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Generation of human umbilical cord vein CD146+ perivascular cell origined three-dimensional vascular construct

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ABSTRACT

Small-diameter vascular grafts are needed for the treatment of coronary artery diseases in the case of limited accessibility of the autologous vessels. Synthetic scaffolds have many disadvantages so in recent years vascular constructs (VCs) made from cellularized natural scaffolds was seen to be very promising but number of studies comprising this area is very limited. In our study, our aim is to generate fully natural triple-layered VC that constitutes all the layers of blood vessel with vascular cells. CD146+ perivascular cells (PCs) were isolated from human umbilical cord vein (HUCV) and differentiated into smooth muscle cells (SMCs) and fibroblasts. They were then combined with collagen type I/elastin/dermatan sulfate and collagen type I/fibrin to form tunica media and tunica adventitia respectively. HUCV endothelial cells (ECs) were seeded on the construct by cell sheet engineering method after fibronectin and heparin coating. Characterization of the VC was performed by immunolabeling, histochemical staining and electron microscopy (SEM and TEM). Differentiated cells were identified by means of immunofluorescent (IF) labeling. SEM and TEM analysis of VCs revealed the presence of three histologic tunicae. Collagen and elastic fibers were observed within the ECM by histochemical staining. The vascular endothelial growth factor receptor expressing ECs in tunica intima; α-SMA expressing SMCs in tunica media and; the tenascin expressing fibroblasts in tunica adventitia were detected by IF labeling. In conclusion, by combining natural scaffolds and vascular cells differentiated from CD146+ PCs, VCs can be generated layer by layer. This study will provide a preliminary blood vessel model for generation of fully natural small-diameter vascular grafts.

1. Introduction

Cardiovascular diseases are leading cause of death or deterioration of the quality of life in worldwide (World Health Organization, 2017). Coronary artery disease constitutes the most common type of cardiovascular diseases and is the leading cause of mortality (Mozaffarian et al., 2015; Wilkins et al., 2017). Today, autologous vessels or prosthetic grafts are used during coronary bypass surgery. Although the autologous vessels are the gold standard for the treatment, they have limited accessibility because of the disease progression and the age of the patient (Bajpai and Andreadis, 2012). On the other hand, clinical use of small-diameter synthetic vascular grafts is inadequate because of the inflammatory response (Grandi et al., 2011), neointimal hyperplasia (Zilla et al., 2007), and thrombosis (Yahagi et al., 2016) resulted in the delay of healing the damaged areas (Pashneh-Tala et al., 2015). Limitations of autologous and synthetic grafts create an urgent need of generating small diameter vascular grafts, completely composed of natural materials, for clinical use.

Vascular tissue engineering approaches aim to mimic vascular layers using natural or synthetic materials with vascular cells (Pashneh-Tala et al., 2015). The small-diameter vascular grafts were first generated by Weinberg and Bell (1986), using purified extracellular matrix (ECM) proteins, vascular smooth muscle cells (SMCs), fibroblasts and, the endothelial cells (ECs) that were embedded in collagen gel. Similarly, Loy et al. (2016), cultured vascular cells in collagen gel (tunica media and adventitia-like layers) and EC monolayer was formed to mimic the intima. Moreover, fibrin was used in gel

Abbreviations: VCs, vascular constructs; HUCV, human umbilical cord vein; SMCs, smooth muscle cells; ECs, endothelial cells; SEM, scanning electron microscopy; TEM, transmission electron microscopy; IF, immunofluorescent; ECM, extracellular matrix; α-SMA, alpha-smooth muscle actin; UC, umbilical cord; PNIPAAm, poly *N*-isopropylacrylamide; MSCs, mesenchymal stem cells; PCs, perivascular cells; HUVEC, human umbilical cord vein endothelial cells; 3D, three dimensional; PGM, Pericyte Growth Medium; MACS, magnetic activated cell sorting; RT, room temperature; FACS, fluorencent activated cell sorting; PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophycocyanin; DMEM-LG, Dulbecco's modified Eagle's medium – Low glucose; CTGF, Connective Tissue Growth Factor; L-AA, L-ascorbic acid; FBS, fetal bovine serum; Pen-Strep, penicillin-streptomycin; WST-1, Water Soluble Tetrazolium-1; EGM, endothelial growth medium; FM, fibroblast medium; VEGF, vascular endothelial growth factor; vWF, von Willebrand Factor

