METHODS ARTICLE

Hemodynamic Characterization of a Mouse Model for Investigating the Cellular and Molecular Mechanisms of Neotissue Formation in Tissue-Engineered Heart Valves

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Decellularized allograft heart valves have been used as tissue-engineered heart valve (TEHV) scaffolds with promising results; however, little is known about the cellular mechanisms underlying TEHV neotissue formation. To better understand this phenomenon, we developed a murine model of decellularized pulmonary heart valve transplantation using a hemodynamically unloaded heart transplant model. Furthermore, because the hemodynamics of blood flow through a heart valve may influence morphology and subsequent function, we describe a modified loaded heterotopic heart transplant model that led to an increase in blood flow through the pulmonary valve. We report host cell infiltration and endothelialization of implanted decellularized pulmonary valves (dPV) and provide an experimental approach for the study of TEHVs using mouse models.

Introduction

RIGHT VENTRICULAR OUTFLOW tract reconstruction is one of the most commonly performed procedures in congenital heart surgery and often requires implantation of a prosthetic heart valve in the pulmonary position. Mechanical heart valves have long durability but require life-long anticoagulation, which is not ideal in children. Xenografts and human cryopreserved valves are characterized by increased early reintervention rates due to strong immunological reactions with subsequent calcification.^{1–3} The lack of growth potential also predisposes children to recurrent surgeries thus increasing morbidity. Decellularized allograft heart valves have been used as tissue-engineered heart valve (TEHV) scaffolds by several investigators with promising results,^{4,5} however, little is known about the cellular mechanisms underlying neotissue formation in these TEHVs.

To better understand this phenomenon, we developed a murine model of decellularized pulmonary heart valve transplantation using the traditional hemodynamically unloaded heart transplant model.⁶ This unique system is advantageous due to the feasibility and availability of genetically manipulated mouse models that recapitulate human cardiovascular disease, including those of the valves.

In addition, mice offer a significant cost saving when compared to large animal models. Furthermore, because the hemodynamics of blood flow through a heart valve may influence its development and subsequent function,⁷ we describe a modified loaded heterotopic heart transplant model that led to an increased right ventricular stroke volume (SV) and blood flow through the pulmonary valve.

In this study, using echocardiography and pressure myography, we perform detailed hemodynamic characterization of both the loaded and unloaded model and compare them to the hemodynamics of the native orthotopic heart. Development of a murine heart valve replacement model would provide a valuable tool for investigating the cellular and molecular mechanisms underlying neotissue formation in TEHV, while the ability to modulate the hemodynamic environment within the model would enable the dissection of the mechanobiological factors underlying this process.

Materials and Methods

Animal care

All animals received humane care in compliance with the National Institutes of Health (NIH) Guide for the Care and Use

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of Laboratory Animals. The Institutional Animal Care and Use Committee at the Nationwide Children's Hospital approved the use of animals and all procedures described in this study.

Donor heart and pulmonary valve harvest

Heart and pulmonary valve harvest was conducted as previously described.⁶ Briefly, C57BL/6 mice (Jackson Laboratories), average weight of 20 g were euthanized by an intraperitoneal injection of 200 mg/kg ketamine (Hospira, Inc.) and 20 mg/kg xylazine (Akorn, Inc.) overdose cocktail. One mouse served as the donor of the pulmonary valve (mouse no. 1), a second mouse served as the heart donor lacking pulmonary valve (mouse no. 2). The heart and pulmonary valves were harvested through an inverted U-shaped anterior thoracotomy under an operating microscope (Leica Application System, LAS V4.3) at 2.5× magnification. Harvest of the pulmonary valve included a 2 mm cusp of the right ventricle with care taken not to injure the pulmonary valve, which appears as a shiny cream-colored film at the junction of the right ventricle and pulmonary artery (PA). After excising the pulmonary valve from mouse no. 2, the harvested pulmonary valve from mouse no. 1 was implanted into the heart of mouse no. 2 as an end-to-end running anastomosis with 10-0 prolene suture. The heart with the implanted pulmonary valve was subsequently transplanted into recipient mouse no. 3 as described in the Implantation of Decellularized Pulmonary Heart Valve and *In Vivo* Functionality section.

