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In vitro and *in vivo* Application of PLGA Nanofiber for Artificial Blood Vessel

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Abstract: Poly(lactic-*co*-glycolic acid) (PLGA) tubes (5 mm in diameter) were fabricated using an electro spinning method and used as a scaffold for artificial blood vessels through the hybridization of smooth muscle cells (SMCs) and endothelial cells (ECs) differentiated from canine bone marrow under previously reported conditions. The potential clinical applications of these artificial blood vessels were investigated using a canine model. From the results, the tubular-type PLGA scaffolds for artificial blood vessels showed good mechanical strength, and the dual-layered blood vessels showed acceptable hybridization behavior with ECs and SMCs. The artificial blood vessels were implanted and substituted for an artery in an adult dog over a 3-week period. The hybridized blood vessels showed neointimal formation with good patency. However, the control vessel (unhybridized vessel) was occluded during the early stages of implantation. These results suggest a shortcut for the development of small diameter, tubular-type, nanofiber blood vessels using a biodegradable material (PLGA).

Keywords: bone marrow cell, differentiation, PLGA, nano fiber, artificial blood vessel.

Introduction

In worldwide, many vascular diseases such as arteriosclerosis, angina pectoris and stroke lead to sudden death of many people. The necessity of artificial blood vessel arose from the difficulties of surgical treatment and pharmaceutical therapy for coronary artery and peripheral vascular disease in above mentioned patients. Additionally same needs were required in the case of the replacement of damaged vessel by various accidents.

Satisfying these clinical requirements in polymeric artificial blood vessel, so many researchers have been concentrating their great efforts by way of developing the tissue engineering tools using decellularized tissues, synthetic biodegradable polymers, cell sheets and biopolymers for scaffold.[1-6] However, such tissue engineered vascular grafts using biodegradable polymer have some disadvantages as overcoming cell origin and supply for ensuring the sufficient amounts of covering the surfaces of artificial vascular graft

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which contact with blood after implantation and avoiding the unendurable patient's pains from surgical treatments. Such problems in tissue engineering tool using patient's own cells can be solved by the adaptation of stem cell (bone marrow cell in this study) as endothelial cell (EC) and smooth muscle cell (SMC) source. [7-13]

In spite of its own good biocompatibility and biodegradability, PLGA scaffolds for tissue engineering made of salt-foaming, salt-leaching, emusion-lyophilization, high pressure gas expansion and phase separation methods can not ensure the enough mechanical strength enduring surgical operation and intricate 3-dimensional structure. Therefore, under the consideration of weak process ability of PLGA, overcoming above weakness of PLGA keeping its original biodegradability and biocompatibility is very important point of developing PLGA blood vessels sustaining its own elasticity and process ability.

Electro spinning of PLGA solution is the unique method of making tube type scaffold for artificial blood vessel which has enough mechanical strength and easy controllability of its diameter, structure and alignment for vascular vessel.[14-18] And after implantation, electro spun tube type nano fiber vessel expected to have good transporting ability of nutrients and oxygen for the living cells because of large surface area, gap ratio and flexibility.

In this study, we made small diameter (~5 mm) PLGA tube through electro spinning method and applied scaffolds for artificial blood vessel by way of the hybridization of smooth muscle cell and endothelial cell differentiated from canine bone marrow under the pre-reported conditions.[13] By the animal experiment using dog, the possibility of artificial blood vessel in small diameter was investigated.

Experimental

Materials and Equipments. PLGA(L-Lactide:glycolide; 7:3) was kindly supplied from META Biomed Co., Ltd. (CheongJu, Korea) and its molecular weight was measured by Waters GPC system, composed of Shodex KD-803 and 802.5 column (Tokyo, Japan) and RI 101 detector, using *N*,*N*-dimethlyformamide (DMF) as eleuent (1 mL/min) and PEG as molecular weight standard. High voltage supplier for electro spinning was purchased from Nano NC Co., Ltd. (Siheung, KyoungGi, Korea). Fibronectin (FN), collagen and fetal bovine serum (FBS) were purchased from Sigma Chemicals Inc.(St. Louis, MO, USA). Ficoll-Paque™ Plus for the separation of lymphocytes from canine bone marrow was purchased from Amersham Biosciences (Uppsala, Sweden).

