Controlled Release of Basic Fibroblast Growth Factor From Gelatin Hydrogel Sheet Improves Structural and Physiological Properties of Vein Graft in Rat

Tomonori Haraguchi, Kenji Okada, Yasuhiko Tabata, Yoshimasa Maniwa, Yoshitake Hayashi, Yutaka Okita

Objectives—Autologous vein grafts are still widely used, but their long-term patency is suboptimal. The objective of the current study was to determine whether wrapping a vein graft in gelatin hydrogel sheet incorporating basic fibroblast growth factor improves their mechanical and physiological properties.

Methods and Results—Autologous femoral vein was interposed into the abdominal aorta in rats. The rats were divided into 3 groups: nontreated grafts (group A), grafts wrapped in basic fibroblast growth factor-free gelatin hydrogel sheet (group B), and grafts wrapped in basic fibroblast growth factor-impregnated gelatin hydrogel sheet (group C). On day 1, endothelial desquamation was observed in group A, and the media in groups A and B were disrupted, staining positive in the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay. In contrast, the media in group C remained intact and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling—negative, associated with activation of MAPK. Graft dilation was significantly inhibited in groups B and C compared with group A, with those in group C showing the smallest degree of neointimal proliferation. At 8 weeks grafts in group C developed neointima with homogeneous elastic laminae, presence of rigid neoadventitia that displayed neovascularity, and the highest blood flow velocity.

Conclusions—Wrapping vein grafts in basic fibroblast growth factor-impregnated gelatin hydrogel sheet improved their structural and physiological properties, and might therefore also improve long-term patency. (Arterioscler Thromb Vasc Biol. 2007;27:548-555.)

Key Words: basic fibroblast growth factor ▪ gelatin hydrogel ▪ vein graft

Recently, coronary arterial bypass grafting is often performed using arterial conduits such as internal mammary artery or radial artery rather than saphenous vein grafts, because of the superior long-term patency of arterial grafts. However, arterial grafts are susceptible to arteriosclerosis, and the available number of arteries for grafting is limited. Moreover, alternatives such as small-caliber synthetic vascular prostheses are still undergoing development for practical use in coronary arterial bypass grafting because of thrombogenicity. Improvement of vein graft patency would therefore be highly beneficial in improving the quality of coronary arterial bypass grafting, as well as surgery for peripheral arterial disease.

Vein graft occlusion is attributable to the neointimal hyperplasia caused by vascular smooth muscle cell (SMC) proliferation. Disruption of endothelium and media of the vein graft by mechanical forces associated with pulsatile blood flow is considered to be one of the causes of such neointimal hyperplasia. In the literature, mechanical perivenous support such as that provided by polytetrafluoroethylene or polyethylene graft stents may protect the endothelium and media of vein grafts from mechanical destruction. We selected gelatin hydrogel sheet (GS) as a substitute for polytetrafluoroethylene or polyethylene because the former is a biodegradable and biocompatible material, and is suitable for wrapping vein grafts because of its pliability.

It has also been shown that pulsatile arterial blood flow may promote apoptosis of vascular SMC. Basic fibroblast growth factor (bFGF) is known to be a potent angiogenic factor, and has also been identified as a survival factor (anti-apoptotic factor) for endothelial cells (ECs) and SMCs of vein grafts. Our previous studies have demonstrated that gelatin hydrogel can bind bFGF by reciprocal electric affinity, and result in the sustained release of bFGF over a period of several weeks. In the current study, we sought to verify our hypothesis that wrapping vein grafts in bFGF-
impregnated GS would preserve vascular endothelium and media both pharmacologically and mechanically, and result in improved long-term patency.

Raf-1 protein kinase is considered to be one of the most important signaling mediators in anti-apoptotic intracellular signaling pathway in ECs via activation of mitogen-activated protein kinase (MAPK). However, little is known about anti-apoptotic signaling pathways in SMCs in the media of vein grafts. In this study, therefore, we also investigated this issue in vein grafts induced by the bFGF released from GS.

