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Retention of an Autologous Endothelial Layer on a Bioprosthetic Valve for the Treatment of Chronic Deep Venous Insufficiency

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Abstract

Purpose—Percutaneous transcatheter implantation of porcine small intestine submucosa (SIS) bioprosthetic valves has been reported as a treatment for chronic deep venous insufficiency. Endothelial progenitor outgrowth cells (EOCs), isolated from whole ovine blood, were evaluated as a source of *in vitro* autologous seeding for SIS endothelialization. Retention of the EOC monolayer was evaluated to test the feasibility of delivering an endothelialized SIS valve.

Materials and Methods—20 bioprosthetic venous valves were constructed from SIS sutured onto collapsible square stent frames and were seeded with ovine EOCs *in vitro*. Retention of the endothelial monolayer through valve loading and delivery (3 valves), *in vitro* flow (3), and *ex vivo* flow (4) was evaluated with immunofluorescent staining and histology compared to paired unmanipulated control valves. In the *ex vivo* shunt loop, venous blood was pulled from an implanted dialysis catheter, through the valve, and returned to the sheep.

Results—Immunofluorescent staining of the EOCs on the valves after *in vitro* seeding revealed a confluent monolayer ($95.6 \pm 2.3\%$ confluent) on each side of the valve. When examined by immunofluorescent staining, the endothelial monolayer remained intact after loading and delivery ($97.1 \pm 1.7\%$) and when subjected to flow in the *in vitro* loop ($96.0 \pm 3.0\%$). Histology of the valves subjected to the *ex vivo* shunt loop revealed retention of the endothelial monolayer.

Conclusion—Endothelial monolayers seeded on SIS were retained under loading and delivery, *in vitro* flow, and *ex vivo* flow. EOCs are a promising cell source for autologous endothelialization of bioprosthetic valves for the treatment of chronic deep venous insufficiency.

Introduction

Chronic deep venous insufficiency (CDVI) of the lower extremities is a major health and medical problem in the United States. CDVI is a disease characterized by incompetent or absent venous valves and, if left untreated, can result in significant pain, edema, and ulceration. Most CDVI patients are not candidates for direct deep vein valve surgical repair,

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neo-valve construction, or valve transplantation (1–3). Thus the need for a percutaneous artificial venous valve is evident (4).

We have been developing and exploring three types of bioprosthetic venous valves in animals (5–8). They consist of a double-leaflet of porcine small intestinal submucosa (SIS) (Cook Biotech, West Lafayette, IN) attached to three different types of square stent frames. Following successful implantation of the bioprosthetic venous valves in animals, we used the first generation clinically in 3 patients and the second generation in 15 patients with advanced CDVI (8–10). Percutaneous transcatheter implantation of the SIS venous valves was shown to improve symptoms of CDVI. The implants remained functional for up to 12 weeks in an ovine model and in patients with CDVI, but, after this time, neointimal hyperplasia limited the function of the valves (8–10).

Animal studies reported by Teebken *et al.* (11) and Pavcnik *et al.* (12) demonstrated that leaflets lined with endothelial cells prevented intimal hyperplasia, thereby prolonging valve functionality. Critical issues in using percutaneous valve therapy are the source of endothelial cells for harvest in patients and the ability to deliver the valve leaflet with its endothelium intact and fully functioning. Endothelial progenitor outgrowth cells (EOCs), which can be isolated from the peripheral blood, are a promising source of autologous endothelial cells for valve therapy (13–15). EOCs can differentiate into mature, fully functional endothelial cells with the protective functions of limiting intimal hyperplasia and providing an anti-thrombogenic surface. *In vitro* EOC-seeding of biomedical devices has been investigated for metal and polymer devices (16–18), however seeding on SIS has not been reported. In order to translate this *in vitro* seeding-technique into a clinical device, certain prerequisites must be met: 1. EOCs must adhere and grow on the SIS surface to form an endothelial monolayer; 2. the endothelial layer must be retained through loading and delivery; and 3. the endothelial layer must remain intact under flow in the venous system. This paper reports the retention of the endothelial layer through loading and delivery, *in vitro* flow, and *ex vivo* flow.

