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Development and Validation of Small-diameter Vascular Tissue From a Decellularized Scaffold Coated With Heparin and Vascular Endothelial Growth Factor

*Min Zhou, *Zhao Liu, *Zhiqing Wei, *Changjian Liu, *Tong Qiao, *Feng Ran, *Yan Bai, †Xuefeng Jiang, and ‡Ying Ding

**Department of Vascular Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School; †College of Chemistry and Chemical Engineering, Nanjing University, Nanjing, China; and ‡Department of Biostatistics, University of Michigan, Ann Arbor, MI, USA*

Abstract: To overcome shortcomings of current small-diameter vascular prostheses, we developed a novel allogenic vascular graft from a decellularized scaffold modified through heparin immobilization and vascular endothelial growth factor (VEGF) coating. The VEGF coating and release profiles were assayed by enzyme-linked immunosorbent assay, the biological activity of modified surface was validated by human umbilical vein endothelial cells seeding and proliferation for 10 days in vitro. In vivo, we implanted either a modified or a nonmodified scaffold as bilateral carotid allogenic graft in canines ($n = 15$). The morphological examination of decellularized scaffolds showed complete removal of cellular components while the extracellular matrix structure remained intact. After modification, the scaffolds possessed local sustained release of VEGF up to 20 days, on which the cells cultured showed significantly higher proliferation rate throughout the time after incubation compared with the cells cultured on nonmodified scaffolds ($P < 0.0001$). After 6 months of implantation, the luminal surfaces of modified scaffolds exhibited complete endothelium regeneration, however, only a few disorderly cells and thrombosis overlay the luminal surfaces of nonmodified scaffolds. Specifically, the modified scaffolds exhibited significantly smaller hyperplastic neointima area compared with the nonmodified, not only at midportion (0.56 ± 0.07 vs. 2.04 ± 0.12 mm^2, $P < 0.0001$), but also at anastomotic sites (1.76 ± 0.12 vs. 3.67 ± 0.20 mm^2, $P < 0.0001$). Moreover, modified scaffolds had a significantly higher patency rate than the nonmodified after 6 months of implantation (14/15 vs. 7/15, $P = 0.005$). Overall, this modified decellularized scaffold provides a promising direction for fabrication of small-diameter vascular grafts.
Key Words: Decellularization—Heparin—Neointimal hyperplasia—Vascular endothelial growth factor—Vascular graft.

Atherosclerosis and heart diseases are still the leading causes of morbidity and mortality worldwide. Therapies for coronary artery and peripheral vascular diseases often require replacement of the damaged vessels with vascular grafts. For reconstruction of large arteries, such as the aorta or iliac artery, the current commercial grafts made from expanded polytetrafluoroethylene (ePTFE) or Dacron are utilized satisfactorily. However, synthetic grafts are not suitable for reconstruction of smaller-diameter (internal diameter [ID] <5 mm) arteries, due to thrombosis, limited reendothelialization, and neointimal hyperplasia, owing mainly to the inherent properties of the synthetic materials (1). Therefore, physicians routinely use autologous vessels for such reconstruction procedures. Unfortunately, the number of appropriate vessels is limited in many patients due to coexisting diseases, size mismatch, or previous procedures. Although considerable research has focused on the development of novel small-diameter vascular grafts for many years, there is still no adequate alternative to the autologous vessels (2).

Recently, a promising new approach for the small-diameter vascular grafts is the use of arterial scaffolds with total depletion of cellular antigens, which have

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Address correspondence and reprint requests to Dr. Min Zhou, Department of Vascular Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Hankou Road, Nanjing 210008, China. E-mail: zhouminnju@gmail.com

shown good mechanical properties and biocompatibility in vivo (3,4). It is observed, however, the acellular luminal surface without endothelial cells (ECs) coverage carries a substantial risk for thrombosis when exposed to the blood directly (5,6). For this reason, most recent studies have focused on the creation of tissue-engineered vascular grafts prepared by recellularizing scaffolds with host vascular cells prior to implantation (7). Especially, the innovative use of stem cells has opened up new avenues for a fully engineered vascular graft (8,9). As we know, however, such kinds of tissue-engineered vessels may only be appropriate for patients with planned surgery, but not for those who need immediate vascular reconstruction procedures, because reseeding procedures cannot be processed in a short time. Therefore, it is clinically meaningful to produce off-the-shelf engineered vessels from the decellularized biological scaffolds.

The alternative research pathway is the engineering of grafts through the surface coating with angiogenic growth factors to enhance the process of migration and proliferation of ECs, so that the "spontaneous" endothelialization would occur in vivo, which may be a more appropriate concept in the clinical setting. The possible use of growth factors in decellularized vascular graft coatings has been recently reported by Conklin et al. (10). They demonstrated that basic fibroblast growth factor coating on the heparin-immobilized decellularized scaffolds (HDSs) significantly increased both ECs and endothelial progenitor cells proliferation, additionally, that seeded cells after only 3 h of attachment were stable under perfusion conditions in vitro.