Materials and Methods

Animals
Ten-week-old Sprague-Dawley rats (CLEA Japan Inc, Tokyo) were used for this study. They were maintained on a light/dark (12/12-hour) cycle at 22°C, receiving normal food and water ad libitum. The handling of laboratory animals and their use in experiments conformed to the Guidelines for Animal Experiment at Kobe University Graduate School of Medicine and the Guide for the Care and Use of Laboratory Animals published by the National Academy Press.

Preparation of bFGF-Impregnated GS
Human recombinant bFGF with an isoelectric point of 9.6 was supplied by Kaken Pharmaceutical Co, Tokyo, Japan. The gelatin used in this study was isolated by an alkaline process from bovine bone with an isoelectric point of 4.9 and molecular mass of 99 000 kDa (Nitta Gelatin Co, Osaka, Japan). GS were prepared by chemical cross-linking of 5% bovine bone gelatin solution with glutaraldehyde. GS of 20 mm × 0.3 mm were prepared and immersed in 50 mmol/L glycine aqueous solution at 37°C for 1 hour to block residual aldehyde groups of glutaraldehyde. The sheets were freeze-dried and sterilized by ethylene oxide gas. Basic fibroblast growth factor was incorporated into GS by impregnation for 30 minutes before implantation, concomitant with exposure to a peptide antibiotic (vancomycin hydrochloride; Meiji Seika Kaisha, Ltd, Tokyo, Japan).

Vein Graft Procedure and Experimental Groups
Rats were anesthetized with pentobarbiturate (5 mg/kg body weight intraperitoneally). The right femoral vein without branches was transected and removed in heparinized saline. After heparinization (400 U/kg body weight intravenously), the abdominal aorta was transected and the femoral vein graft interposed by end-to-end anastomosis using a 9-to-0 polypropylene suture (PROLENE; Johnson & Johnson Gateway, LLC, NJ). GS with or without bFGF was cohesive enough to wrap the vein graft tightly. GS of sufficient length to include both anastomotic sites was used to wrap the graft twice.

A total of 90 rats were divided into three groups as follows: group A received nontreated grafts (n = 29); group B, grafts wrapped in bFGF-free GS (n = 31); group C, grafts wrapped in bFGF-impregnated GS (100 μg) (n = 30). In groups B and C, an antibiotic (vancomycin hydrochloride, Meiji Seika Kaisha, Ltd) was incorporated into GS, whereas in group A the agent was injected into surrounding tissues.

Measurement of Graft Diameter
Graft diameter was defined as the maximum dimension of the transverse minor axis of the vein graft, and the graft dilation was calculated according to the following formula:

\[ \text{Graft dilation ratio} (\%) = \left( \frac{\text{diameter at harvesting} - \text{diameter at implantation}}{\text{diameter at implantation}} \right) \times 100 \]

Measurement of Blood Flow Velocity
The velocity of blood flow through the vein graft was measured using a Transonic flowmeter (Transonic System Inc, NY) and was recorded using PowerLab/MacLab800 (AD Instruments Japan Inc, Nagoya, Japan). It was measured at 5 different sites in the graft, and the mean value was taken as the flow velocity.

Evans Blue Staining
To evaluate the EC desquamation throughout the whole region of vein graft, Evans blue staining was performed based on a previous study by Saria et al. Evans blue (10 mg/kg) (ICN Biomedicals, Aurora, Ohio) was injected intravenously 30 minutes before vein graft harvesting (n = 5 for each group). After perfusion-fixation with 100% methanol, vein grafts including both anastomotic sites were removed and opened longitudinally for macroscopic examination. Then the vein graft was separated from aorta, it was decolorized in 20-fold weight of formamide (Amresco Inc, Solon, Ohio) at room temperature for 48 hours. Evans blue uptake into formamide was determined by determination of absorbance, in which absorbance at 600 nm was measured by using a Gene Quant Pro RNA/DNA Calculator (Amersham-Pharmacia Biotech, Cambridge, UK) and the concentration was determined by the calibration curve.

