The Porcine Aortic Tissue Culture System *in vitro* for Stem Cell Research

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Background and Objectives: Due to the shortage of human donors for transplantation, the use of animal organs for xenotransplantation has come into great interest. Xeno-derived vessels and cardiac valves would be possible alternatives for the patient suffering from cardiovascular diseases. Therefore, we established *in vitro* culture system of a porcine vessel that could be helpful for the research of xenograft and stem cell research.

Methods and Results: We primarily isolated porcine thoracic aorta, cultured square-shaped pieces up to 17 days and analyzed its morphology and characters. The endothelial cells were primarily isolated from cultured porcine aortic pieces and their morphology, function and character were analyzed in order to confirm them as endothelial cells at day 3, 4, 8, 10 and 17. Even at day 17, the morphology exhibited the intact endothelial layer as well as specifically expressed CD31 and von Willebrand factor. The morphology of primarily isolated cells from cultured tissues was identical as an endothelial cell. By flow cytometry analysis, more than 80% of the isolated cells expressed CD31 and up to 80% took up acetyl low density lipoprotein (ac-LDL) until day 10 of tissue culture period even though it decreased to about 50% at day 17 that means they not only showed typical endothelial cell characters but also functioned properly.

Conclusions: We successfully established and optimized a porcine vascular tissue *in vitro* culture system that could be a valuable model for *in vitro* study of xenotransplantation and stem cell research.

Keywords: Xenotransplantation, Stem cell, Primary porcine aortic tissue culture, Endothelial cells, DiI-ac-LDL

Introduction

Cardiovascular diseases have been the leading causes of death and major health problem in the world. The shortage of transplantable human organs has generated great

interest in the use of alternative animal organs such as pig's for xenografts. However, one of the major obstructions is human natural antibody-mediated hyperacute rejection by that natural antibody bind to galactosyl- α -1,3-galactose (Gal α -1,3-Gal) residues on porcine endothelial cells (EC) (1). The endothelium is the first site of contact between a vascularized xenograft and the recipient's immune system. The major barriers to organ xenotransplantation occur at the interface of the recipient blood supply and the vessels of donor organ, where recipient blood flows through donor blood vessels (2). Engagement of the recipient's complement cascade ensues, followed by endothelial cell activation, loss of vascular integrity, and thrombosis (3). If hyperacute rejection is controlled, an acute vascular rejection with mononuclear cell infiltration

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of the graft occurs (4), followed by a T-cell-mediated rejection also directed to the endothelial cells (5).

Endothelial cells play a central role in various biological processes such as coagulation (6, 7), inflammatory responses (8), regulation of vessel wall structure (9) and immunological rejection of xenografts (10-12). Endothelial cells are the first xenogenic cells that interact with the host immune system after organ xenotransplantation. Therefore, it is important to study the molecular mechanisms and immune response of endothelial cells in order to prevent xenograft rejection. Moreover obtaining highly pure and well-characterized endothelial cells would be critical.

The study of endothelial cell and blood vessel is required to be preceded for xenotransplantation. For that reason, in order to facilitate *in vitro* studies of xeno-immune responses, it is essential to establish primary isolation system of endothelial cells consistently maintained for further study of endothelial cells and aortic tissue culture system. In this study, we cultured porcine aortic tissue *in vitro* and confirmed the endothelial layer of cultured tissue was intact and the endothelial cells from those tissues were functioned properly that means an optimized aortic tissue culture system and characterization systems was established for endothelial cells that could be helpful for the research of cardiovascular xenograft and stem cell

research.

Materials and Methods

In vitro porcine aortic tissue culture

The thoracic aorta from adult swine were dissected and discarded the connective and adipose tissue, cut into a rectangular form (5 cm length and 3 cm width) in cold Hank's balanced salt solution with 3× antibiotics followed by be pinning with 21G syringe needles to avoid bending itself (Fig. 1A). Aortic tissues were cultured on culture dishes in DMEM based endothelial cell culture media up to 17 days and samples for analyzing were collected at day 3, 4, 8, 10 and 17 (Fig. 1B). After tissue culture, porcine endothelial cells were isolated as described above. Briefly, aortic tissues were incubated with 0.05% collagenase I for 20 min at 37°C, for a while the endothelium was scraped and digested for 30 min in 0.05% collagenase I. The digest was filtered with nylon mesh, washed and seeded on 35 mm tissue culture dish in DMEM based endothelial cell culture media. The cells were incubated at 37°C in 5% CO₂.

