ELSEVIER

Biomaterials 23 (2002) 805–815

Biomaterials

www.elsevier.com/locate/biomaterials

Advanced technique for long term culture of epithelia in a continuous luminal–basal medium gradient

Karl Schumacher, Raimund Strehl, Uwe de Vries, Will W. Minuth*

Department of Anatomy, University of Regensburg, Universitatstrasse 31, D-93053 Regensburg, Germany

Received 4 October 2000; accepted 2 May 2001

Abstract

The majority of epithelia in our organism perform barrier functions on being exposed to different fluids at the luminal and basal sides. To simulate this natural situation under in vitro conditions for biomaterial testing and tissue engineering the epithelia have to withstand mechanical and fluid stress over a prolonged period of time. Leakage, edge damage and pressure differences in the culture system have to be avoided so that the epithelial barrier function is maintained. Besides, the environmental influences on important cell biological features such as, sealing or transport functions, have to remain upregulated and a loss of characteristics by dedifferentiation is prevented.

Our aim is to expose embryonic renal collecting duct (CD) epithelia as model tissue for 14 days to fluid gradients and to monitor the development of tissue-specific features. For these experiments, cultured embryonic epithelia are placed in tissue carriers and in gradient containers, where different media are superfused at the luminal and basal sides. Epithelia growing on the tissue carriers act as a physiological barrier during the whole culture period. To avoid mechanical damage of the tissue and to suppress fluid pressure differences between the luminal and basal compartments improved transport of the medium and an elimination of unilaterally accumulated gas bubbles in the gradient container compartments by newly developed gas expander modules is introduced. By the application of these tools the yield of embryonic renal collecting duct epithelia with intact barrier function on a fragile natural support material could be increased significantly as compared to earlier experiments. Epithelia treated with a luminal NaCl load ranging from 3 to 24 mmol/l were analyzed by immunohistochemical methods to determine the degree of differentiation. The tissue showed an upregulation of individual CD cell features as compared to embryonic epithelia in the neonatal kidney.

Keywords: Epithelia; Biomaterial testing; Tissue engineering; Gradient perfusion culture

1. Introduction

The generation of perfect skin equivalents [1,2], vascular grafts [3,4,6], insulin producing organoids [5,7], liver [8,9], renal tubular [10,11], urothelial [12], esophageal [13] or tracheal [14] artificial modules for biomaterial research and tissue engineering will only succeed if the epithelial tissues exhibit the necessary degree of functionality and a close structural association with the biomaterials used as artificial extracellular matrix. It is of utmost importance to learn, under realistic in vitro conditions, how tightly epithelial cells are attached to matrices under fluid stress over prolonged periods of time and to monitor their influence on cellular differentiation.

The focus of our interest is the mechanisms of transition leading from an embryonic to a mature epithelium with specific functions. As a model system, we use a defined renal epithelium. In parallel, we investigate the differentiation process in the developing kidney and in isolated embryonic tissue cultured by advanced techniques. Our aim is to adapt the culture conditions as closely as possible to the situation within the kidney. A tissue-specific environment for epithelia can be simulated with tissue carriers in combination with gradient culture containers [15,24]. Almost all kinds of biomaterials such as textiles, filters, foils or natural collagenous membranes can be placed into tissue carriers as an artificial matrix or basement membrane substitute [16]. Then, epithelial cells are seeded and grown to confluency on the individual support

*Corresponding author. Tel.: +49-941-943-2876; fax: +49-941-943-2868.

E-mail address: will.minuth@vkl.uni-regensburg.de (W.W. Minuth).

0142-9612/02/$ - see front matter
PII: S0142-9612(01)00186-7

materials. In the next step, the tissue carriers are placed in a gradient culture container, which is separated into a luminal and a basal compartment by the epithelia grown on the tissue carrier [17,24]. Each compartment can be continuously perfused with fresh medium. Earlier experiments showed the feasibility to culture embryonic renal collecting duct (CD) epithelia. However, it is often found that the barrier function of the epithelium is not fully developed or breaks during the culture period. Therefore, the challenge in gradient perfusion culture experiments is to maintain a functional epithelium which presents a physiological barrier separating the luminal from the basal compartment. Epithelial leaks (Fig. 1a) and edge damage (Fig. 1b) depend on sub-optimal biomaterials and liquid pressure differences caused by the culture conditions [18]. An epithelial leak arises when perfect confluency of the cells on the tissue carrier is not developed or when a perfect sealing between neighboring cells is not present because of cell biological reasons. Edge damage arises at sites where living cells and polymer materials of the individual tissue carrier come in contact. A further problem is that of liquid pressure differences in the gradient culture container (Fig. 1c).

In the present paper we would like to demonstrate, for the first time, how to overcome technical difficulties

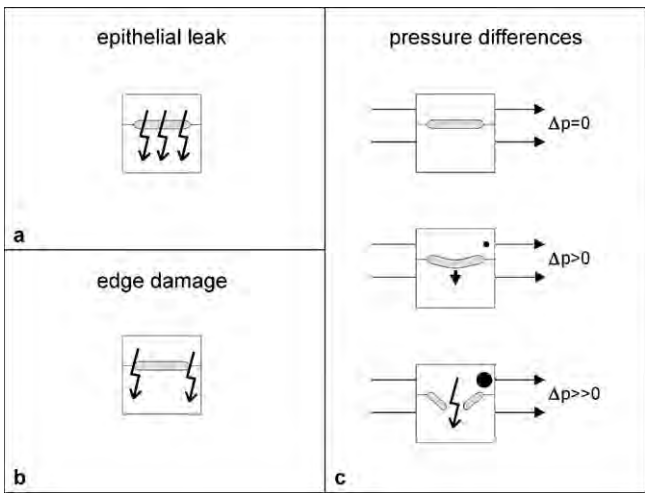


Fig. 1. Schematic illustration of loss of barrier function in epithelia cultured in a gradient container: (a) epithelial leak is present when the epithelium is not able to seal the paracellular shunt; (b) edge damage occurs at sites where living cell come in contact with the polymer material of the tissue carrier; (c) schematic illustration of liquid pressure differences, which may cause tissue damage in a gradient culture container. No tissue damage will occur when pressure is identical at the luminal and basal sides of the gradient culture container ($\Delta p = 0$). In contrast, a small gas bubble (small black dot) at the outlet of the luminal compartment will increase the pressure in the luminal compartment resulting in an extension of the tissue towards the basal side (arrow, $\Delta p > 0$). When the gas bubble increases (big black dot), in diameter an increase in pressure in the luminal compartment is found ($\Delta p \gg 0$). The tissue cannot withstand this pressure and is disrupted. Consequently, the barrier function is lost.

while performing gradient culture experiments with the help of new tools for advanced tissue culture. To minimize the occurrence of harmful gas bubbles, media were transported through specific tubings and newly developed bottle closures. To avoid pressure differences in the gradient culture container a new gas expander module is introduced, which is able to separate gas bubbles from the liquid phase, but does not decrease the content of oxygen in the culture medium. Furthermore, by immunohistochemical methods we monitor the development of embryonic CD epithelia cultured in a gradient container for 14 days. We give information about the state of the art and demonstrate problems and efforts for culturing epithelia under fluid gradient adapted to natural conditions.

