epithelia and for immunohistochemical detection of CD proteins 7 μ m-thick cryosections were prepared with a Cryostat HM 500 (Microm, Walldorf, Germany). For histological control sections were stained with toluidine blue solution (results not shown). The following antibodies were used for immunolabeling: Mab 703 recognizes P cells, while Mab 503 detects IC cells of adult renal collecting duct cell cultures [33]. Mab P_{CD} 9 binds to a 32/39-kDa protein on all of the CD cells of the neonatal kidneys [16]. Immunolabeling was started by fixing the cryosections for 10 min in ice-cold ethanol as described earlier [15]. After several rinses with phosphate-buffered saline (PBS), pH 7.2, the sections were incubated with a blocking solution (PBS) containing 10% horse serum and 1% bovine serum

Fig. 2a–h Immunofluorescence microscopy of CD epithelia, under isotonic culture conditions. Labeling of Mab 703 (a, b), Mab 503 (c, d), peanut agglutinin (PNA) (e, f) and Mab P_{CD} 9 (g, h) on embryonic collecting duct epithelia cultured for 1, or 14 days in a gradient container with standard IMDM on the luminal and basal sides. After day 1 Mab 703 (a), Mab 503 (c) and PNA (e) binding had not developed, while all of the cells were labeled with Mab P_{CD} 9 (g). After day 14 less than 5% of the cells showed binding with Mab 703 (b) and Mab 503 (d), while more than 80% of cells reacted with PNA (f) respectively 100% with Mab P_{CD} 9 (h). *bar* 20 μ m *asterisk* luminal side, *arrow* basal aspect of the epithelium

mab 703



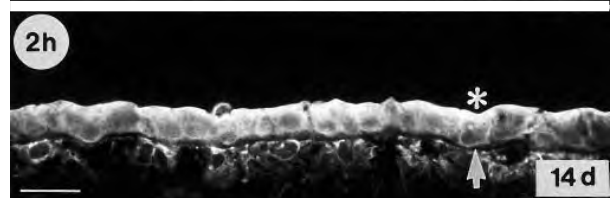
mab 503



PNA



P_{CD} 9



albumin (BSA) for 30 min. The primary antibodies Mab 703 and Mab 503 (each of them diluted 1:100 in blocking buffer) and Mab P_{CD} 9 (undiluted hybridoma supernatant) were incubated for 1.5 h. After several rinses with PBS/1% BSA the sections were treated for 45 min with a donkey-anti-mouse-IgG-fluorescein-isothiocyanate (FITC)-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1:200 in blocking buffer. After being rinsed several times in PBS/1% BSA the specimens were coincubated for 45 min with a PNA-rhodamine conjugate (Vector, Burlingame, Vermont, USA) diluted 1:2000 in PBS to detect β -type IC cell features [20, 21, 27]. To label the nuclei and to determine the percentage of labeled cells in the epithelium 4 μ g/ml propidium iodide (Sigma-Aldrich-Chemie, Deisenhofen, Germany) solution in PBS was used. Following several washes with PBS the sections were embedded in FITC guard (Testoc, Chicago, Illinois, USA) and examined using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

Evaluation

To determine the proportion of immunopositive cells in the cultured epithelia a double labeling procedure was applied. The epithelia were first labeled with the nuclear marker propidium iodide (not shown) and then with a cellular marker such as Mab 703, 503, PNA or Mab P_{CD} 9 (Figs. 2–6). By this method the amount of Mab-labeled and -unlabeled cells within the epithelium could easily be determined. The mean of immunopositive cells is given in the individual experimental series. In total more than 100 epithelia were examined from gradient perfusion culture experiments for the present investigation. Each treatment was repeated more than three times and at least 5 epithelia were analyzed per experimental series.