Fig. 1B showed experimental design of *in vitro* aortic tissue culture. At day 0, aortic tissue was prepared as an above procedure and tissue culture was started. At day 3, 4, 8, 10 and 17 from tissue culture, cultured aortic tissues

Fig. 1. (A) Schematic diagram for porcine aortic tissue culture. (B) Experimental design of *in vitro* aortic tissue culture. At day 0, tissue culture was started (arrow). At day 3, 4, 8, 10 and 17, cultured tissues were collected for analysis (arrow head).

were collected and analyzed as following methods like immunostaining or functional assay.

Characterization of primary porcine endothelial cells of *in vitro* cultured aortic tissue endothelium

Primary cells were cultured onto an 8 well chamber slide in complete media. When cells reached confluence, they were washed with PBS and fixed with a 70% ethanol for 1 hr at -20°C . 4- μm -thick section of cultured porcine aorta was prepared for hematoxylin and eosin (H&E) staining and immunohistochemistry. Frozen tissues sections (4- μm -thick) were fixed with acetone for 10 minutes and air-dried for 20 minutes. The tissue sections were then washed with PBS three times. Tissues or cells were treated 1 mM of H_2O_2 to remove endogenous peroxidase for 30 min at room temperature (RT). The sections were treated with 0.5% of Triton-X100 for 15 minutes at RT and rinsed three times with PBS. After preincubation, samples were incubated with primary antibodies, such as von Willebrand factor (vWF), CD31 (PECAM, platelet/endothelial cell adhesion molecule), vimentin or desmin or α -SMA (smooth muscle actin) overnight at 4°C . vWF and CD31 were used for endothelial cell-specific markers. Vimentin is a characteristic marker of mesenchymal cells, and desmin and α -SMA are specific markers of smooth muscle cells. The sections were washed three times with PBS and incubated with secondary peroxidase conjugated goat anti mouse IgG antibody (1 : 200) or goat anti rabbit IgG antibody (1 : 200) for an hour. Diaminobenzidine (DAB) was added for colorization. The DAB reaction was stopped by flushing with tap water for 10 min. Counterstaining was done with hematoxylin. Dehydration step was followed by a xylene step. Slides were examined with a Nikon microscope and pictures were taken with a Nikon digital camera (Diagnostic Instruments, Tokyo, Japan).

Cell functional assay

Primary cells were incubated with 10 $\mu\text{g}/\text{ml}$ of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labelled Ac-LDL (DiI-ac-LDL, Biogenesis, UK) in growth medium at 37°C for 4 hours. The cells were washed with PBS and imaged using a Nikon inverted microscope with a 200 $\times$ or 400 $\times$ objective and captured using a Spot digital camera (Diagnostic Instruments, MI, USA) for permanent record.

Quantification of the purity and the function of primary isolated porcine endothelial cells by FACS analysis

To quantify purity and function of primary cells, we analyzed the uptake of DiI-ac-LDL. 10 $\mu\text{g}/\text{ml}$ of DiI-ac-

LDL was added to the medium and cells were returned to the incubator for 4 hours in darkness.

Briefly, cells were resuspended in FACS buffer [HBSS, 2% of FBS, 0.05% of Sodium azide] and incubate for 30 minutes on ice in 1 : 100 dilution of monoclonal anti-rat CD31 in darkness. Cells were washed twice with buffer then centrifuged at 500 g for 5 min. Cells were incubated again as above with a 1 : 200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG. Cells were washed, pelleted and resuspended 2 ml of FACS buffer for acquisition on a Becton-Dickinson FACS Calibur using CellQuest software (Becton Dickinson, Mountain View, CA). A minimum of 1×10^4 events were counted in all analyses.