Materials and Methods

Animals

The study involved 6 female domestic sheep (*Ovis aries*; Western crossbreed) weighing 47.7 to 67.1 kg (mean 54.3 kg). Blood was collected for endothelial progenitor cell isolation (6 sheep). The EOC-covered SIS venous valves were tested in an *ex vivo* ovine shunt (one long-term sheep). This study was reviewed and approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University in accordance with guidelines established by the Animal Welfare Act.

General anesthesia was used to minimize discomfort. The sheep was tranquilized with 6 mg/kg IV of Midazolam (Bedford Laboratories Inc., Bedford, OH) and 4 mg/kg Ketamine (Fort Dodge Animal Health, Fort Dodge, IA). The sheep was then intubated and maintained on 2–2.5% isoflurane (Isothesia, Burns Veterinary Supply, Rockville Center, NY), with 2 liters of O₂ either by breathing spontaneously or using a mechanical respirator. Atropine sulfate (6 mg) (American Regent Laboratories, Inc., Shirley, NY) was intravenously injected to control saliva secretion.

Isolation of endothelial progenitor cells from circulating blood

Whole blood was drawn from previously tunneled central heparin-bonded 15 Fr hemodialysis catheter (Spire Biomedical, Bedford, MA) and anticoagulated with acid citrate dextrose (7:1) immediately. EOCs were isolated from whole blood as previously described (19). Isolated mononuclear cells in Endothelial cell growth media (EGM-2, Lonza,

Walkersville, MD) with 20% fetal bovine serum (FBS, Hyclone, Thermo Scientific) were plated on a fibronectin coated Falcon 12-well tissue culture plate (5 to 10×10^6 cells/well, 1.5 mL/well). After 24 hours, the plate was rinsed with phosphate buffered saline (PBS, Gibco) ($\times 3$) and fed with EGM-2 with 20% FBS. The plate was fed everyday for 7 days then 3 days/week for up to 5 weeks while observing for colony formation.

Purification of endothelial outgrowth cells

Colonies of outgrowth (average 1–3 colonies/plate) were isolated and plated onto rat-tail collagen I coated flasks. Flasks were fed 3 days/week with EGM-2 with 10% FBS. EOCs for this study were used at passage 5. EOCs were seeded on glass slides and stained with immunofluorescence to confirm their endothelial cell markers. Seeded EOCs (before fixing) were incubated with acetylated-low density lipoprotein (Di-Ac-LDL, $25 \mu\text{g/mL}$, Invitrogen) for 1h at 37°C . Di-Ac-LDL media was removed and replaced with fresh media and incubated for 10 minutes at 37°C . Cells were rinsed with PBS with calcium and magnesium, fixed with 3.7% paraformaldehyde, and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Additionally, seeded EOCs were fixed and stained for the surface marker VE-Cadherin (Santa Cruz), visualized with secondary antibody (anti-mouse IgG₁ Alexa-488, Invitrogen) and counterstained with DAPI.

SIS valve construction

This bioprosthetic venous valve was similar to the original bicuspid square stent valve (5). The valves were constructed of square stents (0.0075-inch stainless steel wire) and a layer of air dried and then rehydrated SIS sheet. To form the bioprosthetic valve, a 20 mm square piece of SIS was sutured with 7.0 Prolene monofilament to the 10 mm square stent. For consistency, all 20 valves were constructed with the serosal side up and the overhanging cut edge on the mucosal side. A loop of 5.0 Prolene suture was threaded through two diagonal eyes of the square stent to aid in hands free collapse of the valve. The SIS was either stored in sterile water or air dried until needed and then soaked in 10% FBS media overnight prior to use. When needed, the SIS membrane was slit at the diagonal axis of the stent to create the valve opening. The bioprosthetic valve size was designed to fit the flow loop tubing diameter of 6 mm.

