Bioengineering of vascular grafts using multilayer of PLCL nano-fibers with endothelial cells and fibroblast cells.

Boram Lee

Department of Medical Science

The Graduate School, Yonsei University

Bioengineering of vascular grafts using multilayer of PLCL nano-fibers with endothelial cells and fibroblast cells.

Directed by Professor Jong-Chul Park

The Master's Thesis

Submitted to the Department of Medical Science

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

Boram Lee

December 2013

This certifies that the Master's Thesis
of Boram Lee is approved.

Thesis Supervisor: Jong-Chul Park

Thesis Committee Member#1: Soo Hyun Kim

Thesis Committee Member# 2: Eun Jig Lee

The Graduate School
Yonsei University

December 2013

Acknowledgements

I really appreciate all assists to all researchers, students and professors in KIST research team and Yonsei university research lab. Especially, I would like to thanks for supportive professors.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

Abstract

Bioengineering of vascular grafts using multilayer of PLCL nano-fibers
with endothelial cells and fibroblast cells.

Lee, Bo Ram

Deptartment of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong-Chul Park)

Tissue engineering (TE) provides effective way to regenerate damaged or lost tissues by combining cells and biomaterial scaffolds to reconstruct and regenerate new tissue or organ. Diseases of vascular systems are the fatal causes of death and disability in people. Vascular grafts have been used successfully to replace large-diameter (diameter > 6mm) blood vessel, However the patency of small-diameter(diameter <6mm) vascular graft is still disappointing.

We looking forward this study that we can make the successfully performed small diameter vascular graft using nano- fibrous scaffold as ECM construct in native vessels, and co-culture system of endothelial cells and fibroblast cells on the scaffolds provide proper micro-environment.

For investigating the effect of scaffold with HDFs layer for cultured different term, we designed 2D co-culture experiment which is in-direct contact condition. In an indirect contact co-culture of fibroblasts and endothelial cells, it found that confluent HDFs layer influenced the HUVECs tube-like structures. In vitro static culture conditions, It was observed that the nano-fibrous scaffold with fibroblast layer supported HUVECs survival in the middle of vessel structure after 1, 7days in culture. However, the examination described that tubular structure did not maintain after 1month in static culture. Therefore we designed in vivo experiments which is the co-cultured vascular grafts were implanted in the nude mice for 2,4weeks. Histological analysis showed that co-culture system generated cellular infiltration into the multilayer of PLCL fibers and also collagen depositions were observed in the outer area of co-cultured vessel by post operated 4weeks.

Key words : vascular grafts, co-culture, tissue engineering, endothelial cells, electrospining

Bioengineering of vascular grafts using multilayer of PLCL nano-fibers with endothelial cells and fibroblast cells.

Lee, Bo Ram

Deptartment of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jong-Chul Park)

Ⅰ. Introduction

Tissue engineering (TE) provides effective way to regenerate damaged or lost tissue/organs by combining cells and biomaterial scaffolds to reconstruct and regenerate new tissue or organ.[1]

Diseases of vascular systems are the fatal causes of death and disability in people. Vascular grafts have been used successfully to replace large-diameter (inner diameter > 6mm) blood vessel, However the patency of small-diameter (inner diameter <6mm) vascular graft is still undeveloped.[2]

The limitation of tissue-engineered small vascular grafts is the inability to obtain a confluent endothelium on the lumenal surface. Thus any denuded areas on the lumenal surface of vascular grafts may lead to thrombus formation.[3]

Moreover, for the survival of a tissue-engineered construct with size larger than the diffusion limit of oxygen after its implantation in vivo, the tissue has to be vascularized, which means that a capillary network capable of delivering nutrients to the cells has to be formed within the construct .[4] Therefore, angiogenesis has recently been recognized as one of the key challenge in TE.

We looking forward this study that we can make the successfully performed small diameter vascular graft using nano- fibrous scaffold as an ECM construct, and Co-cultured endothelial cells and fibroblast cells on the scaffold provide proper micro-environment.

Nano fibrous scaffold created by electrospinning technique have enormous potential for tissue engineering since they can mimic the structure and function of native extracellular matrix(ECM). Moreover, the electrospun nano-fibers have large surface area which allows for the direct attachment of ECM ligands, growth factors, and other biomolecules onto fiber surfaces to locally modulate cell and to enhance mechanical strength.[3]

Living tissues have complex and well-organized microstructures. Although microtechnology has been used to create in vivo-like cell microstructures in vitro, most available microscale systems are two-dimensional, and few threedimensional (3D) systems have been explored. Microtechnologies offer the new possibility of building structures and devices in dimensional scales close to those of tissues.[5] With the incorporation of microfabrication into biology, research has been carried out on engineering of cell-substrate, cell- cell, and cell-medium interactions on the micrometer scale.[9-11]

It has been reported that, in addition to endothelial cells, other cells, such as fibroblasts, are also involved in the angiogenic process. Therefore, an in vitro cell co-culture model with fibroblasts and endothelial cells has been used to study the mechanisms of angiogenesis, and the results showed that co-culture of endothelial cells with fibroblasts could significantly enhance the angiogenesis of endothelial cells.[7-8] The importance of extracellular matrix formed by fibroblasts on the scaffolds for the angiogenesis in the co-culture system has been reported.[7-8]

Moreover Fibroblasts play an essential role in the angiogenic process through their production of extracellular matrix molecules and through their release of essential growth factors.[8] Therefore, the goal of this study is to develop that the co-culture system to supply most suitable micro-environment for endothelial cell's proliferation and organization into tube-like structures. For this purpose, in this research, we produced a nano-fibrous PLCL scaffold and co-cultured HUVECs and HDFs on the other side of scaffold and made the cylinder-like construct to mimic the vessel structures by rolling the cells-biomaterials sheets. Therefore, co-cultured HUVECs (co-HUVECs) be influenced by contact with HDFs directly and in-directly. We can study direct contact effect through observation of

HUVECs-HDFs interaction with contact layers of 3D construct model. And also we can gather the information about in-direct effect of HUVECs-HDFs interaction by observation of the endothelial cells migration and movement on the scaffold with fibroblast cells.

In this study, a newly designed tissue-engineered blood vessel was fabricated not only to mimic the structure and morphology of the natural vascular structure, but also offered temporary supports and guides for vascular tissue survival, organization, and remodeling.

Ⅱ. Materials and methods

1. Preparation of elctrospun nano-fibrous PLCL scaffold.

PLCL was dissolved in hexafluoro-2-propanol(HFIP) to generate a 9%(wt/v) solution with continuous stirring. Electrospinning was performed with a steel capillary tube, electrically insulated stand. The capillary tube was maintained at a high electric potential for electrospinning. The capillary tube was connected to a syringe filled with the PLCL/HFIP solution. A constant volume flow rate of 1mL/h was maintained using a syringe pump. High voltage of 18 kV was applied when the solution was drawn into fibers and was collected on a porous foil covered collection plate kept at a distance of 18 cm from the needle tip. And then, the electrospun nanofibrous scaffolds were treated with 100% ethanol for 10 min to induce a b-sheet conformational transition, which resulted in insolubility in water. The scaffold was dryed for 2-3days at room temperature. After dried, scaffolds were coated by collagen solution(Collagen type Ⅰ: 1% acetic acid = 1:2 diluted solution) for 24hours at 4℃ temperature because of that scaffolds were hydrophobic state.

