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Review article

Physiological and cell biological aspects of perfusion culture technique employed to generate differentiated tissues for long term biomaterial testing and tissue engineering

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Abstract—Optimal results in biomaterial testing and tissue engineering under *in vitro* conditions can only be expected when the tissue generated resembles the original tissue as closely as possible. However, most of the presently used stagnant cell culture models do not produce the necessary degree of cellular differentiation, since important morphological, physiological, and biochemical characteristics disappear, while atypical features arise. To reach a high degree of cellular differentiation and to optimize the cellular environment, an advanced culture technology allowing the regulation of differentiation on different cellular levels was developed. By the use of tissue carriers, a variety of biomaterials or individually selected scaffolds could be tested for optimal tissue development. The tissue carriers are to be placed in perfusion culture containers, which are constantly supplied with fresh medium to avoid an accumulation of harmful metabolic products. The perfusion of medium creates a constant microenvironment with serum-containing or serum-free media. By this technique, tissues could be used for biomaterial or scaffold testing either in a proliferative or in a postmitotic phase, as is observed during natural development. The present paper summarizes technical developments, physiological parameters, cell biological reactions, and theoretical considerations for an optimal tissue development in the field of perfusion culture.

Key words: Biomaterial testing; tissue engineering; differentiation; dedifferentiation; tissue carrier; perfusion culture container; electrolyte environment.

INTRODUCTION

In biomaterial testing, as well in tissue engineering, a variety of materials and artificial extracellular matrices have been brought into contact with living cells

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in order to study surface and cell interactions [1]. Beside the optimization of metal or polymer implants, the goal is production of living constructs such as skin, cartilage, bone, or tendon for severely injured patients [2–13]. To bridge the temporal loss of important vital functions, there is a need for cultured parenchymal cells from liver, insuline producing pancreatic islets, or renal tubular cells as part of bioartificial modules [14–17]. Apart from these purely clinical applications, almost all kinds of tissue-specific cultures are valuable to pharmaceutical research, biomaterials, and toxicity-long-term-testing for consumer protection. Ideally in an optimal application, the generated tissues will allow almost the same structural, physiological, and antigenic differentiation features as found within the tissues from which they derived.

To date, most of the cultures in biomaterial research and tissue engineering experiments use single cells obtained from a piece of tissue by enzymatic digestion [18, 19]. The cell suspension is then pipetted onto the surface of a flat biomaterial specimen or onto a three-dimensional artificial matrix preferentially made of biodegradable polymers [20–22]. It may seem that few technical limitations exist. However, relatively few papers describe the successful culture of highly differentiated tissues with perfect cellular functions over a prolonged period of time [23–32].

On the surface of a biomaterial, or within the artificial scaffold, the cells have to find an optimal anchoring site where they begin to construct their own tissue-specific matrix for typical structural and functional features to develop. This experimental step sounds very simple, but actually it is the most crucial one. A multitude of papers show that it is in principle possible to harvest tissues that were generated during a prolonged period of time under *in vitro* conditions [33–37]. On the other hand, numerous papers demonstrate that besides typical physiological features, atypical cellular characteristics are developed during culture by dedifferentiation [38–46]. If such imperfect tissues are used for implantation into a patient, rejection or inflammation may result. In case that suboptimal cultured tissues are used for biomaterial or toxicity testing, incalculable responses of the dedifferentiated tissue will make it difficult to draw conclusions for the *in vivo* situation. Since the artificial extracellular matrix and the culture environment can exert a positive, as well as a negative, influence on tissue development, it is important to find a way to individually regulate the degree of differentiation. According to our experiences in the last 10 years, the limited success in generating highly differentiated tissues is due to the suboptimal anchorage of cells and to the stagnant environment present within a culture dish resulting in an unphysiological environment [47].

In this paper we would like to discuss to which degree the use of advanced culture techniques and of individually selected scaffolds placed in tissue carriers in combination with culture under a permanent supply of fresh medium help to improve cellular differentiation.

TECHNICAL PREREQUISITES TO TRIGGER CELLULAR DIFFERENTIATION

Tissue carriers

For biomaterial testing and tissue engineering a variety of supports, biomatrices, or scaffolds made of metals, ceramics, or various polymers such as poly(glycolic acid) or poly(lactic acid) are applied [48–52]. To facilitate handling of these individually selected materials different kinds of tissue carriers were used in our experiments (Fig. 1a, b; Minucells and Minutissue, Bad Abbach, Germany). One type of carrier for example holds any type of scaffold, filter, or net with 13 or 47 mm in diameter. The material is placed into a holder ring and held in place by a tension ring (Fig. 1a) [46, 47]. The second type of carrier holds small pieces of natural extracellular matrices (6 mm in diameter) such as the capsula fibrosa of neonatal rabbit kidney or other organs. The explant is fixed in the carrier like the skin of a drum (Fig. 1b) [52]. To allow cells to multiply and attach, the tissue carriers are then placed into a 24-well plate in a CO_2-incubator. Medium-containing serum or growth factors may be applied for a limited period of time to stimulate cell proliferation in order to produce the necessary amount of cells.

Perfusion culture

Under *in vivo* conditions most tissues are supplied with constant nutrition by capillaries. This physiological system is not sufficiently mimicked by the stagnant environment in conventional culture dishes. Due to this fact, many of our experiments did not perform as expected and showed a loss of function (Figs 5a and 6a) [44, 53, 54]. To overcome these problems tissue carriers were placed into perfusion culture containers (Fig. 1c, d; Minucells and Minutissue). Typically, a constant supply of fresh culture medium enters at the basal side, while the metabolized medium is drained at the upper side of the container. By this method the tissue is continuously supplied with fresh medium, guaranteeing constant nutrition and preventing an unphysiological accumulation of metabolic products.

In a second type of container, culture is possible under a fluid gradient so that a tissue-specific environment can be mimicked (Fig. 1d). The tissue carrier separates the container into luminal and basal compartments. On both sides of the tissue either the same medium or media of different compositions can be superfused. For example, the typical environment of the stomach, small intestine, gall bladder, or kidney can be mimicked by using a hyper- or hypotonic medium on one side, while an interstitial-like fluid is used on the other side. A cartilage environment can be simulated by using culture medium resembling synovial fluid at one side and conventional medium at the other side. A gradient container can hold 1 (Fig. 1d) or 2×3 tissues carriers (not shown). Three tissues may then be used as a control, while the others are utilized in the experimental series.