Specimen Preparation
Harvested vein grafts were rinsed in saline and preserved in 10% formalin. The formalin-fixed grafts were dehydrated in a graded ethanol bath, cleaned in xylene, embedded in paraffin, and sectioned (thickness, 4 μm) for histological analysis.

Immunohistochemistry
Immunohistochemical identification of α smooth muscle actin (α-SMA) and von Willebrand Factor were performed on sections prepared as described, using the enhanced polymer 1-step staining method (enhanced polymer 1-step staining anti-human α SMA/HRP, enhanced polymer 1-step staining anti-human von Willebrand factor/horseradish peroxidase, Dako Japan Co, Ltd, Kyoto, Japan). The presence of phosphorylated Raf-1 (p-Raf-1), phosphorylated Erk1/2, and phosphorylated Elk-1 was assessed using rabbit polyclonal antibodies against p-Raf-1 phosphorylated on tyrosine 340/341 (Santa Cruz Biotechnology, Inc), or on serine 338/339 (Santa Cruz Biotechnology, Inc), phosphorylated Erk 1/2 (Cell Signaling Technology, Inc), and phosphorylated Elk-1 phosphorylated on serine 383 (Cell Signaling Technology, Inc). These first antibodies were diluted 1:100 in antibody diluent (Dako Denmark A/S) and applied to the prepared sections as above overnight at 4°C. The sections were visualized with donkey anti-rabbit Ig conjugated with fluorescein isothiocyanate (Amersham Biosciences, Corp) using fluorescence microscopy (Carl Zeiss Vision, Hallbergmoos, Germany).

TUNEL Staining
Accumulated internucleosomal DNA fragments were detected using an in situ apoptosis detection kit (Apoptosis in situ Detection Kit ApopT; Chemicon International, Inc). The samples were stained with peroxidase substrate (DAB) and counterstained in hematoxylin solution.

Western Blot Analysis
Frozen vein grafts were homogenized in 20-fold weight of tissue protein extraction reagent (Pierce Biotechnology, Inc) and their protein concentration evaluated with a dye-binding assay that involved the use of the Bio-Rad reagent (Bio-Rad Laboratories). Sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer was added to the extracts, and was heated in a boiling water bath for 5 minutes and then centrifuged at 10 000 rpm for 10 seconds. Supernatants were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% polyacrylamide gel, 150 V, 50 minutes.). Electrophoresis of proteins from the gel to a nitrocellulose membrane was performed for 60 minutes at 230 mA. The membrane was then soaked in 5% bovine serum albumin diluted with Tris-buffered saline containing 0.1% Tween20 (MP Biomedicals, Inc, Aurora, Ohio) to reduce nonspecific protein binding, then probed overnight at 4°C with a 1:250 dilution of the first antibodies described in the Immunohistochemistry section. Enhanced chemiluminescence analysis was performed according to the manufacturer’s
instructions (Amersham Biosciences, Corp). Blots were subsequently probed for β-actin (Cell Signaling Technology, Inc) as an additional internal control for equivalent protein loading. The signals were quantified with a densitometer (Kodak EDAS290 System; Eastman Kodak Co, Rochester, NY).

Quantification of Neointimal Area

For area measurement, Elastica van Gieson-stained cross-sections were reviewed using a BX50 microscope (Olympus Optical Co Ltd, Tokyo, Japan) equipped with a charge-coupled device camera (Sony Co Ltd, Tokyo, Japan) and television monitor, and using computer-aided image analysis software (Scion Image, Scion Corp, Bethesda, Md). The neointimal area was determined by subtracting the area of the lumen from the area bounded by the media. The proportion of neointima was determined by using following formula: neointima (%) = neointimal area/area bounded by the media × 100

Evaluation of New Vessels

To evaluate the angiogenic effect of bFGF, the number of neovessels in the adventitia of vein grafts was determined. Vessel counts were obtained in each group at 8 weeks under microscopic examination and using quantitative methods. The analysis was carried out in specimens immunostained for α-SMA and von Willebrand factor, and adventitial vessels >20 μm in diameter were counted.