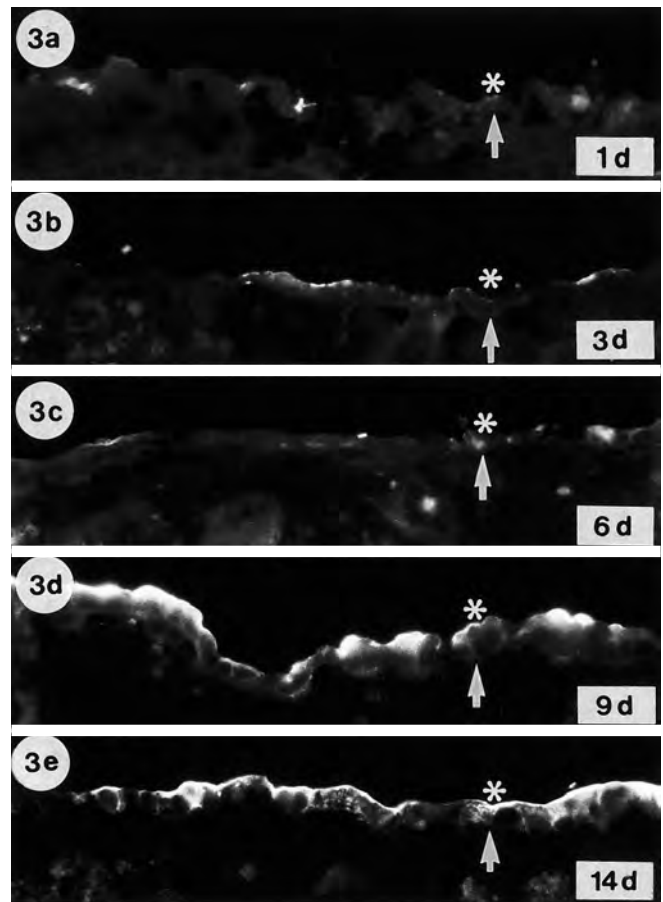
Results

Culture strategy

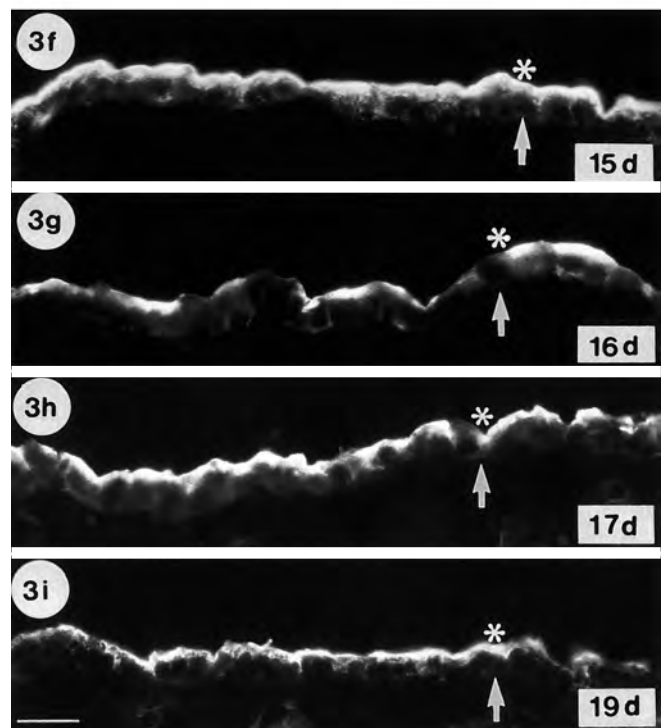
We used embryonic CD epithelia to investigate the mechanisms involved in the development and preservation of differentiation [22]. A prerequisite for reaching a high degree of cellular differentiation under in vitro conditions was that the epithelia be kept in an environment which was optimized as far as possible. Thus, the embryonic tissue was kept on its original extracellular matrix environment in a carrier (Fig. 1a). To mimic a natural fluid exchange, the epithelia were placed in a gradient culture container (Fig. 1b). Six epithelia could be placed in such a container. Three epithelia were run as a control, while the other three were used as the experimental series. The epithelia on the tissue carriers separate the container into an upper and a lower compartment (Fig. 1b, c).

Fig. 3a–i Immunofluorescence microscopy of CD epithelia under gradient culture. Development of Mab 703 antigen expression in embryonic CD epithelia cultured for 19 days in a gradient container. At the basal side of the epithelium standard IMDM was used, while on the luminal side IMDM including 12 mM NaCl and 17 mM Na gluconate was superfused for 14 days. Then on day 14 the medium was changed. Until day 19 standard IMDM was applied. During a lag phase of 6 days (a–c) only minor Mab binding developed, while 70%–90% of cells showed reactivity after 9 (d) or 14 (e) days. When the luminal medium was changed back to standard IMDM for another 5 days 90%–80% (f–i) of cells show intensive labeling with the antibody. *bar* 20 μ m *astersik* luminal side, *arrow* basal aspect of the epithelium

mab 703



switch



Thus, it was possible to superfuse the apical and basal sides of the epithelia with standard IMDM and to mimic isotonic conditions in the first series (Fig. 2).

In a second series of experiments gradient culture conditions were simulated by using standard IMDM at the basal side and IMDM supplemented with additional 12 mM NaCl and 17 mM Na gluconate at the luminal side (Figs. 3–6). This alteration of IMDM results in an adaptation to Na and Cl electrolyte parameters found in the serum of neonatal rabbits (Table 1).

