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| Original Article |

Tissue Regeneration in the Pores of Poly(lactide-*co*-glycolide)-Impregnated Wall of Expanded Polytetrafluoroethylene (ePTFE) Hybrid Grafts

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Abstract : Understanding of relationship between pore properties and tissue regeneration of expanded polytetrafluoroethylene (ePTFE) is important in design of vascular tissue engineering. Tissue regeneration into a micron scale pore of the ePTFE wall was investigated by employing techniques of superficial surface modification of ePTFE, fabrication of the hybrid scaffold composed of biodegradable poly (lactide-*co*-glycolide) (PLGA) and ePTFE, and seeding of vascular cells on its lumen surface. The ePTFE was in advance transformed into a hybrid scaffold by sequential four steps of treatments such as chemical modification of ePTFE surfaces, impregnation of biodegradable PLGA polymer into its wall pores, and coatings of both PLGA polymer on the ePTFE lumen surface and collagens on the PLGA-coated lumen surface. The hybrid scaffold was in advance *in vitro* tissue-cultured with vascular smooth muscle for 12 weeks and stem cells for another 2 weeks on its collagen-coated lumen surface, thus obtaining an *in vitro* tissue-cultured scaffold. This *in vitro* tissue-cultured hybrid scaffold was implanted in a carotid artery of mongrel dog for 4 weeks. The morphologies of the hybrid grafts explanted from the artery were analyzed by light microscopy, scanning electron microscopy and transmission electron microscopy (TEM), focusing on tissue regeneration in the modified pores of ePTFE wall. They demonstrated migration of smooth muscle cells into the PLGA-impregnated/surface-modified pores of ePTFE wall along biodegradation of impregnated PLGA polymer, leading to tissue regeneration in its surface-modified pores. TEM results of the patent hybrid grafts showed both cell organelles and extracellular matrix of the regenerated media tissues in the pore channels of ePTFE wall with 20-30 μm inter-nodal distances.

Key words: *Hybrid scaffold, ePTFE, Vascular graft, TEM*

1. Introduction

Artificial vascular graft has been clinically used to replace the lost or damaged blood vessels.[1] Even though vascular grafts with large diameter (inside diameter ≥ 6 mm) such as expanded polytetrafluoroethylene (ePTFE), poly (ethylene terephthalate) (Dacron) and polyurethane have been employed clinically, small diameter ones (inside diameter < 6 mm) have not been successful due to low blood flow rate and shear rate, leading to graft failures.[2,3] For examples, while clinically available autologous veins and artificial grafts have shown 60-70% patency, vascular grafts such as ePTFE and Dacron have done 47% and 54% patency, respectively, as femoro-popliteal bypass grafts at 5 years.[4-6] No significant extent of either trans-anastomotic or trans-mural endothelialization was not reported even in large diameter ePTFE grafts in patients, requiring development of tissue regeneration on their surfaces.[5]

Numerous methods to improve the patency of the vascular grafts have been tried such as tissue regeneration, delivery of bioactive agents and immobilization of either bioactive growth factors or passive macromolecules on their surfaces.[1,7,8] Delivery of bioactive molecules such as vascular endothelial growth factors (VEGF), heparins and basic fibroblast growth factors (bFGF) has been tried through the wall pores of ePTFE. Cell non-adhesive biocompatible macromolecules such as poly (ethylene oxide) and poly (vinyl alcohol) have been immobilized

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on its surface to repel adhesion of proteins and blood cells for development of passive grafts.[9,10] Immobilization of cell-adhesive oligopeptides such as RGD, REDV and LDV oligopeptides have been also tried to control specific cell adhesion on the graft surfaces.[11,12] Furthermore, bioactive agents such as VEGF, bFGF and heparins have been incorporated on graft surface via either chemical cross-linking or physical adsorption, and the pores of the graft walls were employed to release diverse bioactive molecules to induce tissue regeneration through neo-endothelium formation.[4,8] Seeding of autologous endothelial cells on the graft surface increased patency to more than 70% in 7 years, which was comparable to that of vein grafts.[13]

Recently, biodegradable polymers have been employed for fabrication of vascular scaffolds such as nano-fiber, poly (lactide-*co*-glycolide) (PLGA), poly (ε-caprolactone), gelatin and small intestine submucosa systems.[8,9,14,15] Peters *et al* have tried to incorporate VEGFs in the biodegradable polymers scaffolds.[16] In their work, the scaffolds were implanted into animal arteries, showing increase in neo-angiogenesis over scaffold biodegradation. Stem cells have been employed for vascular tissue regeneration to utilize their differentiations into vascular endothelial cells. Bhattacharya *et al* have seeded bone marrow stem cells on polyester grafts, showing increase in endothelialization and micro-vascularization.[17] Vascular tissue regeneration has been reported by implanting in animal the biodegradable scaffolds such as poly (lactide-*co*-ε-caprolactone) and poly (glycolic acid) with homogeneous bone marrow stem cells, showing promising results of vascular tissue regeneration.[18] These experimental results indicated that combination of biodegradable polymers, stem cells and mechanical stability were very important factors for patency of artificial grafts. At the same time, regeneration of biological tissues should be considered as another important factor for integration of regenerating media tissue with the implanted graft.