Decellularization of pulmonary heart valve

Harvested pulmonary heart valves were placed in an incubation solution containing 10 mM Tris buffer (pH 8.0) plus 0.1% ethylenediaminetetraacetic acid (EDTA; Calbiochem) and 10 KIU/mL aprotinin (Sigma Aldrich) for 1 h. Afterward, the valves were placed in a decellularization buffer containing 0.1% sodium dodecyl sulfate (SDS; Sigma Aldrich) in hypotonic 10 mM Tris buffer with 0.1% EDTA and 10 KIU aprotinin for 48 h at room temperature with continuous shaking (Corning®; LSE Orbital Shaker). The decellularization buffer was changed every 12 h. After decellularization, the valves were washed thoroughly in phosphate-buffered saline (PBS, Gibco®; Life Technologies) for six 10-min intervals. The valves were then transferred into solution containing: 20 µg/mL RNase A (Sigma Aldrich) and 0.2 mg/mL

DNase (Sigma Aldrich) in 4.2 mM magnesium sulfate (Sigma Aldrich), 5 mM Ca^{2+} (Sigma Aldrich), and Tris buffer (pH 7.2). The valves remained on the continuous shaker for 48 h while changing the solution every 12 h. The decellularized tissues were then washed for six 10-min intervals in PBS. The valves were stored at -80°C before implantation.

Implantation of decellularized pulmonary heart valve and in vivo functionality

To evaluate functionality and host cell repopulation, decellularized ($n=6$) and intact native ($n=3$) pulmonary heart valves were heterotopically implanted into 6- to 8-week-old C57BL/6 mice (average weight of 20 g) using the traditional unloaded heterotopic heart transplant model (Fig. 1a) as previously described.⁸ Briefly, after the administration of 5 mg/kg ketoprofen (Fort Dodge®), 100 mg/kg ketamine, and 10 mg/kg xylazine cocktail, the abdomen was shaved, prepped, and draped in the standard sterile fashion. A self-retaining retractor was placed after making a generous mid-line incision. The intestines were eviscerated and wrapped in saline-soaked gauze to facilitate exposure of the retroperitoneum. Peritoneal attachments to the descending colon were incised. Blunt dissection was conducted to expose the infra-renal abdominal aorta and inferior vena cava (IVC) down to the level of aortic bifurcation. Lumbar branches to the IVC and aorta were ligated. Proximal and distal vascular control was obtained with 6-0 silk ties. An aortotomy was made and an end-to-side aorto-aortic anastomosis was performed with 10-0 prolene suture in a running fashion. In a similar manner, a venotomy was performed with an end-to-side anastomosis of the PA to the IVC. Upon releasing the 6-0 silk ties, the patency of anastomosis was evaluated; hemostasis was obtained and the intestine was returned to the abdomen, which was then closed in two layers. Mice were euthanized at either 3 months (native $n=3$; decellularized $n=3$) or 6 months (decellularized $n=3$).

Loaded and unloaded heterotopic heart transplant

The traditional unloaded transplant model is limited in that preload to the heart graft is only about 5% of total blood circulating volume, corresponding to the proportion of cardiac output received by the coronary circulation. In a separate set of experiments, we sought to improve blood flow

FIG. 1. Schematic representation of unloaded (a) and loaded (b) heterotopic heart transplant. Arrows indicate direction of blood flow. AA, ascending aorta; CS, coronary sinus; LA, left atrium; LV, left ventricle; PA, pulmonary artery; R-AbA, recipient abdominal aorta; R-IVC, recipient inferior vena cava; RA, right atrium; RV, right ventricle; SVC, superior vena cava. Color images available online at www.liebertpub.com/tec

through the pulmonary valve by modifying the volume-loaded transplant model described by Asfour *et al.*⁹ The modification involved ligation of the recipient IVC cephalad to the superior vena cava (SVC)-IVC anastomosis (Fig. 1b), increasing preload of the transplanted heart to 50% of the total blood circulating volume. Harvested whole hearts without pulmonary valve implants were transplanted into isogenic donor mice using either the traditional unloaded model ($n=8$) or the modified loaded model ($n=6$). One week after transplantation, ultrasonography and right ventricle pressure measurements were conducted; mice were subsequently euthanized and tissue harvested for histological analysis.