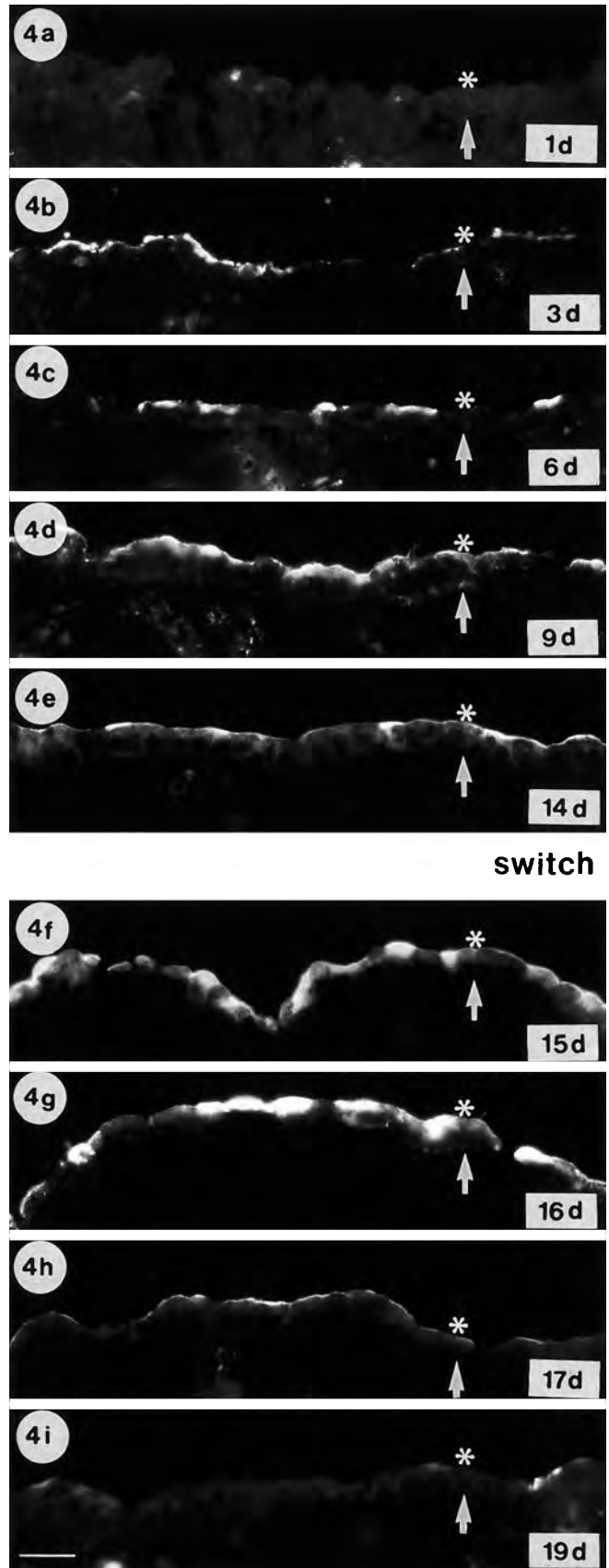
In all cases we examined the appearance of all of the cultured epithelia under the light microscope after they had been exposed to a constant medium flow in the gradient container for a period of 19 days. Over a diameter of 6 mm the surface of the renal explant on the tissue carrier (Fig. 1a) was completely covered by an epithelium. Single epithelia were additionally analyzed by electron microscopy. Independent from isotonic (Fig. 2) or gradient (Figs. 3–6) culture conditions all of the specimens revealed a perfect morphology of isoprismatic cells with distinct polarization (data not shown). Clearly visible tight junctions separated the luminal from the basolateral plasma membrane. A basement membrane was consistently developed as shown in a recent publication [21].

1 Suppressed development of Mab 703 and Mab 503 binding under isotonic culture conditions in a gradient culture container

We analyzed by immunohistochemical methods the primary appearance of Mab 703, Mab 503, PNA and Mab P_{CD} 9 binding in the embryonic epithelia from the first until the 14th day of culture (Fig. 2). In this series of experiments the embryonic CD epithelia were cultured in a gradient container, but under isotonic conditions with standard IMDM at the luminal and basal sides of the tissue (Table 1, Fig. 7a). On day 1 no immunoreaction was found with Mab 703 (Fig. 2a), Mab 503 (Fig. 2c) or PNA (Fig. 2e), while Mab P_{CD} 9 (Fig. 2g) labeled all of the cells within the collecting duct epithelium. On day 14 less than 5% of the cells were reacting with Mab 703 (Fig. 2b) and Mab 503 (Fig. 2d). In contrast, more than 80% of cells showed PNA binding (Fig. 2f) and all of the cells revealed immunohistochemical labeling with Mab P_{CD} 9 (Fig. 2h).