Understanding both the behaviors of tissue regeneration in the pores of ePTFE wall with 20-30 μm inter-nodal distances and the morphologies of the regenerated tissues with cellular organelles had been recognized as one of important issues in developing patent vascular grafts. Our research object was to investigate and understand a possibility of regeneration of media tissue regeneration in the micro-scale wall pores of the hybrid graft composed of both biodegradable PLGA layers and ePTFE, by focusing on the TEM observation of the tissue-regenerated pores of the chemically surface-modified ePTFE. We have previously reported several works of chemical surface modification of ePTFE by both wet chemistry and ion bombardment,[19-22] fabrication of three layers of ePTFE hybrid scaffold and its vascular tissue engineering.[23-26] Hydrophilic surface of ePTFE demonstrated advantageous properties in its applications as vascular scaffolds for tissue engineering, such as inductions of better cell adhesion as well as control of transport of bioactive molecules than those of the hydrophobic ones.[4,8,14,24]

The hybrid ePTFE scaffold was fabricated as a substrate for vascular tissue engineering by coating of biodegradable PLGA on both inner and outer surfaces of the modified ePTFE, by impregnating PLGA into its surface-modified pores. We utilized the properties of hybrid graft scaffolds for tissue engineering such as surface modification, and combination of biodegradable PLGA for tissue regeneration and non-biodegradable polymers. We here reported the behaviors of media tissue regeneration in the PLGA-impregnated wall pores of ePTFE grafts, and their morphologies by TEM. Overall schematic of this work is described in figure 1.

2. Materials and Methods

2.1 Materials and Chemical Agents

Expanded polytetrafluoroethylene (ePTFE) with 4 mm inside diameter and 20-30 μm inter-nodal distance (Gore Associate; AZ, USA) has been employed as a substrate for fabrication of hybrid vascular graft. Poly (lactide-*co*-glycolide) (PLGA, 75:25), benzophenone, sodium hydride (95%), dioxane, ammonium bicarbonate were purchased from Sigma-Aldrich

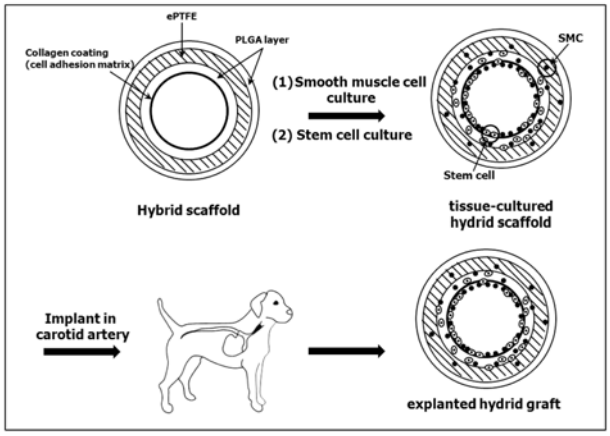

Figure 1. Schematics of media tissue regeneration of hybrid scaffold fabricated with both biodegradable PLGA and non-biodegradable ePTFE graft. After *in vitro* tissue culture of the hybrid scaffold with allogenic SMC for 12 weeks (tissue-cultured hybrid scaffold), autologous bone marrow derived mesenchymal stem cells were *in vitro* tissue-cultured on the SMC tissue-cultured surface for another 2 weeks (tissue-cultured hybrid scaffold with stem cells). Patency of the tissue culture scaffold was evaluated after implant of the *in vitro* tissue-cultured graft in a carotid artery of mongrel dog for 4 weeks (explanted hybrid graft).

Chemical Co. (St. Louis, USA). Dulbecco's modified Eagle medium (DMEM) was purchased from Lonza Korea (Korea). Smooth muscle cells (< passage 7) and bone marrow stem cells (< passage 5) were obtained from carotid artery and marrow of mongrel dogs (age 4, Okgye Farm, Korea), respectively.

2.2 Fabrication of ePTFE Hybrid Vascular Scaffold

Both superficial surface modification of ePTFE and fabrication of ePTFE hybrid scaffold have been reported in previous works in detail.[19-25] In brief, the ePTFE graft has been chemically surface-modified by irradiating ultraviolet light with 366 nm wavelength on the benzophenone (0.86 g) solution containing ePTFE samples in dimethylformamide (80 mL).[19] The treated ePTFE samples (L = 5 cm) were washed with water and then dried under vacuum overnight. 5% PLGA solution with ammonium bicarbonate particles (250-350 μm) were filtered through and then impregnated into the micro-scale pores of the surface-modified ePTFE by using a disposable syringe with one end blocked with a hemostat. Next, the inside and outside surface of the PLGA-impregnated ePTFE samples were coated with the same PLGA solution. After removal of organic solvent in the hybrid scaffolds under vacuum, micro-pores were generated in the biodegradable PLGA layers of the hybrid graft wall by gas forming of ammonium bicarbonates in 37°C water bath for 2 hrs. The hybrid ePTFE scaffolds were dried in vacuum oven (Dae-il Tech, Korea), thus obtaining porous PLGA both in the pores and on the surface layers of the ePTFE hybrid scaffold.[23-25]

2.3 Isolation and Culture of Smooth Muscle Cells

After explant of the carotid artery of mongrel dogs, redundant tissues were removed from the artery by using forceps and blade, and then rinsed with sterile phosphate buffered solution (PBS) in clean bench. The open artery piece (1 cm × 1 cm) was treated with 0.2% collagenase (Sigma Co., USA) for its digestion for 7-10 days. Collection of smooth muscle cells was processed with DMEM containing 10% fetal bovine serum (FBS; Gibco, Australia) and 1% solution of penicillin, streptomycin and glutamine in 5% CO_2 incubator for 7 days. *In vitro* passages were processed, thus obtaining smooth muscle cells of carotid artery. Smooth muscle cells (7^{th} passage) were used for *in vitro* experiment, and analysis of smooth muscle cells followed the methods in our previous report elsewhere.[21, 22]

2.4 Separation and Culture of Autologous Bone Marrow Stem Cells

After mixing PBS and bone marrow from mongrel dog (1:1), the mixture solution was separated with a centrifuge (Hanil Science, Korea) at the rate of 2,500 rpm for 30 min in a 50 mL tube, containing 50% Ficol histopaque-1077 solution (Sigma Co., USA). Another separation process was performed at 1,700 rpm in 4°C for 5 min after washing with the opaque mononuclear cell layers from the previously centrifuged solution. After exclusion of suspended solution, mononuclear cells were added in a mixture solution of 85% DMEM, 10% FBS and 5% antibiotics. Cell dispersion and counting followed and *in vitro* cell culture was performed in 20 mL medium in T-75 culture flask, thus obtaining 2×10^7 cells per flask.