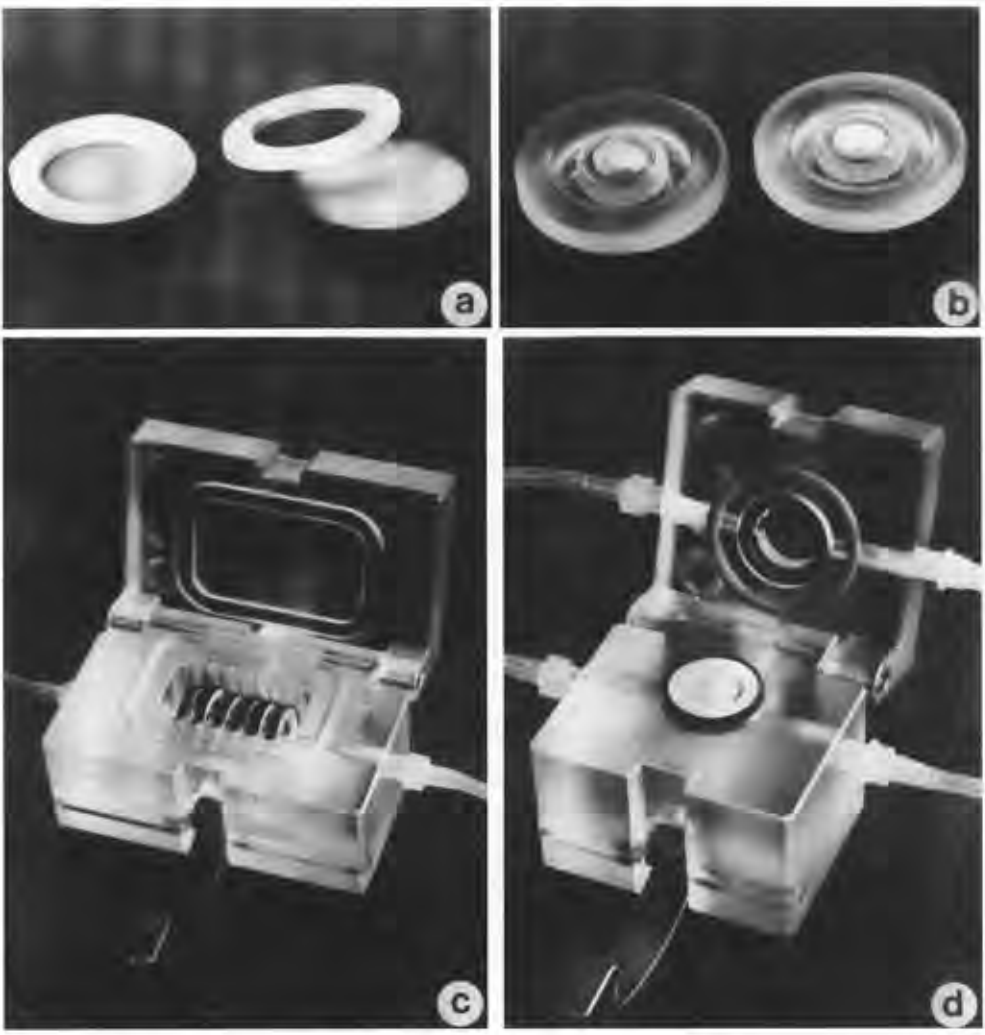

Figure 1. Devices for perfusion culture. (a) Tissue carriers can hold individually selected cell supports or scaffolds with a diameter of 13 or 47 mm for an optimal anchorage of cells. (b) Whole pieces of tissues can be mounted in a second type of carrier. (c) Then the tissue carriers are placed in a perfusion culture container for a permanent exchange of medium. A container can hold six 24-tissue carriers (not shown). At the left side fresh culture medium continuously is pumped into the container. Medium with harmful metabolic products flows out at the upper right side. (d) A gradient perfusion culture container can hold one 6 carriers (not shown) and is connected to two perfusion lines. The closed gradient container allows luminal and basal perfusion with two different media. (e) A peristaltic pump transports the media and a thermoplate with a cover lid provides constant temperature.

PHYSIOLOGICAL PARAMETERS

The perfusion culture system can either be used inside a CO_2-incubator or under atmosphere on a laboratory table with pH-stabilized media (Fig. 1e). Natural and artificial tissues do not develop within days, but weeks, or even longer culture

Figure 1. (Continued).

periods may be required. Due to these long experimental periods it becomes necessary to register environmental parameters in order to control whether the cultures are exposed to optimal conditions.

Temperature

The temperature of the perfusion culture container has to be kept constant. This can be achieved by using a thermo-plate (MEDAX, Kiel, Germany). The temperature is registered by two thermosensors — one on the surface of the plate and another in a special port within the culture container. Typically, a constant temperature can be maintained during 16 h of culture. Once the experiment is started the temperature of the thermo-plate increases and with a short delay, but in parallel, the temperature in the container rises up to the desired value of 36°C. The temperature is then maintained at a constant level very close to 36°C. After switching off the power, the temperature on the plate and within the container declines in parallel to room temperature. With its thick walls the container acts as a buffer to small changes in temperature.

Exchange of fluid in a container

Developing tissues should continuously be provided with fresh medium during culture. In our strategy a peristaltic pump (IPC N8, Ismatec, Wertheim, Germany) transports the medium into the container through thin silicone tubes at perfusion rates of between 0.5 and 5 ml h^{-1}. In the majority of the experiments described herein, fresh medium was pumped through the culture container at a rate of 1 ml h^{-1} for a 2-week culture period or longer. According to our experience, electronic or mechanical fluid perfusion sensors are less suitable to register small volumes exactly. For that reason we used an electronic scale for the exact measurement of

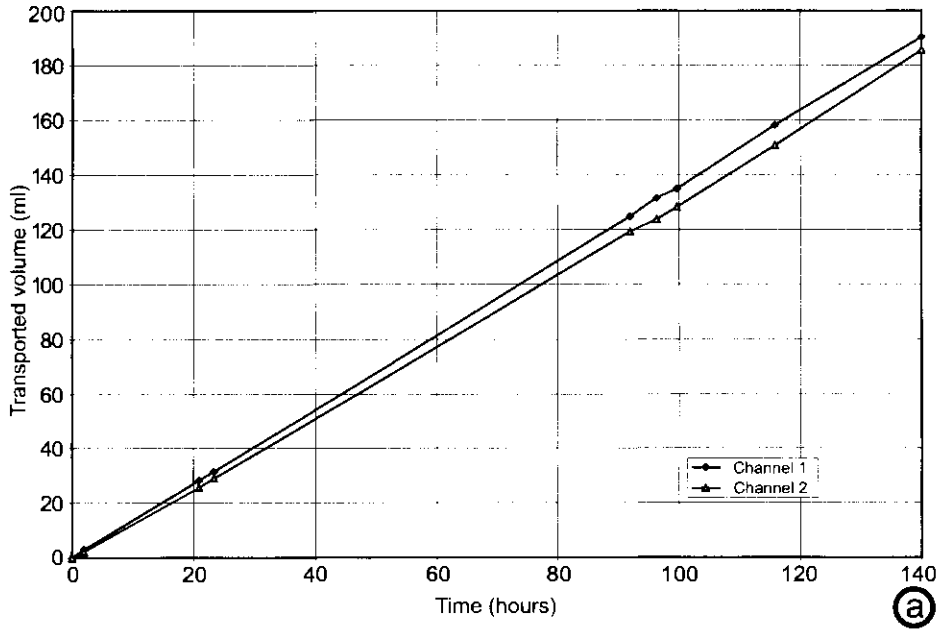

Figure 2. Environmental conditions for perfusion culture. (a) Growing tissues require constant renewal of fresh media. The transport rate of the pump is controlled by wheighting the transported medium by an electronic balance. (b–e) Side view of a culture container demonstrates the exchange of medium: In order to visualize the complete and homogenous exchange of medium the container is filled with acidified yellow culture medium. The container is then perfused with alkalized purple culture medium at a flow rate of 1 ml h^{-1} (b; arrow head). After 20 min the whole basal side of the container is covered with purple medium (c; arrow). In the time between 30 min (d) and 3.5 h (e) the level (arrow) of the purple medium continuously increases. At a flow rate of 1 ml h^{-1} 3.5 h are required for a complete exchange of one container volume. Under these conditions tissue receives constant nutrition, harmful metabolic products do not accumulate and paracrine factors remain on a physiological level. * — clamp to close the container, arrow head — inserted tissue carriers, arrow — indicates the level of fluid exchange.

low flow rates. The waste bottle of a perfusion line was repeatedly weighed to monitor the increase of weight during a certain time period (Fig. 2a). For online measurement an RS 232 bus was used to connect the scale to a personal computer.

In order to visualize the permanent and complete exchange of medium during perfusion, the culture container was filled with acidified yellow culture medium. The container was then perfused with alkalized purple culture medium at a rate of 1 ml h^{-1} (Fig. 2b). After 20 min the whole basal part of the container was filled with purple medium (Fig. 2c). As seen in the side view of the perfusion container in the time between 30 min (Fig. 2d) and 2.5 h (Fig. 2e) the level of the purple medium continuously increases. After 3.5 h the yellow medium in the container was completely replaced by new medium.