Fig. 4a–i Immunofluorescence microscopy of CD epithelia under gradient culture. Development of Mab 503 antigen expression in embryonic collecting duct cells cultured for 19 days in a gradient container. At the basal side of the epithelium standard IMDM, while on the luminal side IMDM including 12 mM NaCl and 17 mM Na gluconate was superfused for 14 days. Then on day 14 the medium was changed. Until day 19 standard IMDM was applied. During a lag phase of 6 days (a–c) only minor MAb binding up to 30% developed, while 70%–80% of cells showed reactivity after 9 (d) or 14 (e) days. When the luminal medium was changed back to standard IMDM for another 5 days immunoreactivity decreased from 80% to 10% (f–i). *bar* 20 μ m *asterisk* luminal side, *arrow* basal aspect of the epithelium

mab 503



2 Development of individual CD cell features under gradient culture conditions

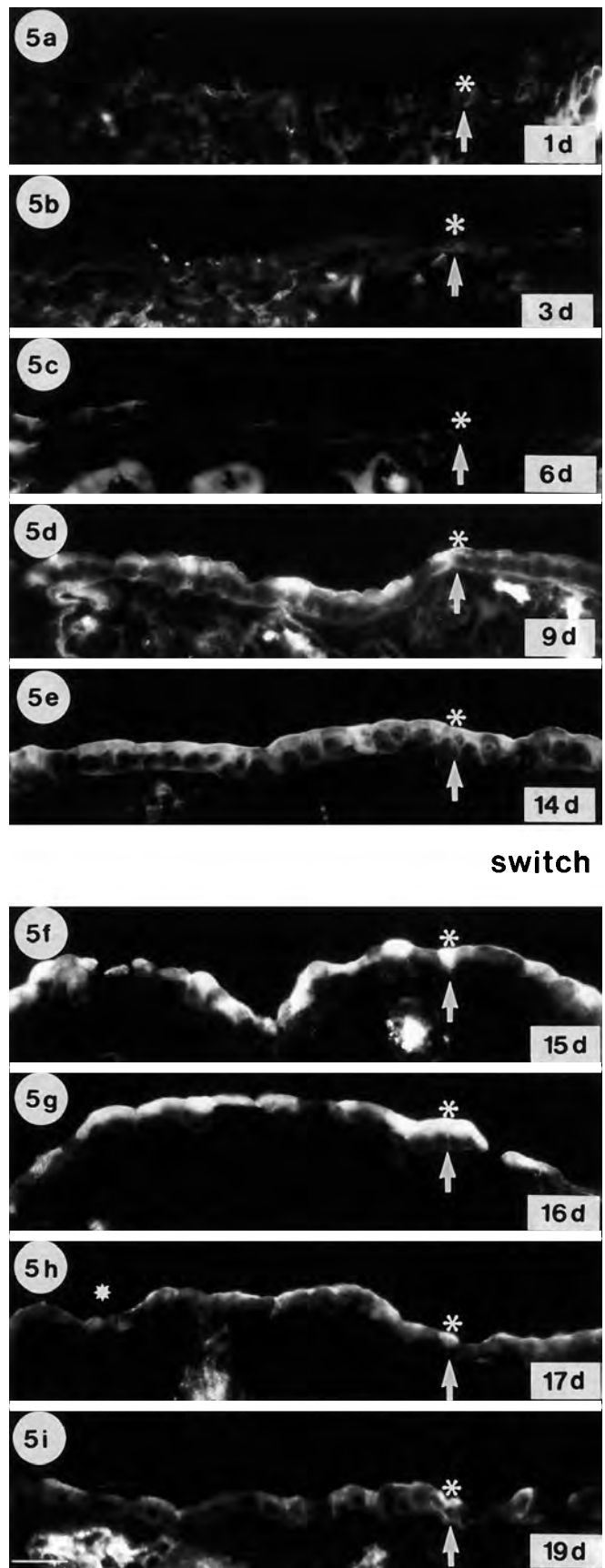
In the second series of experiments the embryonic CD epithelia were cultured under gradient culture conditions. Standard IMDM was used at the basal side, while IMDM altered with additional 12 mM NaCl and 17 mM Na gluconate was superfused at the luminal side for 14 days (Table 1, Figs. 3–6;a–e; 7b). At day 14 IMDM altered with additional NaCl and Na gluconate was replaced with standard IMDM for another 5 days (Table 1, Figs. 3–6;f–i). During the starting period we could observe whether high Na IMDM provoked the developed of individual cell features, while in the culture period we could investigate whether a constant level of antigen expression remained or whether a downregulation occurred.