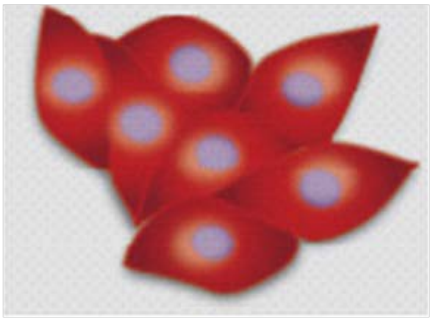
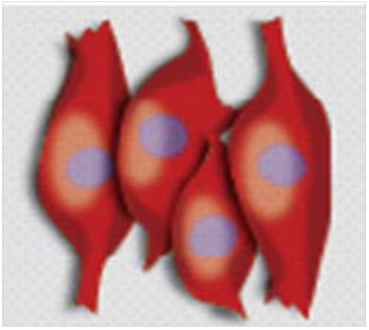
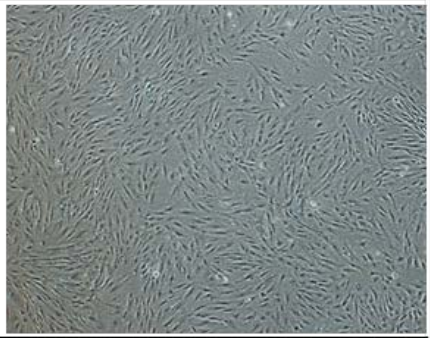
2. Co-culture system of HUVECs and HDF.

HUVECs were maintained in endothelial basal medium-2 (EBM-2) (Lonza, Switzerland). HDFs were maintained in fibroblast growth medium(FGM) (Lonza, Switzerland). The cells were cultured at least a week and were maintained at a humidified atmosphere of 95% air and 5% CO2, 37.5℃. HUVECs below passage 6 and HDFs below passage 12 were used for this study. Before cell seeding, PLCL nanofibrous scaffold were sterilized using a 70% ethanol solution and UV for 30min. HUVEC's cell density was 2.5×10^4 cells/cm and HDF's cell density was 0.5×10^4 cells/cm.

First of all, we seeded the HDFs on scaffold and culture in FGM media for 7days. And then HUVECs were cultured on the other side of scaffold for 7days in ECs medium.

After co-culture processing, tissue-engineered vascular graft were produced using the cell-scaffold combined membrane.

Table 1. Morphology of HUVECs and HDFs

Human umbilical vein Endotherial Cell (Passage 4-6)	Humam dermal Fibroblast Cell (Passage 7-11)
	
	

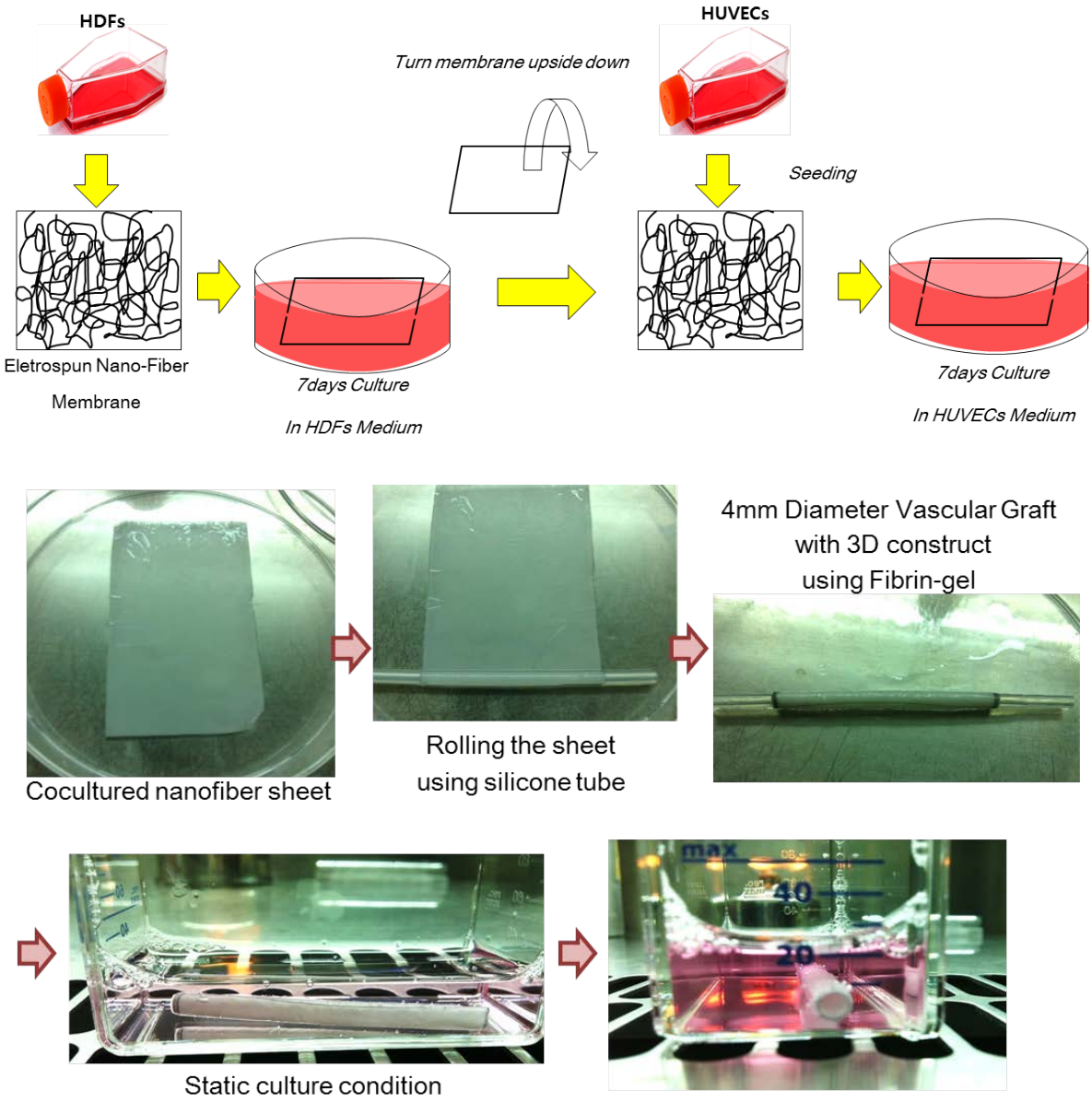


Fig.1. Procedure of co-culture system on the nano-fibrous PLCL scaffolds and method to make the 3D structures of vascular graft with 4mm inner-diameter. After this process the cell-scaffold vascular graft cultured in static condition.

3. CM-DiI : Cellular labeling system

To long-term labeling and tracking of HUVECs, we used the CM-DiI : Cellular labeling system.(CellTracker™ CM-DiI (C7000)).

Stock solutions of lipophilic tracers may be prepared in dimethyl formamide (DMF), dimethylsulfoxide (DMSO), or ethanol at 1–2 mg/mL. DMF is preferable to ethanol or DMSO as a solvent for SP-DiOC. Immediately before labeling, dilute the 1–2 mg/mL solution stock (see Preparing Stock Solutions) into a suitable medium such as Hanks' balanced salt solution (HBSS) or Dulbecco's phosphate-buffered saline (D-PBS).

We incubated cells in the working solution for 5 minutes or less at 37°C, and then for an additional 15 minutes at 4°C. After labeling, wash cells with phosphate-buffered saline (PBS) and resuspend in fresh medium. For adherent cells, labeling in culture while attached results in improved viability compared to labeling after dissociation.

4. Scanning Electron Microscope(SEM)

The surface morphology of cell-scaffold combined samples were viewed under a scanning electron microscope (SEM;NOVA NanoSEM200), with an accelerated voltage of 10 kV. All samples were coated with a sputter coater (BAL-TEC Inc) equipped with a gold target to increase electrical conductivity. The average diameter and diameter distribution were obtained by analyzing SEM images using a custom code image analysis program.

5. Tensile Testing

The tissue-engineered vascular constructs were subjected to tensile testing on an Instron Electropuls mechanical tester (Instron Corporation, Norwood,MA). Each tubular construct (cell seeded, or unseeded) was cut into 10 mm samples, which were mounted between two adapted to the mechanical tester. The distance measured for the unloaded state was used as the gauge length of the samples.

The linear modulus was defined as the slope of the linear portion of the stress-strain curve. Engineering strain was used to measure the deformation of the vascular constructs. Stress-strain curves were plotted and analyzed using a Matlab□script (The Mathworks, Natick, MA, USA) for the calculation of the tensile testing parameters

6. Burst & Leakage Test

To measure burst pressure of cell-scaffold construct, the tissue-engineered vessel was tested by pressure-rheometer. For this test, 20mm length of scaffold were prepared and installed on the pressure-rheometer. Using the same pressure of water-flow with blue ink, we can exam the pressure at burst and leakage point of vascular graft.