In vivo imaging of heart grafts

To determine the right ventricular SV and assess functionality of the pulmonary valve, a high-frequency ultrasound system with pulsed-wave Doppler mode was used (30 MHz; VisualSonics, Inc.). Mice were anesthetized using 1.5% isoflurane (Baxter) vaporized with oxygen at a flow rate of 1 L/min. Body temperature was maintained at 38–39°C. To eliminate poor visualization due to intestinal gas, the midline incision was reopened to eviscerate the intestines and expose the transplanted heart graft. The peritoneal cavity was filled with 0.3 mL of warm normal saline and covered with a piece of a thin waterproof transparent covering while ensuring the absence of air bubbles. Long- and short-axis images (Fig. 4) were acquired in B-mode. From the short-axis view, pulsed-wave Doppler was used to measure the velocity time integral (VTI) at the level of the right ventricular outflow tract and the PA diameter. Right ventricular SV was calculated as the PA cross-sectional area multiplied by the VTI. For comparison, a separate group of age and weight-matched C57BL/6 mice ($n=9$) that had not undergone any surgical intervention were subjected to transthoracic echocardiography (orthotopic heart group).

Right ventricular pressure measurements

Right ventricular systolic and diastolic pressure measurements were obtained by insertion of a 1.2F catheter-tip pressure transducer (Scisense) into the right ventricle. After calibrating the pressure transducer, mice were anesthetized using 1.5% isoflurane with an oxygen flow rate of 1 L/min. Body temperature was maintained at 38–39°C. The abdomen was opened and the intestines eviscerated to facilitate exposure of the heart graft. In the orthotopic heart group, mice were placed on a

ventilator (Harvard Apparatus Model 687) using a tidal volume of 120 μ L and respiratory rate of 148 breaths per minute. In all groups, the apex of the right ventricle was identified and pierced with a 27-gauge needle to facilitate introduction of the pressure catheter. Mean right ventricular systolic and diastolic pressures were obtained for 3 min.

Histological tissue analysis

Mice were perfused with 10% formalin; tissues were harvested and fixed in 10% formalin and paraffin embedded. Four-micron-thick sections were stained with Hematoxylin and eosin (H&E) or Movat's pentachrome stain (American MasterTech) to assess valve morphology, cellular infiltration, and extracellular matrix (ECM) organization following previously established methods.^{10–12} Light-field images were obtained with a Zeiss Axio Imager.A2 microscope (Carl Zeiss).

To further characterize the types of cells that infiltrated our dPVs, tissue sections were assessed for endothelial (CD31) and α -smooth muscle actin (α -SMA)-positive cells. After deparaffinizing, rehydrating, and blocking for nonspecific antibody binding, tissue sections were incubated overnight with rabbit-anti CD31 (1:50, Ab 28364; Abcam) and mouse anti-smooth muscle actin (α -SMA, 1:500, MO851; Dako). Immunofluorescent detection of antibody binding was performed by incubation (1.0 h) with Alexa Fluor 647 goat anti-mouse IgG (1:300, A21235; Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (1:300, A11008; Invitrogen). Cell nuclei were identified by subsequent counterstaining with 4', 6-diamidini-2-phenylindole (DAPI); fluorescent images were obtained with an Olympus IX51 inverted microscope; exposure time was informed by appropriate negative controls.

Statistical analysis

All data are presented as mean $\pm$ standard deviation. One-way layout nonparametric Kruskal–Wallis test was used to evaluate differences among our experimental groups. A *post hoc* Mann–Whitney test was performed to detect significant difference between pairs with Bonferroni–Holm correction for multiple comparisons, when Kruskal–Wallis test was significant. Type I error was strongly controlled at $\alpha=0.05$ for single comparisons and with adjustment for multiple comparisons. The data were analyzed using SAS version 9.3 (SAS Institute).