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form as an alternative to collagen type I to increase elastin and collagen synthesis (Grassl et al., 2003; Gui et al., 2014). Buijtenhuijs et al. (2004) used a 3D tubular scaffold with insoluble elastin and collagen, in which umbilical cord (UC)-derived SMCs proliferate and protect their viability. Researchers have tried to coat the basement membrane with proteins like collagen type IV (Coelho et al., 2010), fibronectin (De Visscher et al., 2012) and heparin (Hoshi et al., 2013; Zhou et al., 2009) in order to facilitate the endothelialisation. Cell sheet engineering method that was introduced by Okano et al. (Owaki et al., 2014), generating cell sheets using smart polymer (poly *N*-isopropylacrylamide; PNIPAAm) coated thermoresponsive surface; has been used for vascular graft production with SMCs and mesenchymal stem cells (MSCs) recently (Backman et al., 2017; Sekiya et al., 2010; Williams et al., 2012). We assume this method can be used as an alternative to coating techniques for endothelialisation.

The CD146+ perivascular cells (or pericytes, PCs) located at the periphery of the vessel walls (Crisan et al., 2008; Nees et al., 2013) are involved in vascular development, maturation, stability, and regulation of blood flow and blood pressure (Kutcher and Herman, 2009; Mendes et al., 2012; Stefanska et al., 2013). Their interaction with the ECs by special junctions and paracrine signalling provides proliferation of ECs (Armulik et al., 2011) and efficient endothelialisation of vascular grafts (Avolio et al., 2017). The PCs have MSC-like properties thus they are good alternative cell sources to terminally differentiated cells for vascular tissue engineering approaches (Crisan et al., 2012; Gokcinar-Yagci et al., 2015). Perivascular cells are isolated from several vascularized tissues and organs like bovine retina, brain, skeletal muscle, adipose tissue, bone marrow, kidney, liver and fetal tissues (placenta and UC) (Gokcinar-Yagci et al., 2015). Among all these sources, human UC is the most favorable source of perivascular cells because it is non-invasive and ethical for being a medical waste (Kajiyama et al., 2015). Human UC perivascular cells expressing SMC markers (Chong et al., 2009), human embryonic stem cell derived pericytes (van der Meer et al., 2013) and, human skeletal muscle derived CD 146+ pericytes (He et al., 2010) have been combined with synthetic or natural scaffolds in a limited number of vascular tissue engineering studies and gave promising results (Chong et al., 2009; He et al., 2010; van der Meer et al., 2013). The human umbilical cord vein (HUCV) CD146+ PC derived vascular cells have not been studied for vascular tissue constructs yet. We suggest that CD 146+ PCs may be good candidates for generating three layered small diameter vascular constructs when combined with human collagen type I, fibrin, elastin, dermatan sulfate, heparin and fibronectin constituting the human natural vascular components.

In our previous study, we gave positive answer to the hypothesis that the CD146+ PCs can differentiate to mesodermal origined SMCs of tunica media (Gokcinar-Yagci and Celebi-Saltik, 2017). Here, we hypothesized that CD146+ PCs can give rise to fibroblasts, that are the main cells of the adventitia by culturing with connective tissue growth factor supplemented differentiation medium. Our second hypothesis is that the vascular tunicae (tunica media and tunica adventitia) can be constructed by combining human collagen type I, elastin, fibrin and dermatan sulfate as natural scaffolds; with SMCs and fibroblasts, differentiated from human CD146+ PCs. We also hypothesized that tunica intima can be generated with HUVEC monolayer obtained with cell sheet engineering method and transferred on human heparin and fibronectin coated tunica media surface. In this study, our objective is to put the three tissue-engineered vascular layers together and culture *in vitro* to generate an intact three dimensional (3D) vascular construct.

2. Materials and methods

2.1. Isolation and characterization of CD146+ perivascular cells

Human UC samples were obtained immediately after delivery from non-complicated pregnant women in Hacettepe University Faculty of Medicine, Department of Obstetrics and Gynecology (n = 3). Hacettepe University Local Non-Interventional Clinical Researches Ethics Committee approved human material use (GO 13/417-27).