EGM-2 (Media for cell culture; containing of EBM-2 500 mL, FBS 10 mL, hydrocortisone 0.2 mL, hFGF-B 2 mL, VEGF 0.5 mL, R^3IGF-1 0.5 mL, ascorbic acid 0.5 mL, hEGF 0.5 mL, GA-1000 0.5 mL, heparin 0.5 mL) and Medium 199 (containing Earle's salt and L-glutamine) were purchased from Cambrex Co.(East Rutherford, NJ, USA) and Gibco (Gaithersburg, USA) respectively. Penicillin-streptomycin solution was purchased from Jeil Biotech Services Inc. (Daegu, Korea). FITC conjugated antibody mouse monoclonal anti-CD 146 was purchased from Chemicon International Inc. (Temecula, CA, USA), mouse monoclonal anti-α-smooth muscle actin (α-SMA, primary antibody), goat anti-mouse IgG (biotinylate secondary antibody) and 3-amino-9-ethylcarbazole (AEC) chromogen were obtained from Sigma Chemicals Inc. (St. Louis, MO, USA).

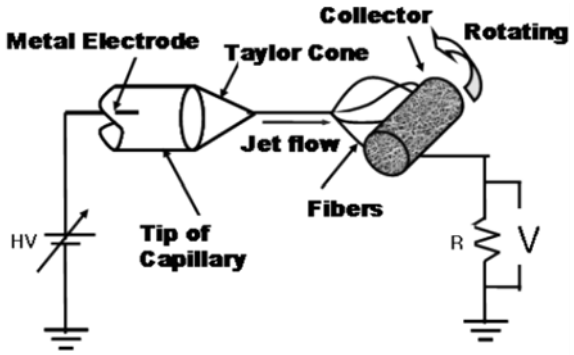

Figure 1. Schematic diagram for electro spinning of PLGA solution in this study.

Manufacture of Blood Vessel. 1 g of PLGA was dissolved in 5 mL of DMF and located in syringe equipped with 23G needle. This syringe was mounted on the syringe pump (KDS 100, KD Scientific, USA) connected with electro spinning machine. Then PLGA solution was electro spun with 2 mL/h under 10 KV of supplied direct voltage between syringe needle and sample collector (Figure 1). After spinning for predetermined time interval, tubes of PLGA nano fiber were obtained with various diameters and length. Before applying to cell culture experiment and animal study, tubes of PLGA nano fiber were sterilized with 70% EtOH aqueous solution. The thickness of PLGA artificial blood vessel was about 300 nm.

Cell Isolation and Differentiation from Bone Marrow.[13] Bone marrow (30 mL from each dog) was aspirated from the humeral of mongrel dogs (male, 20-25 kg) and mixed with same volume of phosphate buffered saline solution (PBS). Mixed bone marrow cells (BMCs) were isolated using a Ficoll-Paque™ Plus density gradient (BMCs: PBS: Ficoll-Paque = 1:1:1.5 in volume) from the buffy coat layer between the Ficoll-Paque reagent and blood plasma component by centrifuge (2,000 rpm, 30 min). Isolated bone marrow mononuclear cells (BMMNCs) were centrifuged again with PBS for 10 min at 2,000 rpm, then washed 3 times in PBS to remove remaining Ficoll-Paque™ Plus reagents. BMC fraction for endothelial cell (EC)-like cells was cultured in EGM-2 (1% penicillin-streptomycin) on fibronectin (1 μg/cm^2) coated TCPS and BMC fraction for smooth muscle cell (SMC)-like cells was cultured in Medium 199

containing 10% (vol/vol) FBS on collagen (1 μg/cm^2) coated TCPS.

Differentiated ECs and SMCs from cultured BMCs were confirmed by immunochemical staining method using FITC conjugated mouse anti-CD146 monoclonal antibody (Ab) and mouse anti-α-smooth muscle actin (α-SMA) monoclonal antibody (primary Ab)/biotinylate goat anti-mouse IgG (secondary Ab) / AEC chromogen respectively.

Cultured and differentiated cell morphologies were observed by optical microscopy (CK40, Olympus, Tokyo, Japan) and fluorescently labeled cells were analyzed under a fluorescent microscope (Axopphot 2, Carl Zeiss, Germany).

