Renal epithelia in long term gradient culture for biomaterial testing and tissue engineering

Will W. Minuth [*], Karl Schumacher and Raimund Strehl

Department of Molecular and Cellular Anatomy, University of Regensburg, D-93053 Regensburg, Germany

Abstract. In the organism epithelia perform perfect barrier functions. Strong rheological and mechanical influences constitute the normal environment of this tissue throughout life. Most epithelia are exposed to different fluids at the luminal and basal sides. To obtain realistic information about tissue development in modern biomaterial testing and tissue engineering it is necessary to mimik the natural environment of epithelia. Cultured cells are brought in contact with an artificial extracellular matrix to determine whether proper development into a functional epithelium occurs. As under natural conditions the cultures have to withstand mechanical and fluid stress over a prolonged period of time in close contact to a selected biomaterial. However, development of tissue-specific features such as polarization, tightness and transport under *in vitro* conditions will only occur, if the biomaterial and the culture conditions support tissue development. Leakage, edge damage and pressure differences during culture have to be avoided so that the natural functions of the growing epithelium can develop. Our aim is to generate functional epithelia derived from renal explants containing stem cells, which are microsurgically isolated and placed into specific O-ring carriers for optimal handling. The cells develop in combination with a collagenous matrix from an embryonic into a functional collecting duct (rCD) epithelium. To achieve optimal culture conditions the tissue is placed in a gradient culture container. A typical environment can be simulated by superfusing different culture media at the luminal and basal sides. Within days epithelia growing inside the gradient container build up a physiological barrier, which is maintained during the whole culture period. The described method allows to investigate the influence of new biomaterials over prolonged periods of time.

Keywords: Stem cells, kidney, 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] constructs is a challenge for biomaterial research and tissue engineering. For all these projects it is important that the different kinds of epithelial tissues exhibit the necessary degree of functionality and a close structural association to the biomaterials used as artificial extracellular matrix. Many questions concerning tissue development in combination with biomaterials can not be answered yet. Consequently, we have to study under realistic *in vitro* conditions how epithelial cells attach tightly to artificial matrices and form functional barriers under fluid or mechanical stress over prolonged periods of time.

In the focus of our interest is the mechanism leading to the transition from an embryonic into a functional renal epithelium and how this transition is influenced by biomaterials. As a model system we use

*Corresponding author: Prof. Dr. Will W. Minuth, Department of Molecular and Cellular Anatomy, University of Regensburg, Universitätstrasse 31, D-93053 Regensburg, Germany. Tel.: +49 (0) 941 943 2876, Fax: +49 (0) 941 943 2868; E-mail: will.minuth@vkl.uni-regensburg.de.

a defined renal collecting duct (rCD) epithelium derived from tissue explants that contain stem cells of neonatal rabbit kidney in combination with advanced perfusion culture techniques.

The basis for our method of tissue generation and biomaterial testing are O-ring carriers. Most biomaterials such as textiles, filters, foils or natural collagenous materials can be placed into these tissue carriers as an artificial matrix or basement membrane substitute [15]. Then explants from the cortex of neonatal kidney are brought in contact with the selected biomaterial. To adapt the culture conditions as closely as possible to the situation within the kidney, we establish a tissue-specific environment for epithelia using tissue carriers that are placed in gradient culture containers [16]. The epithelia on the tissue carrier separate it into a luminal and a basal compartment. Each of the compartments can be continuously perfused with fresh medium. However, it is often found that the barrier function of the epithelium is not fully developed or breaks during the long term 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.

In the present paper we would like to demonstrate how to overcome difficulties performing long term gradient culture experiments with advanced techniques. 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 was introduced, which is able to separate gas bubbles from the liquid phase without affecting the content of oxygen in the culture medium. We monitored the development of embryonic CD epithelia cultured in a gradient container for 14 days by immunohistochemical methods.

