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Human adipose tissue-derived stromal cells in combination with exogenous stimuli facilitate three-dimensional network formation of human endothelial cells derived from various sources

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ABSTRACT

In natural tissues, the nutrition of cells and removal of waste products is facilitated by a dense capillary network which is generated during development. This perfusion system is also indispensable for tissue formation *in vitro*. Nutrition depending solely on diffusion is not sufficient to generate tissues of clinically relevant dimensions, which is a core aim in tissue engineering research.

In this study, the establishment of a vascular network was investigated in a self-assembling approach employing endothelial and mural cells. The process of vascularization was analyzed in constructs based on a carrier matrix of decellularized porcine small intestinal submucosa (SIS). A three-dimensional hydrogel containing Matrigel™, collagen, and respective cells was casted on top of the SIS. Various types of human endothelial cells (hECs), *e.g.* HUVECs, cardiac tissue ECs (hCECs), pulmonary artery ECs (hPAECs), and iPSC-derived ECs, were co-cultured with human adipose tissue-derived stromal cells (hASCs) within the hydrogel. Analyzed hECs were able to self-assemble and form three-dimensional networks harboring small caliber lumens within the hydrogel constructs in the presence of hASCs as supporting cells. Additionally, microvessel assembling required exogenous growth factor supplementation.

This study demonstrates the development of stable vascularized hydrogels applying hASCs as mural cells in combination with various types of hECs, paving the way for the generation of clinically applicable tissue engineered constructs.

1. Introduction

Tissue engineered 3D constructs are mostly cultivated under static conditions. The nourishment of the cells within these constructs depends solely on diffusion. Diffusion across long distances is a slow process, as the time required for diffusion increases in proportion to the square of the distance, for example, one glucose molecule takes 0.5 milliseconds to diffuse through a 1 μm thick capillary wall, but requires 15 h for diffusion through a 1 cm thick myocardial wall [1]. Therefore, prevascularization and perfusion approaches are required to engineer tissues in clinically relevant dimensions. Prevascularization strategies can be classified into four major categories. (1) Bioprinting allows the placement of cell aggregates or matrices containing cells at defined locations with a high level of spatial control and with good control over the initial organization of the vascular network and the lumen diameter [2], but with a limited resolution (20–100 μm) and cell viability

Abbreviations: EBM, endothelial basal medium; EC, endothelial cell; EGM-2, endothelial growth medium; FACS, Fluorescence-activated cell sorting; FCS, fetal calf serum; GFP, green fluorescent protein; hASC, human adipose tissue derived stromal cell; hCEC, human cardiac endothelial cell; hPAEC, human pulmonary artery endothelial cell; HUVEC, human umbilical vein endothelial cell; iCell-EC, iPSC derived endothelial cells from Cellular Dynamics; iPSC-EC, iPSC derived endothelial cells; MACS, magnetic cell isolation and cell separation; PBS, phosphate buffered saline; SIS, small intestinal submucosa

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(40–80%) [3]. (2) With microfabrication, a resolution less than 10 µm can be achieved by applying lithographic masks [4]. Subsequently, the patterned channels are reseeded with endothelial cells. Microfabrication often results in rectangular shapes instead of curved arrangements found in nature [5]. (3) *In vivo* prevascularization uses the body as a natural bioreactor to generate functional microvessels within artificially built tissues. The disadvantages of this approach are the need for surgical intervention and the duration required for vessel ingrowth, which is slow and hence not suitable for thick and metabolically highly active tissues [6]. (4) The most common approach represents the self-assembling of endothelial cells (ECs) and mural cells into vascular networks. Therefore, ECs and mural cells are incorporated into synthetic or natural 3D hydrogels and, if cultured under appropriate conditions, rapid self-assembling into immature microvessels can be observed [6]. Obstacles to establishing a functional perfusion are the capillary-like dimensions of the assembled microvessels, as well as the lack of anastomosis sites. Additionally, the choice of the mural cell source is a critical step. In this study, we apply hASCs as mural cell as these cells are relatively easy to obtain, have a high proliferation capacity and have been shown to be effective in therapeutic angiogenesis for the treatment of ischemic diseases in animal models as well as in several clinical trials (reviewed in [7]). For example, freshly isolated, autologous hASC were injected transendocardially into hearts of no-option patients suffering from ischemic cardiomyopathy resulting in preserved ventricular function, myocardial perfusion, and exercise capacity (PRECISE trial [8]). The feasibility and safety of such treatments was also demonstrated in a phase II trial with bone marrow derived allogeneic mesenchymal precursor cells [9]. The therapeutic effect in these studies may be attributed rather to paracrine effects than differentiation of engrafted stem cells.

In this study, we investigate the self-assembly of HUVECs and other human ECs in combination with hASCs as mural cells. We study the dynamics of the process as well as external influences on endothelial cell network formation in a 3D hydrogel casted onto decellularized small intestinal submucosa (SIS) as a carrier matrix.

