Bridging the gap between traditional cell cultures and bioreactors applied in regenerative medicine: practical experiences with the MINUSHEET perfusion culture system

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Abstract To meet specific requirements of developing tissues urgently needed in tissue engineering, biomaterial research and drug toxicity testing, a versatile perfusion culture system was developed. First an individual biomaterial is selected and then mounted in a MINUSHEET[®] tissue carrier. After sterilization the assembly is transferred by fine forceps to a 24 well culture plate for seeding cells or mounting tissue on it. To support spatial (3D) development a carrier can be placed in various types of perfusion culture containers. In the basic version a constant flow of culture medium provides contained tissue with always fresh nutrition and respiratory gas. For example, epithelia can be transferred to a gradient container, where they are exposed to different fluids at the luminal and basal side. To observe development of tissue under the microscope, in a different type of container a transparent lid and base are integrated. Finally, stem/progenitor cells are incubated in a container filled by an artificial interstitium to support spatial development. In the past years the described system was applied in numerous own and external investigations. To present an actual overview of resulting experimental data, the present paper was written.

Keywords Cell culture · Perfusion culture · 3D culture · Tissue carrier · Bioreactor · Tissue engineering · Biomaterial testing · Biomedicine

Introduction

Nowadays it is standard in the laboratory to culture cells and tissues according to their individual needs by more or less sophisticated techniques. However, 25 years ago proliferating cells were generally kept in a small selection of glass or plastic containers resembling the traditional Petri dish. The problem was that a dish does not meet the requirements of developing tissues. Thus, for the generation of tissues improved culture techniques were needed but at that time attractive bioreactors were not commercially available.

As a consequence, the lack of suitable tools for the generation of specialized tissues was the motivation to start with the construction of the MINUSHEET[®] perfusion culture system (Minuth 1990). The goal was to devise a simple technique, which enables selection of an individual biomaterial for optimal cell adhesion to mount it in a specific holder, to seed cells on it in a 24 well culture plate and finally to transfer it to a series of perfusion culture containers for the generation and long term maintenance of various specialized tissues (Minuth and Rudolph 1990; Minuth et al. 1992a). Considering further the diversity of specialized tissues in an organism on the

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one hand and the variety of biomaterials used in tissue engineering and regenerative medicine on the other hand, it was obvious that only a highly adaptable system could provide those environmental parameters that are demanded for corresponding in vitro experiments.

Technical properties

Mounting a biomaterial in a tissue carrier

The introduced concept is based on a MINUSHEET® tissue carrier, which enables the user to mount a selected biomaterial by his own hands in the laboratory. To stay compatible with a 24 well culture plate, the biomaterial is punched out to a diameter of 13 mm (Fig. 1a). In this coherence it does not matter whether decellularized extracellular matrix, synthetic polymers, ceramics, metals or biodegradable scaffolds are selected. Further on, materials can be used in form of foils, filters, nets, fleeces, foams or solid supports containing small or big pores.

For practical application, a punched out filter is placed in the base part of a MINUSHEET® tissue carrier (Fig. 1b; black ring). By pressing down a tension ring (white ring) the filter is fixed in position. The use of this demonstrated tissue carrier prevents damage of the mounted biomaterial and protects cells during seeding, ongoing development and further experimental manipulation.

The following disinfection of the mounted carrier depends on the chemical composition of the selected biomaterial. Therefore it is either performed by formaline, ethylene oxide gas, irradiation or autoclaving. Subsequently, the tissue carrier can be frozen, stored at room temperature in a sterile box or used immediately for cell seeding.

Seeding of cells

For seeding of cells a sterile tissue carrier mounted with a biomaterial is placed by forceps into a 24 well culture plate (Fig. 1c). In a next step culture medium is slowly added by a pipette so that the surface of the inserted biomaterial is just wetted. Then cells are transferred by a pipette within a small droplet of medium. In a standard set up seeding of cells is performed only on the upper side of a selected

Fig. 1 Application of a MINUSHEET® tissue carrier. **a** First a biomaterial measuring 13 mm in diameter is selected. **b** Then the biomaterial is placed in the black base part of a tissue carrier. Mounting is completed by pressing the white tension ring in the base part. **c** After sterilization the carrier is transferred by forceps to a 24 well culture plate for cell seeding

biomaterial. However, in the case a co-culture experiment is planned, seeding of a second cell type is made after turning the tissue carrier.

For creation of an artificial interstitium isolated cells or a thin slice of living tissue are mounted between two pieces of polyester fleece in a carrier. Further pieces of a collagen sheet can be placed in a tissue carrier like the skin of a drum. These few examples illustrate that in principle numerous kinds of applications exist for mounting a biomaterial in combination with isolated cells or even living tissues in a carrier.

