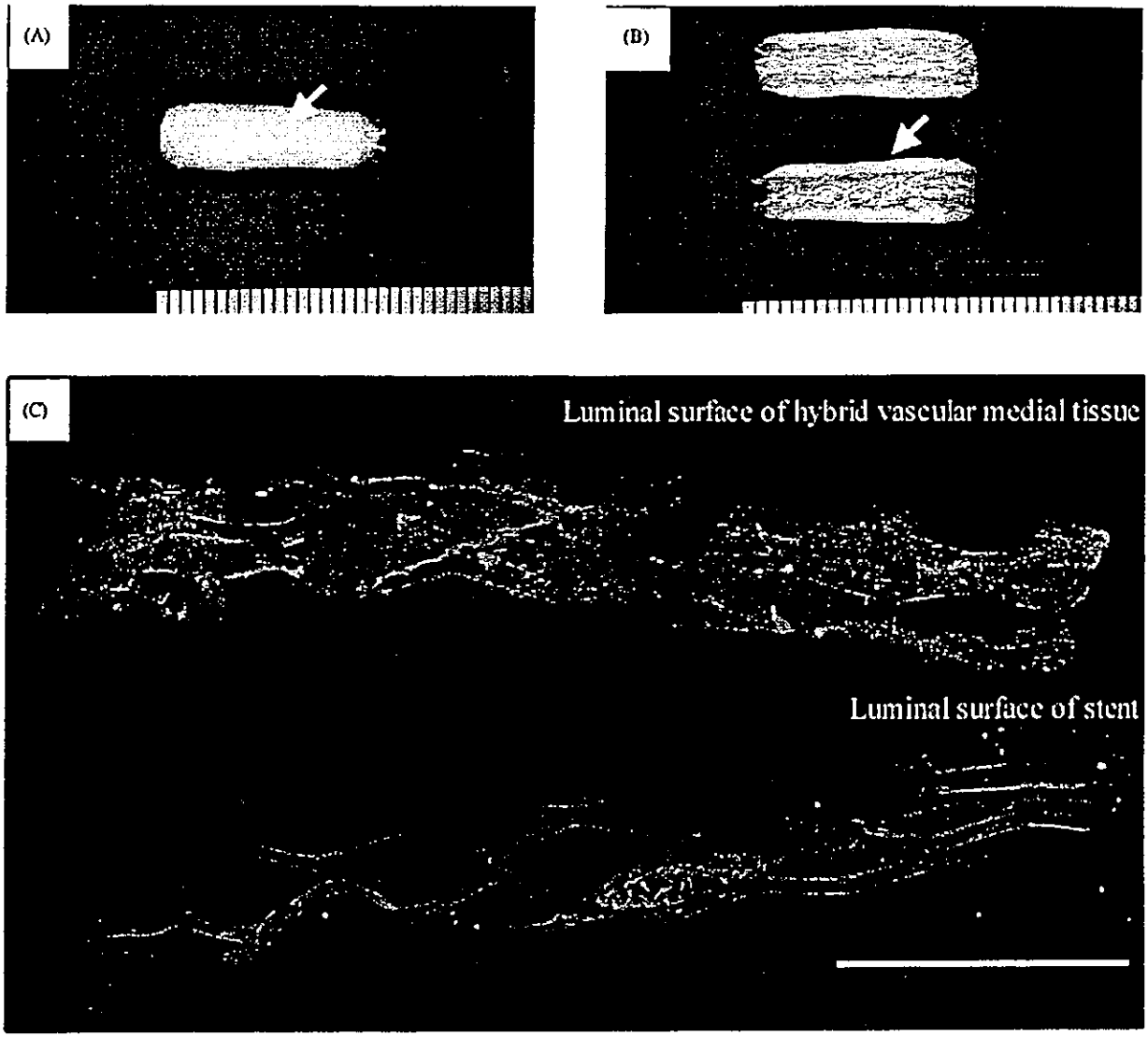


Fig. 6. (A), (B) Gross apparatus of EPC-seeded stent implanted into hybrid vascular medial tissue. EPC-seeded stent attached to the balloon catheter was inserted into the hybrid vascular medial tissue (arrow), and expanded by balloon inflation (A). After balloon deflation and removal of the catheter, hybrid-vascular-medial-tissue-implanted stent was cultured for 7 days. After the culture period, hybrid vascular medial tissue was cut longitudinally ((B), arrow) for further examination. Bar = 1 cm. (C) CLSM examination of the luminal surface of the hybrid-vascular-medial-tissue-implanted EPC-seeded stent and stent surface after 7 days of incubation. Upper micrograph shows that the luminal surface of hybrid medial tissue, from which the implanted stent was detached, was almost fully covered with green fluorescent cells proliferated from DiO-labeled EPCs, except where stent struts were attached. Lower micrograph shows the inner surface of implanted stent also fully covered with DiO-labeled EPCs. Bar = 5 mm.

difficulty in harvesting autologous ECs, which must be obtained from a large vein or fat tissue through additional surgical invasion. In this study, we addressed the circumvention of this problem by using EPCs. Recent studies showed that EPCs, which are derived from the bone marrow, circulate in peripheral blood [25 29]. Harvesting circulating EPCs requires only peripheral blood collection. This minimally invasive harvesting procedure indicates that EPC is a candidate for a new source of cells for vascular tissue engineering. Our previous studies showed that EPCs have comparable antithrombogenic potential such as production of endothelial ·nitric oxide synthase, tissue plasminogen activator, and prostacyclins (prostaglandin $I_2$) to that of mature ECs, as well as high proliferating potential [11], and that a high patency rate of EPC-seeded small-diameter (5 mm) vascular grafts implanted in the canine carotid artery can be achieved [30].

In this study, we fabricated two types of stent devices. One was the EPC-seeded stent. EPCs were seeded on the photocured gelatin-coated stent strut. The photocurable gelatin is a gelatin-based macromer, in which multiple

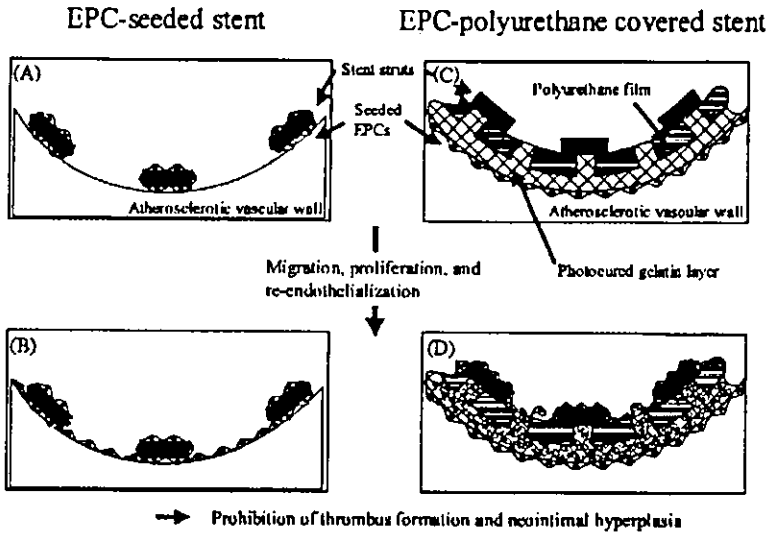


Fig. 7. Hypothetical outcome following implantation of EPC-seeded stent and EPC-polyurethane-covered stent. After implantation of EPC-seeded stent to the atherosclerotic vascular wall, seeded EPCs will migrate from the struts and proliferate to form new endothelium (A), (B). In the case of EPC-polyurethane-covered stent (C), (D), EPCs between the polyurethane film and the arterial wall will migrate to the luminal surface via micropores and proliferate to form new endothelium. This re-endothelialization will inhibit thrombus formation and neointimal hyperplasia.

benzophenone groups are derivatized in a gelatin molecule and photocrosslinked upon UV irradiation [12]. The other was an EPC-seeded microporous SPU graft covered stent. Much larger surface area of the latter stent as compared with the former stent provides high number of cell loading on the stent. In our previous paper, controlled tissue ingrowth through micropores was verified by in vivo study using covered stents without cell loading [31].

Ex vivo expanded EPCs derived from canine peripheral blood completely covered both surfaces of the photocured gelatin-coated stent and SPU film-wrapped covered stent with no difficulty. The adhesion strength of seeded cells was sufficient to maintain an almost confluent layer after balloon expansion. Furthermore, the luminal surface of the hybrid medial tissue, in which a balloon-expanded EPC-seeded stent was deployed, was fully covered with EPCs that migrated from the stent struts and proliferated in adjacent hybrid tissue after 7 days of culture. These results may contribute to the promotion of re-endothelialization by EPCs to repair injured vascular surface, inhibit thrombus formation, and inhibit SMC stimulation. A hypothetical in vivo outcome of the re-endothelialization process is shown in Fig. 7. That is, once the stent is deployed at the atherosclerotic site, EPCs migrate on the blood-flow-facing surface of stent struts and into the denuded vessel surface where they grow, resulting in complete coverage with EPCs which are expected to fully differentiate into ECs. To verify this hypothesis, we have moved on to using EPC-seeded stents in swine coronary arteries with artificially created atherosclerotic walls.

## Acknowledgements

This study is financially supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR), grant no. 97-15 and in part by a grant-in-aid for Scientific Research (A2-12358017, B2-12470277) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors wish to thank Mr. Takaaki Kanemaru for his advice and guidance during SEM examination and Ms. Yumiko Terashima for her contribution in preparing cell culture and hybrid medial tissue.

