J. Biomater. Sci. Polymer Edn, Vol. 12, No. 3, pp. 353–365 (2001)

Long term culture of epithelia in a continuous fluid gradient for biomaterial testing and tissue engineering

WILL W. MINUTH *, RAIMUND STREHL, KARL SCHUMACHER and UWE DE VRIES

Department of Anatomy, University of Regensburg, 93053 Regensburg, Germany

Received 11 May 2000; accepted 10 January 2001

Abstract—Epithelia perform barrier functions being exposed to different fluids on the luminal and basal side. For long-term testing of new biomaterials as artificial basement membrane substitutes, it is important to simulate this fluid gradient. Individually-selected biomaterials can be placed in tissue carriers and in gradient containers, where different media are superfused. Epithelia growing on the tissue carriers form a physiological barrier during the whole culture period. Frequently however, pressure differences between the luminal and basal compartments occur. This is caused by a unilateral accumulation of gas bubbles in the container compartments resulting in tissue damage. Consequently, the occurence of gas bubbles has to be minimized. Air bubbles in the perfusion culture medium preferentially acumulate at sites where different materials come into contact. The first development is new screw caps for media bottles, specifically designed to allow fluid contact with only the tube and not the cap material. The second development is the separation of remaining gas bubbles from the liquid phase in the medium using newly-developed gas expander modules. By the application of these new tools, the yield of embryonic renal collecting duct epithelia with intact barrier function on a fragile natural support material can be significantly increased compared to earlier experiments.

Key words: Kidney; epithelia; biomaterial testing; tissue engineering; differentiation; perfusion; gradient culture container; electrolytes.

1. INTRODUCTION

Epithelia within an organism exhibit specific barrier functions. The epithelial cell layer is closely attached to the basement membrane. The contact between neighboring cells determines the quality of the seal enabling the tissue to control the transepithelial transport. The focus of interest is the generation of skin equivalents [1], vascular grafts [2], insulin producing organoids [3], liver [4, 5], kidney [6], or urothelial [7] support systems. In cases where an artificial matrix is necessary

*To whom correspondence should be addressed. E-mail: will.minuth@vkl.uni-regensburg.de

for construction, the resulting functionality depends on successful integration of epithelial tissue and the applied biomaterial. Consequently, the individual epithelia have to be brought in contact with a variety of biomaterials in order to test their suitability for optimal cell adhesion and differentiation. It is of the utmost importance to learn how tightly attached the cells are to the biomatrix under realistic *in vitro* conditions, to recognize the influence on cellular differentiation and to investigate the ability to resist natural fluid stress over prolonged periods of time.

Tissue-specific conditions for biomaterial testing with epithelia can be simulated in gradient culture containers. Almost all kinds of biomaterials such as textiles, filters, foils, or membranes can be placed into tissue carriers as an artificial basement membrane substitute [8, 9]. The tissue carriers are then placed into a gradient culture container, which is separated into luminal and basal compartments by the epithelia [10]. Earlier experiments showed the feasibility of culturing embryonic renal collecting duct (CD) epithelia in a gradient culture [11].

The challenge for biomaterial testing in combination with gradient perfusion culture is to maintain a functional epithelium which presents a physiological barrier separating the luminal from the basal compartment. However, a crucial experimental problem is that the barrier function of the epithelium frequently does not develop or breaks during the culture period resulting in an unphysiological leakage between the two compartments. Experiments have shown that in most cases the leak is due to pressure differences between the luminal and basal medium compartments leading to microinjuries within the epithelium. This is caused by gas bubbles found to be attached to the inside of the tubing and culture containers.

In the present paper we attempt to demonstrate newly-developed tools used to separate bubbles from oxygen-rich media in order to avoid pressure differences in gradient culture containers. It is shown that by using these simple tools epithelia can be maintained in a luminal–basal medium gradient for long term biomaterial testing.

