Antithrombogenic Modification of Small-Diameter Microfibrous Vascular Grafts

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Objective—To develop small-diameter vascular grafts with a microstructure similar to native matrix fibers and with chemically modified microfibers to prevent thrombosis.

Methods and Results—Microfibrous vascular grafts (1-mm internal diameter) were fabricated by electrospinning, and hirudin was conjugated to the poly (L-lactic acid) microfibers through an intermediate linker of poly(ethylene glycol). The modified microfibrous vascular grafts were able to reduce platelet adhesion/aggregation onto microfibrous scaffolds, and immobilized hirudin suppressed thrombin that may interact with the scaffolds. This 2-pronged approach to modify microfibrous vascular graft showed significantly improved patency (from 50% to 83%) and facilitated endothelialization, and the microfibrous structure of the vascular grafts allowed efficient graft remodeling and integration, with the improvement of mechanical property (elastic modulus) from 3.5 to 11.1 MPa after 6 months of implantation.

Conclusion—Microfibrous vascular grafts with antithrombogenic microfibers can be used as small-diameter grafts, with excellent patency and remodeling capability. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: vascular graft • small diameter • microfibers • biomaterials • hirudin

Arterial replacement is a common treatment for vascular diseases, with more than 500,000 vascular grafts being used in the bypass procedures of coronary and peripheral arteries each year. However, small-diameter synthetic vascular grafts frequently have issues with thrombosis and occlusion. Several methods have been developed to construct tissue-engineered cellular vessels by using vascular cells.1–4 Recently, researchers5–7 have shown that bone marrow cells can be used to construct vascular grafts; bone marrow mesenchymal stem cells can resist platelet adhesion and are antithrombogenic in vivo when seeded onto electrospun fibrous scaffolds.8 Because a cellular graft takes days to weeks to construct and special care needs to be taken during preservation, shipping, and surgery, in this project, we take an alternative approach by fabricating chemically modified acellular microfibrous vascular grafts that can be made available off the shelf.

In the past 2 decades, both decellularized native matrix and synthetic materials have been used to engineer vascular grafts.8–11 Synthetic biodegradable polymers, such as poly(lactic acid), poly(glycolic acid), and their copolymers, have also been used to make porous vascular grafts by using techniques such as solvent casting and particulate leaching.3,12–14 In native blood vessels, collagen and elastin fibers are the major matrix components that provide structural and mechanical support. To simulate the microstructure and nanostructure of native extracellular matrix, researchers15,16 have used an electrospinning technique to fabricate fibrous scaffolds for vascular graft construction.5,17–22 To maintain the patency of vascular grafts, cell seeding or surface modification is needed to generate a nonthrombogenic luminal surface. Although it has been shown that seeding vascular cells and bone marrow cells into the grafts can improve the patency of vascular grafts, whether chemical modification of microfibers can generate nonthrombogenic grafts has not been addressed. Herein, we engineered the chemical property of microfibers to fabricate acellular vascular grafts that are nonthrombogenic and have long-term patency.

Hirudin is a polypeptide (65 to 66 amino acids) derived from the saliva of the medicinal leech Hirudo medicinalis. It is the most potent, naturally occurring, specific inhibitor of thrombin. In this study, we conjugated hirudin to the poly(l-lactic acid) (PLLA) microfibers through an intermediate linker of poly(ethylene glycol) (PEG). The PEG layer was able to reduce platelet adhesion/aggregation onto microfibrous scaffolds, and immobilized hirudin could suppress...
thrombin that may interact with the scaffolds. This 2-pronged approach to modify the microfibrous vascular grafts showed improved patency and facilitated endothelialization, and the microfibrous structure allowed efficient graft remodeling and integration.

