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***In vivo* assessment of two endothelialization approaches on bioprosthetic valves for the treatment of chronic deep venous insufficiency**

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Abstract

Chronic deep venous insufficiency is a debilitating disease with limited therapeutic interventions. A bioprosthetic venous valve could not only replace a diseased valve, but has the potential to fully integrate into the patient with a minimally invasive procedure. Previous work with valves constructed from small intestinal submucosa (SIS) showed improvements in patients' symptoms in clinical studies; however, substantial thickening of the implanted valve leaflets also occurred. As endothelial cells are key regulators of vascular homeostasis, their presence on the SIS valves may reduce the observed thickening. This work tested an off-the-shelf approach to capture circulating endothelial cells *in vivo* using biotinylated anti-KDR antibodies in a suspended leaflet ovine model. The antibodies on SIS were oriented to promote cell capture and showed positive binding to endothelial cells *in vitro*; however, no differences were observed in leaflet thickness *in vivo* between antibody-modified and unmodified SIS. In an alternative approach, valves were pre-seeded with autologous endothelial cells and tested *in vivo*. Nearly all the implanted pre-seeded valves were patent and functioning; however, no statistical difference was observed in valve thickness with cell pre-seeding. Additional cell capture schemes or surface modifications should be examined to find an optimal method for encouraging SIS valve endothelialization to improve long-term valve function *in vivo*.

Keywords

Endothelial cells; venous insufficiency; small intestinal submucosa (SIS); venous valve; Vascular Endothelial Growth Factor Receptor-2

Introduction

In a healthy venous system, one-way blood flow from the extremities to the heart is maintained by a series of bi-leaflet venous valves that prevent blood backflow. Chronic deep venous insufficiency (CDVI) is a disease characterized by venous valves in the deep veins that are unable to close and prevent the backflow of blood.^{(1),(2)} Compression therapy, the current standard of care for venous insufficiency, aids in the healing of ulcers; however, approximately 20% of patients have ulcer recurrence despite ongoing compression therapy^{(3),(4)}. Open surgical treatment options that involve transplanting or ligating functional veins to diseased veins have not demonstrated significant improvements in ulcer healing over compression therapy; furthermore, many CDVI patients are not good surgical candidates due to pre-existing conditions including diabetes mellitus^{(4),(5)}. The inability of current therapies to treat the cause of chronic venous insufficiency has motivated the development of novel treatment methods to restore the function of venous valves.

Transcatheter bioprosthetic venous valves were suggested by Dotter in 1981 as a curative and minimally-invasive therapy for deep venous insufficiency⁽⁶⁾. Work by Pavcnik et al. constructed an artificial valve amenable to percutaneous delivery using decellularized, porcine small intestinal submucosa (SIS) sutured onto a metal frame that could be collapsed into a catheter⁽⁷⁾. After initial studies in sheep, the valve was redesigned to resist migration and to form a better seal with the vein wall⁽⁸⁾. In a clinical trial, 15 patients whose symptoms did not improve with prior standard surgical treatment received these advanced SIS valves. At 3 months post-implantation, 12 patients (80%) showed reduction of symptoms, including the healing of ulcers^{(8),(9)}. Despite these improvements, 13 of the 15 implanted valves were unable to prevent blood backflow after 3 months, and no valves were able to close after 12 months. Characterization of SIS valves implanted in sheep identified that SIS valve leaflets thicken with neotissue due to thrombus formation and intimal hyperplasia; this thickening resulted in reduced leaflet mobility and incomplete valve closure^{(8),(9)}. Therefore, strategies to sustain SIS leaflet mobility and prolong artificial valve function should actively limit thrombus formation and intimal hyperplasia.

Endothelialization of vascular devices is thought to be crucial to long-term resistance of thrombosis and intimal thickening^{(10)–(12)}. Rather than mature endothelial cells, which require invasive procedures and donor site morbidity to obtain, endothelial outgrowth cells (EOCs) are a promising source for autologous endothelialization of vascular devices⁽¹³⁾. EOCs are isolated from a whole blood draw, expanded rapidly *in vitro*, and express mature endothelial cell markers^{(14),(15)}. *In vitro* seeding of biomedical devices with EOCs has been investigated with metal and polymer devices, as well as seeding on a select number of biomaterials^{(13),(16)–(21)}. Prior to our studies published in 2012, the seeding of EOCs on SIS biomaterial had not yet been reported⁽²²⁾. This work optimized the endothelialization of SIS valves and confirmed the retention of an endothelial layer through the manipulations necessary for transcatheter implantation and the application of *in vitro* or *ex vivo* flow in the ovine model.