2. MATERIAL AND METHODS

2.1. Tissue isolation

The generation of embryonic CD epithelia was performed by isolating cortical explants from the kidneys of newborn New Zealand rabbits according to methods described earlier [12]. The explants consisted of a piece of stripped-off capsula fibrosa with adherent CD ampullae, S-shaped bodies, and nephrogenic blastema, which were mounted in tissue carriers (Fig. 1a). For multiplication of cells the carriers were placed in a 24-well plate in a CO_2 incubator (5% CO_2/95% air). 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 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 was medium containing fetal bovine serum applied.

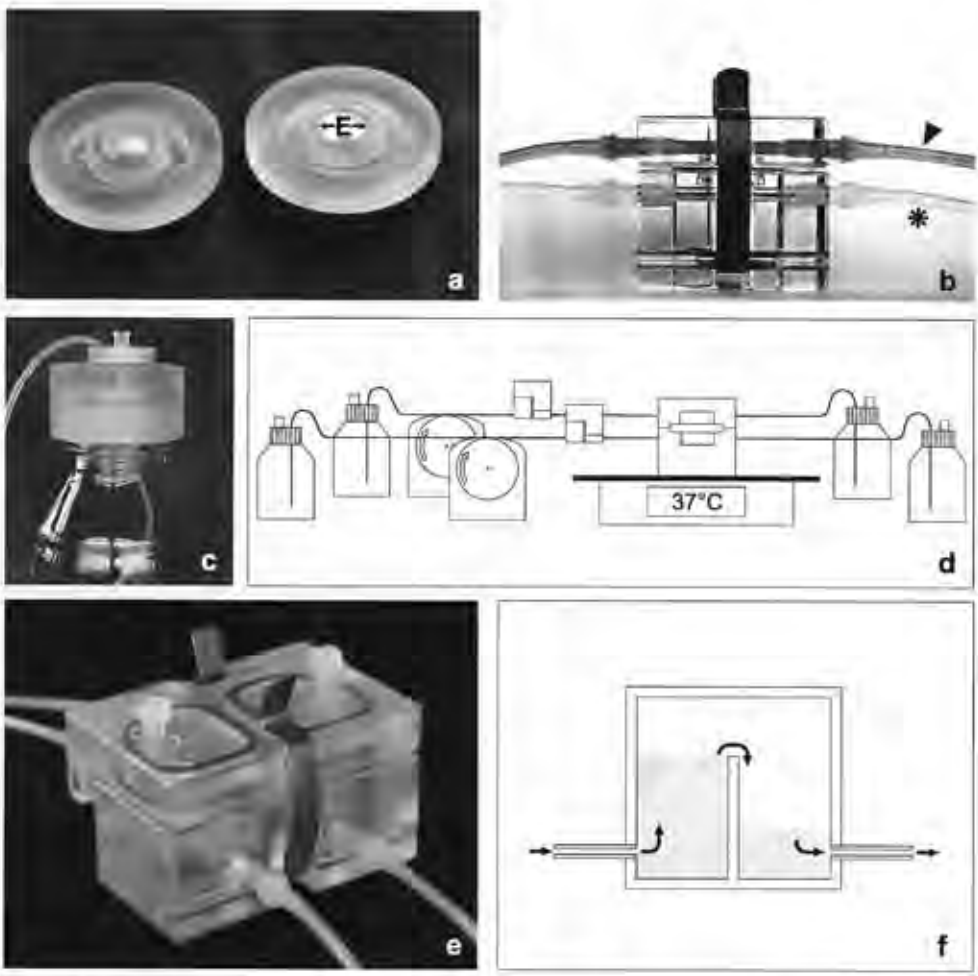


Figure 1. Technical equipment for gradient perfusion culture. (a) Renal explants with an embryonic CD epithelium (6 mm in diameter, E) were fixed in a tissue carrier with an overlapping ring. (b) The tissue carrier was placed in a gradient culture container. IMDM with phenol red was superfused on the luminal side (arrow head), while on the basal side phenol red in IMDM was omitted to allow visual control of the quality of the gradient (asterise). (c) The medium was pumped from the storage bottle through a silicone tube and a newly-developed screw cap. In combination with the gas expander module, this avoids the appearance of gas bubbles. (d) Schematic 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. The media are not reperfused but collected in separate waste bottles. (e) Lateral view of the gas expander module. (f) Schematic illustration of the gas expander function. 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.

