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Circulating Donor Heart Exosome Profiling Enables Noninvasive Detection of Antibody-mediated Rejection

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Background. Endomyocardial biopsy remains the gold standard for distinguishing types of immunologic injury—acute versus antibody-mediated rejection (AMR). Exosomes are tissue-specific extracellular microvesicles released by many cell types, including transplanted heart. Circulating transplant heart exosomes express donor-specific human leukocyte antigen (HLA) I molecules. As AMR is mediated by antibodies to donor HLAs, we proposed that complement deposition that occurs with AMR at tissue level would also occur on circulating donor heart exosomes. **Methods.** Plasma exosomes in 4 patients were isolated by column chromatography and ultracentrifugation. Donor heart exosomes were purified using anti-donor HLA I antibody beads and complement C4d protein expression was assessed in this subset as marker for AMR. **Results.** Three patients had no rejection episodes. Circulating donor heart exosomes showed troponin protein and mRNA expression at all follow-up time points. One patient developed AMR on day 14 endomyocardial biopsy that was treated with rituximab, IVIG/plasmapheresis. Time-specific detection of C4d protein was seen in donor heart exosome subset in this patient, which resolved with treatment. C4d was not seen in other 3 patients' donor exosomes. **Conclusions.** Anti-donor HLA I specificity enables characterization of circulating donor heart exosomes in the clinical setting. Further characterization may open the window to noninvasively diagnose rejection type, such as AMR.

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INTRODUCTION

Cardiac allograft rejection remains one of the major causes of morbidity and mortality in heart transplantation. In the perioperative period and during the first year of transplantation, acute cellular rejection (ACR) episodes can occur in up to 30% of the cases.[1] As more patients with previous exposure to blood transfusions and with higher panel reactive antibodies are undergoing heart transplantation, antibody-mediated rejection (AMR) is also being increasingly diagnosed. Recent consensus statements and more standardization of diagnosis, classification, and treatment of AMR over the past 15 years[2-4] may have also improved diagnosis of AMR, which was likely under-reported previously. Currently, endomyocardial biopsy (EMB) is the clinical gold standard for post-transplant surveillance, but this invasive technique creates potential complications and significant patient discomfort.[5,6] Recently, cell-free donor-derived DNA assay and genome transplant dynamics have been introduced as candidate methods for noninvasive

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diagnosis of graft injury from the recipient plasma.[7] Even though these noninvasive methodologies have reached clinical application, they have not replaced EMB as the primary modality rejection surveillance in heart transplant patients. Also, treatment of rejection episodes typically mandates repeat EMBs to understand efficacy of treatment. For example, at our institution, a heart transplant patient undergoes 20 surveillance EMBs in the first 2 years post-transplant. Therefore, the field is in critical need for development of other biomarker platforms for cardiac allograft surveillance and monitoring for treatment of rejection episodes.

Exosomes are circulating tissue-specific extracellular microvesicles released by many tissue types, including cardiomyocytes, into the peripheral circulation. Exosome profiles may reflect condition-specific stress/injury imposed on their tissue counterparts.[8-10] In the context of transplantation, we demonstrated that circulating transplant tissue-specific exosome profiles might serve as a noninvasive biomarker of acute rejection.[11-13] In a rodent allogeneic heart transplantation model, donor heart exosome profiles predicted early acute rejection with 100% accuracy,[11] before histologic evidence of grade 1R rejection. Given these findings, we embarked on a pilot study in 4 heart transplant recipients in the perioperative period to investigate whether transplant heart exosomes can be reliably profiled from recipient blood in the clinical setting. Three patients had no ACR or AMR, but 1 patient developed early rise in donor-specific antibodies (DSAs) with AMR seen subsequently by EMB, requiring treatment with rituximab, plasmapheresis, and IVIG.

