Published in final edited form as:

Ann Surg. 2008 September ; 248(3): 370–377. doi:10.1097/SLA.0b013e318184dcbd.

Tissue engineered vascular grafts demonstrate evidence of growth and development when implanted in a juvenile animal model

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Abstract

INTRODUCTION—The development of a living, autologous vascular graft with the ability to grow holds great promise for advancing the field of pediatric cardiothoracic surgery.

OBJECTIVE—To evaluate the growth potential of a tissue engineered vascular graft (TEVG) in a juvenile animal model.

METHODS—Polyglycolic acid non-woven mesh tubes (3cm length, 1.3 cm id; Concordia Fibers) coated with a 10% copolymer solution of 50:50 L-lactide and ϵ -caprolactone were statically seeded with 1×10^6 cells/cm² autologous bone marrow derived mononuclear cells (BM-MNCs). Eight TEVGs (seven seeded, one unseeded control) were implanted as inferior vena cava (IVC) interposition grafts in juvenile lambs. Subjects underwent bimonthly magnetic resonance angiography (Siemens 1.5T) with vascular image analysis (<http://www.BioimageSuite.org>). One of seven seeded grafts was explanted after one month and all others were explanted six months after implantation. Neotissue was characterized using qualitative histological and immunohistochemical staining and quantitative biochemical analysis.

RESULTS—All grafts explanted at six months were patent and increased in volume as measured by difference in pixel summation in MRA at one month and six months. The volume of seeded TEVGs at explant averaged $126.9 \pm 9.9\%$ of their volume at one month. MRI demonstrated no evidence of aneurysmal dilation. TEVG resembled the native IVC histologically and had comparable collagen ($157.9 \pm 26.4 \mu\text{g}/\text{mg}$), elastin ($186.9 \pm 16.7 \mu\text{g}/\text{mg}$), and glycosaminoglycan ($9.7 \pm 0.8 \mu\text{g}/\text{mg}$) contents. Immunohistochemical staining and western blot analysis showed that Ephrin-B4, a determinant of normal venous development, was acquired in the seeded grafts six months after implantation.

CONCLUSIONS—Tissue engineered vascular grafts demonstrate evidence of growth and venous development when implanted in the IVC of a juvenile lamb model.

Introduction

The development of a living, autologous vascular graft with growth potential holds great promise for advancing the field of congenital heart surgery. Currently available synthetic vascular grafts, such as polytetrafluoroethylene (PTFE), lack growth potential and present problems related to biocompatibility including thrombosis, ectopic calcification, and increased susceptibility to infection.¹ As a result, they are a cause of significant morbidity and mortality in modern pediatric cardiothoracic operations.² The lack of growth potential of currently used vascular conduits has resulted in the development of two surgical strategies: (1) delaying surgery until a patient has grown to a suitable size to allow for implantation of an adult-sized graft or (2) implantation of an oversized graft. Both strategies have deleterious effects on the patient. Delaying surgery results in prolonged exposure to hypoxia and volume overload. Chronic hypoxia can lead to developmental delay and failure to thrive, while volume overload can cause cardiac failure.³ Use of oversized vascular grafts results in turbulent blood flow and increases the risk of thromboembolic complications, a leading cause of graft failure and post operative morbidity and mortality.⁴

In an initial clinical pilot study, the feasibility of using tissue engineered vascular grafts (TEVG) in the surgical repair of congenital cardiac anomalies has been clearly established.⁵ This study demonstrated an excellent safety profile associated with the use of a TEVG constructed from biodegradable tubular scaffolds statically seeded with autologous bone marrow derived mononuclear cells (BM-MNC) as large caliber venous conduits in a low-pressure, high-flow, circulatory system. Furthermore, this study demonstrated an increase in size of the TEVG when implanted in juvenile recipients raising the question of whether this increase in size was the result of growth or aneurysmal dilation.

In this investigation we evaluated the development of these TEVG using a juvenile lamb model. Specific focus was placed on elucidating the mechanisms by which these grafts increase in size over time. Graft size and morphology were serially evaluated using magnetic resonance imaging (MRI) over a 6-month time course and an extensive histological, biochemical, and molecular analysis of the grafts was performed in order to characterize the neotissue from the perspective of vascular growth and development.