Fig. 2 Versatile use of a MINUSHEET® tissue carrier in a ► perfusion culture container. **a** In a basic version a perfusion culture container can hold six tissue carriers for provision with always fresh medium. **b** In a gradient perfusion culture container the contained tissue is exposed to different fluids at the luminal and basal side. **c** For observation of growing tissue under a microscope a transparent lid and base is integrated in a container. **d** A perfusion culture set up is running in the typical case on a laboratory table and under atmospheric air. A thermo plate maintains the desired temperature of 37 °C. During culture a peristaltic pump transports the medium (1.25 ml/h) from a storage bottle (left side) to the waste bottle (right side). Arrow indicates flow of medium

Analysis of cell distribution

Regardless of whether transparent or non-transparent biomaterials are used, the quality of seeding and resulting cell distribution must be controlled. This can best be achieved by epi-fluorescence microscopy, when specimens were fixed in 70 % ethanol and labeled for example by propidium iodide (Minuth et al. 1994). In such a protocol fluorescent nuclei reflect the distribution of cells on a selected biomaterial. Surprising results were obtained, when MDCK cells were cultured on materials such as glass, polystyrene (Thermanox®), white and black polycarbonate filters. Depending on the selected material the pattern of screened cells was ranging between perfect confluence, atypical dome, cysts and cluster formation.

Selection of a perfusion culture container

Only for the relatively short period of cell seeding a MINUSHEET® tissue carrier is kept in the static environment of a 24 well culture plate. Then a perfusion culture container is selected to offer adherent cells a fluid milieu, which better meets special needs of developing tissue than the static environment of a dish. Further the exact adjustment of a tissue carrier within a perfusion culture container guarantees an equal distribution and consequently a continuous transport of always fresh culture medium, whereby an uncontrollable accumulation of harmful metabolites and an overshoot of paracrine factors during proceeding culture is prevented.

In the basic version of a perfusion culture container up to six tissue carriers can be placed beside each other (Fig. 2a). A continuous fluid flow provides here for example developing connective tissue from all sides with always fresh nutrition and respiratory gas. In a

gradient perfusion culture container the tissue carrier is fixed centrally between the base and the lid. This design enables to transport different media at the luminal and basal side (Fig. 2b). For microscopic observation a

container exhibits a transparent lid and base (Fig. 2c). A further container contains a flexible silicone membrane. When mechanical force by an eccentric rotor is applied, a transmitted stimulus in the interior supports development of cartilage or bone. Finally, a perfusion culture container filled with a polyester fleece as an artificial interstitium makes it possible to investigate spatial development of parenchyma.

Fresh fluid continuum

When cells in combination with a biodegradable biomaterial are kept in culture, it must be considered that both can produce harmful metabolites such as lactic acid leading in turn to an un-physiological accumulation. Thus, an overshoot of such metabolites must be prevented and its concentration has to be kept on a constant low physiological level. Due to this reason developing tissue is exposed in a perfusion culture container to a continuous flow of always fresh medium. To prevent an accumulation of harmful metabolites within a perfusion culture container, quality of medium is measured at the outflow for example by a blood gas analyzer (Nova Biomedical, Rödermark, Germany). According to registered metabolites the rate of medium transport can be adapted to the individual needs of contained tissue. Finally, metabolized medium is not re-circulated but collected in a separate waste bottle.

The transport of culture medium is best accomplished by application of a slowly rotating peristaltic pump (ISMATEC, IPC N8, Wertheim, Germany), which is able to provide adjustable transport rates between 0.1 and 5 ml per hour and channel (Fig. 2d). In personal experiments optimal results were obtained, when medium was transported with 1.25 ml/h for a period of at least 13 days. Further on, for maintaining a defined temperature of 37 °C within a perfusion culture container, a heating plate (MEDAX-Nagel, Kiel, Germany) and a special Plexiglas cover lid (not shown) is used (Fig. 2d).

Stabilization of pH in transported culture medium

Perfusion culture can be performed either in a traditional CO₂ incubator or better on a laboratory table. In the case a CO₂ incubator is used, a culture medium is selected containing a buffer system with a relatively high amount of NaHCO₃. It will maintain in

a 5 % CO₂ atmosphere of an incubator a constant pH between 7.2 and 7.4. However, when such a formulated medium is used in a perfusion culture set up outside a CO₂ incubator, the pH will shift from the physiological range to alkaline values due to the low content of CO₂ (0.03 %) in atmospheric air. In turn contained cells, respectively, tissues are chronically damaged and will finally die.

In principle, most of media are suitable for application in perfusion culture. However, when it is performed outside a CO₂ incubator, the media must be ordered with a strongly reduced NaHCO₃ concentration. Further biological buffers such as HEPES (GIBCO/Invitrogen, Karlsruhe, Germany) or BUFFER ALL (Sigma-Aldrich-Chemie, München, Germany) have to be added for constant stabilization of pH. The necessary amount is determined by admixing increasing concentrations of biological buffer solution (always in the same volume) to an aliquot of medium. Then the medium must equilibrate overnight on a thermo plate at 37 °C under atmospheric air. Finally, the aliquots are measured by an electrolyte analyzer. The data revealed that for example addition of 50 mmol/l HEPES or an equivalent of BUFFER ALL (1 %) to IMDM (Iscove's Modified Dulbecco's Medium, GIBCO/Invitrogen) maintains the pH between 7.3 and 7.4 throughout long term perfusion culture on a laboratory table under atmospheric air (Roessger et al. 2009).