7. Immunofluorescence Staining

For immunocytochemical analysis, the cell-scaffold constructs were gently washed with PBS and were fixed in 4% paraformaldehyde after harvest at desired time points. The fixed samples were rinsed 3 times in PBS and then were incubated in blocking buffer (4% BSA in PBS) for 60 min at room temperature to block non-specific binding. Anti-von Willebrand Factor Antibody(vWF_ MAB3442, diluted 1: 50), CD31 (Endothelial Cell(PECAM-1)_M0823, diluted 1: 20 for ECs were used as primary antibodies.

Scaffolds seeded with ECs and FBs were incubated with different primary antibodies at 4 ℃ overnight respectively. After washing 3 times with PBS, the samples were reacted with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H L) or Alexa Fluor 488 goat anti-mouse IgG (H L) (1: 200 dilution, Invitrogen, CA) for 2h at room temperature. Then the samples were washed 3 times with PBS and were observed under a confocal laser scanning microscope .

8. Histological Analysis

Histologic sections(6μm) were routinely stained with hematoxylin and eosin(H&E) and viewed under light microscopy.

6-micrometer thick sections were cut using a microtome and stained with Masson's trichrome. Connective tissue is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink.

Ⅲ. RESULTS

1. Nano-fibrous PLCL scaffolds.

The morphology and diameter of electrospun PLCL nano-fibrous scaffold were observed by SEM(fig.2). The PLCL scaffolds have a construct of randomly oriented nano-fibers which is 200-500nm of diameter. And SEM image showed that surface of scaffold have a porous network which make the scaffold have a permeability of nutrients and other substance.

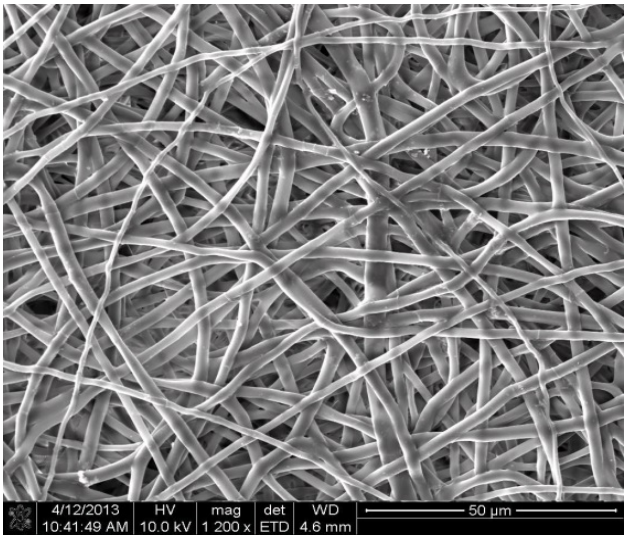


Fig.2. SEM image of Nano-fibrous PLCL scaffolds showing surface construct which is porous networking structures. Scale bar = 50µm.

2. Response of HUVECs on the scaffold with fibroblast layer.

For observation of HUVECs in co-culture environment, the author designed the three experimental groups (HDFs layer in cultured for 3,7 and 14days in HDF's medium)(fig.3). After HDF layers cultured on the scaffold, we also cultured HUVECs on the other side of scaffold for 1day in a HUVEC's medium. After HUVECs in cultured for 1day, the samples were immunofluorescence stained for cd31 and Von Willebrand factor(vWF) to analysis Endothelial Cells.

In this co-culture system, HUVECs and HDFs are cultured an indirect contact conditions. However we considered that effect of growth factor secreted by fibroblast layer on HUVECs appearance in co-culture.

As shown fig.3, confocal images of HUVECs layer showed a network formation of HUVECs at 3, 7days after co-culture conditions. After 7days, HUVECs did not cover on scaffold as mono-layer morphology, but we observed capillary-like network formation of HUVECs by immunofluorescence staining of EC-specific markers such as cd31 and vWF. However, after 14days in culture, HUVECs are cultured randomly on scaffold. In conditions of HDFs in 7days culture, fibroblast layer stimulated the HUVECs into the capillary-like formation.

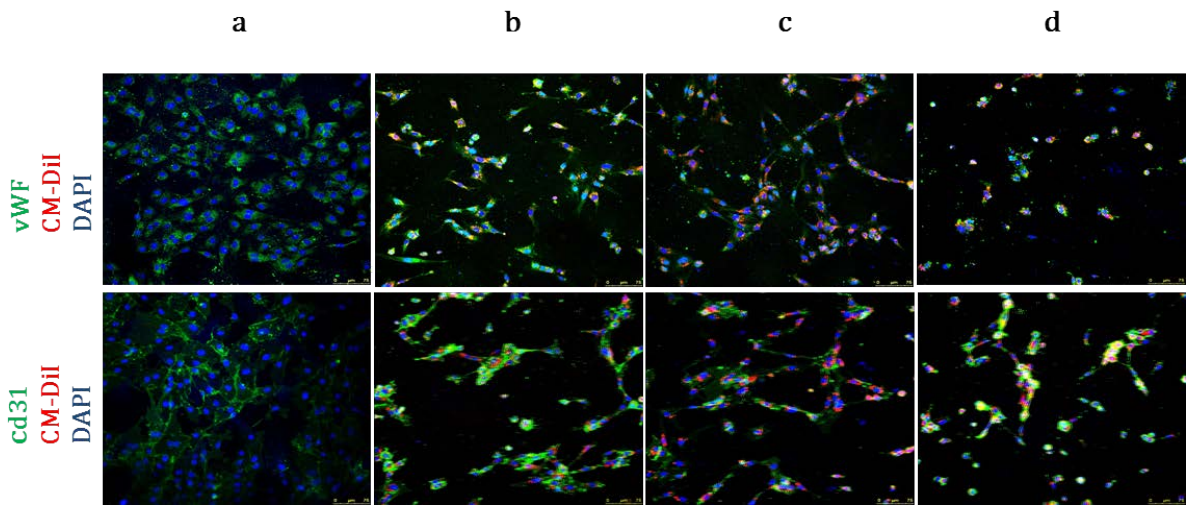


Fig.3. Confocal images of Immunofluorescence staining using antibody such as vWF and CD31 for detection of HUVECs layer from 2D Co-culture system model. Only HUVECs cultured on scaffold as a control models.(a) HUVECs co-cultured on the scaffold with HDFs layer which cultured different time. Fibroblast cells layer cultured for 3days(b), 7days(c) and 14days(d) and then HUVECs cultured for 1day.(a-d) To identify the ECs in the co-culture system CM-DiI cell tagging (red) were used(b-d) and nuclear stained in blue with DAPI. The tube-like network formation of HUVECs was observed during the co-culture of HUVECs and 7days cultured-HDFs.(c) Scale bar =75µm.

After observing of HUVECs morphology on different conditions of HDFs layer, we decided that HDFs layer in 7days cultured is proper conditions to HUVECs angiogenic performance.

The author observed networking morphology of HUVECs in long term culture conditions on scaffold with 7days cultured-HDFs layer(fig.4).

For the HUVECs, the expressions of cd31 antibody were stained by immunofluorescence staining to observed cells structure. After 1 and 3days, the confocal images showed capillary-like network formation. After 7days, There are parallel alignment construct with time in culture. However there are only some of HUVECs left on the scaffold in culture 14days. (fig.4).