Statistical Analysis

Means and standard deviations were calculated for each measured parameter from the A, B, and C groups at 2, 4, and 8 weeks. Student t test was used for comparisons between 2 groups, whereas the multiple comparison test (Tukey-Kramer) was used for comparisons between >2 groups. The data were computed with StatView 5.0.1 (SAS Institute Inc, Cary NC). A value of P<0.05 was considered significant.

Results

Affinity of the GS for Vein Grafts

The GS was attached firmly to the vein graft as early as day 1 after operation (Figure 1A to 1C), and was completely biodegraded by 4 weeks. Inflammatory changes were not discernible in any group at any time period.

Perivenous Stent Effect of GS Wrapping

At implantation, in selected cases (n=5), the blood flow velocity through the vein grafts without GS was found to be reduced by ~40% compared with the adjacent proximal abdominal aorta (Figure 1D). Moreover, the amplitude was smaller and oscillations were damped out in comparison with proximal aorta. However, vein grafts with GS wrapping showed no change of amplitude and frequency of the blood flow wave (Figure 1D).

Endothelial Preservation

Endothelium of the graft in groups B and C showed strong staining compared with group A with antibody specific to von Willebrand factor at day 1 (Figure 2A). In group A, thrombi formed where ECs were desquamated, and were covered with neo-endothelium on day 1 (supplemental IIIA, available online at http://atvb.ahajournals.org). To evaluate EC desquamation throughout the whole region of vein graft, the graft was stained with Evans blue dye. Evans blue is believed to penetrate areas of intima that was not covered by endothelium18,19 and have a high affinity for elastin,20 which constitute elastic laminae. Vein grafts in group A stained more deeply with Evans blue than those in groups B or C (Figure 2B). In accordance with these macroscopic findings, the Evans blue uptake was significantly higher in group A (Figure 2C). In Figure 2B, the proximal and distal aortas were stained more deeply than the vein graft between them. It would be attributed not only to anastomotic manipulations but also to a high degree of affinity of Evans blue for elastin, which is predominantly contained in an artery as elastic laminae. (supplemental Figure IIIB).

Antiapoptotic Effects of bFGF-Impregnated GS on Vein Graft

In groups A and B, the media of graft was disrupted and stained TUNEL-positive as early as day 1, whereas the media in group C remained intact and TUNEL staining was essentially negative (Figure 3). The frequency of TUNEL-
positive cells was significantly lower in group C than in groups A or B (A: 56.4 ± 5.9%, B: 64.6 ± 10.2%, C: 10.4 ± 2.8%). To evaluate the mechanism by which bFGF-impregnated GS exerts its anti-apoptotic effects, immunohistochemistry and Western blot analysis of vein grafts on day 1 were performed. The endothelium in groups B and C stained strongly with both p-Raf-1 (Ser) and p-Raf-1 (Tyr) antibodies compared with group A (Figure 4, left panel arrowheads). Meanwhile in the media, group C showed a striking increase in Raf-1 (Ser), Erk1/2, and Elk-1 phosphorylation by immunohistochemistry, both compared with groups B as well as A (Figure 4, left panel stars). Western blot analysis and subsequent densitometry showed strong activation of Raf-1 (Ser), Erk1/2, and Elk-1 in group C, which corresponded to the results of immunohistochemistry (Figure 4, right panel).

Figure 2. A, Vein graft cross sections on day 1. Staining for von Willebrand factor, magnification ×400. Endothelium of the graft in groups B and C showed strong staining for von Willebrand factor compared with group A. e indicates endothelium. B, Macroscopic findings of Evans blue staining of vein graft on day 1. Vein graft in group A was stained more deeply with Evans blue than in groups B and C. Proximal or distal anastomotic sites were deeply stained with Evans blue in all groups because of intimal desquamation by aortic clamping or anastomotic manipulation. Arrows, anastomotic sites; p-Ao, proximal aorta; v, vein graft; d-Ao, distal aorta. C, Quantitative evaluation of Evans blue uptake of vein graft for each group. The value of group A was significantly higher than the other groups. #P < 0.05.