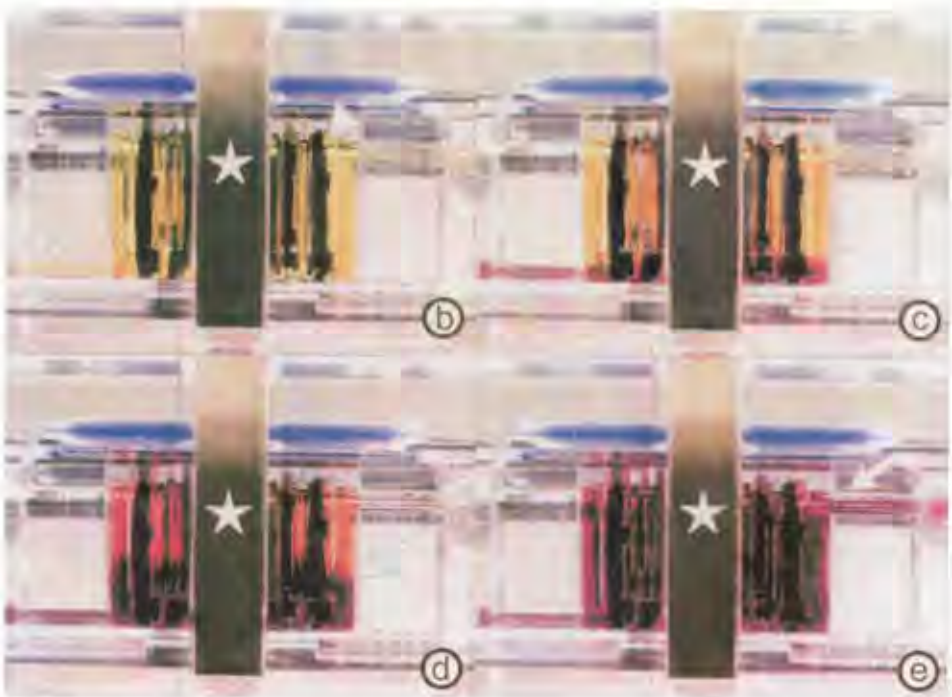

Figure 2. (Continued).

Metabolic parameters in the medium

In most of the experiments described herein, Iscove's modified Dulbecco's medium (IMDM; order # 21980-032; Gibco BRL-Life Technologies, Eggenstein, Germany) without serum was used as the standard medium [55]. Aldosterone (1×10^{-7} M; Sigma-Aldrich-Chemie, Deisenhofen, Germany) as survival factor and 1% antibiotic-antimycotic solution (Gibco BRL-Life Technologies, Karlsruhe, Germany) was added to all culture media. Since the generation of artificial tissues requires prolonged culture periods, the early registration of alterations in the environment that may damage the cells is important. Consequently, metabolic activity was monitored by analyzing the superfused culture medium. A T-piece was mounted in the outlet tube of the container and medium samples were collected using a sterile syringe (Fig. 3). 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. The samples were analyzed immediately with a Stat Profile 9 Plus analyzer (Nova Biomedical, Rödermark, Germany) according to the manufacturer's instructions. Solutions with defined electrolyte concentrations served as controls.

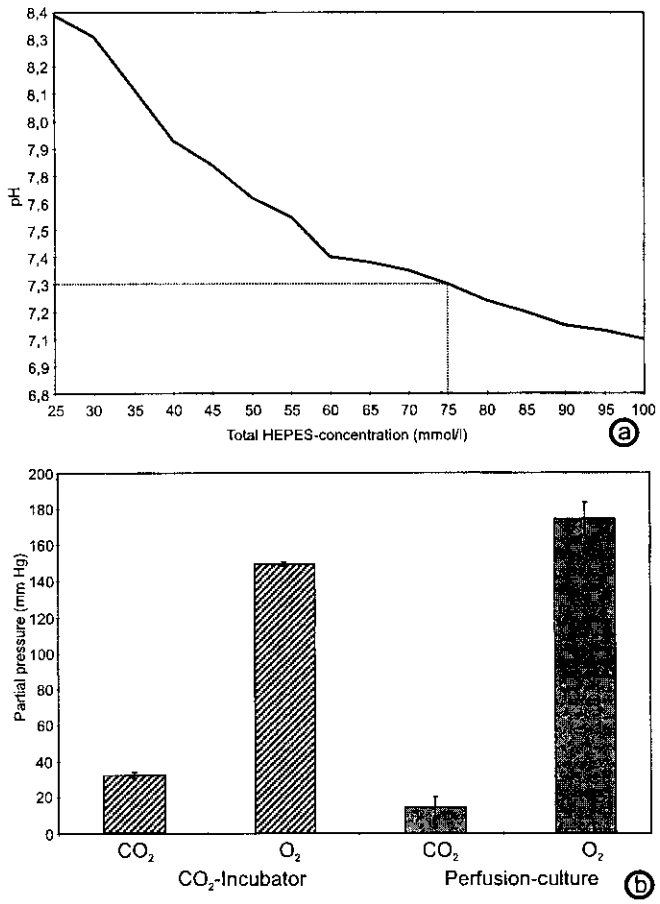

Figure 3. Metabolic parameters in perfusion culture. (a) Perfusion culture runs either in a CO_2-incubator or under air atmosphere on a laboratory table. In this case the culture media have to be pH stabilized. For example, HEPES or BUFFER ALL (Sigma) a different biological buffer have to be added to the culture medium until a stable pH is obtained after equilibration with air on the thermoplate at 37 °C. (b) To obtain optimal equilibration of pH, O_2, and CO_2 in perfusion cultures the media are pumped through thin, gas-permeable silicone tubes, which allow continuous and optimal exchange of gases. Under laboratory air (0.3% CO_2) in IMDM (3024 mg l^{-1} $NaHCO_3$, 75 mmol l^{-1} HEPES) 14.36±5.61 mmHg CO_2 but 174.12±9.17 mmHg O_2 are measured. Under perfusion culture conditions the O_2 partial pressure of the culture medium is considerably higher than in a conventional culture plate. (c) Despite constant perfusion of medium the concentration of produced lactate differs from day to day.

pH-stabilization

Cultures in a CO_2-incubator are usually buffered by a system including a high amount of $NaHCO_3$ and a 5% CO_2 atmosphere to maintain a constant pH of 7.4. If such a medium is used in perfusion culture and outside a CO_2-incubator, the pH shifts from the physiological range to alkaline values due to the low content of CO_2 (0.3%) in room air. For that reason the medium used outside a CO_2-incubator has

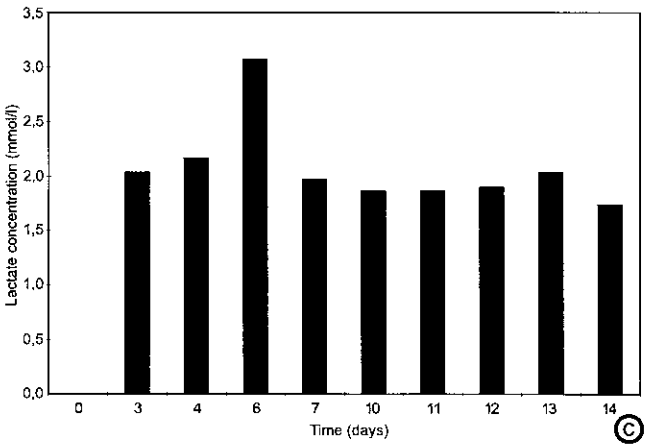

Figure 3. (Continued).

to be stabilized by reducing the $NaHCO_3$-concentration and by adding biological buffers such as HEPES or BUFFER ALL (Sigma-Aldrich-Chemie, Deisenhofen, Germany). Equilibrating the medium with increasing concentrations of HEPES in a culture dish overnight on a thermo plate and under laboratory air atmosphere (Fig. 1e) can be used to adjust the pH to the desired value (Fig. 3a). Addition of 50 mmol l^{-1} HEPES or an equivalent of BUFFER ALL (*c.* 1%) to IMDM will constantly stabilize the pH to 7.4 for the whole culture period under laboratory air atmosphere and without injection of gas into the system.

Oxygen supply

Developing tissues have to receive an optimal oxygen supply. In a CO_2-incubator (95% air/5% CO_2) an average gas partial pressure of 33.60 ± 0.80 mmHg CO_2 and 150.58 ± 2.94 mmHg CO_2 are maintained in fully equilibrated IMDM medium by a Stat Profile analyzer. To obtain optimal equilibration of the pH, pO_2, and pCO_2 in perfusion cultures the media were pumped through long, small-diameter, gas-permeable silicone tubes, which allow a continuous and optimal exchange of gases. Under laboratory air (0.3% CO_2) in IMDM (3024 mg l^{-1} $NaHCO_3$, 75 mmol l^{-1} HEPES) 9.34 ± 2.82 mmHg CO_2 but 174.60 ± 16.33 mmHg O_2 are measured (Fig. 3b). Compared to stagnant cultures in a CO_2-incubator, the oxygen partial pressure in the perfusion culture medium is considerably higher. Actually, the gas-permeable silicone tubes provide a large surface for gas exchange by diffusion due to the small inner diameter of the tubes (1 mm) and its extended length (1 m).