CD146+ PCs were isolated from human UC samples according to our previously described protocol (Gokcinar-Yagci et al., 2016). UC samples were cut into 3–4 cm pieces in length and Wharton's jelly and arteries were removed with sterile forceps. Separated cord vein pieces were sutured at both ends and were incubated in collagenase solution (1 mg/mL, Sigma, USA) at 37 °C for 16–18 h. After washing and centrifugation steps, isolated cells were cultured in Pericyte Growth Medium (PGM, Promocell, Germany) at 37 °C in a 5% CO_2 incubator. Cells that reached 75–80% confluence were trpsinized with a TrypLE (Gibco, USA) solution. CD146+ cells were sorted by magnetic activated cell sorting (MACS) technique using CD146 microbead Kit (Miltenyi Biotec, Bisley, UK) and cultured in PGM. At 80–85% confluence, adherent cells were trypsinized with TrypLE solution and cell viability was checked by trypan blue staining.

Passage three CD146+ PCs were characterized according to their multilineage differentiation capacity requested for MSC characterization and specific marker expression. First, their adipogenic and osteogenic differentiation capacity were evaluated by incubating with adipogenic and osteogenic differentiation medium for 21 days at 37 °C in a 5% CO_2 incubator (Celebi et al., 2010). Differentiation media was refreshed in every three days, osteogenic and adipogenic differentiation capacities were evaluated at 21st day with Alizarin red (Sigma, USA) and Oil red-O (Sigma, USA) staining respectively. Cells cultured in PGM for 21 days were used as control group for differentiation analysis.

Surface markers of passage three CD146+ PCs were analyzed by flow cytometry using our previously described protocol (Gokcinar-Yagci et al., 2016). In brief, the cells (2–5 × 10^5 cells/mL) were suspended in fluorescent activated cell sorting (FACS) buffer and centrifuged at 200*g* for five minutes (min). Then they were incubated for 20 min at room temperature (RT) in the dark with the combination of following fluorescent-conjugated mouse anti-human antibodies: CD146-phycoerythrin (PE), CD73-fluorescein isothiocyanate (FITC), and CD105-allophycocyanin (APC). IgG1-PE, IgG1-FITC and IgG1-APC were the isotype controls. All antibodies were obtained from Becton Dickinson Pharmingen (Mississauga, ON, Canada). After three times of washing steps with FACS buffer, cells were analyzed with FACS Aria using FACS Diva Analysis Software v6.1.2 (BD Biosciences, USA).

2.2. Smooth muscle cell and fibroblast differentiation of CD146+ perivascular cells

CD146+ PCs (n = 3, passage three) were seeded in culture plates as 5 × 10^3 cells/cm^2 and cultured with PGM until confluence. SMC differentiation was induced by culturing the cells for seven days in SMC differentiation medium according to our previously described protocol (Gokcinar-Yagci and Celebi-Saltik, 2017). Medium changes were performed two times weekly.

Fibroblast differentiation of CD146+ PCs was performed with a differentiation medium (Tong et al., 2011). CD146+ PCs (n = 3, passage three) were seeded in culture plates at 5 × 10^3 cells/cm^2 and cultured with PGM until confluence. Fibroblast differentiation was induced by culturing the cells for 21 days in differentiation medium [Dulbecco's modified Eagle's medium – Low glucose (DMEM-LG), 100 ng/mL Connective Tissue Growth Factor (CTGF, Sigma Aldrich, USA), 50 μg/mL L-ascorbic acid (L-AA), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Pen-Strep) at 37 °C in a 5% CO_2 incubator. Medium changes were performed two times weekly.

Fibroblast differentiation of CD146+ PCs was characterized phenotypically by immunofluorescent (IF) labeling with primary mouse anti-human antibodies: 1/60 dilution of anti-tenascin-C (Abcam, UK) and 1/60 dilution of anti-collagen type I (Abcam, UK). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to analyze expression of fibroblast-specific markers (tenascin-C and collagen type I) by incubation of CD146+ PCs with fibroblast differentiation medium for 21 days (n = 3). PCs cultured with PGM (n = 3) were used as negative control, foreskin fibroblasts (ATCC, USA) cultured with fibroblast growth medium (FM, Promocell, Germany) were used as positive control cells. “EZ-10 Spin Column Total RNA Mini-Preps Kit” (Bio Basic Inc., Canada) was used to isolate cellular RNA. Concentration of the RNA was evaluated with NanoDrop ND-1000 spectrophotometer (Thermo Ficher Scientific Inc., USA) at 260 nm. The quality of the extraction