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TISSUE ENGINEERING
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# Intralumenal Tissue-Engineered Therapeutic Stent Using Endothelial Progenitor Cell-Inoculated Hybrid Tissue and *in Vitro* Performance

TOSHIHIKO SHIROTA,[1,2] HISATAKA YASUI,[2] and TAKEHISA MATSUDA, Ph.D.[1]

## ABSTRACT

**Rapid reendothelialization at an atherosclerotic lesion after balloon or stent inflation may be essential for maintaining homeostatic tissue function, which could reduce or prevent restenosis. We devised an endothelial progenitor cell (EPC)-enriched tubular hybrid tissue and mounted it on a small-diameter metallic stent (outer diameter, 1.5 mm), which is used for intravascular angioplasty to atherosclerotic lesions. This study addressed the fabrication technique and *in vitro* performance to verify lumenal endothelialization. A thin collagenous tubular tissue was prepared by contraction of collagen fibers by inoculated EPCs, which were isolated from canine peripheral blood and expanded *ex vivo*, in a collagen gel formed in a mold. An EPC-inoculated hybrid tissue-covered stent, loaded on a balloon catheter, was inserted into a tubular hybrid vascular medial tissue inoculated with smooth muscle cells (SMCs) as an arterial media mimic, and subjected to balloon inflation for enlargement (outer diameter, 3 mm), followed by balloon deflation. The EPC-inoculated hybrid tissue-covered stent tightly adhered to the lumenal surface of the hybrid medial tissue. On culture, EPCs in the hybrid tissue migrated and proliferated to form a completely endothelialized lumenal surface at stented sites as well as sites adjacent to the vascular hybrid medial tissue with the prolongation of culture. This *in vitro* pilot study before *in vivo* experiments suggests that an EPC-inoculated hybrid tissue-covered stent may be a novel therapeutic device for reendothelialization or paving with EPC-enriched tissue at an atherosclerotic arterial wall, resulting in the prevention of restenosis and the rapid formation of normal tissue.**

## INTRODUCTION

A BLOOD VESSEL has an elaborate cell organization to maintain its physiological and biochemical conditions. Vascular endothelium plays a particularly important role in the bioregulation of blood vessels, by providing a nonthrombogenic surface, by acting as a physical and biochemical barrier, and by secreting bioactive substances that control vascular tone and blood flow.[1] Furthermore, endothelium modulates vascular wall remodeling to regulate the cellular behavior of smooth muscle cells (SMCs).[2–4] Once endothelial dysfunction occurs, blood vessels lose their nonthrombogenicity, which induces thrombus formation, and the vessel wall permits plaque formation accompanied by phenotypically altered SMCs, which proliferate and migrate into the intimal layer and produce extensive extracellular matrices (ECMs).[5–9] This complex morphogenesis leads to progressive atherosclerosis. Further endothelial injury or detachment of endothelial cells (ECs) occurs during balloon angioplasty or stenting,[10] which enhances

[1]Department of Biomedical Engineering, and [2]Department of Cardiac Surgery, Graduate School of Medicine, Kyushu University, Fukuoka, Japan.

occlusion or stenosis due to thrombus formation and intimal hyperplasia.[11]

If rapid reendothelialization occurs at atherosclerotic arterial sites after intravascular stenting, the above-mentioned adverse effects are minimized and the regeneration of normal tissue may be enhanced. Indeed, some studies have shown that EC seeding at atherosclerotic lesions in animal experiments reduced restenosis in injured arteries.[12–14] The efficacy of cell delivery to atherosclerotic lesions and the ability to cover the lumenal surface of an injured artery are critical for the therapeutic effectiveness of EC-delivery devices.[15–17] Furthermore, clinical conditions often do not permit the harvest of autologous ECs from the veins of patients with severe atherosclerosis.

An alternative source of autologous EC-equivalent cells is endothelial progenitor cells (EPCs), which are derived from bone marrow and circulate in peripheral blood.[18–21] Cloned EPCs have been studied for cell transplantation therapy[22,23] and small-diameter hybrid vascular grafts.[24–26] Our previous study indicated that human EPCs with high proliferative potential also have antithrombogenic potential comparable to that of mature ECs,[27] and canine EPC-seeded small-diameter vascular grafts implanted in canine carotid arteries showed high nonthrombogenicity as well as high patency.[25]

EPC-seeded metallic stents may be used for mechanical dilation devices with cell delivery. There must be three different types of EPC-seeded stents: one involves the direct seeding of EPCs on stent struts, another is an EPC-seeded synthetic graft-covered stent, and the third is an EPC-inoculated hybrid tissue-covered stent. The former two approaches are reported elsewhere. When such an EPC-seeded stent is implanted in an atherosclerotic lesion, EPCs may migrate to and proliferate on the lumenal surface of the artery, irrespective of the type of stent, resulting in a physical and biological barrier. In this article, we describe a method for fabricating EPC–collagen hybrid tissue-covered stents and examine *in vitro* endothelialization.

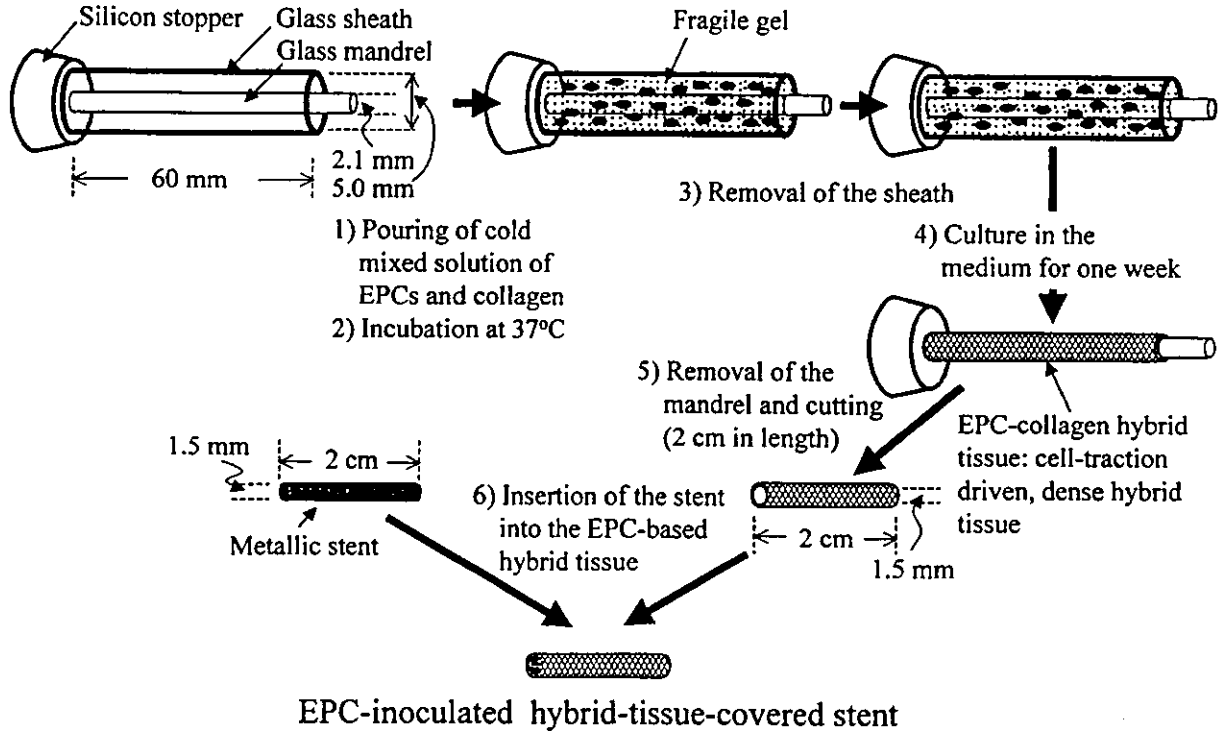


**FIG. 1.** Schematic drawing of the fabrication of the EPC-inoculated hybrid tissue-covered stent. A tubular glass mold, which consisted of an internal glass mandrel and an external glass sheath coaxially fixed at one end with a silicone stopper, had the following dimensions: outer diameter and length of the mandrel, 2.1 and 70 mm, respectively, and inner diameter and length of the sheath, 5.0 and 60 mm, respectively. A cold mixed solution of EPC suspension with medium (0.4 mL, $2.5 \times 10^7$ cells/mL) and 0.4 mL of acid-solubilized bovine dermal type I collagen solution (3 mg/mL) was poured into the space between the sheath and the mandrel ($1.0 \times 10^7$ cells per mold). After thermal gelation at 37°C for 20 min, the outer sheath was removed. The EPC-inoculated collagen gel was cultured in the medium for 7 days, after which a densely packed, thin and dense tubular tissue was formed around the mandrel. After removal of the mandrel, a tubular hybrid tissue (cut into 2-cm lengths) was mounted on a metallic stent.

## MATERIALS AND METHODS

### *Canine endothelial progenitor cell culture*

Animal experiments were conducted in compliance with guidelines of the Committee on Ethics in Animal Experiments at the Faculty of Medicine, Kyushu University (Fukuoka, Japan), the Guidelines for Animal Experiments at the Faculty of Medicine, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Government of Japan. Approximately 15 mL of peripheral blood was collected from the vein of an adult mongrel dog (body weight, 17.5 kg) into a plastic syringe containing 100 USP of sodium heparin (Mochida Pharmaceutical, Tokyo, Japan). This blood was gently layered over a Ficoll gradient (Histopaque-1077; Sigma, St. Louis, MO) and then centrifuged at 400 × *g* for 30 min at 25°C. The resulting peripheral blood mononuclear cells (PBMCs) were suspended in EC basal medium 2 (Clonetics, San Diego, CA) supplemented with 20% fetal bovine serum (FBS; Life Technologies, Rockville, MD) and 0.94% EGM-2MV (Clonetics), which contained human vascular endothelial growth factor, human fibroblast growth factor, human epidermal growth factor, insulin-like growth factor 1, ascorbic acid, and hydrocortisone acetate. PBMCs were seeded in one well (9.5 $cm^2$) of a six-well polystyrene culture plate precoated with type I