2. Materials and methods

2.1. Isolation of tissue

Generation of embryonic renal collecting duct (rCD) epithelia was performed by isolating cortical explants from the kidneys of newborn New Zealand rabbits according to methods described earlier [17]. The explants consisted of a piece of stripped off capsula fibrosa with adherent collecting duct ampullae (renal epithelial stem cells), S-shaped bodies and nephrogenic blastema (renal mesenchymal stem cells), which were mounted on tissue carriers (Fig. 1b; Minucells and Minutissue, Bad Abbach, Germany – www.minucells.de). For multiplication of cells the carriers were placed in a 24-well plate in a CO_2-incubator (5% CO_2/95% air) for 24 hours. During 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 hours the entire surface of the explant (6 mm in diameter) was covered by a monolayer of polarized renal collecting duct (rCD) epithelium. Only for this limited period of time medium containing fetal bovine serum was applied.

2.2. Gradient perfusion culture

24 hours after initiation of the culture the tissue carriers (Fig. 1b) were placed in gradient containers (Fig. 2), which allow 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 (Fig. 2a). 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
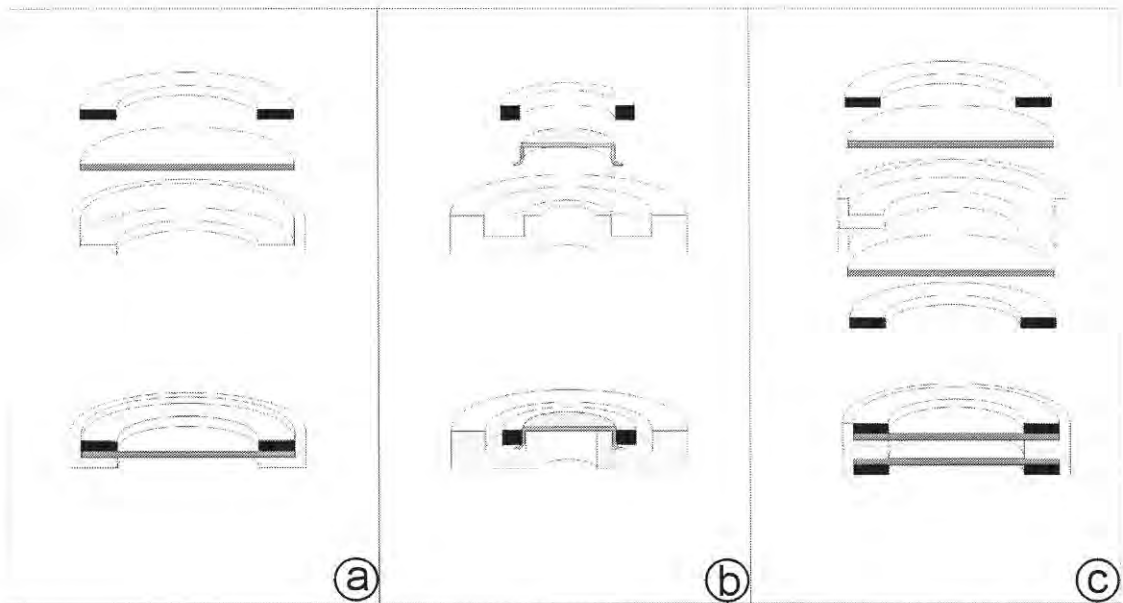
Fig. 1. Schematic and photographic illustration of tissue carriers used in gradient culture experiments. (a) Vertical section through a tissue carrier. A membrane is held in position by a holder and a span ring. The epithelium develops on the surface of the membrane. (b) Vertical section through a tissue carrier for flexible support materials. Collageneous material is placed in a holder and fixed by an overlapping span ring. The epithelium (6 mm in diameter) derived from the embryonic renal CD ampulla was fixed in this kind of tissue carrier. (c) Surface view to a carrier holding 2 biomaterials. In the space between tissue is growing.
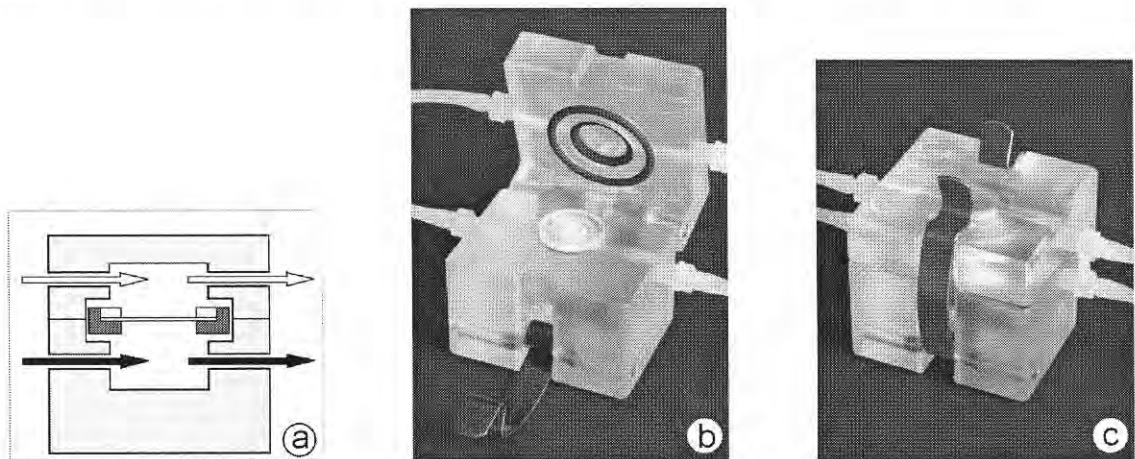