2. Materials and methods

2.1. Tissue isolation

The generation of embryonic collecting duct epithelia was performed by isolating cortical explants from the kidneys of newborn New Zealand rabbits according to methods described earlier [19]. The explants consisted of a piece of stripped off capsula fibrosa with adherent collecting duct ampullae, S-shaped bodies, and nephrogenic blastema, which were mounted in tissue carriers (Fig. 2b; Minucells and Minutissue, Bad Abbach, Germany—www.minucells.de). For the multiplication of cells, the carriers were placed in a 24-well plate in a CO_2-incubator (5% CO_2/95% air). During the culture of the explants in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL Life Technologies, Eggenstein, Germany) including 10% fetal bovine serum (Boehringer, Mannheim, Germany), an outgrowth of cells from the collecting duct ampullae was observed. Within 24 h the entire surface of the explant, 6 mm in diameter, was covered by a monolayer of polarized collecting duct epithelial cells. Only for this limited period of time, the medium containing fetal bovine serum was applied.

2.2. Gradient perfusion culture

Twenty-four hours after initiation of the culture the tissue carriers (Fig. 2) were placed in gradient containers (Figs. 3e, f and 5b), which allow the culture of epithelia in a fluid gradient so that a tissue-specific environment can be simulated (Minucells and Minutissue). A tissue carrier with the developed tissue separated the container into a luminal and a basal compartment. On both sides of the epithelium either the same medium or media of different composition could be perfused. The culture system was used on a laboratory table (Figs. 5a and b). Only few additional equipments were necessary to

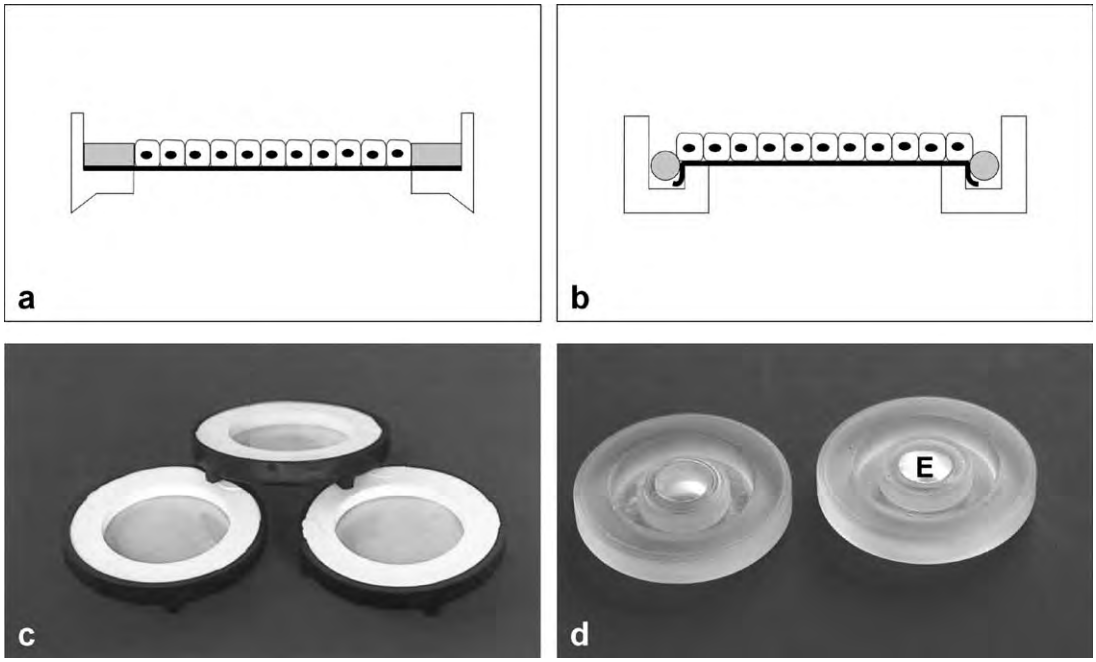


Fig. 2. Schematic and photographic illustration of tissue carriers used in gradient culture experiments: (a) vertical section through a tissue carrier. A membrane is held in position by a holder and a span ring. The epithelium develops on the surface of the membrane; (b) vertical section through a tissue carrier for flexible support materials. Collageneous material is placed in a holder and fixed by an overlapping span ring; (c) surface view to three tissue carriers consisting of a black holder and a white span ring. As an example a polycarbonate membrane 13 mm in diameter individually can be placed into the carrier; (d) surface view of a tissue carrier for flexible collagenous matrices. The epithelium (E; 6 mm in diameter) derived from the embryonic renal CD ampulla was fixed in a tissue carrier and develops on the surface of the artificial matrix.

perform perfusion culture. A thermo plate (MEDAX, Kiel, Germany) including a cover lid maintained a constant temperature of 37°C within the gradient culture container and a peristaltic pump (IPC N8, ISMATEC, Wertheim, Germany) transported the medium through the container.

2.3. Medium

During gradient perfusion culture, Iscove's modified Dulbecco's medium (IMDM; order #21980-032; Gibco BRL-Life Technologies, Eggenstein, Germany) [20] without serum was used as the standard medium. Fresh medium was continuously perfused at a rate of about 1 ml/h for a 2-week culture period. Aldosterone (1×10^{-7} M; Sigma-Aldrich-Chemie, Deisenhofen, Germany) and 1% antibiotic–antimycotic solution (Gibco BRL-Life Technologies) were added to all culture media. Furthermore, up to 50 mmol/l HEPES (Gibco BRL-Life Technologies) was used in the medium to maintain a constant pH of 7.4 in perfusion culture under laboratory room atmosphere (0.3% CO_2). Control epithelia were treated by perfusing standard IMDM at the luminal and basal sides. Experimental series were run with standard IMDM at the basal side, while IMDM containing additional 3–24 mmol/l NaCl was superfused at the luminal side.