Application of PLGA Blood Vessel *in vitro* Cell Culture. For the purpose of cell adhesion on the surfaces of PLGA tubes, ECs were seeded on the inside of fibronectin coated (1 μg/cm^2) PLGA tube (5 mm in diameter; Layer A) at a cell density of 1 × 10^7 cell/mL and cultured under the conditions of EGM-2 media containing 10% FBS and l% (wt/vol) penicillin and streptomycin at 37 °C, 5% CO_2 for 10 days. SMCs were seeded on the inner side of collagen coated (1 μg/cm^2) PLGA tube (6 mm in diameter; Layer B) at a same cell density and cultured under the same conditions above mentioned. The culture medium was changed in every 2 days.

For the investigation of time dependent cell adhesion behaviors on PLGA scaffold *in vitro*, PLGA tubes were fixed with glutaraldehyde solution and dehydrated with graded EtOH at 12 h, 1, 2, 4 and 7 days after cell seeding and observed adhered cell morphology by SEM (JSM 7000F, JEOL, Tokyo, Japan). The cell seeded grafts were maintained *in vitro* in Medium 199 supplemented with 10% (vol/vol) FBS for 1 week prior to implantation.

Application of PLGA Blood Vessel in Animal Model.[13] Bone marrow donor mongrel dogs (20-25 kg) were anesthetized with injection of intramuscular ketamine (30 mg/kg) and intravenous pentobarbital (30 mg/kg) and ventilated with a mixture of O_2, N_2, and isoflurane during the operation. Through a longitudinal midneck incision, common carotid arteries were exposed. Prior to arterial clamping, heparin (100 unit/kg; Choongwae Pharma Co, Seoul, Korea) was administered intravenously.

As shown in Figures 2 and 3, the combined artificial blood vessel having different inner diameter (5 and 6 mm ID, 50 mm in length for tubular PLGA blood vessel layer A and B, respectively) was placed as an end-to-end anastomosis to the common carotid arteries using a 6-0 Prolene suture (Ethicon). PLGA artificial vascular grafts without cell seeding served as controls. The implanted blood vessel patency was monitored in 3 weeks after implantation by the arterial digital subtraction angiography. All care and handling of the animals were provided according to the Guide for the Care and Use of Laboratory Animals of Yonsei University.

Explant Characterization. Midportion segments of the implanted blood vessel were fixed with 10% (vol/vol) formaldehyde solution and dehydrated with a series of ethanol aqueous solution. The samples were fixed in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Excreted elastin and active collagen in the extracted tissue

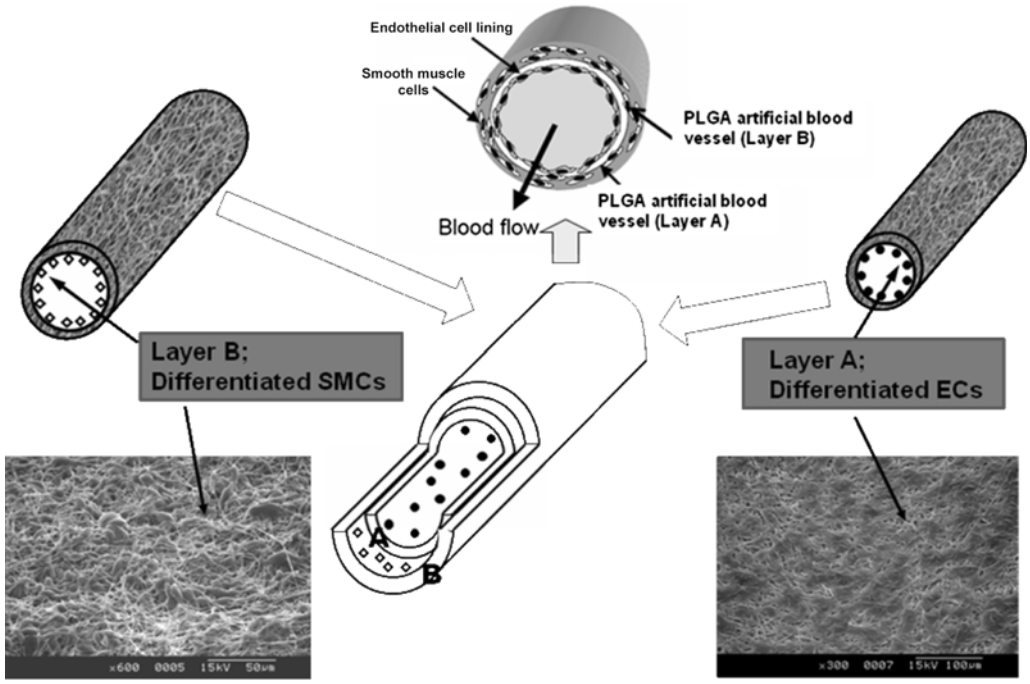

Figure 2. Structure and components of dual layer type artificial blood vessel composed of PLGA nano fiber and differentiated cells (EC and SMC) from canine bone marrow.