Pre-seeding of vascular devices is one method of establishing device endothelialization; however, *in situ* endothelialization enabled by surface modification of acellular biomaterials

could offer greatly improved clinical translatability ⁽²³⁾. Kinase insert domain receptor (KDR), also known as vascular endothelial growth factor receptor 2, is a surface protein unique to endothelial cells and therefore a suitable target for endothelial cell capture ⁽²⁴⁾. Previous work demonstrated the ability of anti-KDR antibodies to capture human umbilical cord vascular endothelial cells *in vitro* under low flow conditions ⁽²⁵⁾. In this work, cell capture more than doubled when the antibody was oriented using a G-protein strategy. However, the G-protein linker would not be suitable for implantation due to its immunogenicity; thus, alternative antibody orientation strategies must be explored. For this work, we employed a biotin-streptavidin system for antibody binding and orientation. Biotin-streptavidin binding is one of the strongest non-covalent bonds in biological systems ⁽²⁶⁾. This work evaluated the capacity of this binding scheme to orient anti-KDR antibodies for capturing endothelial cells on SIS leaflets.

The goal of this research was to promote early endothelialization of SIS, either by pre-seeding *in vitro* or capture *in vivo*, to ultimately reduce the thickening of implanted artificial venous valve leaflets. We hypothesized that early endothelialization of SIS leaflets by either of these two strategies would limit the formation of intimal hyperplasia on bioprosthetic venous valves.

Materials and methods

EOC capture on antibody-modified SIS

Antibody biotinylation—Biotin was covalently attached to the heavy chain of the anti-human KDR antibody (Clone 89106, R&D Systems) using a two-step procedure: oxidation followed by biotinylation. To oxidize the antibody heavy chain, anti-KDR was diluted in 20mM sodium meta-periodate in 0.1M sodium acetate buffer (pH=5.5) for 30 minutes on ice in the dark to convert the vicinal diol groups on the sugars from hydroxyls to aldehydes. After quenching the reaction with glycerol, the antibody was concentrated and excess reagents were excluded using Amicon Ultra-0.5 spin columns with a 10 kDa molecular weight cutoff (Millipore) according to the manufacturer's protocol. To conjugate biotin to the antibody, the antibody was re-diluted in PBS and 1/10th volume EZ-Link™ hydrazide-biotin solution (50mM in DMSO, Thermo Scientific) was added to covalently conjugate biotin to the antibody via a hydrazone bond. Biotinylated antibody solutions were concentrated and excess reagents removed using the Amicon spin columns, and final concentrations were determined using a FluoroProfile™ kit (Sigma Aldrich) according to the manufacturer's protocol. A mouse IgG1 standard antibody (Dako) was used to generate a standard curve.

The specificity of the biotinylation for the heavy chain of anti-KDR was confirmed via western blot. Biotinylated anti-KDR was denatured by heating for 5 min at 95 °C, run on a Novex 4-12% bistris gel (Invitrogen) using standard western blotting techniques. Denaturation of the antibody was validated by identifying distinct bands for the light and heavy chains with ponceau-S staining. The antibody biotinylation reaction was then confirmed using streptavidin-HRP and measurement of the chemiluminescent SuperSignal West Pico Substrate (Pierce). Unmodified antibody was used as a negative control.

Immunofluorescent staining was used to confirm that the biotinylated antibody retained reactivity to EOCs using methods described previously ⁽²²⁾. EOCs were grown to confluence on 8-well chamber slides and fixed with 3.7% paraformaldehyde. EOCs were incubated with either biotinylated anti-KDR or unmodified anti-KDR, followed by Alexa Fluor 568-conjugated streptavidin (Invitrogen) and counterstained with DAPI.

Antibody orientation on SIS—Vacuum pressed SIS sheets were provided by Cook Biotech, Inc. SIS was biotinylated by treating with a 0.5 mg/mL solution of EZ-Link™ Sulfo NHS-LC-biotin (Thermo Scientific) in PBS for 24 hrs at room temperature. Biotinylated SIS was rinsed with water and incubated for 2 hr at room temperature with 0.1 mg/mL streptavidin solution in PBS (Thermo Scientific). SIS samples were then rinsed with PBS-Tween (0.05% Tween 20 in PBS) and incubated with biotinylated anti-KDR solutions overnight at room temperature. Samples were thoroughly rinsed with PBS-Tween prior to experiments.