FIG. 2. Histology of mouse PV before implantation. H&E stain of intact native valve (a), decellularized valve (b), and pentachrome stain of dPV (c). dPV, decellularized pulmonary valves; H&E, Hematoxylin and eosin; PV, pulmonary valve. Color images available online at www.liebertpub.com/tec

FIG. 3. Histological analysis of intact nPV and dPV explants. Representative samples of nPV explanted at 3 months (a–d); dPV explanted at 3 months (e–h) and 6 months (i–l). H&E shows cellularity of dPV at 3 months (e) that is increased by 6 months (i). Pentachrome stain shows ECM and confirms cellular infiltration (b, f, j). Positive α -SMA cells suggesting tissue remodeling are seen in the 3 month dPV explants (g); fewer α -SMA-positive cells in explanted 6-month grafts (k) compared with 3-month grafts suggest physiological rather than pathological remodeling in our model. No α -SMA expression was observed in the nPV (c). CD31 expression reveals endothelialization of all explants (d, h, l). Arrow identifies positive cells. α -SMA, α -smooth muscle actin; ECM, extracellular matrix; nPV, native pulmonary valve. Color images available online at www.liebertpub.com/tec

Results

Histology of decellularized pulmonary valve implants

Compared to intact native pulmonary valves (nPV), cells were not detected within the decellularized mouse pulmonary valves and arteries using H&E staining, however there was presence of ECM as observed with Movat's pentachrome stain (Fig. 2a–c).

Microscopic analysis of decellularized pulmonary valve explants

Explanted dPVs demonstrated host cell repopulation by 3 months posttransplantation with increased cellular infiltrate seen by 6 months (Fig. 3a, e, and i). Pentachrome staining revealed the presence of diversified ECM and confirmed cellular infiltration throughout the valve leaflets (Fig. 3b, f,

FIG. 4. Two-dimensional echocardiography of long- (a–c) and short-axis view (d–f) of orthotopic (a, d), loaded (b, e), and unloaded (c, f) hearts. Schematic illustration of short-axis view (g). PA diameter was measured using short-axis view. Ao, aorta; Thr, thrombus. Color images available online at www.liebertpub.com/tec

FIG. 5. Calculated right ventricular SV of orthotopic ($n=9$), loaded ($n=6$), and unloaded ($n=8$) hearts. SV calculated as: area of PA multiplied by pulmonary valve velocity time interval obtained from two-dimensional echocardiography. SV, stroke volume.

and j). It was noted that 3 months following transplantation, dPV leaflets had thickened morphology similar to embryonic heart valves during stages of remodeling¹² (Fig. 3e–h). By 6 months, the dPVs were less thickened and appeared to take a more elongated shape similar to the nPV (Fig. 3i–l). Immunofluorescent analysis demonstrated the presence of endothelial cells as indicated by CD31 expression in the dPV at 3 and 6 months (Fig. 3h, l). In contrast to the intact nPV, which typically exhibit α -SMA-negative quiescent valvular interstitial cells (VICs), dPVs stained positive for α -SMA at 3 months suggestive of active tissue remodeling (Fig. 3c, g). By 6 months, the degree of positively stained α -SMA decreased, suggesting less activation or quiescence of VICs, similar to the intact nPV (Fig. 3k).

Surgical mortality, echocardiographic, and hemodynamic assessment

Heart grafts were successfully implanted into recipient mice using the modified loaded ($n=6$) and traditional unloaded ($n=8$) models with a surgical mortality rate of 15% and 10%, respectively. Echocardiography demonstrated blood flow in the right and left ventricles of the loaded hearts in contrast to unloaded hearts, which had complete occlusion of the left ventricle by thrombus (Fig. 4a–c). The calculated

right ventricular SV was significantly increased in the loaded model compared with the unloaded model although significantly less than that observed in the orthotopic heart (Fig. 5). Unloaded hearts had significant atrophy of the right ventricle wall compared with loaded and orthotopic hearts as measured by the right ventricle wall thickness (Table 1). No right ventricle dilatation was observed.

Peak velocities across the pulmonary valve were significantly decreased in the unloaded hearts compared with loaded and orthotopic hearts (Table 1). To evaluate right ventricular function, the right ventricular fractional area change was calculated as a marker for right ventricular contractility.^{13–15} There was increased right ventricular contractility in loaded heart compared with orthotopic and unloaded hearts (Table 1).