2.4. Gas equilibration of the medium

Conventional cultures in a CO_2-incubator are usually buffered by a system containing a definite amount of $NaHCO_3$, 95% air and 5% CO_2 to maintain a constant pH of 7.4. If such a medium were used in perfusion culture outside a CO_2-incubator under room atmosphere, pH would shift out of the physiological range into the alkaline range. For that reason medium used outside a CO_2-incubator had to be stabilized by reducing the $NaHCO_3$ content and/or by adding HEPES (Sigma-Aldrich-Chemie, Deisenhofen, Germany). The equilibration for a constant pH under room atmosphere was performed using a 24-well culture plate. In each well 1 ml of culture medium was pipetted. Then to each of the wells an increasing concentration of 10–50 mmol/l HEPES was added. For the following 24 h the culture plate was placed on a thermo plate at 37°C under room atmosphere. After equilibration, the pH in each well was measured with a Stat Profile 9 Plus analyzer (Nova Biomedical, Rödermark, Germany). The HEPES concentration that yielded a pH between 7.2 and 7.4 under room atmosphere could easily be determined. To obtain an optimal equilibration of pH, O_2 and CO_2 in perfusion cultures the media were pumped through 1 mm inner diameter thin, gas-permeable silicone tubes, which allowed the continuous exchange of atmospheric gases.

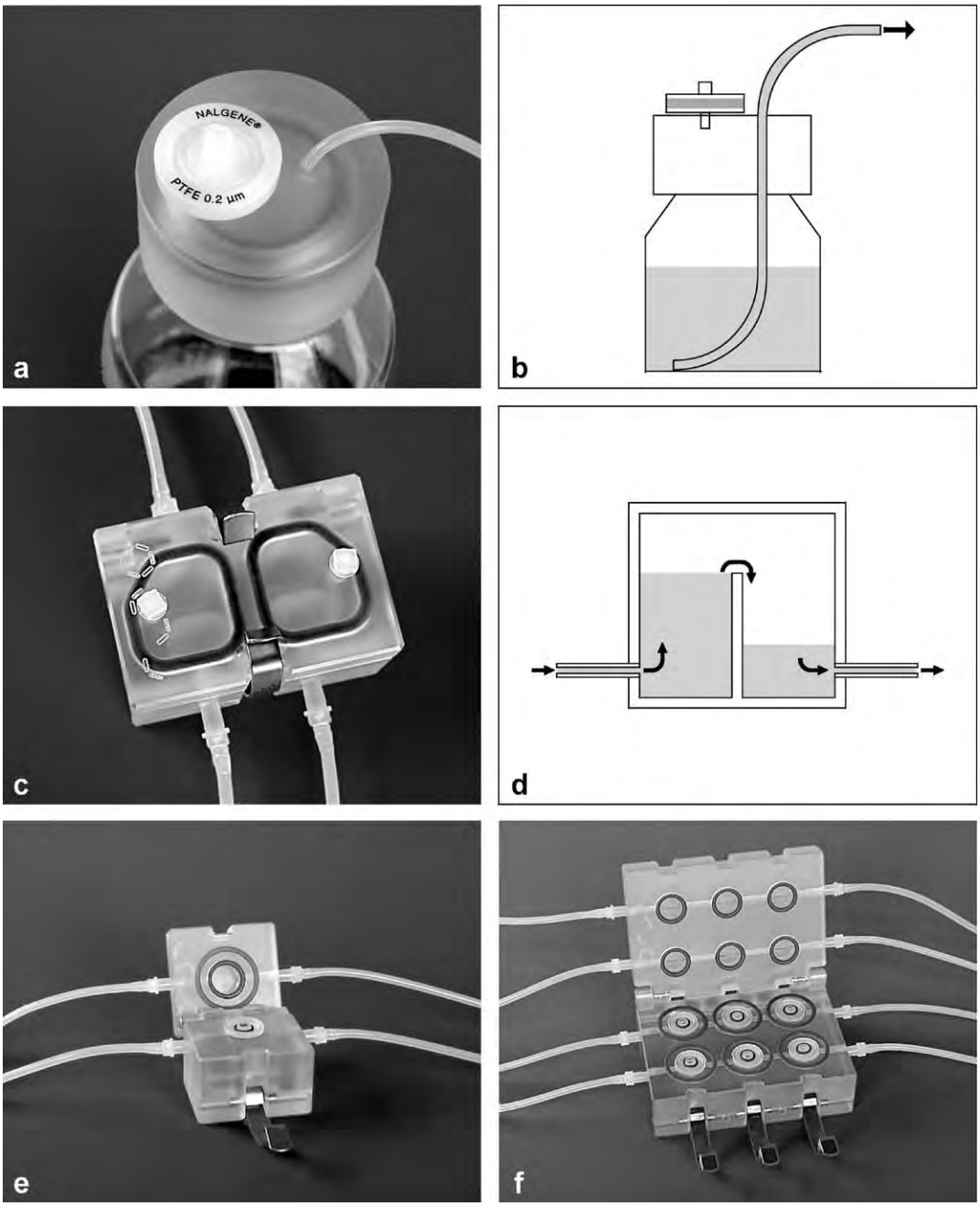


Fig. 3. Advanced technical equipment for gradient culture experiments: (a, b) photographic and schematic illustration of newly developed closures for media bottles to minimize the arise of gas bubbles. The silicone tube for the medium is conducted through the closure without further contact; (c) surface view of the gas expander module consisting of two chambers; (d) lateral view of the gas expander module. Gas saturated medium enters at the left side of the module. The medium crosses a barrier, while gas bubbles collect in the upper half of the container during transportation; (e) the tissue carrier is placed in a gradient culture container; (f) gradient culture container for six tissue carriers.

2.5. Elimination of gas bubbles

In gradient perfusion culture experiments with epithelia, the appearance of gas bubbles had to be minimized. The unequal distribution of gas bubbles in the apical or basal compartment resulted in pressure differences, which caused damage of the tissue. Gas bubbles concentrate preferentially at sites where different materials come in contact. In perfusion culture, the medium was transported from the store bottles by a silicone tube with 1 mm inner diameter. Consequently, to guarantee the sterile closure of the bottles, newly developed screw caps were used, which conduct the tube but do not allow contact of the medium with screw cap material (Fig. 3b).

Furthermore, the elimination of the remaining gas bubbles in the medium could be performed by newly developed gas expander modules (Figs. 3c and d; Minucells and Minutissue).

2.6. Registration of gas bubbles

The appearance of gas bubbles in perfusion lines was detected by a sensor, which was placed on a 1 mm inner diameter glass capillary at the tube of the effluent culture medium (Fig. 4). Floating air bubbles were registered by an infrared (IR) gate sensor (Conrad Electronics, Wernberg, Germany). Registered impulses were logged by a personal computer.