To quantify the binding of the antibody to the biotinylated-SIS surface, anti-KDR was radiolabeled with the isotope ¹²⁵I using Pierce Iodination Beads (Thermo Fisher). Iodination beads (2 beads per 5-500ug protein) were rinsed with PBS and combined with Na¹²⁵I. Antibody was added to the reaction for 15 min at room temperature with agitation. Radiolabeling efficiency was determined with a trichloroacetic acid precipitation. A constant amount of ¹²⁵I labeled antibody was combined with a varied amount of non-radiolabeled antibody to yield the desired concentrations of antibody solution. These solutions were incubated for 2 hrs at room temperature on biotin and streptavidin-bound SIS surfaces. Radioactivity of the samples was quantified using a WIZARD automated gamma camera (PerkinElmer).

To confirm the activity of anti-KDR following attachment to SIS, the amount of ¹²⁵I-labeled recombinant human KDR (rhKDR) bound to the antibody-modified SIS was quantified. Anti-KDR coated SIS samples were made as previously described using concentrations of 10, 20, 40, and 80 µg/mL biotinylated antibody. The rhKDR (R&D Systems) was labeled with ¹²⁵I using Pierce Iodination Beads using the same technique as KDR antibody. The rhKDR solution (0.1 µg/mL) was added to the antibody-modified SIS samples (n=5) and incubated overnight at room temperature. Samples were rinsed with PBS-Tween until rinse solutions were at background levels of radioactivity. The radioactivity of the rhKDR was measured on a WIZARD automated gamma camera.

Suspended SIS leaflet devices—To explicitly test circulating cell capture *in vivo* and eliminate any potential cell migration from the vessel wall, suspended, non-functioning leaflet devices previously designed by our group ⁽²⁷⁾ were deployed via catheter in sheep. All suspended leaflet devices used in this experiment were constructed to fit within the ovine inferior vena cava (IVC), which ranged from 18 to 26 mm in diameter. The device consisted of 2 parts (Figure 1a-c): (1) a rehydrated SIS leaflet was sewn onto a stainless steel frame (14-mm edge lengths) with 7-0 Prolene monofilament sutures (Ethicon; Johnson & Johnson); these SIS leaflets were suspended from (2) a flexible, 25 mm or 32 mm diameter stent frame, with 5-0 Prolene sutures (Ethicon). The mid-line of the SIS leaflet was cut open to create a valve-like opening. As the biologically-derived SIS material has an intrinsic

sidedness with different properties for its serosal and mucosal sides^{(28),(29)}, the SIS was mounted on the frame such that the serosal side of the SIS was always the distal face of the valve for consistency. The suspended SIS leaflets were air dried, coated with anti-KDR capture antibodies (80 µg/mL, measured post-biotinylation with the FluoroProfile™ kit), manually folded along the horizontal axis of the larger frame, and sterilized with ethylene oxide prior to implantation. Control implants followed an identical set of steps excluding the antibody coating.

Devices were constructed to enable implantation of two leaflet devices proximal to the renal veins and two distal. The diameter of the IVC proximal to the renal veins is generally larger than distal to the renal veins; therefore, the outer frames of the leaflet devices were 32 mm and 28 mm for the proximal and distal devices, respectively, to prevent device migration to the heart. The inner leaflets were identical in dimension above and below the renal veins.

Animal Studies—All animal experiments were performed in female domestic sheep (*Ovis aries*; Western crossbreed) and were approved by the OHSU Institutional Animal Care and Use Committee according to the “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care & Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (International Standard Book, Number 0-309-05377-3, 1996). Prior to surgery, each sheep was given preoperative antibiotics, Ancef (cefazolin) 1 gm/day intramuscular. Animals were transported to the Dotter lab in appropriate cages on the morning of the surgery and anesthetized with intravenous midazolam (0.025 mg/kg) + ketamine (0.9 mg/kg). The animals were then intubated and placed on a mechanical ventilator with isoflurane anesthetic (2.0% - 2.5%) in oxygen while being monitored for respiration, corneal and papillary reflex, and muscle tone and placed supine on an angiographic table. The needle puncture area was scrubbed with betadine solution and draped in the sterile fashion. During the surgical procedure, each animal's vital signs (heart rate, respiratory rate, and arterial blood pressure) were continuously monitored. Veins were punctured with a 21 gauge needle and appropriately size catheters were placed. An initial heparin loading dose of 3000 - 5000 units was delivered and additional heparin administered as needed to maintain an activated clotting time > 300 sec. Digital imaging was performed with a mobile cardiac fluoroscopic unit (OEC9800, GE/OEC) at 15 frames per sec and Omnipaque contrast dye (Amersham Health). Vein internal diameter measurements were calibrated with a graduated guidewire (Cook Medical). Proper placement of the implanted devices was confirmed with venograms of the jugular vein or IVC as appropriate.