Fig. 2. Schematic and photographic illustration of a gradient perfusion culture container. (a) A tissue carrier separates the container into a luminal and basal compartment, which can be separately perfused with different media. (b) Opened gradient container with a tissue carrier 13 mm in diameter. (c) Closed gradient container, which can be perfused at the luminal and basal side.

(Fig. 4). Only little additional equipment was necessary to 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. Elimination of gas bubbles in the culture medium

In gradient perfusion culture the appearance of gas bubbles had to be minimized. 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 storage 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 which bottle closure material (Fig. 3a,b). Remaining gas bubbles in the medium were eliminated by newly developed gas expander modules (Fig. 3c,d; Minucells and Minutissue).
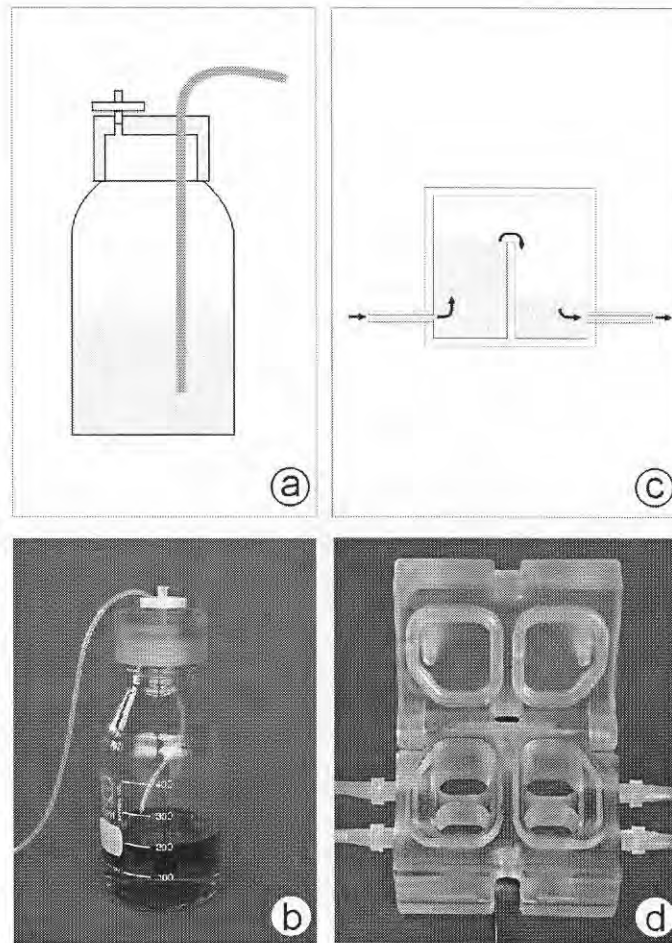

