Donor tissue-specific exosome profiling enables noninvasive monitoring of acute rejection in mouse allogeneic heart transplantation

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ABSTRACT

Objective: In heart transplantation, there is a critical need for development of biomarkers to noninvasively monitor cardiac allografts for immunologic rejection or injury. Exosomes are tissue-specific nanovesicles released into circulation by many cell types. Their profiles are dynamic, reflecting conditional changes imposed on their tissue counterparts. We proposed that a transplanted heart releases donor-specific exosomes into the recipient's circulation that are conditionally altered during immunologic rejection. We investigated this novel concept in a rodent heterotopic heart transplantation model.

Materials and Methods: Full major histocompatibility mismatch (BALB/c [H2-K^d] into C57BL/6 [H2-K^b]) heterotopic heart transplantation was performed in 2 study arms: Rejection (n = 64) and Maintenance (n = 28). In the Rejection arm, immunocompetent recipients fully rejected the donor heart, whereas in the Maintenance arm, immunodeficient recipients (C57BL/6 Prkdc^{SCID}) accepted the allograft. Recipient plasma exosomes were isolated and a donor heart-specific exosome signal was characterized on the nanoparticle detector for time-specific profile changes using anti-H2-K^d antibody quantum dot.

Results: In the Maintenance arm, allografts were viable throughout follow-up of 30 days, with histology confirming absence of rejection or injury. Time course analysis (days 1, 2, 3, 4, 5, 7, 9, 11, 15, and 30) showed that total plasma exosome concentration ($P = .157$) and donor heart exosome signal ($P = .538$) was similar between time points. In the Rejection arm, allografts were universally rejected (median, day 11). Total plasma exosome quantity and size distribution were similar between follow-up time points ($P = .278$). Donor heart exosome signals peaked on day 1, but significantly decreased by day 2 ($P = 2 \times 10^{-4}$) and day 3 ($P = 3.3 \times 10^{-6}$), when histology showed grade 0R rejection. The receiver operating characteristic curve for a binary separation of the 2 study arms (Maintenance vs Rejection) demonstrated that a donor heart exosome signal threshold < 0.3146 was 91.4% sensitive and 95.8% specific for diagnosis of early acute rejection.

Conclusions: Transplant heart exosome profiling enables noninvasive monitoring of early acute rejection with high accuracy. Translation of this concept to clinical settings might enable development of a novel biomarker platform for allograft monitoring in transplantation diagnostics. (J Thorac Cardiovasc Surg 2018;155:2479-89)

Acute rejection leads to distinct changes in the recipient's plasma donor heart-specific exosome pool.

Central Message

Circulating donor-specific exosome profiles in recipient blood herald early acute rejection of cardiac allograft.

Perspective

In heart transplantation, there is a critical need for accurate, noninvasive biomarkers to monitor for immunologic rejection because current clinical standards are based on routine cardiac allograft biopsy. We propose that in an animal of cardiac allotransplantation, donor heart-specific exosome profiling from recipient plasma serves as a noninvasive biomarker for diagnosis of early rejection.

See Editorial Commentary page 2490.

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Abbreviations and Acronyms

ANOVA	= analysis of variance
AUC	= area under the curve
ddcfDNA	= donor-derived cell free DNA
EMB	= endomyocardial biopsy
IgG	= Immunoglobulin G
ISHLT	= International Society for Heart and Lung Transplantation
MHC-1	= major histocompatibility complex-1
ROC	= receiver operation characteristics

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Heart transplantation remains the only curative therapy in patients with end-stage heart failure. Even with major medical advances, immunologic rejection and immunosuppressive regimen-related complications account for the majority of morbidity and mortality in patients undergoing transplant. A recent report by the International Society for Heart and Lung Transplantation (ISHLT) shows that the incidence of rejection events in the first year posttransplant is 25%.¹ The current gold standard of allograft surveillance comprises empiric surveillance and histology-guided endomyocardial biopsy (EMB), which is associated with procedural complications and can consume time and resources from patient and health system standpoints.^{2,3} The frequency of surveillance biopsies varies among centers, with routine biopsies typically being performed weekly within the first month posttransplant and at increasing intervals thereafter. ISHLT reports that a heart transplant patient undergoes 17 EMBs on average during the first 2 years posttransplantation.¹ At our institution, a typical heart transplant recipient undergoes 20 surveillance EMBs during the first 2 years posttransplant, and that is if there are no rejection episodes.

