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Vascularised human tissue models: A new approach for the refinement of biomedical research

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ABSTRACT

Tissue engineering represents a biology driven approach by which bioartificial tissues are engineered through combining material technology and biotechnology. In order to ensure the functionality of *in vitro* cultured cells, culture conditions simulating the natural microenvironment must be created. Therefore (1) sufficient nutrient supply of the cells, (2) co-culture of different cell types, (3) suitable carrier structures (scaffolds) and (4) advanced bioreactor technologies are needed. Bioreactors constitute and maintain physiological tissue conditions at desired levels, enhance mass transport rates and expose cultured cells to specific stimuli. It has been shown that bioreactor technologies providing appropriate biochemical and physiological regulatory signals guide cell and tissue differentiation and influence tissue specific function of bioartificial three-dimensional (3D) tissues. In addition, to safeguard sufficient nutrient supply of complex 3D-bioartificial tissue models, we developed the biological vascularised scaffold (BioVaSc®). The BioVaSc is generated from a decellularized porcine small bowl segment with preserved tubular structures of the capillary network within the collagen matrix. It is the prerequisite for the generation of bioartificial tissues endued with a functional artificial vascular network and has been realized in artificial human liver-, intestine-, trachea- and skin-models. These various human tissue models represent a new technology as alternative to animal experiments for pharmacokinetic (drug penetration, distribution and metabolisation) and pharmacodynamic studies.

1. Introduction

Fast and economic model systems are required to accurately accomplish a careful analysis in the development of new products, including pharmaceuticals, chemicals, cosmetics and foods. These models must provide predictive data on the product actions on the target systems and functions. In general, the safety and effectiveness of new drugs tested in animal models before clinical trials are carried out. This practice has been questioned by the finding that less than 50% of animal tests accurately predict human response (Perel et al., 2007). Additionally, the study of Hackam and Redelmeier (2006) demonstrated a poor replication of even high-quality animal studies into human treatments. Hackam and Redelmeier (2006) lined out that 92% of new drugs fail in clinical trials, even following success in animal tests, as dramatically illustrated by the recent TGN1412 trial.

Already Russell and Burch (1959) postulated the concept of replacement, reduction and refinement of animal studies (3R principle). Since 1980 the German Federal Ministry of Education and Research (BMBF) supports research projects with the goal to reduce animal testing (Diekmann and Hansper, 2001). While replacement and reduction turn out to be problematic, refinement offers more improvement opportunities.

The applicability of the 3R principle is limited by numerous problems. First, most of the available *in vitro* models are applying cell lines of mammalian origin derived from tumours or have a transformed phenotype. The functional and structural features of these cells do not mirror the original tissue, resulting in an altered response to various endogenous and exogenous factors with respect to the real *in vivo* situation. Second, "non-transformed" mammalian-derived *in vitro* models merely consist of primary cell cultures or isolated tissue slices. The *in vitro* survival of such models is limited, thus time-course and dose-response studies are not possible. Third, cells in natural tissues are embedded in three-dimensional (3D) tissue architecture. Artificial two-dimensional cell culture conditions do not address their spatial organization, resulting in distorted cellular functions. For example, polarized cells of the parenchymal tissue, which normally require com-

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plex cellular interactions, may not behave physiologically when adhering to solid substrates, as in the case of conventional culture conditions (Penza et al., 2009).

Tissue engineering can overcome these obstacles. It represents a biology driven approach by which biological tissues are engineered through combining material technology, cell biology and biotechnology. Primary human cells are seeded on matrices that are fashioned from natural materials, or from synthetic polymers. The cell–matrix constructs are cultured *in vitro* to constitute a bioartificial tissue. During *in vitro* applications, bioartificial tissues models serve as test systems for pharmaceutical drug screening and patient specific therapy. Target screening requires testsystems that mimic the human tissues with increasing accuracy in order to optimize the selection of potential effectors. However, tissue engineering of complex 3D tissues and organs is limited by their need of nutrient supply to guaranty tissue survival and physiological tissue function (Walles et al., 2003). Therefore, we devised bioartificial human 3D tissues with an innate vascular and capillary network (Mertsching et al., 2005; Schultheiss et al., 2005). These tissues are based on decellularized porcine small bowl segments whose vascular structures within the cell-free collagen matrix are seeded with human microvascular endothelial cells (mEC). Experimentally, immuno-histochemical staining for vitality and specific endothelial markers (CD31, VE-Cadherin, Flk-1) matched functional findings by insulin dependent FDG uptake predominantly in the region of the former vascular structures. This bioartificial vascularised scaffold (BioVaSc®) was populated with additional primary human cells (hepatocytes (HC) or intestinal epithelial cells) to create *in vitro* vascularised human tissue models. In specially designed bioreactor systems (Mertsching and Hansmann, 2009) the co-cultures of primary human cells on the BioVaSc were maintained under physiological conditions. The complex culture conditions enhance cell adhesion and 3D growth resulting in more physiological tissue differentiation, morphology, metabolic activity and function.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma–Aldrich (München, Germany), Roth (Karlsruhe, Germany), Fluka (Taufkirchen, Germany) and Merck (Darmstadt, Germany). The applied antibodies were obtained from DakoCytomation (Hamburg, Germany). Cell medium was from Biochrom (Berlin, Germany). Fetal bovine serum (FBS) was from Gibco-BRL (Invitrogen, Karlsruhe, Germany). L-Glutamine, modified Eagle medium non-essential amino acids solution (Alpha-AS-MEM), trypsin and gentamycin were from Gibco-BRL (Invitrogen, Karlsruhe, Germany). The used pumps were MCP Standard pumps from Ismatec Laboratoriumstechnik GmbH (IDEX Corporation, Wertheim-Mondfeld, Germany).