Despite the clear functional efficacy of TEVG, the mechanisms underlying vascular neotissue formation remain poorly understood, in part because of an incomplete characterization of the fully formed tissue engineered neovessel. A better understanding of these phenomena will be critical to the continued development of this promising technology and the development the first man-made vascular graft with growth potential.

Materials and Methods

Scaffold

Non-woven polyglycolic acid (PGA) mesh tubes (1.3cm internal diameter, 1.5mm thick) [Concordia Fibers (Coventry, RI)] were cut to a length of 3cm and coated with 10% (w/v) copolymer solution of 50:50 L-lactide and ϵ -caprolactone (P(CL/LA)) in 1,4-dioxane. The conduits were then freeze dried under vacuum pressure. Porosity was determined by imaging with scanning electron microscopy [FEI corp, Model XL-30]. Tensile strength was measured using tensiometry [Instron Model 5543].

Bone marrow derived mononuclear cells

BM-MNC were isolated as previously described.⁶ Briefly, 50 ml of bone marrow was aspirated from the sternum of juvenile Dover lambs into a heparized syringe (100U/ml), diluted 1:4 in phosphate buffered saline (PBS) and passed through a 100 μ m filter to remove any fat or bone fractions. BM-MNC were obtained by centrifuging the sample on a histopaque density gradient (Sigma) for 30 minutes at 2700 rpm. Cells yield and viability were determined by staining the cells with trypan blue and quantifying with a hemocytometer.

Seeding Technique

PGA/P(CL/LA) scaffolds were sterilized in a 10% pencillin/streptomycin solution for 24 hours under direct ultraviolet light. Scaffolds were then washed in sterile PBS and statically seeded with BM-MNCs (approximately 1×10^6 cells/cm²). Grafts were incubated [37°C, 5% CO₂, 95% relative humidity, 760 Torr] for a minimum of two hours in autologous serum.

Juvenile lamb Intrathoracic IVC replacement model

The animal care and use committee at the Yale School of Medicine approved the use of animals for this experiment. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health. Eight TEVGs were implanted as inferior vena cava (IVC) interposition grafts.⁸ Juvenile lambs were anesthetized with 2%–4% isoflurane. The right chest wall was opened along the seventh intercostal space and the IVC was exposed and heparin (100U/kg) was administered intravenously. The TEVG was sewn in as a vascular interposition graft using standard continuous technique. Hemostasis was obtained, and the incision was closed.

Magnetic Resonance Imaging Methods

Postoperatively, each animal was imaged bimonthly for six months. MRI was performed using a Siemens Sonate 1.5 T MR Scanner. 1.5T. MRA was performed using a 3D MPRAGE acquisition with 25cm FOV, 1.1mm slice thickness, 128 slices, TE=3ms, TR=24ms, 2 averages, alpha=45, 192 $\times$ 256 matrix and bandwidth = 220Hz/pixel. The data were analyzed with BioImage Suite software (www.bioimagesuite.org). All images were reoriented so the IVC was parallel to the y-axis of the images; the images were resampled to have dimensions 101 $\times$ 256 $\times$ 101 and isotropic voxel size equal to 0.78125 mm.

Anastamotic sites were identified and confirmed with gross images. We used a non-rigid registration technique to compute regional voxel expansion between image pairs (one and six months post TEVG implantation). These expansion measures were integrated over the area of the graft to yield the average volumetric graft expansion. The change in size between the one month TEVG volume and six month TEVG were expressed as the ratio of the six month TEVG volume over the one month TEVG volume. The same method was used to measure the change in volume of the right pulmonary artery, which served as a control.

Histology and immunohistochemistry

Cross-sectional samples of the TEVG were fixed in 10% formalin and embedded in paraffin. Five micron sections were stained using standard techniques for hematoxylin and eosin, von Kossa (calcium), Masson's Trichrome (collagen), Alcian Blue and Movat's Stains (glycosaminoglycans), and Verhoeff-van Gieson (elastin).

Immunohistochemical staining was performed using standard streptavidin-biotin detection. Primary antibodies were used to identify endothelial cells (rabbit-anti-human von Willebrand Factor (vWF, Dako), and smooth muscle cells (mouse-anti-human smooth muscle actin (SMA, Dako). Both antibodies cross-react with ovine tissue. Positive expression was detected using 3,3-diaminobenzidine (DAB) (Vector) and nuclei were counterstained with hematoxylin. Vessel morphometry including determination of graft wall thickness were determined.