Exosomes are bilayered, membrane-bound nanoparticles (30–200 nm) arising from endosomal compartments called multivesicular bodies. They are secreted by many tissue types into bodily fluids, including blood and urine.^{4–6} Along with surface marker profiles that are identical to their tissue counterparts, exosomes carry stable proteomic and RNA signatures that are condition specific. Exosomes are being extensively studied for their diagnostic potential, but similar to other quantitative

assays based on circulating free proteins and nucleic acids, whole plasma exosome analysis is also associated with a high noise-to-signal ratio because many tissue types contribute to the total plasma exosome pool.⁴ We recently proposed that characterization of tissue-specific exosomes from bodily fluids would improve diagnostic accuracy by reducing the noise-to-signal ratio and therefore serve as a better biomarker. In the context of transplantation, we hypothesized that transplant tissue-specific exosome profiling from recipient circulation would serve as an accurate biomarker platform. To characterize transplant tissue exosomes, we took advantage of 2 concepts: transplanted tissues release distinct donor major histocompatibility complex-1- (MHC-1) specific exosomes into a recipient's circulation, and iatrogenic donor–recipient MHC mismatch introduced from allotransplantation allows for donor-specific exosome profiling from recipient blood. In the context of heart transplantation, we proposed that donor heart-specific exosome profiling would enable noninvasive monitoring of immunologic rejection. We report our investigation of this concept in a heterotopic heart transplantation model.

MATERIALS AND METHODS

Mice

All experiments were conducted in accordance with approved protocols through the University of Pennsylvania Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6 (MHC H2-K^b) immunocompetent wild type and C57BL/6 immunodeficient (Prkdc^{scid}) mice (MHC H2-K^b) served as recipients. BALB/c (MHC H2-K^d) mice served as heart donors. All animals were purchased from The Jackson Laboratory (Bar Harbor, Maine).

Study Design

The 2 study groups were Rejection and Maintenance arms. In the Rejection arm, full MHC mismatch (BALB/c into C57BL/6) heart transplants were performed, resulting in acute rejection with allograft asystole by day 9 to 12 (median, day 11). Recipients were killed for plasma exosome analysis and allograft histology on days 1, 2, 3, 4, 5, 7, 9, 11, 15, and 30. Along with naïve pretransplant recipients, at least 5 transplants were performed for each time point (n = 64 total). In the Maintenance arm, full MHC mismatch heart transplant was performed into C57BL/6 immunodeficient recipients, and because these animals lack T and B cells, they accepted the allograft long-term. Recipients were killed on days pretransplant, 1, 2, 4, 7, 11, and 30 (4 transplants per time point; n = 28 total), and recipient plasma exosome and donor heart histologic analysis was performed. Schematic of the study design is shown in [Figure 1](#).

Heterotopic Heart Transplantation and Posttransplant Monitoring

Animals were anesthetized with ketamine and xylazine, and donors received 200 U heparin after which the heart was explanted. Each recipient's abdominal aorta and inferior vena cava was exposed, and the donor heart pulmonary artery to vena cava and donor aorta to recipient aorta anastomoses were performed to complete the allograft implantation. The heterotopic heart transplantation procedure is available in [Video 1](#). Allograft

FIGURE 1. Heterotopic heart transplantation model. Full major histocompatibility complex-1 (MHC-1) mismatched BALB/c hearts (MHC-1 H2-K^d) were heterotopically transplanted into strain-controlled C57BL/6 (MHC-1 H2-K^b) mice (rejection arm $n = 64$) or immunodeficient C57BL/6 Prkdc^{SCID} (MHC H2-K^b) mice (maintenance arm $n = 28$). Animals of both study arms were monitored for allograft viability and were killed at various time points for plasma exosome analysis (days 1-30).

function was assessed daily via percutaneous palpation and heart rate monitoring until the animal was killed or until asystole. Recipients were killed for plasma and donor heart harvest at the mentioned time points. The plasma sample from each animal was analyzed independently.

Plasma Exosome Isolation

About 300 to 500 μ L whole blood was centrifuged at 500 g to obtain plasma that was passed via size exclusion chromatography column to obtain eluent fractions containing exosomes.⁴ The pooled fractions were filtered through a 100 kDa cut-off membrane and ultracentrifuged at 120,000 g for 2 hours at 4°C. A pellet containing exosomes was resuspended in phosphate buffered saline for downstream analysis.

