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Tissue-associated self-antigens containing exosomes: Role in allograft rejection

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ABSTRACT

Exosomes are extracellular vesicles that express self-antigens (SAgs) and donor human leukocyte antigens. Tissue-specific exosomes can be detected in the circulation following lung, heart, kidney and islet cell transplantations. We collected serum samples from patients who had undergone lung (n = 30), heart (n = 8), or kidney (n = 15) transplantations to isolate circulating exosomes. Exosome purity was analyzed by Western blot, using CD9 exosome-specific markers. Tissue-associated lung SAgs, collagen V (Col-V) and K-alpha 1 tubulin (Kα1T), heart SAgs, myosin and vimentin, and kidney SAgs, fibronectin and collagen IV (Col-IV), were identified using western blot. Lung transplant recipients diagnosed with bronchiolitis obliterans syndrome had exosomes with higher expression of Col-V (4.2-fold) and Kα1T (37.1-fold) than stable. Exosomes isolated from heart transplant recipients diagnosed with coronary artery vasculopathy had a 3.9-fold increase in myosin and a 4.7-fold increase in vimentin compared with stable. Further, Kidney transplant recipients diagnosed with transplant glomerulopathy had circulating exosomes with a 2-fold increased expression of fibronectin and 2.5-fold increase in Col-IV compared with stable. We conclude that circulating exosomes with tissue associated SAgs have the potential to be a noninvasive biomarker for allograft rejection.

1. Introduction

Exosomes are small vesicles (40–100 nm in diameter) generated by reverse budding of early endosomes (ie, multi-vesicular bodies) and secreted by fusion with the cell membrane [1]. All mammalian cells release exosomes; they can be found *in vivo* in most body fluids, including plasma, urine, and saliva. The cargo within an exosome depends upon the cell from which it originated. One function of exosomes is releasing unwanted cell material; .however, they can also transfer proteins, mRNAs, and micro RNAs (miRNAs) to the surrounding milieu. Exosomes from cancer cells have been shown to induce angiogenesis, invasion, and metastasis; they have even been shown to confer drug resistance [2]. After allogeneic heart and skin transplantation, donor-derived exosomes containing donor major histocompatibility complex can cross dress recipient antigen-presenting cells, leading to activation and proliferation of alloreactive T cells via the semi-direct pathway of allorecognition [3,4].

Recent reports from our laboratory [5] and others [6] demonstrated exosomes in the sera and bronchoalveolar lavage (BAL) fluid of human lung transplant recipients (LTxRs) diagnosed with acute and chronic rejection with distinct antigenic properties and presence of mRNA and miRNA. The exosomes isolated from human LTxRs diagnosed with rejection contained not only donor-mismatched human leukocyte antigen (HLA), but also the lung-restricted self-antigens (SAgs) collagen type V (Col-V) and K-alpha 1 tubulin (Kα1T), indicating that exosomes are secreted by transplanted lungs [5]. Another study reported the presence of tissue-specific exosomes following islet cell transplantation, and suggested that exosomes may be biomarkers that allow noninvasive monitoring of immunologic rejection of islet tissues post-transplant [7].

Studies from our laboratory and others have demonstrated immune responses to tissue-restricted antigens in transplant recipients diagnosed with transplant glomerulopathy (TG) [8], chronic rejection following human kidney transplant [9], and coronary artery vasculopathy (CAV) [10,11] after heart transplantation (HTx). Therefore, we hypothesized that tissue-restricted SAgs expressing exosomes may be present not only in the circulation during or prior to rejection of human

Abbreviations: Ab, antibody; BAL, bronchoalveolar lavage; BOS, bronchiolitis obliterans syndrome; CAV, coronary artery vasculopathy; Col-V, collagen type V; DSA, donor-specific antibodies; FN, fibronectin; HLA, human leukocyte antigen; HTx, heart transplant; HTxR, heart transplant recipient; IgG, immunoglobulin G; Kα1T, K-alpha 1 tubulin; LTxR, lung transplant recipient; miRNA, micro RNA; Myo, myosin; PVDF, polyvinylidene difluoride; RTx, renal transplant; RTxR, renal transplant recipient; SAg, self-antigen; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TG, transplant glomerulopathy; Vim, vimentin

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lung and islet transplantations but also in the circulation during or prior to rejection following renal (RTx) and HTx. In this communication, we demonstrate the presence of exosomes containing Kα1T and Col-V in the sera of human LTxRs diagnosed with bronchiolitis obliterans syndrome (BOS), Col-IV and fibronectin (FN) in renal transplant recipients (RTxRs) diagnosed with TG, and cardiac myosin (Myo) and vimentin (Vim) in HTx recipients (HTxRs) diagnosed with CAV.