Further on, beside conventional media well suited culture media for perfusion culture running under atmospheric air are Leibovitz's L-15 Medium and CO₂ Independent Medium. Both were successfully applied under chemically defined conditions (Minuth et al. 2013; Minuth and Denk 2013).

Respiratory gas in transported medium

For enrichment of oxygen (O₂) in a perfusion culture set up medium is pumped through a gas-permeable silicone tube. It provides a large surface for the gas exchange by diffusion due to a thin wall (1 mm), small inner diameter (1 mm) and extended length (1 m). For example, IMDM (3024 mg/l NaHCO₃, 50 mmol/l HEPES) equilibrated against atmospheric air reveals in a standard perfusion culture set up partial pressures of 160 mmHg O₂ and 10 mmHg CO₂ (Minuth et al. 2001; Strehl et al. 2004).

Further the requirement for oxygen depends on specialization of the individual tissue. For that reason

in special cases the concentration of O_2 must be adapted in transported medium. A simple technical solution is a gas exchange module containing a gas inlet and outlet (Strehl et al. 2004). Further a spiral with a long thin-walled silicone tube for medium transport is mounted inside the module. Since the tube of the spiral is gas-permeable, diffusion of gases between culture medium (inside the spiral) and a given atmosphere (outside the spiral) within the gas exchange module takes place during transport. Applying this simple method the gas atmosphere can be adjusted by a constant flow of a specific gas mixture at the outside of the spiral. During run of such an experiment the content of gas at the in- and outflow of a perfusion culture container is controlled by a blood gas analyzer.

Elimination of gas bubbles

During transport of medium gas bubbles will arise in the perfusion culture set up. Problematic is that they impede the flow of medium. Surprisingly, formation of gas bubbles is observed during suction of medium from the storage bottle, during transport at material transitions between tubes and connectors, at the surface of developing tissue and at the outflow of a perfusion culture container. First gas bubbles are small so that they are not visible to the naked eye. However, during transport of medium they increase in size, fuse with each other and form an embolus that can massively impede medium flow. When gas bubbles accumulate inside a perfusion culture container, they cause a regional shortage of medium supply. Finally, formation of gas bubbles in a gradient perfusion container is leading to remarkable fluid pressure changes, although normally two media must be transported at exactly the same speed and pressure (Fig. 2b). Thus, an embolic effect caused by gas bubbles in one of the channels leads to massive pressure differences destroying in turn the barrier function of an interposed epithelium.

To minimize arise of bubbles in a culture set up, a gas expander module is placed before medium is entering the perfusion culture container (Minuth et al. 2004a, b). Inside a gas expander module medium is rising within a small reservoir and expands before it drops down after a barrier. During this process gas bubbles are separated from the medium and collected at the top of the gas expander module. As a result, culture medium leaving the gas expander module stays oxygen-saturated but is free of gas bubbles.

Design and construction

Described MINUSHEET[®] tissue carriers (Fig. 1) and perfusion culture containers (Fig. 2) were not designed for one-off application but for multiple use. Since the tools are exposed to numerous cycles of cleaning and sterilization during years, a special design had to be made and stringent requirements on material quality were necessary. To prevent unwanted cracks and alterations in material surface, tissue carriers were finally produced by injection molding with Pocan[®] thermoplastic polyester resin. The illustrated perfusion culture containers and related equipment such as gas expander and gas exchange modules were produced in a certified workshop by a computerized numerical controlled (CNC) milling machine out of Makrolon[®] polycarbonate.

Featuring development of epithelia

In previous personal experiments it was observed that epithelial cells do spread in a dish very well but often they do not develop expected cell biological features. To support differentiation, environment for epithelia was improved by offering an individual extracellular matrix or biomaterial for adhesion and by provision with always fresh culture medium.

For example, collecting duct (CD) tubule cells derived from the embryonic parenchyma of neonatal kidney were isolated with the associated organ capsule and mounted in a MINUSHEET[®] tissue carrier. For the first time could be observed that these cells develop during subsequent perfusion culture into a polarized epithelium. Immunohistochemistry further demonstrated that harvested epithelia express the same cell biological features as observed in adult Principal (P) and Intercalated Cells (IC) of the collecting duct tubule (Herter et al. 1993; Minuth et al. 1993; Aigner et al. 1994, 1995).

Moreover, perfusion culture experiments gave new insights in the spatial development as well of renal microvasculature and glomeruli (Kloth et al. 1994, 1995, 1998a; Kloth and Suter-Crazzolara 2000) as even of intact gastric glands (Kloth et al. 1998b). In other experiments it was investigated to what extent regeneration can be influenced by engineered microvessels (Frerich et al. 2006, 2008) or isolated endothelial cells (Bakowsky et al. 2005; Hayashi et al.