Nanoparticle Detector Analysis

Exosomes were analyzed on the NanoSight NS300 nanoparticle detector (Malvern Instruments Inc, Malvern, United Kingdom) for quantitation and size distribution of total plasma exosomes and donor heart-specific exosomes. Total plasma exosomes were analyzed on the light scatter mode. For donor heart-specific exosome characterization, a subpopulation of recipient plasma exosomes with surface expression of donor BALB/c MHC (H2-K^d) was profiled using anti-H2-K^d antibody conjugated quantum dot (Biolegend, San Diego, Calif) on the nanoparticle detector fluorescence mode. Mouse immunoglobulin G (IgG) antibody quantum dot (Santa Cruz Biotechnologies, Santa Cruz, Calif) was used as isotype control. Each sample was run in duplicate and each experimental run was duplicated independently. In each displayed panel, the nanoparticle size distribution curve is represented by particle size (nanometers) on the x-axis and nanoparticle concentration ($\times 10^6$ /mL) on the y-axis (Figures 2, B, and 4). The curve in blue represents the total plasma exosome pool distribution, and the red curve represents the subpopulation of donor tissue specific exosomes.

Donor heart exosome signal was quantified as follows:

$$\frac{\text{H2 - K}^d \text{ fluorescence}}{\text{H2 - K}^d \text{ light scatter}} - \frac{\text{Pretransplant H2 - K}^d \text{ fluorescence}}{\text{Pretransplant light scatter}} - \frac{\text{IgG isotype fluorescence}}{\text{IgG isotype light scatter}}$$

Western Blot

Equal quantities of exosome protein and tissue lysates (10 μ g) were run on polyacrylamide gels, transferred on nitrocellulose membrane (Thermo Fisher Scientific, Waltham, Mass). Exosomal fractions and tissue lysate were run on 2 separate blots as indicated by a dashed line in Figure 2, A. The blots were blocked and incubated with the desired primary antibody at concentration per manufacturer's protocol. Horseradish peroxidase coupled secondary antibodies (Cell Signaling Technology, Danvers, Mass) were added and detected through chemoluminescence using ImageQuant LAS 4000 phosphorimager (GE Healthcare, Chicago, Ill).

Histology

Donor heart tissue was cut with cryostat, fixed with 4% paraformaldehyde, and blocked (0.05% Triton X-100; Sigma-Aldrich, St Louis, Mo) before staining with hematoxylin and eosin. For time course characterization of rejection, T cell infiltration was assessed by immunohistochemistry using anti-CD3 antibody. Analysis was performed using Zeiss epifluorescence microscope. Histologic evaluation and ISHLT grading of acute rejection in donor heart was performed by the clinical heart transplant pathologist at our institution.

Statistical Analysis

First, data were checked for normality. Statistical significance for parametric data was assessed by independent sample *t* test (2-tailed) for continuous variables with 2 groups (donor heart-specific exosome signal day 1 vs day 2, donor heart-specific exosome signal day 2 vs day 3, and donor heart-specific exosome signal day 1 vs day 3, all in the Rejection arm) and 1-way analysis of variance (ANOVA) for continuous variables with more than 2 groups (normality and homoscedasticity was assumed for donor heart-specific exosome signal in the

VIDEO 1. Mouse model of heterotopic abdominal heart transplantation. Video available at: [http://www.jtcvsonline.org/article/S0022-5223\(18\)30265-4/fulltext](http://www.jtcvsonline.org/article/S0022-5223(18)30265-4/fulltext).

Maintenance arm, donor heart-specific exosome signal in the Rejection arm from day 3 to 30, and total exosome concentrations from pretransplant time-point to day 30 for both Maintenance and Rejection arms). Additionally, all comparative statistical tests for total exosome concentration and donor heart-specific signal characterization (independent sample *t* test and 1-way ANOVA) were performed using Monte Carlo permutation data resampling. This was done to further statistically validate the significance of the findings with respect to total exosome concentration and donor heart-specific exosome analysis. Therefore, *P* values are reported as “*P*” and “permute *P*” for results of Monte Carlo permutation data resampling analysis. For the receiver operating characteristic (ROC) curve, the true-positive rate (sensitivity) was plotted against the false-positive rate (specificity) to illustrate performance of a binary classifying system (Maintenance vs Rejection arms). A threshold was determined using the Youden index, and likelihood ratio, sensitivity, and specificity were calculated. ROC curves were compared using the method of DeLong and colleagues.⁷ General statistics were assessed using StataMP version 14.2 (StataCorp, College Station, Tex), and scatter plots and NanoSight panels were constructed using Prism version 7.0 (GraphPad, San Diego, Calif). All reported tests were 2-tailed and alpha level was set to 0.05.