Lactate synthesis

Metabolically active cells produce lactate, which is secreted into the medium and harms the cells when present in unphysiological concentrations by causing a pH shift [56, 57]. If a small amount of tissue and a large volume of medium is present,

lactate concentrations in most cases play a neglectable role. However, if the amount of tissue increases and the volume of medium decreases, concentrations of lactate may reach an unphysiological level [58]. Addition of serum to the culture medium can buffer lactate secretion to a certain degree. However, instead of adding serum we continuously eliminated the secreted lactate by perfusion culture. Furthermore, in our experiments it was often observed that during a 14-day culture period varying amounts per day of lactate were found in the perfusion culture (Fig. 3c). Since the medium was not recycled but permanently renewed, damage to the cells was prevented and the level of lactate was kept at physiological concentrations below 0.2 $mg\,ml^{-1}$ by increasing the rate of perfusion according to the physiological need.

CELL BIOLOGICAL REACTIONS OF CELLS AND TISSUES IN PERFUSION CULTURE

Almost all of our perfusion culture experiments were performed under completely serum-free conditions. Recent publications show that cell lines [59], gastric [54], connective [58], renal tubular [60], or vascular [61] tissues can be maintained under perfusion culture over a prolonged period of time and in a so far unknown quality. With the exception of cell lines [59], it can further be shown that all of the experiments failed when performed in conventional culture dishes.

Proliferation vs postmitosis

Due to growth, the proliferation activity of cells is high in developing tissues, while in many adult tissues proliferation becomes rare [62]. Mitotic activity and postmitosis can be discriminated immunohistochemically by the application of the proliferation marker MIB 1. This marker recognizes the Ki 67 protein that is expressed exclusively by dividing cells [63]. For example, in the embryonic kidney large numbers of MIB 1 positive cells were found, while in the adult kidney almost no labeling could be detected [64]. The results showed that most of the cells in adult tissues did not proliferate as expected, but remained in the functional interphase.

The knowledge about natural proliferation activity in the different kinds of tissues is of great importance for the culture strategy used to generate differentiated constructs (Table 1). If the resources for cells are limited, the first steps of the culture protocol is designed for cell propagation, which is stimulated by numerous growth factors or fetal bovine serum [65–75]. However, the features of proliferating cells represent an immature state of differentiation. In contrast, physiological functions are expressed once the cells leave the mitotic cycle and enter the postmitotic interphase [76–79]. Differentiation is initiated by withdrawal of growth factors or fetal serum [46, 80, 81].

Table 1.
Proliferation vs differentiation in biomaterial testing and tissue engineering. The aim is the generation of differentiated tissue constructs that features physiological characteristics comparable to native tissues. The close correlation of cell proliferation and the expression of immature cell characteristics demands for a specific experimental design: Step 1 — proliferation phase for the production of large numbers of cells. Step 2 — initiation of differentiation by reduction of growth factors and the use of serum-free media. Step 3 — maintenance of differentiation by morphogenic factors and specific media

	Step 1	Step 2	Step 3
Aim	Multiplication	Initiation of differentiation	Maintenance of differentiation
Experimentation	Growth factors medium plus serum	Morphogens medium without serum	Adapted media without serum
Reaction of tissue	Quick mitotic cycle	Reduced mitotic cycle	Postmitotic phase
Degree of differentiation	Low	Starting	High

Each cell type and tissue requires its own biomatrix

The quality of generated tissues is highly dependent upon the individual biomatrices to which the cells are attached [82, 86]. This was first shown by coating culture dishes or filter inserts with poly(L-lysine), collagens, laminin, or fibronectin in order to mimic the extracellular matrix [87, 88]. Depending on the selected coating, the cells changed their gene expression, spreading activity, morphological appearance, or extracellular matrix synthesis [89–95]. In our experiments with MDCK cells we recognized that the growth patterns largely depended on the materials used. For example, cells cultured on polystyrene showed a perfect confluent monolayer (Fig. 4a), while cells on polycarbonate demonstrated a tendency to produce small three-dimensional clusters (Fig. 4b). MDCK cells on cellophane showed a heterogenous distribution (Fig. 4c). Only a small part of the cells was in contact with the cellophane, while the rest was growing as three-dimensional tubular structures.

In the past, more than 300 different biomaterials were tested in our laboratory. The general conclusion is that growth on an unknown biomaterial cannot be predicted since the sensitivity of cells in terms of cell anchorage and spreading is unexpectedly large. Thus, standardized artificial extracellular matrices have to be introduced so that the effects of excellent, average, and insufficient adherence can be compared for an individual cell type. An endothelial cell will prefer a matrix with strong adherence along the total attachment site so that the cell shape becomes flat and a thin endothelial cell layer develops [96, 97]. A chondrocyte in constrast needs to settle with a relatively weak adherence so that flattening of the cell is prevented and

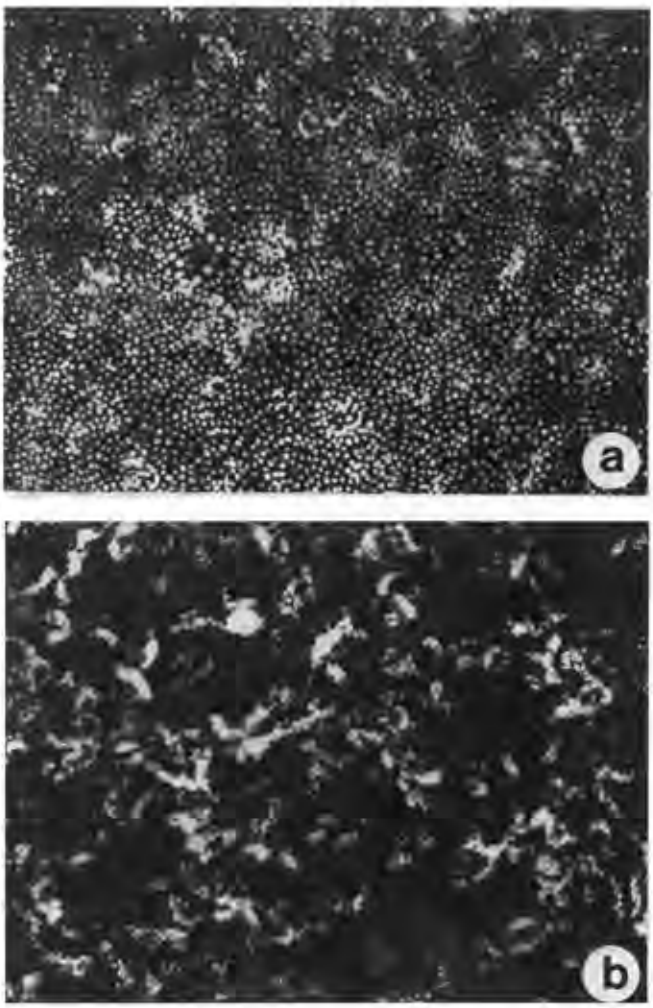

Figure 4. Spatial structure of MDCK cells on different culture supports. To demonstrate the distribution of cells the nuclei are labeled with propidium iodide for fluorescence microscopy. (a) Cells cultured on polystyrene show a perfect confluent monolayer. (b) Cells on polycarbonate demonstrate a tendency to form three-dimensional clusters. (c) Cells on cellophane reveal a heterogenous distribution (bar = 60 μm).

a round shape within the cartilage lacuna can be maintained [98–100]. A newly synthesized tissue-specific extracellular matrix will later completely envelop the chondrocytes. The two opposite examples show that each tissue needs its own and very specific artificial extracellular matrix. This fact has been underestimated but is of great importance, since the external signal of an excellent or a less suitable cell anchorage is mediated to very different cellular pathways via integrins [101–105].