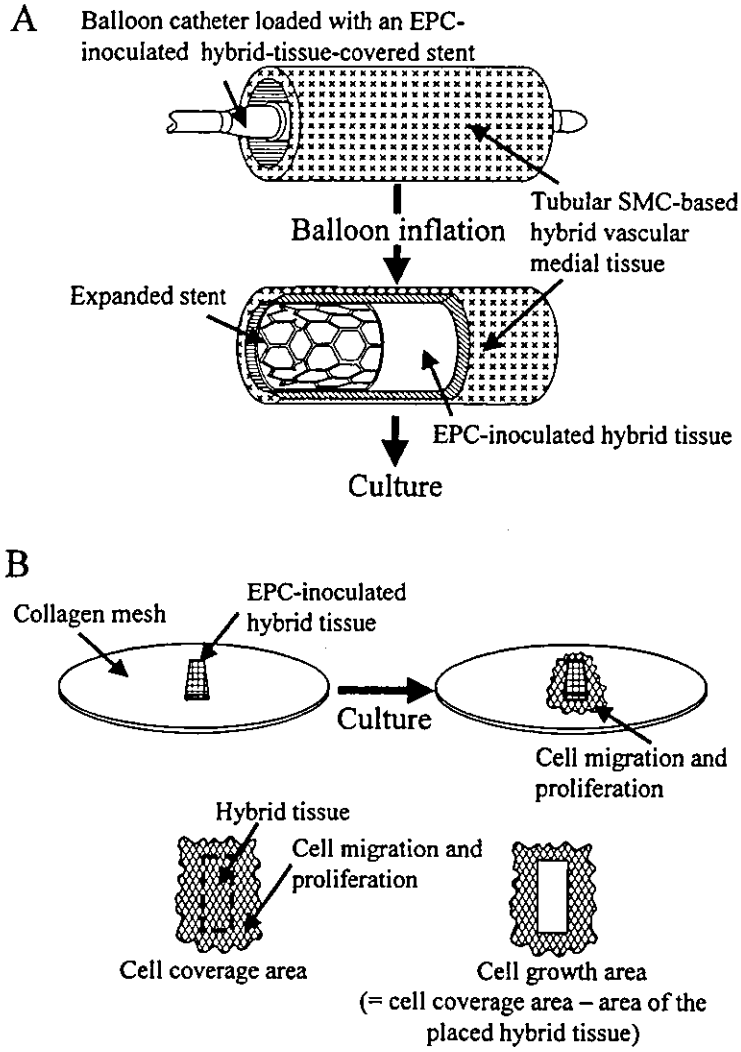


**FIG. 2.** *In vitro* evaluation of endothelialization. (**A**) The hybrid tissue-mounted stent was loaded on a balloon catheter and inserted into a tubular hybrid vascular medial tissue composed of SMCs and collagen. It was then subjected to balloon inflation to enlarge the stent and balloon deflation. The hybrid tissue-mounted stent tightly adhered to the lumenal surface of the medial tissue, and was cultured for 7 days. (**B**) Schematic diagram of the evaluation of two-dimensional growth kinetics. A piece of the hybrid tissue was put on a thin collagen mesh. EPCs in the hybrid tissue migrated and proliferated on the mesh with time. The cell coverage area was defined as the area occupied by cells on the mesh including the hybrid tissue, and the cell growth area was defined as the area where migration and proliferation newly occurred.

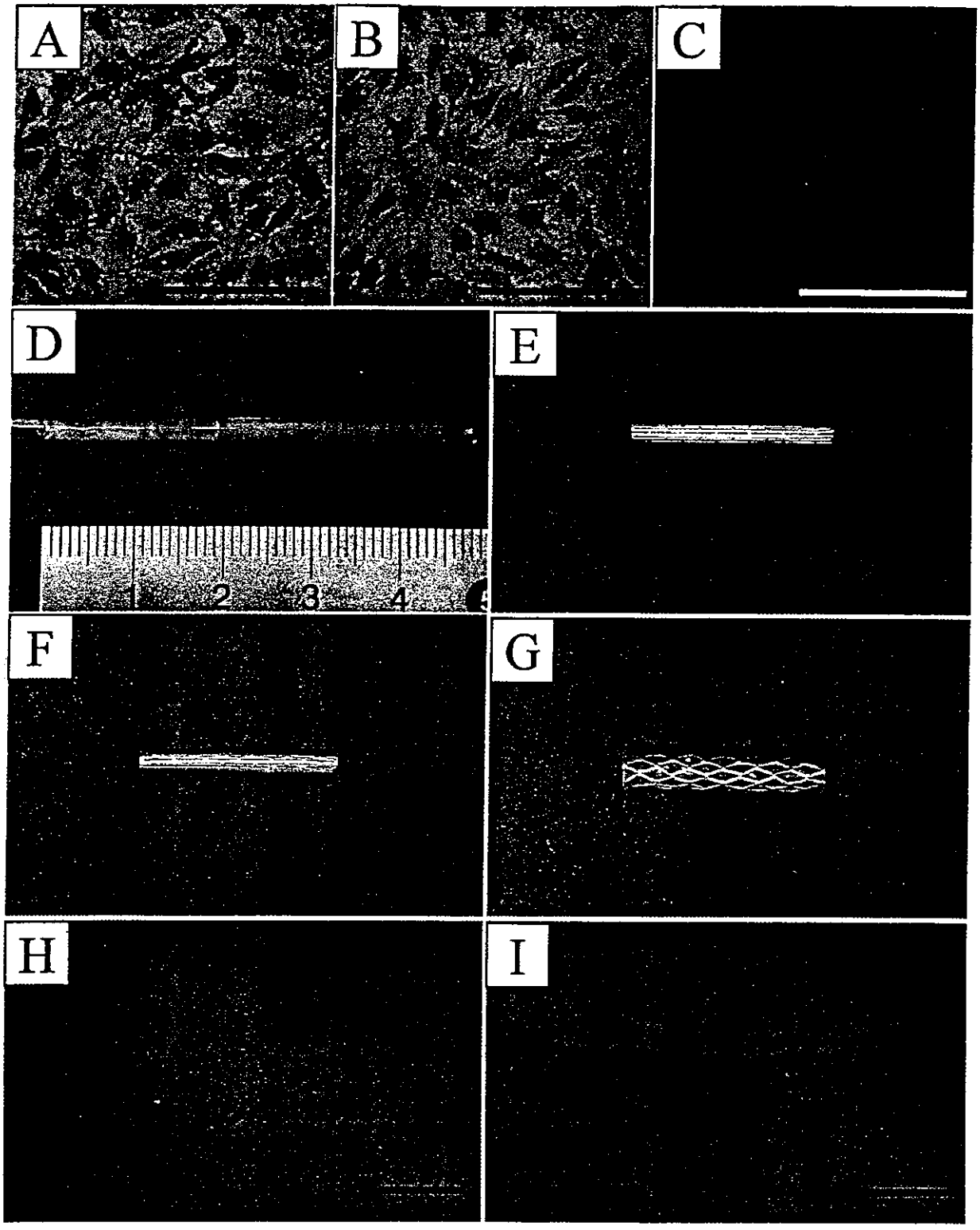


**FIG. 3.** (**A** and **B**) Light micrographs of immunohistochemical staining for vWF (**A**) and Flk-1 (**B**) of second-passage canine EPCs. (**C**) CLSM photograph shows that EPCs took up DiI-acetylated LDL. Original magnification, ×400; magnification bars, 100 $\mu$m. (**D–F**) Fabrication of the EPC–collagen hybrid tissue-covered stent. The gel shrank spontaneously and formed a dense thin tubular tissue around the glass mandrel after 7 days of incubation (**D**). (**F**) The hybrid tubular tissue was mounted on the gold stent (**E**). There were no macroscopic disruptions or cracks on stent expansion (**G**). Magnification bars, 10 mm. (**H** and **I**) Light micrographs of transverse sections show that EPCs and collagen were incorporated in the hybrid tissue homogeneously [(**H**) hematoxylin–eosin, (**I**) Masson's trichrome; original magnification, ×200; magnification bars, 100 $\mu$m].

collagen (collagen-coated microplate 6 well/flat bottom; Asahi Techno Glass, Tokyo, Japan). The culture medium was changed three times a week. After EPCs had grown to occupy approximately two-thirds of the culture plate, they were detached with 0.1% trypsin (Life Technologies) and replated onto a 100-mm polystyrene dish (Corning, Corning, NY). For device fabrication, these EPCs were expanded by subculturing six times (split ratios of 1:2 to 1:4) in 1 month.

### *Characterization of EPCs*

The von Willebrand factor (vWF) and Flk-1 (vascular endothelial growth factor receptor 2) were used as endothelial markers. Second-passage EPCs were stained with primary antibodies (rabbit polyclonal antibody against human vWF [Nichirei, Tokyo, Japan] and mouse IgG against human Flk-1 [Santa Cruz Biotechnology, Santa Cruz, CA]) and then with secondary antibodies (biotinylated goat anti-rabbit IgG antibody [Nichirei] and biotinylated rabbit polyclonal anti-mouse antibody [Nichirei], respectively). Streptavidin combined with immunoperoxidase (Nichirei) and 3,3′-diaminobenzidine-tetrahydrochloride (Merck, Darmstadt, Germany) were used to visualize the immunoreaction products.

Acetylated low-density lipoprotein (LDL) uptake by second-passage EPCs was examined in medium containing Dil-acetylated LDL (20 $\mu$L/mL; Biomedical Technologies, Stoughton, MA) for 4 h at 37°C. Cells were examined with a confocal laser-scanning microscope (CLSM) (Radiance 2000; Bio-Rad, Hercules, CA).

### *Fabrication of an EPC-inoculated hybrid tissue-covered stent*

Expanded EPCs were labeled with a 0.01 mM concentration of the cell tracer 3.3′-dioctadecyloxacarbocyanine perchlorate (DiO), (Vybrant DiO cell-labeling solutions; Molecular Probes, Eugene, OR) for 20 min at 37°C. The fabrication procedure is shown schematically in Fig. 1. A tubular glass mold consisting of an internal mandrel and external sheath coaxially fixed at one end with a silicone tube was constructed with the following dimensions: the outer diameter and length of the inner

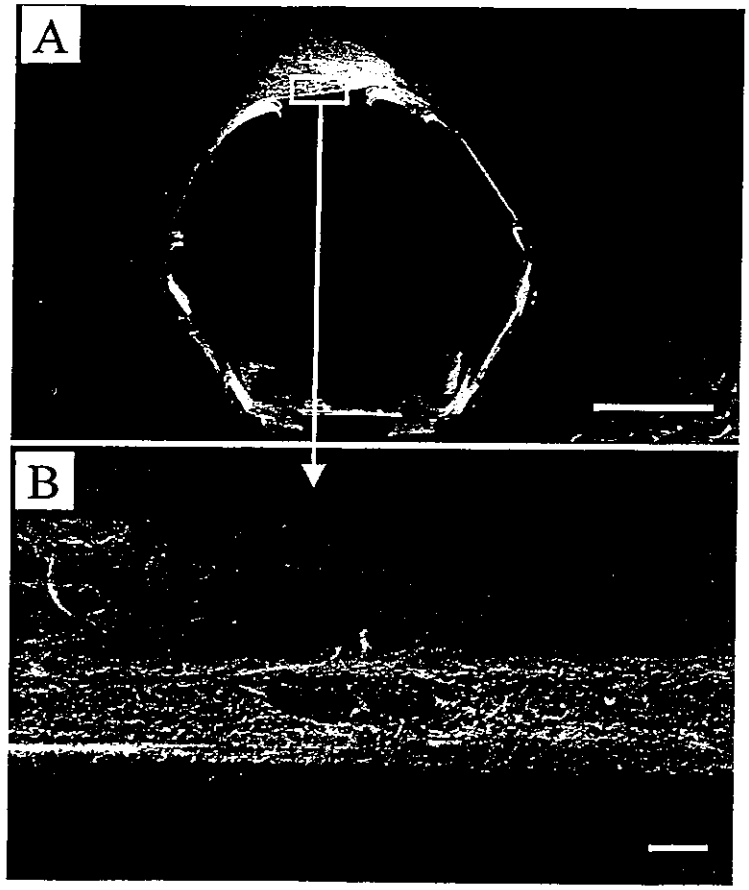


**FIG. 4.** Scanning electron micrographs of the hybrid tissue-mounted stent after expansion show that the hybrid tissue had expanded uniformly with no microscopic disruptions [(A) original magnification, ×20; magnification bar, 1 mm; (B) original magnification, ×1000; magnification bar, 10 $\mu$m].