Currently, a variety of growth factors are known to stimulate, affect, or control the process of angiogenic vessel growth. The most important two are basic fibroblast growth factor and vascular endothelial growth factor (VEGF), with the later being recognized as a more powerful mitogen and chemoattractant for ECs (11). Specifically, it has been shown in a number of studies that VEGF gene or protein infusion increases reendothelialization and therefore, it has been hypothesized that as a consequence of this reendothelialization, neointimal hyperplasia is inhibited (12–15).

The present study aimed to develop a novel small-diameter decellularized vascular graft modified through heparin immobilization and VEGF coating, with the idea to promote endothelialization, inhibit neointimal hyperplasia, and improve patency rate. For that purpose, we determined coating and release actions of VEGF quantitatively, and investigated the effects of coating VEGF on proliferation of human umbilical vein endothelial cells (HUVECs) in vitro. Furthermore, the ability of VEGF-releasing surface in vivo was also tested through long-term animal studies, which will eventually be needed to demonstrate its clinical applicability.

MATERIALS AND METHODS

Preparation and characterization of decellularized scaffolds (DSs)

Decellularized scaffolds were prepared as previously described (7). In brief, freshly harvested canine carotid arteries were decellularized by detergent washes and multiple enzymatic treatments. After cleaning, the grafts were lyophilized and sterilized in ethylene oxide gas. A segment from the scaffolds was fixed in neutral buffered 10% formalin, embedded in paraffin, and sliced for hematoxylin-eosin (H&E) and Masson's trichrome staining. The luminal surface of the DSs was also analyzed by scanning electron microscopy (SEM) as follows: specimens were fixed in 1% buffered glutaraldehyde and 0.1% buffered formaldehyde for 1 and 24 h, respectively, then dehydrated with a graded ethanol series, critical point dried, sputter coated with platinum, and observed by SEM (ISI-SX-40, Akashi, Tokyo, Japan).

Heparin immobilization

Covalent immobilization of heparin to DSs was performed using 1-ethyl-3- (3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (both from Pierce Biotechnology, Rockford, IL, USA) according to Yao et al. (16). Briefly, 1 g heparin, 2 g EDC and 1.2 g NHS were added into 500 mL 2-morpholinoethanesulfonic acid buffer (0.05 M, pH 5.6) for 10 min at 37°C to activate carboxylic acid groups of heparin, then DSs were immersed into the reagent solution for 4 h at 37°C under gentle shaking. Following heparin immobilization, vessels were rinsed in 0.1 M Na_2HPO_4 (2 h), 4 M NaCl (four times for 24 h), and distilled water (three times for 24 h).

Vascular endothelial growth factor coating and release

The HDSs were cut into standard 5-mm-diameter disks, and excess adventitia was gently removed. The disks were incubated with 5 mL recombinant human VEGF165 (R&D Systems, Minneapolis, MN, USA) solution (VEGF concentration varying from 0 to 100 ng/mL) in PBS containing 1 mg/mL bovine serum albumin (Sigma, St. Louis, MO, USA) overnight at room temperature. Thereafter, the concentration of VEGF in the suspension was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) in order to calculate the amount of

coating VEGF. The non-HDSs disks served as the control group. Six specimens of each group were tested at each selected concentration.

The HDSs and DSs disks coated with VEGF as described above (all fabricated at 100 ng/mL VEGF, including the following undescribed) were transferred to 1 mL release medium, which consisted of endothelial cell culture medium (CM) (described below) at 37°C under continuous shaking, and the medium was replaced every 24 h, where the VEGF concentration was measured periodically up to 20 days. Six specimens were tested in each group.

Endothelial cells proliferation experiment

Human umbilical vein endothelial cells were isolated as previously described (7) and cultured in endothelial cell CM, which consisted of a mixture of equal volumes of RPMI 1640 and M199 containing 1% antibiotic–antimycotic and 10% fetal bovine serum (all from Hyclone, Logan, UT, USA). After being passaged twice, cells were detached from the flasks by incubation with trypsin–EDTA solution (Sigma), centrifuged, and resuspended in CM, then counted using a hemocytometer. Approximately 5000 cells (50 μL) were pipetted into each well of the 96-well plate containing a VEGF-coating HDSs (VHDSs) disk, and allowed to attach for 2 h. Subsequently, 100 μL CM was added to each well, the medium was changed every 48 h. Cell proliferation was determined using [^{3}H]thymidine uptake at selected times as described elsewhere (17). Briefly, supernatant CM was replaced by 200 μL CM containing [^{3}H]thymidine (Amersham, Buckinghamshire, UK, 1 mM, 1.75×10^6 dpm/mL). After 6 h incubation, medium was removed and cells were incubated for 10 min using cold 10% trichloroacetic acid solution (TCA), followed by two washes with cold 10% TCA. The precipitate was solubilized overnight in NaOH (1 mL, 1 M). After washing of the surface with NaOH (0.5 mL,1 M), radioactivity in the total volume of 1.5 mL NaOH was measured using a 1414 Winspectral liquid scintillation counter (Wallac, Turku, Finland). The DSs disks served as the control group; six specimens of each group were tested at each selected time.