Fig. 3. Advanced technical equipment for long term gradient culture experiments. (a) Schematic and (b) photographic illustration of newly developed closures for media bottles to minimize the arise of gas bubbles. The silicone tube for the medium is conducted through the bottle closure without further contact. (c,d) View to a gas expander module consisting of 2 chambers to eliminate gas bubbles. Gas saturated medium enters at one side and crosses a barrier, while gas bubbles collect in the upper half of the container during transportation.
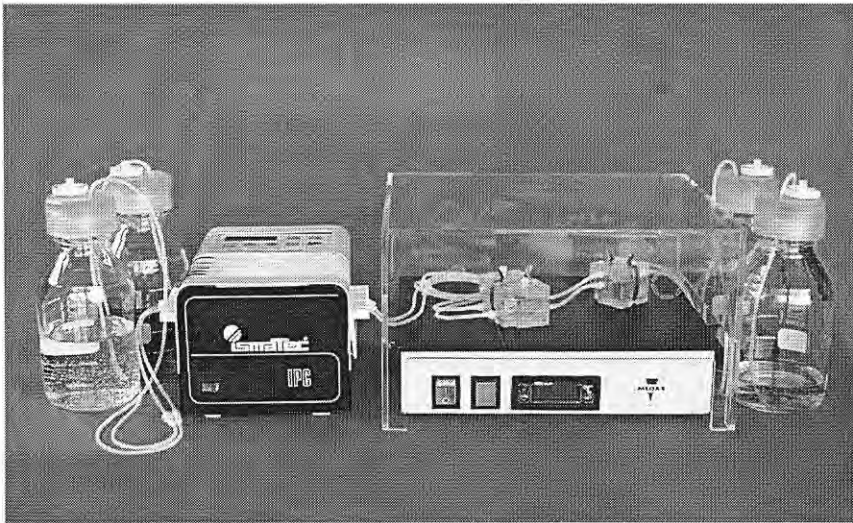
Fig. 4. Technical equipment and physiological parameters for gradient culture. Illustration of the gradient perfusion culture including the gas expander module and the gradient culture container to avoid the appearance of gas bubbles. The media are not reperfused but collected in separate waste bottles. A 8 channel peristaltic pump (IPC N8) transports the medium and a thermo plate gives the right temperature.

2.4. Registration of gas bubbles

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. Floating air bubbles were registered by an infrared (IR) gate sensor (Conrad Electronics, Wernberg, Germany). Registered impulses were logged by a personal computer.

2.5. Adapted medium

During gradient perfusion culture serum-free Iscove's modified Dulbecco's medium (IMDM; order # 21980-032; Gibco BRL-Life Technologies, Eggenstein, Germany) [18] was used as the standard medium. Fresh medium was continuously perfused at a rate of 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 superfused with standard IMDM at the basal side, while IMDM containing additional 3–24 mmol/l NaCl was superfused at the luminal side.

2.6. Gas equilibration of the medium for perfusion culture

Conventional cultures in a CO_2-incubator are usually buffered by a system containing a defined amount of $NaHCO_3$, 95% air and 5% CO_2 to maintain a constant pH of 7.4. If such a medium was 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 or a different biological buffer such as Buffer All (Sigma-Aldrich-Chemie, Deisenhofen, Germany). The equilibration for a constant pH under room atmosphere was performed using a 24-well culture plate. 1 ml of culture medium was pipetted into each well. Then an increasing concentration of 10 to 50 mmol/l HEPES was added to each of the wells. For the following 24 hours the culture plate was incubated 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 optimal equilibration of pH, O_2 and CO_2 in perfusion cultures the media were transported through 1 mm inner diameter and gas-permeable silicone tubes, which allowed continuous exchange of atmospheric gases.

2.7. Physiological parameters

Metabolic activity of the cells was monitored by analyzing the superfused culture medium. Media parameters such as pH, pCO_2, pO_2, osmolarity, lactate and electrolyte concentrations of Na^+, K^+, Cl^- and Ca^{2+} were determined in undiluted 200 μl samples of the culture medium (Fig. 6). 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 compartment, before and after the medium had passed through the container.

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. 2a). To detect unphysiological leaks in 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 applied. Traces of red color within the clear medium showed unphysiological 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 electrolyte measurement with an analyzer (Nova Biomedical, Fig. 6). Medium specimens were collected just before and after the medium had passed the luminal and basal compartment 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 difference in osmolarity between the luminal and basal compartment.