2.5 *In vitro* Tissue Culture

We followed the method of *in vitro* tissue culture of the ePTFE hybrid scaffold reported in our previous papers in detail.[19-22] The hybrid scaffold was in advance sterilized in autoclave. After loading 0.5% water-soluble collagen solution (Bioland, Korea) on the lumen of the sterile ePTFE hybrid scaffold, homogeneous *in vitro* coating was obtained by rotating the prepared samples in a 15 mL conical tube with a rotator (Heidolph, Germany) in 5% CO_2 incubator (Sanyo, Japan) for 1 d. Smooth muscle cells ($p < 7$, 1×10^6 cells/cm^2) from the carotid artery of mongrel dog (age 4) were seeded on the collagen-coated lumen of the hybrid ePTFE scaffold. Homogenous cell adhesion was tried by rotating the cell-seeded samples 90° every 6 hrs for 1 d. *In vitro* cell culture was processed in a 37°C, 5% CO_2 incubator by rotating the sample-containing centrifuge tube at a rate of 50 rpm. Another set of *in vitro* culture was performed with bone marrow stem cells (1×10^6 cells/cm^2) for 2 weeks by employing the same culture procedure as that of smooth muscle cells, thus obtaining tissue-cultured hybrid scaffolds.

2.6 Fluorescence-activated Cell Sorting (FACS) analysis

The cells obtained with 2 mL trypsin-EDTA solution (Lonza, Switzerland) were added in 10 mL medium and then centrifuged at 1,200 rpm for 5 min. The cell aggregates submerged were suspended after removal of upper layer. The aggregates were divided into 1×10^5 cells in 1 mL and then centrifuged at 1,500 rpm for 2 min. After removal of the upper layer of the aggregate solution, the cells were centrifuged in 1 mL PBS with 1% bovine serum albumin (BSA) (Sigma-Aldrich Co., USA) at 1,500 rpm for 2 min. After repeated removal of the upper layer solution, 1 mL PBS containing 1% BSA was loaded. The solution was stored in dark room after addition of fluorescein isothiocyanate (FITC) conjugated with CD34 antibody.[26] The obtained solution was mixed with 1 mL PBS buffer with 1% BSA and then centrifuged at 1,500 rpm for 3 min. 1 mL PBS buffer with 0.1% paraformaldehyde (Sigma Co., USA) was

mixed with the cells in dark environment after removal of the upper layer solution. The cells were analyzed for stem cells by FACS (BD, FACS Calibur™, USA).

2.7 Verification of Smooth Muscle Cells

Smooth muscle cells obtained from the carotid artery of mongrel dogs was stained with anti-smooth muscle-α actin antibody according to the protocol suggested by the vendor (Sigma; St. Louis, MO, USA). The cells were verified by reacting with anti-smooth muscle-α actin antibody for staining of cytoplasm and nucleus with gray and pale purple, respectively, with hematoxylin.

2.8 Cell Proliferation Assay

The number of smooth muscle cells was counted with cell count kit-8 (CCK-8; Dojindo Lab, Japan) by employing 1 mL medium containing both 900 μL culture medium and 100 μL water-soluble tetrazolium salt-8. 100 μL CCK-8 solution was inserted into 900 μL DMEM, and then the cell culture proceeded in the 5% CO_2 incubator at 37°C for 2 hrs. After aliquoting 100 μL medium with CCK-8 solution into a 96 well plate, an optical density of the obtained medium solution was measured at a wavelength of 450 nm by the micro-plate reader (Tecan, Australia).

2.9 Animal Experiment

In vitro tissue-cultured hybrid scaffolds (L = 5 cm) were implanted in the carotid artery of mongrel dogs (age 4; Okgye Farm, Korea). Experimental procedures were described in our previous report.[26] In brief, all animals were treated as below in the Korea University School of Medicine. Fourteen mongrel dogs (20-30 kg) were assigned randomly to the control (ePTFE grafts, n = 6) and experimental groups (tissue-cultured hybrid scaffolds, n = 8). The left carotid artery was exposed under general anesthesia. After an 8 mm ultra-sound probe connected to a transit time flow meter (Quantix/ORTM; Cardiosonix Ltd., Ra'anana, Israel) was placed directly on the carotid artery, hemodynamic parameters (diameter, flow rates, pulsatility index, shear rate, resistance index, and systolic/diastolic ratio) were measured as reported in our previous paper.[26] Heparin (1.0 mg/kg) was administered before the carotid artery was clamped. After resection of a 5 cm carotid artery, graft anastomosis was performed with a continuous 6-0 prolene suture in an end-to-end fashion. After anastomosis, hemodynamic parameters were measured at the native carotid artery of the distal anastomosis.

Low molecular weight heparin (150 units/day) and warfarin (5 mg/day) were administered to both groups from the day of the surgery to the third post-operative day. Warfarin (5 mg/day) was then administered from the fourth postoperative day to the explant day. The anastomosis portion containing both host vessels and tissue-regenerated scaffolds were harvested for observation at 4 week after implantation. From now, the explanted tissue-cultured scaffolds are called as "explanted hybrid grafts".