mandrel, 2.1 and 70 mm, respectively; the inner diameter and length of the outer sheath, 5.0 and 60 mm, respectively. A cold equivolumetric mixed solution of labeled EPCs suspended in EC basal medium 2 supplemented with 20% FBS and 0.94% EGM-2MV ($2.5 \times 10^7$ cells/mL; 0.4 mL) and acid-solubilized bovine dermal type I collagen solution (3 mg/mL, 0.4 mL, CellGen; Koken, Tokyo, Japan) was poured into the space between the sheath and the mandrel of the mold. After incubation at 37°C for 20 min, the outer sheath was removed. The resultant gel was incubated in the same medium for 7 days, and the mandrel was then removed to give a thin tubular hybrid tissue (inner diameter, 1.5 mm) composed of collagen gel containing EPCs. A gold stent (diameter, 1.5 mm; length, 2 cm; a prototype stent supplied by Odensha, Kawasaki, Japan) loaded on a Multi-Link catheter (3.0-mm inflated diameter; Advanced Cardiovascular Systems, Hampshire, UK) was inserted into the tubular EPC–collagen hybrid tissue (cut to 2 cm long). The number of EPCs in the hybrid tissue before and after balloon deployment (inflation pressure, 10 atm; duration, 60 s) was determined after the tissue was digested with collagenase (0.5 mg/mL, Collagenase N-2; Nitta Gelatin, Osaka, Japan). Cell viability was determined by the trypan blue exclusion technique (trypan blue stain, 0.4%; Life Technologies).

### *Fabrication of hybrid vascular medial tissue*

A tubular hybrid vascular medial tissue used for *in vitro* inserted examination was fabricated according to a method reported previously.[28] Briefly, a cold mixture of canine smooth muscle cell suspension ($8 \times 10^6$ cells/mL, 8 mL) in M199 (Life Technologies) supplemented with 10% FBS and 8 mL of bovine dermal type I collagen solution (3 mg/mL) was poured into the space between the sheath (inner diameter, 15 mm) and the mandrel (outer diameter, 3 mm) of a tubular glass mold. After incubation at 37°C for 30 min, the outer sheath was removed and the resultant gel was incubated in the same medium for 7 days.

### In vitro *evaluation of endothelialization*

The EPC-inoculated hybrid tissue-covered stent was inserted into the hybrid vascular medial tissue, as shown in Fig. 2A. The hybrid tissue-mounted stent loaded on the Multi-Link catheter (3.0-mm inflated diameter) was inserted into the hybrid vascular medial tissue, and the balloon was inflated (inflation pressure, 10 atm; duration, approximately 30 s). The hybrid vascular medial tissue and the hybrid tissue-mounted stent were incubated for 7 days in EC-basal medium 2 supplemented with 20% FBS and 0.94% EGM-2MV.

Two-dimensional cell migration and proliferation from the EPC–collagen hybrid tissue were observed on a collagen mesh, as shown in Fig. 2B. A piece of hybrid tissue was cut open (average area, $33 \pm 6.5$ mm$^2$) and placed on a thin collagen mesh, which was produced by tightly compressing collagen gel made by the thermal gelation of an equivolumetric mixed solution of type I collagen solution (3 mg/mL) and M199. The hybrid tissues that had incubated on the collagen mesh for 3 or 7 days was subjected to a determination of two-dimensional cell growth kinetics. Nuclei of the proliferated cells on the collagen mesh were stained with Picogreen dsDNA quantitation reagent (Molecular Probes) and the stained areas were measured by CLSM. In this study, the cell coverage area was defined as the area occupied by stained cells on the collagen mesh, including the hybrid tissue placed on the mesh, and the growth area was defined as the area where migration and proliferation newly occurred.

### *Histological study*

Cell distribution in the EPC-inoculated hybrid tissue-covered stent and the adjacent hybrid vascular medial tissue was determined by CLSM and scanning electron microscopy (SEM) (JSM 840A; JEOL, Tokyo, Japan). DiO-labeled cells in the transverse sections of the specimens were observed by CLSM after fixation. Transverse sections were also stained with vWF antibody. SEM examinations were performed after fixation with osmium

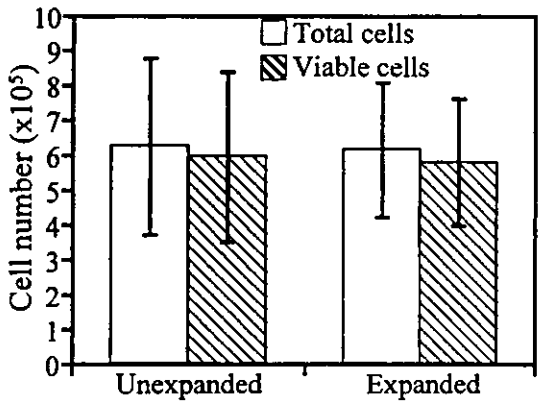


**FIG. 5.** Quantification of cells incorporated in unexpanded and expanded hybrid tubular tissues. Incorporated cells were collected after collagenase digestion of a 2-cm-long hybrid tissue, and counted under a phase-contrast microscope. Cell viability was determined by the trypan blue exclusion technique. The number of incorporated cells in the 2 cm of unexpanded hybrid tissue was $(6.3 \pm 2.5) \times 10^5$, including $(6.0 \pm 2.4) \times 10^5$ viable cells (viability, 95%; $n = 5$). After expansion of the tissue, the numbers of incorporated cells and viable cells were almost identical to those in the unexpanded hybrid tissue [$(6.2 \pm 1.9) \times 10^5$ cells, including $(5.8 \pm 1.8) \times 10^5$ viable cells; viability, 94%; $n = 5$).

tetroxide (Nissin EM, Tokyo, Japan). The specimens for light microscopy were stained with hematoxylin–eosin and Masson's trichrome stain. The thickness of the hybrid tissue was determined by CLSM, where thickness was defined by the average of the maximum thickness and the minimum thickness in two transverse-sectional views.

## RESULTS

Canine peripheral blood mononuclear cells (PBMCs), obtained from peripheral blood by the centrifugal gradient density technique, were cultured by a technique similar to that described previously.[27] Colonies of proliferative EPCs appeared at approximately 10 days, and

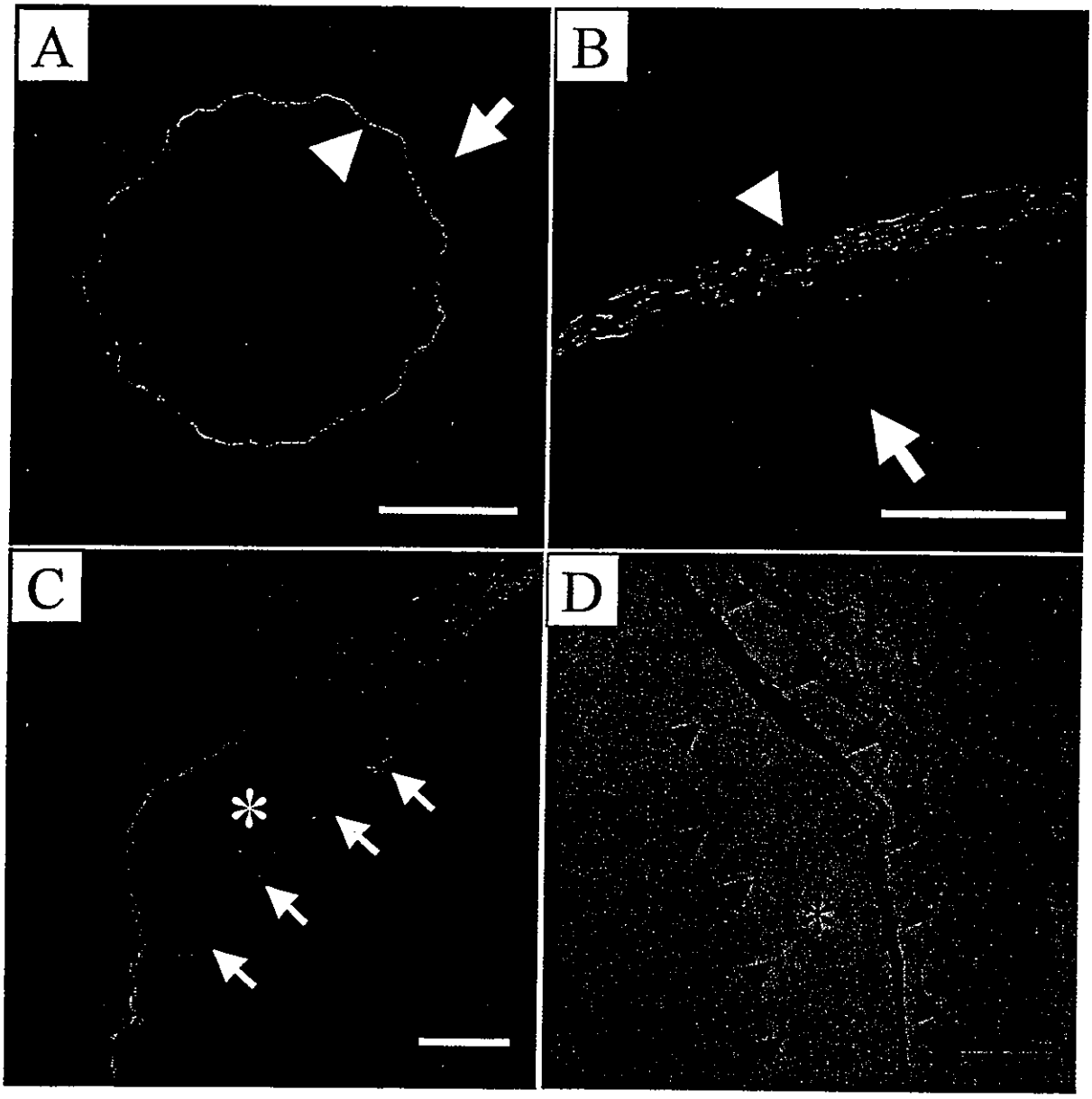


**FIG. 6.** Transverse sections of a hybrid tissue-mounted stent expanded in hybrid medial tissue and cultured for 7 days (**A** and **B**) CLSM images of the transverse section show that a thin DiO-labeled green fluorescent cell layer (arrowhead) uniformly formed on the hybrid medial tissue (arrow) [(**A**) original magnification, ×40; magnification bar, 1 mm; (**B**) original magnification, ×200; magnification bar, 100 μm]. (**C**) CLSM image of a transverse section around the stent strut (asterisk). A thin DiO-labeled cell layer covered the stent strut (arrows). Original magnification, ×100; magnification bar, 100 μm. (**D**) Light micrograph of the transverse section around the stent strut shows that vWF-positive EPCs covered not only the lumenal surface (arrows) of the hybrid medial tissue but also the stent strut (arrowheads). Original magnification, ×100; magnification bar, 100 μm].