Methods

Microfibrous Scaffold Fabrication and Characterization

A 20% weight per volume solution of PLLA (Lactel Absorbable Polymers, Pelham, Ala), with an inherent viscosity of 1.09 dL/g, was formulated using 1,1,3,3,9,9-hexafluoro-2-propanol. The mixture was sonicated for 30 minutes or until all of the PLLA crystals were completely dissolved. Electrospinning was performed, with modifications using a novel setup with a rotating stainless steel mandrel (1-mm diameter and 75 rpm) and a spinneret that automatically moved back and forth in the longitudinal direction of a mandrel to achieve a uniform thickness of the conduit longitudinally. The negative voltage of 4.5 kV was applied to the mandrel, and a positive voltage of 4 kV was applied to the spinneret by using a high-voltage generator (Gamma High Voltage, Ormond Beach, Fla). The electrospinning process was allowed to proceed until an approximately 200-μm wall thickness was achieved.

The conduit was removed from the mandrel and placed into a vacuum desiccator for 24 hours to evaporate any residual 1,1,3,3,9,9-hexafluoro-2-propanol. The quality, thickness, and porosity of the microfibers were inspected using a scanning electron microscope (Hitachi S-5000). The conduit was trimmed into 7-mm-length segments, sterilized in 70% isopropyl alcohol, placed under germicidal UV light for 30 minutes, and washed 3 times with PBS.

Hirudin-PEG Conjugation

Di-olamino PEG (molecular weight, 3350; Sigma Aldrich) was covalently linked through the carboxyl groups on the PLLA microfibers of the grafts by using the following 0-length cross-linkers: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysulfosuccinimide (Pierce Biotechnology, Rockford, Ill). The C-terminus of hirudin (Sigma) was covalently attached to the amine groups on the di-olamino PEG molecules via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysulfosuccinimide. Afterward, the conduits were incubated with a solution of 100 mg/mL glycine in PBS for 30 minutes at room temperature to wash away and block any remaining amine-reactive sites created by cross-linking reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysulfosuccinimide. The conduits were washed with PBS 3 times. To verify that hirudin was linked to nanofibers, immobilized hirudin was stained by using a rabbit antibody against hirudin (American Diagnostica, Inc, Stamford, Conn) and a fluorescein isothiocyanate–conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, Inc, West Grove, Pa).

Platelet Adhesion Onto Microfibrous Scaffolds

Platelet-rich plasma from healthy human volunteers was collected and incubated for 30 minutes on the microfibrous scaffolds with or without conjugation: PLLA, PEG-PLLA, and hirudin-PEG-PLLA. Samples were processed and analyzed using 2 techniques: scanning electron microscopy and immunofluorescence staining with 1:100 mouse antihuman CD41 antibody (Laboratory Vision, Fremont, Calif). The total number of adherent platelets from these 4 images was summed, and the results from multiple experiments from 3 individual donors were subjected to ANOVA. A Holm r test was used to calculate statistical significance for P<0.05.

Animal Studies

All procedures were approved by the Institutional Review Board of the University of California, San Francisco. Female Sprague-Dawley rats (weight, 200 to 240 g) were obtained from the Charles River animal facility. The rats were anesthetized with 2.0% isoflurane in 70% nitrous oxide and 30% oxygen. The right common carotid artery was dissected, clamped, and transected; and the graft was sutured end to end with 10 to 0 interrupted stitches. No heparin or any other anticoagulant was used at any point before, during, or after the implantation procedure. For 1-month studies, 12 animals were used in each experimental group. For 6-month studies, 8 animals were used in each experimental group. After 1 to 6 months, the animals were reanesthetized and the vascular grafts were resected and washed with heparinized saline to remove the remaining blood.

Histological Analysis

For histological analysis, the sample was placed into an optimal cutting temperature (Sigma Aldrich) and cryopreserved at −20°C. Cryosections were taken at 10-μm thicknesses. Immunohistochemical staining was used to analyze the tissue sections with the following primary antibodies: CD31 (BD Biosciences, San Jose, Calif), myosin heavy chain (Santa Cruz Biotechnology), and CD68 (Santa Cruz Biotechnology). Immunohistochemistry images were captured with a microscope (Zeiss Axioskop 2 MOT).