We hypothesized that circulating donor heart exosomes can be characterized in the clinical setting, and that their cargoes would reflect condition-specific injury to transplanted heart. If so, in the index patient with AMR, donor human leukocyte antigen (HLA) specific antibody-mediated complement deposition that occurs on the donor heart tissue would also occur on the circulating transplant heart exosome surface, as they express identical donor HLA profiles. If so, further investigation of this platform may open the window for noninvasive diagnosis of AMR. We report our initial experience with this platform in the clinical setting.

MATERIALS AND METHODS

Human Plasma Samples

University of Pennsylvania Institutional Review Board approval was obtained for collection and analysis of human plasma samples from 4 subjects undergoing heart transplantation. Plasma samples were collected up to 26 days of perioperative follow-up, along with collection of clinical and EMB data. Informed patient consent was obtained in each case.

Exosome Analysis

Exosomes were isolated from thawed plasma sample by size exclusion chromatography along with ultracentrifugation.[12,13] Exosomes were analyzed on the NanoSight NS300 nanoparticle detector on the light scatter mode for quantification and size distribution according to manufacturer's protocols (Malvern Instruments Inc., Westborough, MA). Cryo-electron microscopy analysis of extracellular vesicles was performed at the University of Pennsylvania core facility using the standard protocol.

Affinity Antibody Coupled Bead Purification of Tissue-specific Exosomes

Anti-donor HLA I-specific antibody was covalently conjugated to N-hydroxysuccinimide magnetic beads (Thermo Fisher Scientific, Waltham, MA) and incubated with 50–100 µg protein equivalent of exosomes overnight at 4°C. The bead-bound exosome fractions were separated per manufacturer's protocol and utilized for downstream analysis.

Western Blot

Exosomes were lysed in 1× RIPA buffer with 1× concentration of protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO). Total proteins were separated on polyacrylamide gels, transferred onto nitrocellulose membrane (Life Technologies, Carlsbad, CA). The blot was blocked, incubated with desired antibodies and detected through chemiluminescence using Image quant LAS 400 Phospho-Imager. Antibodies to troponin T, cytochrome c and secondary antibodies conjugated with horseradish peroxidase for anti-rabbit, anti-mouse were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-flotillin 1, and anti-complement C4d antibodies were purchased from Proteintech (Rosemont, IL).

RNA Analysis

Total RNA (12.5–25 ng) isolated from exosomes specific to donor HLA I was isolated using RNeasy mini kit (QIAGEN, Frederic, MD). RNA was reverse transcribed with the SuperScript III one-step RT-PCR system (Life Technologies) for gene expression validation. The primers used were: human cardiac troponin T (forward) 5′- CAT GGA GAA GGA CCT GAA TGA- 3′, (reverse) 5′- CGT CTC TCG ATC CTG TCT TTG- 3′, and human β-actin (forward) 5′-CTGTACGCCAACACAGTGCT-3′, (reverse) 5′- GCTCAGGAGGAGCAATGATC-3′.

RESULTS

Patient A underwent redo sternotomy, heart transplantation; EMB on day 9 showed no ACR or AMR, with negative DSA. Patient B developed primary graft dysfunction post-transplant and required intraoperative central ECMO initiation. DSA titers were negative, and surveillance EMBs performed on postoperative days (PODs) 14 and 21 did not show ACR or AMR. Patient C, a 48-year-old woman with end-stage sarcoid heart disease underwent orthotopic heart transplantation. Surveillance EMB on POD 7 was negative for ACR or AMR, but blood test on POD 8 showed de novo DSA (results made available on POD 12). Echocardiography on POD 12 showed normal left ventricular ejection fraction of 70%. Repeat EMB on POD 14 showed rejection on hematoxylin & eosin staining (Figure 1A), with C4d deposition in interstitial capillaries consistent with AMR by immunofluorescence. She was immediately initiated on rituximab and IVIG/plasmapheresis therapy. Upon completion of 6 doses, repeat EMB on POD 33 showed resolution of AMR and markedly decreased DSA titers (**Table S1, SDC**, http://links.lww.com/TXD/A283). Patient D required intraoperative central ECMO initiation with open chest for primary graft dysfunction. EMB on day 10 showed low grade 1R ACR, with negative DSA.