2.9. Registration of temperature

During long term cultures temperature had to be maintained at a constant level. Temperature was controlled by 2 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. Perfusion rate

During the 14 days 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 an electronic balance was used. Measuring the increase in amount/weight of medium over time in the waste bottles an exact 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. 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 [19]. The antibody was kindly provided by Dr. M. 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 [20].

For the light microscopical assessment 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). Immunolabelling was started by fixing the cryosections for 10 minutes in ice-cold ethanol as described earlier [21]. 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 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 minutes 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 repeated at least three times. More than 5 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.

3. Results and discussion

3.1. Challenge

Gradient culture of epithelia for periods of time is urgently needed in modern biomaterial research. However, the barrier function is frequently developed only to a minor degree or even lost. Major obsta-
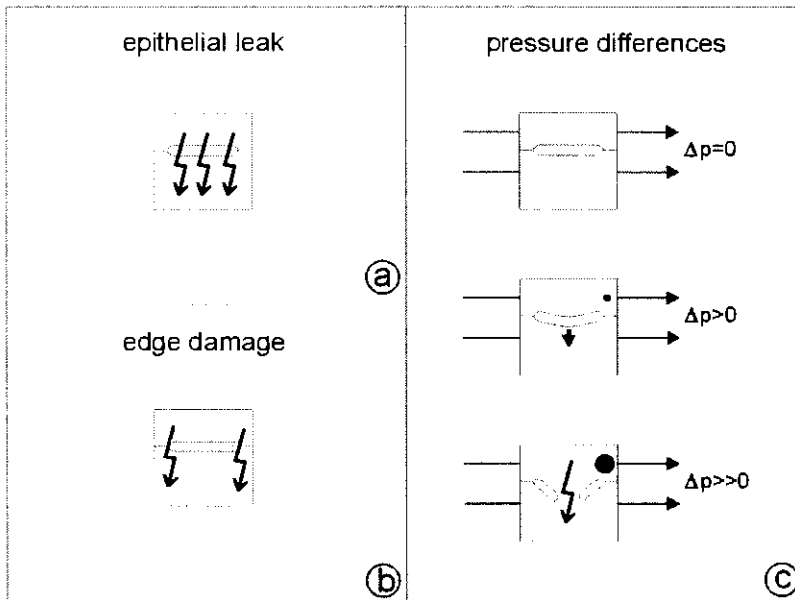
Fig. 5. Schematic illustration of possible loss of barrier function in epithelia cultured in a gradient container. (a) Epithelial leaks appear when the epithelium is not able to seal the paracellular shunt. (b) Edge damage occurs at sites where living cell come in contact with a polymer material. (c) 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 side 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 can not withstand this pressure and is disrupted. Consequently the barrier function is lost.

cles are epithelial leaks (Fig. 5a). They arise if perfect confluency of the cells on the selected biomaterial is not developed or if perfect sealing between neighboring cells is not present due to cell biological reasons. Edge damage (Fig. 5b) leads to a loss of barrier function and is seen at sites where living cells and suboptimal polymer materials come in contact [22]. Exept for epithelia contacting air all other epithelia in our organism exhibit a barrier function, where they are exposed to different fluids at the luminal and basal side. To mimic such a situation under *in vitro* conditions with primary cultures and in combination with new biomaterials appears to be a simple task, but the experimental realization proves rather difficult. First of all cultured epithelia have to withstand the superfusion of media and they have to maintain the physiological barrier between the luminal and basal compartments in the culture container. Uncontrolled mixing of the luminal and basal media must not occur.

3.2. Continuous supply of medium to epithelia

Gradient perfusion culture was performed outside a CO_2-incubator on a laboratory table exposed under room atmosphere (Fig. 4). A peristaltic pump transported the medium from the storage into the waste bottle so that no recirculation occurred. This guaranteed constant nutrition and oxygen supply including permanent elimination of harmful metabolic products.