En Face Immunofluorescence Staining for Endothelial Cells

Freshly explanted grafts were transected longitudinally and fixed with 4% paraformaldehyde for 15 minutes. The samples were washed with PBS, blocked with 1% BSA, and incubated with mouse anti-rat CD31 antibody and Alexa-Fluor 488 secondary antibody. The samples were washed and mounted onto a slide such that the luminal surface of the graft was in contact with the coverslip.

Mechanical Testing

Freshly explanted vascular grafts were cut into 3-mm-wide ring segments. The tensile strength in the circumferential direction of these rings was tested by using a custom-built soft tissue tester. Two 0.016-inch-diameter stainless steel rods were placed through the lumen of the ring segment of the graft and attached to polycarbonate compression grips. The sample was then loaded onto the mechanical tester, and the applied force and deformation were recorded every second via software (Labview v4.0; National Instruments, Austin, Tex). To obtain stress, the cross-sectional area of the sample was measured by histological analysis of adjacent cross sections and calculated by using the following formula: 2(width of the ring × wall thickness). The elastic modulus was calculated based on the applied force, graft deformation, and the dimensions (thickness and width) of the rings.

Results

Direct Fabrication of a Microfibrous Tubular Graft With a Microstructure Similar to Native Matrix Fiber

By directly electrospinning polymer fibers onto a rotating mandrel, we successfully made microfibrous tubular grafts (Figure 1A). This is significant progress toward making seamless microfibrous grafts. The PLLA microfibers made by electrospinning formed a structure (Figure 1B) similar to native matrix fibers. The average diameter of the fibers was approximately 2 μm. As seen in the image, the electrospinning process resulted in a highly porous and random structure of fibers, which is an excellent tissue-engineering scaffold.

Microfibers Could Be Modified by Using PEG and Hirudin

PEG is capable of creating a brushlike layer onto various surfaces and was used to create a protein- and platelet-repulsive surface on the PLLA microfiber scaffold. We first
conjugated PEG onto microfibers and then linked hirudin to the end of the PEG molecule (Figure 1C). The purpose was to use PEG to resist protein adsorption and platelet adhesion and to use hirudin to inactivate thrombin that reached the luminal surface of the vascular grafts. The successful conjugation of PEG and hirudin onto microfibers was confirmed by immunostaining for hirudin, which showed the coating of hirudin on individual microfibers (Figure 1D).

**PEG and Hirudin-PEG–Conjugated Surfaces Reduced the Number and Aggravation of Adherent Platelets**

To determine whether the grafts modified by PEG and hirudin-PEG can reduce platelet adhesion, platelets were incubated with untreated grafts and PEG- or hirudin-PEG-modified grafts. As shown in Figure 2A through F, both CD41 staining for platelets and scanning electron microscopy showed that microfibrous scaffolds with conjugated PEG and hirudin-PEG had fewer platelets on their surfaces than untreated microfibrous scaffolds. The reduction of platelet adhesion on PEG and hirudin-PEG microfibrous scaffolds was statistically significant (Figure 2E), which may help reduce the possibility of early in vivo graft failures as the result of thrombosis and platelet adhesion/activation. The similar reduction of platelet adhesion by PEG and hirudin-PEG suggested that PEG contributed to this antiplatelet adhesion property.

In addition, scanning electron microscopy demonstrated the morphological characteristics of the platelets on the microfibrous PLLA scaffolds. The adherent platelets on the untreated surfaces appeared to have “spiky” protrusions and pseudopods, indicating that they were activated and aggravated by contact with the microfiber sample (Figure 2D). In contrast, fewer adherent platelets were found on the surfaces modified by PEG or hirudin-PEG, and these platelets did not have the same spiky protrusions as seen on the control samples (Figure 2E and F).

**PEG- and Hirudin-PEG–Modified Grafts Improved Patency Rates In Vivo**

To compare the antithrombogenic property of the vascular grafts in vivo, untreated microfibrous grafts and the grafts modified with PEG or hirudin-PEG were implanted into the...
common carotid artery of rats for 1 month. Patency was determined by ultrasonography and necropsy. Graft patency was determined by the unobstructed flow of blood through the graft. At 1 month, 6 (50%) of 12 untreated grafts were patent, 9 (75%) of 12 PEG grafts were patent, and 10 (83%) of 12 hirudin-PEG grafts were patent. These results suggest that both PEG and hirudin improved the patency rate.