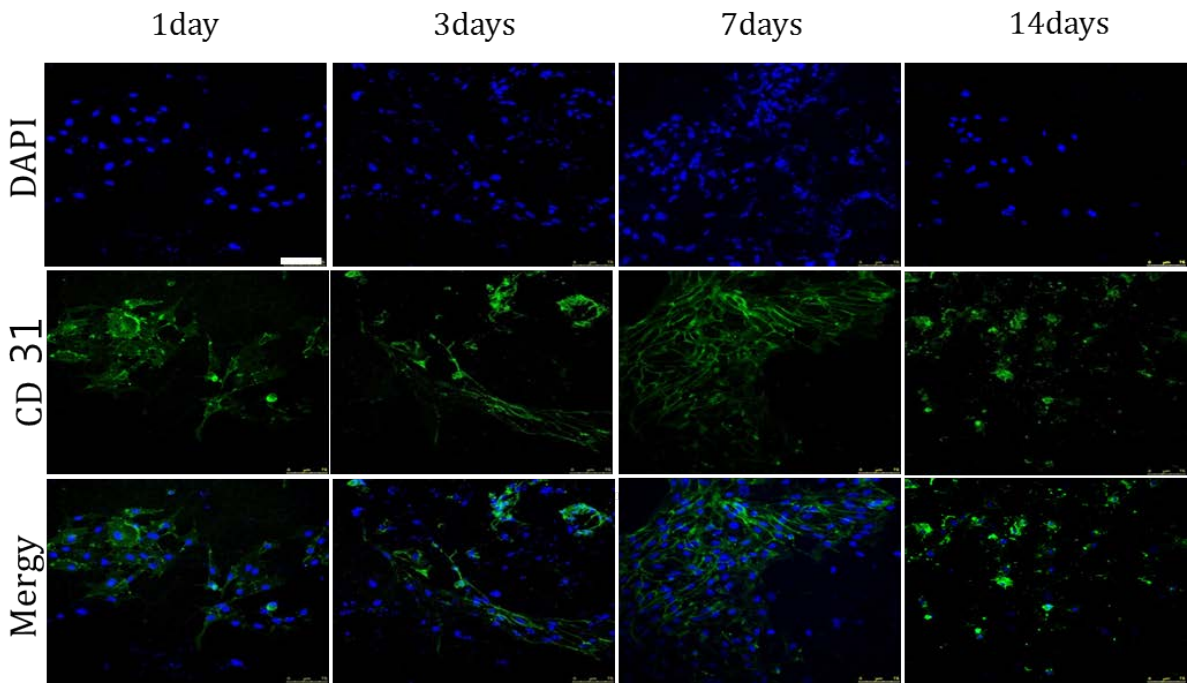


Fig.4. Confocal images of Immunofluorescence staining by CD31(green) for HUVECs layer from 2D co-culture system model. HUVECs cultured on the scaffold with 7days cultured-HDFs layer for different time.(HUVECs cultured for 1day, 3days , 7days and 14days.) There are parallel alignment construct with time in culture(3,7days). After 14days in culture, there are only some of HUVECs on the scaffold. Nuclear stained in blue with DAPI. Scale bar =75μm.

3. Co-cultured small diameter vascular graft.

After 1day in culture, we observed the cross-section of tissue-engineered vascular graft(fig.5). The lumen of graft is clear and each layer adheres well together. The co-cultured vascular grafts have vessel-like structure which is 4mm diameter of cylindricality construct.

Fig.5. Cross-section of small diameter vascular graft after 1day in static culture.

4. Biological characteristics of cells-scaffold vascular graft in vitro static culture.

The small diameter vascular graft were cultured in static conditions (1,7,14,28 days of culture in HUVEC medium). The 3D vessel-like structure was 4mm in diameter, 200μm in thickness for 10 layers of cells-scaffold sheet.(fig.6).

In immunofluorescence staining analysis, it was observed that the nano-fibrous scaffold with fibroblast layer supported HUVECs survival in the middle of vessel structure after 1, 7days in culture. However, after being static culture for 14days, there was few cells survival in the middle part of co-cultured vascular graft. This examination described the only cells in a lumen and outer layer survival after 1 month in static culture. Also the tubular structure did not maintain after 1month in static culture.(fig.6)

Confocal images of the cross-sections of vascular grafts in vitro static cultured model and native vessels immunofluorescence-stained by CD31(green) to observed HUVECs layer. This analysis demonstrated that only for 1day and 14days of cultured models showed that endothelium remodeled in the lumen layer of cell-scaffold constructs. And native vessel structures revealed endothelium in each layer.(fig.7)

In vitro static culture conditions are hard for cells survival especially the middle portion of 3D constructs because of poor supplement of nutrients and other substances.

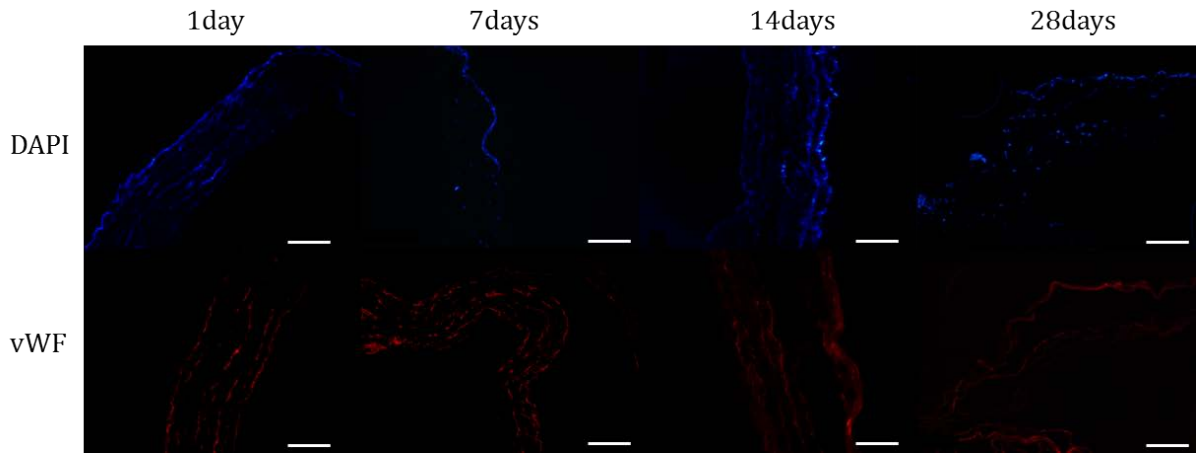


Fig.6. Immunofluorescence staining using Anti-von Willebrand Factor antibody(vWF, red) to observed HUVECs from the cross-sections of the vascular grafts of in vitro static cultured model. We observed the vascular graft after 1day, 7days, 14days and 28days in vitro static culture. After 1day and 7days in vitro static culture, there are alive-HUVECs layer in the middle of vessel structures. However, in vitro 3D model in 14days cultured showed that there are few endothelial cells survival in the middle part and after 1month in static culture, we can observed HUVECs in outer and lumen part of vascular graft only. Nuclear were stained by DAPI.(blue) Scale bar = 100μm.