2009). To evaluate perspectives of living conservation, human gingival epithelium was kept in long term perfusion culture (Lehmann et al. 1997; Lauer 2009). Co-culture of human oral keratinocytes with osteoblast-like cells gave new insights for performance of hard and soft tissue reconstruction in future (Glaum et al. 2010).

Factors influencing reproductive aging and the development of fertilized eggs were screened with anterior pituitary gland cells (Zheng et al. 2007), oviduct epithelium (Reischl et al. 1999) and endometrial cells (Tiemann et al. 2005). New protocols for an optimal matrix coating and adaptation to continuous medium flow were elaborated for hepatocytes (Fiegel et al. 2004; Schumacher et al. 2007; Du et al. 2008; Xia et al. 2009). Regeneration of urothelium was analyzed in combination with newly developed stent materials (Sternberg et al. 2004). Finally, effects of newly developed drugs on ciliary beat frequency (CBF) were elaborated with the help of nasal epithelium kept in perfusion culture (Dimova et al. 2005). Reconstruction of cornea became possible by modulation of environment under dynamic culture conditions (Wu et al. 2014). Finally, reactions of retinal pigment epithelium kept in perfusion culture could be registered after laser irradiation by two-photon microscopy (Miura et al. 2013).

Renal epithelia exposed to a gradient

Past and present experiments revealed that gradient perfusion culture answers unsolved questions in developmental biomedicine. During the embryonic and early fetal period epithelia are still exposed to the same fluid at the luminal and basal sides due to still leaky barrier characteristics. However, in maturing epithelia a tight junction complex and up-regulated transport features form a functional barrier. To investigate such processes, a MINUSHEET® tissue carrier with epithelial cells seeded on different biomaterials was mounted in a gradient perfusion culture container (Fig. 2b). Transportation of different fluids through the lid and base part of the container produces a specific environment for epithelia. When this strategy was followed, for example intact renal barriers could be generated (Dankers et al. 2010, 2011).

Application of a gradient perfusion culture container made it further possible to investigate the

influence on differentiation of different fluid composition at the luminal and basal sides of embryonic renal collecting duct (CD) epithelia (Minuth et al. 1992b, 1997a, b, 1999, 2001, 2005a; Steiner et al. 1997; Schumacher et al. 2002a; Minuth et al. 2009a). In the course of performed experiments it was detected that development of a CD epithelium starts with an unexpected long latent period of three days and needs at least 10 days for up-regulation of typical signs of differentiation. Further on, development can be triggered by increasing concentrations of NaCl administered at the luminal side. In such an electrolyte gradient over days typical epithelial cell characteristics such as TROMA I (Cytokeratin Endo-A; Fig. 3a), cingulin (Fig. 3b) or Na/K ATPase $\alpha 5$ (Fig. 3c) were up-regulated. Most interestingly, when fluid with an increased NaCl concentration at the luminal side was replaced against a low NaCl concentration, achieved characteristics were down-regulated within few days. This result illustrates that a luminal-basal electrolyte gradient maintains functional features within renal epithelia.

Challenging experiments were performed with hydrogel mounted in a MINUSHEET® tissue carrier

Fig. 3 Features of a renal collecting duct (CD) epithelium kept for 13 days in a gradient perfusion culture container. At the luminal side IMDM + aldosterone (1×10^{-7} M) + 15 mmol/l NaCl, while at the basal side IMDM + aldosterone (1×10^{-7} M) was transported. Immunohistochemistry shows that an intense label for tissue-specific markers such as **a** TROMA I, **b** cingulin and **c** Na/K ATPase $\alpha 5$ is present. Site of the basal lamina is marked by an asterisk, while lumen is indicated by an arrow

to substitute the glomerular basement membrane. In this experimental set up endothelial cells are seeded on the one side, while podocytes were growing on the other side. When those co-cultures were mounted in a gradient perfusion container, development of an intact urine-blood barrier has taken place so that related functions can be tested under advanced culture conditions (Bruggeman et al. 2012). Further on, testing of special bilayered scaffolds with tailorable properties in perfusion culture helps to optimize long term adherence and special differentiation of renal epithelial cells (Mollet et al. 2014).

Finally, epithelia kept in gradient perfusion culture illustrated that commercially available media often do not contain all of the compounds normally needed for optimal cell differentiation. As a consequence, for special demands culture media have to be adapted by addition of defined electrolyte concentrations so that an adequate degree of differentiation is achieved (Schumacher et al. 1999, 2002b).

