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Expression profiles of angiogenesis-related proteins in prevascular three-dimensional tissues using cell-sheet engineering

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ABSTRACT

The prefabrication of endothelial cell network assembly (ECNA) in tissue-engineer multi-layered cell-sheets, known as *in vitro* prevascularization, is beneficial strategy for inducing anastomosis with the host vasculature after transplantation. However, the mechanisms of neovascularization via transplanted prevascular cell-sheets are unknown. This study investigated neovascularization process and angiogenesis-related protein secretion by prevascular cell-sheets. Prevascular (ECNA-positive) double-layered fibroblast (FB) sheets were created by sandwiching human aortic endothelial cells (HAECs) between two human dermal FB sheets. As the ECNA-negative control, FBs-sandwiching double-layered FB sheets were used. Two types of cell-sheets were subcutaneously transplanted into immune-deficient rats. At 3 days after transplantation, induction of the newly-formed microvessels near the host vasculature was observed in the ECNA-positive cell-sheet. In contrast, no neovessel was observed in the ECNA-negative cell-sheet at 1 week after transplantation. Consequently, the secretion of angiogenesis-related proteins in conditioned media of each cell-sheet cultured for 3 days were compared. The levels of hepatocyte growth factor (HGF), placenta growth factor (PlGF) and matrix metalloproteinase-9 (MMP-9) significantly increased in the ECNA-positive cell-sheets. These results suggested that these molecules might involve in neovascularization after the transplantation of prevascular cell-sheets. These findings may contribute to understanding its mechanism.

1. Introduction

Cell-sheet technology is a tissue engineering methodology without the use of biodegradable scaffolds [1–4]. Cell-sheets are harvested from confluently cultured cells on temperature-responsive culture dishes covalently grafted with a temperature-responsive polymer, poly (*N*-isopropylacrylamide), by lowering culture temperature without by enzyme digestion [5,6]. Additionally, due to the presence of intact deposited extracellular matrices that are produced during *in vitro* cultivation, harvested cell-sheets can be easily reattached to other surfaces such as other cell-sheets and the host tissues. Cell-sheet-based clinical therapies have been performed to the treatments for corneal epithelial disease [7], esophageal stricture after endoscopic submucosal dissection [8], and dilated cardiomyopathy [9]. Moreover, by using this technology, functional multi-layered three-dimensional (3-D) cell-dense tissues including cardiac, hepatic, and pancreatic tissues are also created by layering cell-sheets for regenerative medicine [10–12].

A rapid and sufficient vascularization is crucial for the survival and function of engineered 3-D cell-dense tissues after transplantation. The prefabrication of endothelial cell network assembly (ECNA) in engineered tissues before transplantation, which is known as *in vitro* prevascularization, has been attempted as an approach to achieving the vascularization [13]. ECs are three-dimensionally co-cultured with other cell types *in vitro*, resulted in the organization of these cells into ECNA that often contains lumens spontaneously. Moreover, these transplanted 3-D tissues can anastomose by developing interconnections to the blood vessels of host tissue [13–15]. Therefore, the *in vitro* prevascularization of engineered tissues is beneficial for inducing functional anastomosis with the host vasculature.

On the other hand, our laboratory has also reported that an original approach for initiating *in vitro* prevascularization in regenerative multi-layered 3-D tissues by a cell-sheet-based sandwich co-culture system using temperature-responsive culture dishes and a cell-sheet manipulator [16,17]. Human umbilical vein endothelial cells (HUVECs) were sandwiched between cell-sheets such as myoblast sheets and fibroblast (FB) sheets,

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resulted in the creation of prevascular layered cell-sheets. In the case of prevascular quintuple-layered myoblast sheets, the networked HUVECs were connected to the host vessels at one week after transplantation, and the partially formed microvessels were found to contain erythrocytes in the engrafted cell-sheets. Therefore, our prevascularizing strategy contributes to the development of transplantable 3-D cell-dense tissues for regenerative medicine. However, the mechanism of neovascularization after the transplantation of prevascular layered cell-sheets is hardly understood.

This study created a scaffold-free prevascular 3-D tissue by sandwiching human aortic endothelial cells (HAECs) between human dermal FB sheets using a cell-sheet-based sandwich co-culture system and investigated the loci and progression of neovascularization after subcutaneous transplantation in immune-deficient rats. Furthermore, the secretion rates of angiogenesis-related proteins from ECNA-positive/-negative cell-sheets were investigated for understanding the mechanism of transplanted prevascular cell-sheet-induced neovascularization.

2. Materials and methods

2.1. Cell culture

Human dermal fibroblasts (FBs) derived from neonatal foreskin and human aortic endothelial cells (HAECs) were purchased from Lonza (Walkersville, MD). Human FBs and HAECs were cultured at 37 °C in a humidified atmosphere of 95% air with 5% CO_2 in a commercially-available growth media, FGM-2 (Lonza) and EGM-2 (Lonza), respectively.