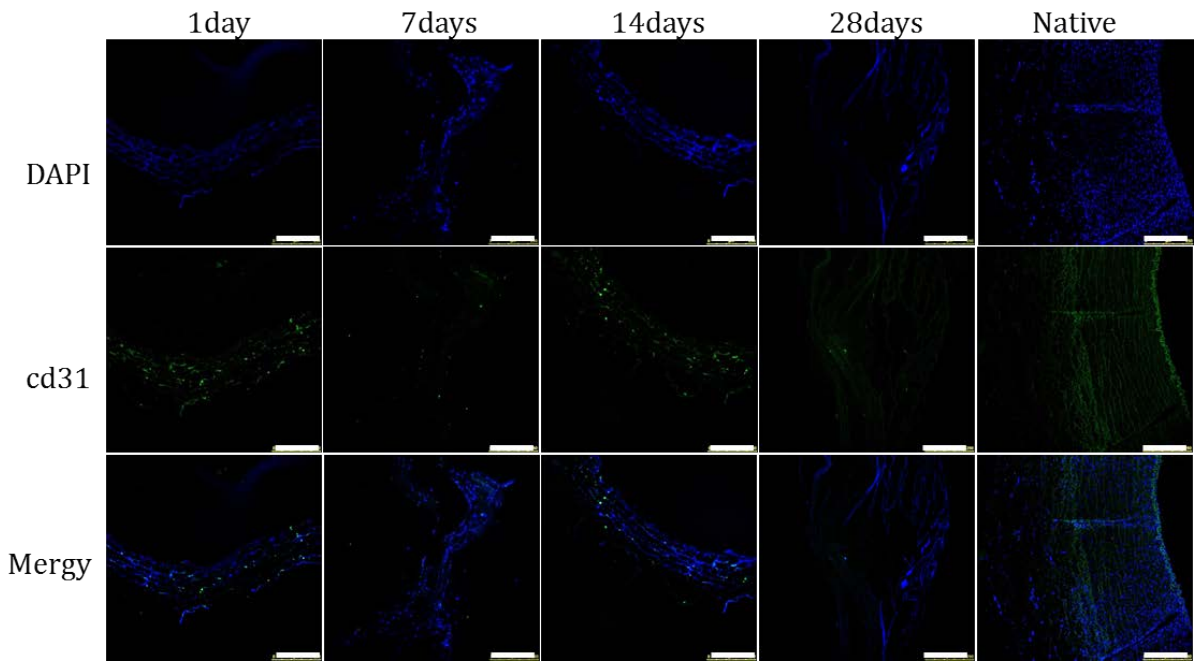


Fig.7. Confocal images of the cross-sections of vascular grafts in vitro static cultured model and native dog aorta immunofluorescence-stained by CD31(green) to observed HUVECs layer. We observed the vascular graft after 1day, 7days, 14days and 28days in vitro static culture. Only 1day and 14days of cultured models showed that endothelium remodeled in the lumen layer of cell-scaffold constructs. And native vessel structures revealed endothelium in each layer. Nuclear were stained by DAPI.(blue) Scale bar = 250μm

Hematoxylin and Eosin(H&E) staining and Masson's trichrome(MT) staining of the cross-sections in the anastomosis portion of the grafts with co-cultured system in vitro static culture. And native vessels of dogs were observed by same histological staining protocols for control model. The connective organization and cells remodeling structure were revealed by histological staining analysis.(fig.8)

In histological analysis, it was observed that almost every layer of cells even middle part of cells-scaffold vascular graft were survival and construct the connective organization in 1,7 and 14 days of static culturing.(fig.8) and there are network structure of connective materials in static culture for 14days.(blue arrow) However, after being culture in 28days, a few of survival layers were revealed in part of outer and lumen of vascular graft which were contacted media directly.

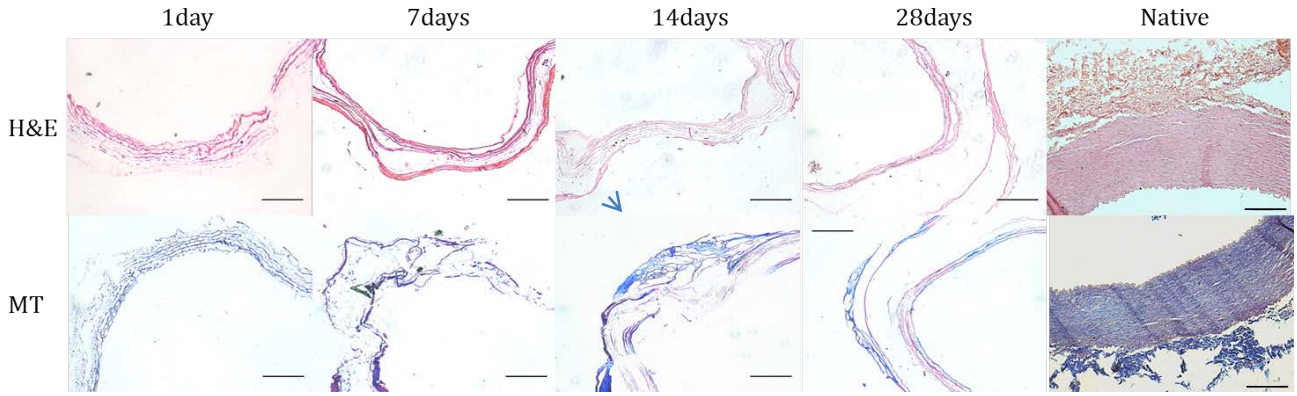


Fig.8. Hematoxylin and Eosin(H&E) staining and Masson's trichrome (MT) staining of the cross-sections of tissue-engineered vascular grafts with co-cultured cells(HUVECs and HDFs) following In vitro static culture. And native dog aorta were observed by same histological staining for control model. H&E staining image demonstrated networking structures in 14days cultured model.(blue arrow) However, after 28days in vitro static culture, the middle part of 3D vascular structure without cells and ECMs such as collagen and connective materials. Scale bar = 200μm

In SEM observation, after static culture in time, the each layer of tissue-engineered vessels was separated. The cell-layer of lumen to 5^{th} layer and outer following in vitro static culture for 1, 7 days can separated. However, after 14days and 1month in culture, each layer of vascular grafts was not divided from other layer because of cell-cell and cell-scaffold adhesion. In 3D co-culture model, it was possible to investigate the HDF-HUVEC interaction from direct contact between HDF and HUVEC layers. (fig.9)

After 1day in culture, HUVECs grown and covered on a scaffold with what appeared to be mono-layer. Lumen's surface which was contact medium directly showed that HUVECs layer were thicker than middle part. But still, only few cells attached on outer scaffold. However, after 7days in static culture, all the layers of tissue-engineered vessel were covered with thick, multi-layer of HUVECs especially lumen an outer. (fig.9)

Similar to the results obtained in the HUVECs layer, it was observed that mono-layer of HDFs were revealed after 1day in culture and thick, multi-layer of HDFs were showed after 7days in culture. However, both of cell types, the middle part of vessel have hard environment to survival than lumen and outer because of nutrient supply.(fig.9)

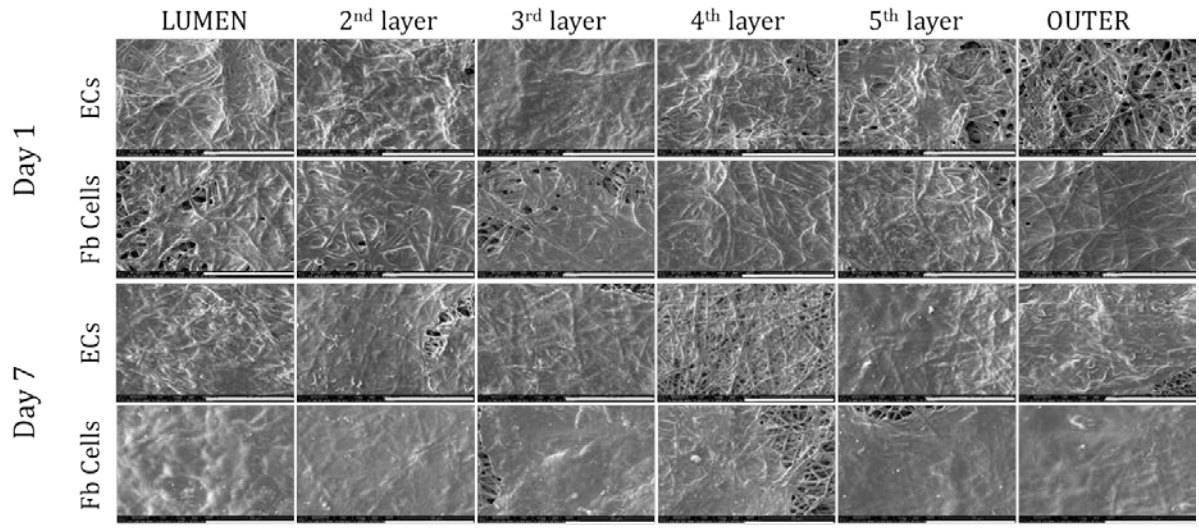


Fig.9. SEM images of each layer of 3D- small diameter vascular graft following in vitro static culture. We can separated each layer only for 1day and 7days of static cultured model. After 14days of culture, each layers stick together so hard therefore it can't be divided and observed for SEM examination. It was observed that mono-layer of HUVECs and HDFs were revealed after 1day in culture and thick, multi-layer of HDFs and HUVECs were showed after 7days in culture. However it was observed that the middle part of 3D constructs are hard conditions for cells survival in static culture conditions. Scale bar = 50μm

5. Mechanical properties of cells-scaffold vascular graft in vitro static culture.

To measure the mechanical properties of the small diameter vascular graft, we do some experiment such as burst pressure test, tensile strength test.