Results

Establishment of porcine aortic tissue culture *in vitro*

Morphology of cultured porcine aortic tissue was intact during *in vitro* culture up to 17 days. Histological findings of aortic tissues showed that endothelial layer was intact (Fig. 2P~T). To characterize whether the endothelial layer was intact, the cultures were stained using endothelial specific markers, such as vWF and CD31. The endothelial cell was maintained their characteristics and undamaged up to 17 days. vWF was highly expressed in the surface of aortic tissue for 10 days, showing prominent perinuclear cytoplasmic staining, but tended to decline after 10 days (Fig. 2A~E). CD31 was expressed in the endothelium, showing junctional staining consistent with plasma membrane but showed decreased expression after 10 day (Fig. 2F~J). The cells within the intimal layer positively with α -smooth-muscle actin, a characteristic of smooth muscle cells (Fig. 2K~O), and the characters were evident for up to 17 days.

Characterization of primary endothelial cells from cultured porcine aortic tissues

Endothelial cells were isolated from cultured aortic tissue at day 3, 4, 8, 10 and 17. The cells from cultured aortic tissues exhibited a cobblestone morphology that is typical characteristic of endothelial cells and their morphology was maintained (Fig. 3). Endothelial phenotype was confirmed endothelial specific markers, CD31 and vWF. Isolated cells expressed vWF in cytoplasmic granules (Fig. 4A~E) and CD31 on the cell-cell membrane borders (Fig. 4F~J). During tissue culture, most cells were positive in vWF and CD31. α -SMA and desmin, specific markers for smooth muscle cells, were expressed at day 3 and 4, but their expression disappeared after day 8 (Fig. 4I~T). In

Fig. 2. Characterization of cultured porcine aortic tissue. Tissues were stained with antibodies for von Willebrand's factor (vWF), CD31 (platelet/endothelial cell adhesion molecule, PECAM), α -smooth muscle actin (α -SMA), respectively. Nuclei were counterstained with hematoxyline (Magnification $\times 400$). H&E, hematoxyline and eosin.

spite of tissue culture for 17 days, endothelial cells of aortic tissue remained intact.

Functional analysis of primary endothelial cells established from explant cultures of porcine aortic tissues

To test the function of primary endothelial cells from cultured porcine aortic tissues, primary endothelial cells were isolated from cultured aortic tissues for 8, 10 and 17 days. The cells were double-labeled with CD31 and DiI-ac-LDL for flow cytometry. Approximately, 80% of cells from cultured aortic tissue from 8 to 10 days were expressed CD31 and took up DiI-ac-LDL. At day 17, over 50% of cells were still shown positive for CD31 and DiI-ac-LDL. The cells from cultured tissue showed their function was decreased from 80% to 50% at day 17 (Fig. 5). Therefore in spite of tissue culture up to 17 days, the optimistic porcine aortic tissue culture should be less than 17 days according to our conditions.

Discussion

Cardiovascular disease, including coronary artery and peripheral vascular disease, is the leading cause of mortality in the world. Surgical replacement or bypass surgery is the most common intervention for coronary and peripheral atherosclerotic diseases. But many patients do not have suitable autologous vessels.

An alternative approach for vessel grafting is to engineer vessels by using scaffolds based on either a biodegradable or decellularized matrix. However, the materials that are commonly used lack growth potential, and long-term results have revealed several material-related failures, such as stenosis, thromboembolization, calcium deposition, and risk of infection (13). Previously, organ cultures such as heart valves were developed to study the biological characteristics of native aortic valves cultured *in vitro* and understand fundamental valve pathogenesis (14-17). An organ culture maintains cells within their na-

Fig. 3. Morphology of primary endothelial cells isolated from cultured aortic tissues with different culture periods (day 3, 4 and 6) under phase contrast microscope. Cell culture was carried out as mentioned in Materials and Methods.