We confirmed isolation of an extracellular microvesicle pool enriched in exosomes in all 4 patients, validated by electron microscopy (Figure 1B), nanoparticle detector analysis (Figure 1C), and by staining for canonical exosome markers

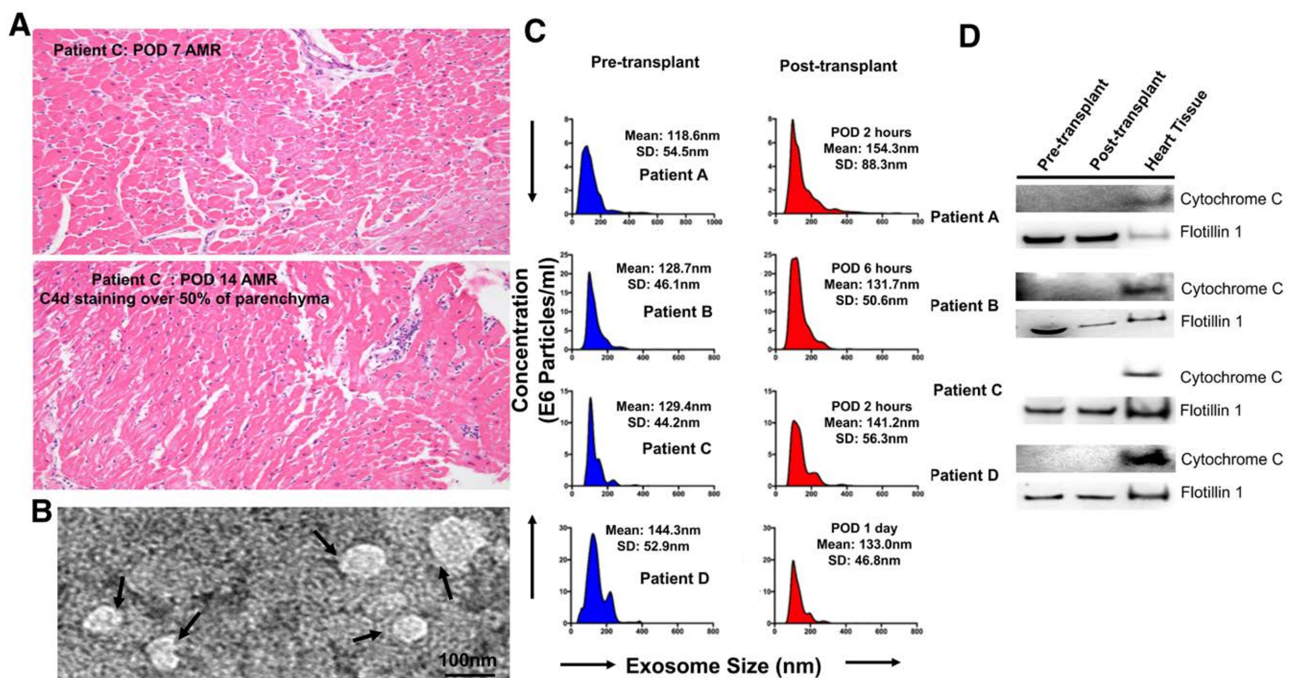

FIGURE 1. Whole plasma exosome profiles are unchanged pre- and post-transplant. A, Endomyocardial biopsy (EMB) on AMR patient, POD 7 and POD 14 showed presence of C4d staining in parenchyma with Hematoxylin and Eosin staining. B, Electron microscopy of plasma extracellular vesicles revealed that the majority of nanoparticles isolated were in the size range of exosomes. C, Representative nanoparticle detector analysis of plasma extracellular microvesicles from the 4 transplant patients are shown. Size distribution of particles is primarily in the exosome range. D, Western blot analysis of plasma microvesicles showed expression of canonical exosome marker flotillin 1 but absence of apoptotic body/cellular debris marker cytochrome c. AMR, antibody-mediated rejection; HLA, human leukocyte antigen; POD, postoperative day.