2.10 Hematoxylin & Eosin Y (H&E) Stain

The explanted hybrid grafts were visualized by light microscopy for observation of nucleus, extra-cellular matrix formation and their distributions after staining with H&E. The stain was performed in a routine procedure after the explanted hybrid grafts were processed with 10% neutral buffered formalin for 1 day and then stored in a refrigerator in PBS.[24-26] After paraffin embedding, 5 μm slide sections were prepared (Tissue Embedding; Leica: Germany) with a microtome (RM2245; Leica, Germany). The sections were treated with xylene and graded ethanols, and then stained with H&E. The stained sections were visualized with a light microscopy (CK40-F200; Olympus: Japan) and their images were stored via an image processor (UTHSCSA Image Tool 3.0) supplied by the Samwoo Science Co. (Seoul, Korea).

2.11 Scanning electron microscope (SEM)

Explanted hybrid grafts were sputter-coated with gold in plasma for 60 sec, thus forming approximately 200 Å thickness, as reported in our previous paper.[20] The samples fixed on an aluminum stage were inserted into a vacuum chamber of SEM (JSM-6400; JEOL Ltd, Japan). Morphological images of the pores, outer and inner surfaces of the samples were observed by magnifying 10 - 1,000 times under vacuum.

2.12 Transmission Electron Microscope (TEM)

The vascular tissue blocks were fixed in a mixture solution containing 4% formaldehyde and 1% glutaraldehyde in 0.1 M PBS (pH 7.4) for at least 2 hrs at room temperature, and then washed in 0.1 M PBS three times per 15 min. The obtained samples were fixed again in 1% osmium tetraoxide (OsO_4) in 0.1 M PBS at room temperature for 2 hrs by mixing equal quantities of 2% aqueous OsO_4 and 0.2 M PBS and used immediately. En bloc stain was processed with 2% aqueous uranyl acetate for 1 hr at room temperature to prevent uranyl acetate from being precipitated as it was photo-reductive, and then washed in distilled water 2-3 times for 5 min each. Dehydration was performed with graded ethanol from 50 to 100% for 10 to 15 min each and then propylene oxide 3 times for 15 min, according to the suggested protocol. Further dehydration was processed with a mixture of EMBed 812 and

propylene oxide (1:1) overnight at room temperature in tightly capped vials to prevent moisture from coming into specimen. 100% EMBed 812 was used at room temperature for 2 hrs and then caps were removed from the vials to allow any remaining propylene oxide to evaporate.

Dry samples were embedded in beam capsules and polymerized in a 65°C oven for more than 1 d for sectioning. Thick sections (0.5-1.0 μm) were cut, and transferred via a steel loop to a drop of water on a slide and dried it on a slide warmer. Toluidine blue was stained for 1 to 3 min, and then sections were observed under microscope. After cutting thick ultrathin sections (60-90 nm), the sections were collected onto grids, which stored in a grid box. The grids were allowed to air-dry for overnight before staining. They were stained with uranyl acetate for 30 min and then lead citrate for 10 min. The morphologies of the samples were observed under TEM (JEM-2010; Jeol Ltd., Japan).

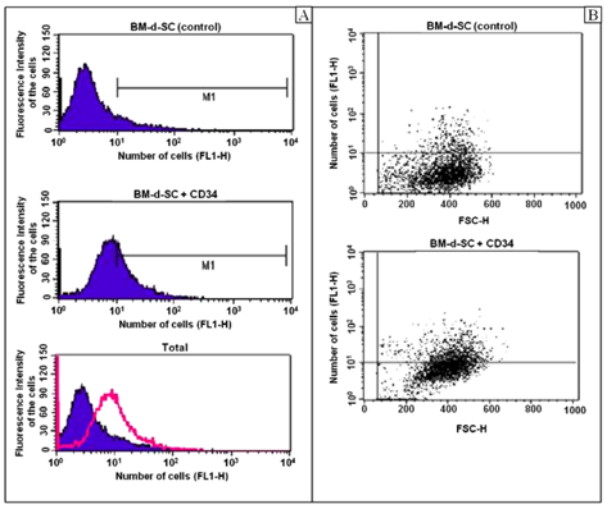

Figure 2. FACS analysis of stem cells derived from canine bone marrow through CD34+ antibody.

3. Results and Discussion

3.1 Characterizations of Unmodified ePTFE Graft and Hybrid Scaffolds

Morphologies of the unmodified ePTFE and hybrid scaffolds have been reported in our previous papers.[20-26] While the unmodified ePTFE graft showed the morphologies of its wall pores with both 20-30 μm inter-nodal distances and inter-connected fibers,[20] the chemically modified one had gray colors and hydrophilic surfaces, inducing quicker transport of bioactive molecules such as heparin and albumin through the pores of ePTFE wall.[19,21] Morphologies and both *in vitro* and *in vivo* tissue engineering of the hybrid scaffolds have been also reported in our previous works.[22-26]

3.2 Analysis of Smooth Muscle Cells

Verification of smooth muscle cells from the carotid artery of mongrel dogs was confirmed by staining with anti-smooth muscle-α actin antibody. Hematoxylin staining of the isolated cells induced their reaction with anti-smooth muscle-α actin antibody, thus showing staining of cytoplasm and nucleus with gray and pale purple, respectively. The negative control cells showed nucleus staining only, thus indicating that the cells were smooth muscles (data not shown) as reported in our previous works.[22-25]

3.3 FACS analysis of Bone Marrow Derived Stem Cells

Percentage of the cells with CD34+ antibody was measured by FACS among the separated and cultured cells from the bone marrow of the mongrel dogs.[27] When the peaks of the cells with CD34+ antibody (Fig 2A-a) were compared with those of the cells without (Fig 2A-b) CD34+ antibody, the peaks of the cells with CD34+ antibody showed their shifts to right position on the spectrum (Fig 2A-c). Since M1 indicated the percentage of the peak-shifted cells with fluorescence on its surface, the percentage of the CD34+ tagged stem cells derived from bone marrow was calculated as 37.5% after removing non-specifically reactive cell group from mononuclear cells. Compared to that of the control groups without antibody, the peak of the cells with CD34+ antibody was measured on right hand side of the control, indicating 34.4% of the bone marrow derived stem cells (Fig 2B).