Pigment epithelium in combination with retina

Retina is a complex neural cell composition that is delimited by a pigment epithelium. Since typical morphological features cannot be maintained in static environment of a dish, intact retina was mounted in a tissue carrier for perfusion culture in a gradient container (Framme et al. 2002; Spiegel et al. 2002; Saikia et al. 2006; Jian and Jingbo 2007; Hamilton et al. 2007; Hammer et al. 2008; Kobuch et al. 2008). These experiments illustrated that the pigment epithelium and neighboring neurons maintain a perfect morphology for a culture period of at least 10 days. On the one hand these exciting findings illustrate novel perspectives for safety testing of newly developed pharmaceuticals designed for intraocular application. On the other hand these experiments give rise to new opportunities for investigating the wide field of retina inflammation, aging, degeneration and repair by the help of an adequate culture system (Klettner and Roider 2009, 2012; Klettner et al. 2009; Miura et al. 2010; Treumer et al. 2012). In this coherence, for Example, molecular regulation of vascular endothelial growth factor secretion and cell biological reactions after fucoidan exposure were investigated (Klettner et al. 2013, 2014; Dithmer et al. 2014).

Blood-retina and blood–brain barrier

Blood-retina and blood–brain barriers are of special interest for the transport of new medicines. It has been shown that a MINUSHEET® tissue carrier in combination with a gradient perfusion container is an ideal tool to elaborate special features of these barrier functions under in vitro conditions closely adapted to nature (Steuer et al. 2004, 2005; Hamilton and Leach 2011). In turn, those experiments gave new insights in molecular permeation and expression of multidrug resistance protein (P-gp) and multidrug resistance-associated protein (MRP).

Blood-air barrier

Lung epithelial cells (pneumocytes) cover alveoli in the lung. Their specific environment in form of a blood-air barrier can be simulated by use of a gradient perfusion container (Gueven et al. 1996). For this special purpose pneumocytes and endothelial cells were seeded for example on a polycarbonate filter and then transferred to a gradient perfusion culture container. During these experiments development of the tight junction complex was registered sealing in turn the blood-air barrier. Also typical features of polar differentiation within the epithelia were up-regulated. It was further shown that gradient perfusion culture in combination with pneumocytes and endothelial cells is a valuable model to investigate dose-controlled exposure of airborne particles. Finally, to elaborate characteristics of barrier transport and mechanisms of repair after alveolar injury a dose controlled air–liquid interface (ALI) was created by the use of A549 cells and kept in gradient perfusion culture (Tippe et al. 2002; Bitterle et al. 2006; Maier et al. 2008; Nandkumar et al. 2014).

Blood-gas barrier

A swim bladder assures that a fish can adjust its weight to the water pressure and in turn to float. Culture experiments with fish swim bladder gas gland were successfully performed by the application of a gradient perfusion container (Prem and Pelster 2000). In those experiments cells of gas gland were cultured on a filter at the interface between gas on one side and culture medium on the other side. The harvested epithelia showed a typical polarity and functionality as

it is known from the swim bladder gas gland in the living fish.

Testing new drugs

Orally administered drugs have to pass the epithelial barrier in the digestive tract before entering the interstitium within the organism. To test the transport of newly developed drugs across such an epithelial cell layer, long term gradient perfusion culture experiments were performed (Kloth et al. 1999, 2000). Experiments with Caco-2 cells in gradient perfusion culture demonstrated development of a tightly sealing epithelium cell layer. Further it was shown that in gradient perfusion culture reproducible results are achieved much earlier than observed in traditional 21 day static cultures. Also the permeability coefficient of several model medicines across a Caco-2 cell layer in gradient perfusion culture was approximately twofold higher than observed under static culture conditions (Masungi et al. 2004, 2009).

Renewal of epidermis/gingiva

The regeneration of epidermis and surgical repair of skin is an especially important subject in actual biomedicine. In order to evaluate a cost-effective engineering of full-thickness skin grafts and the treatment of ulcers, epidermis equivalents were investigated by the help of gradient perfusion culture (Kremer et al. 2001). In these experiments composite grafts of INTEGRA[®] matrix and human keratinocytes could be successfully generated in a gradient container. In a different context it was shown that development of a gingival epithelium (Lauer 2009; Hagedorn et al. 2009) or co-culture of keratinocytes and osteoblast-like cells in a perfusion container reveals much better results than obtained under static culture conditions (Glaum et al. 2010; Glaum and Wiedmann-Al-Ahmad 2013).

Regeneration of renal parenchyma

An increasing number of patients is suffering from acute and chronic kidney diseases. For this purpose the implantation of stem/progenitor cells and regeneration of damaged parenchyma are of special interest. Thus, to test developmental capacity renal, stem/progenitor cells were mounted between layers of a polyester

fleece to simulate an artificial interstitium during perfusion culture (Minuth and Schumacher 2003; Minuth et al. 2004a, b, 2005b). Scanning electron microscopy (Fig. 4a), label by fluorescent Soybean Agglutinin (SBA) (Fig. 4b) and semi-thin sections (Fig. 4c) illustrate the successful generation of renal tubules during 13 days in perfusion culture (Heber et al. 2007; Hu et al. 2007; Minuth et al. 2007a).

