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TOPICAL REVIEW

Technical and theoretical considerations about gradient perfusion culture for epithelia used in tissue engineering, biomaterial testing and pharmaceutical research

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Abstract
Epithelia act as biological barriers, which are exposed to different environments at the luminal and basal sides. To simulate this situation and to improve functional features an *in vitro* gradient perfusion culture technique was developed in our laboratory. This innovative technique appears to be simple at first sight, but the performance needs practical and theoretical knowledge. To harvest intact epithelia after a long-term gradient culture period of many days, leakage, edge damage and pressure differences in the system have to be avoided so that the epithelial barrier function is maintained continuously. Unexpectedly, one of the major obstacles are micro-injuries in the epithelia caused by gas bubbles, which arise during transportation of the medium or due to respiration of the cultured tissue. Gas bubbles randomly accumulate either at the luminal or basal fluid flow of the gradient perfusion culture container. This phenomenon results in fluid pressure differences between the luminal and basal perfusion compartments of the gradient container, which in turn leads to damage of the barrier function. Consequently, the content of gas bubbles in the transported culture medium has to be minimized. Thus, our technical concept is the reduction of gas bubbles while keeping the content of oxygen constant. To follow this strategy we developed a new type of screw cap for media bottles specifically designed to allow fluid contact only with tube and not with cap material. Furthermore, a gas expander module separates gas bubbles from the liquid phase during transportation of the medium. Finally, a new type of gradient culture container allows a permanent elimination of transported gas bubbles. Application of this innovative equipment optimizes the parallel transportation of fluid in the luminal and basal compartments of a gradient culture container.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Epithelial tissue cultures prepared from skin [1, 2], endothelia [3, 4], organ parenchyme [5, 6], urothelial [7, 8], esophageal [9, 10] and tracheal [11, 12] structures are important experimental tools for biomedical research. They offer the unique possibility of optimizing the differentiation profile of tissue engineered grafts, of obtaining insight concerning cellular reaction to new biomaterials or of following alterations of metabolic activity after acute or chronic application of newly developed pharmaceuticals.

In the last decade, numerous efforts have been made to culture functionally intact epithelia. In contrast, published results demonstrate that epithelia in culture often do not develop the expected degree of functionality [13–15]. One reason for insufficient development is the exposure of epithelial cells to the same medium on the luminal and basal sides in a conventional culture dish, which represents a non-physiological situation and results in a permanent biological short-circuit current. This phenomenon in turn correlates with sub-optimal development of polarization and/or a loss of specific features by dedifferentiation (figure 1(*a*)).

The quality of cultured epithelia is highly dependent on the selected biomaterial [16–18], cellular attachment [19], intercellular communication [20, 21] and the environmental conditions during culture [22, 23]. All these factors have to complement one another in order to support differentiation, while preventing the development of atypical characteristics by dedifferentiation. Since it is known that not only growth factors but also a variety of environmental stimuli influence development, it is of outmost importance to offer tissue-specific conditions for cultured epithelia as much as possible [18, 24–28].

In the present review we demonstrate the state of the art in culturing epithelia under fluid gradients and discuss related challenges and efforts. Furthermore, we present our approach to overcome the methodical difficulties associated with gradient perfusion culture by innovative technical equipment developed in our laboratory over the last few years.

2. Results and discussion

2.1. Toward a tissue-specific environment

For decades various types of epithelia have been cultured in modified Petri-dishes to use them as *in vitro* models (figure 1(*a*)). However, frequently epithelial cells do not develop the required degree of functionality when cultured in a stagnant environment. A further factor of limitation is the unequal distribution of nutrients and oxygen supply. The side of epithelia resting on the bottom of the dish receives a significantly reduced supply of nutrients and oxygen compared to the side facing the culture medium. In each case this is a non-physiological situation for the growing epithelia, which results frequently in a sub-optimal development. Thus, the challenge is to improve the culture conditions by creating an epithelium-specific environment.

An environment in a more physiological sense can be simulated by using filter inserts (figure 1(*b*)) [10, 29, 30].

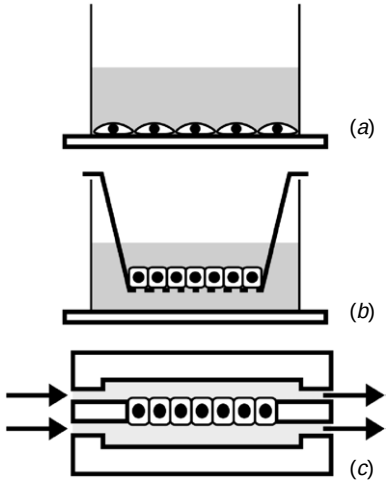

Figure 1. Schematic illustration of different culture conditions for epithelia. (*a*) Sub-optimal development of epithelial cells at the bottom of a culture dish. (*b*) Epithelial cells cultured in a filter insert. (*c*) Epithelium cultured in a gradient container with constant luminal and basal perfusions of fresh medium (arrow).

The epithelial cells grow here on a membrane in a cylindrical holder. The growing cells can be provided with media of different compositions at the luminal and basal sides. However, long-term maintenance of a physiological gradient for the epithelia is problematic with this technique due to the stagnant environment and the small volume of medium supply. The gradient between the luminal and basal medium can therefore not be maintained over prolonged periods of time.

To simulate a typical environment for epithelia in long-term experiments, a gradient perfusion culture system was invented and designed in our laboratory (figure 1(*c*)) [31, 32]. It allows the simulation of individual needs necessary for epithelia to achieve a high degree of cellular differentiation over prolonged periods of time [33–36]. For example, gradient perfusion culture made the *in vitro* generation and modulation of a collecting duct epithelium derived from renal stem cells possible for the first time [33–36].

By now various other types of epithelia have been cultured using this gradient perfusion culture system. Examples are the successful pharmaceutical testing with epithelial cell lines [37], the establishment of a blood–retina barrier [38, 39], the maintenance of retinal pigment epithelium (RPE) in response to laser application [40], the engineering of skin equivalents [41] and finally the modified dentin barrier test [42].