3.4 Cell Adhesion Assay

Cell adhesion on the inner and outer surfaces of the employed samples was observed with SEM after *in vitro* cell culture for 7 days. We observed both surface-coated collagen on the surface of the porous PLGA layer before cell culture, and adhesion and spreading of smooth muscle cells on their surfaces after *in vitro* cell culture for 7 days (data not shown) as reported in our previous paper.[23]

3.5 *In Vitro* Media Tissue Regeneration

To regenerate effectively media tissue of the hybrid scaffolds, smooth muscle cells of the carotid artery of mongrel dog were *in vitro* cultured for 8 weeks by seeding them on the surface of collagen-coated porous biodegradable PLGA scaffold at a density of 1×10^6 cells/cm^2. When we measured the cell numbers with CCK-8 assay, *in vitro* absorption intensity was increased more than 3 times during 7 weeks compared with that of 1 week (Fig 3). H&E staining of the tissue-cultured samples showed regeneration of media tissues by observing nucleus and

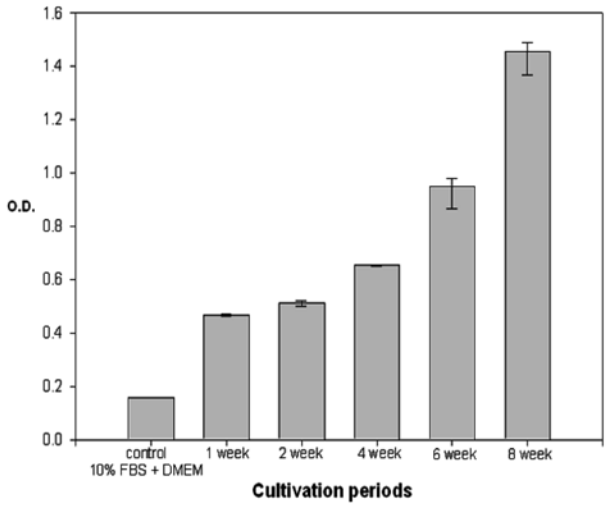

Figure 3. Proliferation of smooth muscle cells on the collagen-coated hybrid scaffolds by CCK-8 measurement.

cytoplasm, as well as the porous PLGA scaffold remaining on the lumen and outside surfaces of the hybrid scaffolds, i.e. less than 100 μm thickness. We observed that while the cells were stained with gray, the PLGA scaffolds were observed as white in color (Fig 5A).

3.6 Patency of *In Vitro* Tissue-cultured Hybrid Scaffolds in Mongrel Dog

After harvesting the tissue-cultured hybrid scaffolds either with or without stem cells implanted in the carotid artery for 4 weeks, their proximal, mid and distal sites were grossly observed with three kinds of methods such as eyes, digital camera and microscopes. Blood clotting and inflammatory responses of the sample surface were observed with microscopes, and then its patency was analyzed with a digital camera (Fig 4). When we observed the lumen of the tissue-cultured hybrid scaffolds implanted for 1 week by the digital camera, the proximal area showed good patency (Fig 4B), but their mid (Fig 4C) and distal (Fig 4D) areas showed worse results, leading to graft failure. However, the tissue-cultured hybrid scaffolds with stem cells implanted for 4 weeks showed patency in all the sites of grafts, i.e. proximal (Fig 4F), mid (Fig 4G) and distal (Fig 4H) areas. Detailed analysis of the explanted hybrid grafts had been made in our previous report.[26]

3.7 Histological analysis of the Explanted Hybrid Grafts

The proximal, medial and distal locations of the tissue-cultured hybrid scaffolds implanted for 1 week were analyzed with H&E stain. Its proximal location showed formation of loose neo-intima and blood clot. The samples were patent (Fig 5C), however, and both distal and medial areas showed similar extent of neo-intima formation (Fig 5D). Next, we evaluated the morphologies of the tissue-cultured scaffolds with stem cells after implanted in the carotid artery for 1 week, which were *in vitro* tissue-cultured in advance with both autologous smooth muscle cells for 12 weeks and then autologous stem cells for 2 weeks. When we stained the micro-sections of proximal, medial and distal areas with H&E, media tissues were regenerated on their lumen by autologous smooth muscle cells. Media tissue was also regenerated in the pores of the ePTFE

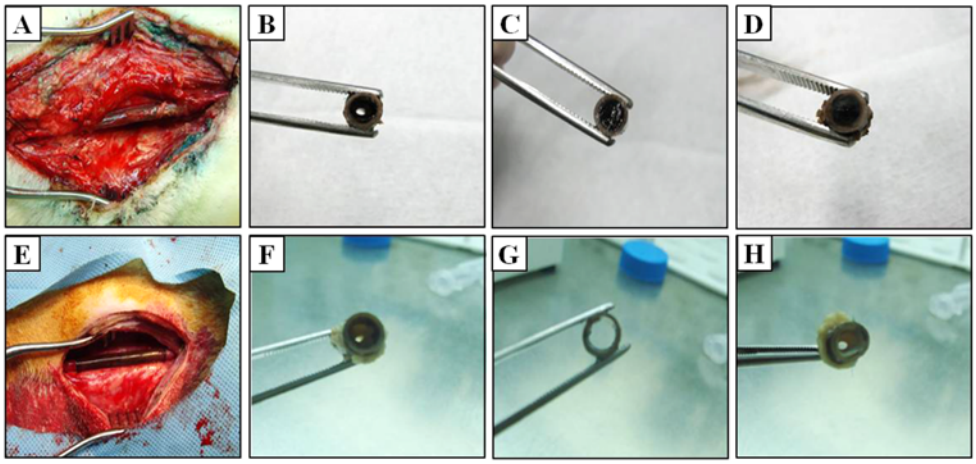

Figure 4. Gross observation of the tissue-cultured hybrid scaffolds either without or with stem cells after their implants in a carotid artery of a mongrel dog (A, E). The tissue-cultured hybrid scaffolds without stem cells were harvested at 1 week after implant (B, C and D), the tissue-cultured hybrid scaffold with stem cells were implanted for 4 weeks (F, G and H), (B and F = proximal; C and G = medial; D and H = distal).