tive microstructural environment, and thus offers greater potential. It was reported that bovine mitral valve endocardium lined by endothelial cells remained after culturing for 6 days (15). Native porcine aortic valves cultured in an *ex vivo* pulsatile organ culture system maintained the native extracellular matrix composition of the leaflets while preserving the morphology and cell phenotype. In human, a pulsatile flow system *in vitro* using biodegradable patch scaffold was developed for cardiovascular surgery that provides biochemical and biomechanical signals in order to regulate autologous, human-tissue development *in vitro* (18). The application of patches or grafts in cardiovascular surgery, and particularly in pediatric cardiac surgery, is a widely accepted surgical technique for repair or reconstruction of cardiovascular structures (19, 20). Currently, the patch materials used clinically are limited to prosthetic materials, autologous pericardium, and allogenic or xenogenic (glutaraldehyde-fixed) pericardium (20). All materials used for patch repair or reconstruction have limitations such as their inability to grow, repair and remodel. Aneurysm formation and the inability of patches to grow or remodel are important sources of morbidity and mortality after repair or reconstruction of cardiovascular structures, especially in children and young adults. Tissue engineering is proposed as a solution (18). As the vasculogenic potential of mature endothelial cells is not

sufficient for therapeutic purposes (particularly when isolated from adult tissue), various endothelial progenitor cells were isolated and tested for their vasculogenic/therapeutic activity (21, 22).

As another approach to meet the demands of patients waiting for xenograft where pigs most have been used, xenotransplantation must be more studied for preventing xenograft rejection that is happened between recipient endothelial cells and donor immune components.

Endothelial cells were retained on the surfaces of cultured leaflets with no remodeling of the leaflets for a period of 48 hours (17). However, vessel tissue itself has not yet been cultured. Here we directly cultured porcine aortic tissues and confirmed their survival even after 17 days, the tissue cultures, tissues had intact endothelia and primary isolated cells from the pieces of direct porcine aortic tissues showed typical cobblestone morphology as endothelial cells. Endothelial cell specific markers, CD31 and vWF, were positive by immunohistochemistry throughout 17 culture days. Overgrowth of contaminating cells, such as fibroblast was seen for the first 3~4 days as cells were positive in desmin and α -SMA. But after 8 days, it was shown that cells had high purity as endothelial cells by immunohistochemistry and double-detection for CD31 and DiI-ac-LDL by FACS analysis. From day 8 to day 10, cells over almost 80% double-expressed CD31 and DiI-ac-

Fig. 4. Characterization of primary endothelial cells isolated from cultured porcine aortic tissue. Cells were isolated from cultured aortic tissues with different culture periods (day 3, 4, 8, 10 and 17) and stained with antibodies to von Willebrand factor (vWF), CD31 (platelet/endothelial cell adhesion molecule, PECAM), desmin, α -smooth muscle actin (α -SMA). Nuclei were counterstained with hematoxyline (Magnification $\times 400$).

Fig. 5. Functional analysis of primary endothelial cells isolated from cultured porcine aortic tissue. (A) Double-staining of primary porcine endothelial cells with antibody for CD31 and DiI-ac-LDL. Cells were isolated from cultured aortic tissues with different culture periods (day 8, 10 and 17). Representative profiles of CD31 and DiI-ac-LDL expression in endothelial cells are shown in the dot plots. (B) Quantification of CD31 and DiI-ac-LDL expression with FACS analysis. Negative control (NC) was treated only with secondary antibody. Each experiment was triplicated. Results are typical of multiple experiments with 10,000 cells for each labeling condition.

LDL by FACS analysis. The phenomenon was decreased to 50% at day 17. The maximal culture period with optimistic condition of direct-porcine aortic tissue culture would be 8 to 10 days under our basal culture condition. Therefore, we successfully established that the porcine aortic direct-tissue culture system in this study can serve as a valuable *in vitro* experimental model for vascular xenotransplantation as well as a solution for shortage of suitable vessels. Furthermore, our tissue culture system facilitates the study of xeno-organs such as porcine vessels for vessel regeneration.

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Potential conflict of interest

The authors have no conflicting financial interest.

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