In the past few years, the technical state of gradient perfusion culture has been improved continuously by our group, but it is in our view not yet fully optimized. First important steps are being taken to understand how to maintain epithelia in a permanent fluid gradient over prolonged periods of time in order to evaluate cellular adherence, differentiation and epithelial tightness in comparison with the *in vivo* situation. Specific gradient perfusion culture conditions have been developed for different types of epithelia, which allow them to develop cell biological features closely resembling their functional counterparts found within the organism. In

this context, we have been learning about close attachment of epithelial cells on selected biomaterials as supports and resistance to fluid stress. During gradient perfusion culture the epithelia have to tolerate different fluid compositions at the luminal and basal sides over prolonged periods of time.

2.2. Principles of gradient perfusion culture

The described strategy to generate functional epithelia for gradient perfusion culture consists of three principal steps: (i) biological material can be used in the form of either an intact piece of flat tissue such as retina [40] or single cells such as cell lines [37, 43]. The biological specimens are grown on natural supports or synthetic filters as basal lamina substitutes. (ii) To protect the developing epithelia from mechanical damage during experimental handling the support materials are placed into specifically designed tissue carriers (figures 2(*a*), (*b*)). (iii) To ensure optimal nutrition and maintenance of epithelia the tissue carrier is placed into a gradient perfusion culture container (figure 2(*c*)).

2.3. Selection of a suitable support improves development

In gradient perfusion culture experiments epithelia have to develop on individually selected support materials as substitutes for the tissue-specific basal lamina (figures 2(*a*), (*b*)) [33–36, 43, 44]. One prerequisite for optimal epithelial development is the positive interaction of cells with the synthetic filter material selected to be used as a basal lamina substitute. To obtain confluent growth of epithelial cells the support material has to show full biocompatibility.

The spectrum of prospective materials is extremely wide. More rigid support materials such as nitrocellulose, polycarbonate or other polymer materials available in the form of membranes, foils or meshes can be applied to improve the mechanical stability of the artificial basal lamina substitute. Such rigid materials later can better withstand deformation during experimentation resulting from pressure differences in the gradient perfusion culture container (figure 2(*a*)) [35, 36]. A selected support material is then placed into the lower part of the tissue carrier and is held in place by a tension ring. Many of the described materials are commercially available as discs measuring 13 mm in diameter. Others have to be excised by a punching tool in order to fit into a tissue carrier. Another type of carrier can hold sheets of natural extracellular matrices. In this case the matrix is held in place on the tissue carrier by a silicone ring like the skin of a drum (figure 2(*b*)). For example, the fibrous capsule of the kidney can be used, if special care is taken during handling and experimentation. The selected support material must also be permeable and show enough porosity to allow free exchange of molecules. Before cell seeding the selected support material must be able to withstand a sterilization procedure without losing its specific properties.

To quickly analyze the growth pattern of cells seeded onto a support DAPI or propidium iodide labeling can be performed. The nuclear staining pattern of individual specimens is then analyzed under the fluorescence microscope. Epifluorescence analysis can be applied for all materials regardless of whether the selected support is transparent or non-transparent [43].

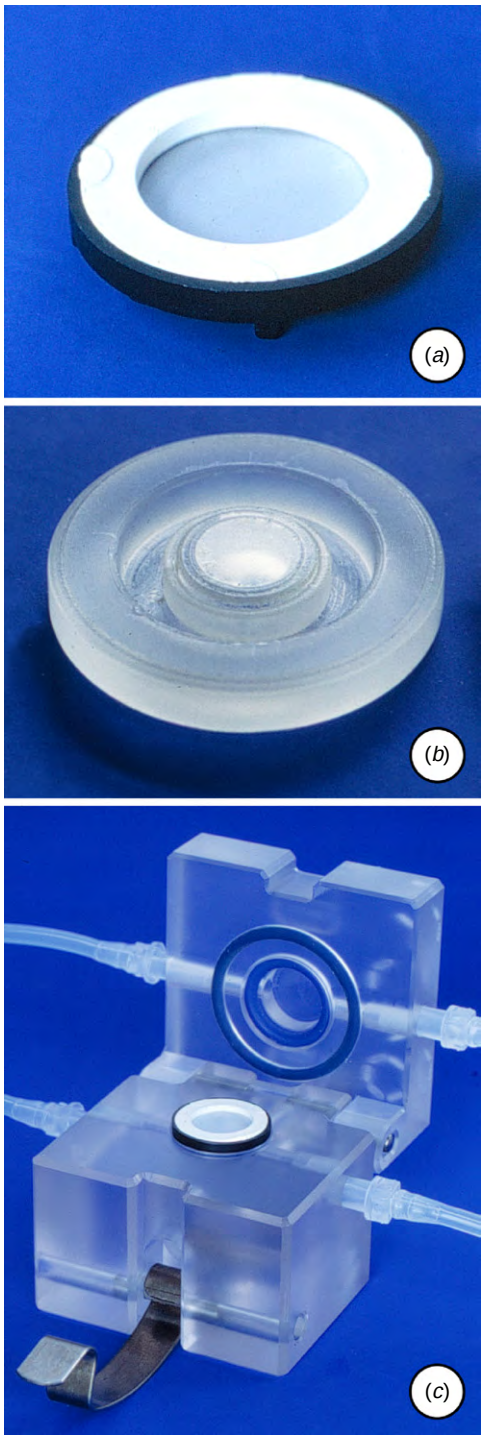

Figure 2. Illustration of different types of tissue carriers and a gradient culture container. (*a*) Inflexible materials such as filters with 13 mm diameter are placed in a holder and fixed by a tension ring. (*b*) Flexible matrices are held in place like the skin on a drum. (*c*) The gradient perfusion culture container consists of a basal and upper part. The tissue carrier is placed in the space between, thus dividing the two compartments.