2. Material and methods

2.1. Ethics statement

Patient material was processed following approval of the Ethics Committee at Hannover Medical School and after obtaining written informed consent from the patients. All tissues were used anonymously for this study. The generation and application of patient-derived induced pluripotent stem cells was approved by the Ethics Committee at Hannover Medical School.

2.2. Animal care

This study was approved by the Institutional Review Board and the local Animal Protection Committee, and was conducted according to local government regulations (#10/0214; #11/0458) and Committee protocols of Hannover Medical School and the Research Advisory Committee. All animals received humane care in compliance with the European Convention on Animal Care.

2.3. Preparation of small intestinal submucosa (SIS)

The preparation of decellularized small intestinal submucosa (SIS) was performed as previously described [10,11]. In brief, porcine small intestinal segments were isolated from German landrace pigs (18–25 kg) and stored in undiluted Braunol (7.5% povidone-iodine solution in water, B. Braun) at 4 °C. *Tunica mucosa* and *tunica serosa* of intestinal segments were mechanically removed, followed by a chemical decellularization in 1% Triton X-100 in 10 mM TRIS, pH 7.5 under continuous shaking (90 rpm) at room temperature for 24 h. Afterwards, SIS was washed with Ampuwa water for 24 h under continuous shaking, followed by washing with phosphate buffered saline (PBS) supplemented with 1 g/L Vancomycin, 100 mg/L Gentamicin, and 2.5 mg/L Amphotericin B under continuous shaking for 10 days at room temperature (daily change). Finally, SIS was sterilized by 150 Gy gamma-ray irradiation and stored in PBS at 4 °C until further use for a maximum of 6 months. Before use, SIS was cut open along the longitudinal axis, fixed in a metal frame with the submucosal side facing up and covered with culture medium.

2.4. Human umbilical vein endothelial cells (HUVECs)

HUVECs obtained from mixed donors were purchased from Lonza. Cells were cultured in Endothelial Growth Medium (EGM-2: EBM-2 with supplements, Lonza). Passages 4–6 were used for all experiments.

2.5. Isolation of adult human cardiac endothelial cells (hCECs)

hCECs were isolated from atrial appendages. Surrounding fat tissue was removed from the atrial appendage, the cardiac tissue was mechanically and subsequently enzymatically dissociated using the Neonatal Rat Heart Dissociating Kit (Miltenyi) according to manufacturer's instructions. Dissociated single cells were cultured in EGM-2. For enrichment of hCECs, the human CD31 Microbead Kit (Miltenyi Biotec) was used according to manufacturer's instructions and cultivated in EGM-2. Cells were used from passages 4 to 6.

2.6. Isolation of adult human pulmonary artery endothelial cells (hPAECs)

Small pieces of pulmonary artery were treated with 0.2% collagenase type II (380 U/mg, Worthington) solution. Samples were incubated for 10 min at 37 °C under shaking. Dissociated cells were collected by centrifugation of the supernatant at 300 rcf for 5 min after terminating the enzymatic digestion with DMEM containing 20% FCS. Cells were re-suspended in EGM-2 supplemented with 10% FCS and plated onto a fibronectin-coated 12-well plate. For purification, cells were subjected to MACS using the human CD31 Microbead Kit according to manufacturer's instructions. CD31 positive cells were seeded into a fibronectin-coated 6-well plate and cultured in EGM-2 containing 10% FBS. Cells were used from passages 5 to 7 for the 3D hydrogel construct generation.

2.7. Induced pluripotent stem cell derived endothelial cells

These cells were either purchased from Cellular Dynamics International (iCell-ECs) or generated in-house. For the differentiation of ECs from hiPSCs, an in-house generated hiPSC line (hCBiPS2_AAVS1eGFPC18) carrying an enhanced green fluorescent protein (GFP) under the control of a CAG promoter was used [12] [13]. hiPSCs were differentiated towards an endothelial cell lineage according to a protocol published by Patsch and colleagues [14]. Differentiated cells were purified by the application of the human CD31 Microbead Kit according to manufacturer's instructions and subsequently cultured in EGM-2 in fibronectin-coated cell culture flasks.

iCell-ECs were cultured on fibronectin-coated cell culture flasks in iCell endothelial maintenance medium as recommended by the company. Cells from passages 3 to 6 were used for the experiment.

2.8. Isolation of adult human adipose tissue derived stromal cells (hASCs)

Human adult adipose tissue-derived stromal cells (hASCs) were isolated from human tissues that were obtained from patients undergoing abdominoplasty. Isolation was performed according to a previously published protocol [15] with minor modifications. In brief, fat and connective tissue were minced, followed by addition of 10 mL Collagenase type II solution (760 U/mL, Worthington) per 20 mL of