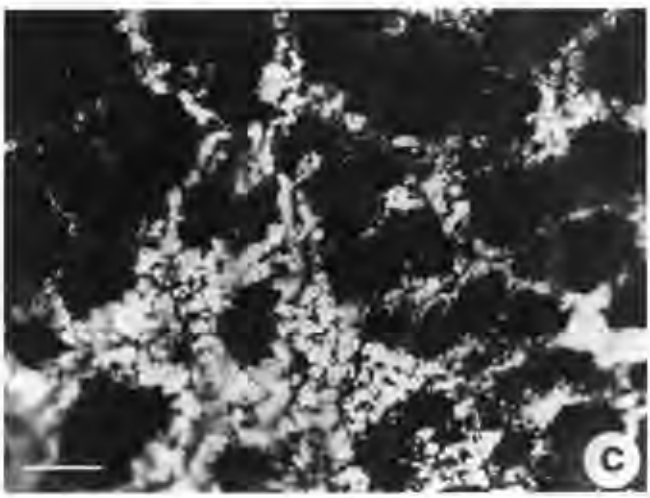

Figure 4. (Continued).

Harmful extracellular metabolites

Not only cultured tissues, but also a variety of biomaterials or artificial scaffolds made for tissue engineering, liberate harmful metabolites by erosion or biodegradation. Many of the presently used materials consist of biodegradable copolymers made, for example, of lactic and glycolic acids [56, 106, 107]. Even though these biomaterials are considered to be very biocompatible, they may affect proper tissue development. This is due to the liberation of acidic degradation products. Especially inside of a developing 3-dimensional scaffold, these acidic metabolites may accumulate in unphysiological concentrations and may cause severe cell damage when the tissue is kept in the stagnant environment of a culture dish. The liberation of degradation products was registered as a function of pH shift in a culture dish for 20 days without cells [58]. It was observed that during this period the pH of the medium decreased from 7 to 2.5. At the same time an increase in lactate from 0 to 0.18 mg ml^{-1} was measured. Thus, the unphysiological pH decrease was caused by the release of degradation products. In contrast, when the scaffold was used in a perfusion culture container, accumulation of liberated lactate and acidification of the medium were avoided. By this improvement, cartilage tissue constructs were successfully generated in perfusion containers under stabilized culture conditions and could be maintained for up to 85 days [129]; a success explained by the constant supply of nutrients to the cultures and the permanent elution of acidic degradation products from the polymers.

Agressive cellular products require perfusion culture

Cultures of the stomach mucosa could serve as an excellent tool for the investigation of drug delivery processes or for surgical research. For example, if an artificial esophageal tissue is engineered it has to form a unit with the gastric mucosa.

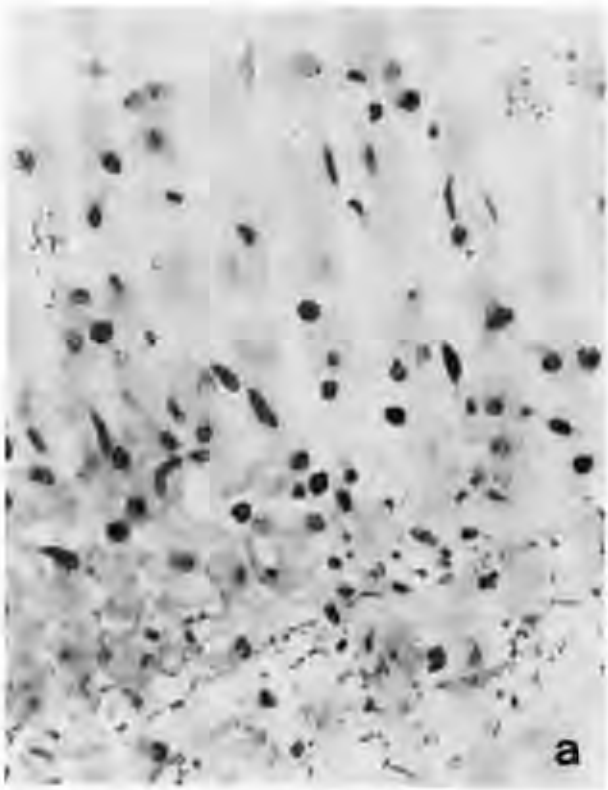

Figure 5. Semithin sections of stomach tissue cultured under stationary and under perfusion culture conditions. The wall of the corpus region of neonatal rabbits was mounted on tissue carriers and was cultured for 5 days in serum-free IMDM. (a) Semithin sections of stomach mucosa cultured in stagnant environment show the complete distruction of the tissue. An epithelial lining could not be detected. The remainings of the tissue consist of few spindle shaped cells and a loose scaffold of extracellular matrix. The production of mucus ceased during the first days of culture. (b) Perfusion culture reveals an excellent morphological and functional preservation of all tissue compounds after 5 days. The mucosa shows a typical composition consisting of a single layer of mucus-producing epithelial cells resting on the lamina propria (L. p.) and a densely packed lamina muscularis mucosa (L.m.). Numerous granules can be observed in the apical portion of the epithelial cells indicating active production of mucus during the whole culture period (bar = 30 μm).

However, the simulation of this process under *in vitro* conditions is difficult to realize. Culture of the stomach mucosa is an ambitious task due to the secretion products of the stomach glands. Proteases and HCl are secreted in the culture fluid. It leads to self-digestion of the mucosa under stationary culture conditions. The effect can be slowed by the addition of serum which serves as a buffer for the agressive secretion products [108].

Therefore, we were interested to know whether perfusion culture might improve the culture conditions, so that serum-free culture medium could be used for mature tissue [54]. Large pieces of stomach wall tissue (diameter: 13 mm, thickness:

Figure 5. (Continued).

2–3 mm) were prepared from neonatal rabbits. To prevent the tissue from curling up the samples were mounted in a tissue carrier (Fig. 1a). For control, the tissue was kept under conventional stationary conditions where the culture fluid was exchanged daily. Tissue cultured in the stagnant environment lost its typical morphological and functional features within the first days of culture (Fig. 5a). The epithelial lining of the mucosa was completely destroyed. The remaining tissue consisted of few spindle shaped cells and a loose scaffold of fibrillar extracellular matrix. In contrast, the tissue samples cultured serum-free for 5 days under continuous medium perfusion (flow rate 4 ml h^{-1}) maintained the typical cellular composition of the stomach mucosa (Fig. 5b). All tissue layers such as the lamina epithelialis or muscularis were well preserved. Numerous dark granules were detected at the apical cell poles of the epithelial cells indicating active mucus secretion. This result was confirmed by the viscous consistence of the culture fluid that had passed through the culture container. By this live conservation of tissue it should be possible to investigate questions concerning the development of new surgical suture or scaffold materials as well as drug delivery processes without the interference of an organism.

Parenchymal and stromal tissue interactions

In biomaterial research and tissue engineering thin pieces of tissue are nourished by diffusion, while nutrition and oxygen supply become insufficient when the thickness of the growing tissue increases with time. Conceivable is a coupling of the growing tissue to natural microvessels or artificial capillaries, such as hollow fibers, in order to improve the nutrient and oxygen supply of the tissue [109]. In this context it is important to learn about the mechanisms by which natural or artificial capillaries can be integrated into developing tissue, to collect information on parenchymal–stromal interactions, and on the question in how far implanted biomaterials interfere with this process.

The investigation of these problems using a simple endothelial monolayer in a flat culture dish seemed less suitable to us, since the formation of functional blood vessels within tissues depends on the coordination of a series of subsequent differentiation steps in a 3-dimensional organization [110]. Firstly, it requires the cooperation of cells of the parenchyme and the stroma. Secondly, both cell populations are essential sources for the production of the specific extracellular matrix and the paracrine growth or differentiation factors to integrate the vessels.

An organo-specific microvessel model in perfusion culture helped to answer a part of these questions [111]. Thin pieces of embryonic tissue of the neonatal rabbit kidney were isolated and brought into culture. To maintain the 3-dimensional organo-specific environment, the application of proteases for the isolation of cells was avoided. The extracellular matrix, as well as the specific cellular composition of the tissue, were not altered by the preparation protocol. The pieces were then placed in tissue carriers (Fig. 1b) and were cultured in serum-free medium under conventional stationary conditions (Fig. 6a) or under continuous medium perfusion (Fig. 6b, c). The comparison of the tissue integrity following the different culture methods revealed a poorly preserved tissue structure after stationary culture (Fig. 6a), while perfusion-cultured explants showed no necrotic lesions (Fig. 6b).