During long term culture of epithelia in a gradient container we continuously controlled the physiological environment (Fig. 6a,b). An individual example was taken before the basic culture medium IMDM reached the container. A stable pH of 7.4 was found under room atmosphere air during perfusion cul-
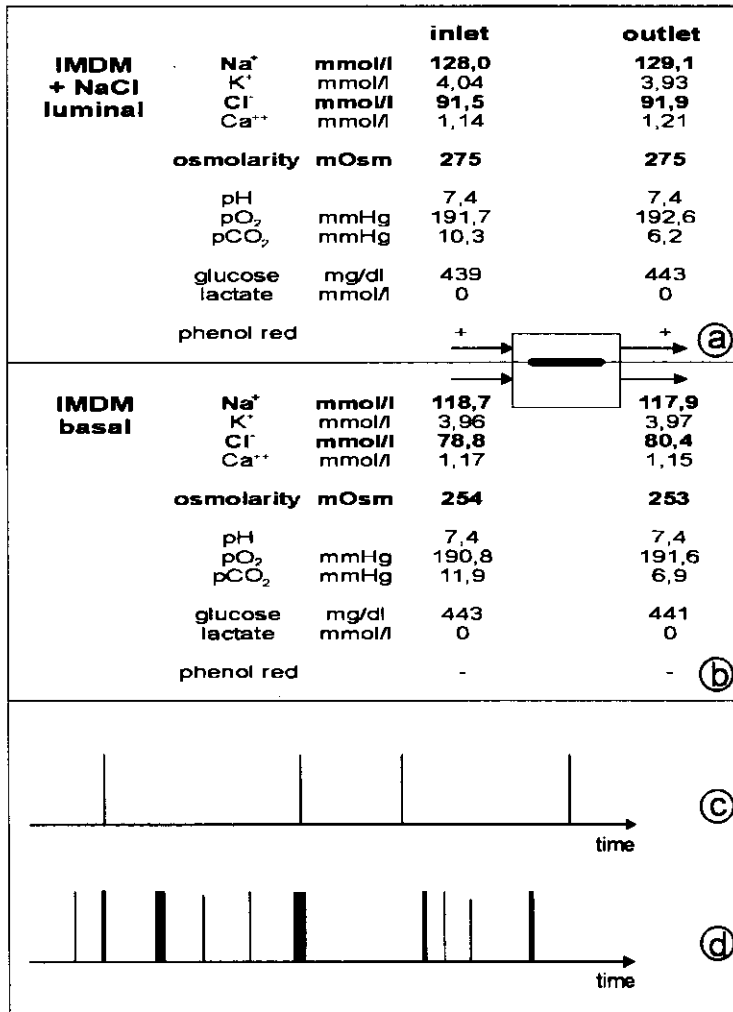
Fig. 6. Physiological parameters before (a) and behind (b) a gradient culture container. (c) and (d) Registration of gas bubbles passing through a gradient culture container by a infrared (IR) gate sensor over a 96 hours period. The frequency and size of registered gas bubbles are indicated by black 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 (c) as compared to controls without bubble elimination (d).

ture. As compared to a CO_2-incubator a relatively low content of 11 mm Hg CO_2 was detectable because of the low content of CO_2 (0.3%) in air. In contrast, a high amount of 190 mm Hg 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 medium before and after the container. The content of 440 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 unphysiological levels after the container could not be observed. It shows that an harmful influence of this metabolite will not occur during the culture period because of its continuous elimination. This aspect is especially important, when biodegradable biomaterials are used in the experiments.

3.3. Functional barrier

To measure the maintenance of epithelia with an intact barrier function media containing phenol red was applied at the luminal, while medium without phenol red was used at the basal side (Fig. 6a,b). Only those epithelia were used for further experimentation which maintained the fluid gradient without mixing luminal and basal fluids. Maintenance of a fluid gradient further could be controlled by comparison between the Na^+ or Cl^- content, respectively the osmolarity between the luminal and basal compartment. The luminal culture medium consistently contained higher concentrations of NaCl and higher osmolarity than the medium at the basal side. By this simple method the maintenance of the epithelial barrier was monitored in combination with physiological parameters.