To study the effect of volume loading on the hemodynamics of the heterotopic heart grafts, right ventricular systolic and diastolic pressure measurements were obtained. A significant increase in right ventricular systolic pressure was noted in the transplanted heart grafts compared with orthotopic hearts. There was no significant effect on diastolic pressure (Table 2).

Histological analysis of explanted heart grafts

At 1-week postimplantation, heart grafts were explanted for histological analysis of the pulmonary valve and ventricles. Seven of eight unloaded pulmonary heart valves and five out of six loaded pulmonary valves examined were patent on microscopic evaluation (Fig. 6a–c). Masson's Trichrome stain of the ventricles revealed epicardial fibrosis suggestive of cardiac remodeling, which was likely secondary to ischemia/reperfusion injury (images not shown). Movat's pentachrome of pulmonary heart valves showed ECM and valve interstitial cell distribution (Fig. 6d–f).

Discussion

The present study provides a potentially powerful experimental tool for the study of the cellular and molecular mechanisms underlying neotissue formation in decellularized TEHV using murine models. Intermediate to long-term outcomes of decellularized pulmonary valve allografts in human clinical trials are promising^{4,5}; however, the availability of heart valve allografts remains a significant limiting factor. The use of decellularized xenogenic heart valves in children has controversial results. While some

TABLE 1. TWO-DIMENSION ECHOCARDIOGRAPHIC INDICES OF RIGHT VENTRICLE AND PULMONARY VALVE FUNCTION

Echo parameters	Orthotopic heart (N)	Loaded heart (L)	Unloaded heart (U)	p-Value
RV wall thickness (mm)	0.41 ± 0.06	0.47 ± 0.11	0.29 ± 0.05^a	0.0121
RV Fractional area change (%)	59.4 ± 15.8	75.9 ± 14.4	59.0 ± 17.7	0.0485
VTI (mm)	22.3 ± 5.4	12.7 ± 2.9^b	6.8 ± 5.1^c	0.0012
PA diameter (mm)	0.86 ± 0.11	0.62 ± 0.11^b	$0.44 \pm 0.06^{c,d}$	0.0009
Peak velocity (mm/s)	437.05 ± 136.60	354.15 ± 100.15	136.64 ± 93.75^e	0.0102

^aU versus L, p -value=0.014.

^bN versus L, p -value=0.0077.

^cU versus N, p -value=0.0034.

^dU versus L, p -value=0.0367.

^eU versus N, p -value=0.0053.

L, loaded ($n=6$); N, orthotopic ($n=9$); PA, pulmonary artery; RV, right ventricle; U, unloaded ($n=8$); VTI, velocity time integral.

TABLE 2. PRESSURE MEASUREMENT IN THE RIGHT VENTRICLE OF ORTHOTOPIC, UNLOADED, AND LOADED HEARTS

<i>RV pressure indices</i>	<i>Orthotopic heart (N)</i>	<i>Loaded heart (L)</i>	<i>Unloaded heart (U)</i>	<i>p-Value</i>
RV systolic pressure (mmHg)	24.0 ± 7.4	42.3 ± 21.1	63.5 ± 27.3 ^a	0.0177
RV diastolic pressure (mmHg)	2.6 ± 6.1	4.4 ± 7.1	10.9 ± 13.3	0.55

^aU versus N, *p*-value = 0.0131.

authors show promising results at 3 years follow-up,² others report high early conduit failure rate^{1,3} making the pursuit for the ideal TEHV conduit especially for children and young adults paramount. Decellularized TEHVs based on biodegradable synthetic materials may serve as suitable replacement of allografts and xenografts, but preclinical study in nonhuman primate and ovine models are characterized by valvular regurgitation due to leaflet shortening and reduced leaflet coaptation.^{16–19}