2.7. Physiological parameters

The metabolic activity of the cells was monitored by analyzing the superfused culture medium. Media parameters such as pH, pCO_2, pO_2, lactate, osmolarity and electrolyte concentrations of Na^+, K^+, Cl^- and Ca^{2+} were determined in undiluted 200 µl samples of the culture medium. Through a T-connection in the tubing, samples were analyzed in a Stat Profile 9 Plus analyzer according to the manufacturer's instructions (Nova Biomedical). Solutions with defined electrolyte concentrations served as controls. Specimens of medium were collected in the luminal and basal compartments, before and after the medium had passed through the container (Figs. 5c and 6).

2.8. Search for barrier leaks in the epithelia during culture

The tissue carriers separated the gradient culture container into a luminal and a basal compartment (Fig. 5b) To detect nonphysiological leaks in the tissue the luminal compartment was perfused with IMDM containing phenol red (order #21980-032; Gibco BRL-Life Technologies). At the basal side IMDM without phenol red (order #21056-023; Gibco BRL-Life Technologies) was used. Traces of red color within the clear medium showed nonphysiological leakage of the epithelia. Thus, only experiments that maintained a perfect separation of red and clear media in the waste were declared successful and used for evaluation.

Control for barrier leaks was further performed by the electrolyte measurements with an analyzer (Nova Biomedical; Fig. 6). Medium specimens were collected just before and after the medium had passed the luminal and basal compartments of the gradient container. Since, in the present experiments, the luminal medium contained more Na or Cl than the culture medium at the basal side the stability of the gradient could be controlled by comparing the Na or Cl concentrations and the differences in osmolarity between the luminal and basal compartments.

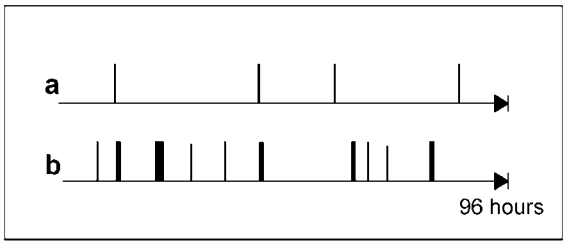


Fig. 4. Registration of gas bubbles passing through a gradient culture container by an infrared (IR) gate sensor over a 96 h period. The frequency and size of registered gas bubbles are indicated by black bars. It can be shown that the amount of gas bubbles is drastically decreased by the use of newly developed bottle closures and a gas expander module (a) as compared to controls without bubble elimination (b).

2.9. Registration of temperature

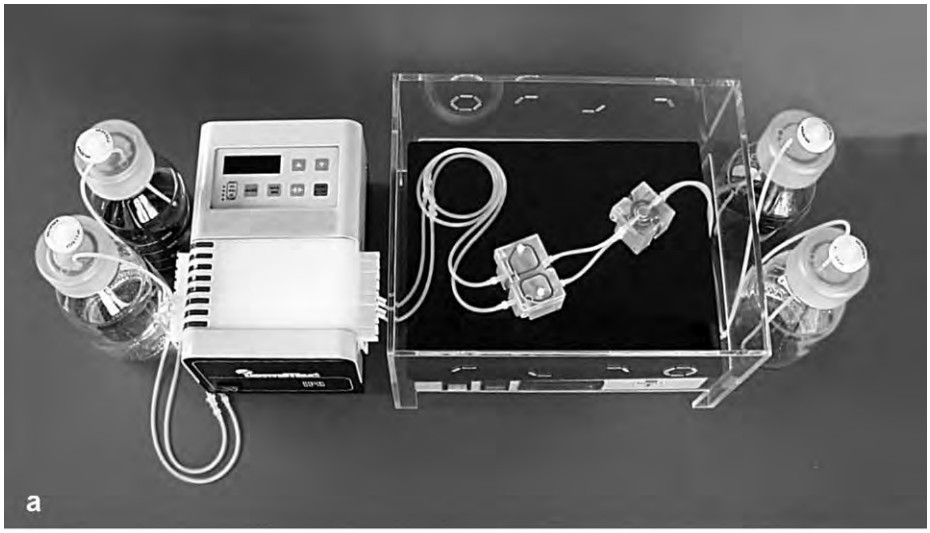
During long term cultures temperature had to be maintained at a constant level. Temperature was controlled by two thermoresistors (Conrad Electronics), one of which was mounted on the surface of the thermo plate, while the other was integrated into a reference culture container. Both temperatures were continuously logged by a personal computer.

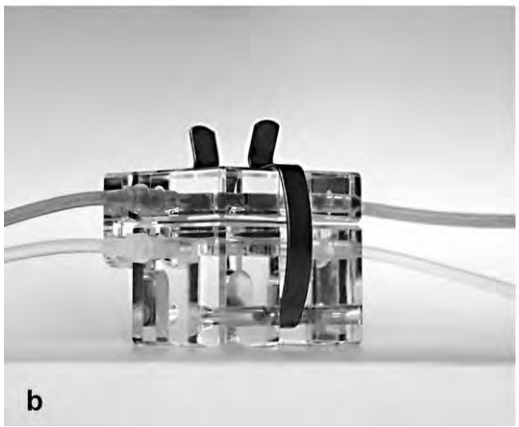
2.10. Registration of perfusion rate

During the 14 days of the culture period it was important to control the amount of medium transported from the storage to the waste bottles. Since it was impossible to find autoclavable sensors to register low perfusion rates at the level of 1 ml/h, a simple electronic balance was used. On measuring the increase in amount/weight of medium over time in the waste bottles a registration of individual perfusion rates became possible.

2.11. Detection of cellular differentiation

During culture the embryonic cells developed features of adult epithelial tissue. To register the primary appearance of individual collecting duct cell characteristics we used a set of markers reacting specifically in adult renal tissue (Fig. 7). A monoclonal antibody against Na/K ATPase developed by D.M. Fambrough was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD. Mab 703 recognizes 40, 48, 51, 60 and 99 kDa proteins on P cells identified on adult cultured renal collecting duct cells [21]. The antibody was kindly provided by Dr. M.