with absence of cytochrome c (apoptotic body marker) by Western blot (Figure 1D). In assessing total plasma exosome quantity and size distribution (median size 100 nm), there were no significant differences comparing the preoperative to postoperative values, and amongst the postoperative values between patients (Figure 1). Collectively, this suggests that heart transplantation, subsequent to initiation of immunosuppression, and postoperative maintenance on ECMO, does not significantly alter the total circulating quantities and size distribution of exosomes.

We have previously shown that transplant tissue-specific exosomes can be enriched from peripheral blood total exosome pool using anti-donor HLA I-specific antibody-conjugated beads.[11,12] We assessed the validity of this methodology in purifying circulating donor heart-specific exosomes in the clinical setting (Figure 2A). To confirm enrichment of donor-specific exosomes, we assessed for expression of cardiac myocyte specific marker, troponin T, mRNA in the putative enriched transplant heart exosome subpopulation. Troponin mRNA was detectable specifically in the donor heart exosome subset by RT-PCR in the postoperative recipient plasma samples in all 4 patients (Figure 2B–E), but not in pre-transplant and isotype control samples. In patient B, who was maintained on ECMO for the first 8 days, persistent troponin mRNA signal was seen, suggesting a continuous production of exosomes by the transplanted heart, even though the organ was not functional to sustain systemic circulatory demand.

In all 4 cases, we also successfully purified an exosome subset carrying cardiac troponin protein (Figure 2F–I). This demonstrates that the donor heart releases exosomes right after allograft implantation (earliest time point checked was 2 hours after release of aortic cross-clamp), and can be reliably tracked over the postoperative follow-up. Importantly, they carry cardiac tissue-specific markers, including troponin, as part of their intraexosomal cargo. Furthermore, Western blot and RT-PCR analysis suggest that even during primary graft dysfunction, the transplanted heart releases exosomes.

Out of the 4 patients studied, patient C was the only one who developed rejection requiring additional immunosuppressive therapy, with AMR confirmed on day 14 EMB. As hypothesized above, we assessed for time- and patient-specific presence of complement C4d in the circulating transplant heart exosome subset (Figure 2F–I). C4d was first detected on day 7 sample in patient C only (Figure 2H), with possibly a much lower detection on day 14 plasma sample, the latter sample obtained after initiation of AMR therapy. This suggests that donor HLA expressed on circulating transplant heart exosomes may be susceptible to the same process of DSA mediated complement fixation at the tissue level that is crucial in the pathogenesis of AMR. Furthermore, treatment of AMR led to an immediate drop in C4d detection in the donor heart exosome subset, which was consistently undetectable on further follow-up. Correspondingly, DSA titers markedly decreased and remained nearly undetectable with AMR treatment, temporally correlating with C4d detection and loss in the donor heart exosomes. The other 3 patients did not show any signs of AMR by EMB, and did not develop DSA during postoperative follow-up. Accordingly, C4d was not detected in the donor heart exosome subpopulation in patients A, B, and D.

DISCUSSION

This pilot study validates that the methodologies established for characterization of transplant tissue-specific exosomes in animal models can be translated to the clinical