Surgical implantation and explant characterization

This investigation was performed according to the Guide for the Care and Use of Laboratory Animals and was approved by the Ethical Committee of Researches of Nanjing University.

The VHDSs grafts ($n = 15$) were implanted as carotid artery interposition grafts in mongrel dogs (female, approximately 20 kg). Anesthesia was induced with injection of intramuscular ketamine (30 mg/kg) and intravenous pentobarbital (30 mg/kg), and then maintained with isoflurane and oxygen. Through a longitudinal midneck incision, bilateral common carotid arteries were exposed. Prior to arterial clamping, heparin (100 U/kg) was administered intravenously. The grafts were placed as an end-to-end anastomosis to the common carotid arteries using a 7-0 prolene suture. The DSs grafts ($n = 15$) were implanted in a similar manner in the contralateral side as controls. The length of both graft segments was approximately 4–5 cm. The arterial flow was reestablished and the closure was sutured by layers. All animals received aspirin (100 mg daily) postoperatively. The implanted graft patency was monitored by the arterial digital subtraction angiography every month.

After 6 months of implantation, all the grafts were explanted for histological examination. The midportion and distal anastomotic sites were stained with H&E and Masson's trichrome, and the luminal surface was analyzed with SEM. The degrees of neointima formation were evaluated by histomorphometry using computer-assisted planimetry system (Image Pro Plus 3.0.1 software, Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

Quantitative data were expressed as mean ± SD. Analysis of variance (ANOVA) was performed to assess the difference of two disks across all concentrations (DSs vs. HDSs) or time points (DSs vs. VHDSs). Pearson's chi-squared test was employed to compare the patency rate between the modified and nonmodified scaffolds. In addition, unpaired Student's *t*-test was conducted to compare the difference of the neointima area at two sites between VHDSs and DSs grafts. All analyses were performed using SAS software (version 9.1, SAS Institute, Inc., Cary, NC, USA). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Characterization of DSs

Decellularized scaffolds of 3 mm in ID were prepared by removing cellular components and leaving the native extracellular matrix of the arteries (Fig. 1A). H&E staining did not show any signs of remaining nuclear material in the vessel walls (Fig. 1B). Masson's trichrome staining showed that the extracellular elements such as elastin and collagen were well preserved (Fig. 1C). SEM confirmed the removal of cellular debris, while the basic extracellular microstructure remained intact (Fig. 1D).

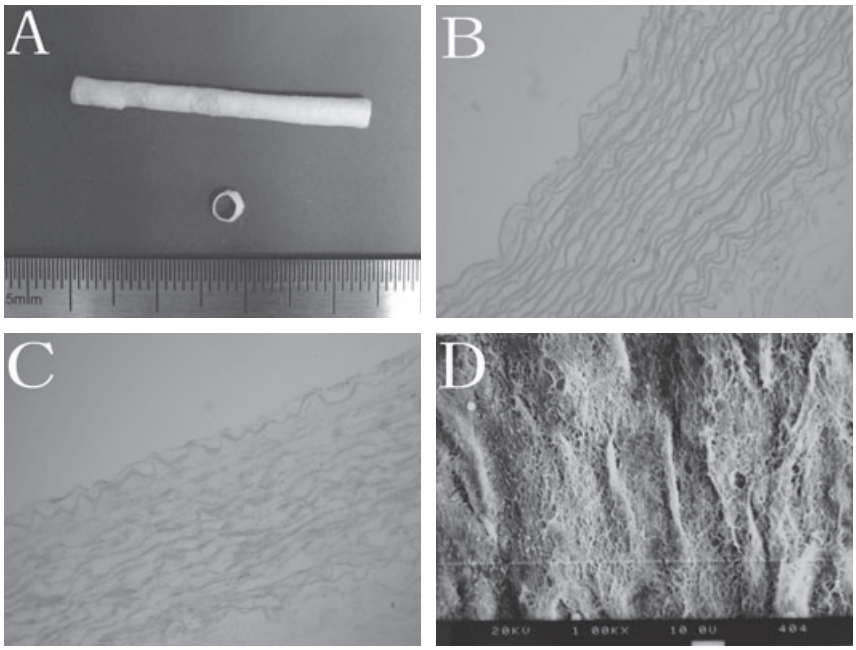

FIG. 1. Characterization of decellularized vascular grafts. (A) Gross view of decellularized graft. The scale is in centimeters. (B) H&E staining showed complete removal of cellular components from canine carotid artery (×50). (C) Masson staining showed well-preserved extracellular matrix in the decellularized graft (×50). (D) Scanning electron micrograph of the luminal surface of the matrix (×1000, the scale bar indicates 10 μm).

Vascular endothelial growth factor coating and release

We calculated the amount of coating VEGF from concentration differences between before and after reactions tested by ELISA methods. Figure 2 showed that the coating of VEGF to both HDSs and DSs disks presented a positive correlation with the VEGF concentration used for incubation, and for the highest VEGF concentration, 100 ng/mL, the VEGF coated to HDSs and DSs disks amounted to 10.04 ± 1.10 and 4.98 ± 0.93 ng/disk, respectively. There was a significant difference of the amount of VEGF coating between HDSs and DSs disks throughout all VEGF concentrations ($P < 0.0001$, $n = 6$ for each group at each concentration).