2.4. Tissue carriers

In order to prevent damage of the growing epithelia by a one-sided supply of medium at the bottom of a culture dish, our technical solution is the placement of the selected support

material within a tissue carrier made of Procan® (figure 2(*a*)) or polycarbonate (figure 2(*b*)) during experimental manipulation. This allows an exact orientation of the support within the tissue carrier. Since the tissue carrier rests on small protrusions, it allows medium supply at the luminal and basal sides, when used at the bottom of a culture dish. Most importantly, the tissue carrier makes an exact geometrical placement of the developing epithelium possible within a gradient perfusion culture container (figures 1(*c*), 2(*c*)). It guarantees uniform supply of medium to the luminal and basal sides. Thus, the tissue carrier stabilizes the developing tissue, prevents mechanical damage and facilitates handling during transport or manipulation.

2.5. Gradient perfusion culture container

Under *in vivo* conditions epithelia are continuously supplied with fresh nutrients and oxygen, while metabolic waste products are eliminated. This physiological situation cannot be mimicked sufficiently in the stagnant environment of conventional culture dishes. Trying to overcome these problems tissue carriers are therefore used in combination with gradient perfusion culture containers (figures 1(*c*), 2(*c*)). All these types of containers were designed in our laboratory. They are CAD-constructed and CNC-machined out of polycarbonate (Makrolon®) in a specialized workshop. During fabrication the machined surfaces are treated with specific lubricants. This treatment makes the surfaces non-attractive for cell attachment and causes cells to grow exclusively on the basal lamina support and not to spread across the tissue carrier and the inner surface of the gradient perfusion culture container. A gradient container typically can hold one (figures 1(*c*), 2(*c*)) or six (not shown) tissue carriers. Culture experiments under visual control can be performed using a special microscope gradient container, in which the tissue carrier with a diameter of 13 mm is mounted between a transparent lid and base part, so that continuous microscopical observation becomes possible (not shown).

Since the tissue carrier is held in place between the base part and the lid of a gradient perfusion culture container (figures 1(*c*), 2(*c*)) its luminal and basal sides can be superfused with individual media mimicking a natural environment for epithelia. Fresh culture medium is continuously transported into the container at one side, while the metabolized medium is removed at the other side of the container. On its passage through the gradient culture container the medium flows across the upper and lower sides of the epithelium in the tissue carrier. In this way the developing tissue is continuously supplied with fresh medium guaranteeing constant nutrition and preventing a non-physiological accumulation of metabolic products or an excess of paracrine factors. Metabolized medium is collected as waste and is not reperfused during the experiment.

Finally, the medium environment can be adapted to the maturation state of the cultured epithelia. For example, to mimic an environment for immature epithelia, the gradient container can be perfused with identical media at the luminal and the basal sides. In contrast, an environment for adult epithelia can be simulated by altering the medium composition on the luminal side, while standard medium is used on the basal side. Applying this culture design the epithelia are continuously supplied with fresh culture medium and are exposed to a fully defined and constant fluid gradient environment over prolonged periods of time. However, during the entire culture period the epithelia have to withstand the luminal–basal medium gradient as well as the mechanical forces and fluid stress which occur under *in vitro* conditions.

2.6. Continuous transport of culture medium

In our opinion developing epithelia have to be supplied with a continuous flow of fresh, oxygenated culture medium in order to obtain constant nutrition, to avoid unstirred fluid layers and to achieve a high degree of histotypical differentiation. This is best accomplished using a slowly rotating peristaltic pump designed to deliver very low adjustable pump rates of 0.1–5 ml per hour (figure 3). As revealed in earlier experiments, it is favorable for the development of epithelia to continuously perfuse medium at a rate of 1 ml h^{-1} for a 2 week culture period using a IPC N8 peristaltic pump (ISMATEC, Wertheim, Germany).

2.6.1. Unidirectional transport of medium. Fresh culture medium is continuously supplied from a storage bottle to the gradient culture container at a constant transport rate. The metabolized medium is collected in separate waste bottles and consequently not recirculated (figure 3). This guarantees constant nutrition and oxygen supply as well as a continuous elimination of harmful metabolic products.

2.6.2. Housing. Gradient perfusion culture experiments are performed at a constant temperature of usually 37 °C. This can be achieved by placing the gradient perfusion culture container into an incubator. The disadvantage, however, is the limited accessibility inside the apparatus. An alternative is to use a thermo plate (MEDAX-Nagel, Kiel, Germany) covered by a removable lid (figure 3). Another solution for epicritical experiments is to incubate the gradient perfusion culture container in a temperature-controlled water bath.

2.6.3. Media for gradient perfusion culture. Publications from several laboratories show that gradient perfusion culture is performed with commonly used media such as HBSS, DMEM, IMDM or others [35, 37, 38, 40]. Depending on the experimental design some of the media contain serum as a supplement, while in other series serum-free media are applied. A medium typically used for gradient perfusion culture in our laboratory is serum-free Iscove's modified Dulbecco's medium (IMDM; order no. 21980-032; Gibco BRL-Life Technologies, Eggenstein, Germany). It is, e.g., used for the successful culture of embryonic collecting duct (CD) epithelia derived from renal stem cells [33, 35, 36]. In this specific case aldosterone (1×10^{-7} M; Sigma-Aldrich-Chemie, Deisenhofen, Germany) is added as a necessary maintenance factor. In addition a 1% antibiotic–antimycotic solution (Gibco BRL-Life Technologies) is used to prevent the growth of microorganisms.