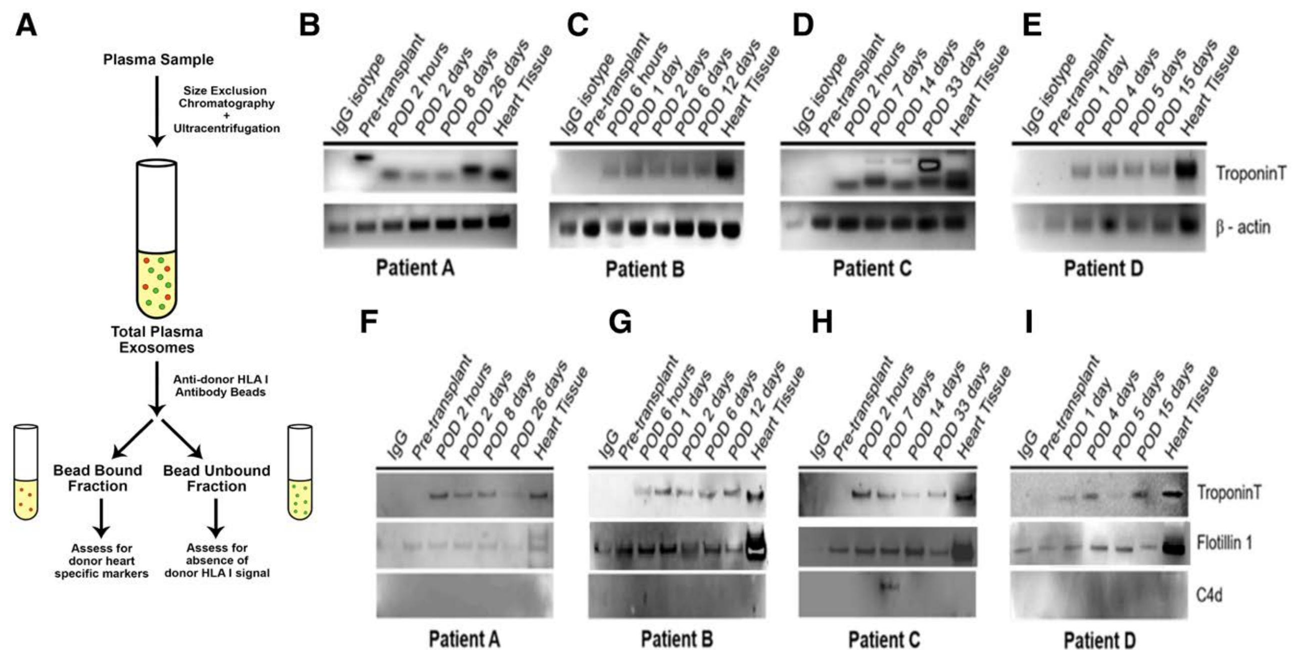

FIGURE 2. Schematic representation of donor-specific exosome capture using anti-donor HLA I antibody conjugated beads is shown (panel A). Circulating donor heart-specific exosomes carry cardiac-specific marker, troponin T, as part of its intraexosomal cargo. Donor specific heart exosomes were enriched from recipient plasma using anti-donor HLA I-specific antibody-conjugated beads. RT-PCR analysis for bona fide cardiomyocyte mRNA marker, troponin T, is shown for all 4 patients (panels B–E). In all cases, IgG isotype antibody-conjugated beads (negative control) and pre-transplant samples did not show any enrichment of troponin T mRNA. In all 4 subjects, transplant heart exosomes showed troponin T mRNA expression in all post-transplant samples. Human heart tissue positive control is shown. β-actin mRNA control is also shown. High de novo DSA titer with EMB positive AMR in Patient C correlates with time- and patient-specific expression of complement C4d in donor heart exosomes (panels F–I). Donor exosome cargo was analyzed for expression of troponin T protein by Western blot analysis in all 4 patients (A–D). In all 4 patients, troponin T expression was seen in post-transplant samples only, as early as 2h post-implantation. Pre-transplant samples and IgG isotype antibody bead-bound fractions (negative control) did not show expression of troponin T. In patient C, where POD 8 blood sample showed de novo DSAs, selective C4d expression was seen in POD 7 donor heart exosome fraction, with markedly decreased expression after initiation of plasmapheresis/IVIG as seen on POD 14 sample. Human heart tissue was used as positive control for troponin T and flotillin. AMR, antibody-mediated rejection; DSA, donor-specific antibody; EMB, endomyocardial biopsy; HLA, human leukocyte antigen; POD, postoperative day.