In a separate experiment, the release of coating VEGF from scaffolds was measured for 20 days (Fig. 3). Initial release of VEGF from DSs disks was faster compared with that from HDSs. During the first 5 days, $74.40 \pm 5.78\%$ of VEGF released from DSs disks, whereas only $35.70 \pm 4.29\%$ released from HDSs disks. After 20 days, a total of $98.03 \pm 2.03\%$ VEGF released from HDSs disks, while only $73.98 \pm 4.72\%$ released from DSs disks. The difference of VEGF release between DSs and HDSs disks was statistically significant throughout

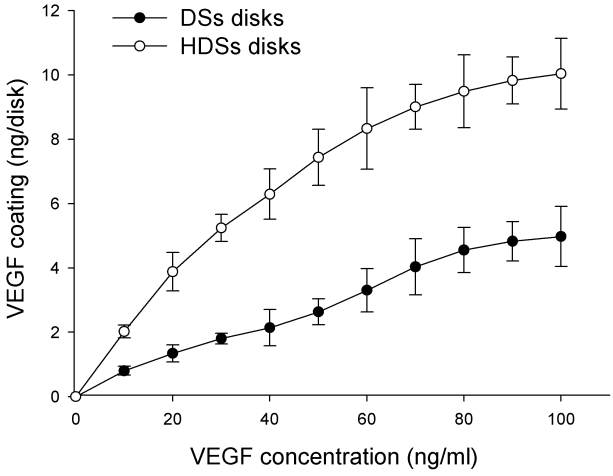

FIG. 2. Coating of VEGF to HDSs and DSs disks as a function of the VEGF concentration ($n = 6$, mean ± SD).

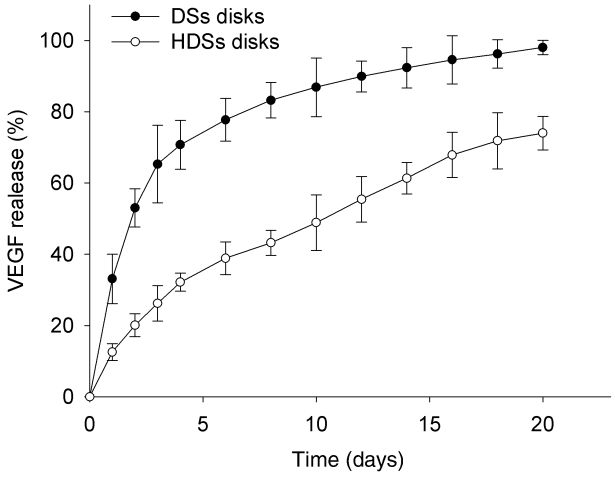

FIG. 3. Release of VEGF from HDSs and DSs disks in endothelial cells culture medium over 20 days (n = 6, mean ±SD).

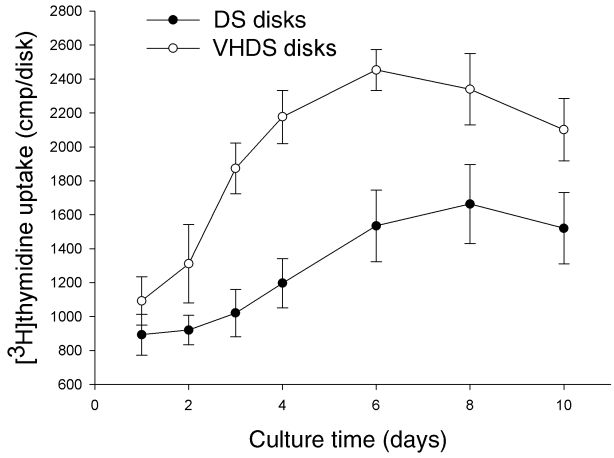

FIG. 4. [^{3}H]Thymidine uptake of HUVECs cultured on VHDSs and DSs disks during 10 days after seeding (n = 6, mean ± SD).

the time ($P < 0.0001$, $n = 6$ for each group at each time point).

Proliferation of HUVECs

HUVECs proliferation was measured using [^{3}H]thymidine uptake by monitoring the rates of cell DNA synthesis. Figure 4 demonstrated that there was almost no increase in [^{3}H]thymidine uptake on DSs disks during the first 3 days, indicating low cell proliferation. In contrast, [^{3}H]thymidine uptake increased over control during the initial 6 days on VHDSs disks, and there was little additional proliferation at later times, reflecting contact inhibition. HUVECs cultured on VHDSs disks showed a significantly higher [^{3}H]thymidine uptake compared with those on DSs disks throughout the days after seeding ($P < 0.0001$, $n = 6$).