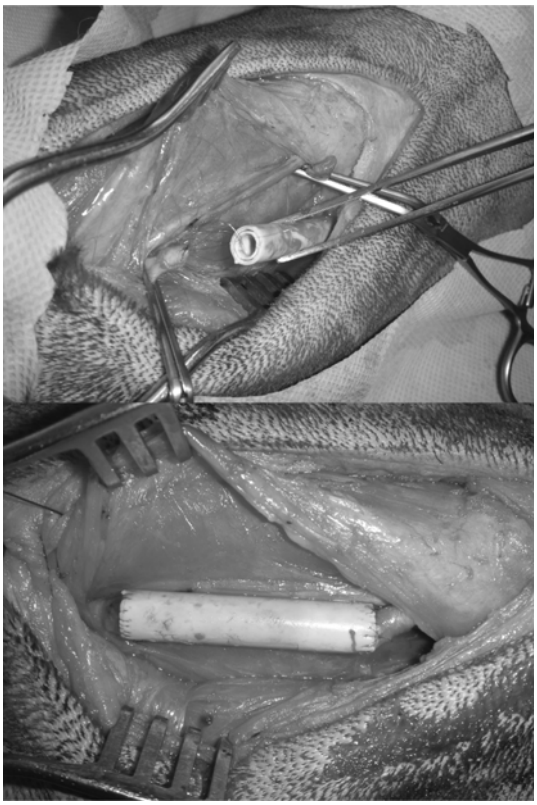

Figure 3. Photographs of surgery for artificial blood vessel implantation.

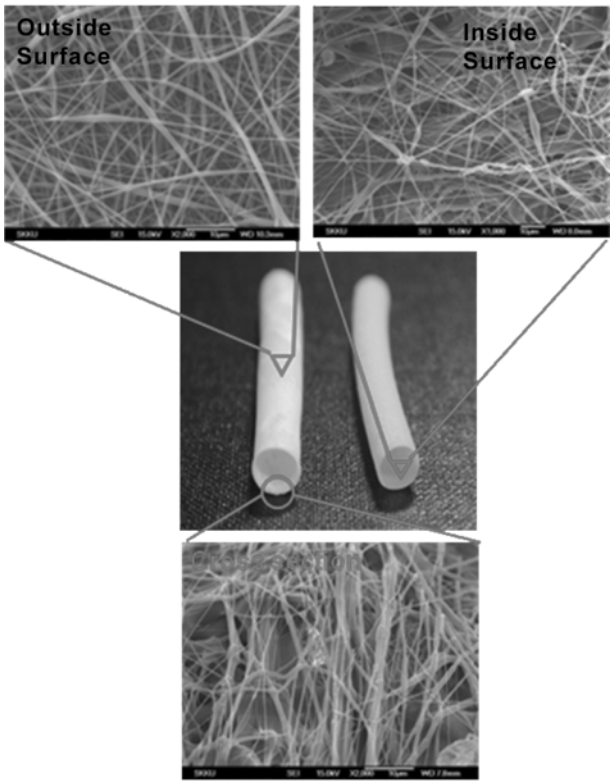

Figure 4. Surface morphologies of micro structures for synthesized tube type blood vessel of PLGA nano fiber.

sections was confirmed by well known staining tools with van Gieson and Masson trichrome method.

Results and Discussion

Physical and Chemical Characteristics of PLGA Scaffolds. The molecular weight of PLGA measured by GPC was 130,000 in number averaged molecular weight. As shown in Figure 4, electro spun tubular type nano fiber of PLGA blood vessels were shown relatively flexible tube shape and connected open pored structure, and their mean diameter of nano fibers was 800 ± 21 nm. Their mechanical strength was 323 MPa (tensile modulus) and about 100 times of that of natural blood vessel in human.[19,20] Therefore this type of PLGA blood vessel was sufficient for implantation through surgical treatment in animal model.