Figure 3. Vein graft cross-sections on day 1. Elastica van Gieson staining (a–c), magnification ×40. Elastica van Gieson staining (d–f), magnification ×200. TUNEL staining (g–i), magnification ×200. The media of groups A and B are disrupted (a, b, d, e) and show positive TUNEL staining (g, h brown), but were maintained intact in group C with no TUNEL staining (c, f, i).
Neointimal Progression

Elastic laminae, which were stained black with Elastica van Gieson, had emerged in the neointima in each group by 4 weeks (Figure 5A). However, the appearance of elastic laminae was patchy and the laminar structure of the media had disappeared in group A (Figure 5A a, d). In group B, the neointima was thicker than group C and the laminar structure was poorly defined as in group A. (Figure 5A b,e). In group
C, the structure of the graft wall, especially the internal elastic lamina, remained intact (Figure 5A f arrows). Moreover, the neointima in group C was thinner than in the other groups (Figure 5A c,l) and, statistically, the percentage of neointima was significantly lower (Figure 5B).

Graft Dilation
There were no significant differences in the graft diameter at implantation (group A, 1.62±0.20 mm; group B, 1.59±0.25 mm; group C, 1.66±0.17 mm). However, the graft dilation rates in groups B and C were lower than in group A at every phase of the study thereafter with one exception of group B at 8 week (Figure 5C).

Graft Blood Flow Velocity
There were no significant differences in mean blood flow velocity among the 3 groups at 2 and 4 weeks. However this value was significantly higher in group C at 8 weeks than in the other groups (Figure 5D).

Vein Graft Remodeling
More than one-third of the grafts in group A formed dumbbell-like aneurysms after 4 weeks, but such aneurysmal changes were scarcely observed in groups B and C (Figure 6A). The elastic laminae appeared somewhat patchy in group A and B, but were expressed throughout the neointima which covered the entire lumen evenly in group C (Figure 6B). Expression of elastic laminae appeared to correlate with neointimal αSMA antibody staining in all groups (Figure 6B and 6C, arrows). Furthermore, the “neoadventitia” was constructed more robustly with collagen-rich tissue at 8 weeks in group C (Figure 6B). Such neoadventitia was accompanied by vasa vaso- luminae are present throughout the neointima, and cover the whole lumen evenly in group C. The “neoadventitia” was also constructed more robustly with collagen-rich tissue in group C. C. Serial sections immunostained for αSMA, magnification ×40. Note that the sites where elastic laminae appeared as black in (B) correspond to the sites stained dark brown with αSMA immunostaining (white and black arrowheads). D, The mean number of new vessels in the adventitia of each group. The number of experiments performed is indicated in each column. The value for group C was significantly higher than for the other groups. #P < 0.05.

Discussion
An arterial graft differs from a vein graft in the thickness of the media and content of elastic laminae. These properties are known to contribute to the structural stability and mechanical performance in vessels exposed to high pressure. Vein grafts transplanted into the arterial circulation are therefore exposed to higher than accustomed circumferential pressure stretch.

Circumferential pressure stretch is believed to contribute to degradation of the vein graft endothelium. Liu et al reported that vein grafts exposed to arterial blood flow lose up to 60% of the ECs and SMCs within 12 hours of implantation.22 Consistent with that, the present study demonstrated that the endothelium of vein grafts without GS (group A) was desquamated and the media disrupted on day 1. EC desquamation induces thrombus formation, which leads to neointimal progression.

Stooker et al demonstrated that perivenous polytetrafluoroethylene stenting support inhibited graft over-dilation and EC damage when vein grafts were exposed to arterial blood flow in vitro.5 Moreover, Lardenoye et al demonstrated that extravascular stent placement resulted in strong inhibition of vein graft atherosclerosis in hypercholesterolemic transgenic mice.6 Such stenting effects can also be achieved when gelatin hydrogel was applied, as in the present study. Vein grafts supported by GS (groups B and C) maintained normal blood flow velocity and characteristics. Moreover, endothelium of groups B and C remained intact and the graft dilation ratio was lower than in group A.