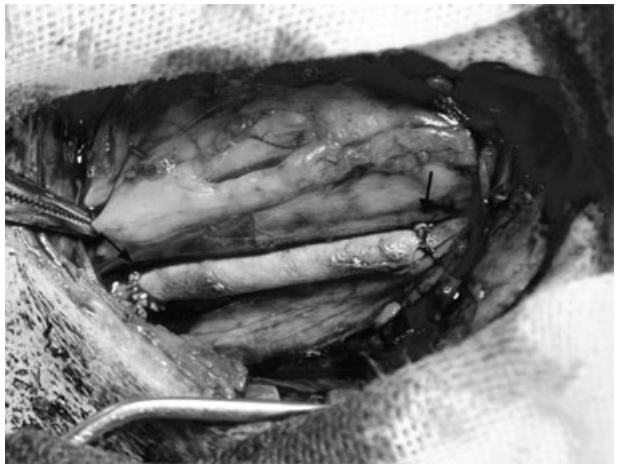

FIG. 5. Surgical implantation of the vascular grafts. The grafts were interposed to common carotid arteries by the end-to-end anastomosis in canine models. The arrowheads indicate the anastomotic sites.

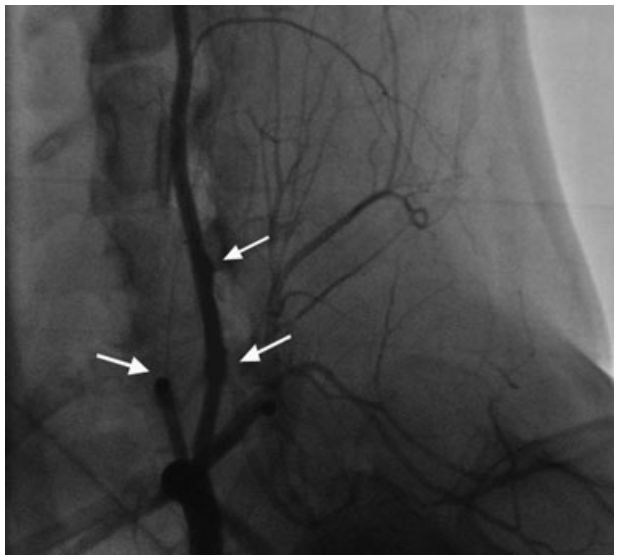

FIG. 6. Angiogram of the grafts at 6 months after implantation. The VHDSs graft maintains patency (right side), while the DSs graft was occluded (left side). The arrowheads indicate anastomotic sites.

Surgical implantation and explant characterization

Segments of the canine common carotid arteries were replaced by either VHDSs or DSs grafts without matrix rupture or deformation (Fig. 5). The animals were periodically investigated by digital subtraction angiography after implantation (Fig. 6). Out of the 15 VHDSs grafts, 14 maintained patency for up to 6 months, one occluded within the first month due to thrombus formation. However, only seven DSs grafts were still patent when explanted at 6 months, the other eight were observed with thrombotic occlusion within the first 2 months after implantation (Fig. 7). The patency rate between VHDSs grafts and DSs grafts differed significantly ($P = 0.005$).

The patent grafts were retrieved at 6 months for histologic analyses. The H&E staining showed VHDSs exhibited endothelium regeneration on the luminal surface and a dense population of fibroblast-like cells in the inner layers of the media (Fig. 8A), but only a few poorly organized cells and thrombotic deposit overlay the luminal surfaces of DSs, and massive disorderly cells were present throughout the reconstituted wall (Fig. 8B). SEM observation revealed that the luminal surfaces of both grafts were covered with a confluent monolayer of cobblestone-like cells, but the orientation highly parallel to the direction of arterial flow observed in VHDSs was not seen in the DSs (Fig. 8C,D). Masson's trichrome

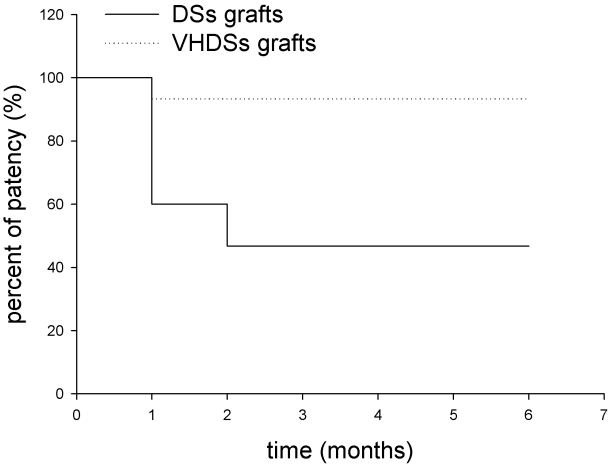

FIG. 7. Long-term patency rate of VHDSs and DSs grafts (both $n = 15$).

staining displayed that collagen and elastin were reconstituted during the 6 months in both grafts (Fig. 9) and the subsequent morphometric analysis revealed that the VHDSs grafts ($n = 14$) exhibited significantly smaller hyperplastic neointima area compared with the control DSs grafts ($n = 7$), not only at midportion (0.56 ± 0.07 vs. 2.04 ± 0.12 mm^2, $P < 0.0001$), but also at distal anastomosis (1.76 ± 0.12 vs. 3.67 ± 0.20 mm^2, $P < 0.0001$) (Figs. 9 and 10).