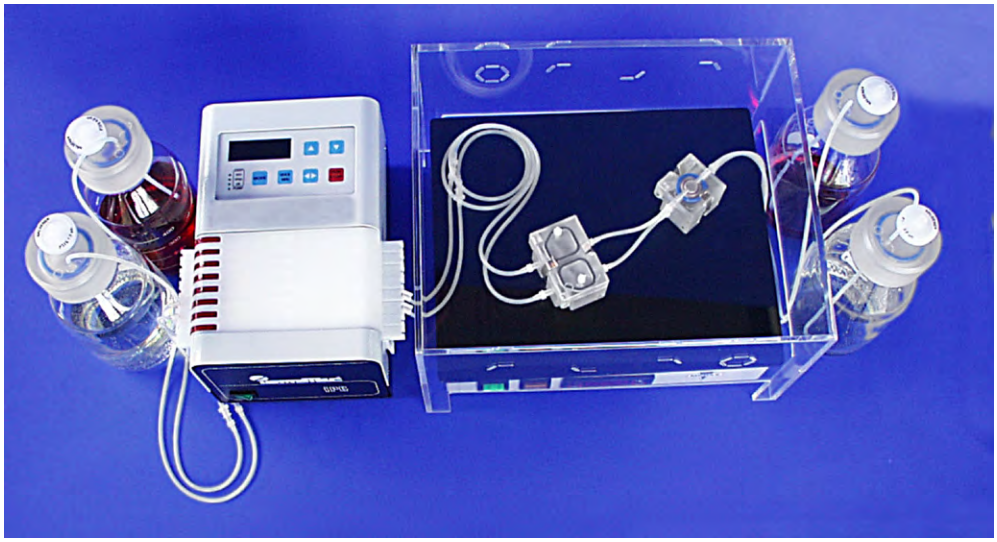

Figure 3. Perfusion culture setups are self-contained and can be used on a laboratory table. A peristaltic pump (left side) transports the media (1 ml h^{-1}) to a gas expander module and onward to a gradient container. The waste medium is collected in the bottles on the right side. A heating plate and a lid maintain the desired temperature.

2.6.4. Buffer for pH maintenance. Conventional culture media for use 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 is used in gradient perfusion culture outside a CO_2-incubator under room atmosphere, pH will shift out of the physiological into the alkaline range. To be used outside a CO_2-incubator the medium therefore has to be stabilized by reducing the $NaHCO_3$-content and/or by adding a biological buffer.

Equilibration of a culture medium to maintain a constant pH under room atmosphere can be performed experimentally using a 24 well culture plate. Each well is filled with 1 ml of the selected culture medium. Then an increasing concentration from 10 to 50 mmol l^{-1} HEPES (Nr 15630-056, Gibco BRL-Life Technologies) or 0.1–1.5% Buffer All (Nr B-8405, Sigma-Aldrich-Chemie) is added to each of the wells. For the following 24 h the culture plate is incubated on a thermo plate at 37 °C under room atmosphere. After equilibration the pH in each well is measured with a pH meter or with a whole blood analyzer such as the Stat Profile 9 Plus (Nova Biomedical, Rödermark, Germany). The HEPES respectively Buffer All concentration, which yields a medium pH between 7.2 and 7.4 under room atmosphere, can easily be determined in that way. Determining the pH by observation of the phenol red indicator alone is not recommended, since phenol red is not sensitive enough to indicate small pH shifts around the physiological range between pH 7.2 and 7.4.

2.6.5. Medium oxygenation. Gradient perfusion culture can either be performed inside a CO_2-incubator or under room atmosphere on a laboratory table (figure 3). To obtain optimal equilibration of pH, O_2 and CO_2 the buffered gradient perfusion culture media are transported through 1 m long highly gas-permeable silicone tubes (Minucells and Minutissue) with 1 mm inner and 3 mm outer diameter to allow optimal exchange of gases. To allow continuous control of the physiological environment throughout an experiment, medium for analysis is aspired with a sterile syringe through a T-connection in the tubing directly before or after the gradient perfusion culture container. Analysis of an individual IMDM specimen shows following parameters: pH 7.4; pO_2 190 mmHg; pCO_2 12 mmHg; Na^+ 118 mmol l^{-1}; K^+ 4 mmol l^{-1}; Cl^- 80 mmol l^{-1}; Ca^{2+} 1.15 mmol l^{-1}; glucose 446 mg dl^{-1}; lactate 0 mmol l^{-1}; osmolarity 253 mOsm. Since the epithelia are exposed to a luminal and basal fluid environment, specimens of media are collected from the luminal as well as the basal medium.

To avoid changes in the saturation with respiratory gases the fluid specimens must be analyzed without delay, e.g. in an analyzer such as the Stat Profil 9 Plus analyzer. In a CO_2-incubator (95% air/5% CO_2) for example, average partial pressures of 33 mmHg CO_2 and 150 mmHg O_2 are maintained in fully equilibrated IMDM medium. Compared to these values a relatively low partial pressure of 12 mmHg CO_2 is detectable in fully equilibrated perfusion culture medium under room atmosphere due to the low CO_2 content (0.3%) in atmospheric air. In contrast, a high O_2 partial pressure of 190 mmHg is found in the perfusion culture medium after equilibration against atmospheric air during transport from the storage bottle to the gradient perfusion culture container in silicone tubes. Thus, compared to stagnant cultures in a CO_2-incubator the oxygen partial pressure in the perfusion culture medium is considerably higher.

As shown above, the analysis of the used perfusion culture medium (IMDM) at the outlet of the gradient culture container reveals a remaining content of 415 mg dl^{-1} glucose. This indicates that the exchange of culture medium is high enough to prevent the limitation of aerobic physiological processes by a decline in glucose concentration. Furthermore, no unphysiological accumulation of lactate can be detected in the used medium at the outlet of the container at a medium transport rate of 1 ml h^{-1} as shown for example in gradient

culture experiments with embryonic renal collecting duct epithelia [35, 36]. Due to the continuous elimination of lactate in perfusion culture no harmful influence of this metabolite will occur during the culture period under normal conditions.

However, an increasing amount of metabolically active cells in combination with a biodegradable support material such as e.g. polylactide may liberate lactate, which will accumulate in the medium. Unphysiologically high concentrations of liberated lactate in turn can lead to a shift in pH that would harm the cultured epithelia. In this case the medium transport to tissue volume ratio has to be increased in order to improve lactate elimination. Thus, the medium transport rate has to be elevated, if high lactate levels are measured in the culture medium used.