The development of microvascular endothelial cells was monitored by using a monoclonal antibody generated in our laboratory [37]. Immunohistochemistry showed that the integrity of the blood vessel chords within the tissue was not maintained at all under stationary culture conditions (Fig. 6a) and poorly developed under perfusion ones (Fig. 6b). The continuous removal of metabolic products and the presence of tissue-specific extracellular matrix alone were thus not sufficient to promote the development of the existing vessel structures. Obviously the serum free culture medium lacked supplements like growth and survival factors necessary for the preservation of the already formed vessels or for stimulation of vessel growth. Therefore a number of soluble factors was tested for their angiogenic potency [37, 61]. It was found that explants kept for 13 days in perfusion culture and in the presence of aldosterone and vitamin D_3 developed numerous broad vessel-like structures (Fig. 6c). As in the nephrogenic zone of neonatal rabbit kidney, these endothelial cell cords run in parallel towards the fibrous organ capsule. In culture they broadened and elongated considerably. Despite this proliferative activity the

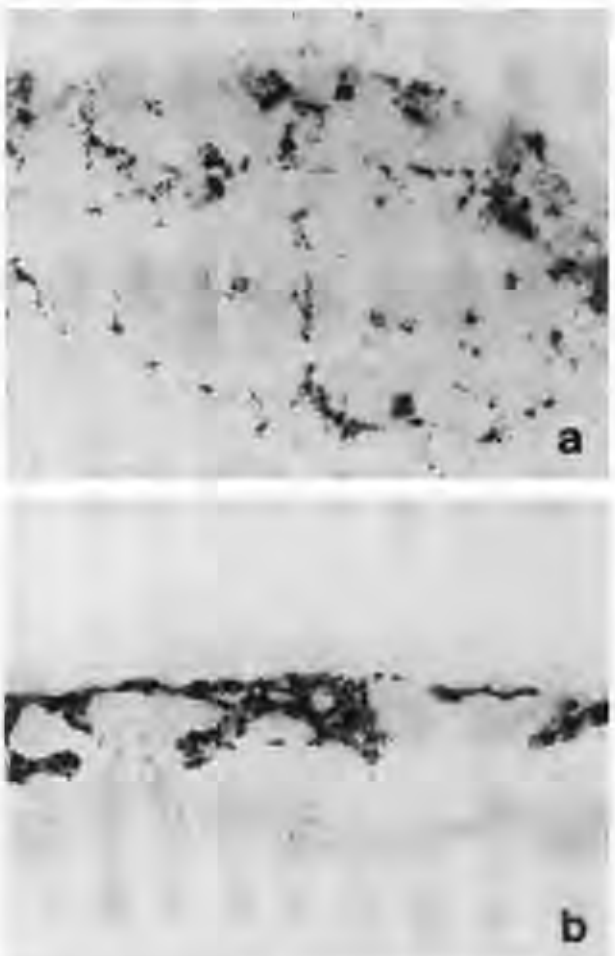

Figure 6. Modulation of renal microvascular system under different culture conditions. Tissue was prepared from kidneys of neonatal rabbits. The nephrogenic zone of the renal cortex was prepared by stripping off the fibrous organ capsule. The application of proteases was avoided, thus the natural composition of the extracellular matrix and the cellular elements was preserved. Explants were mounted in tissue carriers to ensure overall access to the culture medium. (a) Mounted explants were cultured serum-free for 13 days under conventional stationary conditions. The tissue samples show large necrotic lesions. Immunolabelling with mab EC1 shows that blood vessels have disappeared, only single endothelial cells and small cell clusters are detected. (b) Necrotic lesions were not detected in cortex explants cultured under continuous medium perfusion. However, the blood vessels of the nephrogenic zone are desintegrated. Endothelial cells are arranged in large clusters. We conclude that the organo-specific extracellular matrix is not sufficient to maintain blood vessel integrity. Obviously growth or differentiation factors are lacking. (c) Supplementation of the perfusion culture medium with the hormones aldosterone (1×10^{-7} M) and vitamin D_3 (1×10^{-9} M) results in coordinated endothelial cell proliferation. The vessel streaks of the nephrogenic zone have broadend, elongated and maintained their typical spatial organization as observed in the neonatal kidney (bar = 60 μm).

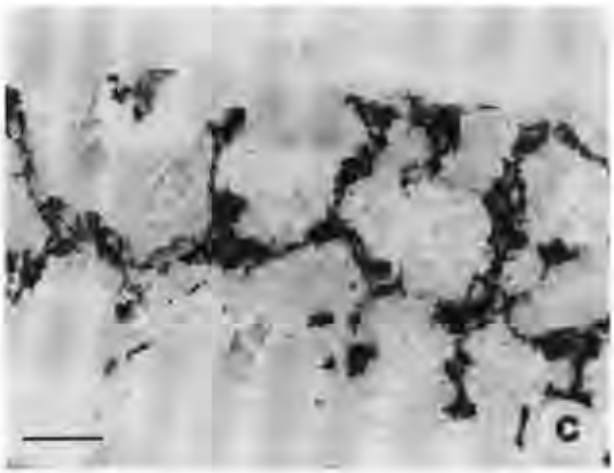

Figure 6. (Continued).

proliferating endothelial cells maintained their typical spatial organization. The most interesting question for ongoing experiments now is whether the premature renal vessel strands can be integrated into scaffold materials or into artificially-generated parenchymal cell populations prepared from kidney or other organs.

Switching on gene products by learning from adult tissues

Tissue engineering conducted within animals generally shows less problems in the generation of functional tissues than experiments performed under pure *in vitro* conditions [112, 113]. For example, experiments using connective tissues such as cartilage or bone have a common problem, in that the synthesis of the pericellular matrix is altered in culture. The cells change their program for the synthesis of extracellular matrix proteins and switch to synthesis of collagen type I instead of type II [114–116]. After secretion, the extracellular matrix proteins have to be polymerized outside the cells to form a strong structure able to resist mechanical stress. However, in culture the extracellular matrix remains weak since atypical compounds are synthesized and the extracellular polymerization and composition of the matrix is insufficient. To improve the situation, different culture techniques with various morphogenic supplements have been tested, but up to date the problem is not solved. Consequently, we have to learn not only to handle isolated cells on a scaffold, but we should also become capable of culturing whole pieces of cartilage or bone as an intact piece of tissue exhibiting a high degree of cellular and extracellular differentiation features. Most interestingly, up to date we found only one paper describing the successful culture of pieces of native cartilage [117]. Not a single paper was found showing the successful culture of pieces of bone.

Each culture medium leads to an individual cell type

It is obvious that for decades cells and tissues have been cultured in a few predefined culture media. Despite this fact some other media could probably be more useful for biomaterial testing and tissue engineering. In one set of experiments for example, embryonic renal collecting duct epithelia were exposed to six different culture media under serum-free conditions in perfusion culture for 14 days (Fig. 7) [118]. During that period proliferation was downregulated and the epithelial cells remained in the postmitotic phase, as observed in the adult kidney (Table 1). Morphological differences in the experimental series were not observed. To monitor individual protein expression by immunohistochemical methods, the monoclonal antibodies (mab) 703 and 503 recognizing principal (P), and intercalated (IC) cell features were used respectively [119]. We found that mab 703 binding on CD cells (P cell feature) may correlate with the Na content in the different culture media. Culture in IMDM with the lowest Na content showed 5–10% mab 703 binding cells. M 199 with intermediate Na content resulted in 30–40% mab 703 binding cells, while all the other media with a high Na content produced between 95 and 100% mab 703 binding cells (Fig. 7). In contrast, mab 503 binding (IC cell feature) did not correlate with the increasing Na concentrations of the different media. We concluded that without the influence of growth factors in perfusion culture, each of the media produced a very specific type of epithelium. The result showed that cultured epithelia in a postmitotic phase display an unexpected sensitivity not only for the scaffold but also for the electrolyte environment.