Perfusion culture experiments in combination with an artificial interstitium and chemically defined media further showed that application of different kinds of polyester fleeces results in various patterns of spatial tubule development (Roessger et al. 2009). A new finding was that formation of tubules can be induced by aldosterone, while antagonists such as spironolactone or canrenoate prevent development (Minuth et al. 2007b, 2008, 2010a; Minuth and Denk 2008). When the contact between the mineralocorticoid receptor (MR) and heat shock protein 90 is disturbed by geldanamycin, formation of intact tubules is reduced, while atypical features arise in form of cell clusters.

At that time it was a fully new aspect in biomedicine that a polyester fleece used as an artificial interstitium can be principally applied for the regeneration of renal parenchyma (Blattmann et al. 2008; Minuth et al. 2009b). All up to date performed experiments yet point out that development of tubules is triggered by interactions between their basal lamina, newly synthesized fibers of the extracellular matrix and fibers of the polyester fleece (Minuth et al. 2010a, b, c, d; Miess et al. 2010; Glashauser et al. 2011).

However, performed experiments dealing with regeneration of renal parenchyma also inform that intact development of renal tubules is not self-evident but can be paralleled by arise of abnormal cell and extracellular matrix features, as it was recently detected (Minuth and Denk 2012, 2014a, b).

Finally, by keeping slices of adult kidney within a polyester interstitium during perfusion culture it became possible to investigate splicing of the Na–K–2Cl cotransporter NKCC2 adapted to typical renal environment (Schießl et al. 2013).

Stabilizing survival after transplantation

Before an implantation is made, stem/progenitor cells are normally kept in the beneficial atmosphere of a CO₂ dependent culture medium. In contrast, when an implantation has been performed, they are exposed to

Fig. 4 Generation of renal tubules at the interface of a polyester interstitium after 13 days by perfusion culture. **a** Scanning electron microscopy demonstrates development of numerous renal tubules (T). **b** Fluorescent label for Soybean Agglutinin shows numerous tubules developing within an artificial interstitium. **c** Semithin section after Richardson staining shows generated tubules in oblique, respectively, vertical view between fibers of the polyester fleece (PF). Site of the basal lamina is marked by an *asterisk*, while lumen is indicated by an *arrow*

unbalanced interstitial fluid of diseased renal parenchyma. To investigate buffering of this harsh transition, renal stem/progenitor cells were exposed to conventional IMDM (Fig. 5a) in comparison to CO₂ Independent Medium (Fig. 5b) or Leibovitz's L-15 Medium (Fig. 5c) (Minuth et al. 2013; Minuth and Denk 2013). Analysis by transmission electron microscopy after fixation by conventional glutaraldehyde solution showed polar differentiation and typical

features of transporting tubule cells. Formation of an excess of vacuoles as an indicator for toxicity was not observed. In so far the results demonstrate that CO₂ Independent Media or Leibovitz's L-15 Medium reflect an advantageous fluid microenvironment for isolation, implantation and initial development of renal stem/progenitor cells.

Engineering of connective tissue

A broad research field in regenerative medicine is the interaction between cells derived from connective tissue and a selected scaffold used as a substitute for extracellular matrix. In those culture set ups a variety of biodegradable biomaterials is applied. Especially in these experiments perfusion culture helps to prevent an overshoot of harmful metabolites by continuous elimination and keeps in turn fluid environment on a constant level.

Connective tissue barrier

Regarding connective tissue research it is barely considered that it can exhibit essential barrier functions. Experiments related to such barriers were performed for example with dentin discs mounted in a MINUSHEET[®] gradient perfusion container during culture (Schmalz et al. 1996, 1999, 2001, 2002; Camps et al. 2002; Galler et al. 2005; Demirci et al. 2008; Vajrabhaya et al. 2009; Ulker and Sengun 2009; Sengün et al. 2011; Ülker et al. 2013a, b; Kim et al. 2013a, b). In this series of experiments it was shown that polymerized dental resin materials release residual monomers, which may interact with pulp tissue. In so far gradient perfusion culture appears to be an appropriate technique for exploring long term toxic effects under realistic in vitro conditions (Sengün et al. 2011; Korsuwannawong et al. 2012; Kim et al. 2013a, b; da Silva et al. 2014). A further innovative approach is tooth regeneration that was investigated by allogeneic stem cells (Wei et al. 2013).

In addition, new information about permeability and degradation of gelatine membranes seeded with fibroblasts on one side was obtained by culture in a gradient perfusion container (Dreesmann et al. 2008). In a similar culture set up a cell-type specific four-component hydrogel was evaluated for the generation of hyaline cartilage and vertebral disc repair (Aberle et al. 2014).