2.6.6. Modulation of gas content. Epithelial tissues have highly individual oxygen requirements [45]. For that reason it is important to individually adjust the respiratory gas content in perfusion cultures. A popular method for medium oxygenation is to bubble a pressurized gas mixture through the medium in the storage bottle. An enormous disadvantage of this method is the formation of gas bubbles in the transported medium. These bubbles accumulate along the medium transport path and can cause pressure differences within the system. This method can also introduce infections caused by contaminated gases. Thus, the technical challenge is to obtain maximum oxygen saturation in the medium while avoiding the formation of gas bubbles.

Consequently, a gas exchanger module was developed in our laboratory as a technical solution, which houses a long thin-walled silicon tube for medium to pass through (figure 4(*c*)). The tubing is curled into a spiral inside the exchanger module which features a gas inlet and outlet. The tubing is highly gas permeable and guarantees optimal diffusion of gases between culture medium and surrounding atmosphere inside the gas exchanger module. The desired atmosphere within the gas exchanger is maintained by a constant flow of a specific gas mixture through the module. This way the gas partial pressures of oxygen, carbon dioxide or any other gas in the medium can be adjusted by diffusion under absolutely sterile conditions. By maintaining a defined carbon dioxide concentration in the medium this method can even be employed to adjust medium pH via the bicarbonate buffer instead of using alternative CO_2-independent buffer systems.

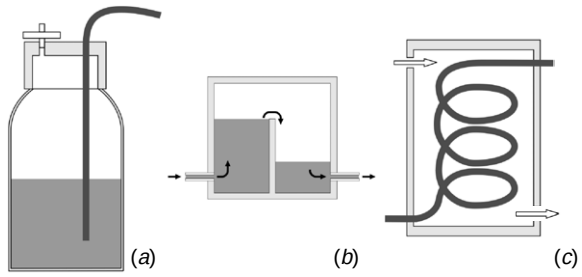

Figure 4. Schematic illustration of advanced technical equipment for gradient perfusion culture experiments. (*a*) Schematic illustration of newly developed closures for media bottles to minimize the formation of gas bubbles. A continuous piece of silicone tubing is conducted through the closure to allow medium transport without connectors. (*b*) Schematic lateral view of the gas expander module. Gas saturated medium enters at the left side of the module. The medium crosses a barrier, allowing gas bubbles to separate and collect in the upper half of the container during medium transport. (*c*) Schematic view of a gas exchanger module to adjust the content of respiratory gases in the perfusion culture medium. The module consists of a housing and a long thin-walled gas permeable silicone tube for medium to pass through. The desired gas atmosphere within the exchanger is maintained by a constant flow of a specific gas mixture. Equilibration of the culture medium occurs through the highly gas permeable tubing inside the module.

2.7. Technical–biological interface

Gradient perfusion culture appears quite simple at first sight. Closer examination, however, reveals that it is a sophisticated technique, which requires knowledge, experience and extensive training in order to produce intact epithelia *in vitro* after a culture period of 14 days. The success of experiments depends on the one hand on the technical equipment such as a suitable support within a tissue carrier placed inside the gradient perfusion culture container. On the other hand, it depends on the proper growth of epithelial cells and their ability to establish the epithelial barrier. Only an optimal interaction between the technical presuppositions and the growing epithelial cells will later result in the development of an intact physiological barrier.

For the simulation of such epithelium-specific culture conditions a tissue carrier containing a support with attached cells is placed in a gradient perfusion culture container (figures 1(*c*), 2(*c*)). Thus, the support material and the tissue carrier separate by constructing the gradient perfusion culture container into a luminal and a basal compartment. However, the presence of an intact barrier function cannot be expected immediately and constantly. The crucial problem is that the epithelial cells grow on a membrane, which vibrates depending on the environmental pressure. Thus, handling of the tissue carrier and its insertion into the gradient container may produce mechanically micro-injuries in the growing epithelium, which can result in cell damage and consequently leakage of the biological barrier. However, the biological barrier will only become perfect when the growing epithelial cells form a biological seal in combination with the support material used as basal lamina substitute including the tissue carrier and gradient container.

2.8. Embryonic and adult environment

During development epithelia are first exposed to an environment consisting of the same fluid at the luminal and basal sides (figure 5(*a*)). This situation changes during maturation as neighboring cells develop intercellular seals. A functional barrier will be present when the luminal and basal aspects of the epithelium become exposed to different fluid environments (figure 5(*b*)). Mature epithelia exhibit this selective barrier function throughout their lifespan and thereby control the transport of molecules from one side to the other.

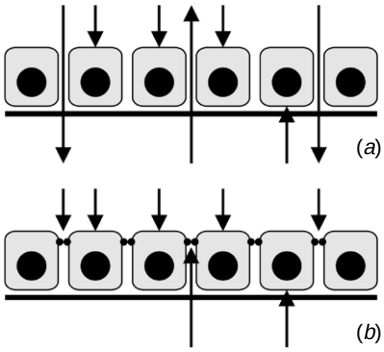

Figure 5. Schematic illustration of epithelial development. (*a*) An immature epithelium does not yet provide a barrier function. The tight junctions are not fully developed, therefore the uncontrolled passage of molecules is possible via the paracellular shunt (large arrows). (*b*) In the mature state the tight junctions are fully sealed and the paracellular shunt is closed. The epithelial barrier is intact and the epithelial cells mediate selective passage of molecules (short arrows).

After insertion of a tissue carrier into a gradient container the apical and the basal sides of the epithelium can be superfused with media of identical or individual composition. However, since the epithelium is still developing, a physiological barrier between the luminal and basal compartments is not established yet. Consequently, an uncontrolled exchange of fluid between the luminal and basal compartments of the epithelium may occur initially until the epithelium has further matured. During culture the epithelial cells spread out further, whereby sites of edge damage and mechanical damage are sealed so that leakage between the luminal and basal sides is decreased.