Seeding SIS surface

The SIS valve was presoaked in EGM-2 with 10% FBS overnight. In a 24-well coated with agarose and equipped with an o-ring, sheep EOCs were seeded on the serosal side of the SIS valve at a density of 9×10^5 cells/cm² in EGM-2 with 10% FBS at a concentration of 1.2×10^6 cells/mL. After 24 hours the valve was flipped into a second agarose-coated well equipped with an o-ring and was seeded on the mucosal side with the same number of sheep EOCs. After an additional 24 hours the valve was placed vertically in a 48-well plate with fresh media for a final 24 hour incubation. To analyze percent confluence, leaflets were incubated in fixative solution (3.7% paraformaldehyde in PBS). After 45 minutes of incubation at room temperature, the EOCs on the valve were permeabilized, blocked with 10% goat serum (Invitrogen). The cytoskeleton was stained with Rhodamine Phalloidin 568 (Invitrogen) and the nuclei with Hoechst 33342 (Invitrogen). Immunofluorescence was evaluated using a 20 $\times$ objective on a Zeiss Multiphoton Confocal microscope (Zeiss, Thornwood, NY). The serosal side of the valve was imaged first; then the focal plane was changed to transverse the depth of the SIS while keeping the xy direction constant, and the mucosal side was imaged. The percent confluence was quantified as the percentage of surface area covered by the cells on the SIS. ImageJ (NIH) was used to measure the area without cells which was then subtracted from 100% to give the percent confluence. The percent confluence is reported as the mean of each condition (12 representative images analyzed per condition) plus or minus the standard deviation. Statistical significance

compared to the unmanipulated controls was determined using the Student's t-test with Excel (Microsoft).

Delivery system

The modified 20 cm long delivery system consisted of a transparent 12 Fr introducer sheath (Cook Medical) and a dilator of similar size. The EOC-covered bioprosthetic venous valve was front-loaded in the sheath before deployment. The SIS valve was deployed with a 10 Fr dilator.

Evaluation of loading and delivery

Seeded valves (n=3) were loaded into the delivery catheter and expanded into a tube of fixative solution (3.7% paraformaldehyde in PBS). Leaflets were stained and imaged as described above using confocal microscopy. Samples were compared to unmanipulated controls that underwent the identical fixing and staining protocol without loading and delivery.

***In vitro* flow loop**

The *in vitro* flow loop was constructed from transparent hemodialysis tubing with an internal diameter of 6 mm (Fresenius USA, Lexington, MA). A side line tube with a 16 Fr sheath for SIS valve deployment was connected to the flow loop. The flow loop was driven by a peristaltic pump (7523-60, MasterFlex, Vernon Hills, IL) with an Easy Load II Head (77201-60) to simulate venous circulation. A Transonic Small Animal Flow Meter (T106, Transonic Systems Inc.[®], Ithaca, NY) was used to monitor flow. Testing was done with the flow model in a horizontal position through which Hyskon flow media (20% dextran/dextrose solution in EGM-2 with 10% FBS to simulate the viscosity of blood) at 35–37 °C was circulated continuously.

***In vitro* flow test**

EOC-seeded bioprosthetic venous valves (n=3) were loaded into the delivery catheter and expanded into the *in vitro* flow loop. The internal resistance of the *ex vivo* shunt was approximated by the application of a clamp downstream of the valve. A final flow rate of 85 mL/min was applied for 20 min. The flow rate of 85 mL/min was selected to simulate a wall shear stress of venous blood flow (2 dynes/cm²). The 20 minute flow exposure was selected because the loss of adhered EOCs would most likely occur after deployment and initial exposure to flow. The valve was removed, by cutting the loop on either side of the valve, and fixed in paraformaldehyde for 1 hour. Samples were stained using the above protocol and compared to unmanipulated controls that underwent identical fixing and staining protocol without loading and delivery. A portion of flow and control samples were saved for histology. The histology samples were processed and embedded in paraffin blocks. The valves were sectioned in 5 µm slices across the leaflet and stained with hematoxylin and eosin (H&E).