The vascular graft in static culture for 14days showed that they have higher mechanical properties. Those higher physical characteristics relied on the ECM remodeling in the 3D tissue-engineered vascular graft. After 1 month in static culture, mechanical properties down regulated from the result mentioned before.(fig.10) We considered the reason of this phenomenon that cells survivability of middle part were down regulated as shown before at the results of biological analysis.

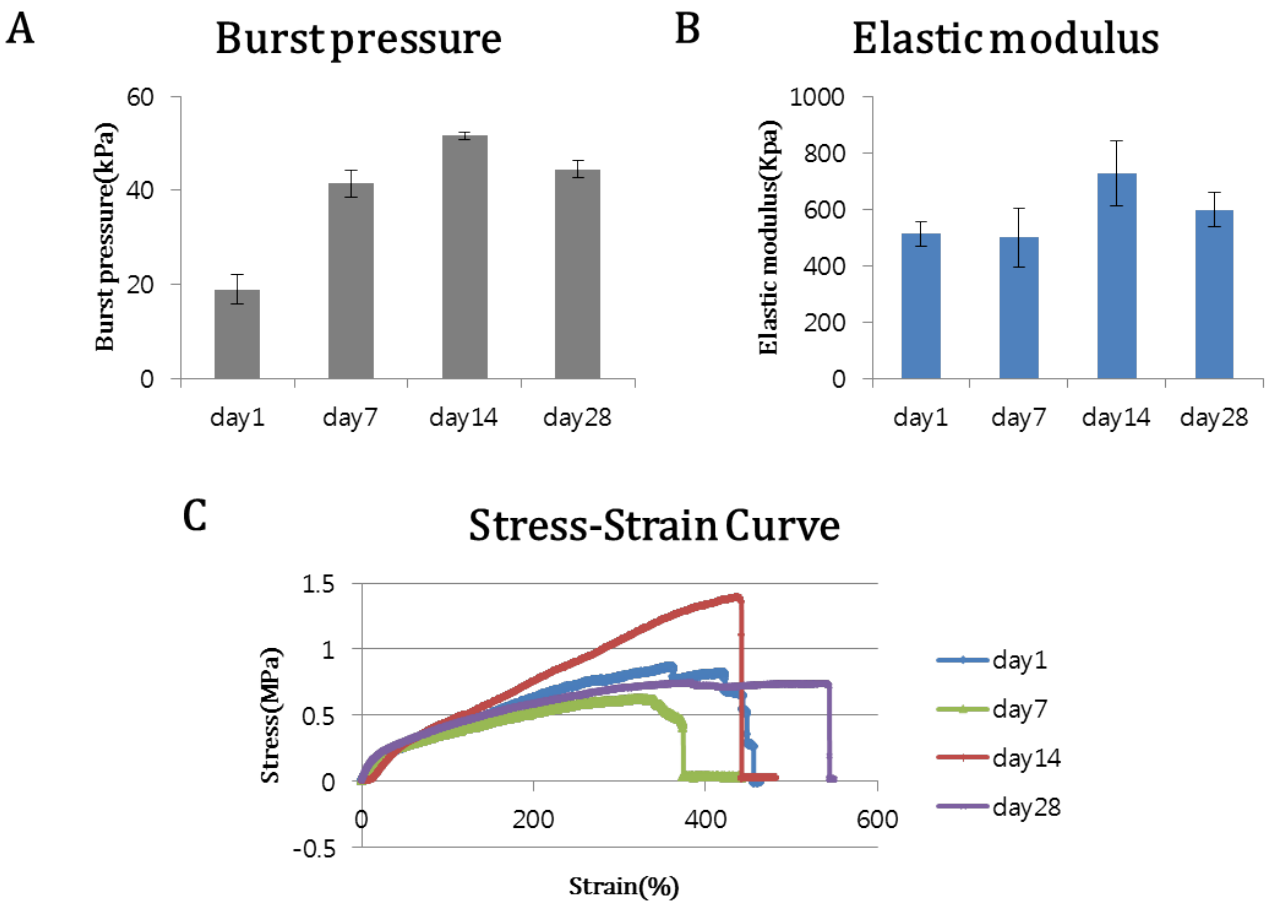


Fig.10. Mechanical properties of cells-scaffold vascular graft in vitro static culture.

(A) Burst pressure test of the grafts with co-cultured cells (HUVECs and HDF cells) In vitro static culture. Burst pressure results demonstrated that the 3D structure of vascular graft has most durable mechanical properties for 14days in culture.

(B,C)Stress-strain curve and Elastic modulus of the cell-scaffold complex vascular graft with co-culture system (HUVECs and HDFs) following In vitro static culture. Significant tensile strength value such as stress and elastic modulus was observed for 14days in vitro culture. And then, after 1 month in vitro culture, both values are down-regulated.

6. Characterization of tissue-engineered vessel cultured in vivo conditions.

We then produced two type of vascular graft which were made of PLCL membrane with co-culture of HUVECs and HDFs(co-cultured vessel) and PLCL membrane with mono-culture of HDFs(mono-cultured vessel). After 1day in static condition, the vascular grafts were implanted in the subcutaneous area of nude mice.

Post- operative in 2weeks and 4weeks, two types of vascular grafts were explanted and examined by histology analysis. The histology analysis results of vascular grafts showed that co-culture system generated cellular infiltration into the multilayer of PLCL fibers and also collagen depositions were observed in the outer area of co-cultured vessel by post operated 4weeks. (fig.11A)

Zoom at the graft area stained by CD31 looking for the endothelial cells layer and lumen following 14days post-implantation. Images of co-cultured vessel revealed endothelial cell layer remodeled at the luminal area and other multiple layers. The results indicated that co-cultured-HUVECs survival each layer and then the endothelial cells reconstruct into micro vessel-like structure, which means that survival of endothelial cells on the each layer surface effect on the survival of tissue-engineered vessel through delivering nutrients better. (fig.11B-a, left) a lot of cells survival in the mono-cultured vessel however endothelial cells layer are observed only outer layer which contact with native tissues. (fig.11B-a, right)

Double cross-section staining for CD31(endothelia cell maker) and α-SMA demonstrated smooth muscle cell positioning in the outer area of vessel structure following 28days post-implantation. EC layer remodeled in each layer surface(CD31, green) and fibroblast cells differentiated into smooth muscle-like cells and the cells layer were detected by α-SMA in co-cultured vessel. Same stained images of mono-cultured vessel showed lots of survival cells on each layer.(fig.11B-b)

DNA content within the co-cultured vessels increased to three times from 14days to 28days sample. (fig.11C)