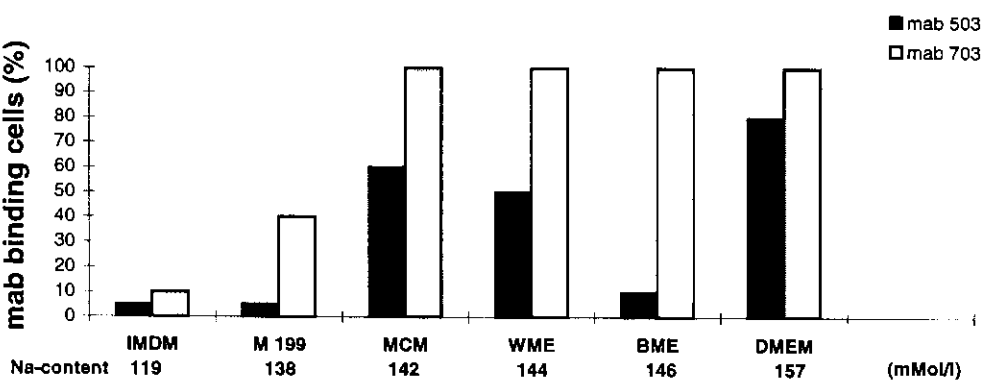

Figure 7. Development of renal collecting duct epithelia kept in 6 different serum — free culture media and under perfusion culture. IMDM with 119 mmol l^{-1}, M 199 with 138 mmol l^{-1}, MCM with 142 mmol l^{-1}, WME with 144 mmol l^{-1}, BME with 146 mmol l^{-1}, and DMEM with 157 mmol l^{-1} Na. Differentiation is monitored by immunohistochemical labeling cryostate sections of the tissue with mab 503 and mab 703. Both antibodies label distinct cell populations of the mature renal collecting duct epithelium [119]. Each medium induces its specific differentiation profil. Thus, medium composition represents a specific trigger for the differentiation of the epithelial cells.

Electrolyte sensing mechanism for differentiation

During embryonic development, epithelia are exposed to an environment consisting of the same fluid at the luminal and basal side. During functional maturation this environment changes. After the epithelia have formed a functional barrier, the luminal and basal aspect of the cells are confronted with different fluids. In contrast, in biomaterial testing within a culture dish, epithelial cells are fed the same medium on the luminal and basal side. This is an unphysiological situation for the epithelia because they are exposed to a permanent biological short circuit current, which frequently results in a minor development of polarization and a loss of functional features [44].

To elucidate cell-biological and technical requisites for the construction of an artificial renal tubular element, the differentiation profile of epithelia cultured under embryonic and adult perfusion culture conditions has to be investigated. To mimic an embryonic environment the epithelia can be perfused with the same medium on the luminal and basal side of a gradient container. An adaption to a physiological environment of the adult organism was simulated in a gradient container by culturing embryonic CD epithelia with increasing Na concentrations on the luminal side, while standard medium was used on the basal side (Table 2) (Fig. 1d). For gradient culture experiments, the electrolyte parameters of the culture medium had to be adapted by adding NaCl and Na-gluconate [120]. Comparing IMDM with the serum of neonatal rabbits as a model fluid for the interstitial space, unexpectedly large differences in the concentration of electrolytes were observed. For example, serum contains 137 mmol l^{-1} Na^+, while in IMDM only 120 mmol l^{-1} Na^+ were measured (Table 2). Similarly, large differences were found when comparing K^+ Cl^-, and Ca^{2+} concentrations.

Simulation of an embryonic environment for renal CD epithelia in a gradient container with standard IMDM on both the luminal and basal side revealed only 5–10% of cells positive for mab 703 (Fig. 8c) and mab 503 from day 1 until day 19 (Fig. 9a) [121]. In contrast, mimiking an adult environment in a gradient container using IMDM containing additional 12 mmol l^{-1} NaCl/17 mmol l^{-1} Na-gluconate on the luminal side, dramatic changes occurred in the epithelium (Figs 8d

Table 2.
Physiological parameters of media for renal tissue kept in stationary and perfusion culture. IMDM contains 75 mmol l^{-1} HEPES to maintain a constant pH of 7.4. An individual experiment is given

pH = 7.4	pO_2 (mmHg)	pCO_2 (mmHg)	Na^+ (mmol l^{-1})	K^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	Glucose (mg dl^{-1})	Osmolarity (mOsm)
Stationary	150.5	33.6	112.3	4.2	83.3	1.2	422	241
Perfusion	174.6	9.34	120.4	4.1	86.9	1.2	393	255
Gradient								
Basal	172.2	10.8	120.6	4.0	86.6	1.1	370	255
Luminal	171.6	11.4	144.4	3.9	97.0	0.8	375	297

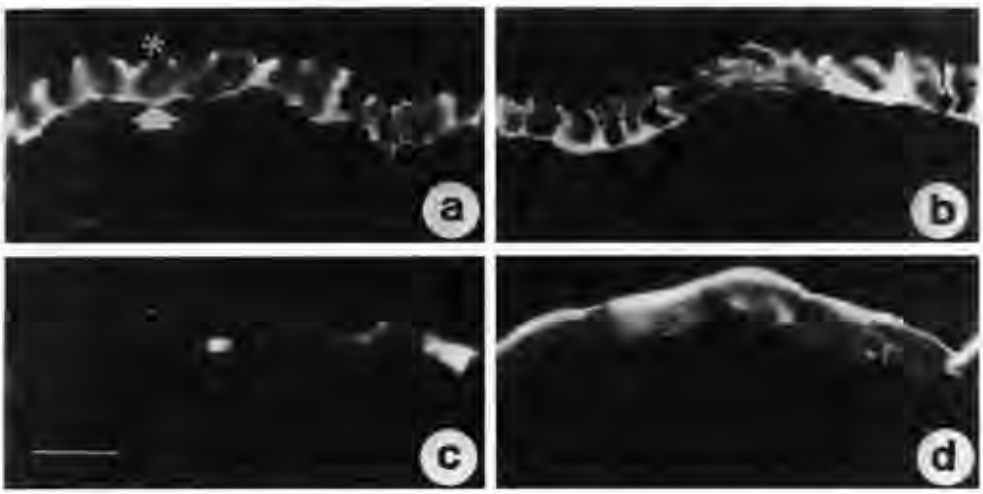

Figure 8. Influence of the electrolyte environment on the differentiation status of renal collecting duct epithelia kept in a gradient culture container for 14 days with same (a, c) and different (b, d) media at the luminal and basal side. As revealed by immunohistochemistry all cells are positive for Na/K ATPase (a) and 10% are positive for mab 703 (c), when IMDM is applied on both sides of the epithelium. In contrast, epithelia cultured with a luminal NaCl load show 80 to 90% mab 703 positive cells (d), while all cells are positive for Na/K ATPase (b) (bar = 20 μm).

and 9b). Mab 703 binding was found on less than 5% of the cells within the cultured epithelium on day 1, and from days 3 to 6 only 10% of the cells were positive. Then on day 9 > 70% and on day 19 > 90% of the cells became positive for mab 703 (Fig. 8d). Mab 503 was found on less than 5% of the cells on day 1, then from days 3 to 6, 30% immunopositive cells were present. A further increase of immunopositive cells was registered at days 9 and 14 so that up to 70% and 80%, respectively of the cells were positive for the antibody after day 19 (Fig. 9b). For control, the expression of Na/K ATPase was not altered in epithelial cells confronted with identical (Fig. 8a) or different electrolyte concentrations (Fig. 8b).