2.1 Primary appearance and maintenance of Mab 703 binding. Mab 703 binding is localized on P cells of CD cell cultures derived from adult rabbit kidney [33]. On cultured embryonic CD epithelia the antigen was found on less than 5% of all of the cells on day 1 (Fig. 7b, Fig. 3a). By days 3 (Fig. 3b) and 6 (Fig. 3c) only 10% of the cells were positive. Then on day 9 more than 70% (Fig. 3d) and on day 14 more than 90% (Fig. 3e) of the cells became positive of Mab 703. Switching the culture medium at the luminal side from high-Na IMDM to standard IMDM did not affect the antigen expression. From day 15 until day 19 (Fig. 3f–i) between 80% and 90% immunopositive cells were found. As compared to cultures with standard IMDM (Fig. 2a, b) high-Na IMDM induced the development of Mab 703 binding. Changing the extracellular fluid environment back to standard IMDM did not affect antigen expression in this experimental series.

2.2 Primary appearance and subsequent disappearance of mab 503 binding. Mab 503 recognizes IC cells of CD cell cultures derived from adult rabbit kidney [33]. On cultured embryonic CD epithelia binding of the Mab was found on less than 5% of the cells on day 1 (Fig. 7b; Fig. 4a). On day 3 (Fig. 4b) and on day 6 (Fig. 4c) 30% immunopositive cells were found. A further increase of immunoreactivity was found on day 9 (Fig. 4d) and day 14 (Fig. 4e), when up to 70% and 80% of the cells, respectively were positive for the antibody. When the high Na IMDM was switched to standard IMDM at the luminal

Fig. 5a–i Immunofluorescence microscopy of CD epithelia under gradient culture, showing development of PNA expression in embryonic collecting duct cells cultured for 19 days in a gradient container. At the basal side of the epithelium standard IMDM was applied, while on the luminal side IMDM including 12 mM NaCl and 17 mM Na gluconate was superfused for 14 days. Then on day 14 the medium was changed. Until day 19 standard IMDM was applied. During a lag phase of 6 days (a–c) only minor Mab binding up to 5% was developed, while 40%–100% of cells showed reactivity after 9 (d) or 14 (e) days. When the luminal medium was switched back to standard IMDM for another 5 days immunoreactivity decreased from 90% to 30% (f–i). *bar* 20 μ m, *asterisk* luminal side, *arrow* basal aspect of the epithelium

PNA



side of the gradient container on day 15 (Fig. 4f) and day 16 (Fig. 4g) 80% immunopositive cells were still detected. However, on day 17 (Fig. 4h) the immunoreactivity decreased to 50% positive cells. Then on day 19 (Fig. 4i) only 10% labeled cells were found. Thus, replacing high Na IMDM with standard IMDM on day 14 resulted in a dramatic decrease of immunopositive cells in the cultured epithelium.

2.3 Primary appearance and subsequent disappearance of PNA binding. PNA binding is localized on the β -type IC CD cells of the adult rabbit kidney [27]. On cultured embryonic CD epithelia PNA binding was found on less than 1% of the cells on day 1 (Figs. 5a, 7b). On day 3 (Fig. 5b) and 6 (Fig. 5c) less than 5% positive cells were detected. In contrast, 40% positive cells were found on day 9 (Fig. 5d). Nearly all of the cells were positive on day 14 (Fig. 5e). When the high Na IMDM was switched to standard IMDM on day 15 (Fig. 5f) and day 16 (Fig. 5g), 90% and 80% PNA-positive cells, respectively, were found. On day 17 (Fig. 5h) the percentage of labeled cells decreased to 70%. Finally on day 19 (Fig. 5i) only 30% of the cells remained positive for PNA binding. As compared to cultures with standard IMDM (Fig. 2e, f) high-Na IMDM did not affect the development of PNA binding. PNA binding is exclusively triggered by aldosterone [19–22]. However, despite the presence of aldosterone in all media the switch from high-Na IMDM to standard IMDM resulted in a constant downregulation of PNA binding.

2.4 Constant mab P_{CD} 9 binding. Mab P_{CD} 9 labels all of the collecting duct cells in the neonatal rabbit kidney [16]. Starting from day 1 (Figs. 6a, 7b) until day 14 (Fig. 6e) the antibody labeled all of the collecting duct cells. When the high-Na IMDM was switched to standard IMDM from day 15 (Fig. 6f) until day 19 (Fig. 6i) no disappearance of the antigen was observed. Consequently, the change of the culture medium did not affect the expression of the antigen.

Summarizing the time course of development of Mabs 703 (Figs. 3a–c), 503 (Figs. 4a–c) or PNA (Fig. 5a–c) binding the embryonic CD epithelia showed after days 1, 3 and 6 none or only minor immunoreactivity. In contrast, after days 9 and 14 (Figs. 3d, e; 4d, e; 5d, e) numerous cells with an intensive label were found. For the first time it became obvious to us that the development

