In vitro human model for tumor target screening in Cancer Research

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ABSTRACT

Monolayer and suspension cultures have limited predictive value for target screening especially for nanomaterials. Target screening requires testsystems that mimic the human cancer with increasing accuracy in order to optimize the selection of potential effectors.
Tissue engineering is not only the generation of tissues it should also identify biological principles of cellular behaviour.
We developed a new 3D vascularised test system with a functional artificial vascular network embedded in a collagen matrix. This bioartificial vascularised matrix can be additional populated with tumor cells to create ex vivo vascularised tumor-like structures.
This model offers the possibility to simulate physiological drug application and a human 3D testsystem to established nanomaterials/systems for cancer research/therapy.

Keywords: Tissue Engineering, target screening, nanomaterials

INTRODUCTION

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. It offers the potential to create replacement structures from biodegradable scaffolds and autologous cells for reconstructive surgery. Applied in vitro, bioartificial human tissues allow the generation of functional 3-dimensional cell-systems mimicking the microenvironment of potential human target tissues which eventually better reflect the in vivo behaviour of tested cells than currently applied cell-based test systems. Their application in drug research may help to make drug development more efficient and reduce the research and development expenses. Additionally, they represent promising models for individualized oncologic therapy. Patient-specific therapeutic in vitro test systems can be established for specific tumor entities. Furthermore bioartificial 3-D human tumour tissues might be interesting models for basic research projects revealing new insight into mechanisms of organogenesis and expression of malignancy.

METHODS

Our system is based on decellularized porcine small bowl segments and preserved tubular structures of the capillary network within the collagen matrix which is functional associated with one small vein and artery (vascularised matrix).
A 10 to 15 cm long segment of jejunum was obtained from a donor animal (German landrace pig; age: 3 months; body weight 23 kg) under general anesthesia (trapanal/fentanyl infusion) via a median laparotomy. The donor was killed at the end of the operation by an anesthesia overdose. The animal 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 (Experiment #02-504). The intestinal lumen was flushed with 500 ml NaCl 0,9% at 4°C containing antibiotic solution (neomycin 3250 IE and bacitracin 250 IE), immediately after explantation. Then, the scaffold was decellularized after mechanical removal of the small bowl mucosa using a modified method of Meezan [1] and kept in cell-type specific medium at 37°C until reseeding. Modifications were as follows. For decellularization, the small bowel segment was incubated in 1% sodiumazid solution (2 hours at 4°C) under shaking conditions. To remove the cellular proteins, an incubation in 1m sodiumchlorid solution containing 2000 U DNase Type I (2 hours at 37°C) followed. To dissolve lipid-membran-proteins, the tissue was incubated twice in sodiumdeoxycholat/0,1% sodiumazid solution (5 hours at 4°C). Sterilization was guaranteed by 100 Gray γ-radiation.

This vascularised matrix enables the generation of a functional artificial vascular network. Therefore the vascular network is populated with endothelial cells.

For vascular endothelialization the scaffold was transferred into a specially designed circulating perfusion set up and its arterial pedicle was filled with 2 ml EBM-2 culture medium containing 5×106 trypsinized micovascular endothelial cells. Cells were allowed to attach to the scaffold matrix for 30 min. No iterative cell seeding were performed. A non-pulsatile medium perfusion rate of 0.8 ml/min was implemented using a roller pump (IPC, Ismatec, Glattburg, CH) and steadily increased for 72 hours to 1.5 ml/min . This increased rate was maintained unchanged throughout the entire experimental duration of 21 days [2]. Thereafter tumor cellines as for example A549 (lung tumor) or BLM (invasive growing skin tumor) are seeded on the collagenmatrix in Coculture with the endothelial cells.

Immunhistochemical staining for cell characterization was performed by use of the avidin-biotin-peroxidase technique. Endothelial cells were characterized by the expression of CD31, VE-Cadherin and Flk-1, A549 by the expression of EGFR, CD 90. A goat anti-mouse antibody served as a secondary antibody. Streptavidin-peroxidase conjugate was applied, and final staining was performed with diaminobenzidine.