Excessive circumferential stretch may also induce apoptosis of SMCs in the media of vein grafts.23 Basic fibroblast growth factor is believed to be an anti-apoptotic factor for vein graft SMCs and ECs,9,10 as well as a potent angiogenic factor. We have previously demonstrated that GS impregnated with bFGF allows controlled release with continuing bioactivity for 1 week or more, and that comparable efficacy could not be achieved by direct injection of bFGF.11,12 Accordingly, we hypothesized that wrapping vein grafts in GS-impregnated bFGF might prevent structural degradation of the vein graft by serving not only as a supporting...
perivenuous stent, but also as a pharmacological (anti-apoptotic) sustained-release system. As expected, vein graft media in group C on day 1 did remain TUNEL-negative, and intact, unlike in groups A and B.

Many growth factors the receptors for which are protein tyrosine kinases stimulate the MAPK pathway by activating first the GTP-binding protein Ras, and then the Ras-dependent protein kinase cascade formed by Raf-1 (MAPK kinase kinase), MEK1/2 (MAPK kinase), and Erk1/2 (MAPK).24,25 The signal activated by MAPK is transmitted to intranuclear transcription factors such as Elk-1, resulting in cell growth and differentiation. Therefore, Raf-1 protein kinase has been identified as an integral component of the Ras/Raf/MEK/Erk signaling pathway in the anti-apoptotic process.13 It contains a central activation domain that can be phosphorylated and activated by either p21-activated protein kinase at amino acids Serine338 and 339 (p21-activated protein kinase site) or Src kinase at amino acids tyrosin340 and 341 (Src site).26,27

Regarding ECs protection, Alavi et al demonstrated that bFGF selectively promotes phosphorylation at the p21-activated protein kinase site (Ser338/339) of Raf-1, whereas vascular endothelial growth factor induces phosphorylation at the Src site (Tyr 340/341).13 In the current study the endothelium in group C was stained with anti- p-Raf-1 (Tyr 340/341)- as well as with p-Raf-1 (Ser 338/339)-specific antibody, and the same was true of group B. This would suggest that it is not only the bFGF from GS but also the GS itself that plays a prominent role in EC protection in association with some autocrine growth factors (eg, autocrine bFGF or autocrine vascular endothelial growth factor10,28,29). In addition, the endothelium in groups B and C showed both integrin-linked kinase overexpression as well as Akt activation in immunohistochemistry (supplemental Figure II). Considering that integrin-linked kinase is believed to function as the effector of the PI3K/Akt signal pathway, integrin-mediated EC attachment to extracellular matrix would also be important for EC survival.30,31

Immunohistochemical findings on day 1 showed that compared with other groups the media in group C showed stronger activation of Raf-1 (Ser338/339), Erk1/2, and Elk-1, which is a terminal target and substrate for activated MAPK.32,33 Western blotting also demonstrated that Raf-1 (Ser338/339), Erk1/2 and Elk-1 were highly phosphorylated in group C. These results suggest the media would be preserved by the anti-apoptotic functions of bFGF released from GS, operating via activation of MAPK.

Does the anti-apoptotic effects of bFGF on medial SMCs have an impact on neointimal SMC progression? Yamashita et al conducted studies in a rabbit carotid artery interposition model, and showed that neointimal thickness of external jugular veins transplanted with antisense bFGF was decreased as a result of inhibition of MAPK activation.34 Hu et al also demonstrated inhibition of neointimal hyperplasia of mouse vein grafts, which is incubated with suramin (a platelet-derived growth factor receptor antagonist) and then interposed into the carotid artery enveloped with a mixture of suramin and pluronic gel.35 However, in our study the percentage of neointima in group C was significantly lower than in the other groups. The seemingly contradictory results of these studies might be attributable to different methods and means of administration of growth factor or its antagonist. In our study the neointima showed strong activation of MAPK in either the presence or absence of bFGF released from GS (data not shown), probably because of the autocrine growth factors described. The first 2 studies demonstrated direct inhibition of neointimal hyperplasia of vein graft by growth factor antagonist, whereas the objective of our study was to achieve structural stability of vein graft, which leads to both minimized and optimized neointimal formation.