Validation of Endothelial Cells and Smooth Muscle Cells. Cell morphology of ECs and SMCs after 2-4 passage of primary culture was well keeping their own shape (Figures 5(A) and 5(B)). Differentiated ECs and SMCs from bone marrow were also confirmed by immune staining of CD 146 and α-SMA for EC and SMC respectively as the results of Figures 5(C) and 5(D) (fluorescence lighting for ECs and red color expression for SMCs after differentiation). These results showed that ECs and SMCs for artificial vascular graft through tissue engineering tool can be obtained from bone marrow.

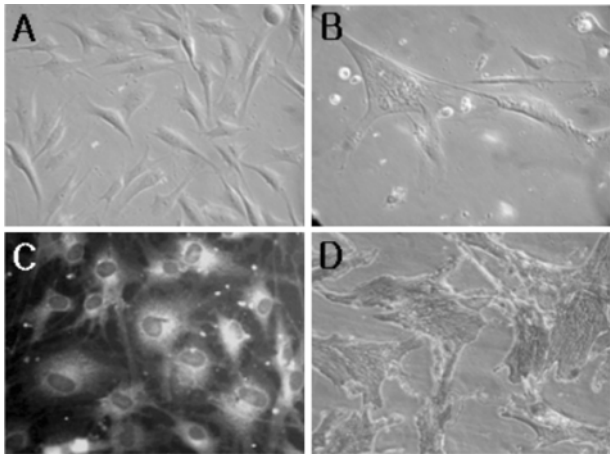

Figure 5. Cell morphologies of differentiated EC and SMC from bone marrow. (A) and (B); Optical microscopic pictures of EC and SMC respectively, (C); Immunochemical staining picture of EC with CD-146 antibody, (D); Immunochemical staining picture of SMC with α-SMA antibody.

***In vitro* Cell Adhesion.** By SEM observation, well adhered cells (EC and SMC) were observed on the surface of artificial blood vessel which coated with collagen (SMC) and fibronectin (EC) as previously mentioned, and more cells were found at the both edge sides of artificial blood vessel comparing to center of it and proliferated after 7 days culti-

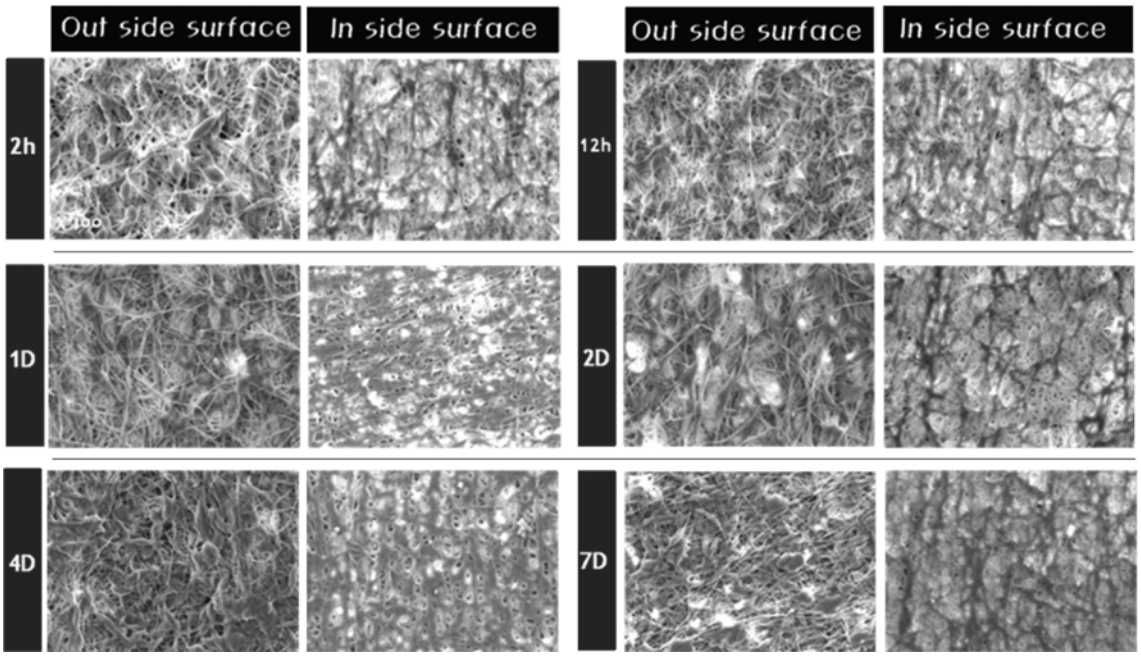

Figure 6. Cell morphologies of cultivated SMC on the surface of Layer B at predetermined culturing time for 7 days, observed by optical microscopic.