Na^+	**mmol/l**	**116,7**
K^+	mmol/l	4,0
Cl^-	**mmol/l**	**78,7**
Ca^{++}	mmol/l	1,11
Osmolarity	**mOsm**	**250**
pH		7,4
pO_2	mmHg	193,8
pCO_2	mmHg	11,8
Glucose	mg/dl	431
Lactate	mmol/l	0

c

Fig. 5. Technical equipment and physiological parameters for gradient culture. (a) Illustration of the gradient perfusion culture line including the gas expander module and the gradient culture container for the luminal and basal perfusion of medium. In combination with the gas expander module this avoids the appearance of gas bubbles. The media are not reperfused but collected in separate waste bottles. An eight channel peristaltic pump (IPC N8) transports the medium and a thermo plate gives the right temperature. (b) Lateral view of a gradient container. IMDM with phenol red was superfused at the luminal side, while at the basal side phenol red in IMDM was omitted to allow visual control of the quality of the gradient. (c) Physiological parameters of an individual experiment of IMDM measured before reaching a gradient culture container.

Tauc, Department of Cellular and Molecular Physiology, University of Nice, France. Mab anti-cytokeratin 19 was produced in the laboratory of Prof. Dr. R. Moll, Marburg, Germany. Antibodies recognizing COX 1 and 2 were obtained from Santa Cruz Biotechnology, California, USA.

For the light microscopical monitoring of cell development and immunohistochemical detection of collecting duct proteins 7 μm cryosections of the tissue were prepared using a Cryostat HM 500 (Microm, Walldorf, Germany). Immunolabeling was started by fixing the cryosections for 10 min in ice-cold ethanol as described earlier [22]. Following several washing steps 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 albumin (BSA) for 30 min. The primary antibodies (each of them diluted 1 : 100 in blocking buffer) were incubated for 1.5 h. Following several washes with PBS containing 1% BSA the sections were treated for 45 min with a donkey-anti-IgG–fluorescein–isothiocyanate (FITC)-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA) diluted 1 : 200 in blocking buffer. Following several washes in PBS the sections were embedded in Slow Fade Light Antifade Kit (Molecular Probes, Oregon, USA) and examined using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

2.12. Evaluation

In total, more than 30 epithelia were examined in gradient culture experiments for the present investigation. To obtain an objective result, each treatment was

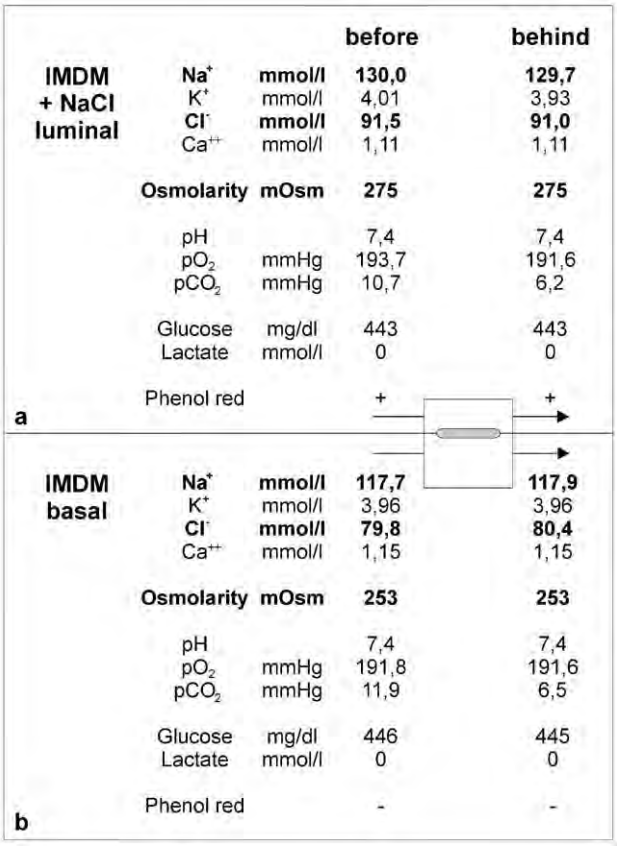


Fig. 6. Individual measurement of the maintenance of a luminal–basal medium gradient after 10 days. Before and after passing the gradient culture container, medium was collected at the luminal and basal sides. During the whole culture period the luminal (130 mmol/l Na) and basal (117 mmol/l Na) gradient is maintained indicating an intact barrier function of the cultured collecting duct epithelium. The luminal–basal gradient of Na, Cl and osmolarity indicates a perfect barrier function of the cultured epithelia.

repeated at least three times. More than five epithelia were analyzed per experimental series. A minimum of 50 vertical cryosections per individual group was examined. In the text and figures the mean numbers of labeled cells as compared to unlabeled cells within the epithelium is given.