***Ex vivo* venous shunt in the sheep model**

The *in vitro* flow loop was attached with two stopcocks to both lumens of the central double lumen hemodialysis catheter on the back of the sheep to form an *ex vivo* venous shunt loop. For the safety of the sheep, on the day of the experiment, the animal was placed in a stanchion, allowing the attachment and operation of the flow loop without sedation. The stanchion also prevented the sheep from moving and harming itself. Ten minutes prior to each run, we administered 100 IU/kg of heparin to anti-coagulate the sheep, the administration of which was repeated hourly during the experiment. The flow loop was

filled with sterile saline prior to attachment to the catheter, the SIS valve was delivered, and the peristaltic pump flow was applied to fill the loop with blood.

Ex vivo flow test

EOC-seeded bioprosthetic venous valves (n=4) were loaded into the delivery catheter and expanded into a saline filled flow loop. Flow was applied to fill the loop with blood. The flow rate was ramped up to 65 mL/min (a flow rate of 85 mL/min was desired to simulate a wall shear stress of venous flow of 2 dynes/cm², however due to peripheral resistance in the sheep, the maximum attainable flow rate was 65 mL/min, which corresponded to a wall shear stress of 1.5 dynes/cm²). After 30 minutes of blood flow the line was flushed with saline to rinse the loop and valve. The 30 minute flow exposure was selected because loss of adhered EOCs would most likely occur after deployment and initial exposure to flow. The valve was removed by cutting the flow loop on either side of the valve and placed in paraformaldehyde fixative for 1 hour. The valves were stained using the above protocol and compared to unmanipulated controls that underwent the identical fixing and staining protocol without loading and delivery. A portion of flow and control samples were saved for histology as described above.

Results

Twenty bioprosthetic venous valves were constructed of collapsible square stents covered with SIS and sutured to provide leaflets (Figure 1a). Once collapsed and drawn into the sheath, the bioprosthetic valve was deployed by advancing the dilator and pulling back the sheath (Figure 1b).

Sheep EOCs were isolated from whole blood and cultured. EOCs were seeded on glass slides and stained with immunofluorescence to confirm their endothelial cell markers. EOCs uptook acetylated-low density lipoprotein (Ac-LDL) and stained positive for the surface marker VE-Cadherin. Bioprosthetic venous valves were seeded with sheep EOCs over a 72 hour dual-sided protocol at a seeding density of 9×10^5 cells/cm². Endothelialization was evaluated with immunofluorescent staining of the actin cytoskeleton and the nuclei of the attached cells using confocal microscopy. The optimized seeding protocol resulted in a confluent monolayer of EOCs ($95.6 \pm 2.3\%$ confluent) with the characteristic cobblestone morphology of mature endothelial cells (Figure 3a,b).

Retention of the endothelial monolayer was evaluated through loading, delivery, and *in vitro* flow using immunofluorescent staining and confocal microscopy to evaluate the maintenance or loss of EOCs. After loading and delivery, the surface of the leaflet was $97.2 \pm 1.9\%$ confluent (Figure 3c,d), which is not statistically different than the unmanipulated controls (Student's t-test, $p=0.15$). There was no detectible removal of the monolayer and the EOCs retained their endothelial morphology. After *in vitro* flow, the surface of the leaflet was $96.0 \pm 3.0\%$ confluent (Figure 3e,f), not statistically different than the unmanipulated controls ($p=0.94$). There was no detectible loss of the endothelial monolayer from *in vitro* flow.

To test the *ex vivo* retention of the endothelial monolayer, the *in vitro* flow loop was attached to both lumens of the implanted catheter to allow for blood to be continuously drawn from and returned to the sheep. Placement of all 4 seeded bioprosthetic venous valves was successful after deploying into the saline filled loop (Figure 1c) and under *ex vivo* flow (Figure 1d) with no tilting or migration. Histology was performed on the *ex vivo* valve and sections were stained with hematoxylin and eosin (H&E) (Figure 4). Histology revealed endothelial cells were retained along the valve surface, however there was some cellular

infiltration into the SIS by red and white blood cells as well as isolated cellular adhesion over the endothelial monolayer.

