Application of the Tissue-Engineered Plant Scaffold as a Vascular Patch

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ABSTRACT: Tissue-engineered plant scaffolds have shown promising applications in in vitro studies. To assess the applicability of natural plant scaffolds as vascular patches, we tested decellularized leaf and onion cellulose in a rat inferior vena cava patch venoplasty model. The leaf was decellularized, and the scaffold was loaded with poly(lactic-co-glycolic acid (PLGA)-based rapamycin nanoparticles (nanoparticles). Nanoparticle-perfused leaves showed decreased neointimal thickness after implantation on day 14; there were also fewer CD68-positive cells and PCNA-positive cells in the neointima in the nanoparticle-perfused patches than in the control patches. Onion cellulose was decellularized, coated with rapamycin nanoparticles, and implanted in the rat; the nanoparticle-coated onion cellulose patches also showed decreased neointimal thickness. These data show that natural plant-based scaffolds may be used as novel scaffolds for tissue-engineered vascular patches. However, further modifications are needed to enhance patch strength for artery implantations.

INTRODUCTION

Polyester and expanded poly(tetrafluoroethylene) (PTFE) vascular grafts have been widely used for revascularization and have made great contributions in patients for more than 50 years. However, small-diameter (<6 mm) vascular grafts continue to have a very low long-term patency after surgery. Autogenous blood vessels remain the preferred choice for conduits owing to their higher patency rate, particularly when a “no touch” vein graft harvesting technique is used. Apart from the commonly used polyester and expanded PTFE grafts, biological vascular grafts, such as cryopreserved allografts, human umbilical vein grafts, and xenografts, have also been developed and used in vascular surgeries. In our previous patch angioplasty study in the rat model, we showed that the inferior vena cava (IVC) patch had a thick neointima, and that the pericardial patch with covalently attached rapamycin could decrease neointimal thickness.

Advancements have been made in in vitro plant-based scaffold research recently. A study reported decellularizing spinach and parsley, recellularizing with human endothelial cells, and cultivating for 21 days; they demonstrated the potential of decellularized plants as scaffolds in tissue engineering. Another study reported using decellularized spinach leaf scaffolds in stem cell growth and differentiation in bone tissue engineering.

We have previously shown that pericardial patches can be coated with poly(lactic-co-glycolic acid (PLGA)-based rapamycin nanoparticles (nanoparticles) to inhibit neointimal hyperplasia in a rat IVC patch venoplasty model. The decellularized human saphenous vein patch and rat thoracic aorta patch can also be coated with heparin or PD-1 antibody to decrease neointimal thickness; although derived from different materials, these patches exhibit a similar healing process. We hypothesized that plant-based patches could be used as vascular patches and may also exhibit a similar healing process. We selected to develop scaffolds from two plant tissues: leaf and onion cellulose. We hypothesized that decellularized leaf and onion cellulose can serve as novel biological scaffolds and that the scaffold could facilitate drug delivery. We assessed the biocompatibility of the plant materials as novel sources of vascular grafts and drug-delivery scaffolds, given that this is a novel application of plant scaffolds in vascular research.

RESULTS

Plant Leaf. The green plant leaf turned white after decellularization and bleaching and turned red after perfusion with rhodamine water. Immunofluorescence study showed red fluorescence from the leaf. Scanning electron microscopy (SEM) showed a consistent diameter of the PLGA nanoparticles.
The hydrogel solidified in 1 min (Figure 1E). The patches were then placed into the rat IVC. On day 14, the patches were incorporated into the rat IVC; no thrombosis formation was seen. Hematoxylin–eosin (HE) staining showed a notably thinner neointima in the nanoparticle-perfused patches than in the control patches (Figure 2A,B). Fewer cells infiltrated into the interstitial space between the leaf fibers of the patches (Figure 2A,C). Immunohistochemistry showed a line of CD31-positive cells on the luminal surface of the neointimas in both groups; furthermore, α-actin-positive cells were also seen in the neointimas of both groups (Figure 3A). Fewer CD68-positive cells and PCNA-positive cells were seen in the neointima in the nanoparticle-perfused patches than in the control patches (Figure 3A–C).