2.2. Decellularization of the porcine scaffold

A porcine jejunal segment was obtained from a 3-month-old pig for scaffold generation. All animals received human care in compliance with the Guide for Care and use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996) after approval from our institutional animal protection board. Our experimental protocol to obtain a porcine jejunal segment has been published previously (Mertsching et al., 2005, 2009; Schultheiss et al., 2005). For chemical decellularization, we connected the arterial pedicle to a recirculating perfusion circuit of a bioreactor and perfused it for 75 min with 4% sodium desoxychylate monohydrate solution (Sigma–Aldrich, Hamburg, Germany) at a rate of 2.3 ml/min (Fig. 1A). To remove cell residues and sodium desoxychylate, we perfused the decellularized porcine jejunal segment for 75 min with phosphate buffered saline solution (Sigma–Aldrich, Hamburg, Germany) at a rate of 3.9 ml/min and a pressure of 80–100 mm Hg. Then, the decellularized porcine jejunal segment was incubated in 150 ml DNase I-solution (200 U/ml) (Roche, Penzberg, Germany) at room temperature over night. For removal of all chemical residues, the porcine scaffold was incubated 7 times for 4 h in 4 °C cold phosphate buffered saline solution. To effectuate tissue sterility, the scaffold was γ-irradiated with 25 kGy over night (BBF Sterilisationsservice GmbH, Rommelshausen, Germany).

2.3. Histological characterisation of the acellular porcine jejunal matrix

Segments of the acellular porcine jejunal matrix were incubated for 1 h in Bouin's reagent (formaldehyde & picric acid) (Sigma, Munich, Germany) and embedded in paraffin for histological and immuno-histochemical analysis. Human skin served as control. Hematoxylin and Eosin staining was done according to standard protocols. For immuno-histochemical characterisation, the tissue was stained using the EnVisionTM technique by the presence of CD 31 (MCA 1746, Serotec, Düsseldorf, Germany), Flk-1 (sc-6251, Santa Cruz, Heidelberg, Germany), Mdr-1 (AM391-5M, DCS, Darmstadt, Germany), Na–Ca-ATPase (sc-4569, Santa Cruz, Heidelberg, Germany), VE-cadherin (sc-6458, Santa Cruz, Heidelberg, Germany), VEGF (M 0616, Dako, Hamburg, Germany) and Zo-1 (M7044, Dako, Hamburg, Germany). Endogenous peroxidase was blocked with peroxidase blocking solution (Dako, Hamburg, Germany) and unspecific binding was blocked by the use of Antibody Diluent (Dako, Hamburg, Germany). For detection a EnVisonTM System + HRP (Dako, Hamburg, Germany) was used. The staining reaction was done with DAB and slides were counterstained with Hematoxylin (Sigma, Munich, Germany). The Feulgen-reaction was used as a qualitative marker to detect DNA-residues in the decellularized porcine tissue. For this, the tissue was processed with a commercial DNA-staining-kit according to the manufacturer's protocols (Merck, Darmstadt, Germany). For all stainings isotype control were done. Only stainings with negative isotype controls were analysed.

2.4. Characterisation of the acellular porcine jejunal matrix is detailed described in Mertsching et al. (2009)

The water content and dry weight of the native and the decellularized porcine tissue were determined ($n = 9$). For protein-content determination, tissue segments were dissolved in 0.5 M acetic acid (Merck, Darmstadt, Germany) at room temperature over night on a horizontal shaker (Greiner Bio-One, Kremsmünster, Austria) at 200 rpm. Thereafter, collagens, glycosaminoglycans (GAG) and proteoglycans and elastin were quantified using the SicrolTM Soluble Collagen, the Blyscan™ Sulfated Glycosaminoglycan, and the FastinTM Elastin assays (all Biocolor, Newtownabbey, UK), respectively. For DNA-quantification, a laboratory kit was used (DNeasy Blood & Tissue, Qiagen, Hilden, Germany).

2.5. Generation of the bioartificial vascularised scaffold (BioVaSc)

The vascular structures remaining within the decellularized jejunal segment were reseeded with human microvascular endothelial cells (mEC) using a specially designed recirculating perfusion set-up (Fig. 2). The cell preparation steps were published previously (Linke et al., 2007; Mertsching et al., 2009). Microvasculare ECs were cultured in endothelial cell basal medium (EBM)-2 supplemented with hydrocortisone (100 μg/500 ml cul-