When finally a confluent, sealing monolayer is reached, the typical environment of e.g. stomach, small intestine, gall bladder or kidney epithelia can be mimicked by using a hyper- or hypotonic medium on the apical side, while an interstitial-like fluid is used on the basal side.

2.9. Development of an intact epithelial barrier

A suitable support material for optimal cell growth, a tissue carrier and a gradient perfusion culture container are only the technical prerequisites to generate an epithelial barrier. Most importantly, the biological seal has to be produced by the epithelial cells themselves. This development is a rather complex cell biological process.

Like all basic tissues, epithelial tissue consists of a cellular and an extracellular compound. In brief, an epithelium starts to develop by the formation of cell–cell contacts, followed by the adhesion to the extracellular matrix and definition of polarization [46]. Subsequently the synthesis of a basal lamina starts, which usually occurs in cooperation with cells of the lamina propria found underneath the epithelium. At this early point of development the epithelium has not reached complete physiological epithelialization and has therefore not built up a tight barrier function yet (figure 5(*a*)). Since the tight junctions are still not functionally established, the paracellular shunt is open and allows uncontrolled passage of electrolytes and other molecules through the epithelium. Consequently, the fluid environment at the future luminal and basal sides is more or less equal.

In the next step a complex concert of influences such as paracrine factors, growth factors, hormones, nutrition, respiratory gas, metabolites and biophysical stress such as e.g. hydrostatic pressure or fluid osmolarity stimulates the development toward a functional epithelium. As a consequence, the tight junctions are functionally closed and the paracellular shunt is controlled (figure 5(*b*)) [33, 34]. Starting from this point of development the epithelial cells control which electrolytes or other molecules are allowed to pass the barrier. Once the development of a sealed epithelium is finished, the luminal and basal fluid environments are completely separated by the established barrier and start to differ in their composition.

With the exception of epithelia which are luminally exposed to air such as skin, lung and cornea all other epithelia in the organism act as a barrier between two differently composed fluids at their luminal and basal sides. To mimic such a situation in perfusion culture, different culture media are transported in parallel through the luminal and basal compartments of the gradient container. To ensure equal transport rates parallel channels of a peristaltic pump are used. Now the epithelia have to withstand the resulting rheological forces and furthermore they have to maintain a physiological barrier between the luminal and basal compartments in the gradient container as it is known from the organ or tissue site where they were derived [47].

Surprisingly, the barrier function of the cultured epithelia may not be sufficiently developed or even break down during gradient perfusion culture. This again results in a non-physiological leakage between the luminal and basal compartments of the epithelial barrier. Experiments have shown that leaks arise due to very different reasons, which include insufficient confluency of cells, epithelial edge damage or micro-injuries caused by accumulating gas bubbles which lead to fluid pressure differences between the luminal and basal compartments.

2.10. Reasons that disturb the barrier function

The proper development of epithelia can be disturbed by various influences. This in turn can lead to loss of the functional seal or mechanical damage to the epithelial barrier.

In order to maintain a fluid gradient, it is essential that the growing epithelium on the tissue carrier keeps up a perfect seal (figure 6(*a*)). Mixing of the two media must not occur. This often fails in practice due to epithelial damage occurring during the experimental period (figures 6(*b*)–(*e*)). Edge damage is found at sites where epithelial cells, basal lamina support and the polymer material of the tissue carrier come into contact (figure 6(*b*)). An epithelial leak appears when the cultured cells do not grow to perfect confluency on the support or do not develop complete sealing between neighboring cells for cell biological reasons (figure 6(*c*)). Mechanical damage (figure 6(*d*)) is usually caused during experimental handling. Unexpectedly, the majority of leaks within epithelial

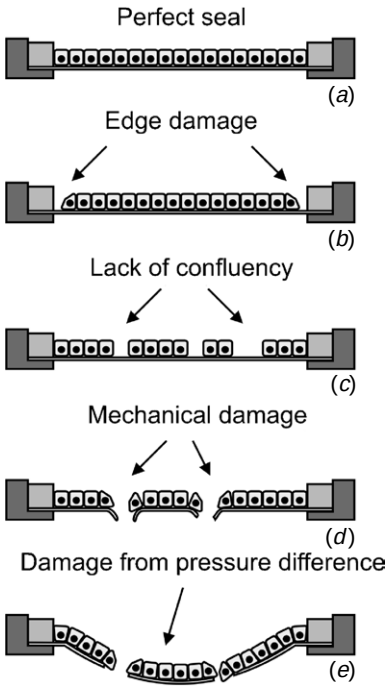

Figure 6. Schematic illustration of epithelial growth, epithelial leakage and possible types of damage to the epithelia in a gradient perfusion culture container. (*a*) To build up a functional barrier it is essential that the growing epithelium produces a perfect seal on the tissue carrier. (*b*) Edge damage occurs at sites where living cells, support and polymer material of tissue carrier come in contact. (*c*) Lack of confluency of cells is often due to a sub-optimal support material or to missing ingredients in the culture medium. (*d*) Mechanical damage is mostly caused during handling or by accumulating gas bubbles. (*e*) Liquid pressure differences have to be avoided in the gradient perfusion culture container, because they can lead finally to the rupture of the cultured epithelium.

tissues in gradient perfusion culture are caused by liquid or embolic pressure damage (figure 6(*e*)). Severe liquid pressure differences between the luminal and basal compartments of the gradient container can build up due to embolic obstruction of one of the tubings by air bubbles. This causes differences by erratic breaks in the fluid continuum, which in turn leads to massive mechanical damage to the cultured epithelial tissue (figure 8).

As long as the epithelia are supported by mechanically rigid matrices such as nitrocellulose or polycarbonate filters, the transport of media will not severely affect the barrier function (figure 2(*a*)). However, many cultured epithelia are kept on mechanically more fragile supports (figure 2(*b*)). They can only be well preserved in the gradient perfusion culture container as long as pressure differences between the luminal and basal compartments are avoided (figure 8(*a*)).