3.4. Elimination of gas bubbles

A perfect atmosphere for epithelia cultured in a gradient container is obtained when no pressure difference is present between the luminal and basal compartment (Fig. 5c; $\Delta p = 0$). However, since oxygen-rich media are used, gas bubbles within the tubing present a major problem for long term gradient perfusion cultures. Gas saturated culture medium is transported by slowly rotating peristaltic pumps. During this process gas separates from the liquid phase and randomly accumulates in the form of gas bubbles. Their location and amount in the gradient culture container or within tubes with effluent culture medium can not be predicted. The bubbles remain attached for some period of time and increase in diameter. Finally, having reached a certain size the air bubbles lead to fluid pressure differences between the luminal and basal medium compartment comparable to an embolus in a small blood vessel. In gradient perfusion culture this initially causes a protrusion of the tissue to the side of lower pressure (Fig. 5c; $\Delta p > 0$). An increasing pressure difference will finally result in the disruption of the tissue (Fig. 5c; $\Delta p \gg 0$).

To avoid liquid pressure differences in the gradient container it is necessary to minimize the occurrence of air bubbles in the system. Bubbles preferentially occurred at sites where 2 different polymer materials come in contact along the conducting flow path. Consequently we constructed bottle closures, which conduct the medium through the closure in a silicone tube thus avoiding contact (Fig. 3a,b). Experiments showed that the use of the newly developed closures significantly decreased the occurrence of gas bubbles during media transport.

To further eliminate gas bubbles we developed a gas expander module (Fig. 3c,d). The module consists of 2 chambers. The medium rises a small reservoir, then drops down across a barrier before leaving the container. During this process air bubbles are separated from the medium and collected at the top of the container. Combining the newly developed bottle closures with a gas expander module we found that the occurrence of gas bubbles in the culture medium could be drastically reduced (Fig. 6c) compared to media transport without these tools (Fig. 6d). Most important in this connection is, that reduction of gas bubbles by the described method does not affect the content of oxygen in the medium.

3.5. Triggering epithelial development

We elucidate mechanisms triggering the development from an embryonic towards a functional rCD epithelium in gradient culture experiments. Earlier experiments showed that the terminal differentiation of rCD epithelia can be influenced by 2 very different stimuli. Administration of a steroidal hormone such as 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]. It could be shown that peanut lectin (PNA) binding was upregulated depending on aldosterone application [24].

Recent experiments further revealed that a chronical electrolyte load such as administration of additional NaCl could modulate cell biological features of adult principal (P) and intercalated (IC) cells [20,25,26]. P_{CD} Amp 1, an antigen occurring exclusively in the embryonic CD ampulla was downregulated in these experiments demonstrating the maturation of the epithelia after a chronic NaCl exposure [27].