2.2. Gradient perfusion culture

Twenty-four hours after culture initiation the tissue carriers were placed in gradient containers, which allow epithelia culture in a fluid gradient so that a tissue-specific environment can be simulated (Fig. 1b; Minucells and Minutissue, Bad Abbach, Germany) [11, 13]. A tissue carrier with the developed tissue separated the container into luminal and basal compartments. On both sides of the epithelium either the same medium or media of different compositions could be perfused. The culture system was used on a laboratory table (Fig. 1d). Little equipment was necessary to perform perfusion culture. A thermo plate (MEDAX, Kiel, Germany) 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, IMDM [14] without serum was used as the standard medium. Fresh medium was continuously perfused at a rate of about 1 $ml\,h^{-1}$ 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 $mmol\,l^{-1}$ 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 side. Experimental series were run with standard IMDM on the basal side, while IMDM containing additional 3–24 $mmol\,l^{-1}$ NaCl was superfused on 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 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, the 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).

2.5. Elimination of gas bubbles

To eliminate gas bubbles specifically-modified screw caps (Fig. 1c) in combination with newly developed gas expander modules (Fig. 1e, f) were employed (Minucells and Minutissue). Registration of air bubbles in perfusion lines was performed by a sensor, which was placed on a 1-mm inner diameter glass capillary at the tube of the effluent culture medium (Fig. 2a). Floating air bubbles were registered by

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

2.6. Physiological parameters

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 gradient container (Fig. 3).

2.7. Search for barrier leaks in the epithelia during culture

The tissue carriers separated the gradient culture container into a luminal and basal compartment (Fig. 1a, b). To detect unphysiological leaks in tissue, the luminal compartment was perfused with IMDM containing phenol red. At the basal side IMDM without phenol red was used. 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.

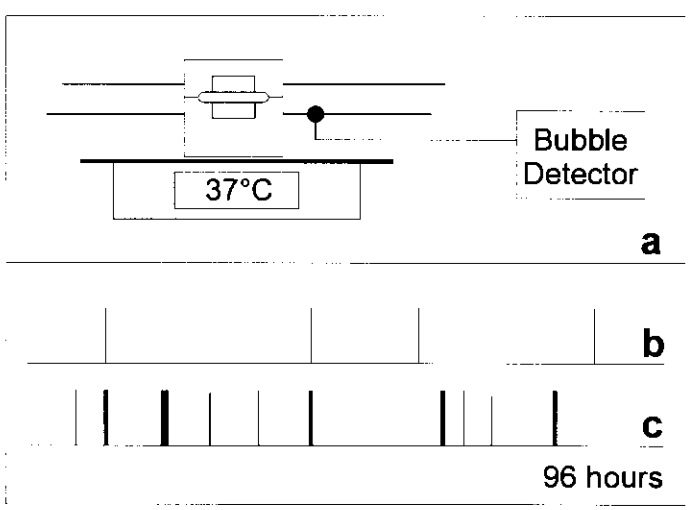
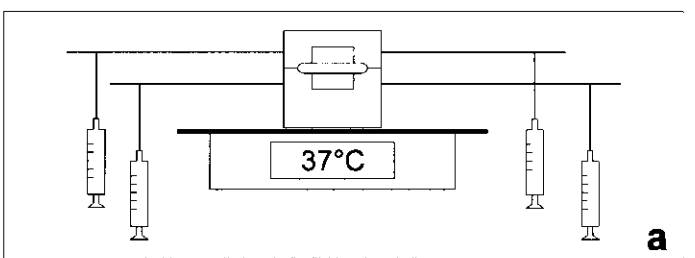


Figure 2. Registration of gas bubbles passing through a gradient culture container by a infrared (IR) gate sensor over a 96-h period (a). The frequency and size of registered gas bubbles are indicated by black bars. It can be shown that the amount of gas bubbles is drastically decreased by the use of a gas expander module (b) as compared to controls without bubble elimination (c).

Control for barrier leaks was further performed by the electrolyte measurements with an analyzer (Nova Biomedical, Figs 3 and 4). 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 on 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 compartments.