IVC implant procedure—Four suspended SIS leaflet devices were implanted into each of three domestic sheep (51-65 kg). Each animal had 2 control SIS devices and 2 SIS devices coated with oriented anti-KDR. In each animal, one of the leaflet devices proximal to the renal veins was anti-KDR coated and the other an unmodified control. The order of devices in the IVC was evenly distributed between treatment groups to mitigate possible differences in cell capture due to placement location within the IVC. The IVC was accessed via a femoral puncture and angiography was used to measure the vein width and determine implant location.⁽³⁰⁾ The suspended leaflet device was front-loaded inside a 5 cm-long

chamber of a 12 Fr delivery catheter. After deployment, the suspended SIS leaflet device self-expanded across the width of the vein. Angiography was used to confirm placement at implant (Figure 1d). The three sheep were sacrificed at 2 weeks to determine the effects of antibody modification on the early endothelial cell capture and remodeling of the suspended SIS devices. At explant, angiography was used to identify any positional shifting and confirm proper flow through the vein. After exsanguination, the IVC was flushed with 1-2 L heparinized saline. The IVC was accessed and the suspended leaflet devices were removed and immediately placed in 3.7% paraformaldehyde.

Histology—After 24 hrs fixation in 3.7% paraformaldehyde, the two leaflets of each explanted device were separated, one half placed in a new solution of 3.7% paraformaldehyde and the other half placed in 10% formalin for histology. The leaflet stored in formalin was cut in half perpendicular to the wire frame and mounted in paraffin with the cut face of each half of the leaflet parallel to the histology cut surface. Sections were stained according to standard procedures with hemotoxylin and eosin and Masson's trichrome. The leaflets that remained stored in paraformaldehyde were measured using digital calipers (Fisher Scientific) for the thickness at both the original cut surface of the valve and at the opposite end. These thickness measurements were averaged for each leaflet. A one-way analysis of variance (ANOVA) with Tukey's post hoc was used to determine differences between treatment groups, with $p < 0.05$ considered significant.

EOC pre-seeded SIS valves

EOC isolation and characterization—Ovine EOCs were collected, isolated, and purified as described previously.⁽²²⁾ Briefly, outgrowth colonies were isolated from a culture of the mononuclear cell layer of a whole blood draw. EOCs were expanded in endothelial growth media (EGM-2, Lonza) with a final fetal bovine serum (FBS, Hyclone, Thermo Scientific) concentration of 10%. Cells were used at passage 5 for all studies.

Flow cytometry was used to confirm the presence of typical endothelial cell surface markers. After dissociation from culture flasks with TrypLE (Life Technologies), EOCs were resuspended at a concentration of 1×10^6 cells/mL and blocked using 5% mouse serum for 20 min on ice. A master mixture of the antibodies PE/Cy7-anti-CD146 (MCAM, BD Pharmingen), AlexaFluor647-anti-KDR (VEGFR2, BD Pharmingen), and FITC-anti-CD31 (PECAM, Biolegend) was prepared with each antibody being used at the concentration recommended by the manufacturer. Samples were incubated in the multicolor antibody master mixture for 30 min on ice in the dark, washed twice in PBS with 1% FBS and transferred into a 96 well plate. Fluorescent intensity measurements were acquired using a MACSQuant analyzer (Miltenyi Biotec). Samples stained with a single antibody were used to perform automated compensation in FlowJo v.X. Fluorescent minus one (FMO) controls were used in channels where emission overlap caused the background fluorescence to be evidently higher than unstained samples after compensation.

SIS device construction—SIS bioprosthetic valves were constructed with custom dimensions to match each sheep's unique anatomy. Angiography was used to identify the maximum diameter of the left and right jugular vein in each of six sheep (9.8-12.3 mm).

Valves were constructed by suturing SIS to a custom made stainless steel frame with 7-0 Prolene monofilament sutures. The frame was constrained prior to suturing on the SIS with a Prolene loop to customize the dimensions of expansion upon delivery. After the constraining Prolene loop was removed, a slit was cut across the SIS mid-line to create the valve opening.