**Onion Patches.** Onion cellulose is transparent before decellularization (Figure 4A). SEM revealed a consistent shape on the surface of the onion patch (Figure 4B). HE staining also showed a similar shape (Figure 4C). After nanoparticle conjugation, SEM showed a smooth surface of the onion cellulose, and the nanoparticles could be seen on the surface (Figure 4D). After harvesting on day 14, HE staining revealed a much thinner neointima in the nanoparticle-conjugated patches than in the control patches (Figure 5A,B). Fewer cells infiltrated into the interstitial space between the fibers in the patches (Figure 5A,C). Immunohistochemistry showed a line of CD31-positive cells on the luminal surface of the neointimas in both groups; α-actin-positive cells were also seen in the neointimas in both groups (Figure 6A). Fewer CD68-positive cells and PCNA-positive cells were seen in the neointima in the nanoparticle-conjugated patches than in the control patches (Figure 6A–C).

**DISCUSSION**

In this study, we showed that decellularized plant tissue including leaf and onion cellulose can be used as vascular patches or natural drug-delivery systems in a rat IVC patch venoplasty model. We also showed that decellularized leaf vasculature can be a scaffold for nanoparticle delivery to inhibit venous neointimal hyperplasia in rats. The plant patch induced...
a similar healing process as the bovine pericardial patch, decellularized human saphenous vein patch, decellularized rat thoracic aorta patch, and polyester patch. Although the autologous graft is the first choice among vascular grafts, it is not suitable for every patient. Therefore, biological and prosthetic vascular grafts are also used in clinical applications. However, immune rejection or the risk of animal-transmitted diseases, in the case of biological vascular grafts from animals such as bovine or porcine pericardial patches, remains a risk. Although using a combination of CRISPR-Cas9 and transposon technologies for genome engineering of pigs for greater compatibility with the human immune system enables safe and effective porcine xenotransplantation, this technology would require a long time for translation to preclinical research. Plants are now attracting notable attention given the variety and the natural structure. Although plant and animal cells are different, they also share some similarities.

Three-dimensional (3D) cellulose scaffolds produced by decellularizing apple hypanthium tissue can be used for in vitro 3D cultures of different cells. A study showed that these cells can adhere, invade, and proliferate in the cellulose scaffolds, retain high viability even after 12 continuous weeks of culture, and achieve cell densities comparable to those of other natural and synthetic scaffold materials. Plant-based scaffolds present many advantages over several biomaterials; these can modify cell phenotype or affect cellular response to external stimuli and mediate changes in cell behavior. Furthermore, the physical properties of the various plant scaffolds can be matched with the diverse physiological functionalities of cells and human tissue constructs. The use of decellularized spinach leaf 3D scaffolds has been reported; while these present challenges associated with artificial scaffolds, their surface properties and the pore shapes are effective for stem cell binding, growth, and proliferation. Decellularized apple, carrot, and celery-derived tissues as scaffolds have been investigated for the regeneration of more tissue types, such as adipose tissue, bone tissue, and tendons. However, these pioneer studies are all in vitro studies, and in vivo studies are lacking. Because the vascular graft heals via a complex process, we implanted the decellularized plant as a vascular patch and demonstrated its potential future applications.

We demonstrated that cells migrated to and infiltrated the decellularized fibers of the leaf and onion cellulose after implantation as a patch, which is similar to our previous observation that cells infiltrated into the patches made from other materials. We also showed rapid neointima formation after patch implantation in the IVC. PLGA-based rapamycin nanoparticles can inhibit venous neointimal hyperplasia, whereas PLGA-based TGF-β1 nanoparticles can be considered to decrease pseudoaneurysm formation. We explored the leaf as a natural drug-delivery scaffold, and observed notably thinner neointima formation in the nanoparticle-perfused leaf. We used PLGA-based rapamycin nanoparticle-coated onion cellulose patch and showed a thinner neointima in the IVC angioplasty. This result shows that onion cellulose can be successfully modified as a vascular scaffold, and the plant leaf could be a promising drug-delivery system.