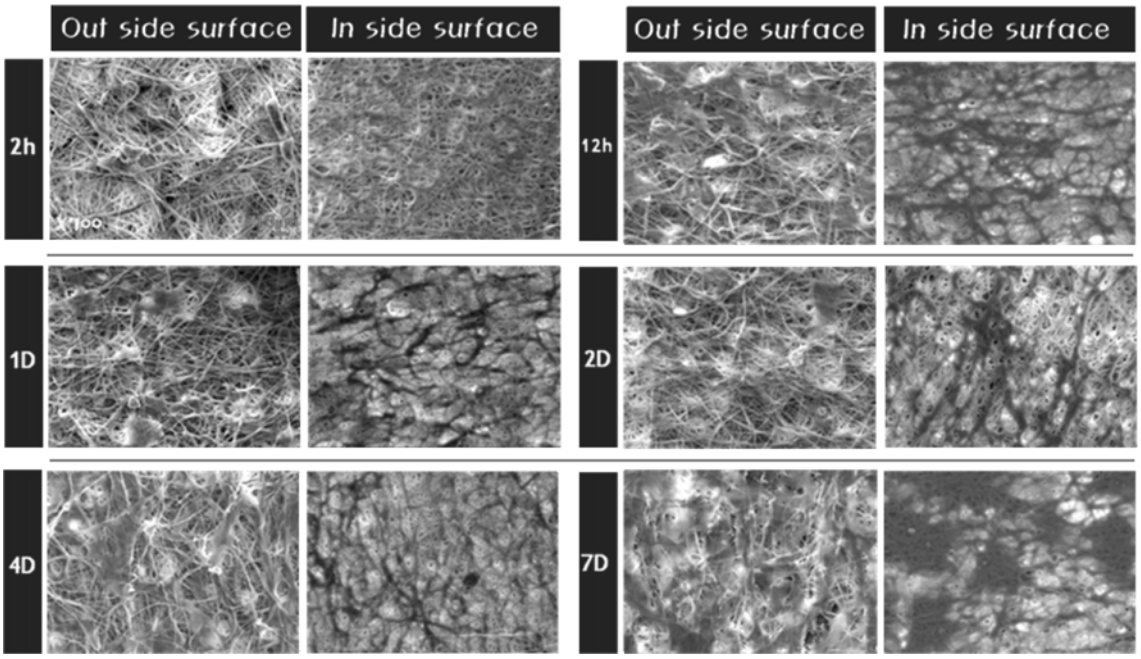

Figure 7. Cell morphologies of cultivated EC on the surface of Layer A at predetermined culturing time for 7 days, observed by optical microscopy.

vation (Figures 6, 7). In spite of SMC and EC cells were seeded inside of Layer A and B, smaller amount of SMC and EC cells were also observed outside of Layer A and B in early stage of cultivation. This means that the hydrophilic characteristics of tubular type PLGA nano fiber blood vessel enhanced cell adhesion, migration and proliferation compared to the commercialized PLGA scaffold (hydrophobic).

Vascular Tissue Regeneration *in vivo*. The H&E-stained sections showed reconstruction of the artery, with structures very similar to those of the native artery (Figure 8(H)). In the biopsy results as Figures 8(F) and 8(G) (in rectangular region), we can observe novel micro vessel in Layer A gen-