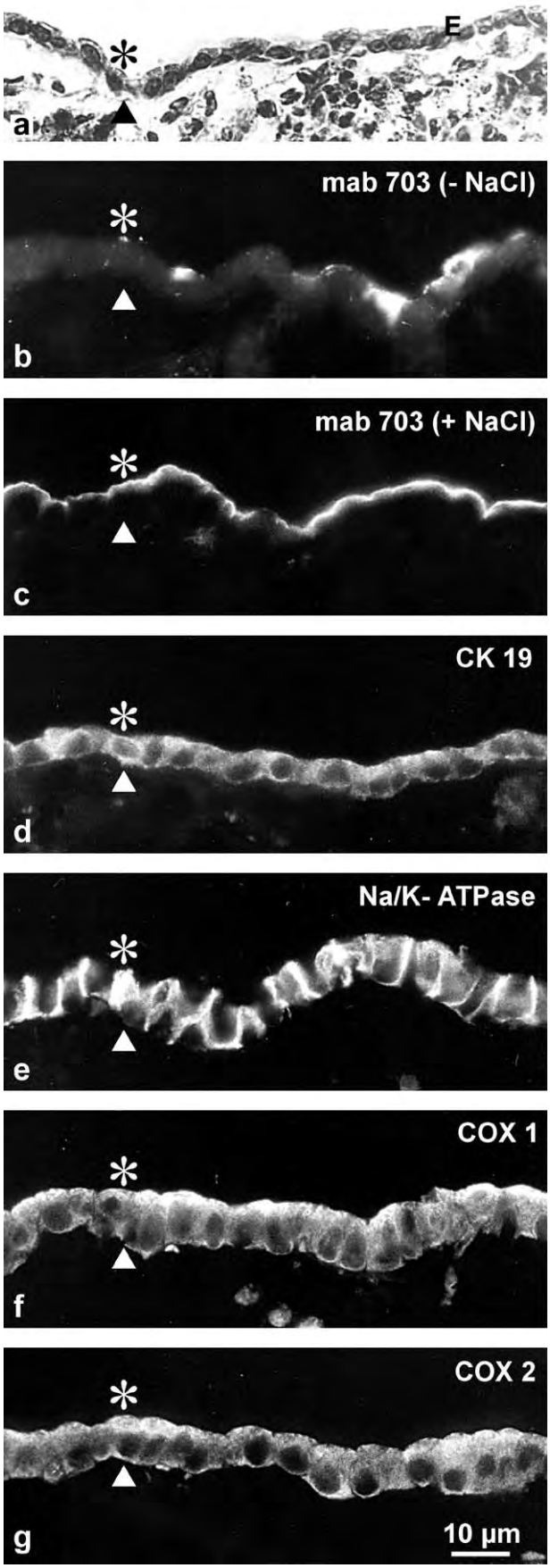


Fig. 7. Cellular differentiation profile of embryonic collecting duct epithelia in gradient culture: (a) cryosection of a CD epithelium cultured for 14 days in a gradient container. The epithelium rests on a kidney-specific collagenous matrix; (b) culture of the epithelium with IMDM at the luminal and basal sides results in only 10% mab 703 immunopositive cells; (c) IMDM containing 130 mmol/l Na at the luminal side reveals at least 90% mab 703 immunopositive cells; (d) alls cells of the CD epithelium show immunolabel with anti-cytokeratine 19; (e) basolateral immunolabeling with anti Na/K ATPase on all of the epithelial cells; (f) all cells show immunolabel for COX 1; (g) all cells within the epithelium react with anti-COX 2. Asterisk—luminal side, arrow head—basal aspect of the epithelium.

3. Results

3.1. Oxygen-rich media versus gas bubbles

Culture of renal epithelia can be performed in a gradient container, which allows a permanent medium exchange at the luminal and basal sides of the tissue (Figs. 3e, f, 5a and b). No problems for the preservation of epithelia will arise as long there is no pressure difference between the luminal and basal sides (Fig. 1c). However, the media are pumped through gas-permeable silicone tubes, which allow saturation with 193 mmHg oxygen (Fig. 5c). This is advantageous for the tissue, but disadvantageous for the medium transport. During long term perfusion culture we often observed the occurrence of gas bubbles at unpredictable sites within the tubes, connectors or the culture container. They increased in size over time and left the sites of accumulation to appear unpredictably in the effluent tubes of the gradient container. The unequal distribution of air bubbles either in the luminal or basal compartment produced pressure differences that frequently resulted in the damage of tissue so that the barrier function was lost (Fig. 1c).

To avoid liquid pressure differences in the gradient container we had to minimize the occurrence of air bubbles in the system. Bubbles preferentially occurred at sites where two different polymer materials along the conducting flow path come in contact. Consequently, we had to construct bottle closures, which conducted the medium in a silicone tube through the closure, but did not allow contact with the medium (Figs. 3a and b). Experiments showed that the use of the newly developed closures significantly decreased the occurrence of gas bubbles during media transport.

For the further elimination of gas bubbles, we developed a gas expander module (Figs. 3c and d). The module consists of two chambers. The medium rises in a small reservoir, then drops down a barrier when leaving the container (Fig. 3d). During this process air bubbles were separated from the medium and collected at the top of the container. Combining the newly developed screw caps with the gas expander module it was observed that the occurrence of air bubbles in the culture medium could be drastically reduced (Fig. 4a) as compared to media transport without these tools (Fig. 4b).

3.2. Physiological environment

Gradient perfusion culture was performed outside a CO_2-incubator on a laboratory table exposed to room atmosphere. An electronically regulated thermo plate with a coverlid maintained the right temperature for the culture container and a peristaltic pump transported the medium into the waste bottle so that no recirculation occurred. This guaranteed constant nutrition and oxygen supply including the permanent elimination of harmful metabolic products. During long term culture of epithelia in a gradient container we continuously controlled the physiological environment (Figs. 5c and 6). An individual example is shown before the basic culture medium IMDM reached the container (Fig. 5c). A stable pH of 7.4 was found under room atmosphere air during perfusion culture. As compared to a CO_2-incubator a relatively low content of 11.8 mmHg of CO_2 was detectable because of the low content of CO_2 (0.3%) in air. In contrast, a high amount of 193 mmHg of O_2 was measured by equilibrating the media in silicone tubes during transport from the storage bottle to the container. Since the epithelia were exposed to a luminal and basal fluid environment specimens of media were collected from the luminal and basal media before and behind the container (Fig. 6). A high concentration of 415 mg/dl glucose indicated that the exchange of culture medium is high enough so that a decline in glucose will not limit aerobic physiological processes. Also an accumulation of lactate to nonphysiological levels behind the container could not be observed. It shows that a harmful influence of this metabolite will not occur during the culture period because of its continuous elimination.

To measure the maintenance of epithelia with an intact barrier function medium containing phenol red was applied at the luminal (Fig. 5b and 6a), while medium without phenol red was used at the basal side (Fig. 6b). Only those epithelia were used for further experimentation which maintained the fluid gradient without mixing luminal and basal fluids. The maintenance of a fluid gradient, further, could be registered by a comparison between the Na and Cl contents, respectively, and the osmolarity between the luminal and basal compartments (Fig. 6). The luminal culture medium contained constantly higher concentrations of Na, Cl and osmolarity as compared to the medium at the basal side. By this simple method, the maintenance of the epithelial barrier could be monitored in combination with the physiological parameters.

3.3. Cellular differentiation

Epithelia cultured in a gradient container were analyzed by immunohistochemical methods to determine the development of specific features (Fig. 7a). Immunohistochemistry demonstrated that culture of the embryonic tissue with the same media at the luminal and basal sides resulted in less than 10% mab 703 positive cells within the epithelium when a marker for facultative protein expression was used (Fig. 7b). In contrast, when 12 mmol/l NaCl were added to the luminal IMDM nearly all cells showed immunostaining after mab 703

incubation (Fig. 7c). Markers for constitutive protein expression such as cytokeratine 19 (Fig. 7d) or Na/K ATPase (Fig. 7e) showed that all cells within the epithelium were positive. Important enzymes within the renal collecting duct system are COX 1 and 2 [33]. Most interestingly, all cells within the epithelium were positive for immunolabeling by anti COX 1 (Fig. 7f) and anti COX 2 (Fig. 7g) antibodies. This result was obtained independent of low or high NaCl concentrations added to the luminal culture medium (Figs. 7b and c).