To further determine the long-term remodeling of the patent grafts, hirudin-PEG–modified grafts were implanted for 6 months. At 6 months, 6 (86%) of the 7 implanted hirudin-PEG grafts were patent. Based on histological analysis, the failed grafts were clogged with thrombus, indicating that the imperfect patency rate was related to easiness of clotting in 1-mm grafts.

The patent grafts from the hirudin-PEG group are shown in Figure 3 as representatives. Figure 3A shows a stereomicrograph image of an implanted graft moments after the anastomosis was completed. The porous structure of the graft was immediately filled with red blood cells and other cellular components, and the color of the graft changed from milky white to a red. The interrupted suture technique did not result in any bleeding or leakage at the anastomotic sites. Good blood flow was observed at both the proximal and distal ends of the graft.

After 1 month, there was visible angiogenesis in the wall of the graft (Figure 3B). The presence of newly formed microvessels indicates the integration of the graft into the host’s vasculature. In addition, it suggests that the graft is becoming a living part of the host and that angiogenesis is necessary to supply nutrients, oxygen, and other diffusible chemicals to the local cells that reside within and around the graft. The amount of angiogenesis was slightly less in the 6-month grafts (Figure 3C), suggesting that the angiogenesis was part of a short-term wound-healing process in the graft.

A suture site and the characteristics of the graft after 1 month were captured by a stereomicroscope (Figure 3D). The luminal surface was presented by transecting the graft along the longitudinal direction. The anastomotic sites were free of thrombosis and intimal hyperplasia.

Graft patency was also monitored by using Doppler Duplex (Figure 3E) and MRI (Figure 3F), showing a normal flow rate in the patent grafts.

Endothelialization, Cellular Infiltration, and Organization

The patent grafts (either in the untreated group or in the hirudin-treated group) showed similar histological results. Therefore, only hirudin-treated grafts are shown as representatives. Patent grafts (exemplified by hirudin-PEG grafts in Figure 4) showed few signs of thrombosis and/or intimal hyperplasia on the luminal walls of the graft at either the 1-month point (Figure 4A) or the 6-month point (Figure 4B). Neotissue formation on the outer surface of the grafts was significant at both points. The neotissue in the 1-month samples had a highly porous and loose tissue structure on the outside of the graft (Figure 4C). On the other hand, the 6-month sample had dense neotissue with extracellular matrix alignment in the circumferential direction (Figure 4D). The nucleus staining revealed that there were cells within the walls of the graft and the neotissue in the outer layer (Figure 4E and F), suggesting that the graft is capable of supporting cellular ingrowth.

Endothelialization on the luminal surface is important to maintain the long-term patency of vascular grafts. All patent samples had complete endothelial coverage at the 1-month
The newly formed capillaries and microvessels were evident in the neotissue of the graft (Figure 5A, C, and D), which suggests the remodeling of the grafts. Immunofluorescent en face staining revealed that endothelial cells (ECs) had aligned in the flow direction and had a morphological appearance similar to ECs in native arteries (Figure 5B).

Smooth muscle cell (SMC) presence and organization are also important for the long-term stability of vascular grafts. Interestingly, SMC staining showed that SMCs were mostly in the neotissue surrounding the grafts at 1 month (Figure 5E) and 6 months (Figure 5F) after implantation. The 6-month sample (Figure 4F) had a clearly defined band of SMCs that are highly organized and aligned in the circumferential direction. There was no sign of a neointima or intimal hyperplasia in these patent grafts.

The inflammatory responses to the PLLA vascular graft were monitored via immunostaining of CD68, a cell surface marker for monocytes and macrophages (Figure 5G and H). There were few CD68-positive cells within the walls of the graft, suggesting minimal inflammatory responses to the grafts.