RESULTS

Donor BALB/c MHC Is Specifically Expressed on BALB/c Tissues and Their Exosomes

We confirmed that plasma exosomes isolated from naïve donor BALB/c mice express H2-K^d MHC class I antigens on their surface and that the signal is undetectable in both naïve C57BL/6 wild type and C57BL/6 immunodeficient (Prkdc^{SCID}) recipient-naïve mouse plasma exosomes. First, to validate that we were successfully isolating exosomes using the described methodologies, we performed Western blot analysis of plasma exosome protein content. In both BALB/c and C57BL/6 exosomes, expression of canonical exosome markers flotillin-1 and CD63 was noted, without expression of cytosolic marker cytochrome *c* (Figure 2, A). In addition, BALB/c exosomes specifically expressed H2-K^d MHC compared with C57BL/6 exosomes. To demonstrate MHC-specific surface expression, we performed nanoparticle detector analysis. This showed that MHC class I antigens are expressed on an exosome’s surface, and are specific to the MHC of its tissue counterpart (Figure 2, B).

Heterotopic Heart Transplantation

Given the above results, full MHC-mismatched heart transplants were performed in both study arms. Within the Maintenance arm, recipients showed normal allograft function through follow-up (Figure 3). In the Rejection arm, deterioration in function (by abdominal palpation) was noted with asystole occurring between days 9 and 12 (median, 11 days) (Figure 3). On nanoparticle detector analysis,

FIGURE 2. Donor BALB/c major histocompatibility complex (MHC) is specifically expressed on BALB/c tissues and their exosomes. A, Western blot analysis of plasma exosomes from BALB/c and C57BL/6 samples. H2-K^d specificity was only seen in BALB/c samples, not in C57BL/6 samples. Samples tested on Western blot showed exosome markers CD63 and flotillin-1, but absence of cellular/apoptotic body marker cytochrome C. B, Nanoparticle detector analysis for anti-BALB/c (H2-K^d) antibody specificity. A distinct H2-K^d signal was seen in BALB/c donor plasma samples, not in C57BL/6 samples or immunoglobulin G (IgG) isotype controls.

FIGURE 3. Allograft viability by Kaplan-Meier test. Animals were followed for cardiac allograft viability 1 to 30 days.

total plasma exosome distribution and quantity was similar at all measured time points in the Maintenance arm (Figure 4, A) and donor BALB/c heart exosome signal distribution was also similar throughout follow-up (Figure 4, A). In the Rejection arm, total plasma exosomes were similar at all time points, but the donor heart exosome signal decreased significantly by day 2, further dropped by day 3, and remained low at levels equivalent to naïve recipient controls (Figure 4, B).

Donor Heart Exosome Signal Herald Early Acute Rejection

Having noted changes in donor heart exosome signal with immunologic rejection, we assessed the time sensitivity and accuracy of the transplant tissue exosome platform in this model. First, we checked whether total plasma exosomes show immunologic rejection-specific or time-specific changes in their profiles. In both study arms, at all tested time points, including pretransplant samples, total plasma exosome quantities were similar ($P = .278$ for the Rejection arm and $P = .157$ for the Maintenance arm by omnibus testing) (Figure 5, A). This demonstrated that total plasma exosome quantitation was neither sensitive nor specific for noninvasive monitoring of the cardiac allograft conditional status, and immunologic rejection does not alter total plasma exosome quantity or size distribution.

Next, we assessed the accuracy of donor heart exosome profiling. To validate specificity, we tested for BALB/c exosome signal in naïve pretransplant C57BL/6 wild type and C57BL/6 immunodeficient animals, and noted absence of a BALB/c exosome signal (Figure 5, B pretransplant values). However, a donor exosome signal was detectable through all posttransplant time points in the Maintenance arm ($P = .538$ by ANOVA), validating signal specificity (Figure 5, B). The donor heart exosome signal remained stable over follow-up, suggesting that stable allograft function without rejection leads to a stable donor heart exosome

signal in recipient plasma. Given this, we assessed whether immunologic rejection would lead to changes in the transplant heart exosome signal. We compared the kinetics of plasma donor heart exosomes during allograft rejection to time-matched samples in the Maintenance arm (Figure 5, B). On day 1, donor heart exosome signals were similar between the 2 groups ($P = .280$); however, a significant decrease in the signal was noted by day 2 in the Rejection arm ($P = 2 \times 10^{-4}$). Donor exosome signal further decreased on day 3 ($P = 3 \times 10^{-6}$ compared with day 1; $P = 6 \times 10^{-5}$ compared with day 2), but was unchanged in the Maintenance arm. The donor exosome signal remained low and unchanged after day 3 in the Rejection arm ($P = .217$ for day 3 to day 30 by ANOVA), similar to the pretransplant levels (Figure 5, B). To statistically further validate the diagnostic potential of the donor heart exosome platform compared with total plasma exosome analysis, we performed permutation testing of the total exosome values and donor heart exosome signal values in the Rejection and Maintenance arms. Results of permutation testing showed no differences in total exosome quantities over follow-up within each group and between the 2 study arms. But it validated that there was a significant difference in donor heart exosome signals between days 1 to 2, 1 to 3, and 2 to 3 (Table 1). Taken together, this demonstrated that donor heart exosome characterization enables noninvasive detection of early acute rejection with high sensitivity and specificity in this model.