subsequent subculture resulted in exponential growth to produce $1.3 \times 10^8$ cells within 2 months of venipuncture. Immunohistochemical staining for endothelial markers of second-passage EPCs showed that almost all of the cells (>98%) were positively stained with vWF antibody (Fig. 3A) and Flk-1 antibody (Fig. 3B). Fluorescence micrographs showed that EPCs took up DiI-acetylated LDL (Fig. 3C). The endothelial phenotype determined by the same immunohistochemical analysis remained constant for 12 passages.

The mixed solution of canine EPC suspension and type I collagen, which was poured into a glass mold, induced the spontaneous formation of a transparent, fragile gel. Immediate removal of the outer sheath from the mold and subsequent incubation in the medium for 7 days produced a dense, elastomeric tubular hybrid tissue (Fig. 3D). The inner diameter and wall thickness of the hybrid tissue were approximately 1.5 mm and $125 \pm 12.0$ $\mu$m ($n = 5$), respectively. The metallic stent (outer diameter, approximately 1.5 mm; length, 2 cm; Fig. 3E) was inserted into the hybrid tissue cut to a length of 2 cm (Fig. 3F), and expanded by an inserted balloon. There was neither cracking nor breaking of the enlarged hybrid tissue (Fig. 3G). SEM observations showed that after stent expansion, the hybrid tissue was expanded uniformly with no microscopic disruptions (Fig. 4A and B). Light microscopic observations of the hybrid tissue showed that the EPCs and collagen were homogeneously distributed throughout the tissue (Fig. 3H and I). The number of cells in the 2 cm of hybrid tissue before and after balloon expansion, which was determined after the collagen matrix was digested with type I collagenase, was $(6.3 \pm 2.5) \times 10^5$ cells [viable cells, $(6.0 \pm 2.4) \times 10^5$ cells; viability, 95%; $n = 5$)] and $(6.2 \pm 1.9) \times 10^5$ cells [viable cells, $(5.8 \pm 1.8) \times 10^5$ cells; viability, 94%; $n = 5$; Fig. 5), respectively. There was no further cell death observed at 3 days after expansion (data not shown).

This EPC-inoculated hybrid tissue-covered stent was expanded in hybrid tubular vascular medial tissue, which is a cell traction-driven, dense SMC-incorporated collagen tube (inner diameter, approximately 3 mm; outer diameter, approximately 5.5 mm; length, approximately 3 cm), and cultured for 7 days. A thin EPC-inoculated hybrid tissue was adhered tightly to the inner surface of the hybrid medial tissue. CLSM examination showed that a DiO-labeled green fluorescent cell layer was uniformly formed on the lumenal surface of the hybrid medial tissue with an average thickness of $61 \pm 18$ $\mu$m (n = 5; Fig. 6A and B). In addition, these fluorescent cells migrated and proliferated to cover the stent struts (Fig. 6C). Positive immunostaining for vWF showed that the cells that cover the stent struts were EPCs (Fig. 6D). SEM examination of the lumenal surface of the hybrid tissue-mounted stent-inserted hybrid medial tissue showed that EPCs completely covered the lumenal surface (Fig. 7A), and at high magnification the surface of the stent struts and the lumenal surface of the hybrid tissue were both completely covered by the EPC layer (Fig. 7B and C).

Cell migration to and proliferation in adjacent hybrid tissue were investigated with collagen mesh, on which was placed an EPC-inoculated collagen hybrid tissue (average area of the sample tissue, $33 \pm 6.5$ mm$^2$) accord-

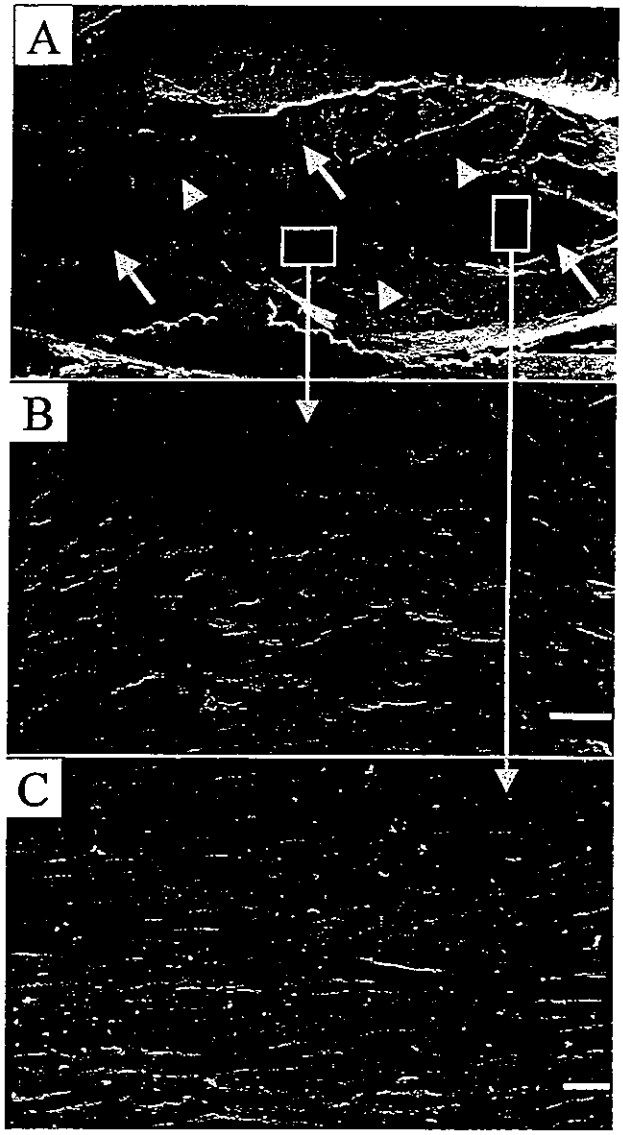


**FIG. 7.** SEM examinations of the lumenal surface of the EPC-inoculated hybrid tissue stent in the hybrid medial tissue after 7 days of incubation. (A) Lumenal surface view of the stent shows that EPCs completely covered both the lumenal surface of the hybrid tissue (arrows) and the stent struts (arrowheads). Original magnification, ×100; magnification bar, 500 $\mu$m. (B) Surfaces of the stent struts were completely covered by a monolayer of EPCs (original magnification, ×250; magnification bar, 50 $\mu$m). (C) The lumenal surface of the hybrid tissue was completely covered by a layer of EPCs (original magnification, ×500; magnification bar, 20 $\mu$m).

ing to the method shown schematically in Fig. 2B. EPCs migrated to the collagen mesh and proliferated with incubation (Fig. 8A and B). The area of cell growth was 12 ± 6.0 $mm^2$ at 3 days and 42 ± 10 $mm^2$ at 7 days ($n$ = 4 for each; Fig. 8C).

## DISCUSSION

EC dysfunction or the detachment of ECs from an injured artery induces thrombus formation on the lumenal surface, and such thrombi release many bioactive substances including thrombin,[9] platelet-derived growth factor,[5,7] and fibroblast growth factor,[8] which promote mitosis, migration, and phenotypic alteration of SMCs to produce massive extracellular matrices at the site of injury.[29] The formed thrombi can also act as scaffolds in which SMCs accumulate.[30] This uncontrollable SMC behavior is induced locally by the substances released from the thrombus and results in the formation of neointimal hyperplasia.[31–35] Therefore, rapid and complete reendothelialization may be needed to prevent atherosclerosis- and angioplasty-induced thrombus formation and neointimal hyperplasia. The enhancement of reendothelialization has been studied by several techniques. In particular, autologous EC seeding is an especially attractive strategy because the seeded ECs should directly and physiologically contribute to endothelialize the denuded artery without any critical adverse effects. Indeed, previous animal studies of EC seeding have indicated the rapid reendothelialization of denuded arteries and a reduction in the area of neointimal hyperplasia.[12–14] Rapid reen-