2.8. Detection of cellular differentiation

During culture the embryonic cells developed adult epithelial tissue features. To register the primary appearance of individual CD cell characteristics a set of markers



Before				After	b
Luminal	Basal			Luminal	Basal
130,0	**117,7**	**Na^+**	**mmol/l**	**129,7**	**117,5**
4,01	3,96	K^+	mmol/l	3,93	3,96
91,0	**80,4**	**Cl^-**	**mmol/l**	**91,5**	**79,8**
1,11	1,15	Ca^{++}	mmol/l	1,11	1,15
275	**253**	**Osmolarity**	**mOsm**	**275**	**253**
7,44	7,42	pH		7,46	7,44
193,7	191,8	pO_2	mmHg	191,6	191,6
10,7	11,9	pCO_2	mmHg	6,4	6,5
443	446	Glucose	mg/dl	443	445
0	0	Lactate	mmol/l	0	0
+	-	Phenol red		+	-

Figure 3. Physiological parameters of an individual experiment in media measured before and after a gradient culture container. (a) Schematic illustration of sites where media specimens were collected. (b) On the luminal side IMDM containing 130 mmol l^{-1} Na and 91 mmol l^{-1} Cl was superfused, while on the basal side standard IMDM with 117.7 mmol l^{-1} Na and 80.4 mmol l^{-1} Cl was applied. The luminal–basal gradient of Na, Cl, and osmolarity indicates a perfect barrier function of the cultured epithelia.

were used reacting specifically in adult renal tissue (Fig. 5). 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, IA, USA. Mab 703 recognizes 40, 48, 51, 60, and 99 kDa proteins identified on adult cultured principal (P) cells of the renal CD [15]. The antibody was kindly provided by Dr. M. Tauc, Department of Cellular and Molecular Physiology, University of Nice, France.

For the light microscopical monitoring of cell development within 14 days of culture, 7-μm cryosections were prepared using a Cryostat HM 500 (Microm, Walldorf, Germany). Immunolabelling was begun by fixing the sections for 10 min in ice-cold ethanol as described earlier [16]. 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 monoclonal antibodies (mab), anti Na/K ATPase and mab 703 (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

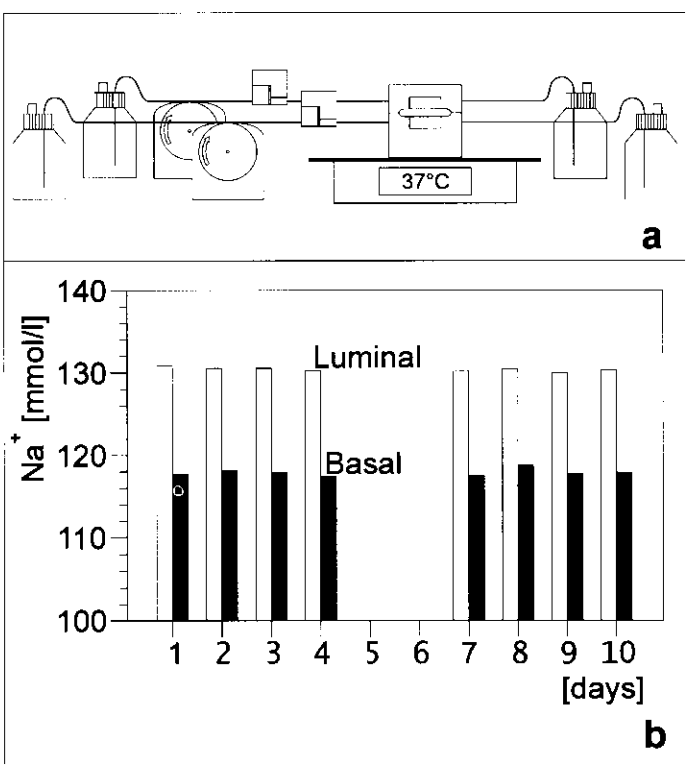


Figure 4. Maintenance of a luminal–basal medium gradient over 10 days. After passing the gradient culture container, medium was collected on the luminal and basal side (a). During the whole culture period the luminal (130 mmol l^{-1} Na) and basal (117.7 mmol l^{-1} Na) gradient is maintained indicating an intact barrier function of the cultured CD epithelium (b).