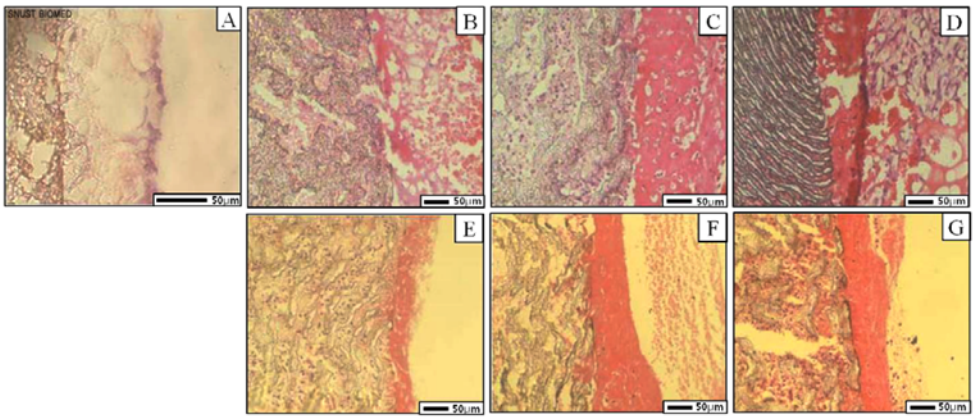

Figure 5. Cross sectional H&E staining of the tissue-cultured hybrid scaffolds either without (B, C, D) or with stem cells (E, F, G) for 1 week after their implants in the carotid artery, where proximal = B and E; medial = C and F; distal = D and G. The hybrid scaffolds showed tissue formation by SMC (A).

walls, which were previously impregnated with biodegradable PLGA polymers (Fig 5E-G). The thickness of the regenerated media tissue was increased as it went from proximal (Fig 5E) to medial (Fig 5F) and distal (Fig 5G) positions, but the samples were patent. The lengths of the media tissue of the explanted hybrid graft implanted for 1 week were measured as 54.3, 108.1 and 111.8 μm at the locations of the medical, medial and distal locations, respectively, over 6.6 μm of its initial PLGA layer.

3.8 Morphological Observation of the Explanted Hybrid Grafts with SEM and TEM

The SEM data verified tissue regeneration of the tissue-cultured hybrid scaffolds, which were tissue-cultured in advance with stem cells and then implanted in the carotid artery for 4 weeks. The scaffolds were previously *in vitro* tissue-engineered with autologous smooth muscle cells and then autologous stem cells for 12 and 2 weeks, respectively (Fig 6). Cell adhesion and spreading of the explanted hybrid grafts were observed on its lumen surface with cell aggregations on some locations (Fig 6A), and the tissues on both medial and proximal areas had similar morphologies (Fig 6B). The smooth connection between the regenerated distal tissue and the host artery was observed on their lumen surface (Fig 6C).

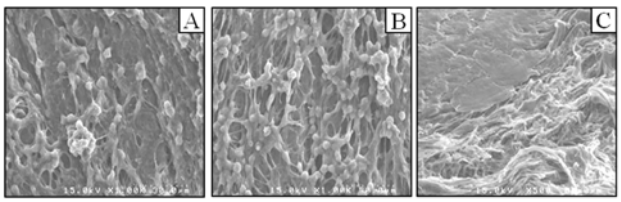

Figure 6. Surface morphologies of hybrid grafts by SEM (×1000), where A = proximal, B = medial, C = distal. The tissue-cultured scaffold with stem cells was implanted in the carotid artery of the mongrel dog for 4 weeks.

Next, detailed morphological analyses of the explanted hybrid grafts were performed with TEM. The results showed that the lumen of the control carotid artery of mongrel dogs had nucleus and diverse organelles of the cells, and the extracellular matrix under the lumen cell monolayer had connective tissues with collagens, elastic fibers and muscle tissues, which are characteristic morphologies of vascular tissues (Fig 7A). The ePTFE graft wall was observed in white as designated as G in figure 7. The explanted hybrid grafts showed adhered cells on the lumen surface, which showed their characteristic organelles such as glycogen, mitochondria, endoplasmic reticulum and others (Fig 7B). These organelles were similar to those of the control tissue. The proximal and distal anastomoses of the explanted hybrid graft implanted for 4 weeks were observed by magnifying up to 5,000 times for analysis of internal structure of the tissue-engineered grafts (Fig 7C-D). The impregnated biodegradable PLGA polymers in the ePTFE wall pores of the tissue-cultured hybrid scaffolds were replaced with regenerating tissues. The pore space was filled with regenerated tissues and they were adhered on its chemically modified surfaces. The observed regenerated tissue had nucleus and specific organs such as mitochondria and endoplasmic reticulum in its cell cytoplasm, showing migration of media tissues along the chemically modified pore surfaces of the ePTFE wall. The regenerated connective tissues showed formation of collagens, elastic fibers and muscle tissues similar to those of normal blood vessels.