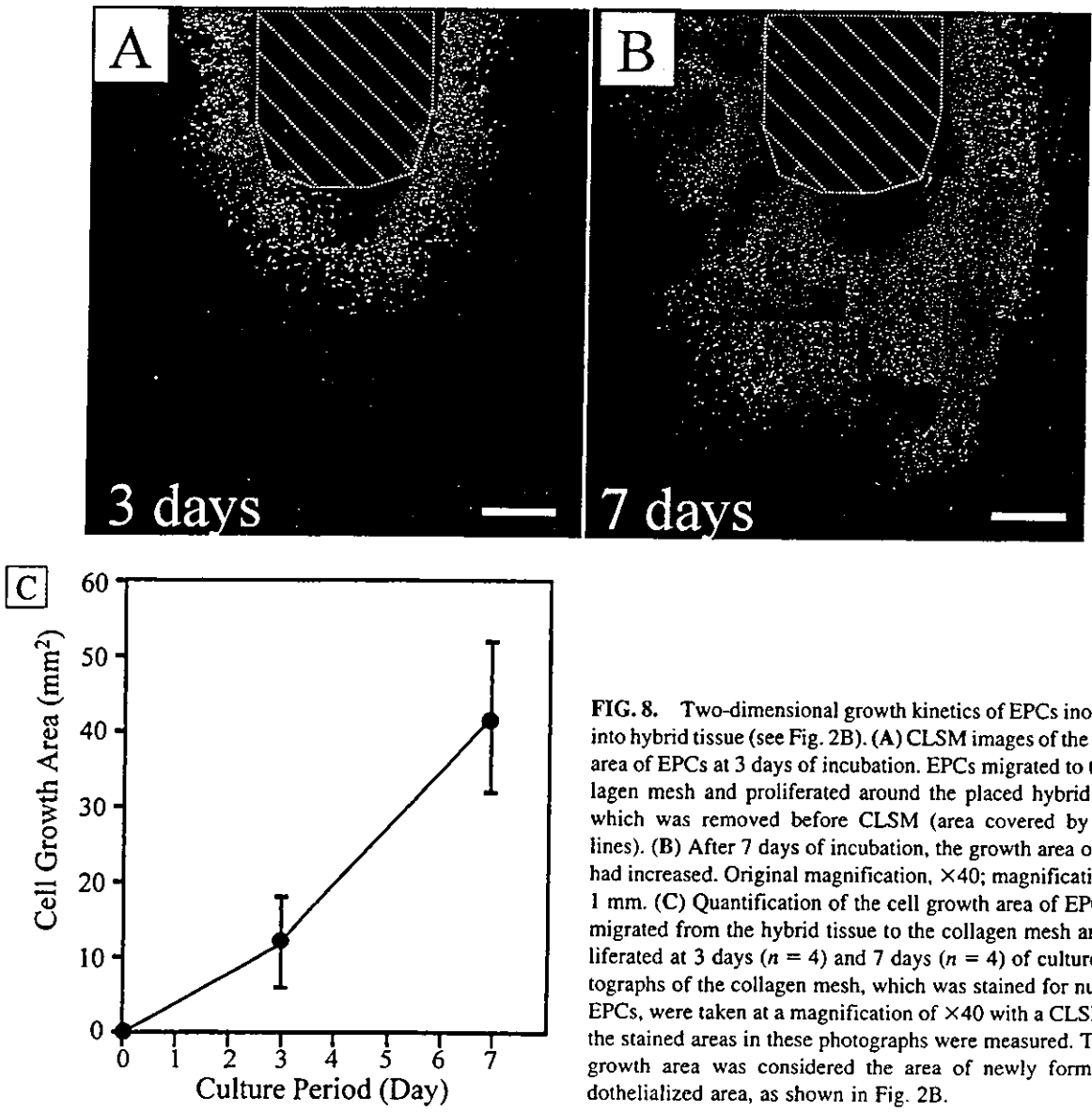


**FIG. 8.** Two-dimensional growth kinetics of EPCs inoculated into hybrid tissue (see Fig. 2B). (**A**) CLSM images of the growth area of EPCs at 3 days of incubation. EPCs migrated to the collagen mesh and proliferated around the placed hybrid tissue, which was removed before CLSM (area covered by dotted lines). (**B**) After 7 days of incubation, the growth area of EPCs had increased. Original magnification, ×40; magnification bar, 1 mm. (**C**) Quantification of the cell growth area of EPCs that migrated from the hybrid tissue to the collagen mesh and proliferated at 3 days ($n$ = 4) and 7 days ($n$ = 4) of culture. Photographs of the collagen mesh, which was stained for nuclei of EPCs, were taken at a magnification of ×40 with a CLSM, and the stained areas in these photographs were measured. The cell growth area was considered the area of newly formed endothelialized area, as shown in Fig. 2B.

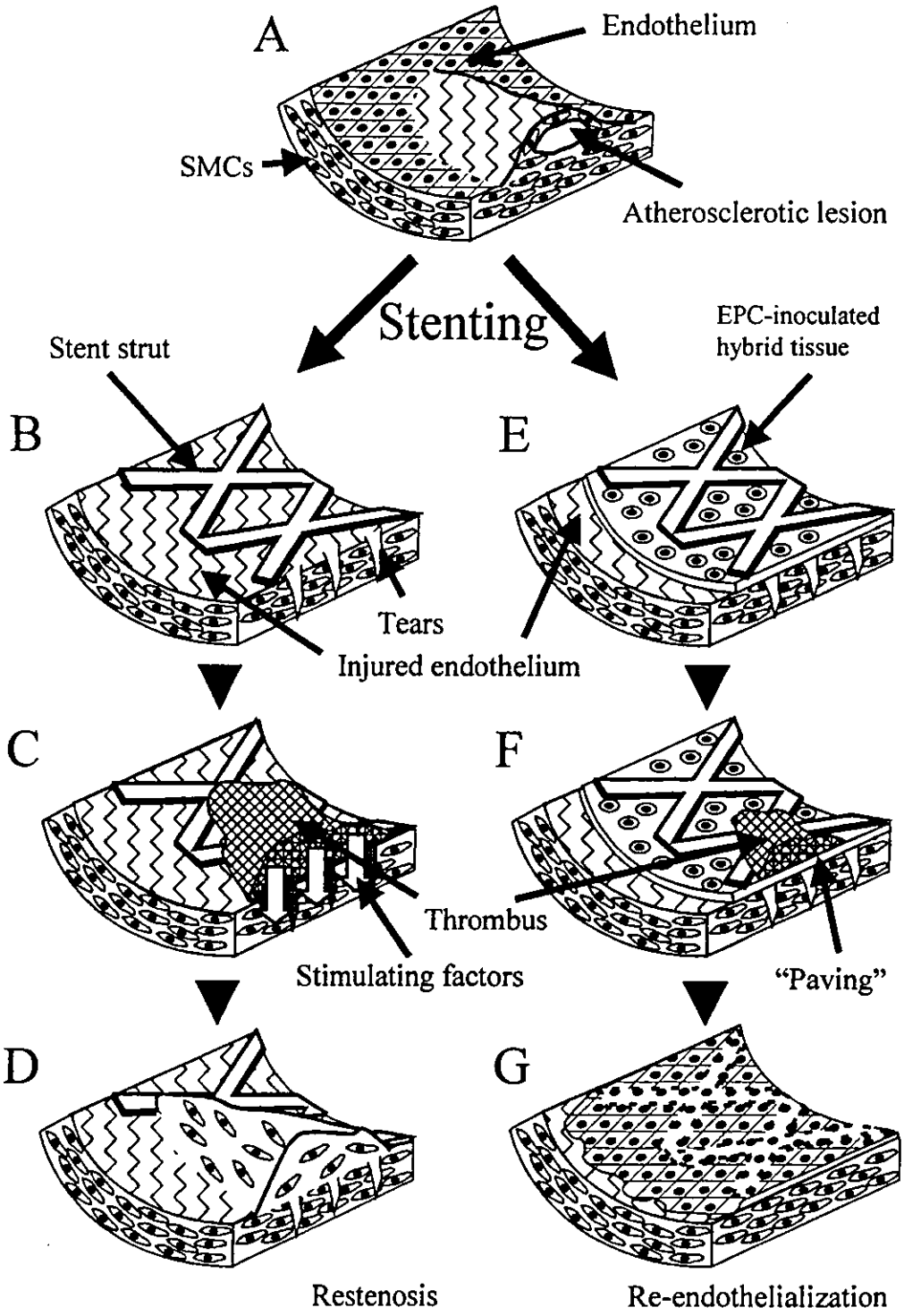


**FIG. 9.** A hypothetical outcome following implantation of an EPC-inoculated hybrid tissue-covered stent. (**A**) Schematic representation of an atherosclerotic lesion. (**B** and **E**) Stenting with or without the EPC-inoculated hybrid tissue induces endothelium injury and dissection of the vascular wall. (**C**) Thrombus formation occurs because of endothelial injury. Formed thrombus releases stimulating factors, which induce SMC migration, proliferation, and production of extracellular matrices. (**D**) Restenosis results from stimulated SMCs. (**F**) In contrast, the EPC-inoculated hybrid tissue showed minimal thrombus formation and "paved" medial SMCs in response to stimulating factors. (**G**) Highly concentrated EPCs may rapidly form new endothelium (reendothelialization), which may result in normalization of the lesion.

dothelialization depends on how many and how securely ECs are delivered to an atherosclerotic site.[15-17] Thus, the EC delivery technique is critical, because the arterial wall is continuously exposed to high hydrodynamic shear stress blood flow, which inhibits the attachment of seeded ECs. Previous studies sought to improve the efficacy of the attachment of seeded ECs to the lumenal surface of an atherosclerotic lesion by using various delivery devices, including a double-balloon catheter[12] that can cut off arterial flow during cell seeding, or a cell/glue matrix[13] to improve EC adhesion to the vascular wall. However, the rate of cell attachment to the lumenal surface of the artery is affected by the amount of time that arterial flow can be cut off, which is greatly limited, especially in the coronary artery. Another factor that affects endothelialization is the number of delivered cells. To harvest a sufficient number of autologous ECs, large vein or tissue microvessels must be harvested conventionally. However, the need for surgical invasion may hamper the clinical use of the EC-seeding techniques. Thus, a less invasive autologous EC source is necessary for applying the EC-seeding methods in practical cardiovascular medicine.

To address these problems, our proposed procedure and device uses tissue-engineering and progenitor cell technology. It has been shown that few EPCs circulate in the peripheral blood.[18-21] EPCs were used as a cell source in this study because they could be obtained with only minimal invasiveness. EPCs, which are not mature ECs but become so under a differentiation-inducing process, have antithrombogenic potential similar to ECs, as was verified for human EPCs,[27] and *in vivo* antithrombogenicity was shown by canine EPCs-seeded small-diameter vascular graft[25] in our previous studies. A tissue-engineered hybrid tubular tissue was prepared by the cell traction-driven process using EPC-inoculated collagen gel, similar to a method described previously.[36] The delivery of this hybrid tissue and its adhesion to the vascular wall lumenal surface was achieved with an intralumenal angioplastic device, an expandable metallic stent. This intralumenal tissue-engineered therapeutic device could deliver a large number of cells for rapid reendothelialization.

Our results showed that thin tissue (wall thickness, $125 \pm 12.0\ \mu m$) exhibited mechanical elasticity for stent expansions ranging from 1.5 to 3 mm in diameter with no macroscopic or microscopic tears (Figs. 3 and 4). Such elasticity is suitable for percutaneous implantation to small arteries including the coronary artery. Such expandable thin hybrid tissue may completely "pave" or cover atherosclerotic, EC-denuded vascular walls.