Although decellularized allograft heart valves have been used as TEHV scaffolds with success,^{4,5} little is known about the cellular and molecular mechanisms underlying neotissue formation in these TEHVs. To study the mechanisms leading to neotissue formation in decellularized heart valve grafts, we established a mouse model of pulmonary valve transplant⁶ and subsequently implanted decellularized mice pulmonary heart valves into isogenic mice recipients. We show host cell infiltration and endothelialization of the valve leaflet at 3 and 6 months postimplant. Whereas explanted intact nPV retained an elongated structure and had no α -SMA-positive cells detected, decellularized pulmonary valves (dPV) at 3 months appeared thickened, reminiscent of embryonic valvular morphology.¹² In addition, tissue remodeling of the valve was evidenced by the presence of α -SMA-positive cells, which has been shown by several investigators to be activated valve myofibroblasts.^{20–23} Failure

of α -SMA-positive cells to undergo apoptosis or become quiescent following heart valve tissue remodeling is one mechanism that leads to valve fibrosis and stenosis.^{20,24,25} In our study, we observed fewer α -SMA-positive cells in explanted 6-month grafts compared with 3-month grafts, suggesting physiological and not pathological remodeling in our model. In tissue-engineered vascular graft constructs, our group showed that neovessel regenerates from adjacent host blood vessels and is independent of seeded cells,^{26,27} a mechanism we believe may be applicable to our current findings. Alternatively, it is possible that the transplanted dPV loses viability over time; however, further work is required to delineate the origin and fate of infiltrating host cells in TEHV recipients. We believe that our model is well suited for longitudinal studies to characterize the natural history of neo-valve formation, and this is the focus of current research efforts.

It is postulated that the hemodynamics of blood flow through a heart valve influences development and subsequent function through mechanobiological mechanisms.⁷ Therefore, we sought to establish a mouse heterotopic heart transplant model that would increase blood flowing through the pulmonary valve and enable us to investigate the effect of altering hemodynamic forces on neotissue formation. We report a modified loaded heart transplant model that significantly increased right ventricular SV. Contrary to our

FIG. 6. Histological analysis of pulmonary heart valves. H&E reveals patent valves in the orthotopic heart (a), loaded heart transplant model (b), and unloaded heart transplant model (c). Pentachrome staining shows ECM and valvular interstitial cell distribution; (d) orthotopic heart; (e) loaded heart transplant model; and (f) unloaded heart transplant model. RVOT, right ventricular outflow tract. *PA to left atrium anastomosis. Color images available online at www.liebertpub.com/tec

expectation, hemodynamic analysis of the transplanted hearts revealed that despite a patent pulmonary valve and low flow state, there was significant increase in right ventricle pressure gradient compared with the orthotopic heart. Furthermore, this pressure gradient was augmented in the presence of PA or valve occlusion. PA diameters of the transplanted hearts were significantly less than that of the orthotopic heart. We speculate by Poiseuille's law that the decrease in PA diameter with subsequent increase in resistance led to the elevated right ventricle pressure gradient. The reason for a decrease in PA diameter in the transplanted heart is unclear, but may be inherent to the surgical technique where the PA becomes stretched while creating the anastomosis. Hemodynamically, the heterotopic heart transplant model represents a high-pressure model; blood flow is influenced by the nature of the PA anastomosis. Hence, the heterotopic heart transplant model may be modulated to create different hemodynamic states that may influence neotissue formation, valvular function, and cardiac remodeling.

Conclusions

We report host cell infiltration and endothelialization of implanted decellularized pulmonary heart valves in a murine model of heterotopic heart transplant. Although ovine models are considered standard for the study of heart valve degeneration, our unique murine system is advantageous due to the feasibility and availability of genetically manipulated mouse models that can study the role of specific genes, proteins, and cell types involved in neo-valve formation. Furthermore, the hemodynamics of the mouse model can be effectively modulated, providing a useful tool to investigate the biomechanical mechanisms of neo-valve development. Future studies will determine the role of cell-seeding in TEHV remodeling, delineate the origin and fate of infiltrating host cells in TEHV recipients, and lead to the development of the ideal biodegradable synthetic TEHV that will ultimately be tested in larger animal models.

Acknowledgments

Tissue processing and H&E staining were performed by the Morphology Core at Nationwide Children's Hospital. The authors would like to thank Yongjie Miao for his help with statistical analysis.

Disclosure Statement

The present study was funded by the National Institutes of Health R01098228 to C.B., the American Heart Association Scientist Development Grant 13SDG16840035, and National Institutes of Health K99HL116769 to A.J.T. No writing assistance was utilized in the production of this article.

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Received: January 10, 2015

Accepted: April 10, 2015

Online Publication Date: May 29, 2015