Eph-B4 Analysis

Immunofluorescence—Samples were fixed with 4% paraformaldehyde, embedded in OCT, and six millimeter sections were cut. Primary antibodies included Eph-B4 (Santa Cruz), and vWF (Dako). Alexa Fluor 488 and 568 were used for fluorescence detection. All samples were counterstained with DAPI. Images were captured with an Axioimager A1 (Carl Zeiss) under identical conditions.

Western blotting—Native IVC and TEVG were pulverized on dry ice, immersed in lysis buffer, and centrifuged for 60 minutes at 4°C and then at 12,000 g for 10 minutes at 4°C. Samples analyzed were typically 40 µg.

Biochemical Analysis

TEVG sections were frozen and lyophilized for quantitative biochemical analysis.

Collagen—Collagen content was determined by measuring hydroxyproline content as previously described.⁹ Briefly, native IVC and TEVG were digested at 120°C in NaOH (1.33 N). Each sample was then incubated in chloramine T dissolved in n-propanol and acetate citrate buffer (25 minutes, room temperature), followed by incubation in Ehrlich's reagent (65°C, 20 minutes). Total collagen content was determined by multiplying the hydroxyproline content by a factor of 7.5.

Elastin—Elastin content was determined using a Fastin® colometric assay (Biocolor Assays, Inc.). Briefly, four to six milligrams of sample tissue was digested three times in 0.25 M oxalic acid (> 90°C, 60 minutes). Each digestion included an elastin-precipitating agent; the precipitate was reacted with an elastin-binding dye that was detected with spectrometry (513 nm) and compared to a standard curve.

Glycosaminoglycans—Glycosaminoglycan content was determined in triplicate using a Blyscan® colorimetric assay (Biocolor Assays, Inc.). Four to six milligrams of tissue were digested in papain. Precipitates were missed with a glycosaminoglycan-binding dye that was detected by spectrometry (656 nm) and compared to a standard curve.

Results

Scaffold

The scaffold was approximately 80% porous with pore sizes ranging from approximately 5–200 µm.

The ultimate tensile strength of the unseeded scaffold measured 9.16 ± 0.62 MPa with an elastic modulus of 0.35 ± 0.01 MPa.

Cells

The BM-MNC isolation method described above resulted in the isolation of between $1-10 \times 10^7$ cells with cell viability in excess of 90% as determined using hemocytometry and trypan blue exclusion. Cells were seeded on the scaffold and implanted as an interposition graft in the suprahepatic IVC in juvenile lambs. All lambs fully recovered from the procedure and grew and developed normally.

MRI

Our analysis using non-rigid registration of the three-dimensional serial images revealed overall graft enlargement over six months. All grafts increased in size as determined by volume measurement. Mean corrected graft growth in volume was measured to be $126.9 \pm 9.9\%$ over six months (Figure 1). The mean corrected growth for the right pulmonary artery over the same period in time was $140 \pm 12\%$.

Histology and immunohistochemistry

Qualitative histological analysis performed at six months revealed a laminated structure with three layers that correspond to the intima, media and adventitia (Figure 2). Graft wall thickness measured 1.1 mm, which was similar to the native IVC wall thickness (1.0 mm). The inner layer was composed of a monolayer of vWF staining cells consistent with the formation of an endothelial monolayer. The inner endothelial monolayer was surrounded by concentric layers of SMA-positive cells consistent with an organized medial layer of smooth muscle cells. The outer structural layer was rich in extracellular matrix including areas positively staining with Movat stain demonstrating collagen, Verhoeff-Van Gieson stain demonstrating elastin, and Alcian blue stain demonstrating glycosaminoglycans (Figure 3). The von Kossa stain demonstrated minimal areas of ectopic calcification scattered throughout of the graft.

Expression of Eph-B4 in TEVG—Since Eph-B4 is a determinant of veins that has been described during embryonic and young animal development, we examined whether Eph-B4 was present on TEVG in our lamb model.^{10, 11} Eph-B4, a venous marker, was not expressed in the TEVG prior to implantation but was expressed strongly in the endothelium of the TEVG six months after implantation. Eph-B4 expression co-localized with vWF expression, indicating its expression in the endothelial lining.^{10, 11} Western blot analyses confirmed the Eph-B4 expression in TEVG. Eph-B4 protein was detected in the six month TEVG, although less than in the native IVC (Figure 4).