DISCUSSION

Small-diameter vascular synthetic grafts exhibit a high rate of failures due to the lack of an antithrombogenic monolayer in direct contact with circulating blood (5,6). Seeding and sodding with autologous vascular cells on the luminal surface of graft prior to implantation have provided a much higher patency rate than non-cell-seeded grafts (7–9). However, this technology requires time-consuming and labor-intensive procedures, so that such a tissue-engineered approach cannot be utilized in the emergency cases. Therefore, in vivo rapid spontaneous endothelialization technology has been long awaited. Regarding spontaneous endothelialization of vascular grafts in vivo, currently there are three major mechanisms proposed (18): (i) the migration of ECs inward across the anastomosis from the native vessel; (ii) ECs coverage derived from the ingrowth of capillaries through porous grafts; and (iii) the deposition of circulating progenitor ECs onto the luminal surface of the graft.

In this study, accordingly, we incorporated porous decellularized arterial scaffolds (to enable cell transmural ingrowth and adhesion) and angiogenic VEGF165 (for recruitment of native ECs and circulating endothelial progenitor cells) into a vascular graft design, which was considered to be a promising solution for rapid self-endothelialization in vivo.

The DSs would be an ideal candidate for a vascular graft. Our histological analysis showed that the cellular components were completely removed from the DSs, leaving the porous biological three-dimensional (3-D) architecture, which could provide physiologically proper microenvironment for vascular cell repopulation (1,9). And previous studies have shown that mechanical properties such as burst strength,

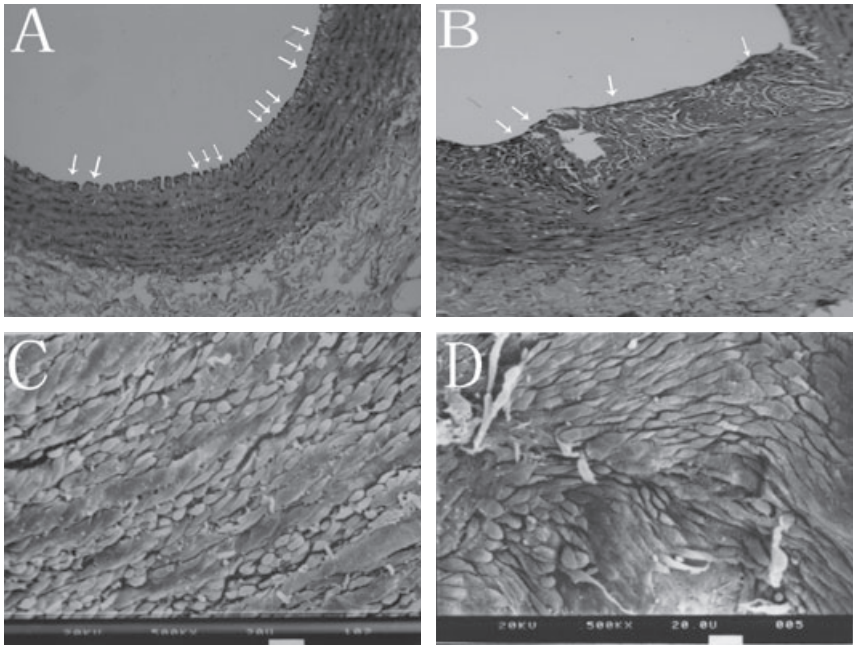

FIG. 8. Histological analyses of intimal surface of the grafts retrieved 6 months after implantation. HE staining of midportion of the retrieved VHDSs graft (A) and DSs graft (B); scanning electron photomicrographs of intimal surface of VHDSs graft (C) and DSs graft (D).

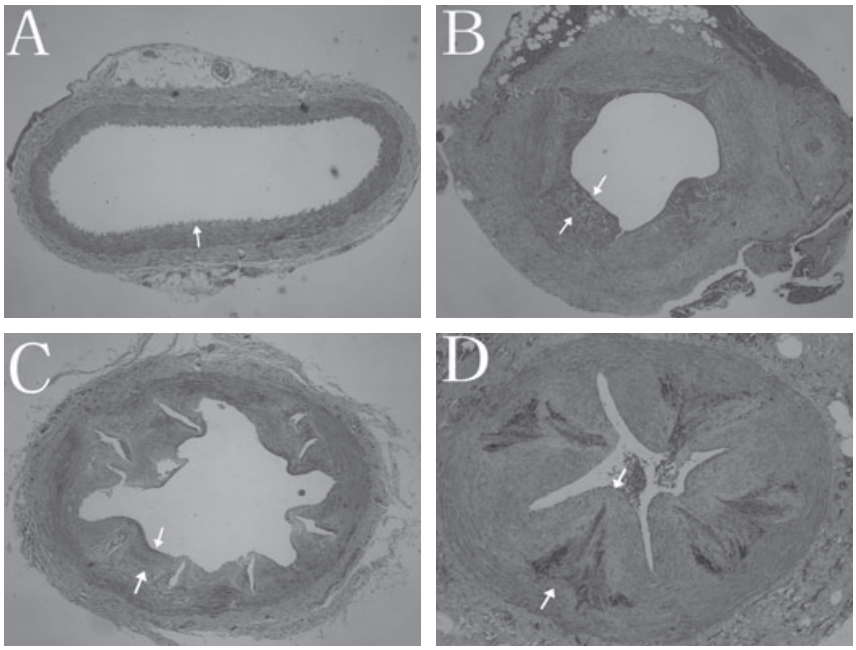

FIG. 9. Masson trichrome staining comparing degree of neointimal hyperplasia at 6 months after implantation. (A) midportion of VHDSs graft; (B) midportion of DSs graft; (C) distal anastomotic site of VHDSs graft; (D) distal anastomotic site of DSs graft. White arrows indicate neointimal formation.