SIS seeding—SIS valves were pre-seeded with autologous EOCs as previously described with minor modifications⁽²²⁾. Briefly, a 10 cm petri dish was agarose coated, and a sterile customized metal frame was used to raise the SIS off of the bottom surface and to allow for easy device turning (Figure 1e). Devices were soaked overnight in cell culture media, and the serosal side was seeded first with 10^6 cells/valve (100 μ L/leaflet, 0.5×10^6 cells/leaflet). After a 1hr incubation at 37 °C, the device was flipped and 10^6 cells/valve were seeded on the mucosal side. Additional media was added after 1hr. Devices were implanted 48hrs after seeding the serosal side.

Jugular vein implant procedure—Six sheep were each implanted with two valves – one into each jugular vein. One valve in each animal was EOC-seeded and the other was an unmodified control. Valves were delivered via catheter as described and tested previously *ex vivo* with slight modification⁽²²⁾. To collapse and load the valve into a 12 Fr catheter, a 7-0 Prolene loop was created by feeding the suture through opposing corners of the frame distal to the valve orifice (Figure 1f). Drawing the loop into the catheter resulted in gentle valve collapse and proper loading for delivery (Figure 1g). Valve placement was confirmed with angiography at the time of implantation (Figure 1h). After 3 months, valves were evaluated for function (i.e., patency and competency) via angiography, as well as thickness and cellular remodeling using histology. At explantation, contrast was injected distal to the valve to confirm the valve remained patent. Injecting contrast proximal to the valve allowed for the assessment of competency, defined as the ability of the valve to block retrograde flow. After sacrifice and exsanguination, the vein was cut above and below the valve, rinsed with PBS, and placed immediately into 10% formalin. The distal end of the valve was marked with a suture to preserve orientation for embedding.

Histology—Samples were fixed in 10% neutral buffered formalin for 48 hrs and then transferred to 70% ethanol until all samples were collected. Explants were embedded whole in methacrylate and then sectioned along the axial axis of each leaflet with a diamond blade (Alizee, Inc.). Sections were stained according to standard procedures with hematoxylin and eosin, as well as von Willebrand factor (vWF) and smooth muscle actin (SMA) via immunohistochemistry.

Results

Sheep EOC characterization

Multicolor flow cytometry was used to characterize sheep EOC surface marker expression (Figure 2). The sheep EOCs expressed the typical endothelial markers CD31, CD146 (MCAM), and KDR. There was a strong positive staining for CD146 and a weak positive staining for CD31 and KDR, which was likely due to limited cross-reactivity of the anti-human antibodies against sheep cells.

Antibody orientation

The anti-KDR antibody was successfully biotinylated selectively on the heavy chain and remained reactive after modification. The western blot analysis determined that 1) streptavidin was non-reactive towards the unmodified antibody, and 2) streptavidin predominantly bound the biotinylated antibody on the heavy chain (Figure 3b). The biotinylated anti-KDR also remained reactive to the ovine EOCs (Figure 3c). The attachment of biotinylated anti-KDR to SIS was quantified using radiolabelled antibody. The amount of biotinylated antibody bound to the SIS increased linearly with increasing concentrations of biotinylated antibody (Figure 4a). Using radiolabeled rhKDR also showed that rhKDR binding to antibody-modified SIS increased with increasing concentrations of biotinylated antibody with a plateau occurring at an anti-KDR concentration of 40µg/mL (Figure 4b).

In vivo EOC capture using anti-KDR antibody

All suspended SIS leaflet devices remained in place in the IVC as shown in Figure 1d for the duration of the study. One minor migration occurred during implantation, which caused two suspended leaflets to fuse together over the two-week implantation. One unmodified leaflet and one antibody-coated leaflet each developed a blood clot that blocked flow thorough the leaflets. There was no statistical difference in the thickness of SIS leaflets modified with anti-KDR compared to unmodified SIS leaflets (Figure 5). The remodeling response varied greatly between animals with some leaflets demonstrating extensive thickening, while others maintained thin leaflet dimensions.

In vivo assessment of EOC-pre-seeded SIS valves

At sacrifice after 3 months, angiography indicated that all valves remained in their original position in the jugular vein as seen in Figure 1h with no migration. Angiography also revealed that all valves remained patent (Figure 6a), which was confirmed by observing the explanted valves (Figure 6d). Valve competency could not be measured in some cases where the implanted valve obstructed the side branch of the jugular vein immediately proximal to the valve that was required to inject contrast. Of valves that allowed for proximal contrast injection, all valves appeared competent (Figure 6b and c), though the injection of contrast dye may have influenced valve closure.