Because the current standard for monitoring rejection in cardiac transplantation is based on histologic grading of ventricular EMB, we investigated the biomarker potential and time sensitivity of the transplant tissue exosome platform in comparison to histology-based grading criteria of acute rejection. Allograft myocardium was analyzed by hematoxylin and eosin staining and by immunohistochemistry for T cell infiltration using anti-CD3 antibody. On the Maintenance arm, no signs of allograft injury or T cell infiltration was noted at any of the time points (Figure 6, A). Within the Rejection arm, grade 0 rejection was noted for days 1 and 2 (Figure 6, B), when the donor heart exosome signal had already significantly decreased (Figures 4, B and 5, B). Grade 1R to 2R rejection was seen by day 5 and progressed to grade 3R rejection by day 9. Multifocal T cell infiltration was evident in day 7 to 9 histology specimens. These histologic results were in accordance with the transplant heart functional status, which revealed median time of allograft activity of 11 days. Collectively, these results demonstrate that donor the heart exosome signal significantly decreases before histologic evidence of early myocyte injury and T cell infiltration (grade 1R).

Given these promising results, we generated a ROC curve to statistically assess the biomarker potential of the transplant heart exosome platform to predict early acute rejection (<1R). We generated ROC curves for a binary

FIGURE 4. Recipient plasma exosome profiles during acute rejection on the NanoSight Nanoparticle detector (Malvern Instruments Inc, Malvern, United Kingdom). Total recipient plasma exosome quantities (blue) versus recipient donor major histocompatibility complex (MHC)-I-specific pool (red) normalized to exosome protein quantity. A, Maintenance arm. B, Rejection arm.

outcome using all Rejection arm time points (day 1-30) as rejection, and all Maintenance arm time points (day 1-30) as no rejection. Area under the curve (AUC) values for total exosome quantity (AUC, 0.659 ± 0.080), median exosome size (AUC, 0.677 ± 0.085), and mean exosome size (AUC, 0.679 ± 0.083) showed poor diagnostic accuracy (Figure 7). Donor heart exosome profiling showed that a signal threshold cut-off < 0.3146 was 91.4% sensitive and 95.8% specific (AUC, 0.934 ± 0.030) with a likelihood ratio of 21.9 for diagnosing acute rejection in this model (Figure 7). Taken together, this validated the superior biomarker potential of donor heart-specific exosome

profiling for time-sensitive diagnosis of early rejection, compared with whole plasma exosome analysis.

DISCUSSION

The clinical gold standard for diagnosis and surveillance of acute and chronic cellular rejection remains histologic analysis by EMB. Unlike other fields, such as kidney and liver transplantation, where circulating creatinine levels and liver enzyme parameters are routinely used in clinical settings as biomarkers to monitor graft organ status, although with limited accuracy, to date no biomarker-based platform has been widely accepted in the clinical

FIGURE 5. Acute rejection leads to distinct changes in donor pool but not total exosome quantity. Each data point represents a single animal. A, There were no differences in total plasma exosome quantity ($P = .278$ for rejection arm and $P = .157$ for maintenance arm). B, On day 1, donor heart exosome signals were similar between the 2 groups ($P = .280$); however, a significant decrease in the signal was noted by day 2 in the Rejection arm ($P = 2 \times 10^{-4}$). Donor exosome signal further decreased on day 3 ($P = 3 \times 10^{-6}$ compared with day 1; $P = 6 \times 10^{-5}$ compared with day 2), but was unchanged in the Maintenance arm. With acute rejection, donor exosome signal remained low and unchanged after day 3 ($P = .217$ for day 3 to day 30), similar to the pretransplant levels.

setting of heart transplantation. Although there are few biomarker platforms that are being studied, such as peripheral blood mononuclear cell gene expression profiling (AlloMap [CareDx, Brisbane, Calif]⁸) and donor-derived-cell free DNA quantitation,⁸ these methodologies have not infiltrated clinical practice to replace EMB as the first-line diagnostic tool. Measurement of circulating protein markers such as creatine kinase, creatinine kinase-myocardial band, and troponin as biomarkers, which are routinely used for diagnosis of myocardial infarction, are of little value for timely, accurate diagnosis of acute cardiac

allograft rejection.⁹ Our investigation proposes an entirely different and novel biomarker platform in heart transplantation based on profiling of transplant tissue-specific exosomes from recipient circulation. In this study, we report the first validation of this concept in a murine heart transplantation model. We demonstrate that a cardiac allograft releases a distinct pool of donor MHC-specific exosomes into recipient circulation that is maintained at a steady state under conditions of allograft acceptance and immunodeficiency; circulating donor heart exosomes can be tracked and quantified reliably under conditions of allograft maintenance; and acute immunologic rejection leads to time-sensitive and accurate changes in donor heart exosome profiles, thus enabling noninvasive monitoring for condition specific changes causing allograft injury. We believe that these promising results warrant further detailed investigations into the biomarker potential of this platform in cardiac transplantation diagnostics.