2.2. Preparation of cell-sheet manipulator

A cell-sheet manipulator is composed of gelatin gel, a plunger devise, and a plunger-guiding cover (Fig. S1). The top surface of plunger is coated with hydrogel for harvesting a cell-sheet. The gelatin gel is prepared as described previously [16]. Briefly, gelatin powder from porcine skin (Sigma, St. Louis, MO) was dissolved in Hanks' balanced salt solution (HBSS) (Sigma) at 55 °C until a homogenous 7.5wt% gelatin solution was attained. After being neutralized with 1N NaOH, the gelatin solution was sterilized by filtration through a 0.45-μm Millex-HV filter (Millipore, Cork, Ireland) and poured into a silicone rubber mold, and then the plunger was put in the mold. The plunger was cooled on ice for completing gelation, resulting in gelatin coating on the top surface of plunger. For harvesting a cell-sheet, gelatin-coated plunger was placed onto confluently cultured cell layer in a temperature-responsive culture dish (UpCell™) (CellSeed, Tokyo, Japan) with the plunger-guiding cover.

2.3. Cell-sheet-based sandwich co-culture

Scaffold-free prevascular 3-D tissues were prepared using a cell-sheet-based sandwich co-culture system as described previously [18]. As ECNA-positive cell-sheets, double-layered FB sheets containing networked HAECs were created as illustrated in Figure S1. As the control layered cell-sheets (ECNA-negative FB-only cell-sheets), FBs, instead of HAECs, were sandwiched between two FB sheets. In this study, FBs within 5th passage and HAECs up to 3rd passage were used .To prepare FB sheets, FBs were seeded to an Upcell dish with 35 mm in diameter at a density of 8.9×10^4 cells/cm^2 and cultured for 1 day at 37 °C. One day before sandwiching between two FB sheets, HAECs or FBs were sparsely seeded to an UpCell dish with 35 mm in diameter at a concentration of 2×10^4 cells/cm^2. For monitoring the cellular behavior of inserted cells in the layered cell-sheets, sparsely cultured HAECs or FBs were labeled with 10 μmol/L CellTracker Green CMFDA (Invitrogen, Carlsbad, CA) immediately before sandwiching between two FB sheets.

The cell-sheet manipulator was placed onto the surface of FB monolayer and incubated for 30 min at 20 °C to allow harvesting. Then, the FB monolayer was harvested with the manipulator and transferred onto sparsely cultured HAECs labeled with a fluorescent reagent. After being incubated for 15 min at 20 °C for harvesting HAECs, the manipulator having the FB layer with HAECs was transferred onto another FB monolayer. The manipulator with the dish was incubated for 50 min at 20 °C, resulted in the harvest of a HAECs-inserting double-layered FB sheet with gelatin gel. Then, the cell-sheet with gelatin gel was separated from the plunger and placed onto a fetal bovine serum (FBS)-coated UpCell dish with 60 mm diameter (CellSeed). After being incubated at 20 °C for 2 h until re-adhering, the transferred cell-sheet with gelatin gel was incubated twice in HBSS at 37 °C for 15 min to remove gelatin gel by melting. Eventually, HBSS including the melted gelatin solution was removed, and fresh EGM-2 medium was added to the dish. After being cultured at 37 °C for 3 days, the transferred cell-sheet was incubated at 20 °C, allowing the prevascular layered FB sheet to detach itself from the temperature-responsive culture surface.

The cell images were obtained using a fluorescence microscope (TE-2000-U) (Nikon, Tokyo, Japan) integrated with an Axio Vision imaging system (Carl Zeiss, Hallbergmoss, Germany). The images of harvesting layered cell-sheets were obtained by a fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera system (Nikon, Tokyo).

2.4. Immunocytostaining and lectin-based staining

Cells were washed with phosphate buffered saline (PBS) (Sigma) and fixed with 4% paraformaldehyde solution (Muto Pure Chemicals, Tokyo) for 30 min. After being permeabilized with 0.2% Triton X-100 in PBS for 10 min, the cells were blocked with 1% bovine serum albumin (BSA) (Sigma) in PBS for 1 h.

For immunostaining human FBs and HAECs, the specimens were treated with a 1:200 dilution of anti-prolyl-4-hydroxylase monoclonal antibody (clone 5B5) (Abcam, Cambridge, UK) or a 1:40 dilution of anti-human CD31 monoclonal antibody (clone JC/70A) (DakoCytomation, Carpinteria, CA) for 1 h, and visualized with Alexa Fluor conjugated secondary antibody (Invitrogen) at a 1:500 dilution. For visualizing HAECs, the specimens were treated with a 1:100 dilution of rhodamine-conjugated Ulex europaeus agglutinin I (UEA-I) (Vector Laboratories, Burlingame, CA) for 1 h. The nuclei were counterstained with Hoechst 33342 (Wako Pure Chemicals, Tokyo). Cell images were observed by the fluorescence microscope and analyzed with an Axio Vision imaging system.

2.5. Subcutaneous transplantation of layered cell-sheets

Animal care and handling were performed according to the "Principles of Laboratory Animal Care" as advocated by the Tokyo Women's Medical University Animal Experiment Committee. F344 nude rats (6–8 weeks old) (CLEA, Tokyo, Japan) were anesthetized with inhaled isoflurane at a dose of up to 3.5%. The dorsal skin was cut and then opened. Layered FB sheets were placed onto the dorsal subcutaneous tissues for engrafting without suture. The transplanted cell-sheets were covered with a silicone rubber membrane (2.0 × 2.0 mm, 0.5 mm in thickness). Then, the skin incisions were closed. The images of subcutaneous tissue after transplantation were obtained using the fluorescence stereomicroscope equipped with the digital camera system. At 1 week after transplantation, the engrafted cell-sheets were excised.