compliance, and suture retention strength are not significantly different between decellularized tissue matrices and native vascular tissues (5). Additionally, others have reported allogenic DSs show low immunogenicity in vivo (19), and more resistance to infection than PTFE graft when used as hemodialysis access (20).

Furthermore, we immobilized heparin to the DSs using EDC and NHS as cross-link agents. During this process, the carboxyl groups on the heparin are activated to succinimidyl esters, which react with amino functions on the collagen and elastin to zero length cross-links (5). This modification leads to covalent linkage of heparin to the DSs, which unlike the ionic linkage of heparin used by several other groups (21), appears to be more stable and long lasting (22). In addition, previous results demonstrated that HDSs eliminated blood clot formation in vitro (5), and subsequent animal studies indicated it reduced the thrombogenicity in vivo as well (6). Moreover, heparin is not only known for its anticoagulant properties, but also interacts with a variety of growth factors that have heparin-binding domains, thus induce as a substrate for binding growth factors and development for controlled release systems (23).

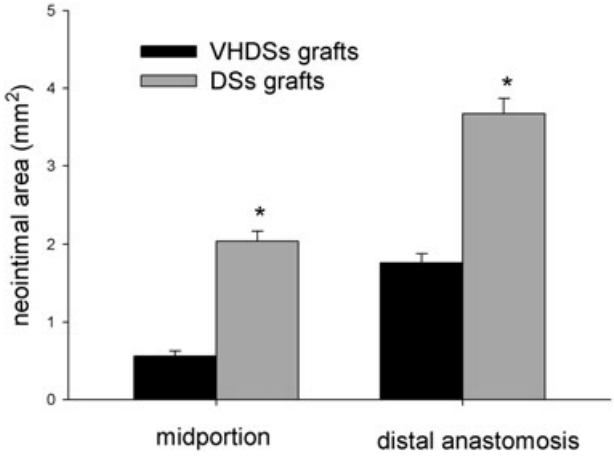

FIG. 10. Comparison of neointima area at midportion and distal anastomotic sites of VHDSs ($n = 14$) and DSs ($n = 7$) grafts at 6 months after implantation (*$P < 0.0001$ when compared to VHDSs at the same site).

Among the heparin-binding growth factors, VEGF is of particular interest in tissue engineering due to its angiogenic properties. The alternative splice variant of VEGF with 165 amino acids (VEGF165) is characterized by a significantly high mitogenic activity and ability to promote growth of ECs both in vitro and in vivo (11). The VEGF165 is secreted as a disulfide-linked homodimer with two identical heparin-binding sites, which induces the strong biological affinity for heparin (24). Besides, heparin interactions also prolong VEGF165 activity by sequestration away from natural degradatory pathways and by restoring lost function following damage under oxidative conditions (25).

To make sure whether the immobilized heparin had a large impact on the VEGF coating behavior, we measured the amount of VEGF coated on both HDSs and DSs under various concentrations; the results showed a positive correlation between the

coating amount and reaction concentrations on both scaffolds, but significantly more VEGF coated on HDSs compared with DSs, probably due to high affinity between heparin and VEGF (16,23).

As the short half-life of free VEGF in vivo is degraded by proteolysis within 10–20 min after a bolus injection (11), most of the envisioned applications may require longer period of sustained release. We also investigated the release profiles over a period of 20 days by ELISA in vitro. The results showed that the VEGF release from HDSs was more gradual than that from DSs. After 20 days of incubation, VEGF retention on HDSs was approximately 26% (2.61 ng) versus 2% (0.10 ng) on DSs. Therefore, HDSs presented VEGF release over longer time than DGs. We interpreted this as VEGF was biologically bound to heparin molecular of HDSs through its heparin-binding domains, and hence became more stable and lasting. Our result was in good agreement with the previously published data. Yao et al. (16) observed a period of 21 days sustained release of radioactively iodinated VEGF (^{125}I-VEGF) from heparin-immobilized collagen matrices by liquid scintillation counting. And Pike's group (23) reported that the heparin cross-linked and modified hydrogels were capable of both storing and providing sustained release of the VEGF over 42 days, and this prolonged release could be controlled with less than 1% heparin content.