The carboxyfluorescein diacetate succinimidyl ester (CFDA SE) is a non-fluorescent molecule, which passively diffuses into cells. Cleaved by intracellular esterases the succinimidyl ester reacts with intracellular amines forming fluorescent conjugates, which are retained throughout cell development, life or even cell division. 500µg of CFDA SE powder was solved in 90 µl of dimethylsulfoxide (DMSO) obtaining a 10 mM stock solution. For labelling we used a 10 µM solution in a total volume of 15 ml Phosphate Buffered Saline (PBS) w/o magnesia (Mg) and calcium (Ca), 5mM EDTA. The freshly isolated endothelial cell solution was centrifuged (600 rpm, 10 minutes), the supernatant was removed and the pellet was resuspended in the prewarmed labelling solution. Cells were incubated at 5% CO2, 37°C for 15 minutes. The uptake of CFDA SE into the cells was stopped by 250 µl fetal bovine serum (FBS) followed by an additional incubation for 30 minutes. Following centrifugation (600 rpm, 5 min), the supernatant was removed and the pellet resuspended in 15 ml prewarmed PBS w/o Mg and Ca, 5 mM EDTA, 0,1% bovine serum albumine (BSA) . This procedure was repeated twice. During the second cycle the cell pellet was resuspended in supplemented EBM2 medium. Cellular labeling was investigated in a cell culture microscope using a standard fluorescent filter at 492 and 517 nm.

Positron emission tomography (PET). Endothelialized BioVaM matrices were positioned within 20 cm from the center of a high resolution dedicated PET fullring scanner (HR+, Siemens, Erlangen, Germany / CPS, Tennessee, Knoxville) and perfused with prewarmed culture medium at 30°C. 40±7.2 MBq of radiolabeled 2-[18F]-fluoro-2`-desoxy-glucose (FDG) were injected into the arterial pedicle. “Washing in” and “washing out” of the medium was allowed for 90 min each and transmission and emission scans were performed after each washing step over 10 min. Thereafter, the cell culture medium was replaced by glucose free PBS containing 1 IU/ml Insulin and 40±8.5 MBq of FDG and the PET procedure was repeated. Acellular matrices served as controls (n=3). After correction for physical decay, attenuation and scatter as measured by a delayed coincidence channel, the data sets were reconstructed by means of iterative algorithm using a 256 matrix, with 2.0 mm Gaussian pre-reconstruction filtering using 6 iterations and 16 subsets. After triple washing by PBS rinsing at 25ºC (identical for the negative and positive controls), the scaffolds were thin layer scanned. The resulting PET-images and thin layer-scans were evaluated by means of regions of interest (ROI) technique.

RESULTS AND DISCUSSION

To obtain a biological scaffold with a preserved feeding artery, a draining vein, and a functional capillary network for graft supply, we developed a special harvesting procedure in animals. Our decellularization procedure left behind a dense layer of cross linked collagen and elastin fibers evolved from the stratum compactum, the small intestinal submucosa (SIS), the tunica serosa, and the tunica muscularis externa with its arterial and venous network. HE staining was used to survey the cellular state of each processed matrix. Our decellularization process resulted in remaining acellular tubular network of 20 to 200 µm diameter (Fig. 1A, B) connected to the venous and arterial pedicle, respectively, with a basal membrane and elastica interna [3]. Semi quantitative DNA detection was applied as molecular marker for scaffold decellularization. Its results confirmed our histological findings. Our scaffold thickness was 0.2±0.01 mm. First clinical experience shows, that the BioVaM is well tolerated and supports an extensive tissue maturation process following implantation [4]. Reendothelialization of vascular structures. The reseeding of the remaining tubular structures of the vascular network within our biological scaffold with endothelial cells was the main objective of this study. Numerous cell types and growth factors have been suggested for endothelial reseeding of variable matrices or scaffolds [5]. We differentiated allogenic porcine bmEPC that were isolated from bone marrow aspirates on our acellular biological matrix. Also endothelial cells are easy to obtain, isolate, and culture from vascular biopsies, they tend to dedifferentiate resulting in a dysfunctional endothelial lining [6]. Additionally microvascular endothelial cells were isolated and tested for seeding in the vascular structures. Recently, stem and bone marrow derived progenitor cells were successfully

cultured and induced to differentiate into endothelial cells [7-9]. To evaluate, whether the seeded bmEPCs differentiated into endothelial cells, we evaluated the tissue samples for the expression of endothelial specific markers CD31, VE-Cadherin and Flk-1 by immunohistochemistry and western blot analysis. Anyway bmEPCs and microvascular endothelial cells generate an active antithrombotic surface that facilitates transit of plasma and cellular constituents. For proper function of the endothelium, integrity is required, i.e. mainly ensured by endothelial cell-to-cell junctions. A functional vascular endothelial lining is of paramount importance to avoid graft thrombosis and failure [6].