Thickness measurements of explanted valve leaflets revealed no difference between valves pre-seeded with EOCs and unmodified SIS control valves (Figure 7a). The remodeling response was highly animal-dependent with thicker pre-seeded valves seen in animals with thicker control valves (Figure 7b). The prevalence of smooth muscle cell infiltration into EOC-pre-seeded SIS seen with histology was very broad, ranging from a uniform thin layer just under the endothelial layer (Figure 8a) to permeating through the entire thickness of the neotissue (Figure 8b). Staining the histology sections for vWF, the presence of an endothelial layer was confirmed on all implants regardless of pre-seeding. These results indicate that tissue remodeling was prevalent in all groups, but surprisingly, the original SIS material was not degraded during the 3 month implantation in the majority (9/12) of valves.

Discussion

Venous insufficiency currently affects over 2.5 million Americans, and approximately 20% of patients develop skin ulceration ^{(1),(2),(31)}. Venous ulcers resulting from CDVI cause an estimated loss of 2 million workdays per year in the US ^{(1),(2)}. The development of an artificial venous valve amenable to transcatheter delivery would provide a novel treatment for chronic deep venous insufficiency capable of treating underlying venous valve incompetence in patients that are poor candidates for open surgery. This work builds upon prior studies using a bioprosthetic venous valve with leaflets made from SIS, and attempted to reduce leaflet remodeling and hyperplasia that hindered the long-term function of implanted valves. Venous valve replacement using allografts or autografts in animals, where the valve endothelium remained intact, did not demonstrate the intimal hyperplasia observed with the SIS venous valve ^{(32),(33)}. As this type of valve replacement is a similar therapeutic strategy to SIS valve insertion, this result suggests the endothelial cell lining is a key facilitator for maintaining valve function. Furthermore, in studies using SIS for pulmonary valve reconstruction in which extensive *in situ* leaflet endothelialization existed, there was constructive remodeling with limited intimal hyperplasia and good long-term function ^{(34),(35)}. Thus, we hypothesized that early endothelialization of SIS leaflets, either by capturing circulating cells or pre-seeding SIS with EOCs, would limit the development of intimal hyperplasia on bioprosthetic venous valves while also providing anti-thrombogenic activity. Previous studies have demonstrated that EOCs can regulate platelet accumulation and coagulation factor activation similar to mature endothelial cells ⁽³⁶⁾, and EOCs have been seeded onto vascular grafts to improve hemocompatibility ^{(13),(20),(21),(37)–(41)}. However, the utility of covering SIS with EOCs to reduce hyperplasia on SIS bioprosthetic venous valves had not been investigated.

The first part of this work sought to attach a capture antibody targeting the endothelial surface protein KDR onto the biomaterial SIS to capture EOCs from blood and accelerate endothelialization of the SIS leaflets. Attaching capture antibodies to surfaces by methods that orient the antigen binding domains outward from the surface have been shown to significantly increase the efficacy of the bound antibodies ^{(25),(42),(43)}. To apply this strategy to SIS, we successfully biotinylated anti-KDR specifically on the heavy chain, enabling the oriented conjugation of the antibody to streptavidin-coated SIS surfaces. In our *in vitro* studies of adhering biotinylated anti-KDR to SIS, we were surprised to find that the quantity of bound antibody did not reach a plateau. We attribute this to the large effective surface area of SIS, which is increased by its fibrous texture and porosity. Therefore, the highest concentration of antibody tested (approximately 80 µg/mL) was used for all future studies.

With both of the *in vivo* studies, there was significant animal-to-animal variability with regard to the remodeling and thickening of implanted SIS leaflets. As such, no clear differences were seen between treatment groups and the unmodified SIS controls. In cases such as stent deployment or vascular grafting, intimal thickening is thought to result from endothelium disruption causing insufficient inhibition of SMC proliferation ^{(10)–(12)}. In our antibody-mediated capture studies, we hypothesize that a lack of inhibitory signaling from the captured cells similarly permitted intimal thickening. The animal-specific nature of intimal thickening may therefore indicate inter-animal variability in a number of factors

including circulating quantities of EOCs, resulting in differential efficacy of cell capture, or differential production of inhibitors of SMC proliferation such as NO. Previous studies have indicated that certain disease states can result in measureable differences in circulating EOCs between human subjects ^{(44)–(46)} as well as altered eNOS activity ⁽⁴⁷⁾, indicating that variability in these attributes may exist, though the underlying mechanism is unclear.