Although there was no significant difference in mean blood flow velocity among the 3 groups until the first 4 weeks of the study, values in group C were significantly higher at 8 weeks. These findings could be attributable to the stent-like effect by the rigid “neoadventitia” produced by bFGF from GS. For the period up to 4 weeks, GS might serve as a perivenuous stent in both groups B and C. At 8 weeks GS had become completely biodegraded in group B. However, by then, in group C only, angiogenesis and collagenses effect of bFGF had developed the rigid neoadventitia organized with collagen-rich tissue and vasa vasmorum-like neovascularity.36

The observation period of up to 8 weeks in the current study is too short to provide information bearing on long-term patency of vein grafts in real-life coronary arterial bypass grafting surgery, and we could not detect differences in graft patency rates among the three groups. However, neointimal characteristics were distinctly different among the three groups. It is noteworthy that there are 2 distinct phenotypes of SMCs, a contractile and a secretory (proliferating) phenotype. In this study, we observed both temporal and spatial correlation between the appearance of the elastic laminae and αSMA, which is believed to develop predominantly in contractile SMCs.37 Neointima covered with homogeneous elastic laminae, particularly in group C, therefore could be considered nonproliferative and to have a better potential for long-term patency. It is indicated by Hu et al that there are large numbers of vascular progenitor cells in the adventitia of vein grafts, which can migrate to the neointima38 (supplemental Figure I). It could be speculated that such progenitor cells were differentiated by bFGF from the GS and contributed to the ideal neointimal formation.

Acknowledgments
We thank Drs S. Inoue and M. Yamamoto of the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Science, Kyoto University, for valuable discussion and advice.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2007;27:548-555; originally published online December 14, 2006;
doi: 10.1161/01.ATV.0000254811.11741.2b
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/3/548

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**Materials and Methods**

Immunohistochemical staining for ILK and phosphorylated Akt

Immunohistochemical identification of integrin-linked kinase (ILK) and phosphorylated Akt (p-Akt) was carried out on sections prepared as described in the section “Immunohistochemistry” using rabbit anti ILK1 antibody (Cell Signaling Technology, Inc., MA) and rabbit anti p-Akt (Thr308) monoclonal antibody (Cell Signaling Technology, Inc., MA) (Diluted 1:100). The sections were visualized with donkey anti-rabbit Ig conjugated with FITC (Amersham Biosciences, Corp., NJ) using fluorescence microscopy (Carl Zeiss Vision, Hallbergmoos, Germany).

**Figure Legends**

**Figure I**

Immunohistochemical staining with vWF on the vein graft in each group at 8 weeks. magnification ×40. In Group C, not only endothelium, but also the neoadventitia was stained positive for vWF. This finding would suggest the presence of abundant endothelial progenitor cells in the adventitia of vein grafts.

**Figure II**
Vein graft cross sections in each group on day 1. Sections were stained with EVG, ILK1, and p-Akt antibodies, magnification ×200. Bar in top left panel indicates 20mm. The endothelium in Groups B and C was strongly stained with both ILK and p-Akt antibody. m: media

Figure III

(A) Vein graft cross sections on day 1 in Group A. HE staining (a) and vWF staining (b), magnification ×400. Thrombus had formed at loci where ECs were desquamated (arrowhead), and neo-endothelium had also formed (arrows). lu:lumen, m:media, e:endothelium, th:thrombus, ne:neo-endothelium. (B) Macroscopic findings of Evans blue staining of an intact vein (a) and of an intact artery (b) on day 1. The vein was scarcely stained with Evans blue dye, while the artery was faintly stained with it. It would suggest that an artery is more easily stained with Evans blue for abundance of elastin.
Fig. I

Immunohistochemistry for vWF at 8 weeks
Fig. III

**A**
- Thrombus formation

**B**
- Evans blue staining
- HE staining
- intact vein
- intact artery

**A**
- a
- lu
- th
- e
- m

**B**
- a
- b
- lu
- ne
- th
- e
- m

**Other**
- vWF staining