4. Discussion

4.1. Maintaining an epithelial barrier

Except for epithelia contacting air, all other epithelia in our organism exhibit a barrier function, where they are exposed to different fluid media at the luminal and basal sides. To mimic such a situation under in vitro conditions appears to be a simple task, but the experimental realization proves rather difficult. First of all, the epithelia have to withstand the superfusion of media and they have to maintain the physiological barrier between the luminal and basal compartments in the gradient container as is known from the organ where they are derived from. Uncontrolled mixing of the luminal and basal media must not occur. A perfect atmosphere for epithelia cultured in a gradient container is obtained when no pressure difference is present between the luminal and basal compartments (Fig. 1c; $\Delta p = 0$). However, since oxygen-rich media are used, gas bubbles within the tubing present a major problem for long term gradient perfusion cultures. A gas saturated culture medium is transported by slowly rotating peristaltic pumps. During this process, gas separates from the liquid phase and randomly accumulates in gas bubbles. Their location and amount in the gradient culture container or within tubes with effluent culture medium cannot be predicted. The bubbles remain attached for some period of time, while they increase in diameter. Finally, after reaching a certain size the air bubbles cause differences in fluid pressure between the luminal and basal medium compartments similar to an embolus in a small blood vessel. In gradient perfusion culture it first causes a protrusion of the tissue to the side of lower pressure (Fig. 1c; $\Delta p > 0$). An increasing pressure difference will finally result in the disruption of the tissue (Fig. 1c; $\Delta p \gg 0$). The actual series of experiments showed the feasibility of culturing embryonic epithelia in a permanent fluid gradient. In individual series of experiments up to 80% epithelia with intact barrier function could be harvested.

4.2. Upregulation of individual cell features

Performing gradient culture experiments, we elucidate mechanisms triggering the development from an embryonic towards a functional renal epithelium. Earlier experiments showed that the terminal differentiation can be influenced by two different stimuli. Administration of aldosterone resulted in the upregulation of individual collecting duct proteins including the functional tight sealing and the establishment of an amiloride sensitive Na transport [23]. Further, it could be shown that peanut lectin (PNA) binding was upregulated depending on aldosterone application [25]. Recent experiments revealed that a chronical NaCl load was able to upregulate cell biological features of adult principal (P) and intercalated (IC) cells [26,27]. In addition, P_{CD} Amp 1, an antigen occurring exclusively in the embryonic CD ampulla was downregulated, which demonstrates that the epithelia matured after a chronic NaCl exposure into an adult state [28].

The present series of experiments with embryonic renal CD epithelia could only be performed with advanced gradient culture technique. The continuous application of NaCl to the luminal culture medium revealed different actions on renal collecting duct antigen expression. Mab 703 recognizes an apical localized protein in P-cells in adult renal CD [21]. The expression of this protein is influenced distinctly by the addition of 12 mmol/l NaCl to the luminal culture medium (Figs. 7b and c) [29]. Furthermore, prostaglandins regulate a variety of cellular functions concerning epithelial salt and water transport, smooth muscle tone and immune cell function [30]. Two different prostaglandin-forming cyclooxygenases are identified. COX 1 is known as the constitutively expressed cyclooxygenase [31], while COX 2 is described as the inducible form of the enzyme. The expression is regulated by different stimuli [32] including salt diet in rats. Yang showed a distinct increase in COX 2 protein levels in the inner medulla of high salt diet treated rats, while COX 1 expression remained unaffected [33]. In contrast, our results revealed that neither COX 1 nor COX 2 expression was influenced by a further NaCl application to the culture medium. The immunohistochemical profile showed that all cells within the cultured epithelium were immunopositive for COX 1 (Fig. 7f) and COX 2 (Fig. 7g). Furthermore, Yang displayed that both isoforms were at lower levels expressed in a culture of collecting duct cell lines in a stagnant environment [33]. However, in gradient perfusion culture we noticed a high expression of both isoforms of cyclooxygenase in all cells of the CD epithelium. Further stimuli of COX 2 expression are glucocorticoids and mineralocorticoids. Zhang showed that inhibition of the mineralocorticoid receptor with spironolactone caused upregulation of renal cortical COX 2 in rat [34]. In our model

representing the collecting duct system of the rabbit kidney, aldosteron application to the culture medium had no influence on the expression of both isoforms. In consequence, we found that by the stimuli known for renal CD development the expressions of COX 1 and 2 could not be influenced. We further conclude that at least for the period of CD maturation the COX 1 and 2 enzymes belong to the constitutively expressed protein family. These data are found to be in accordance with the expression profile of cytokeratine 19 (Fig. 7d) and Na/K-ATPase (Fig. 7e), which are localized in all CD epithelial cells.

In the present experiments we used CD epithelia as a model system to elaborate the cellular differentiation profile during development in a gradient culture container. It is imaginable that a variety of other complex epithelia such as the gastric mucosa, urothelium or endothelium can be cultured for biomaterial testing and tissue engineering under the gradient conditions described [35,36]. In contrast to other authors working with cell lines [37], we could not generate the necessary degree of differentiation with primary cultures in the stagnant environment of a culture dish. We had to apply advanced technique for an improved development of the tissue. While performing these experiments we obtained an unexpected amount of information about tissue maturation. Before, we could not imagine how sensitively the tissue reacts with respect to differentiation features to the offered environment.

References

[1] Van Dorp AGM, Verhoeven MCH, Van der Halt-Van der Meij TH, Koerten HK, Ponec M. A modified culture system for epidermal cells for grafting purposes: an in vitro and in vivo study. Wound Rep Reg 1999;7:214–25.

[2] Gustafson CJ, Kratz G. Cultured autologous keratinocytes on a cell-free dermis in the treatment of full-thickness wounds. Burns 1999;25:331–5.

[3] Waybill PN, Hopkins LJ. Arterial and venous smooth muscle cell proliferation in response to co-culture with arterial and venous endothelial cells. Journal of Vascular and International Radiology 1999;10:1051–7.

[4] Surowiec SM, Conklin BS, Li JS, Lin PH, Weiss VJ, Lumsden AB, Chen C. A new perfusion culture system used to study human vein. J Surg Res 2000;88:34–41.

[5] Papas KK, Long RC, Sambanis A, Constantinidis I. Development of a bioartificial pancreas: effect of oxygen on long-term entrapped βTC3 cell cultures. Biotechnol Bioeng 1999;66(4): 231–7.

[6] Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, Langer R. Functional arteries grown in vitro. Science 1999;284:489–93.

[7] Coppelli A, Ariva C, Giannarelli R, Marchetti P, Viacava P, Naccarato AG, Lorenzetti M, Cosimi S, Cecchetti P, Navalesi R. Long-term survival and function of isolated bovine pancreatic islets maintained in different culture media. Acta Diabetol 1996;33:166–8.

[8] Ohshima N. Tissue engineering aspects of the development of bioartificial livers. J Chin Inst Chem Ingrs 1997;28(6): 441–53.