Fig. 5 Transmission electron microscopy demonstrates renal tubules generated at the interface of a polyester interstitium after 13 days of perfusion culture in **a** Iscove's Modified Dulbecco's Medium, **b** CO₂ Independent Medium and **c** Leibovitz's L-15 Medium. In all cases generated tubules exhibit a polarized epithelium. Neighboring cells are separated by a tight junction complex (arrow head). The basal lamina is indicated by an asterisk

Repair of hyaline cartilage

A challenge in tissue engineering is the treatment of cartilage defects by implantation of chondrocytes seeding within a biodegradable scaffold. The use of a MINUSHEET[®] tissue carrier has demonstrated that it can be a great help to investigate seeding of chondrocytes on selected scaffold materials. Moreover, it has been shown that use of a perfusion culture container improves cell biological quality of growing cartilage, when the medium is permanently renewed (Sittinger et al. 1994; Bujia et al. 1994, 1995; Sittinger et al. 1996, 1997). In those experiments basic data about the degradation process in various scaffold materials could be raised (Capitán Guarnizo et al. 2002). Knowing about exact kinetics of the degradation process a stepwise modification of scaffold materials became possible. As a result the risk of tissue repulsion after implantation was decreased by the application of those optimized scaffold materials (Rotter et al. 1998, 1999; Kreklau et al. 1999; Duda et al. 2000, 2004; Haisch et al. 2002; Gille et al. 2005). It was finally shown that electrospun polymer scaffolds have proven to be particularly advantageous (Schneider et al. 2011, 2012).

In this coherence it was also detected that application of natural extracellular matrix such as a collagen sponge does not improve the quality of generated cartilage (Fuss et al. 2000). In contrast, scaffold materials with modified polyethylene coating (Röpke et al. 2007) or a gelatine-based Spongostan[®] (Anders et al. 2009) revealed much more cartilage specific features than observed without surface treatment. In this context it was observed that synovial fibroblasts are able to adapt synthesis of extracellular matrix (Steinhagen et al. 2010). Finally, engineering of cartilage constructs by means of perfusion culture revealed to be an ideal model to investigate parameters affecting destructive joint diseases (Schultz et al. 1997; Risbud and Sittinger 2002; Bücheler and Haisch 2003).

Formation of bone

Not only for cartilage but also for bone engineering MINUSHEET® perfusion culture technique was successfully applied. For example, developmental capacity of osteoblasts and osteocytes was investigated with ceramic materials (Uemura et al. 2003; Wang et al. 2003; Leukers et al. 2005a; Yeatts and Fisher 2011; Bernhardt et al. 2011), decellularized spongy bone (Seitz et al. 2007), collagen membranes (Rothamel et al. 2004) and mineralized collagen (Gelinsky et al. 2004; Bernhardt et al. 2008). Further hydroxyapatite scaffolds (Leukers et al. 2005b; Detsch et al. 2008; da Silva et al. 2010a, b), poly-D,L-lactic-co-glycolic acid (PLGA) sheets (Shearer et al. 2006), iron based metals (Quadbeck et al. 2010), bioactive glass (Yue et al. 2011), textile chitosan (Heinemann et al. 2008, 2009, 2010), 3D biphasic calcium phosphate scaffolds (Rath et al. 2012) and other biocorrosible bone replacement materials (Farack et al. 2011) were successfully applied in combination with perfusion culture.

Special focus was directed to production of optimized scaffold materials to stimulate cell colonization and formation of extracellular matrix (Mateescu et al. 2012; Campos et al. 2013). An important observation for clinical application was that bone development can be influenced by the process of sterilization, when scaffold material is applied consisting of poly(D,L-lactic-co-glycolic acid (PLGA) (Shearer et al. 2006). A further aspect was to elaborate by perfusion culture why formation of a biofilm occurs on titanium surfaces (Astasov-Frauenhoffer et al. 2012).

Osteoblasts in combination with a scaffold often form thick layers of tissue. A recurrent problem is that unstirred and consequently harming layers of fluid within growing tissue develop. For compensation the continuous provision with nutrition and oxygen must be substituted by transport of medium in pulses or by feedback loops so that bone constructs with an acceptable cell biological quality can develop (Volkmann et al. 2008, 2012).

Development of muscular tissue

Normally a car is not suitable for all terrains. In analogy, only three papers were found dealing with the regeneration of muscular tissue in combination with

the MINUSHEET® perfusion culture system. In detail, when a layer of gastric mucosa was mounted in a tissue carrier and kept in a perfusion culture container, it was observed that not only gastric glands but also smooth muscular tissue are developing within the lamina propria (Kloth et al. 1998b). Further on, when formation of vessels in brain was investigated, the seeding of cerebral pericytes on selected biomaterials resulted in a high expression of site-specific pericytic aminopeptidase N/pAPN (Ramsauer et al. 1998). Finally, proliferation of smooth muscle cells was investigated on special electrospun polymer scaffolds (Rüder et al. 2012).

Generation of nervous tissue

A central problem in neurology research is the escape of dopamine synthesis during the course of Parkinson's disease. As a consequence, to investigate external influences on dopamine synthesis in mesencephalic neurons, MINUSHEET® perfusion culture was successfully performed (Blöchl and Sirrenberg 1996). For example, it was demonstrated that neurotrophins stimulate the release of dopamine via Trk and p75LntR receptors. Further it could be demonstrated by perfusion culture with hippocampal neurons and cells of the pheochromocytoma cell line PC 12 that admixture of exogenous neurotrophins has positive feedback effects on secretion of synthesized neurotrophins. This pathway seems to be triggered by an activation of tyrosine kinase neurotrophin receptors (Canossa et al. 1997). It was further shown that alterations in sodium concentration play an important role in secretion of neurotrophins (Hoener 2000).