Fig. 6a–i Immunofluorescence microscopy of epithelia under gradient culture. Kinetics of Mab P_{CD} 9 binding development in embryonic collecting duct cells cultured for 19 days in a gradient container. At the basal side of the epithelium standard IMDM was applied, while on the luminal side IMDM including 12 mM NaCl and 17 mM Na gluconate was superfused for 14 days. Then on day 14 the medium was changed. Until day 19 standard IMDM was applied. From day 1 until day 14 (a–e) all of the collecting duct cells show immunolabeling with the antibody. Even when the luminal medium was switched back to standard IMDM for another 5 days (f–i) all of cells retained the intensive label with the antibody. *bar* 20 μ m, *asterisk* luminal side, *arrow* basal aspect of the epithelium

P_{CD} 9

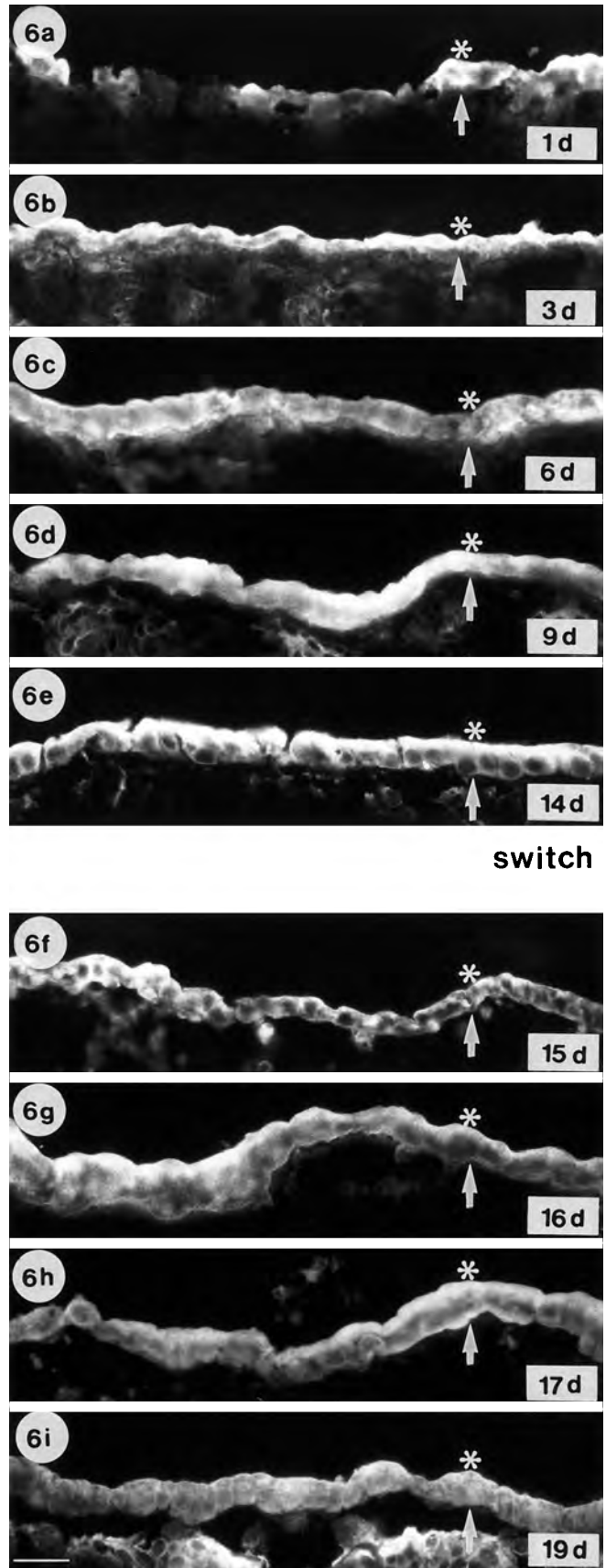
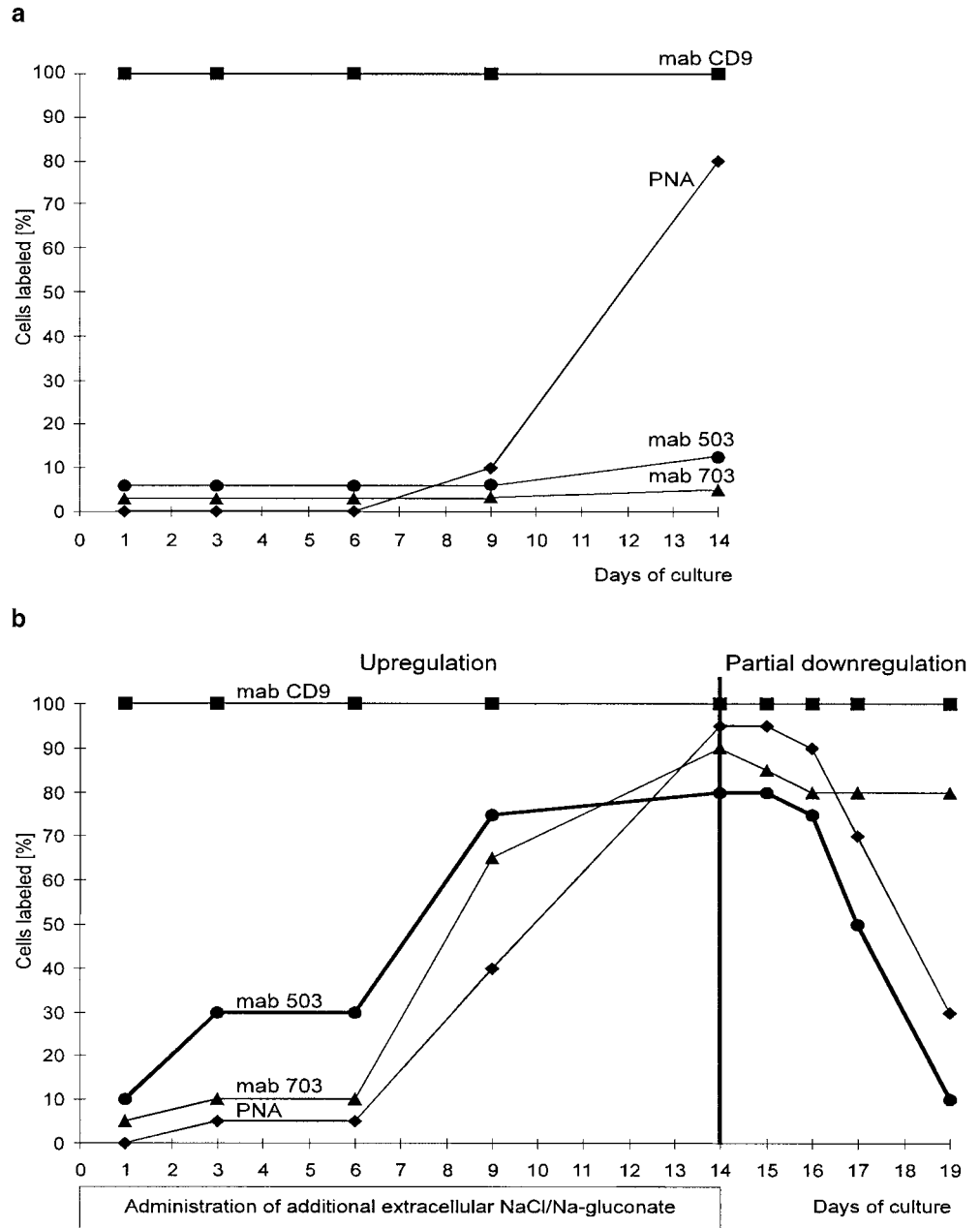