Furthermore, this hybrid tissue could attach highly concentrated cells to the arterial wall. The expanded hybrid tissue (length, 2 cm) contained $(5.8 \pm 1.8) \times 10^5$ viable cells (Fig. 5), and the implanted cell density was estimated to be $3.1 \times 10^5$ cells/cm$^2$ (assuming that the expanded stent diameter was 3 mm). Thus, the number of cells was much higher than that needed to obtain maximal endothelialization based on previous studies on seeding ECs in solutions.[16,17]

*In vitro* culture in hybrid vascular medial tissue showed that inoculated EPCs migrated and proliferated to completely cover the lumenal surface, including the surface of the stent struts, within 7 days (Figs. 6 and 7). These results suggest that implanted EPCs will rapidly form a fully endothelialized lumenal surface on the injured arterial wall, and this enhanced endothelialization will prevent thrombus formation and restenosis. Furthermore, an evaluation of the two-dimensional growth kinetics of EPCs inoculated in the hybrid tissue on the collagen mesh showed that migrated EPCs endothelialized the surrounding area, and the endothelialized area increased with time (Fig. 8). A hypothetical *in vivo* outcome of the reendothelialization process is shown in Fig. 9. An extensive implantation study using swine coronary arteries to verify this hypothesis is now underway.

In conclusion, a new therapeutic procedure using a tissue-engineered device was explored to "pave" or "adhere" EPCs to the lumenal surface of atherosclerotic sites. These highly proliferative EPCs are expected to rapidly provide full endothelialization.

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Address reprint requests to:
*Takehisa Matsuda, Ph.D.*
*Department of Biomedical Engineering*
*Graduate School of Medicine*
*Kyushu University*
*Maidashi, Higashi-ku*
*Fukuoka 812-8582, Japan*

*E-mail:* matsuda@med.kyushu-u.ac.jp

# Canine endothelial progenitor cell-lined hybrid vascular graft with nonthrombogenic potential

Hongbing He, MD[a,b]
Toshihiko Shirota, MD[a,c]
Hisataka Yasui, MD, PhD[c]
Takehisa Matsuda, PhD[a]

**Objective:** We sought to fabricate a compliant engineered vascular graft (inner diameter of approximately 4.5 mm and length of 6 cm) lined with endothelial progenitor cells derived from circulating peripheral canine blood and to verify its nonthrombogenicity potential in vivo.

**Methods:** Autologous circulating endothelial progenitor cells derived from the peripheral veins of 6 adult mongrel dogs were isolated by using a density gradient method. The cells were proliferated in vitro in EGM-2 culture medium, prelined on the luminal surface of in situ–formed collagen type I meshes as an extracellular matrix, and wrapped with a segmented polyurethane thin film with multiple micropores as a compliant scaffold. After canine carotid arteries were bilaterally implanted with these grafts for 1 and 3 months, microscopic observation, histologic staining, and immunochemical staining were performed to evaluate morphogenesis.

**Results:** After 33.3 ± 10.5 days of culture in vitro, 4.2 ± 1.2 × $10^6$ endothelial progenitor cells were obtained. Eleven of the 12 engineered vascular grafts were patent. The grafts possessed smooth, glistening, and ivory-colored luminal surfaces at the predetermined observation period up to 3 months. The intimal layer was covered with confluent, cobblestone-like monolayered cells that were positively stained with factor VIIIB–related antigen. The thickness of the neoarterial walls was approximately 300 μm at 3 months after implantation. A few smooth muscle cells were observed in the medial tissue, and fibroblasts dominated the adventitial tissue.

**Conclusion:** Circulating endothelial progenitor cells could be a substitute source of endothelial cells for endothelialization on small-diameter-vessel prostheses to ensure nonthrombogenicity.

From the Departments of Biomedical Engineering[a] and Cardiac Surgery,[b] Graduate School of Medicine, Kyushu University, Fukuoka, and the Department of Bioengineering,[c] National Cardiovascular Center Research Institute, Osaka, Japan

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Address for reprints: Takehisa Matsuda, MD, Department of Biomedical Engineering, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku grbf, Fukuoka 812-8582, Japan (E-mail: matsuda@med.kyushu-u.ac.jp).

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Well-controlled tissue regeneration, healing, and vital function underlying the nonthrombogenic potential of tissue-engineered small-diameter vascular grafts can be achieved with combinations of a proper selection of key element materials, a fabrication process technology of grafts, and a proper cell-seeding technique and use of an appropriate source of vascular cell types. It has been verified by many research groups that an autologous endothelial cell (EC)–lined graft has proved antithrombogenicity. However, despite the experimentally proved nonthrombogenicity of endothelialized hybrid grafts, only a limited clinical application of such grafts has been reported, although Deutsch and colleagues[1] have been continuously implanting endothelialized grafts under well-controlled clinical criteria using a defined technique. Although significant advancement in vascular tissue engineering has been made with ECs, harvesting ECs from a patient remains a problem of great concern.

On the other hand, recent studies evidenced that mature ECs and immature endothelial-like cells float in circulating peripheral blood.[2-5] Mature ECs are derived

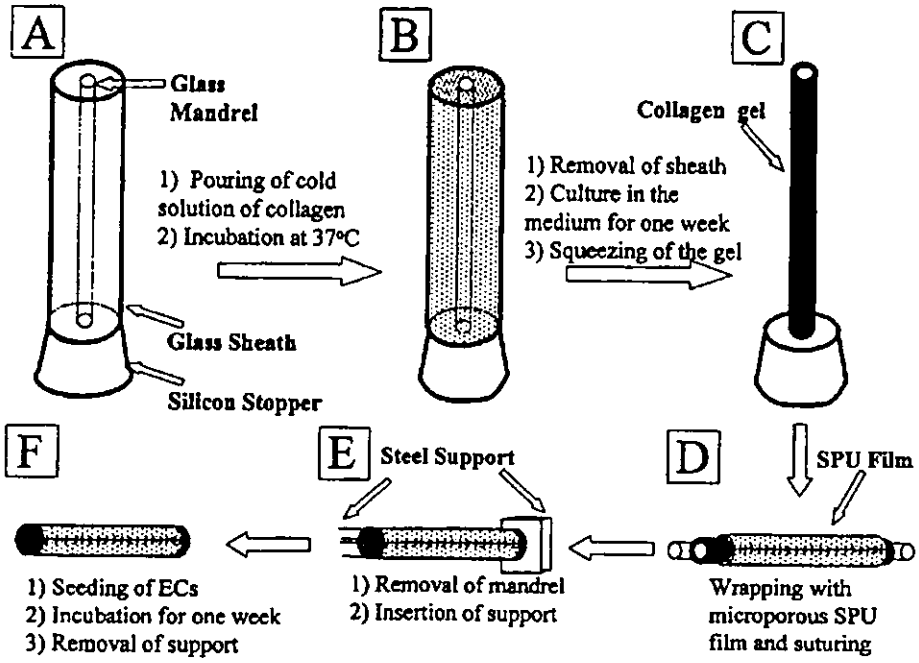


**Figure 1. Schematic of fabrication processes for the artificial vascular graft that is lined with an in situ–formed type I collagen mesh as the matrix and wrapped with the SPU as the scaffold.**

from those that fall out from the vascular wall (fall-out ECs). The possibility of the existence of fall-out ECs has been discussed over the years, and the fall-out hypothesis recently has been verified experimentally.[6] Endothelial progenitor cells (EPCs), which fall out from the bone marrow,[3,4,7] are expected to have much higher proliferative potential than mature fall-out ECs.[4] It is anticipated that when EPCs are harvested from peripheral blood and effectively proliferate, they serve as a very promising alternative source of lining for cells. This eliminates the need for surgical removal of the veins from which ECs are usually harvested for tissue-engineered vascular grafts. Recently, Kaushal and coworkers[8] reported that ovine EPC–seeded, decellularized porcine iliac vessels have been remodeled to provide a functional vessel on implantation and inherent functional properties, especially nitric oxide (NO) production in response to physiologic stimuli, suggesting that EPCs are being differentiated to mature ECs with implantation time and strongly implying that the EPC is an alternative source of nonthrombogenic cell lining.

In this study EPCs were harvested from canine peripheral blood and cultured. A compliant graft, which was assembled with in situ–prepared collagen fiber meshes and a microporous segmented polyurethane (SPU) tubular film, was seeded with EPCs. The preliminary in vivo implantation study was conducted up to 3 months.

## Materials and Methods

### Harvesting and Culturing

The investigations were performed according to the "Principles of Laboratory Animal Care" (formed by the National Society for Medical Research) and the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication no. 56-23, revised 1985). Peripheral blood (15 mL/sample) was collected from 6 adult male mongrel dogs (weight, 27.5 ± 3.1 kg) by means of venipuncture, placed in 50-mL polypropylene centrifuge tubes (IWAKI; Asahi Techno Glass, Tokyo, Japan), and anticoagulated with heparin sodium (final concentration, 200 U/mL; Novo Corp, Tokyo, Japan). The anticoagulated blood was diluted 1:2 with Hanks solution (Life Technologies Inc, Rockville, Md) containing 1 mmol/L ethylenediamine tetraacetic acid and 0.5% bovine serum albumin.