Discussion

A percutaneously implantable bioprosthetic venous valve that remains fully functional over time is an attractive alternative to direct venous valvular reconstruction or transplantation (5, 10). Sutured to a square stent, SIS provides a biomaterial for seeding EOCs on bicuspid SIS-valves. The cusp consists of a free border SIS leaflet and a parietal part (*in vitro* tube). Once expanded in the tube, the bioprosthetic valve leaflets of the cusp have two sides. One side is formed by the serosal face of the SIS and the other by the mucosal face. The air-dried and then hydrated SIS used in this laboratory investigation was approximately 55 μm thick, approximately 2 times thicker than the natural venous valve. All 10 of the deployed valves had good self-expansion and centering of the valve leaflets.

A two-sided seeding protocol was developed to endothelialize both sides of the bioprosthetic venous valves. The serosal side was seeded first for consistency, and then the valve was flipped into a second well equipped with an o-ring to avoid contact between the seeded serosal side and the bottom of the well which could shear off cells or retard growth. In addition, the seeding wells were coated with agarose to avoid migration of EOCs from the serosal side to the bottom of the well. While optimizing EOC-seeding on the valve, it was necessary to use a large number of cells (9×10^5 cells/cm) due to the slit cut across the diagonal of the valve which allowed cells to pass through the valve. Cutting this slit after cell seeding resulted in significant cell-loss and disruption of the endothelial monolayer due to handling.

To examine the role of an autologous endothelial monolayer in the reduction of intimal hyperplasia formation on a bioprosthetic venous valve, it is necessary to show that an *in vitro* seeded endothelial monolayer can be transplanted intact. We sought to determine the retention of the seeded monolayers of EOCs on the SIS leaflets through the various manipulations of working with a percutaneous transcatheter delivery valve. In particular, loading and delivery of the bioprosthetic valve, which requires collapsing the frame followed by the advancement and retraction of a sheath over the valve, could shear off cells from the seeded faces of the SIS. We determined that the endothelial monolayer was retained through loading and delivery as evidenced by confocal microscopy and histology.

In developing a bioprosthetic venous valve, we needed to ensure the retention of EOCs under venous flow conditions (1 to 2 dynes/cm² wall shear stress). To mimic *in vivo* venous flow in our *in vitro* flow loop, the viscosity and flow rate of the circulating fluid were matched to venous blood conditions. A dextran/dextrose solution approximated the viscosity of blood (0.035 poise) and the flow rate was calculated to 85 mL/min to give a wall shear stress of 2 dynes/cm², the higher estimate of *in vivo* venous flow. Under these conditions, the EOCs were retained on the bioprosthetic venous valve through *in vitro* flow when measured by confocal microscopy and histology.

Retention of EOCs under *ex vivo* flow was next examined by connecting the *in vitro* flow loop to the two lumens of the hemodialysis catheter implanted into the sheep. Several considerations were taken into account in the design of the *ex vivo* flow loop: 1. The EOC-seeded bioprosthetic venous valve was delivered into a saline filled loop to minimize the exposure of the surface to standing blood before blood was circulated through the loop; 2. The sheep was anticoagulated in order to limit the influence of loosely adherent red blood cells, platelets, and leucocytes; 3. The blood flow rate through *ex vivo* shunt was 65ml/min to produce a wall shear stress of 1.5 dynes/cm²; and 4. Immunofluorescent staining of the *ex*

vivo valve was not possible because imaging was complicated by loosely-adhered circulating cells during blood exposure in the loop.