During gradient perfusion culture the media are transported through gas permeable silicone tubes to allow saturation with oxygen. This high content of dissolved gas in the culture medium is physiologically advantageous for the cultured tissue, but technically disadvantageous with respect to medium transport. The formation of gas bubbles at random sites within the tubes, connectors or inside the gradient container on the epithelium is often observed during long-term perfusion culture. The gas bubbles increase in size over time and can leave the site of accumulation to appear unpredictably in other parts of the gradient perfusion culture system. This again can result in unequal distribution of gas bubbles either in the luminal or basal compartment and lead to pressure differences by capillary forces, which in turn result in damage to the tissue and loss of the barrier function (figures 6(*d*), (*e*)).

Medium transport is normally achieved by drawing the medium up from the bottom of a storage bottle through tubing connected to the inside of the bottle cap. The medium then passes through ports in the cap before reaching the tubing connected to the outside of the cap, which in turn leads on toward the gradient perfusion culture container. In such a scenario the pump's suction force has to be high in order to overcome the difference in elevation within the storage bottle as well as the capillary forces resulting from thin silicone tubing. In conventional bottle caps this high negative pressure in combination with low pump rates of 1 m h^{-1} can lead to aspiration of gas through the tubing connectors, which in turn causes numerous gas bubbles within the medium.

2.11. Absorbing pressure differences

The technical challenge is to transport oxygen-rich media without formation of gas bubbles. For this special purpose we designed new bottle caps, which eliminate the need for connectors and allow the transport of gas saturated media from the storage bottles to the gradient culture container in continuous silicone tubing (figure 4(*a*)) [35, 48]. One small opening in the cap allows a continuous piece of silicone tubing to pass through, avoiding material transitions along the fluid path. This method considerably reduces gas bubble formation. Another small opening in the screw cap holds a sterile filter allowing gas to enter the storage bottle as medium is drawn from it.

By the use of newly developed screw caps the formation of gas bubbles can be significantly reduced but not fully prevented (figure 4(*a*)) [44]. Consequently, remaining gas bubbles have to be eliminated before reaching the gradient perfusion container without altering the oxygen concentration in the liquid phase. For this special purpose we constructed a gas expander module, which is inserted into the perfusion line between the storage bottle and the gradient perfusion container (figures 3, 4(*b*), 7(*a*)). Within the module the culture medium has to cross a barrier, which leads to a separation of gas bubbles from the liquid phase (figure 4(*b*)). During this process gas bubbles are eliminated, while the content of oxygen within the medium remains unaffected.

Moreover, the gas expander module acts as an absorber for pressure differences between the luminal and basal compartments of the gradient perfusion culture container (figure 7(*b*)). It consists of two separate chambers for the culture media destined for the luminal and basal compartments of the gradient perfusion culture container. Each of these chambers can be ventilated through a port at the top. Furthermore, it can be coupled with the port on the parallel chamber of the gas expander module. This way the individual

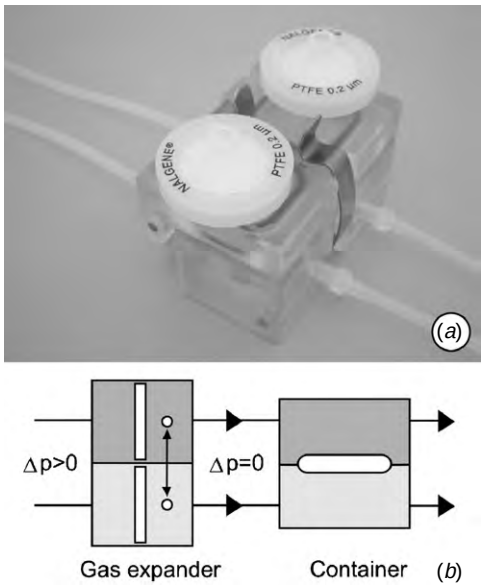

Figure 7. Use of a gas expander module in gradient perfusion culture. (*a*) Photograph shows the module with sterile filters attached to the ports at the top of the module. (*b*) The two individual channels of the module can be bridged to avoid pressure differences in the luminal and basal parts of the gradient perfusion culture container. Arrows indicate direction of flow.

channels of a gradient culture setup can be bridged to obtain identical pressures at the luminal and basal sides.

2.12. Optimal transport of fluid inside the gradient container

During the slow transport of culture media gas frequently separates from the liquid phase and randomly accumulates in the form of bubbles within the gradient culture container. However, gas bubbles can also arise due to the respiratory activity of the cultured epithelia. With time they can increase in diameter. From time to time some of them leave the gradient culture container toward the effluent tubing, where they lead to embolic obstruction of the medium outflow. However, perfect gradient perfusion culture only occurs in the absence of a pressure difference between the luminal and basal compartments (figure 8(*a*); $\Delta p = 0$). As the obstruction randomly affects only the basal or the apical fluid transport path the resulting pressure difference causes the epithelium to protrude toward the side of lower pressure (figure 8(*b*); $\Delta p > 0$). An increasing pressure difference can finally result in the mechanical disruption of the epithelial barrier (figure 8(*c*); $\Delta p \gg 0$).

To avoid the accumulation of gas bubbles to a critical size within the gradient perfusion culture container, our technical contribution is to locate the medium inlet and outlet to the tangential aspects of the tissue carrier (figure 9(*a*)) [49]. When the gradient container is turned from its base to its lateral side, medium enters now at the lowest point, while the outlet is located at the highest point of the fluid compartment to facilitate the outflow of gas bubbles (figure 9(*b*)). Due to this improved flow geometry gas bubbles cannot accumulate but are continuously eliminated during medium transport. Thus, the combination of newly constructed bottle caps (figure 4(*a*)),

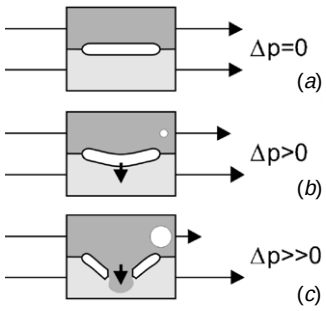

Figure 8. Schematic illustration of loss of barrier function in epithelia cultured in a gradient perfusion culture container. (*a*) No epithelial tissue damage will occur as long as pressure is identical at the luminal and basal sides of the gradient culture container ($\Delta p = 0$). (*b*) In contrast, for example a small gas bubble (small dot) at the outlet of the luminal compartment will increase the pressure in the luminal compartment resulting in an extension of the tissue toward the basal side (arrow, $\Delta p > 0$). (*c*) As the gas bubble grows in diameter (big dot) a further pressure increase in the luminal compartment occurs ($\Delta p \gg 0$). The tissue eventually cannot withstand this pressure and is disrupted. Consequently the barrier function is lost.

gas expander modules (figures 4(*b*), 7) and the redesigned gradient perfusion culture container (figure 9) leads to a drastic reduction of gas bubbles throughout the culture period.