Mononuclear cells were isolated from the blood sample by using a density gradient method with Histopaque-1077 (Sigma Chemical Co, St Louis, Mo) according to the manufacturer's instructions. Cells obtained from the plasma-Histopaque-1077 interface were washed 3 times by means of centrifugation at 250*g* for 10 minutes with MCDB 131 medium (Lite Technologies Inc), containing 5 U/mL heparin sodium. The cells resuspended in EGM-2 culture medium (Clonetics Inc, San Diego, Calif) were inoculated into a culture dish precoated with type I collagen (IWAKI, Asahi Techno Glass) and cultured in EGM-2 in a humidified atmosphere of 5% $CO_2$ at 37°C. The culture medium was freshened twice a week. When the primary cells reached approximately two thirds of the growth area, the cells were subcultured with 0.01% trypsin (Life Technologies Inc) phosphate-buffered

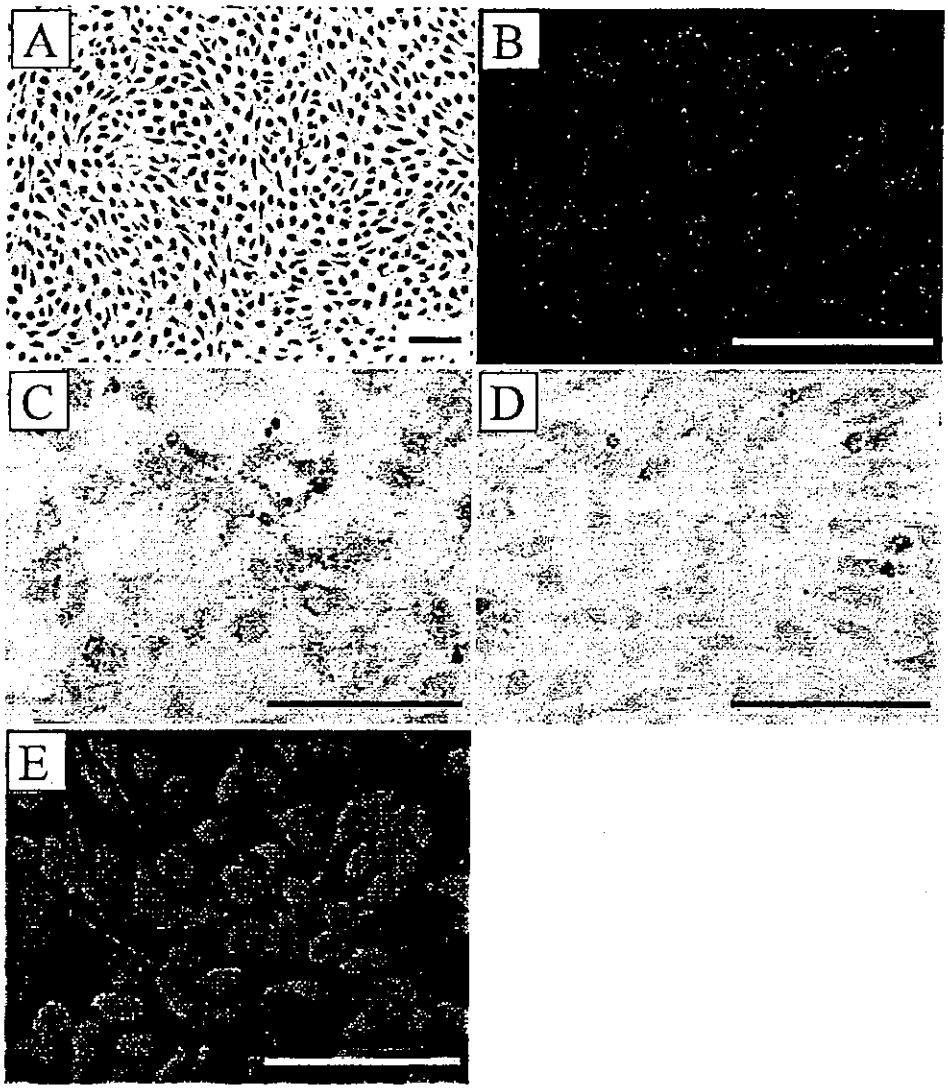


**Figure 2. Blood-derived circulating EPCs in subculture: *A*, phase-contrast microscopy (original magnification 100×); *B*, positive uptake of fluorescence-labeled acetylated low-density lipoprotein (confocal scanning microscopy; original magnification 400×); *C*, positive immunochemical staining of factor VIII–related antigen (light microscopy; original magnification 400×); *D*, positive immunochemical staining of Flk-1 (light microscopy; original magnification 400×); *E*, intracellular NO production proved by means of intracellular staining with an NO-specific indicator, DAF-2DA. *Scale bars* = 100 μm.**

saline solution and inoculated into a nonpretreated polystyrene culture dish (IWAKI, Asahi Techno Glass) with a split ratio of 1:4 to 6. The immunohistologic staining of the rat monoclonal anti-factor VIII–related antigen (Dako Corp, Glostrup, Denmark) and mouse monoclonal anti-human Flk-1 (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), as well as the uptake of fluorescence-labeled acetylated low-density lipoprotein (DiI-acetylated-LDL; Biomedical Technologies Inc, Stought, Mass) were performed to identify the subcultured cells. Diamino-fluorescein-2 diacetate (DAF-2DA; Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan), which is a membrane-permeable, intracellular, NO-specific green fluorescence indicator, was used to detect NO expression.[9] EPCs immersed in serum-free M199 containing 5 μmol/L DAF-2DA for 60 minutes at 37°C were fixed and examined with a confocal laser scanning microscope (Radiance 2000; Bio-Rad Laboratories Inc, Hercules, Calif).

### Cell Growth Curve

The number of subcultured cells in the culture dish was determined by using the method described by Zilla and associates.[10] Briefly, by using a convert microscope, photographs were taken in 10 randomized areas at a magnification of 200× with instant film (Polaroid 667, Cambridge, Mass). The number of cells in each

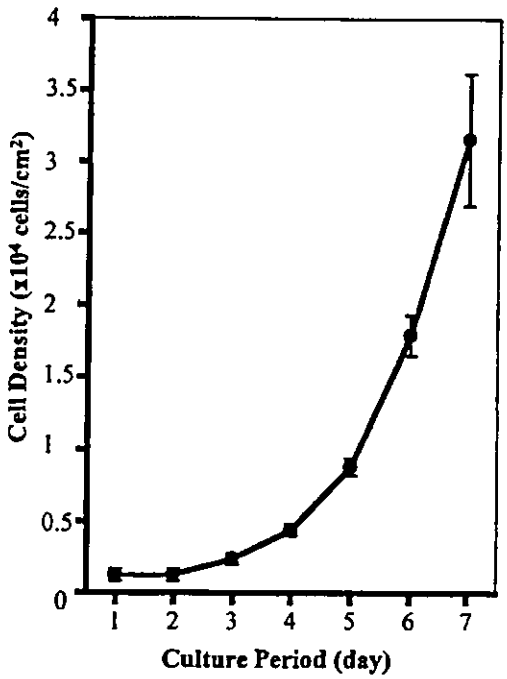


**Figure 3. Growth curve of the circulating EPCs in subculture. Each result is expressed as the mean ± SD. The PDT in the exponential stage was 23 to 29 hours.**

photograph was counted. The cell density was calculated from the average number of cells in the 10 photographs. The population doubling time (PDT) was determined on the basis of the growth curve (from the first day after subculture to the seventh day, n = 3).

### Fabrication of the Artificial Vascular Graft

The procedure followed for fabrication of the compliant artificial vascular graft was our previously reported method.[11] Briefly, a cold mixture of 8 mL of M199 (Life Technologies Inc) culture medium and 8 mL of acid-solubilized bovine dermal type I collagen solution (0.3%, CELLGEN; Koken Corp, Tokyo, Japan) was used to fill the space between the sheath (inner diameter of 15 mm) and mandrel (outer diameter of 5 mm) of a tubular glass mold, as shown in Figure 1. After subsequent thermal gelation at 37°C for 30 to 60 minutes, the sheath was removed. The resultant collagen gel was allowed to stand in the medium for an additional week. The collagen gel around the glass mandrel was squeezed to expel water; wrapped with a 6-cm-long SPU (thickness of 100 μm; Shidame Co, Ltd, Tokyo, Japan) in which the pore-to-pore distance and the pore size were 1 mm and 100 μm, respectively; and then sutured by using the surgical continuous suture technique. Details of the in-house preparation of micropores by using the excimer laser ablation technique were described in our previous work.[12] The collagen gel was designed 3 mm longer than the SPU film wrapping at both ends to prevent the formation of a false aneurysm in the anastomotic sites after interposition. Protruding parts were everted on both ends of the graft to encapsulate the edges of the wrapping and were fixed with 4 sutures circumferentially. The protocol for prelining the inner surface of the vascular graft with EPCs was described in detail in our previous article.[13] Briefly, after 2 mL of EPC-containing M199 solution ($1.0 \times 10^6$ cells/mL) was poured into the lumen of the tissue, the graft was placed horizontally and incubated at 37°C for 20 minutes. Then the graft was rotated 90°, and additional EPC-containing M199 solution was poured into the lumen of the graft. This process was repeated 4 times to achieve confluent density of ECs on the luminal surface of the graft. After 2 days of incubation in M199 supplemented with 20% fetal bovine serum, an EPC-seeded graft was obtained. An intraluminal mechanical support with 6 stainless steel wires, which was described in our previous article,[13] was used when EPCs were seeded. The support was removed before implantation.

### Graft Implantation and Retrieval

General anesthesia was induced by means of injection of intramuscular ketamine (Ketaral 50, 15 mg/kg; Sankyo Co, Ltd, Tokyo, Japan) and intravenous pentobarbital (Nembutal, 19 mg/kg; Dainippon Pharmaceutical Co, Ltd, Osaka, Japan) and maintained by means of intermittent injection of pentobarbital. Bilateral paratracheal incisions were made along the trachea, and both common carotid arteries were dissected, which yielded a segment approximately 10 cm in length. The end-to-end interposition of the prepared artificial vascular graft was performed by running 6-0 polyprolene sutures into bilateral common carotid arteries of the same dog from which the cells were isolated. Neither anticoagulant nor antiplatelet agents were administered, except for the intraoperative heparin (100 U/kg) injected intravenously. The implantation observation period was predetermined to occur at 1 and 3 months. Graft patency was evaluated with manual palpation of the pulse and direct observation of blood flow through the grafts at the time of death. The graft, together with approximately 1- to 2-cm native arteries at both ends, was fixed at 150 mm Hg of pressure for 2 hours with 300 mL of fixative after flushing with 500 mL of phosphate-buffered saline solution containing 100 U/mL heparin for approximately 30 minutes to 1 hour. Then the graft was longitudinally opened, photographed, and subjected to further evaluation.

### Scanning Electron Microscopic Examination

After postfixation in 1% osmium tetroxide, freeze-drying, and subsequent coating by sputtering with platinum, the luminal surfaces of the samples were observed with a scanning electron microscope (S-4000; Hitachi Corp, Tokyo, Japan).

### Histologic Examination

Longitudinally prepared histologic sections were subjected to histologic staining by using the following stains. First, hematoxylin-and-eosin stain was used for general evaluation. The thickness of the neoarterial wall, defined as the thickness of the tissue between the EPC monolayer and the inner surface of the SPU film, was measured from the distal anastomostic site to the midportion of the graft interval of 0.5 cm. Five metric points for each graft were projected with the aid of an image processor (original magnification 100×). Second, Masson trichrome stain was used for collagen. Third, Alcian blue stain was used for proteoglycans. A monoclonal mouse antibody against an anti-factor VIII–related antigen (for EPCs), anti-vimentin (for fibroblasts), and anti-smooth muscle