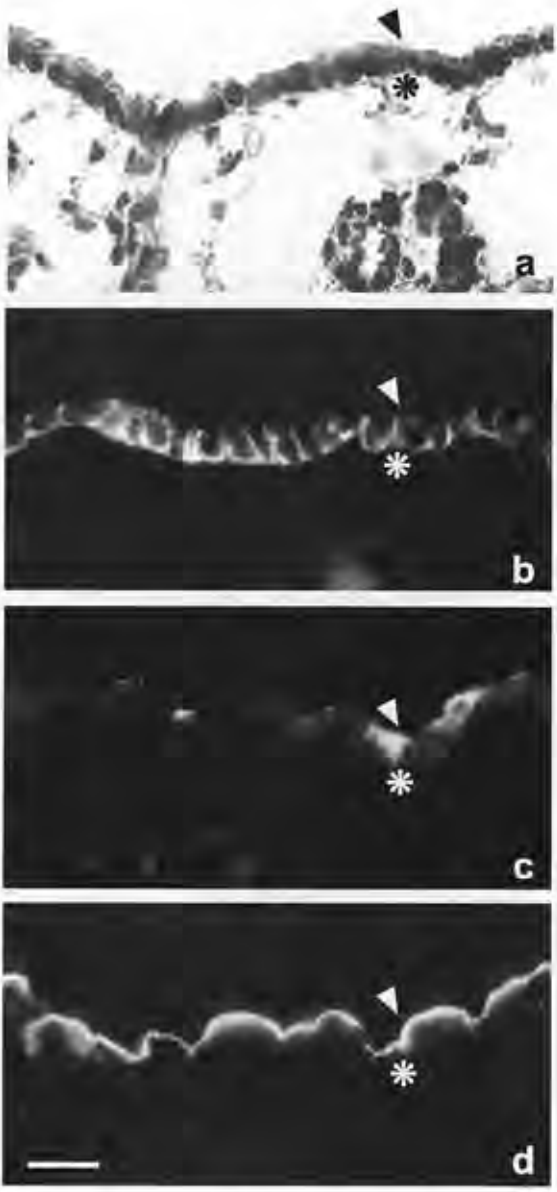


Figure 5. Cellular differentiation of an embryonic collecting duct epithelium in gradient culture. (a) Cryosection of a CD epithelium cultured for 13 days in a gradient container. The epithelium rests on a kidney specific matrix. (b) Culture with the same or with different media on the luminal and basal side results in basolateral immunolabeling with anti Na/K ATPase on all of the epithelial cells. (c) Standard IMDM with 117.7 mmol l^{-1} Na and 80.4 mmol l^{-1} Cl on the luminal side of the epithelium shows only 10% mab 703 immunopositive cells. (d) IMDM containing 130 mmol l^{-1} Na and 91 mmol l^{-1} Cl on the luminal side reveals 90% mab 703 immunopositive cells (bar = 10 μm: (*) — basal aspect, → — luminal side of the epithelium).

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. Following several washes in PBS the sections were embedded in FITC guard (Testoc, Chicago, IL, USA) and examined using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany).

2.9. Evaluation

In total, more than thirty 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 five epithelia were analyzed per experimental series. A minimum of fifty 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

Gradient culture experiments with epithelia often fail due to an insufficient barrier function or mechanical damage during the experimental period [17–19]. As observed in earlier experiments, the majority of leaks within thin tissue layers is caused by air bubbles leading to pressure differences in the tubes of the effluent culture medium, which show similarities to an embolus in a capillary and result in tissue damage. Consequently, the appearance of air bubbles has to be minimized in gradient perfusion culture experiments without altering the oxygen concentration in the liquid phase. In our experimental setup, gas-saturated media were pumped from the storage bottles via silicone tubes and newly developed screw caps (Fig. 1c). The closure conducts the tube so that the medium does not come in contact with the cap material. Then the medium was pumped into a gas expander module, which separates bubbles from the liquid phase of the culture medium (Fig. 1e, f). Monitoring throughout a culture period of 96 h showed a drastic reduction of gas bubbles (Fig. 2b) compared with experiments without a module (Fig. 2c). In contrast to earlier experiments, up to 80% of epithelia with an intact barrier function could be harvested.