Fig. 7a, b Time course of Mab 703, Mab 503, Mab P_{CD 9} and peanut agglutinin (PNA) binding development on embryonic collecting duct epithelia cultured in a gradient container. **a** Isotonic conditions were simulated by perfusing standard IMDM at the luminal and basal sides of the container. **b** Gradient culture conditions were simulated by superfusing at the basal side of the epithelium standard IMDM, while on the luminal side IMDM plus 12 mM NaCl and 17 mM Na gluconate was superfused for 14 days. On day 14 the medium was switched. Until day 19 standard IMDM was applied. Mab P_{CD 9} labeling is constantly developed in all of the embryonic epithelial cells. In contrast, Mab 703, Mab 503 and PNA binding appears after a lag phase of 6 days in a minority of cells. Between days 9 and 14 between 80% and 90% of cells are labeled by the markers. After switching the culture medium back to standard IMDM Mab P_{CD 9} and Mab 803 labeling remain constant, while the amount of Mab 503 and PNA binding cells is dramatically decreased. A mean of 3 experimental series is given



of cell features started with an unexpectedly long lag period between 3 and 6 days (Fig. 7b). Between days 6 and 14 the expression of the individual features increased continuously.

Discussion

Different mechanisms modulate differentiation

We investigate factors affecting the transition from an embryonic into an adult renal CD epithelium with its different cell types. This functional development of renal epithelia depends on the one hand on structural elements such as the extracellular matrix [32] and on the other

hand on soluble morphogenic substances such as vitamin A derivatives [37], growth factors or hormones [3, 20, 39]. In addition to these very heterogenous developmental stimuli it was found that not only changes in the pH or osmolarity [9, 26, 30, 40], but also variations in the extracellular electrolyte composition [23] play an important role in the primary appearance and preservation of individual cell features.

Primary appearance of individual cell features is induced by the extracellular fluid environment

We isolated embryonic renal CD cells from neonatal rabbit kidneys and brought them into culture. Experiments

in conventional culture dishes with a stagnant environment had failed and typical features had not been developed [22]. Consequently, we improved the culture conditions to obtain a sufficiently high degree of differentiation [19, 21]. The embryonic epithelia were placed in tissue carriers and in gradient containers (Fig. 1). Culture medium was permanently superfused on the luminal and basal sides as observed under natural conditions.