The time course of development showed that mab 703 and mab 503 labeling of the embryonic CD epithelia was developed to a minor degree until day 5 (Fig. 9b). Then after day 6 the development increased and reached a maximum after day 10. It is obvious that the development of CD cell features started after an unexpectedly long latent period. The latent period was paralleled by a complete loss of mitotic activity in the epithelium, as observed in the maturing kidney [64]. This result is a clear indication that the development of differentiation features cannot be expected within a few days but takes at least 2 weeks. When at day 14 IMDM containing additional NaCl was replaced by standard IMDM for another 5 days (Fig. 9c), a considerable decrease of mab 503 binding cells was found, while the amount of mab 703 binding cells remained constant. Thus, not only the upregulation but also the preservation of mab 503 antigen expression was controlled by the extracellular electrolyte environment. This finding shows that without the use of

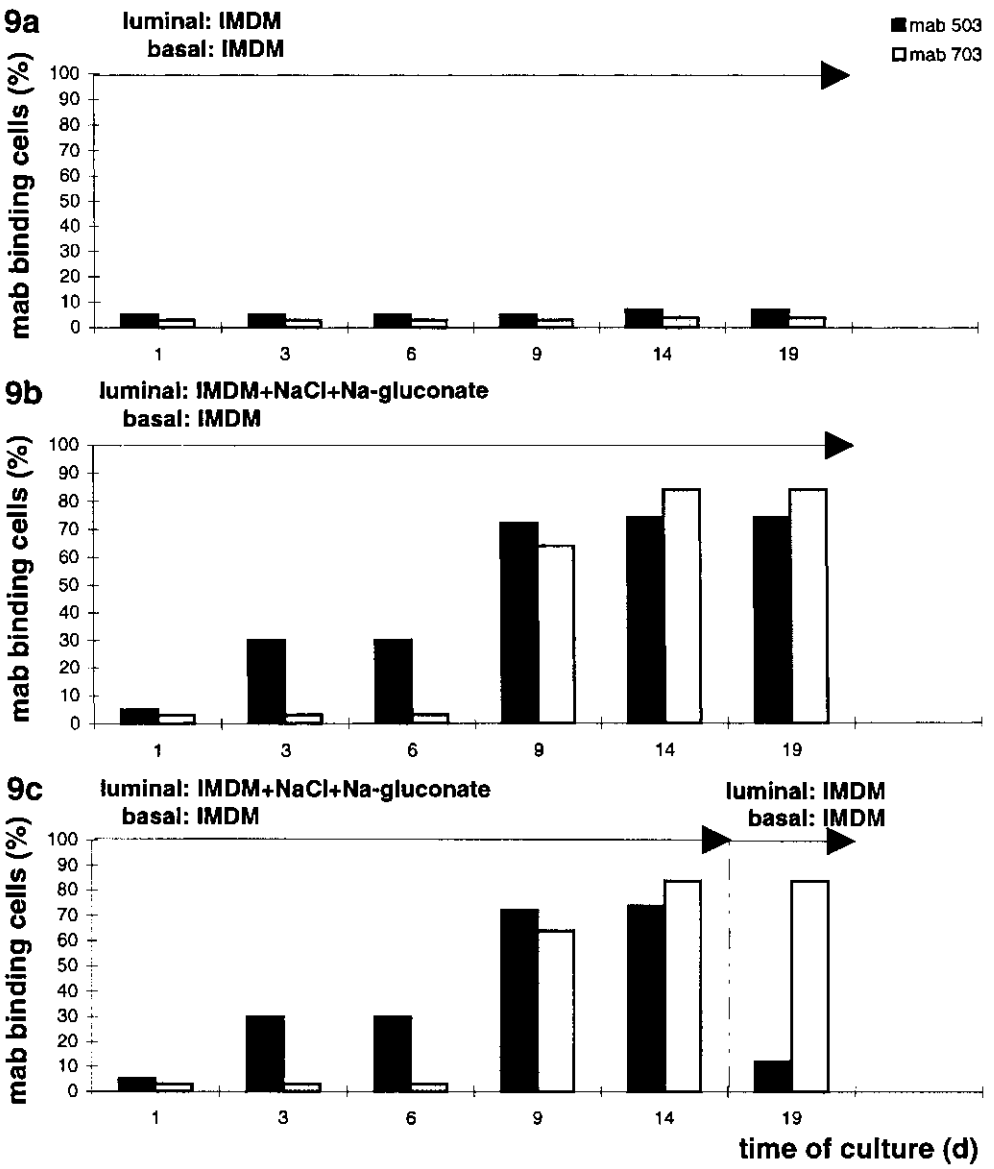

Figure 9. Perfusion culture of renal epithelia in a gradient container shows the influence of the media on differentiation. (a) Development of CD epithelia features under isotonic culture conditions after 1–14 days in a gradient container with standard IMDM on the luminal and basal sides. At day 1 until day 19 mab 703 and mab 503 binding is detected only on less than 5–10% cells. (b) Development of mab 703 and 503 antigen features in embryonic collecting duct epithelia cultured for 14 days in a gradient container. At the basal side of the epithelium standard IMDM was used, while on the luminal side IMDM including additional 12 mmol l^{-1} NaCl and 17 mmol l^{-1} Na-gluconate was superfused. 70 to 80% mab 703 and 503 binding cells are found until day 14. (c) Downregulation of mab 503 binding features. On day 14 the NaCl load was reduced to standard levels. Until day 19 again standard IMDM was applied on both sides of the tissue. Mab 503 binding on cells is downregulated within 4 days, while mab 703 binding remained unchanged.

growth factors the development of epithelia can be triggered by the electrolyte environment in such a sensitive manner which we did not expect [122]. On one hand we concluded that adapting the electrolyte composition of the culture fluid to the specific requirements of the tissue should considerably improve the quality of constructs generated for biomaterial testing and tissue engineering. On the other hand liberation of electrolytes in biomaterials and scaffolds may influence the proper development of tissues in an unexpectedly sensitive manner.

THEORETICAL CONSIDERATIONS AND FUTURE DEMANDS FOR BIOMATERIAL TESTING EXCLUSIVELY UNDER *IN VITRO* CONDITIONS

Summarizing the results of recent research in tissue biology and of experiments presented here it becomes evident that we are at the beginning and not at the end of a phase towards the generation of optimal tissues for improved biomaterial testing and tissue engineering. To achieve an optimal degree of cellular differentiation, certain technical prerequisites are necessary for the culture of differentiated artificial tissues. According to our previous experimental results, cellular differentiation under *in vitro* conditions is not determined by a single morphogenic soluble factor, but depends on a series of biophysical respectively physiological stimuli, which interact in a hierarchic manner (Table 3). Of critical importance for the differentiation status of a cell is first of all the composition of the natural or artificial extracellular matrix [123–128]. Consequently, our working hypothesis is that cell anchorage is at the top of this hierarchy, because it influences all of the following developmental steps in a positive or negative way. For example, if in a first experimental step an optimal cell anchorage is provided, then in consequence an optimal degree of cell differentiation can be expected. In contrast, if suboptimal anchorage is used only suboptimal differentiation will be observed in further experimental steps. A suitable extracellular matrix promotes cellular differentiation by driving cells from mere survival to an active functional state. According to these considerations an optimal composition of the artificial extracellular matrix

Table 3.
Obvious hierarchy of cell anchorage, perfusion of medium, hormonal supplement, and electrolyte environment triggering differentiation in tissue culture. Each experimental step acts and results in an improved degree of tissue specific features. In contrast, each non-optimal treatment results in suboptimal degree of differentiation

Environment	Optimal	Suboptimal	Average	Bad	Minimal
Biomatrix	+	+	+	−	−
Perfusion	+	+	−	−	−
Morphogens	+	+	+	+	−
Electrolytes	+	−	−	−	−
Features	++++	−+++	−−++	−−−+	−−−−

is a contribution to ensure excellent cell anchorage and is the base for all following procedures to reach a high degree of cellular differentiation.

In pharmaceutical research, biomaterial and toxicity testing for consumer protection [130–133] is a need for advanced biomatrices, tailored to fit the specific demands of highly specialized cells. These materials must promote the differentiation of cells that were first propagated under proliferative stimuli and which are then maintained under postmitotic conditions, as observed in our organism. The adaption of the culture medium to promote the specific functions of the tissue is the next step to further improve the quality of tissue constructs. Perfusion culture devices are needed to create a tissue-specific environment ensuring a continuous supply of nutrients and removal of metabolites and paracrine factors. The research of the past years provided new insights into the tremendous importance of cell anchorage and the composition of the extracellular fluid on the physiological function of cells and tissues. Ten years ago people did not realize how sensitive tissues react to their environment.

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