4. Conclusion

Understanding both pore properties of ePTFE wall and the

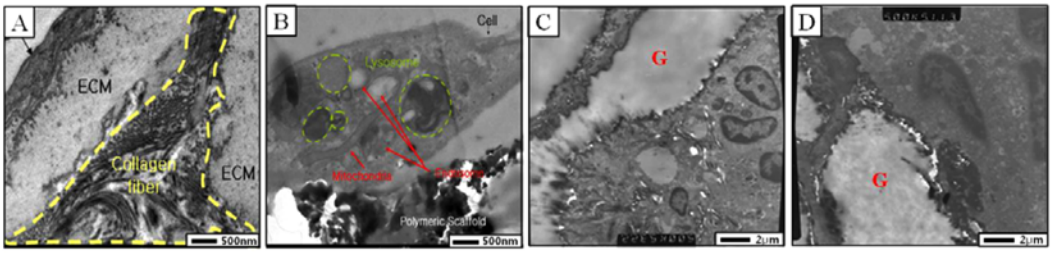

Figure 7. TEM morphologies of carotid artery of mongrel dog showing ECM and collagen fibers (A), the tissue-culture hybrid scaffolds with specific cell organelles of mitochondria, endosomes and lysosomes (B), and the tissue-cultured hybrid scaffolds with stem cells (C and D), which were harvested at 4 weeks after their implants in the carotid arteries of mongrel dogs. G and ECM indicated non-biodegradable ePTFE graft wall and regenerated extracellular matrix, respectively.

behaviors of tissue regeneration both on the lumen and in the wall pores of ePTFE graft is important for controlling pore sizes of ePTFE[28], induction of vascular tissue regeneration and prevention of graft failure by both formation of neointima and prevention of seroma formation.[29,30] *In vitro* media tissue regeneration of 4-mm ePTFE hybrid scaffolds was evaluated by TEM after seeding both smooth muscle cells of carotid artery of mongrel dogs and their bone marrow stem cells on the collagen-coated porous PLGA surface of the hybrid scaffold lumen. The resulting hybrid graft showed in our previous report higher cell adhesion, leading to tissue regeneration along biodegradation of PLGA layers both on the lumen layer and in the wall pores of the hybrid scaffolds. These grafts showed stable mechanical properties during blood circulation *in vivo*.[26] The obtained *in vitro* tissue-cultured scaffolds showed good patency in the carotid artery of the mongrel dog, when implanted for 4 weeks. The explanted hybrid grafts showed higher patency in proximal area rather than medial and distal ones, when grossly observed by the digital camera and histology. Histological analysis and the SEM morphologies showed smooth tissue formation at its anastomosis by connecting regenerating tissues with host one. The TEM results especially showed morphologies of the regenerated tissue and ePTFE wall in submicron scales, containing cell organelles and extracellular matrix such as collagens, in both in the pores of ePTFE and inside the implanted hybrid grafts. Furthermore, tissue regeneration was observed by replacing the impregnated biodegrading PLGA layers on its modified lumen surface. And the regenerated tissues at the anastomoses of the implanted hybrid graft were smoothly connected with adjacent host tissues as observed by SEM. The seeded cells on the lumen surface of the hybrid scaffolds seemed to be migrated into the chemically surface-modified pores of the ePTFE wall with 20-30 μm intermodal distance, which is distinctive from that of the unmodified hydrophobic surface.

The morphologies of the regenerated media tissues in the pore channels of the ePTFE wall showed characteristics of blood vessels in submicron scales as observed by TEM. The adhered cells on the lumen of the hybrid scaffold seemed to migrate and form a new tissue in its surface-modified pore space. The regenerated tissues in the wall pores showed their specific organelles such as nucleus, mitochondria and endoplasmic reticulum as well as connective tissues with collagens, elastic fibers and muscle tissues. These results indicated that the regenerated media tissues in these micron scale pores of ePTFE wall seemed to be composed of both the smooth muscle cells migrated possibly from its lumen and their extracellular matrix components. The regenerated tissue showed morphologies with collagen similar to those of the control artery tissues but seemed to have loose collagens. Both tissue regeneration in the pores of ePTFE wall of the hybrid scaffolds and smooth connection of the regenerated tissues with host tissues may be from the adequate design of hybrid scaffold, i.e. match up of hybrid graft lumen with that of the host blood vessel and higher degrees of cell adhesion on the collagen coated on the scaffold lumen at initial stage, leading to tissue regeneration in the ePTFE wall pores over implant time. In our previous experiment, coating of collagen on the hybrid graft lumen was important for induction of cell adhesion and subsequent *in vitro* tissue formation.[21,22] Both the chemically modified, hydrophilic ePTFE surface and the biodegradable PLGA layer impregnated in the ePTFE pores of the hybrid graft wall might have facilitated proliferation and migration of the adhered cells into its pores without additional delivery of bioactive molecules from its inner and outer side such as growth factors and nutrients. Nutrients from adjacent host tissues and circulating blood might help the seeded cells proliferate and migrate into the wall pores. Transport of bioactive molecules such as heparin and albumin through the wall pores were reported in our previous experiment by comparing their transports in the unmodified hydrophobic pore surface and the

chemically modified and hydrophilic pore surfaces of the ePTFE wall.[22] In that work, we showed quicker transport of heparin molecules through the surface-modified, more hydrophilic pore of ePTFE graft wall than that of the unmodified one. Tissue regeneration in these small pore channels of the hybrid graft was possible by the combined effects of surface modification of ePTFE wall pores, impregnation of biodegradable PLGA into the surface-modified wall pores, and higher adhesion of smooth muscle cells on the collagen-coated surfaces. The adhered cells might be stimulated to be migrated into the pores by the effects of cell adhesion on the hydrophilic surfaces of the modified ePTFE, cell migration to migrate by biodegradation of the impregnated PLGA and delivery of required biomolecules such as nutrients from host tissue and circulating blood into the wall pores of ePTFE.