[9] Bader A, Frühauf N, Tiedge M, Drinkgern M, De Bartolo L, Borlak JT, Steinhoff G, Haverich A. Enhanced oxygen delivery reverses anaerobic metabolic states in prolonged sandwich rat hepatocyte culture. Eptl Cell Res 1999;246:221–32.

[10] Humes HD, Buffington DA, MacKay SM, Funke A, Weitzel WF. Replacement of renal function in uremic animals with a tissue-engineered kidney. Nat Biotechnol 1999;17:451–5.

[11] Fey-Lamprecht F, Gross U, Groth TH, Albrecht W, Paul D, Fromm M, Gitter AH. Functionality of MDCK kidney tubular cells on flat polymer membranes for biohybrid kidney. J Mater Sci Med 1998;9:711–5.

[12] Ludwikowski B, Zhang YY, Frey P. The long-term culture of porcine urothelial cells and induction of urothelial stratification. British Journal of Urology Int 1999;84:507–14.

[13] Miki H, Ando N, Ozawa S, Sato M, Hayashi K, Kitajima M. An artificial esophagus constructed of cultured human esophageal epithelial cells, fibroblasts, polyglycolic acid mesh, and collagen. American Society for Artificial Internal Organs J 1999;45:502–8.

[14] Goto Y, Noguchi Y, Nomura A, Sakamoto T, Ishii Y, Bitoh S, Picton C, Fujita Y, Watanabe T, Hasegawa S, Uchida Y. In vitro reconstitution of the tracheal epithelium. Am J Respir Cell Mol Biol 1999;20:312–8.

[15] Minuth WW, Steiner P, Strehl R, Schumacher K, de Vries U, Kloth S. Modulation of cell differentiation in perfusion culture. Exp Nephrol 1999;7:394–406.

[16] Minuth WW, Majer V, Kloth S, Dermietzel R. Growth of MDCK cells on non-transparent supports. In Vitro Cell Dev Biol Anim 1994;30:12–4.

[17] Steiner P, Strehl R, Kloth S, Tauc M, Minuth WW. In vitro development, preservation of specific features of collecting duct epithelial cells from embryonic rabbit kidney are regulated by the electrolyte environment. Differentiation 1997;62:193–202.

[18] Stockmann M, Gitter AH, Sorgenfrei D, Fromm M, Schulzke JD. Low edge damage container insert that adjusts intestinal forceps biopsies into using chamber systems. Pflügers Arch 1999;438: 107–12.

[19] Minuth WW. Neonatal rabbit kidney cortex in culture as tool for the study of collecting duct formation, nephron differentiation. Differentiation 1987;36:12–22.

[20] Iscove NN, Melchers F. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. J Expt Med 1978;147(3): 923–33.

[21] Tauc M, Koechlin N, Jamous M, Quaghi E, Gastineau M, Le Moal C, Poujeol P. Principal, intercalated cells in primary cultures of rabbit renal collecting tubules revealed by monoclonal antibodies. Biol Cell 1992;76:55–65.

[22] Strehl R, Trautner V, Kloth S, Minuth WW. Existence of a dense reticular meshwork surrounding the nephron inducer in neonatal rabbit kidney. Cell Tissue Res 1999;298:539–48.

[23] Gross P, Minuth WW, Ketteler M, Frömter E. Ionic conductances of cultured principal cell epithelium of renal collecting duct. Pflügers Arch 1988;412:434–41.

[24] Minuth WW, Aigner J, Kloth S, Steiner P, Tauc M, Jennings ML. Culture of embryonic renal collecting duct epithelium in a gradient container. Ped Nephrol 1997;11:140–7.

[25] Minuth WW, Fietzek W, Kloth S, Stöckl G, Aigner J, Röckl W, Kubitza M, Dermietzel R. Aldosterone modulates the development of PNA binding cell isoforms within renal collecting duct epithelium. Kidney Int 1993;44:337–44.

[26] Emmons C, Kurtz I. Functional characterization of three intercalated cell subtypes in the rabbit outer cortical collecting duct. J Clin Invest 1994;93:417–23.

[27] Kim J, Tisher CC, Madsen KM. Differentiation of intercalated cells in developing rat kidney: an immunohistochemical study. Am J Physiol 1994;266:F977–90.
[28] Strehl R, Kloth S, Aigner J, Steiner P, Minuth WW. P_{CD} Amp 1, a new antigen at the interface of the embryonic collecting duct epithelium and the nephrogenic mesenchyme. Kidney Int 1997;52:1469–77.
[29] Schumacher K, Strehl R, Kloth S, Tauc M, Minuth WW. The influence of culture media on embryonic collecting duct cell differentiation. In Vitro Cell Dev Biol Anim 1999;35;8:465–71.
[30] Williams CS, Mann M, Dubois RN. The role of cyclooxygenase in inflammation, cancer, and development. Oncogene 1999;18:7908–16.
[31] Williams CS, Dubois RN. Prostaglandin endoperoxide synthase: why two isoforms? Am J Physiol 1996;270:873–92.
[32] Harris RC, McKanna JA, Akai Y, Jakobson HR, Dubois RN, Breyer MD. Cyclooxygenase 2 is associated with the macula densa of rat kidney and increases with salt restriction. J Clin Invest 1994;94:2504–10.
[33] Yang T, Singh I, Pham H, Sun D, Smart A, Schnermann JB, Briggs JP. Regulation of cyclooxygenase expression in the kidney by dietary salt intake. Am J Physiol 1998;274:F481–9.
[34] Zhang MZ, Harris RC, McKanna JA. Regulation of cyclooxygenase-2 (COX 2) in rat renal cortex by adrenal glucocorticoids and mineralcorticoids. Proc Natl Acad Sci USA 1999;96(26): 15280–5.
[35] Takimoto Y, Nakamura T, Yamamoto Y, Kiyotani T, Teramachi M, Shimizu Y. The experimental replacment of a cervical esophageal segment with an artificial prothesis with the use of collagen matrix and a silicone stent. J Thorac Cardiovasc Surg 1998;116(1):98–106.
[36] Negrete HO, Lavelle JP, Berg J, Lewis SA, Zeidel ML. Permeability properties of the intact mammalian bladder epithelium. Am J Physiol 1996;271(4):F886–94.
[37] Gstraunthaler G, Holcomb T, Feifel E, Liu W, Spitaler N, Curthoys NP. Differential expression and acid-base regulation of glutaminase mRNAs in gluconeogenic LLC-PK(1)-FBPase(+) cells. Am J Physiol 2000;278(2):F227–37.