Biochemical Analysis

Biochemical assays—All TEVG contained similar amounts of collagen when compared to native ovine IVC (157.9 ± 26.4 $\mu\text{g}/\text{mg}$ and 136.7 ± 13.8 $\mu\text{g}/\text{mg}$ dry weight, respectively). Interestingly, the unseeded TEVG on average contained more collagen than the native IVC or seeded TEVG.

Elastin content of seeded TEVG averaged 186.95 ± 16.7 $\mu\text{g}/\text{mg}$ dry weight of tissue. This represents a 47.2% increase in elastin content over the unseeded scaffold, which contained 126.97 ± 36.78 $\mu\text{g}/\text{mg}$ dry weight. However, the seeded grafts contained less elastin at six months compared with native ovine IVC (365.78 ± 44.55 $\mu\text{g}/\text{mg}$).

Glycosaminoglycan content of tissue-engineered scaffolds was routinely greater than native IVC. There was no statistically significant difference between seeded and unseeded specimens,

each reaching glycosaminoglycan levels between $9.7 \pm 0.8 \mu\text{g/mg}$ and $11.8 \pm 4.3 \mu\text{g/mg}$ dry weight respectively (Figure 3). Native IVC contained $4.9 \pm 0.2 \mu\text{g}$ glycosaminoglycan/mg dry weight.

Discussion

Previous clinical studies investigating the use of TEVG in the surgical repair of congenital cardiac anomalies have demonstrated that the conduit created by seeding autologous BM-MNC onto a biodegradable tubular scaffold fabricated from polyglycolic acid fiber mesh coated with a 50:50 copolymer of L-lactone and ϵ -caprolactone increased in size over time.⁵ In this investigation we evaluated the growth potential of TEVG by implanting them as IVC interposition grafts in a juvenile lamb model in an attempt to determine if this increase in size represented growth or aneurysmal dilation. We serially monitored graft volume *in vivo* using MRI prior to harvesting the grafts. We subsequently harvested the conduits and characterized the resulting vascular neotissue in an attempt to identify any pathological changes and to compare the neotissue with native tissue. Results of this study demonstrate that the TEVG functioned well without evidence of graft thrombosis, rupture, or significant ectopic calcification. Using serial MRI we were able to demonstrate that the tissue engineered vascular graft increased in size proportional to the native right pulmonary artery. Using qualitative immunohistochemistry we were also able to show that by six months the TEVG resembled the native IVC including similar wall thickness and possession of a laminated tubular morphology with an inner monolayer of endothelial cells surrounded by several layers of smooth muscle cells concentrically arranged circumferentially around the lumen. Furthermore using quantitative biochemical analysis we showed that the extracellular matrix of the TEVG resembled the extracellular matrix of the native IVC resulting in a TEVG with a biomechanical profile similar to the native IVC. Interestingly, by six months, TEVG expressed Eph-B4, a marker of venous differentiation, suggesting the formation of venous vascular neotissue when the TEVG is implanted in the venous circulation. This marks the first time a TEVG has demonstrated molecular evidence of normal vascular development.

Arteries and veins are defined in the adult by their functional and anatomical differences.¹⁵ However, the molecular differences between arteries and veins are established before the onset of circulation.¹⁶ Molecular differences between arterial and venous endothelial cells have been demonstrated during embryonic development in the chick, mouse and zebrafish.^{16–18} There are several critical molecular determinants of blood vessel development, the most well described of which are Ephrin-B2 for arterial development, and Eph-B4 for venous development.^{19–21} The Eph receptors comprise the largest of the 14 families of receptor tyrosine kinases in mammals and are activated by ligands of a similarly large Ephrin family. After their initial discovery as orphan receptors by Bennett et al in 1995, Eph receptors were found to have matching Ephrin ligands and early research efforts determined that Ephrins and Ephs participate in developmental processes.²² Remarkably, in contrast to other ligands that are uniformly expressed throughout the circulatory system, Ephrin-B2 was found to be specifically expressed only by arteries while Eph-B4, one of its receptors, is expressed only on veins.^{11, 16, 23} These data provided one of the first examples of a genetic distinction between these two vessel subtypes and suggested that Ephrin-mediated interactions may be essential for angiogenesis.^{15, 24} Our data, that Eph-B4 is not present in the preimplantation TEVG but is expressed by 6 months in the developing TEVG, strongly support the concept that the TEVG is growing with “normal” patterns of gene expression. The increased expression of Eph-B4 may be due to the juvenile environment of the young lambs; since transfer of adult veins into adult arterial environments is associated with loss of Eph-B4 expression.²⁵ We believe that induction of Eph-B4 expression in the growing TEVG is the first example of non-malignant Eph-B4 induction after post-natal development.