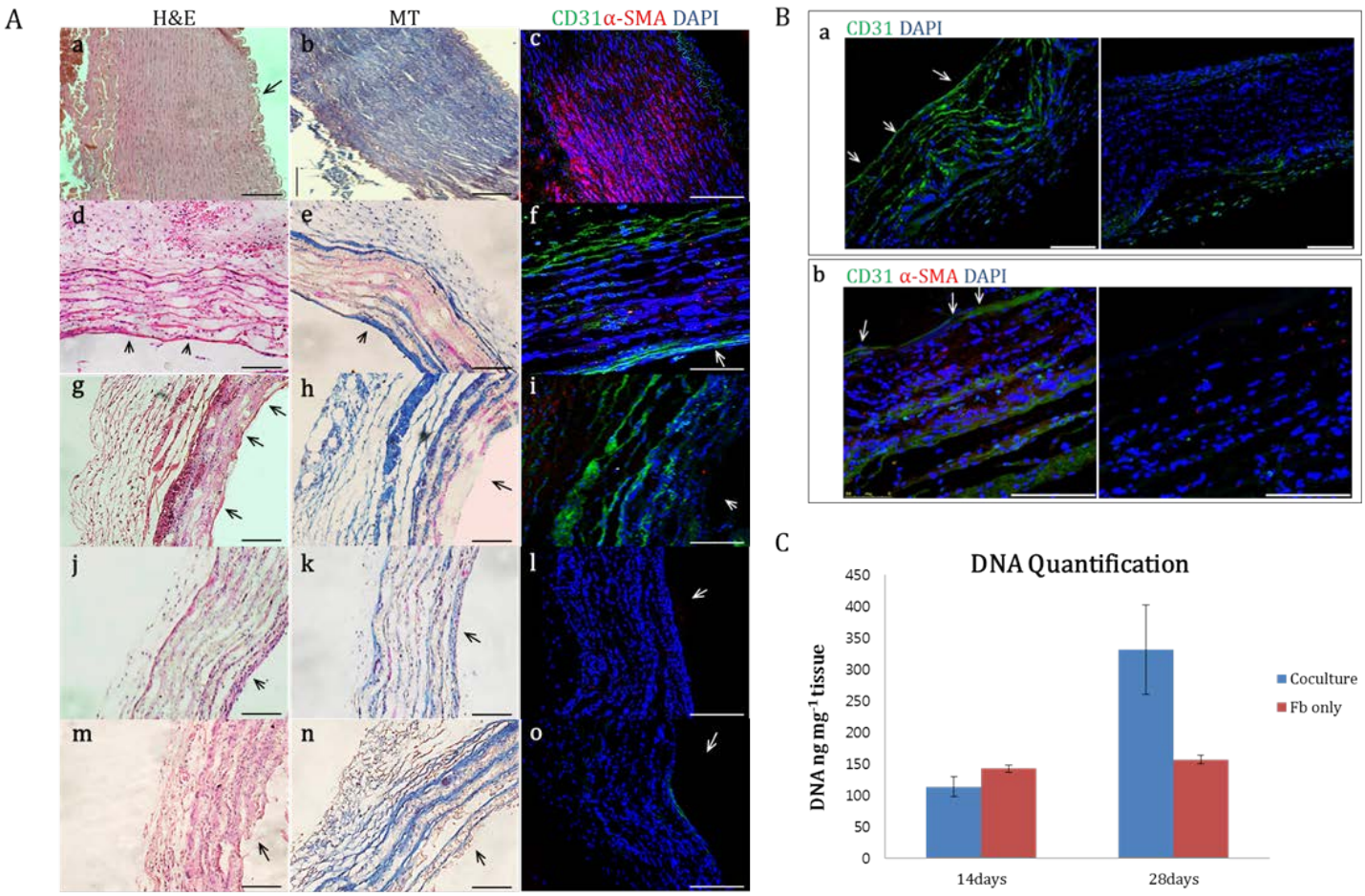


Fig.11. Characterization of tissue-engineered vessel cultured in vivo conditions.

(A) Representative images of native dog aorta are observed by (a)H&E staining, (b) Masson's trichome staining, (c) Immunofluorescence staining(the endothelial layer stained with Anti-von Willebrand Factor antibody and medial layer stained with α -SMA antibody). Representative images of co-cultured graft implanted for 2weeks are observed by (d)H&E staining, (e) Masson's trichome staining, (f) Immunofluorescence staining. Representative images of co-cultured graft implanted for 4weeks are observed by (g)H&E staining, (h) Masson's trichome staining, (i) Immunofluorescence staining. Representative images of human dermal fibroblast cells only cultured graft implanted for 2weeks are observed by (j)H&E staining, (k) Masson's trichome staining, (l) Immunofluorescence staining. Representative images of human dermal fibroblast cells only cultured graft implanted for 4weeks are observed by (m)H&E staining, (n) Masson's trichome staining, (o) Immunofluorescence staining. Black and white arrows indicate lumen. Scale bar = 100μm.

(B) Immunofluorescence staining images of engineered-vessel. (a)Zoom at the graft area stained by CD31 looking for the endothelial cells layer and lumen following 14days post-implantation. Cocultured-vessel construct revealed endothelial cell layer in luminal area and other multiple layer.(left) a lot of cells survival in the fibroblast cells-cultured-vessel however endothelial cells layer are observed only outer layer which contact with native tissue. Arrows indicate lumen. (b) Zoom at the graft area stained by CD31 looking for the endothelial cells layer and α-SMA showed smooth muscle-like cells following 28days post-implantation. Fibroblast cells differentiated into smooth muscle-like cells and the cells layer were detected by α-SMA in cocultured-vessel.(left) Same stained images of fibroblast cells-cultured-vessel showed survival cells on each layer.(right) Arrows indicate lumen. Scale bar = 100μm.

(C) DNA content quantification of co-cultured grafts and human dermal fibroblast cells only cultured grafts.(n=3)

IV. DISCUSSION

It is well known that, in tissue-engineered vessel field, the endothelium construct on the luminal surface.[3] Moreover the survival of small vascular graft with co-cultured cells in long term are influenced by vascularization and tissue-like structure remodeling.[12]

In this study, to determine the effect of cell-cell interactions between fibroblast cells and endothelial cells, we designed co-culture system using electrospun PLCL nano-fiber for scaffold. As we know, in the tissue-engineered blood vessel study, endothelial cells are critical factor. Not only that anti-thrombotic property of endothelium but also remodeling of tissue and vessel structure on the tissue-engineered vessel and moreover the survival of vessel after implantation environment.

Using the nano-fibrous membrane, we are looking for that the elastic properties were up-regulated and provide the similar environment to native extra cellular matrix (ECM) for cells attachment and proliferation. Moreover we found that PLCL electrospun scaffold maintain the phenotype of ECs and Fb cells and permeability of nano-fibrous membrane have an effect on HUVECs were contact with co cultured HDFs.[3] Moreover the SEM images of PLCL nano-fibrous scaffolds showed that surface of scaffold have a porous network which make the scaffold have a permeability of nutrients and other substance.(fig.2) Therefore we are looking for that PLCL scaffolds are supply more proper environment for cells survival.

For investigating the effect of scaffold with HDFs layer in cultured for different term, we designed 2D co-culture experiment which means in-direct contact condition. In an indirect contact co-culture of fibroblasts cells and endothelial cells, it found that confluent HDFs layer influenced the HUVECs tube-like structures. (fig.3 and4) It has already described in other biomaterials systems that fibroblast cells actively promote vascularization through secretion of angiogenic growth factors or through differentiation into mural cells which provide direct physical contact to ECs.[12-16]

It can be demonstrated by paracrine method that the VEGF secreted into medium by fibroblasts may affect the HUVEC angiogenesis.[7]

However, indirect contact co-cultures could not maximally mimic the in vivo situation, while the direct contact co-cultures can be used to determine the mechanisms of cell-to-cell interactions, including junction communications and secretion of paracrine factors. [17] For observation about that

direct cell-cell contact in a co-culture system, we produced the 3D construct of vascular graft with HDFs and HUVECs in vitro static culture and in vivo situation. We expect that understand an important role in the communication of cells in co-cultured in direct contact. Thus, direct contact of HDF and HUVEC appears necessary to confirm the effects in the communications between HDF and HUVEC for angiogenesis.[7]

In vitro static culture conditions, It was observed that the nano-fibrous scaffold with fibroblast layer supported HUVECs survival in the middle of vessel structure after 1, 7days in culture. However, after being static culture for 14days, there was few cells survival in the middle part of co-cultured vascular graft. This examination described the only cells in a lumen and outer layer survival after 1 month in static culture. Also the tubular structure did not maintain after 1month in static culture. This results are examined by immunocytochemical analysis and histology analysis.(fig.6-9)

To measure the mechanical properties of the small diameter vascular graft, we do some experiment such as burst pressure test, tensile strength test. The vascular graft in static culture for 14days showed that they have higher mechanical properties. However, after 1 month of static culture, mechanical properties down-regulated which are similar results from histology analysis.(fig.10)

From the results of 3D structure of cells-biopolymer vessels examination, we considered that in vitro conditions are not enough to survival for cells in the middle portions of vessel structure in 1month.