Mechanical strength is critical for the long-term stability of the grafts. We performed mechanical tests by using rings of the nonimplanted grafts and the explanted grafts (Figure 6A). The representative stress-strain curve of explanted grafts at the 1- and 6-month points is shown in Figure 6B. The elastic modulus of the grafts before implantation was about
Hirudin-PEG did not decrease platelet adhesion further compared with the PEG surface, suggesting that the resistance to platelet adhesion could be attributed to the PEG layer. Consistently, in vivo studies showed that the grafts modified with PEG and hirudin-PEG significantly improved the patency rate of the grafts. The slightly higher patency rate of the hirudin-PEG grafts (10/12) suggests that hirudin may have an additional beneficial effect (eg, inhibition of thrombin activity).

Endothelialization and sufficient mechanical strength are 2 critical aspects for maintaining the long-term stability of vascular grafts. The chemically modified grafts were able to prevent early thrombosis and to allow ECs to form a monolayer so that long-term patency was achieved. Both histological and en face staining showed excellent endothelialization in our grafts at the 1- and 6-month points, contributing to long-term patency. The significant increase of the mechanical strength of the grafts at 6 months suggests that there was an increase of matrix synthesis in and surrounding the grafts in vivo. Based on histological analysis, it is likely that the neotissue on the outer surface of the grafts contributed significantly to the enhanced mechanical strength. The values of elastic modulus for the grafts are similar to those of native arteries in rats (tens of MPa).

The microfibrous grafts were integrated well into native vasculature, supported by the evidence of angiogenesis and SMC recruitment in the outer layer of the graft. The minimal inflammatory response suggests that the PLLA microfibers are biocompatible materials for vascular regeneration. It is possible that cells could migrate into the grafts from both inside and outside surfaces. For example, ECs on the luminal surface could be from microvessels outside the graft, the arteries at 2 ends of the graft, and circulating cells (eg, endothelial progenitor cells) in the bloodstream. Additional work needs to be performed to investigate the origin of ECs. The organization of the SMCs in the neotissue outside of the graft is an interesting observation. These SMCs could be from the arteries connected to the grafts or from the surrounding tissues. This is in contrast to the previous observation of SMCs underneath the endothelium in mesenchymal stem cell–seeded nanofibrous grafts. It is likely that cell-seeded grafts allow better cell recruitment and infiltration into the grafts. Alternatively, because those cell-seeded grafts had fiber alignment in the circumferential direction, the alignment of fibers in the cellular grafts could facilitate cell infiltration into the 3D structure of the scaffold. These possibilities need to be examined in future studies, and the electrospun scaffolds could be engineered to further increase cell infiltration.

In the case of other synthetic materials, there were reports on the use of hirudin to modify synthetic polyester (Dacron) and expanded polytetrafluoroethylene grafts. For example, a previous study showed the successful conjugation of hirudin onto the synthetic polyester surface, but in vivo performance was not reported. Interestingly, direct coating of expanded polytetrafluoroethylene grafts with hirudin also resulted in excellent patency in larger (4-mm-diameter) grafts. A potential disadvantage of noncovalent coating is the lack of control of surface-binding efficiency and hirudin retention on the surface. It will be useful to compare the...
retention of hirudin on the luminal surface of the grafts with and without conjugation under hemodynamic conditions.

The use of bioabsorbable microfibrous scaffolds for vascular grafts makes it possible to regenerate native blood vessels. The slow degradation rate of biopolymers, such as PLLA, maintains the mechanical strength of the grafts long enough and allows gradual replacement of synthetic scaffolds by native matrix with time. The microfibrous structure provides a mechanically stable module until cellular infiltration and scaffold remodeling eventually lead to a tissue-like product capable of self-support. In this study with 6-month implantation, no significant degradation of the grafts was observed because the half-degradation time of PLLA is longer than a year.

The methods to directly fabricate tubular constructs by using electrospinning make it possible to scale up the production of more uniform and better-quality grafts, opposed to the rolling technique previously used. In vitro and in vivo analysis of the functional performance of the microfibrous vascular grafts provides insight into the mechanisms of the remodeling and regeneration of a blood vessel with microstructure. The ability to create antithrombogenic small-diameter vascular grafts makes it more feasible to offer vascular grafts available off the shelf.

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Disclosures
None.

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