The continuous application of NaCl to the luminal culture medium showed different effects on renal collecting duct antigen expression. Mab 703 and mab 503 recognize apically localized proteins in P and IC cells in adult renal CD cells [19]. The facultative expression of these proteins is influenced distinctly by the addition of rather small concentrations of NaCl (6 to 12 mmol/l) to the luminal culture medium (Fig. 7) [28]. Immunohistochemistry demonstrated that culture of the embryonic tissue with the same media at the luminal and basal side resulted in less than 10% mab 703 (Fig. 7a) and mab 503
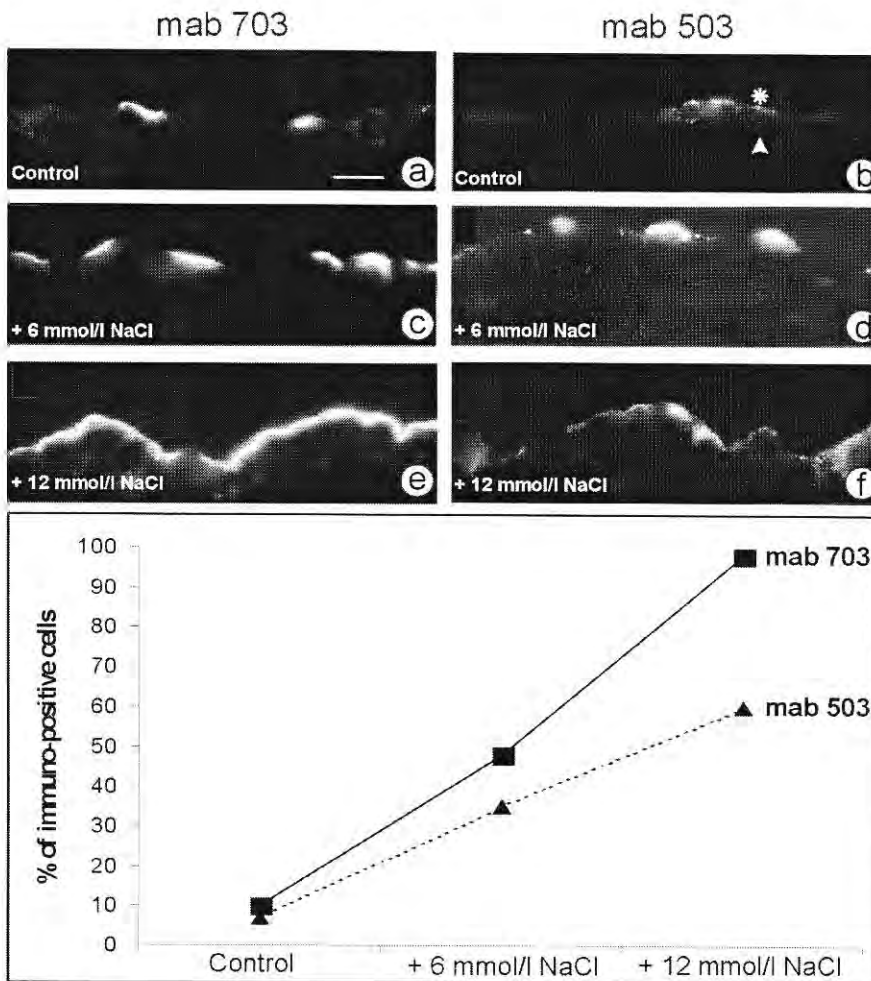

Fig. 7. Cryosections of CD epithelia cultured for 14 days in a gradient container. Each epithelium rests on a kidney-specific collagenous matrix. (a,b) Culture of the epithelium with IMDM at the luminal and basal side results in only 10% mab 703 (a) and mab 503 (b) immunopositive cells. (c,d) Addition of 6 mmol/l NaCl to IMDM at the luminal side reveals 45% mab 703 (c) and 30% mab 503 (d) immunopositive cells. (e,f) Addition of 12 mmol/l NaCl to IMDM at the luminal side reveals 100% mab 703 (e) and 60% mab 503 (f) immunopositive cells. All cells of the CD epithelium show immunolabel for cytokeratine, Na/K ATPase, COX 1 and COX 2. Asterisk – luminal side, arrow head – basal aspect of the epithelium.

(Fig. 7b) positive cells within the epithelium. Addition of 6 mmol/l NaCl yielded 45% mab 703 (Fig. 7c) and 30% mab 503 (Fig. 7d) positive cells. Most interestingly, when 12 mmol/l NaCl were added to the luminal IMDM nearly all cells showed immunostaining after mab 703 incubation (Fig. 7e), while 60% were positive for mab 503 (Fig. 7f). This experimental series shows a concentration-dependent effect of altered culture medium. In contrast, markers for constitutive protein expression such as cytokeratine 19 or Na/K ATPase demonstrated in the experimental series that all cells within the rCD epithelium remained positive. Important enzymes within the renal CD system are COX 1 and 2. All cells within the cultured epithelium were also positive for COX 1 and COX 2 antibodies. Thus, the results with constitutively expressed markers were obtained independent of low or physiological NaCl concentrations added to the luminal culture medium (data not shown).

4. Conclusions

In the present experiments we used embryonic rCD 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 optimized biomaterial testing and tissue engineering under the gradient culture conditions described [29,30]. As compared to earlier culture experiments [31] we had to apply advanced techniques for an improved development of the tissue. The experiments showed the feasibility of culturing embryonic epithelia in a permanent fluid gradient for extended periods of time necessary for biomaterial testing. In individual series of experiments up to 80% epithelia with intact barrier function could be harvested. Performing all these experiments we obtained an unexpected amount of information about epithelial tissue development growing in combination with different biomaterials. Before we could not imagine how sensitive the tissue responds to the environment offered.

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