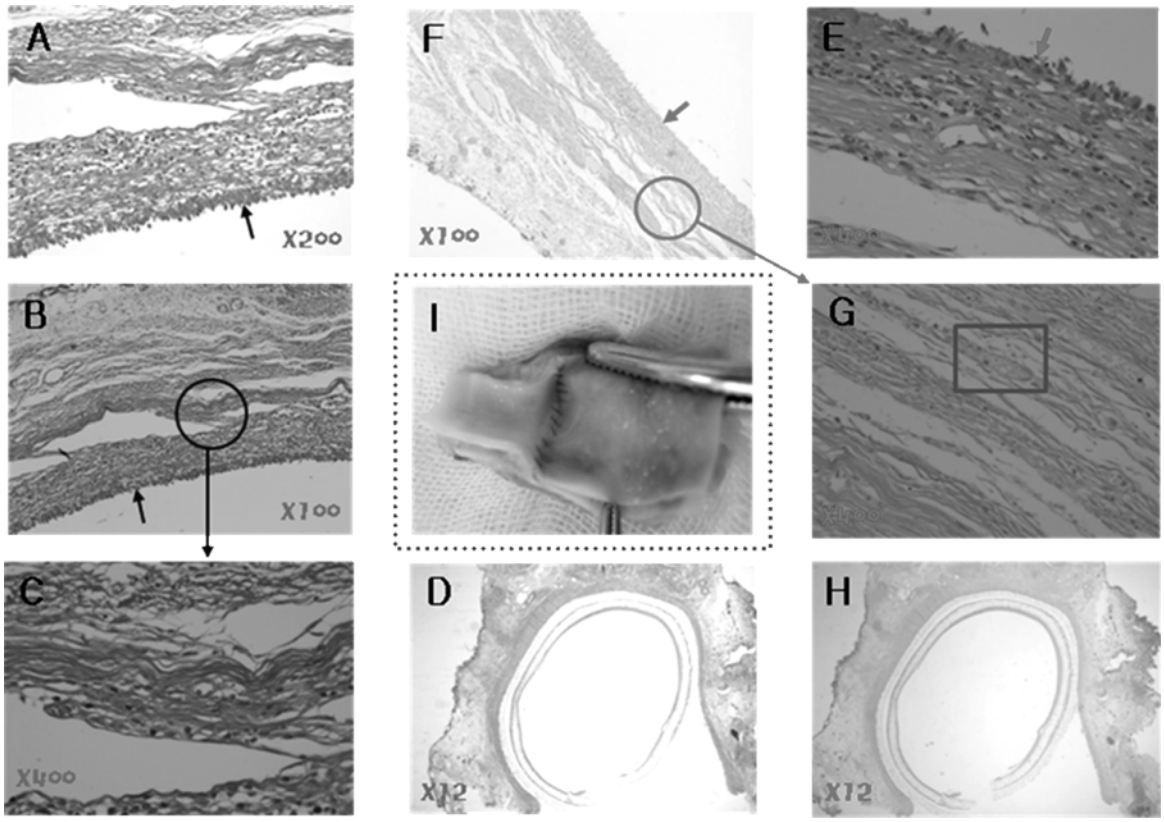

Figure 8. Histological pictures of PLGA blood vessel after implantation (3 weeks), observed by optical microscopy. (A) to (D); stained by Masson trichrome staining method, (E) to (H); stained by H & E staining method. (I); Inside morphology of explanted PLGA blood vessel after implantation.

erated endothelial cell line during biodegradation of PLGA for 3 weeks. This shows that supplies of nutrient and oxygen were fully enough for the proliferation of seeded ECs in Layer A. But in Layer B, newly formed micro vessels were not observed even after PLGA biodegradation. Meanwhile, by van Gieson method staining, elastin (one of major ECMs in artery) did not displayed internal elastic lamina and external elastic lamellae layers in the seeded blood vessel within 3 weeks (data not shown).

This was mainly due to the lack of full growth of SMC on the Layer B of artificial blood vessel. SMCs differentiated from bone marrow cells *in vitro* conditions were exposure to the *in vivo* environment without any other growth factors after implantation, but these cells were surrounded two layers of PLGA artificial blood vessel and such environment was not enough for supplying nutrients or oxygen to proliferate seeded SMCs. Additionally degraded products of PLGA by enzymatic hydrolysis during implantation period turn to be acidic materials (such as lactic acid or glycolic acid in narrow space between two layers of blood vessel) and produce bad effects to the growth of SMCs especially. Such phenomena were reported many articles[21-25] using biodegradable polymer blood vessel in animal models. In spite of the absence of elastin layer in our PLGA dual layer model vessel conduit, good patency and well reserved morphology of inside of blood vessel were obtained after 3 weeks implantation as shown in Figure 8(I).