Our study has some limitations. The onion leaf and cellulose are not adequately strong; hence, modification of onion cellulose is needed for use in aortic angioplasty. A longer observation period might be needed to understand the long-time reaction. Finally, our finding that decellularized onion plant leaf and cellulose effects healing by infiltrating different host cells infiltration and via neointimal reendothelialization.
CONCLUSIONS

Decellularized plant leaf and onion cellulose can both be used as vascular patches in the rat venoplasty model. Their surfaces can be modified or used as drug-delivery scaffolds. This study demonstrates the broad potential applications of plant scaffolds as medical biomaterials. Further modifications to enhance patch strength are needed for their implantation in the artery.

METHODS

The study was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University. All animal care procedures complied with the Guide for the Care and Use of Laboratory Animals. NIH guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 Rev. 1985) were followed.

Scaffold Decellularization and Coating. For leaf decellularization, the leaf was incubated in 10% sodium dodecyl sulfate (SDS) buffer for 24 h, followed by washing with phosphate-buffered saline (PBS), and then with a 10% sodium chlorite bleach in a deionized water solution for 12 h. Subsequently, it was washed with PBS to completely remove the detergent.

Decellularized onion skin scaffolds were then used for coating or for implantation.

Fabrication of PLGA-Based Nanoparticle. We added 100 mg of PLGA into 1 mL of ethyl acetate and allowed the polymer to dissolve overnight. Rapamycin was directly added to the polymer solution and vortexed. In a test tube, 2 mL of 0.3% w/v vitamin E-TPGS was added, followed by 1 mL of the polymer solution. The solution was then vortexed, which resulted in the emulsification of the solution and hardening of the nanoparticles. The hardened nanoparticles were split into two centrifuge tubes and centrifuged. The supernatant was discarded; 15 mL of diH2O was added to completely resuspend the nanoparticles. The nanoparticles were transferred to a tube and frozen at −80 °C for 30 min. After 72 h of

Figure 5. Onion cellulose without (control) or with PLGA-based rapamycin nanoparticle coating harvested from the rat IVC venoplasty model at day 14. (A) Photographs of hematoxylin–eosin stained onion cellulose patches after venoplasty at day 14. The first row shows low-power photographs (scale bar: 1 mm). The second to fourth rows show high-power photographs showing the neointima and cells infiltrating into the patch (scale bar: 100 μm; n = 3). (B) Bar graph showing the neointimal thickness in the venoplasty models at day 14 (t-test, *p = 0.0019; n = 3). (C) Bar graph showing the cells infiltrating into the patch in the venoplasty models at day 14 (t-test, *p = 0.0102; n = 3).

Figure 6. Neointima of the control or PLGA-based rapamycin nanoparticle-coated onion cellulose patches harvested from IVC venoplasty models at day 14. (A) Photograph of immunohistochemical staining for CD31, α-actin, CD68, and PCNA (scale bar: 100 μm). (B) Bar graphs showing CD68-positive cells in the neointima (*p = 0.0160, t-test; n = 3). (C) Bar graph showing the PCNA-positive cells in the neointima (*p = 0.0137, t-test; n = 3).
lyophilization for a 5 mL volume, the lyophilized particles were stored in a paraffilm-wrapped tube at −80 °C.\textsuperscript{24}

**Hydrogel Fabrication.** The hyaluronic acid–sodium alginate (HA/SA) scaffold was fabricated by reacting sodium SA, HA, and CaCO\textsubscript{3} as previously described.\textsuperscript{28,29} Briefly, SA (3.2 × 10\textsuperscript{4} to 2.5 × 10\textsuperscript{5} Da\textsuperscript{2}, Sigma-Aldrich) was dissolved in DMEM/F12 culture medium (HyClone, Hyclone Laboratories) to yield a 0.5% solution; subsequently, HA (4000 Da, Sigma-Aldrich) was distributed in the SA solution in a 1:4 (samples labeled as SA4HA1) or 1:2 (samples labeled as SA2HA1) concentration ratio. Then, the CaCO\textsubscript{3} suspension was introduced into the HA/SA solution, followed by gluconic acid/lactone solution (GDL; Sigma-Aldrich) to obtain a Ca\textsuperscript{2+} and COO− ratio of 1:2. The molar ratio of Ca\textsuperscript{2+} and GDL was also 1:2. After adding GDL, the crosslinking reaction was triggered, and finally, the HA/SA hydrogels were obtained after a 0.5 h reaction.