During the 13-day culture period in a gradient container it was important to obtain information about how far the tissue could maintain its barrier function to separate the luminal from the basal fluid compartment. Two simple methods gave clear information about the quality of the fluid gradient. On the luminal side of the epithelium, culture medium with phenol red was perfused, while clear medium was used on the basal side. If a leak was present at the start or during the course of the culture period, mixing of red and clear culture media could be easily recognized. In all of these cases the ongoing experiment was cancelled and the cultured material was discarded.

A more accurate control of the luminal–basal medium gradient was obtained by measuring several medium parameters in an electrolyte analyzer (Fig. 3). In a

culture experiment for example, the basal side was perfused with standard IMDM (117.7 mmol l^{-1} Na; 80.4 mmol l^{-1} Cl), while the luminal side of the epithelium was perfused with IMDM containing 130 mmol l^{-1} Na and 91 mmol l^{-1} Cl during the whole culture period. Physiological control of an intact physiological barrier function was performed by recording medium parameters before and after a gradient culture container at the luminal and basal side (Fig. 3a). A typical example is given in Fig. 3b. An intact fluid gradient could be measured by 130 mmol l^{-1} Na at the luminal side vs 117.7 mmol l^{-1} Na at the basal side. Accordingly, the luminal and basal compartments contained 91 vs 80.4 mmol l^{-1} Cl. Osmolarity was 275 vs 253 mOsm. In contrast, since only NaCl was added to the luminal culture medium, K or glucose measurements did not reflect a gradient situation but showed identical values. Performing these measurements immediately before and after the gradient culture container throughout the whole culture period of 10 days, information about the epithelial barrier function could be obtained without any recognizable influence or damage to the epithelia (Fig. 4).

Light microscopical examination of the tissue showed that the complete surface of the renal explant, 6 mm in diameter, was covered by a polarized epithelium after exposure to a constant medium flow in the gradient container for a period of 13 days (Fig. 5a). This result was obtained in the control series (Fig. 5c) when standard IMDM was superfused at the apical and basal side, as well as in the series when medium containing 130 mmol l^{-1} Na was superfused on the luminal side of the epithelium (Fig. 5d). Independent of the treatment, all of the epithelial cells demonstrated the primary expression of Na/K ATPase (Fig. 5b). Only a few cells showed binding of mab 703 when standard IMDM was applied on the luminal and basal side (Fig. 5c). However, the amount of mab 703 binding cells changed drastically when the epithelia were exposed to a luminal NaCl load in a gradient container (Fig. 5d). More than 90% of the cells showed a strong luminal labeling.

4. DISCUSSION

To culture epithelia on an artificial biomatrix in a fluid gradient and under permanent perfusion with different media at the luminal and basal sides, appears to be a simple task, but the experimental realization proves rather difficult. First of all the epithelia have to withstand the perfusion culture conditions and maintain a physiological barrier between the luminal and basal compartments in the gradient container. Mixing of the two media must not occur. Secondly, a presupposition for perfect gradient culture is that no pressure difference develops between the luminal and basal compartment (Fig. 6a; $\Delta p = 0$). Thirdly, since oxygen-rich media are used, gas bubbles within the tubing present a major problem for long-term gradient perfusion cultures. During the slow media transport, gas separates from the liquid phase and randomly accumulates in gas bubbles. Their location and amount in the gradient culture container or within effluent tubing can not be predicted. The bubbles remain attached for some period of time, when they increase