Three weeks after endovascular seeding and permanent perfusion, the luminal surface of the supplying artery and vein is lined with a cellular monolayer staining positive for CD31, which mediates adhesion between cells that express CD31. Its expression is limited to endothelial cells, platelets, leukocytes, and their precursors and it has been shown to be highly specific and sensitive for vascular endothelial cells. Within the scaffold matrix, CD31 positive structures are unequally distributed. No positive reaction was detected in acellular controls, excluding non-specific antibody-reactions with the matrix and remaining porcine endothelial cells in the scaffold.

Analogical findings were obtained for VE-Cadherin and Flk-1. VE-Cadherin is an endothelial-specific trans-membrane protein that promotes hemophilic cell adhesion mechanically connecting endothelial cells providing the structural base for interendothelial mechanical stability. It plays a morphogenic role in vascular development by participating in contact inhibition of VEGF signaling and its expression is required for the normal organization of the vasculature in the embryo [10]. Flk-1 is a high-affinity VEGF-2 receptor and is found on differentiated endothelial progenitor cells. It produces a positive endothelial proliferation signal during developmental blood vessel formation.

Western blot analysis confirmed the immunhistological findings. The simultaneous expression of 3 specific endothelial markers on our reseeded biological scaffold indicates the successful EC seeding of the vascular structures. Vitality of vascularized scaffoldwas addred by FDG PET using a dedicated fullring scanner facilitating general graft survey with no need for tissue dissection. As viable cells take up 2`-[18F]-fluoro-2`-desoxy-glucose (FDG), localized vitality can be in principle visualized by positron emission tomography (PET). To increase the resolution of detected activity distribution, additional thin layer scanning was performed. PET images showed 3D activity accumulation within the proximity of the reseeded arterial pedicle. Only radioactive background was seen in acellular controls. PET imaging allows quantification of the PET signal. Maximum uptake ratios for the reseeded biological matrix (perfused with buffer-medium) was 11±0.7 vs. 2±0.9 for the non-seeded matrix. In addition to this, PET scanning revealed the functional integrity of the endothelial vascular lining, thereby supporting our immunhistochemical morphological findings. Cellular FDG uptake can be stimulated by insulin exposure and we applied this principle as an additional test for functional cellular vitality. Insulin stimulation amplified the FDG PET uptake in reseeded matrices by a factor of 4.2±1.1. No increase was detectable in acellular controls. These findings amplify our previous findings that the reseeded matrices are populated with viable EC.

The approaches presented here enable the generation of a functional artificial vascular network in a porcine scaffold (Fig. 1A-D). The generated biological vascularized scaffold may serve as a universal scaffold for tissue engineering .

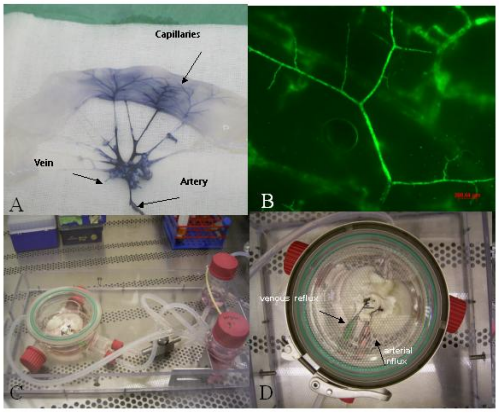

Figure 1: A) vascularized matrix with stained capillary network B) seeded capillaries of the matrix with fluorescence marked liver endothelial cells, C) Liver module with vascularized matrix and pressure sensor, D) 3D human vascularised testsystem with arterial and venous access

Pharmaceutical research is hampered by limited predictive value of routinely applied in vitro and in vivo drug screening models for clinical efficacy. In drug development, the common approach of pharmaceutical industry is to screen small-molecule librabries for function and toxicity in biochemical based or ligand binding high throughput essays [11]. In general enzymes and 2-dimensional cell lines are used in those cell-based assays. The obtained results are of limited biological relevance, since the 2-dimensional cell systems do not adequately mimick the 3-dimensional environment in healthy and tumour tissues [12].

Our bioartificial vascularised matrix can be additional populated with tumour cells to create ex vivo vascularised tumour-like structures (Fig. 1 C,

D). The system offers the option to administer the substances as well as nanomaterials in arterial inflow and the influence of the tumor like tissue macroscopically, on cellular level and molecular (Fig. 1 C, D). Human derived tumor cells are co-cultivated with the endothelial cells in such a way, that they can form a physiological filtration barrier. The medium is conducted over capillaries seeded with endothelial cells to the tumor cells. Anti - angiogenic therapy molecules as antibodies are now tested in the system.

This model offers the possibility to simulate physiological drug application and a human 3D testsystem to established nanomaterials/systems for cancer research/therapy.

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