2.13. Detection of transepithelial leakage during long-term culture

Electrophysiological registration of transepithelial resistance proved less suitable to control the maintenance of a barrier function during long-term gradient culture experiments, since electrode fouling, resulting tissue damage and a decreased degree of cellular differentiation were observed. Addition of radiolabeled molecules such as inuline to the luminal culture medium in combination with detection of radioactivity in the lower fluid path is possible. However, it is not preferred since simple, non-radioactive fluorescent molecules can be employed as well to monitor the quality of the epithelial barrier throughout a long-term gradient perfusion culture period.

During culture, growing epithelia on the tissue carrier have to maintain an intact physiological barrier between the luminal and a basal compartments of the gradient perfusion culture container. One simple and cost-saving method to detect non-physiological epithelial leaks is to superfuse phenol red containing IMDM (order # 21980-032; Gibco BRL-Life Technologies) at the luminal compartment, while IMDM without phenol red (order # 21056-023; Gibco BRL-Life Technologies) is used at the basal side. Photometrically recorded traces of phenol red indicator in the clear medium will indicate mixed media and consequently leakage of the epithelia.

Thus, only experiments in which perfect separation of red and clear media in the waste bottles is maintained for 14 days are declared successful and used for further analytical evaluation. Another way of detecting epithelial leakage is to collect medium samples just before and after the luminal and basal compartments of the gradient perfusion container

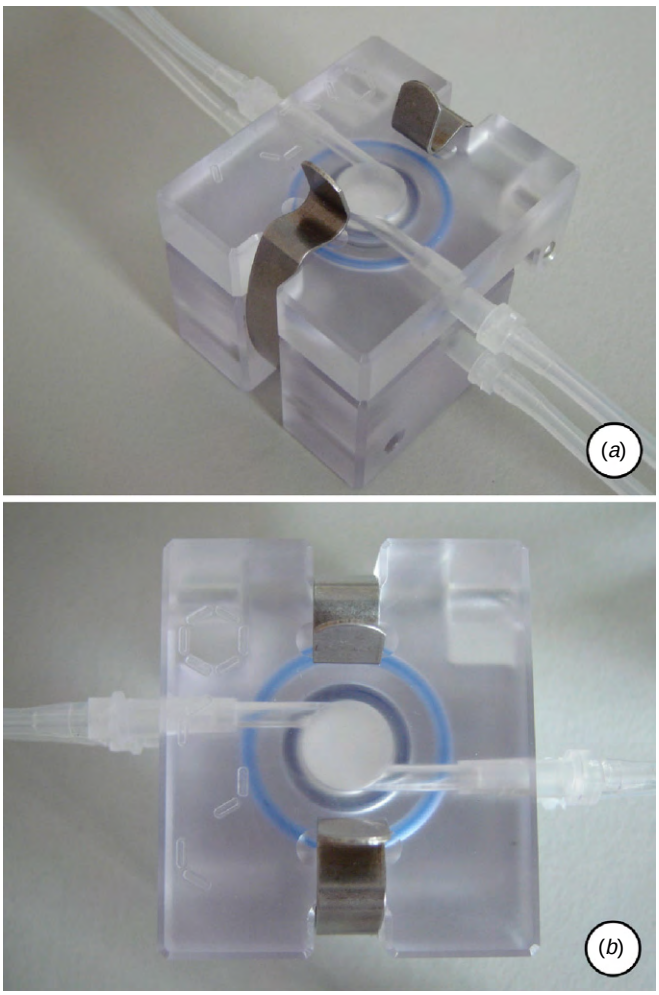

Figure 9. Photographs showing a redesigned gradient perfusion culture container. (*a*) Medium inlet and outlet are located at the tangential aspects of each tissue carrier. (*b*) When the gradient container is placed on its lateral side, medium enters now at the lowest point (right side), while the outlet (left side) is found at the highest point of the fluid reservoir. This innovative flow geometry leads to a continuous and efficient elimination of gas bubbles.

and to analyze these samples in a blood electrolyte analyzer as described above. As the media used in the luminal and basal fluid paths differ in their electrolyte composition, the stability of the fluid gradient can be controlled by comparing the concentrations of key electrolytes (e.g. Na or Cl) or medium osmolarity in the luminal and basal compartments before and after the gradient perfusion culture container [33, 34].

3. Conclusions

In summary, during the last few years we have developed a versatile modular culture system for the generation of epithelial tissues with optimal functional characteristics under gradient perfusion conditions. With respect to the numerous factors that influence epithelial tissue development the culture system was designed to allow individual control of a number of important environmental parameters. That way the microenvironment within the gradient perfusion culture container can be fine-tuned to meet the physiological needs of individual types of epithelia. An innovative construction principle allows the application of gradient perfusion culture containers at minimal financial expenditure and the individual modules can be combined as needed to build a number of advanced customized culture set-ups. A further goal is to close the experimental gap between conventional cell cultures and animal experiments by providing a possibility to culture functionally differentiated epithelia for extended periods of time [50–53]. These cultures can combine different cell types to allow the study of cellular communication, optimal epithelial tissue development and the long-term effects of newly developed pharmaceuticals.

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