In the study using anti-KDR to facilitate EOC capture and accelerate endothelialization, we hypothesize that the trend toward thicker suspended leaflets with anti-KDR-coated SIS was due to the antibody binding strategy. It is possible that the inclusion of streptavidin in our conjugation chemistry led to an immune response that resulted in increased cellular adhesion and remodeling of the SIS. Additionally, variable immune responses between animals may have contributed to the animal-to-animal variability seen in these experiments. This result motivates a need for alternate site-specific binding strategies with reduced immunogenicity, such as copper-free click chemistry ⁽⁴⁸⁾. Interestingly, other groups using antibody-coated stents to accelerate EOC capture have shown results similar to ours, where *in vitro* data demonstrates increased EOC capture on antibody-modified surfaces ^{(49),(50)}, but clinical trials evaluating stent restenosis did not demonstrate significant improvements in clinical outcomes ^{(51)–(53)}. Factors including sub-optimal antibody selection and the inhibitory effect of thrombus formation on cell capture may have reduced the efficacy of antibody-mediated endothelialization in these cases. Therefore, using antibody-coated surfaces to accelerate endothelialization may have limited clinical utility, and alternate mechanisms of enhancing endothelialization, including EOC-specific capture aptamers or EOC-promoting growth factors, should continue to be explored ⁽²³⁾.

As an alternate approach to antibody-mediated EOC capture, we investigated if pre-seeding SIS valves with EOCs would reduce valve remodeling and hyperplasia. However, our data suggest that pre-seeding the valves with EOCs did not appreciably reduce the thickening of implanted valve leaflets. Numerous groups have previously demonstrated that EOCs have a lower gene expression of endothelial nitric oxide synthase (eNOS) compared to aortic ECs ^{(54)–(56)} or umbilical vein ECs ⁽⁵⁷⁾. Therefore, it is possible that endothelialization with EOCs might have provided insufficient expression of eNOS to suppress SMC proliferation and minimize the formation of intimal hyperplasia. Pre-seeding the valve leaflets with alternatively-sourced endothelial cells that exhibit higher eNOS expression may result in sufficient NO to reduce SMC proliferation. Additionally, pre-conditioning EOCs with fluid shear stress has been shown to cause cellular alignment and increase NO production and therefore is another promising method to enhance NO release from EOC-seeded SIS valves ^{(56),(58),(59)}. Genetic engineering of EOCs to overexpress either eNOS⁽⁶⁰⁾ or thrombomodulin⁽⁶¹⁾ may also increase EOC-mediated inhibition of intimal hyperplasia. Alternatively, different EOC seeding conditions may need to be established. In this study both pre-seeded valves and unmodified SIS controls were soaked in endothelial growth media including 10% fetal bovine serum. Despite thorough washing with saline prior to implantation, it is possible that one of the growth factors contained within the media or serum could have been retained in the SIS and contributed to the lack of difference between pre-seeded valves and bare SIS controls.

An additional difference between this work and previous studies using SIS in a bioprosthetic venous valve was the use of vacuum-pressed SIS. Overall, this version of SIS appeared to have a more uniform texture and reduced starting thickness. We attribute these physical characteristics to alterations in the SIS manufacturing process, most notably the inclusion of a vacuum press process as opposed to lyophilization. Interestingly, this vacuum-pressed SIS seemed to have slower degradation, as evidenced by the uncharacteristic presence of non-degraded SIS in the histology of valves explanted after 3 months. To assess the kinetics of endothelialization and remodeling of vacuum-pressed SIS, future studies with multiple time points are needed. Although constructive remodeling of SIS has been demonstrated when SIS is utilized in pulmonary valve reconstruction ^{(34),(35),(62)}, differences in blood flow dynamics and adjacent cellular populations between the pulmonary valve and the jugular vein may have contributed to the detrimental thickening seen in our studies.

In conclusion, this work utilized two different strategies to enhance early endothelialization of bioartificial SIS venous valves. The first strategy utilized an oriented capture antibody targeting KDR to accelerate endothelialization *in situ*, while the second strategy utilized pre-endothelialized SIS valve leaflets. In both strategies, the remodeling response and thickening of the implanted SIS devices varied greatly between animal subjects. Furthermore, no significant differences were seen between unmodified control SIS devices and those that had been coated with either anti-KDR or EOCs to promote endothelialization. The lack of improvement from these strategies may be due to immune responses to the anti-KDR attachment strategy that may have limited the efficacy of the capture antibody, and the pre-seeded EOCs may not have produced sufficient nitric oxide to limit intimal ingrowth. Future studies will look at alternate strategies to orient capture antibodies to SIS, as well as methods to increase nitric oxide production by EOCs, possibly by flow pre-conditioning

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Figure 1: SIS Device Construction and Deployment.