Two other biomarker platforms are being extensively studied for noninvasive cardiac allograft monitoring. The AlloMap test, based on profiling of expression of 11 genes in peripheral blood mononuclear cells, was studied in a randomized trial for noninferiority to EMB in distinguishing grade 0 (quiescence) from grade $\geq 2R$ rejection (moderate to severe rejection).¹⁰ The study excluded patients in the first 6 to 12 months after heart transplantation, when the risk of acute cellular rejection is greatest.¹⁰ The test correctly identified 84% of moderate-to-severe rejection episodes.¹⁰ Although the study reported noninferiority on their composite primary outcome, the results showed that the gene-expression profiling strategy can be associated with up to 68% increase in risk (hazard ratio, 1.04; 95% confidence interval, 0.67-1.68).¹⁰ Furthermore, of the 34 rejection episodes detected in the patients randomized to AlloMap testing surveillance, only 6 rejection episodes were detected on the basis of elevated gene expression score.¹⁰ Therefore, the AlloMap test has been adopted by some cardiac transplant programs for monitoring in patients beyond the first 6 to 12 months after transplantation, but it has not gained wide acceptance in the clinical realm to replace EMB.

Another platform being studied is quantitation of circulating donor-derived cell free DNA (ddcfDNA).^{8,11} Unlike the AlloMap test, this platform has potential universal application in transplantation diagnostics because it is based on the concept that acute rejection leads to increased levels of ddcfDNA. Two studies investigating this idea in heart transplant patients showed that ddcfDNA enabled distinction of grade 0 versus moderate-to-severe rejection on ROC analysis with an AUC of 0.83, sensitivity of 58%, and specificity of 93% for ddcfDNA threshold of 0.25%.^{8,11} Whereas the AUC was 0.6 for distinguishing grade 0 versus mild (grade 1R) rejection. Furthermore, the accuracy of the test worsened in patients

TABLE 1. Independent sample *t* test/1-way analysis of variance (ANOVA) omnibus testing versus Monte Carlo permutation data resampling

Variable	<i>P</i> value (ANOVA/ <i>t</i> test)	<i>P</i> value (Monte Carlo permutation)
Total exosome concentration in Maintenance arm (pretransplant to day 30)	.157	.320
Total exosome concentration in Rejection arm (pretransplant to day 30)	.278	.290
Total exosome concentration in Maintenance vs Rejection arm	.254 (by <i>t</i> test)	.241 by logistic regression
Donor heart-specific exosome signal day 1 Maintenance vs day 1 Rejection	.280	.552
Donor heart-specific exosome signal Maintenance arm (day 1-30)	.538	.336
Donor heart-specific exosome signal day 1 vs day 2	.0002 (2×10^{-4})	.0076 (7.6×10^{-3})
Donor heart-specific exosome signal day 2 vs day 3	.00006 (6×10^{-5})	.004 (4×10^{-3})
Donor heart-specific exosome signal day 1 vs day 3	.000003 (3×10^{-6})	.0045 (4.5×10^{-3})
Donor heart-specific exosome signal Rejection arm (day 3-30)	.217	.265

of older age. Therefore, although the ddcfDNA platform holds promise for development of noninvasive diagnostic platform in heart transplantation, these data demonstrate that other novel biomarker platforms should be carefully investigated.

Conceptually, profiling circulating cardiac allograft exosomes encompasses the strengths associated with the above noninvasive diagnostics, and also addresses some of the concerns associated with them. Similar to ddcfDNA, our proposed platform also has potential universal application. Recently, we reported on the diagnostic potential of profiling transplant islet tissue-specific exosomes as a novel biomarker platform in islet transplantation, and validated

extension of this idea in kidney transplantation because we successfully quantified donor kidney-specific exosomes from recipient blood and urine.⁴ Unlike ddcfDNA quantities, which exist at very low levels in the blood and require relatively complex methodologies, donor heart exosome signal levels were easily detectable using simple methodologies in this study. Whereas other studies distinguished grade 0 from moderate-to-severe rejection, our proposed platform enabled detection of early stages of acute rejection with high accuracy. Lastly, donor heart exosome profiling provides another layer of characterization that might enable further improvement of its accuracy and time sensitivity. In addition to quantitative changes, the proteomic and RNA

FIGURE 6. Histologic International Society for Heart and Lung Transplantation (ISHLT) grading of acute allograft rejection. Corresponding hematoxylin and eosin sections are shown with CD3 stains. A, maintenance arm. B, rejection arm.