**Perfusion of Leaf Vasculature with the Rapamycin Nanoparticles.** Rhodamine water was slowly injected into the main trunk of the leaf using a 1 mL syringe. Approximately, 0.5 mL of rhodamine water could be injected into the leaf, and the leaf vasculature turned red immediately after injection. The leaves were photographed directly under an immunofluorescence microscope. The hydrogel with the nanoparticles was injected in a similar fashion.

**Coating Onion Cellulose with Rapamycin Nanoparticles.** Onion cellulose coated with rapamycin was immersed in an HA solution and coated in a similar fashion as previously described.\textsuperscript{28} Briefly, after washing thrice with PBS (5 min/wash), the HA-coated samples were immersed in a PLGA-based rapamycin nanoparticle solution (2 mg/mL; Zhaoke, Hefei, China) that was also advance-activated in a water-soluble carbodiimide solution (15 min) and incubated at 37 °C for 6 h.\textsuperscript{27}

**Assessment of Rapamycin Bonding.** The morphology of the decellularized onion skin was observed under an SEM to determine whether the nanoparticles bonded with the skin surface. The procedures followed were as described previously.\textsuperscript{28} After freeze-drying, the samples were fixed on a small bracket, gold sprayed, and observed under the SEM (Auriga, Zeiss, Germany).

**Animal Model.** Male Sprague–Dawley rats (aged 6–8 weeks) were used. The aorta and IVC patch angioplasty models were performed as previously described.\textsuperscript{13} Microsurgical procedures were performed aseptically using a dissecting microscope (Nikon, Japan). Control and rapamycin nanoparticle-perfused leaves, control (decellularized but uncoated), and rapamycin nanoparticle-coated onion cellulose patches (approximately 3 × 1.5 mm\textsuperscript{2}) were implanted into the rat infrarenal IVC using continuous 10-0 nylon sutures. Rats were sacrificed on postoperative day 14, and the patches were explanted for analysis. No immunosuppressive agents, antibiotics, antiplatelet agents, or heparin were administered at any time.

**Histology Staining.** Rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate, and tissues were fixed with transcardial perfusion of PBS followed by that of 10% formalin. Tissue was removed and fixed overnight in 10% formalin followed by a 24 h immersion in 70% alcohol. Tissue was then embedded in paraffin and sectioned (4 μm thickness). Tissue sections were deparaffinized and stained with HE stain (Baso, Zhuhai, China) according to the manufacturer’s recommendations. Neointimal and adventitial thickness were measured as we previously described.\textsuperscript{29}

**Immunohistochemistry.** Sections were heated in a citric acid buffer (pH 6.0, Beyotime, Shanghai, China) at 100 °C for 10 min for antigen retrieval. Sections were then treated with 0.3% hydrogen peroxide for 30 min and incubated overnight at 4 °C with primary antibodies. After overnight incubation, the sections were incubated with appropriate secondary antibodies for 1 h at room temperature and treated with 3,3′-diaminobenzidine tetrahydrochloride horseradish peroxidase Color Development Kit (Beyotime, Shanghai, China) to detect the reaction products. Finally, the sections were counterstained with hematoxylin (Baso, Zhuhai, China). Positive cell numbers were counted and reviewed by three blinded professional pathologists.

**Immunofluorescence.** Tissue sections were deparaffinized and then incubated with primary antibodies overnight at 4 °C. The sections were incubated with secondary antibodies for 1 h at room temperature; subsequently, sections were stained with the fluorescent dye 40,6-diamidino-2-phenylindole (Solarbio, Beijing, China) to stain cellular nuclei.

**Primary Antibodies.** Primary antibodies included anti-CD68 (Abcam, ab31360; IHC, 1:50), anti-CD31 (R&D, AF3628; IHC, 1:100), anti-α-actin (Abcam, ab5694; IHC, 1:200), and anti-PCNA (Abcam, ab29; IHC, 1:100).

**Statistical Analyses.** Data are expressed as means ± standard errors of mean. Statistical significance for these analyses was determined using t-tests (Prism 6; GraphPad Software, La Jolla, CA). P-values < 0.05 were considered significant.

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