This study possesses several inherent limitations. The most significant limitation is lack of a standard definition of growth as opposed to aneurysmal dilation. For the purpose of this investigation we defined growth as an increase in size without evidence of pathological change in morphology. From the translational perspective this distinction is more semantic than real so long as the TEVG do not increase in size beyond the native vessels or result in rupture. It is important to note that neither increase in graft size beyond the native vessel nor rupture of a TEVG has been reported when TEVG were implanted in low pressure circulatory systems such as the venous, pulmonary or Fontan circulation.^{5, 12} A second limitation is that the IVC is a capacitance vessel whose size is directly related to the hydration status of the graft recipient. In order to minimize this effect, we standardized the animals feeding and hydration regimen in order to prevent dehydration or over-hydration at the time of imaging. Identification of the anastomotic sites became more challenging as the vessels grew. In order to validate our selection of the proper anastomotic sites the MRI images were compared to digital images of the IVC taken immediately at sacrifice on the same day as the six-month MRI. Finally there were two options available to us in terms of quantifying vessel growth. The obvious method would have been to have performed a manual segmentation of graft volume from the images at each time point and simply compare the volumes directly. This, however, suffers from the uncertainty in manually defining the edges of the grafts (i.e. where the graft blends in to the native vessel) separately at each image, and could be a major source of error in the comparison. The method we choose, instead defined the graft only in one time point and used a non-rigid registration algorithm to map this graft to the next time, relying on image similarity as the criterion for the mapping. The maps are relatively smooth and result in a method that is relatively robust to the exact delineation of the graft.

Previous studies have demonstrated the growth potential of other types of TEVG in the pulmonary circulation.^{13, 14} These studies, however; used a different cell source, seeding the biodegradable tubular scaffolds with autologous endothelial cells and myofibroblasts instead of BM-MNC. Additional work by our group has demonstrated that the TEVG described in this study resulted in the formation of neovessels that not only resembled native vessels morphologically and histologically but also functionally possessing a smooth muscle layer that was responsive to pharmacological stimulation and a functional endothelium that inhibits thrombosis.¹² Taken together these studies suggest that tissue engineering technology offers the ability to create vascular grafts that are living structures capable of growth, repair and remodeling in response to their local environment milieu. Continued investigation into the mechanisms underlying the process of vascular neotissue formation is essential in order to gain insights that will enable us to rationally design improved TEVG and in order to promote the continued translation of this promising technology and enable it to reach its full potential for advancing the surgical treatment of congenital heart disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was sponsored by generous grants from the American Surgical Association Foundation and the National Institutes of Health (HL83980, HL079927).

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Figure 1.

(A) Photograph of TEVG IVC interposition graft *in situ* after six months implantation.

(B) Corresponding MRI of same TEVG six months after implantation. (C) 3-D rendering of the outside surface of the vascular graft at time one month (red) and six months (green) following rigid alignment of the surfaces. Note the relative expansion of the graft over time.

Figure 2. Histology and immunohistochemistry of TEVG explanted after six months in comparison to native IVC. vWF, von Willebrand factor; SMA, smooth muscle actin; VK, Von Kossa stain.

Figure 3.

(A) Quantitative biochemical analysis of TEVG (B) Qualitative IHC analysis of the ECM of the TEVG (100X)

Figure 4.

(A) Immunofluorescence demonstrating co-localization of Eph B-4 and vWF in TEVG (B) Immunofluorescence controls (C) Western blotting demonstrating Eph B-4 expression in BM-MNC (bone marrow derived mononuclear cells), TEVG, & IVC, and graph showing densitometry.