FIGURE 7. Characterization of test accuracy. Receiver operating characteristic (ROC) curves for donor heart-specific exosome signal (AUC, 0.934 ± 0.030), total exosome quantity (AUC, 0.659 ± 0.080), median exosome size (AUC, 0.677 ± 0.085), and mean exosome size (AUC, 0.679 ± 0.083). Donor heart exosome profiling showed that a signal threshold cut-off $<0.3146\%$ was 91.4% sensitive and 95.8% specific for diagnosing acute rejection.

(especially microRNA) cargoes of exosomes are also altered based on the conditional stimulus or stress placed on their tissue counterparts. MicroRNAs are small, noncoding RNAs that bind to the 3' untranslated region of the target messenger RNA. Although outside the scope of this study, we have previously demonstrated that acute rejection of transplanted tissue leads to distinct changes in their exosome protein and RNA signatures.⁴ Future studies analyzing the exosome cargoes of transplanted heart-specific exosomes might reveal distinct microRNA and proteomic markers associated with cardiac allograft rejection. If so, this would further improve the accuracy and time sensitivity of the proposed noninvasive platform.

Toward a better understanding of microRNA cargo profiles in the context of solid organ transplantation, a recent study by Dewi and colleagues¹² found a significant increase of exosomal microRNA miR-142-3p content in the total serum exosome pool of heart transplant recipients under conditions of acute cellular rejection versus no rejection. Acute cellular rejection was also associated with differential regulation and enrichment of miR-92a-3p and miR-339-3p.¹² Further analyses suggested that miR-142-3p, a hematopoietic tissue-specific microRNA, is released from T cells upon stimulation by means of exosomal transport (miR-142-3p significantly increased in the exosomal fraction but not in supernatant of T cell culture) and could modify expression of its target gene messenger RNA, RAB11FIP2, in endothelial cells.¹² Such a T cell-endothelial cell axis via an exosomal shuttle may contribute to T cell-mediated graft rejection. The ability of exosomes to shuttle functional microRNA between cells implicates their functional roles in gene regulation, gene expression, and intercellular communication. Future studies characterizing transplant heart exosome RNA cargoes may facilitate understanding of their diagnostic potential, and their functional roles in the immunologic rejection process. In fact,

studies have shown that exosomes might play a significant role in donor antigen trafficking and in the formation of the immune synapse.^{13,14}

Lastly, although we hypothesized and anticipated that donor heart exosome signal would change with acute rejection, we were a little surprised to see significant decrease in the signal occurring so early in the rejection process, when histology still showed grade 0 (quiescence) rejection without any myocyte damage. In accordance with these findings, we also noted similar changes in donor exosome profiles in a murine xenogeneic islet transplantation model, where transplant tissue exosome levels decreased before any injury to the transplanted islet mass was seen.⁴ Because the output we are measuring is circulating transplant exosome levels, mechanistically it is not known whether the signal drop early during rejection is due to decreased production of exosomes by the transplanted tissue, increased consumption of the transplant exosomes by recipient immune cells or other cell types, or a combination of both. If this is a consumptive process mediated by immune cells, then to some extent the data in the Maintenance arm argue against this idea because the recipients in this group were deficient of T cells and B cells, and yet the transplant heart exosome levels reached steady state by day 1 and remained at the same level as day 1 levels in the Rejection arm, without further increase. One would expect the levels to rise more in an immunodeficient state, rather than reaching steady state so early. A recent report showed that as part of the acute rejection process, donor exosomes are important for presentation of donor antigens to recipient alloreactive T cells in lymphoid organs by the recipient dendritic cells in a phenomenon known as cross-dressing.¹³⁻¹⁶ Quite possibly, this mechanism of alloreactive T cell activation, which can occur early during the rejection process, may mediate suppression of further production of exosomes by the transplanted tissue even before there is targeted injury to the allograft. Because exosomes are important mediators of donor tissue-immune cell interactions, future studies investigating the mechanistic aspects of this interface may open avenues for targeted therapeutics using donor-specific exosomes to manipulate the recipient immune response. Lastly, in this investigation we examined immunologic rejection-specific changes in transplant heart exosomes, but we are cognizant that recipient immune cell-specific exosome profiling might further improve the diagnostic accuracy of tissue-specific exosome platforms. During injury to a transplanted heart with acute rejection, there is also stimulation of alloreactive T cells, which may lead to increased production of T cell exosomes. Concomitant characterization of T cell-specific exosome profiles would further enhance accuracy of this platform. T cell exosome cargo analysis may also provide mechanistic insights into the physiologic roles of exosomes in the immune synapse.