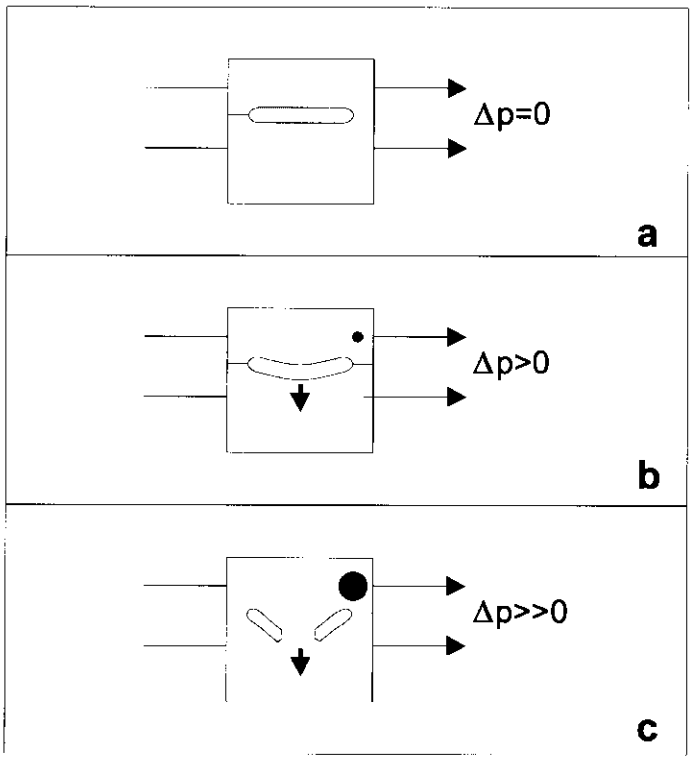


Figure 6. Schematic illustration of liquid pressure differences, which may cause tissue damage in a gradient culture container. (a) No tissue damage will occur when the pressure is identical on the luminal and basal sides of the gradient culture container. (b) In contrast, a small gas bubble (•) at the outlet of the luminal compartment will increase the pressure in the luminal compartment resulting in an extension of the tissue towards the basal side (→). (c) When the gas bubble increases (●) in diameter an increase in pressure in the luminal compartment is found. The tissue can not withstand this pressure and is disrupted. Consequently the barrier function is lost.

in diameter. Finally, when reaching a certain size, the air bubbles cause differences in fluid pressure similiar to an embolus in a small blood vessel. In gradient perfusion culture, it causes a protrusion of the tissue to the side of lower pressure (Fig. 6b; $\Delta p > 0$). An increasing pressure difference can finally result in the disruption of the tissue (Fig. 6c; $\Delta p \gg 0$).

Consequently, the main problem in gradient perfusion culture experiments is to maintain a constant physiological barrier function of the epithelia in the container. As shown in earlier investigations, the generated epithelium develops an aldosterone-dependent Na transport with tight characteristics [12, 13] including principal (P) and intercalated (IC) cell features as observed within the kidney [10, 11, 15]. However, edge damage and microinjuries may lead to leakage [20, 21]. Electrophysiological transepithelial registration to control the maintenance of a barrier function in long term culture experiments proved less favorable, since electrode fouling, tissue damage, and a minor degree of cellular differentiation was observed

in earlier experiments. The addition of radioactive molecules such as inuline to the luminal culture medium did not seem suitable. Instead two simple procedures were employed to monitor the quality of the epithelial barrier. One was optical control using media with different colors on the luminal and basal sides. The other was the continuous control of the gradient expressed by a difference in electrolyte concentration between the luminal and basal compartments (Fig. 4). Combining both methods, it was possible to obtain information about a functional barrier throughout the whole culture period.

The goal is to generate epithelia on individually-selected biomaterials as substitutes for the tissue-specific biomatrix (Fig. 5) [22]. In the presented experiments very fragile biomatrix material such as the fibrous kidney capsule was used as a mechanical support for the epithelia. A more rigid material made of nitrocellulose, polycarbonate, or other polymers will improve the mechanical properties and withstand an extension of the tissue due to pressure differences in the system (Fig. 6) [23]. The present technical status is that we are learning to keep renal epithelia in a permanent fluid gradient to evaluate cellular adherence, differentiation and tightness in comparison to the in vivo situation. According to our experiences it will be possible to culture other types of epithelia on individually selected biomaterials, so that they resemble their functional correlates found within the organism as closely as possible. However, when biomaterials are tested it has to be taken into consideration that not only the test material but also the cellular environment interacts with the tissue as shown in previous investigations [24–26].