Perfusion culture was also applied to investigate differences in secretion between nerve growth factor and brain-derived neurotrophic factor (Griesbeck et al. 1999). Further SH-SY5Y human neuroblastoma cells exhibited differentiation into a neuronal-like state, when long term perfusion culture was applied (Constantinescu et al. 2007). In those experiments the cultures were kept for more than 2 months in an active state. In other series of experiments RAT-1 fibroblasts were investigated expressing *Cypridina noctiluca* luciferase (CLuc) driven by the promoter of the circadian clock gene *Mma11* (Yamagishi et al. 2006). The experiments revealed that the CLuc reporter assay in combination with the applied perfusion culture is an

appropriate technique to test newly developed medications. Impressing results were obtained, when fish pituitary explants were kept in perfusion culture to investigate vasotocin and isotocin release (Kalamar-Kubiak et al. 2011).

Maintenance of retina

Retina has a complex neural cell microarchitecture that is delimited by a pigment epithelium. Previous experiments have shown that typical morphological features cannot be maintained when culture is performed in static environment of a dish. For that reason intact retina was mounted in a tissue carrier to incubate it in a gradient perfusion culture container (Framme et al. 2002; Spiegel et al. 2002; Saikia et al. 2006; Jian and Jingbo 2007; Hamilton et al. 2007; Hammer et al. 2008; Kobuch et al. 2008). Those experiments revealed that retina neurons and the pigment epithelium maintain a perfect morphology for a culture period of at least 10 days. These new findings illustrate new perspectives for safety testing of newly developed pharmaceuticals designed for intraocular application.

In addition, these experiments give rise to new opportunities for investigating the wide field of retina inflammation, aging, degeneration and repair by the help of an adequate culture system (Klettner and Roider 2009; Klettner et al. 2009, 2012; Miura et al. 2010; Treumer et al. 2012. In this coherence for example molecular regulation of vascular endothelial growth factor secretion and cell biological reactions after fucoidan application were investigated (Klettner et al. 2013, 2014; Dithmer et al. 2014).

Conclusion

To improve the environment for cells and developing tissues under in vitro conditions, the MINUSHEET® perfusion culture system was developed 25 years ago. To stay versatile, a biomaterial for optimal cell adhesion is selected, mounted in a tissue carrier and then transferred to a 24 well culture plate. Seeding of cells is performed in static environment, while generation of tissue is made in various types of perfusion culture containers. To prevent an overshoot of paracrine factors a continuous transport of always fresh culture medium is performed. In the meantime

numerous groups utilized the introduced system. A multitude of published papers illustrates that a variety of specialized tissues can be produced in an excellent cell biological quality urgently needed in tissue engineering, biomaterial research and advanced pharmaceutical drug testing.

Final remarks

In 1992 the project received the Philip Morris research award ‘Challenge of the Future’ in Munich/Germany.

To introduce developed tools on the market, Katharina Lorenz-Minuth founded non-profit-oriented Minucells and Minutissue Vertriebs GmbH (D-93077 Bad Abbach/Germany, www.minucells.com) by private sources.

Up to date more than 250 papers were published dealing with the MINUSHEET® perfusion culture system. A list of these different culture set ups is given in the data bank ‘Proceedings in perfusion culture’:

<http://www.uni-regensburg.de/Anatomie/Minuth/proceedings.htm>

For correct use of the MINUSHEET® perfusion culture system W.W. Minuth and L. Denk wrote a book entitled ‘Advanced Culture Experiments with Adherent Cells: From single cells to specialized tissues in perfusion culture’. Open access publishing, University of Regensburg, 2011, ISBN Nr. 978-3-88246-355-2, 417 pages. URN: ubn:de:bvb:355-epub-313392. This manuscript can be downloaded as PDF file without costs and further obligations:

<http://epub.uni-regensburg.de/31339/>

Data raised by the MINUSHEET® perfusion culture system were earlier reviewed:

Minuth WW, Denk L, Glashauser A (2010) A modular culture system for the generation of multiple specialized tissues. *Biomaterials* 31:2945–2954

Minuth WW, Denk L (2012) Supportive development of functional tissues for biomedical research using the MINUSHEET® perfusion system. *Clin Transl Med* 1:22

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Conflict of interest A series of patents (DE 10 2004 054 125, DE 39 23 279, DE 42 00 446, DE 42 08 805, DE 44 43 902, DE

19 530 556, DE 196 48 876 C2, DE 199 52 847 B4, US 5 190 878, US 5 316 945, US 5 665 599, J 2847669, DE 10 2005 002 938, PA 10 2005 001 747.9) demonstrate that Will W. Minuth is the inventor of the MINUSHEET® perfusion culture system. W. W. Minuth and L. Denk declare no competing interests or financial conflicts.

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