Instead of immunofluorescent staining and confocal microscopy, the endothelial monolayer on the *ex vivo* valve was evaluated with histology alone. Using histology, there was no possibility of evaluating the endothelial monolayer in an *en face* dimension due to surface roughness, so cross-sections of the tissue were cut and stained with H&E. The narrowness of these 5 μm slices were a limitation of the histology process and additionally, during processing, portions of the endothelial monolayer lifted off of the SIS adding a complication for capturing the true structure of the endothelial monolayer directly after fixation. Although the cross-sections gave a limited view of the bioprosthetic valve surface, they provided insight into the maintenance of the endothelial monolayer and revealed the influx of circulating cells into the SIS. The infiltrating cells might be migrating through the endothelial monolayer. The EOC attachment strength to the SIS could be improved by longer incubation times or preconditioning the EOCs with flow.

There are potential limitations of this study. While others have used sheep models to illustrate the potential of EOCs to endothelial vascular devices (20), there is no definitive evidence to support the assumption that sheep EOCs represent a valid model of human EOCs, due to variable endothelial reactions in different species. Additionally, the sample size in this initial study with 10 valves tested under loading, delivery, *in vitro* and *ex vivo* flow conditions compared with 10 unmanipulated controls was small. The histological analysis of evaluating the endothelial monolayer is limited due to the small cross-sectional thickness (5 μm) of each section and the lack of an *en face* image that because of surface roughness is unattainable. Despite these possible limitations, the findings reported herein reveal that the *in vitro* coating of the bioprosthetic venous valve with EOCs is a promising strategy to help guide vascular remodeling after implantation.

In conclusion, EOCs are a promising source for autologous endothelialization of bioprosthetic venous valves. The results of this laboratory investigation are encouraging and warrant further studies to test the ability of this endothelial monolayer to limit the formation of intimal hyperplasia in animals.

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Fig 1. Placement of a bioprosthetic valve constructed from SIS sutured onto a collapsible square stent frame into a flow model. **A** . Nonrestricted valve 10 mm in length attached to a 5.0 Prolene monofilament loop with dilator inside the sheath. **B** . Valve front- loaded into a 12 F transparent guiding sheath. **C** . Valve deployed into *ex vivo* shunt flow loop filled with saline and **D** . blood.

Fig 2. Flow loop model. **A.** Photograph of *in vitro* flow loop driven by peristaltic pump and monitored with transonic flow probe **B.** *In vitro* flow loop attached with two stopcocks to both lumens of the hemodialysis catheter on the back of the sheep to form an *ex vivo* venous shunt.

Fig 3.

Preservation of an autologous endothelial monolayer on valve surfaces before loading (**A** and **B**), after loading and deployment (**C** and **D**), and after exposure to flow *in vitro* (**E** and **F**). Confocal microscopy images of EOCs on both sides of the bioprosthetic valves. Immunofluorescent staining of actin filaments (red, rhodamine phalloidin) and nuclei (blue, Hoechst). Scale bars = 100 μm . **A.** Mucosal and **B.** serosal faces before manipulation. EOCs have typical cobblestone appearance. **C.** Mucosal and **D.** serosal faces after loading and deployment demonstrate intact endothelial monolayer. **E.** Mucosal and **F.** serosal faces after *in vitro* flow show preservation of endothelial monolayer.

Fig 4.

Histology images of native sheep jugular valve compared to bioprosthetic valves coated with EOCs. Hematoxylin and eosin (H&E) stain of nuclei (blue), SIS and red blood cells (pink). Scale bars = 50 μ m. Some lifting of endothelial monolayer occurred in processing. **A.** Native sheep jugular valve leaflet. Both valve surfaces are coated by endothelial cells. **B.** Bioprosthetic valve before manipulation. Note elongated nuclei indicated with arrow. **C.** Bioprosthetic valve after *in vitro* flow with endothelial monolayer intact on both sides of the leaflet. **D.** Bioprosthetic valve after *ex vivo* blood flow. Note elongated endothelial cell nuclei on valve surfaces compared to the round nuclei of circulating cells from blood, which adhered primarily to the outside and to a smaller degree to the inside of the SIS leaflet.