INFORMATION

For tissue culture information please contact: http://www.minucells.de

REFERENCES

1. B. Gron, A. Andersson and E. Dabelsteen, *APMIS* **107**, 779 (1999).
2. C. Chen, M. E. Halkos, S. M. Surowiec, B. S. Conklin, P. H. Lin and A. B. Lumsden, *J. Surg. Res.* **88**, 26 (2000).
3. K. K. Pappas, R. C. Long, A. Sambanis and I. Constantinidis, *Biotechnol. Bioeng.* **66**, 219 (1999).
4. X. Liu, E. L. Lecluse, K. R. Broudwer, L. S. L. Gan, J. J. Lemasters, B. Stieger, P. J. Meier and K. L. R. Brouwer, *Am. J. Physiol.* **277**, G12 (1999).
5. A. Bader, N. Fruhauf, M. Tiedge, M. Drinkgern, L. DeBartolo, G. Steinhoff and A. Haverich, *Exp. Cell Res.* **246**, 221 (1999).
6. H. D. Humes, S. M. MacKay, A. J. Funke and D. A. Buffington, *Kidney Int.* **55**, 2502 (1999).
7. B. Ludwikowski, Y. Y. Zhang and P. Frey, *BJU Int.* **84**, 507 (1999).
8. W. W. Minuth, V. Majer, S. Kloth and R. Dermietzel, *In vitro Cell Dev. Biol. Animal* **30**, 12 (1994).
9. F. Fey-Lamprecht, T. Groth, W. Albrecht, D. Paul and U. Gross, *Biomaterials* **21**, 183 (2000).
10. W. W. Minuth, J. Aigner, S. Kloth, P. Steiner, M. Tauc and M. L. Jennings, *Ped. Nephrol.* **11**, 140 (1997).

11. P. Steiner, R. Strehl, S. Kloth, M. Tauc and W. W. Minuth, *Differentiation* **62**, 193 (1997).
12. W. W. Minuth, *Differentiation* **36**, 12 (1987).
13. W. W. Minuth, P. Steiner, R. Strehl, K. Schumacher, U. de Vries and S. Kloth, *Exp. Nephrol.* **7**, 394 (1999).
14. N. N. Iscove and F. Melchers, *J. Exp. Med.* **147**, 923 (1978).
15. M. Tauc, N. Koechlin, M. Jamous, E. M. Quaghi, M. Gastineau, C. LeMoal and P. Pouleol, *Biol. Chem.* **76**, 55 (1992).
16. K. Schumacher, R. Strehl, S. Kloth, M. Tauc and W. W. Minuth, *In vitro Cell Dev. Biol. Animal* **35**, 465 (1999).
17. A. G. M. Van Dorp, M. C. H. Verhoeven, T. H. VanDerNat-VanDer Meij, H. K. Koerten and M. Ponec, *Wound Rep. Reg.* **7**, 214 (1999).
18. T. Naiki, H. Sugiyama, R. Tashiro and T. Karino, *Biorheology* **36**, 225 (1999).
19. F. Jow and R. Numann, *J. Membrane Biol.* **171**, 127 (1999).
20. M. Stockmann, A. H. Gitter, D. Sorgenfrei, M. Fromm and J. D. Schultzke, *Eur. J. Physiol.* **438**, 107 (1999).
21. M. Walser, *Am. J. Physiol.* **219**, 252 (1970).
22. W. W. Minuth, J. Aigner, B. Kubat and S. Kloth, *Exp. Nephrol.* **5**, 10 (1997).
23. W. W. Minuth, K. Schumacher, R. Strehl and S. Kloth, *J. Biomater. Sci. Polymer Edn* **11**, 495 (2000).
24. E. Wintermantel and H. Suk-Woo, *Biokompatible Werkstoffe und Bauweisen — Implantate für Medizin und Umwelt*, p. 87 (1998).
25. A. Atala, *Urol. Clin. N. Am.* **25**, 39 (1998).
26. M. Vert, G. Schwach, R. Engel and J. Coudane, *J. Control. Rel.* **53**, 85 (1998).