And then we produced two type of vascular graft which were made of PLCL membrane with co-culture of HUVECs and HDFs(co-cultured vessel) and PLCL membrane with mono-culture of HDFs(mono-cultured vessel) looking forward to investigated the effect of direct contact between HDFs and HUVECs in vivo situation. After 1day in static condition, the vascular grafts were implanted in the subcutaneous area of nude mice. Post- implantation in 2weeks and 4weeks, the vessels were examined by histological analysis. The in-vivo model described here provides a suitable assay for observation of co-culture system generated cellular infiltration into the multilayer of PLCL fibers and also collagen depositions were observed in the outer area of co-cultured vessel by post operated 4weeks. Moreover EC layer reconstructed in each layer surface(CD31) and fibroblast cells differentiated into smooth muscle-like cells and the layer were detected by α -SMA in co-cultured

vessel.(fig.11) It has already described in other biomaterials systems that fibroblast cells differentiated into smooth muscle-like cells which provide direct physical contact to ECs.[12]

Ⅴ. CONCLUSION

We described here an elctrospun nano-fibrous PLCL scaffold with co-culture system of HDFs and HUVECs to improve the cells survival and remodeling the vessel structure of small diameter vascular grafts. From 2D and 3D co-culture experiment, direct contact of HDF and HUVEC appears necessary to confirm the effects in the communications between HDF and HUVEC for angiogenesis. Therefore we are looking for that PLCL scaffolds are supply more proper environment for cells survival in culture.

REFERENCES

1. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249-57.
2. Soo Hyun Kim, Sang-Heon Kim, young ha kim. Tissue Engineering for Blood Vessel. J Tissue Eng Regen M 2006;Vol.3, No.1:13-20.
3. Haifeng Liu a, Xiaoming Li a, Gang Zhou a, Hongbin Fan b, Yubo Fan a. Electrospun sulfated silk fibroin nanofibrous scaffolds for vascular tissue engineering. Biomaterials 32 2011; 3784-93.
4. Malda J, Rouwkema J, Martens DE, Le Comte EP, Kooy FK, Tramper J, et al.Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs:measurement and modeling. Biotechnol Bioeng 2004;86:9–18.
5. Wei Tan, Tejal A. Desai. Microscale multilayer cocultures for biomimetic bloodVessels. J Biomed Mater Res 2004; 72A:146–160.
6. Wu HC, Wang TW, Kang PL, Tsuang YH, Sun JS, Lin FH. Coculture of endothelial and smooth muscle cells on a collagen membrane in the development of a small-diameter vascular graft. Biomaterials 2007; 28:1385–92.
7. H. Li , J. Chang a. Bioactive s0ilicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect. Acta Biometer 9 2013;6981–91.
8. Sorrell J.M, Baber M.A, Caplan A.I. A Self-Assembled Fibroblast-Endothelial Cell Co-Culture System That Supports in vitro Vasculogenesis by both Human Umbilical Vein Endothelial Cells and Human Dermal Microvascular Endothelial Cells. Cells Tissues Organs 2007;186:157–68.
9. Liu VA, Bhatia SN. Three-dimensional photopatterning of hydrogels containing living cells. Biomed Microdevices 2002;4:257–66.
10. Li N, Tourovskaia A, Folch A. Biology on a chip: Microfabrication for studying the behavior of cultured cells. Crit Rev Biomed Eng 2003;31:423–88.
11. Chiu DT, Jeon NL, Huang S, Kane RS, Wargo CJ, Choi IS, et al. Whitesides GM. Patterned deposition of cells and proteins onto surfaces by using three-dimensional microfluidic systems. Proc Natl Acad Sci USA 2000;97 : 2408-13.

12. Ayelet Lesman, Jacob Koffler, Roee Atlas, Yaron J. Blinder, Zvi Kam, Shulamit Levenberga. Engineering vessel-like networks within multicellular fibrin-based constructs. Biomaterials 2011 : 7856-69.
13. Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, DarlandDC, et al. Engineering vascularized skeletal muscle tissue. Nat Biotechnol 2005;23:879-84.
14. Caspi O, Lesman A, Basevitch Y, Gepstein A, Arbel G, Habib IH, et al. Tissue engineering of vascularized cardiac muscle from human embryonic stem cells. Circ Res 2007;100:263-72.
15. Kunz-Schughart LA, Schroeder JA, Wondrak M, van Rey F, Lehle K, Hofstaedter F, et al. Potential of fibroblasts to regulate the formation of threedimensional vessel-like structures from endothelial cells in vitro. Am J Physiol Cell Physiol 2006;290:C1385-98.
16. Chen X, Aledia AS, Ghajar CM, Griffith CK, Putnam AJ, Hughes CC, et al. Prevascularization of a fibrin-based tissue construct accelerates the formation of functional anastomosis with host vasculature. Tissue Eng Part A 2009;15: 1363-71.
17. Cenni E, Perut F, Baldini N. In vitro models for the evaluation of angiogenic potential in bone engineering. Acta Pharmacol Sin 2011;32:21–30.

Abstract(in Korean)

PLCL 의 전기방사를 이용하여 개발한 지지체에 Human Umbilical Vein Endothelial Cells 과 Human Dermal Fibroblast Cells 의 Co-culture system 을 이용한 소구경 인공혈관의 개발.

<지도교수 박종철>

연세대학교 대학원 의과학과

이보람

인간에게 있어서 심장, 혈관과 혈액으로 구성된 순환기관의 역할은 매우 중요하다. 구성 요소 중 한 가지 만이라도 그 기능을 상실하게 되면 몸에 큰 이상을 가져오기 때문에, 그 기능을 대체할 수 있는 대용물의 개발과 연구가 끊임없이 지속되어 오고 있다.

혈관은 내경의 크기에 따라 대구경 혈관(>6mm)과 소구경 혈관(<6mm)으로 나뉜다. 임상용 소구경 동맥용 인공혈관은 아직 개발되어지지 않고 있다. 현재 소구경 인공혈관의 한계점은 항혈전성과 탄성력이 취약한 점, 감염, 비성장성을 들 수 있다.

이러한 한계점을 극복하기 위하여 본 연구에서는 생 분해성 고분자인 Poly(L-lactide-co-ε -caprolactone)(PLCL)(50:50)을 nano-fibrous 형태로 전기방사한 지지체에 혈관 내피 세포과 섬유아세포를 공배양(co-culture)하여 조직공학적인 방법으로 생체 적합성을 지니는 혈관을 제작 할 예정이다.

본 연구의 궁극적인 목표는 소구경 혈관의 형태와 구조와 흡사하게 인공혈관을 제작 하는 데에서 그치는 것이 아니라 더 나아가 혈관 자체의 생존과 조직의 재생을 돕는 조건을 찾는 것을 목표로 하고 있다.

각 세포들의 간접 접촉 환경에서 섬유아세포가 혈관 내피세포의 배양 형태에 미치는 영향에 대하여 실험한 결과, 7 일간 섬유아세포를 배양한 뒤에 혈관 내피 세포를 공배양 하였을 때 관 모양으로 배양되는 것을 관찰하였다.

또한 혈관 내피세포와 섬유아세포의 직접 접촉 환경에서의 영향을 확인하기 위해 3D 구조의 원통형태로 혈관 구조물을 만들어 in vitro 환경에서 실험한 결과, 14 일간 배양하였을 때 가장 좋은 결과를 나타내었고 28 일 이후에는 혈관 구조물의 가장 안쪽부터 세포가 괴사하는 것을 확인 할 수 있었다.

따라서 in vivo 환경에서 인공혈관의 재형성 및 생존 등을 확인하기 위해 2 주,4 주 동안 배양한 결과, 세포의 공배양을 통하여 만든 인공 혈관에 경우에 조직의 재 형성이 일어난다는 것을 확인할 수 있었다.

핵심되는 말 : 소구경 혈관, 조직공학, 세포 공배양, 혈관 내피 세포, 전기방사