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References

1. SC Lee, SS Lee, KS Kim, *et al*., Evaluation of vascular graft porosity using newly designed device, *Biomater Res*, **13**, 2 (2002).
2. WC Johnson, KK Lee, A comparative evaluation of polytetrafluoroethylene, umbilical vein, and saphenous vein bypass grafts for femoral-popliteal above-knee revascularization: a prospective randomized department of veterans affairs cooperative study, *J Vasc Surg,* **32**, 2 (2000).
3. JW Mall, AW Philipp, A Rademacher, *et al*., Re-endothelialization of punctured ePTFE graft: an *in vitro* study under pulsed perfusion conditions, *Nephro Dial Transplant,* **19**, 1 (2008).
4. OE Teebken, A Haverich, Tissue engineering of small-diameter vascular grafts, *Eur J Vasc Endovasc Surg*, **23**, 6 (2002).
5. S Ravi, EL Chaikof, Biomaterials for vascular tissue engineering, *Regen Med*, **5**, 1 (2010).
6. X Wang, P Lin, Q Yao, *et al*., Development of small-diameter vascular grafts, *World J Surg*, **31**, 4 (2007).
7. MS Hahn, MK Mchale, E Wang, *et al*., Physiologic pulsatile flow bioreactor conditioning of poly(ethylene glycol)-based tissue engineered vascular grafts, *Ann Biomed Eng,* **35**, 2 (2007).
8. U Westedt, M Kalinowski, M Wittmar, *et al*., Poly(vinyl alcohol)-graft-poly(lactide-co-glycolide) nanoparticles for local delivery of paclitaxel for restenosis treatment, *J Control Release*, **119**, 1 (2007).
9. JA Hubbell, Bioactive biomaterials, *Curr Opinion in Biotechnol,* **10**, 2 (1999).
10. H Shin, S Jo, A G Mikos, Biomimetic materials for tissue engineering, *Biomaterials,* **24**, 24 (2003).
11. Zisch AH, Lutolf MP, Ehrbar M, *et al*., Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth, *FASEB J,* **17**, 15 (2003).
12. SH Lim, SW Cho, JC Park, *et al*., Tissue-engineered blood vessels with endothelial nitric oxide synthase activity, *Biomed Mater Res B Appl Biomater,* **85**, 2 (2008).
13. T Haraguchi, K Okada, Y Tabata, *et al*., Controlled release of basic fibroblast growth factor from gelatin hydrogel sheet improves structural and physiological properties of vein graft in rat, *ATVB,* **27**, (2007).
14. S de Valence, JC Tille, JP Giliberto, *et al*., Advantages of bilayered vascular grafts for surgical applicability and tissue regeneration, *Acta Biomate,* **8**, 11 (2002).
15. F Kuwabara, F Narita, A Yamawaki-Ogata, *et al*., Long-term results of tissue-engineered small-caliber vascular grafts in a rat carotid arterial replacement model, *J Artif Organs,* **15**, 4 (2002).
16. MC Peters, PJ Polverini, DJ Mooney, *et al*., Engineering vascular networks in porous polymer matrices, *J Biomed Mater Res,* **60**, 4 (2002).
17. V Bhattacharya, PA Mcsweeney, Q Shi, *et al*., Enhanced endothelialization and micro-vessel formation in polyester grafts seeded with CD34+ bone marrow cells, *Blood,* **95**, 2 (2000).
18. MA Koo, JK Kang, MH Lee, *et al*., Stimulated migration and penetration of vascular endothelial cells into poly (L-lactic acid) scaffolds under flow conditions, *Biomater Res*, **18**, 7 (2014)
19. I Noh, SL Goodman, JA Hubbell, *et al*., Chemical modification and photograft polymerization upon expanded polytetrafluoroethylene, *J Biomater Sci,* **9**, 5 (1999).
20. YJ Choi, MS Kim, I Noh, Modification of expanded polytetrafluoroethylene surface with low-energy nitrogen-Ion-beam irradiation, *J Korean Phy Soc,* **50**, 5 (2007).
21. I Noh, ER Edelman, Smooth muscle cell ingrowth of a surface-modified ePTFE vascular graft, *Key Eng Mater, 2***88**, 367 (2005).
22. YJ Choi, SH Moon, MS Kim, *et al*., Evaluation of the media tissue regenerated hybrid vascular graft, *Tissue Eng Regen Med,* **3**, *1 (2006).*
23. YJ Choi, I Noh, Media tissue regeneration of the hybrid expanded polytetrafluoroethylene vascular graft via gelatin coating, *J Curr Appl Phys,* **5**, 5 (2005).
24. I Noh, Y J Choi, Y Son, *et al*., Diffusion of bioactive molecules through the walls of the medial tissue-engineered hybrid ePTFE grafts for applications in designs of vascular tissue regeneration, *J Biomed Mater Res,* **79A**, 4 (2006).
25. YJ Choi, MS Kim, I Noh, Tissue regeneration of a hybrid vascular graft composed of biodegradable layers and non-biodegradable layer by static and pulsatile flows, *Key Eng Mater,* **342**, 61 (2007).
26. KH Son, YH Fang, YJ Choi, *et al*., Evaluation of the hemodynamics of a tissue-engineered hybrid graft, *J Artif Organs,* **34**, 1 (2010).
27. T Asahara, T Murohara, A Sullivan, *et al*., Isolation of putative progenitor endothelial cells for angiogenesis, *Science* **14**, 275 (1997).
28. KL Boyd, S Schmidt, TR Pippert, *et al*., The effects of pore size and endothelial cell seeding upon the performance of small-diameter e-PTFE vascular grafts under controlled flow

conditions, *J Biomed Mater Res,* **22**, 3 (2004).
29. JP Ko, YH Liu, JJ Chu, *et al*., Effective treatment for recurrent perigraft seromas of upper arm polytetrafluoroethylene grafts: report of two cases, *Chang Gung Med J,* **26**, 440 (2003).
30. J Zanow, U Kruger, U Settmacher, *et al.,* Treatment of perigraft seroma in expanded polytetrafluoroethylene grafts by sequential fibrin sealing of the outer graft surface, *Ann. Vasc Surg,* **24**, 8 (2010).