heart transplantation setting. Furthermore, consistent and specific detection of cardiac troponin in the circulating donor heart exosome subset enables reliable and accurate noninvasive tracking of transplant heart exosomes from the total plasma exosome pool This opens the window to better understand in the future whether transplant heart exosome quantities and, more importantly, their intraexosomal RNA and protein cargoes will accurately reflect condition-specific changes inflicted on the transplant heart, such as AMR, ACR, and myocarditis. In animal transplantation models, we found this to be the case that transplant exosome profiles enabled noninvasive diagnosis of rejection, before histologic evidence of injury to the graft.[11-13]

The findings in this study support our hypothesis that components of the AMR pathogenesis at the tissue level may be reflected in the circulation. Furthermore, as exosomes play a role in immune regulation, understanding transplant tissue exosome protein and RNA cargoes may provide mechanistic insights into the AMR process. In support of this idea, we recently demonstrated that circulating stem cell-specific exosome microRNA cargoes predicted cardiac functional recovery in a stem cell transplant model for treatment of ischemic myocardial injury.[13] If so, this platform would facilitate a more detailed readout of AMR in vivo, then it would enable noninvasive monitoring for AMR and its treatment efficacy.

Interestingly, C4d detection on circulating donor heart exosomes on POD 7 sample temporally correlated with de novo DSA detected on POD 8 sample, whereas EMB performed on POD 7 was negative for AMR. This might suggest that DSAs may bind to allogeneic HLA on circulating transplant tissue exosomes triggering early C4d deposition. DSA capture by circulating exosomes may be one of the ways for allograft protection from AMR. From a mechanistic standpoint, this idea may warrant further investigation.

The index patient with AMR was treated with rituximab, IVIG, and plasmapheresis therapy starting POD 14. Analysis of plasma sample obtained after initiation of therapy showed decreased troponin content in donor heart exosome fraction, along with decreased C4d deposition at time point when EMB was positive for AMR (Figure 2F–I). This might be due to increased clearance of donor heart exosomes by recipient immune system caused by complement deposition, or decreased production of exosomes by the cardiac allograft due to injury secondary to AMR. Better understanding of the temporal relationship between AMR at the allograft tissue level and associated changes in circulating transplant exosome profiles may provide a noninvasive window into this process.

Lastly, we note that transplant heart exosomes were detected in circulation in patients B and D, who developed primary graft dysfunction requiring postoperative arteriovenous ECMO. Patient B was maintained on ECMO for 7 days, and during this time troponin signal at the protein and mRNA levels was detected in donor heart exosome subpopulation. This suggests that a transplanted heart with primary graft dysfunction still releases exosomes into the

peripheral circulation. Future studies analyzing proteomic and microRNA cargoes of transplant heart exosomes during the period of primary graft dysfunction and biventricular recovery may provide a noninvasive window into the mechanisms underlying this condition in solid organ transplantation that is associated with significant morbidity and mortality.

If future investigations of transplant exosome platform show promising results as a candidate noninvasive biomarker for monitoring rejection, it would be important to understand if it would help improve diagnostic accuracy of current platforms. Several research groups have studied the diagnostic role of donor-derived cell-free DNA for solid organ transplants including kidney, heart, liver and lung.[7,14-16] Allomap has been studied extensively, and is utilized in clinical practice, although it has not replaced EMB.[17-19] Combination of these platforms may provide adequate accuracy to help minimize the need for EMB-based monitoring. Compared to Allomap or cell-free DNA which give a single value per time point, an added potential advantage of exosome platform is that it can measure multiple relevant diagnostic parameters for each time point, such as donor exosome quantity, protein cargo, mRNA and microRNA cargoes.

In summary, we report the proof of concept that circulating donor heart exosomes can be characterized in the clinical setting. Analysis of transplant heart exosome cargo may provide a noninvasive window into immunologic processes such as AMR. Future investigations will help elucidate the potential relevance of this platform in transplant diagnostics.

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