In order to validate the biological activity of the coating VEGF, we exposed HUVECs to both VHDSs and DSs disks. The cell proliferation was tested by [^{3}H]thymidine uptake, which was used to measure cell DNA synthesis rather than cell number, because at times investigated, this measurement would only indicate entry of cells into S phase of cell cycle (17). The results demonstrated that the [^{3}H]thymidine uptaken by HUVECs cultured on VHDSs was higher than that for DSs throughout the days after seeding, due to the higher proliferation rate of cells induced by coating VEGF. At confluence, the number of rapidly dividing HUVECs decreased and as a result [^{3}H]thymidine uptake declined, irrespective of the seeding surface. This result indicated that the VEGF released from HDSs would be able to promote ECs proliferation in vitro, meanwhile implying the possible efficacy of this technique in vivo.

As expected, the animal experiments demonstrated that VHDSs grafts had a significantly higher patency rate than DSs grafts (14/15 vs. 7/15, $P = 0.005$) after 6 months of implantation. All of the occluded grafts were found within 2 months after implantation, due to thrombus formation probably caused by the thrombogenicity of extracellular matrices. We hypothesized that both cross-linked heparin and early endothelialization enhanced by coating VEGF contributed to reduce the in vivo thrombogenicity, and improved early patency rate of VHDSs grafts. The histological analysis of the patent grafts retrieved at 6 months revealed a high degree of reendothelialization in VHDSs grafts, and conversely, only a few disorderly endothelium and even thrombosis deposited on the luminal surface of the control grafts. Although we were not able to evaluate the reendothelialization at an early stage or analyze it quantitatively, the effects of VEGF on reendothelialization of vascular graft and injured artery in vivo were becoming increasingly clear (12–15).

The most important finding, in our opinion, is the observation of the inhibition of neointimal hyperplasia in VHDSs grafts. Figures 9 and 10 demonstrated a dramatic decrease of intimal hyperplasia at both midportion and distal anastomotic sites of VHDSs compared with DSs ($P < 0.0001$ for both sites). We hypothesized that the mechanism of neointimal hyperplasia inhibition was principally through VEGF reduced proliferating action on ECs which led to the reendothelialization of grafts, and that this reendothelialization secondarily inhibited neointimal hyperplasia. As we know, ECs are able to secrete many antiproliferative factors, such as nitric oxide and vascular natriuretic peptides, which modulate vascular smooth muscle cells (VSMCs) growth (12,13). A lack of ECs might promote abnormal vascular growth, such as thrombosis, hyperplasia, and obstruction. On the contrary, the presence of normal ECs might lead to the normal growth of VSMCs, and normal vascular regeneration in the long run. Additionally, a recent study discovered that VEGF may inhibit neointimal VSMCs hyperplasia independent of its mitogenic action on ECs (26). Moreover, we cannot exclude the effects of the immobilized heparin, which was found to inhibit the proliferation of VSMCs both in vivo (27) and in vitro (28). Meanwhile, our findings are in substantial agreement with lots of previous research. For example, Asahara et al. (12) showed that local delivery of VEGF protein accelerated reendothelialization and attenuated neointimal hyperplasia in rat carotid balloon injury model. Further experiments studying stent implantation in rabbit iliac arteries have shown that intravenous VEGF protein accelerated reendothelialization, decreased neointima formation, and reduced mural thrombosis (13). There is also evidence of reduction in rabbit vein graft intimal hyperplasia following topical VEGF administration (14). Recently, Hutter et al. (15) proved that VEGF overexpression accelerated endothelial repair and

inhibited neointima formation after arterial injury, while conversely, sequestration of exogenous and/or endogenous VEGF by VEGF-trap delayed reendothelialization and significantly increased neointima size. However, several other publications demonstrated the complex effect of VEGF delivery. For instance, Randone et al. (18) reported that pretreated ePTFE grafts with synthetic extracellular matrix and VEGF resulted in better endothelialization, but also resulted in undesired higher smooth muscle cells density and neointimal hyperplasia. Moreover, Walpoth et al. (29) impregnated ePTFE with fibrin and VEGF, and the following animal study showed that fibrin and VEGF produced the effect of increasing the narrowing at anastomosis and neointimal growth beyond that seen in uncoated grafts, while the patency rates were not significantly different.

Keeping this in mind, we compared Walpoth's experiment system with ours, and noted that many details were different, particularly, in the graft material (ePTFE vs. DS), VEGF coating media (fibrin vs. heparin), mechanism of VEGF release (undescribed vs. local sustained release), animal model (pig vs. canine), and end point time (1 month vs. 6 months), all of which were considered probably to contribute to the contrary results. Anyway, because there is some discordance in the literature, which makes it difficult to provide a definitive conclusion concerning the potential beneficial effect of VEGF, further studies are therefore necessary in order to establish the therapeutic efficacy of VEGF delivery.

CONCLUSIONS

This study exhibited a novel small-diameter decellularized vascular graft immobilized by heparin and coated with VEGF. The graft surface possessed sustained VEGF release over a prolonged period of time, and sufficiently enhanced HUVECs proliferation in vitro. Specifically, the in vivo results add to those already published that VEGF may provide more rapid endothelialization, and inhibit neointima hyperplasia, subsequently improving the patency rate of vascular grafts. However, certain critical characteristics, such as infection, calcification, aneurismal dilation, and vasoreactivity, need to be assessed in further studies.

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