(a-d) Suspended SIS leaflet devices were modified with anti-KDR and implanted in the inferior vena cava of a sheep to capture circulating EOCs *in vivo*. (a and b) The non-functioning valve-like devices were suspended from a steel frame designed to suspend the valve in the sheep inferior vena cava to test cell capture in the absence of cell migration onto the devices. (d) Angiography was used to confirm the placement of the suspended devices. (e-h) SIS venous valves were pre-seeded with EOCs and implanted in sheep jugular veins. (e) SIS valves were mounted onto a circular frame for uniform seeding of EOCs. (f and g) The valves could then be pulled into a catheter using a suture loop. (h) Valves were placed in the jugular veins of sheep and the position was confirmed using angiography.

Figure 2: Flow cytometry characterization of sheep EOCs.

Compared to controls (filled histograms), multicolor stained sheep EOCs (unfilled) demonstrated strong positive staining for CD146, and weak positive staining for CD31 and KDR.

Figure 3: Biotinylation of anti-KDR antibody.

(a) Ponceau stain indicates the total protein of the heavy and light chains for both the unmodified anti-KDR antibody (column 1) and the biotinylated anti-KDR antibody (column 2). (b) Streptavidin HRP preferentially bound to the heavy chain of the biotinylated anti-KDR antibody. Activity of the biotinylated antibodies was confirmed with staining of EOCs. (c) The biotinylated anti-KDR antibody adhered to fixed EOCs as detected by AlexaFluor 568-conjugated streptavidin (top) with DAPI indicating total cells (bottom). Inset contains negative control of unmodified anti-KDR stained EOCs. Scale bars = 100 μ m.

Figure 4: Confirmation of SIS modification.

(a) SIS punches modified with biotin and streptavidin were exposed to I^{125} radiolabeled biotinylated KDR. Radioactivity of the punches was measured to give a quantification of bound biotinylated anti-KDR. (b) Modified SIS punches were incubated with increasing concentrations of biotinylated anti-KDR and then exposed to I^{125} radiolabeled recombinant human KDR. Radioactivity of the punches was measured to give a quantification of bound KDR protein. All data is presented as the mean $\pm$ standard deviation, $n=4$.

Figure 5: Thickness of suspended SIS devices post-implantation.

(a) Suspended devices that were constructed with leaflets of either unmodified SIS or SIS modified with anti-KDR were implanted into sheep inferior vena cava for 2 weeks, and the leaflet thickness was measured at explantation. Data is presented as the mean + standard deviation, $n=3$. Panels b and c show a representative device constructed with unmodified SIS, while d and e show an anti-KDR modified SIS device. (c and e) Mason's trichrome staining was used to highlight the collagenous SIS material (blue) surrounded by a cellular remodeling response. Scale bar = 100 μ m.

Figure 6: Representative angiography of EOC-seeded SIS devices in sheep jugular veins and of a valve post-explantation.

(a) Contrast dye injected distally to the valve demonstrated that both valves were patent. (b and c) Contrast dye injected proximally to the valves did not regurgitate through the valve, suggesting competent valves were able to prevent retrograde blood flow. (d) Representative photograph of an SIS valve explanted after 3 months.

Figure 7: Thickness of explanted SIS valves post-implantation.

(a) Bioartificial SIS venous valves that were either unmodified or pre-seeded with EOCs were implanted in sheep jugular veins for 3 months, and the thickness of the valve leaflets were measured at explantation. Data is presented as the mean + standard deviation, $n=3$. (b) A plot comparing the thickness of unmodified SIS valves compared with the contralaterally implanted EOC-seeded valves indicates that the degree of thickening varied greatly between animals but was similar between treatment groups.

Figure 8: Immunohistochemical staining of SIS valves.

(a and b) EOC-seeded valves were stained for smooth muscle actin; this stain showed that some valves had relatively little smooth muscle cell infiltration (a), while others had robust smooth muscle cell infiltration and proliferation on the valve leaflet (b). (c and d) Von Willebrand factor staining of unmodified SIS valves (c) and EOC-seeded valves (d) demonstrated that all valves regardless of treatment developed an endothelial cell lining on the luminal surface of the leaflet by the end of the 3 month implant period. Scale bars = 500 μm (a and b) or 50 μm (c and d).