Study Limitations

Although we compared the proposed platform with other noninvasive platforms currently being studied in heart transplantation, we recognize that other platforms have been analyzed in clinical settings, our report is the first investigation of a novel concept in a small animal model, and it was done from a conceptual standpoint only. Therefore, drawing comparisons of the findings in our study to other biomarker platforms should be seen in this light. Given that this is the first investigation of the proposed concept, we designed a relatively simple model of cardiac allograft rejection, where immunodeficient recipient mice were used for the Maintenance arm. In a clinically more relevant model, future studies will analyze donor heart exosome profiles in immunocompetent recipients on maintenance immunosuppression to understand rejection-specific changes.

CONCLUSIONS

We report the first validation of a novel biomarker platform based on transplant tissue-specific exosome profiling that may have clinical translational potential in the field of cardiac transplantation diagnostics. Initial investigation in an animal model demonstrated that donor heart exosome profiles predict early acute rejection with high accuracy. These results warrant further investigation of this concept in greater detail in animal models of heart transplantation, and validation and translation of this concept in clinical settings.

Webcast

You can watch a Webcast of this AATS meeting presentation by going to: https://aats.blob.core.windows.net/media/17AM/2017-05-02/RM310/05-02-17_Room310_0730_Habertheuer.mp4.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: heart transplantation, MHC mismatch, allograft rejection, donor-specific exosomes, exosome profiling

Discussion

Dr Keki Balsara (St Louis, Mo). That was a wonderful presentation in which you really used a very simple and elegant model of transplantation to help answer an important question, which is how can one detect early acute rejection in a noninvasive fashion. I have a couple of questions for you.

The first one is, can you speak a little bit to the mechanism by which these major histocompatibility complex exosomes are being expressed, specifically trying to determine if this is driven purely by rejection or are there other drivers at play that might confound some of the information that you have uncovered?

Dr Habertheuer (*Philadelphia, Pa*). In terms of exosome release, we know that exosomes are formed in the so-called multivesicular endosome and that exosomes then are released by fusion with the cellular membrane. Because exosomes basically traverse a lot of major cellular compartments, exosomes then

carry many cell-specific markers from their tissue of origin, such as major histocompatibility complex molecules.

In terms of change of that signal, we really don't know exactly whether the organ releases fewer exosomes if it is stressed or if the consumption of exosomes increases due to the rejection event. That is an important question for us to understand in future studies, to understand what actually happens to those exosomes.

But the theory of exosomes actually being absorbed by an immune cell, the maintenance arm in these animals that are deprived of B and T cells argues a little bit against that. So it is more likely that the release of exosomes is decreased if the organ is stressed.

Dr Balsara. I realize this is a heart transplant model, but has it been examined in other solid organ transplants? Is this something that is specific to heart transplant or is it across all solid organ transplants?

Dr Habertheuer. We believe the beauty of that platform is that it actually could be universally used in other transplant types. To our knowledge and because that was our idea, that was not studied by other groups, and what we did is we followed kidney transplant patients, as an example, for solid organ transplant. I think we did 5

patients over the course of 6 months; none of those patients has rejected so far. But in the setting of clinical islet transplantation, we followed patients over the course of a couple of years and we were able to detect rejection with the same sensitivity and specificity as presented in the mouse heart transplant model and in this very study, well before glucose parameters became abnormal. So it seems to us that it is a platform that can be universally used in transplantation.

Dr Craig Selzman (*Salt Lake City, Utah*). Two things. One would be related to antibiotic-mediated rejection, this is mostly cellular, so any thoughts that you have with this technology? Also, what is your perspective on this versus AlloMap (CareDx, Brisbane, Calif), which is

Food and Drug Administration approved? It is a different way of looking at it, but how would you see these 2 technologies working?

Dr Habertheuer. To answer the question with AlloMap first, and I am referring to the New England trial, the AlloMap test has a low sensitivity, to my knowledge, and it was approved for differentiation of no rejection versus grade 2R, so moderate-to-severe rejection only. In that New England trial, it correctly identified only 6 out of 34 patients based on the test alone. So that is actually quite low sensitivity for a biomarker platform. We showed in that test that our sensitivity and specificity is much, much higher, although we are not at the level yet where we compete with AlloMap.