Previous experiments showed that embryonic CD epithelia can be cultured in a container for weeks exhibiting a morphology comparable to the situation within the kidney [1, 2, 21, 22, 23]. Unknown however are the principal mechanisms by which single characteristics of the heterogeneously composed epithelium arise during maturation of the kidney. With the exception of aldosterone [20] no other hormones, growth factors or morphogens were shown to have an influence on individual embryonic CD cell differentiation [39]. Ongoing *in vitro* experiments now demonstrate that the primary appearance of individual CD cell features in the embryonic CD epithelium is additionally triggered by the fluid environment. When the embryonic epithelia are exposed to the same medium at the luminal and basal side (Fig. 2) cells surprisingly develop quite different features as in epithelia exposed to a luminal-basal medium gradient (Figs. 3–6). The experiments gave clear indications that an electrolyte sensor for cytodifferentiation exists in the embryonic CD epithelia.

Epithelia cultured under isotonic conditions in the gradient container develop intensive PNA binding on more than 90% of the cells during the 14-day culture period (Fig. 5). The extensive PNA binding is caused by the administration of aldosterone and is independent of the extracellular electrolyte environment [20]. In contrast, the development of Mab 703 and Mab 503 binding on the cells could not be stimulated solely by the use of standard IMDM or by the administration of aldosterone (Fig. 2, 7a). Less than 5% of the cells developed immunoreactivity for the antibodies. This minor expression of CD features during culture under isotonic conditions (Fig. 2) could be changed completely by exposure of the embryonic epithelia to a fluid gradient (Figs. 3–5; 7b). Differentiation of renal CD epithelia was started by replacing the luminal standard IMDM in the gradient culture container with IMDM containing additional 12 mM NaCl and 17 mM Na gluconate (Table 1). For the first time we observed that the appearance of individual cell features did not start immediately but after an unexpectedly long lag period of 6 days (Fig. 7b). As revealed by immunohistochemistry and a monoclonal antibody Ki 67 [7] the lag period is paralleled at day 1 by high mitotic activity in the CD epithelium, which is completely down-regulated at day 3 indicating that a postmitotic state comparable to the kidney is reached (data not shown). The *in vitro* development took an expectedly long time of 10–12 days until the cellular features were stabilized (Figs. 3–5). An explanation for this long developmental period cannot be given at the moment.

Preservation of differentiation

Little knowledge is available about whether acquired cell features remain stable in the maturing CD epithelia or if they have to be maintained permanently by the extracellular electrolyte environment. New information in this field was obtained by experiments in which the epithelia were first treated with IMDM altered by additional NaCl and Na gluconate followed by a switch to standard IMDM (Figs. 3–6). These experiments showed that the primary appearance of Mab 703 (Fig. 3) and Mab 503 (Fig. 4) is induced by the extracellular electrolyte environment. In contrast, if the high Na load is omitted after the 14th day Mab 503 (Figs. 4f–i) and PNA (Figs. 5f–i) binding cells were found to be diminished within the next 5 days (Fig. 7b). Most astonishingly, Mab 703 binding remained present until the 19th day despite the lack of Na load for 5 days (Fig. 3f–i). Thus, preservation of Mab 503 binding is controlled by the extracellular electrolyte environment, while Mab 703 binding is not affected. Mab P_{CD} 9 binding was affected neither in its up- nor its down-regulation by the extracellular environment (Fig. 6). Thus, our experiments showed that in the embryonic CD epithelia it is possible to induce the primary appearance of individual cell features by IMDM altered with additional NaCl and Na gluconate (Fig. 7b). In contrast, when the medium is switched back to standard IMDM features, such as Mab 503 (Figs. 4f–i) or PNA (Fig. 5f–i) binding are downregulated, while Mab 703 (Fig. 3f–i) or Mab P_{CD} 9 (Fig. 6f–i) binding remain stable. This is the first experimental hint that differentiation features within renal CD epithelia are developed and preserved by the extracellular electrolyte environment. In our experiments we expected increasing concentrations of NaCl in the culture medium to induce more P than IC cell features since abundant Na increases the number of Na channels during kidney development [28, 36]. However, we found that both P and IC cell features in the embryonic collecting duct were influenced.

Generation of a hybrid cell type

Within the cultured CD epithelia the great majority of cells was able to develop binding of Mab 703, Mab 503 and PNA. Thus, β -type IC cell characteristics, but simultaneously also P cell features were developed. This fact can only be explained by the possibility that a hybrid cell type is growing under *in vitro* conditions, as was shown in an earlier publication [2]. For kidney development it indicates that a common precursor cell type for P and IC cells exists.

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