A significant amount of collagen (the other major ECMs in artery) was present in the grafts, as evaluated by Masson trichrome staining shown in Figures 8(A), (B) and (C).

Graft Patency. To determine the graft patency, the animals were periodically investigated by arterial digital subtraction angiography after implantation. Patency of the seeded vascular grafts was significantly improved by differentiated canine bone marrow (Figures 9(E) and 9(F)). By the SEM observation of inside surface of Layer A and B, we can find out the newly formed endothelium lumen (Figures 9(C) and 9(D)) and proliferated SMC layer (Figures 9(A) and 9(B)) regenerated from differentiated cells (SMCs and ECs) from bone marrow as cell source. Such an well formed neointimal layer revealed good patency in short period (3 weeks) and could promise the possibility of long term patency of tubular type PLGA nano fiber blood vessel.

In contrast, the non-seeded vascular graft (control) showed thrombus formation and graft occlusion within 1 week (data not shown).

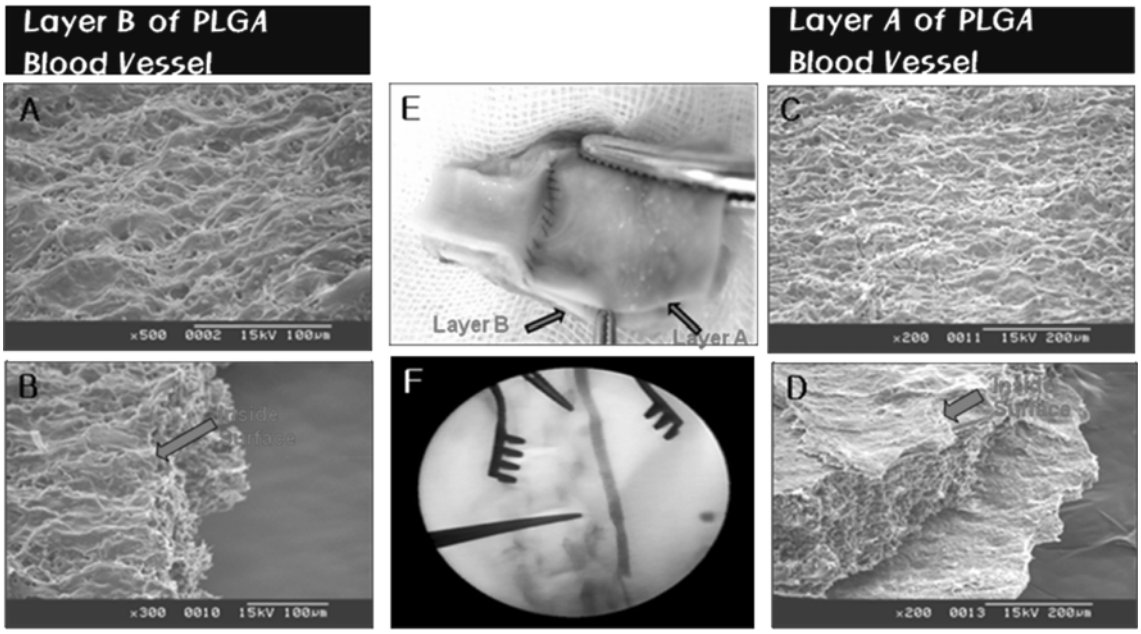

Figure 9. SEM evaluation and angiogram of PLGA blood vessel, (A) and (B); SEM pictures of Layer B of extracted PLGA blood vessel after implantation, (C) and (D); SEM pictures of Layer A of retrieved PLGA blood vessel after implantation, (E); Inside morphology of explanted PLGA blood vessel after implantation. (F); Angiogram of PLGA blood vessel 2 weeks after implantation, the vessel maintains patency without occlusion.

Conclusions

In this study, we made tubular type PLGA scaffold for artificial blood vessel by electro spinning method, and hybridized this dual layered blood vessel with ECs and SMCs differentiated from dog bone marrow. Artificial blood vessels made by tissue engineering tool were implanted and substituted with artery in adult dog for 3 weeks, hybridized blood vessel showed good patency and control vessel (unhybridized vessel) showed occlusion. These results will introduce a shortcut to develop small diameter-tubular type nano fiber blood vessel using biodegradable material (PLGA).

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