2. Methods

2.1. Sample collection

This study was approved by the Institutional Review Boards at Washington University School of Medicine and St. Joseph's Hospital. All patients provided informed consent. Peripheral blood samples were collected from patients who underwent LTx, HTx, or KTx. For this preliminary study, 30 LTxRs, 8 HTxRs, and 15 RTxRs were available. Of the 20 LTxRs, 10 were clinically diagnosed with BOS, and 10 were in stable condition with functioning allografts and 5 patients developed DSA and 5 patients without DSA considered as stable. Of the 8 HTxRs, 5 were diagnosed with CAV; 3 were stable with no evidence of rejection. Nine of the RTxRs had biopsy-proven TG as described by Angaswamy et al. [8], and the remaining 6 RTxRs had a biopsy that confirmed the absence of rejection pathology and a well-functioning kidney (Table 1).

2.2. Exosome isolation

Circulating exosomes were isolated from serum samples (200 µl) using ultracentrifugation followed by sucrose cushion. Purity was confirmed by electron microscopy and CD9 staining was performed as we have described previously [5]. In brief, serum was centrifuged at 2000*g* followed by 10,000*g* for 30 mins at 4 °C to remove cell debris. Following this, serum was diluted with PBS and centrifuged to 100,000*g* for 70 min at 4 °C to isolate exosomes. Exosome pellets were suspended in phosphate buffered saline and exosome concentration was analyzed using the bicinchoninic acid method. Calnexin is used as a negative control [12]. All of the exosomes used in this study did not contain calnexin.

Table 1
Demographic data of patients.

	Chronic rejection	Stable
Lung		
Total No (N = 30)	20	10
Age (Years)	53.3 ± 15.1	51.4 ± 15.8
Male	8 (80%)	5 (50%)
female	2 (20%)	5 (50%)
End stage disease		
COPD	4 (40%)	4 (4%)
IPF	3 (30%)	4 (40%)
CF	3 (30%)	2 (20%)
DSA +	5	
DSA −		5
Heart		
Total No (N = 8)	5	3
Age (Years)	52.5 ± 36.7	56.6 ± 19.7
Male	4 (90%)	3 (100%)
female	1 (10%)	0
Etiology		
CAV	5 (100%)	3 (100%)
Kidney		
Total No (N = 15)	9	6
Age (Years)	41.7 ± 18.1	49.1 ± 21.2
Male	8(90%)	
female	1(10%)	6(100%)
Cause of ESRD		
Diabetes	3	1
PKD	3	2
Reflux/obstruction	1	1
FSGS	2	2

Data represented as mean ± stddev or %. COPD: Chronic Obstructive Pulmonary Disease; IPF: idiopathic pulmonary fibrosis CF: cystic fibrosis; DSA: Donor specific antibodies; CAV: cardiac allograft vasculopathy; ESRD: end stage renal disease, FSGS, focal segmental glomerulosclerosis; PKD, polycystic kidney disease.

2.3. Tissue-associated SAgs

Circulating exosomes were isolated from human LTxRs and analyzed for the presence of the lung-associated SAg, Kα1T, which is a gap junction protein to which antibodies (Abs) develop during rejection; and for the presence of Col-V, which is a sequestrated antigen normally intercalated by Col-I and exposed during rejection after LTx [13]. We selected two self-proteins to which immune responses have been shown to develop by us [10] and by others [11,14] during HTx rejection: cardiac Myo and Vim. For RTxRs, we selected two renal-restricted SAgs: Col-IV, a renal tubular basement membrane protein [15], and FN, to which immune responses have been shown to develop in RTxRs diagnosed with TG [8].

2.4. Western blot analysis

To determine the presence of tissue-associated SAgs in exosomes, we performed Western blot analysis using specific Abs to Col-V, Kα1T, cardiac Myo, Vim, Col-IV, and FN. Ten µg of proteins (lung, heart, and kidney) were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. To detect protein in the isolated exosomes, we used primary Abs to lung-associated SAgs: anti-rabbit Col-V (Abcam, ab7046) and anti-mouse Kα1T (Santa Cruz, 12462-r) immunoglobulin G (IgG); Abs to cardiac-associated SAgs: anti-mouse-Myo (Abcam, ab15-100) and anti-mouse Vim (BD Pharmingen, 550513); Abs to kidney-associated SAgs: FN (Sigma, F3648) and anti-Col-IV (Abcam, ab-6586). Goat anti-rabbit/mouse IgG (Cell Signaling, 7074S; Jackson, 115-035-062) conjugated with horse peroxidase was used as secondary Abs. Blots were developed using enhanced chemiluminescent immunoblot detection kit. J Image Software (NIH) was used for densitometry analysis and semi-quantitation of the signal.

2.5. Statistical data analysis

Graph Pad Prism 6 (GraphPad Software, Inc, CA) was used to perform data analysis. Optical density of exosomes containing lung, cardiac and kidney SAgs as well as for the differences between BOS and stable LTxRs, CAV and stable HTxRs, TG and stable RTxRs were compared using Mann-Whitney or two-tailed student's *t*-test. Statistical data in each cohort was expressed as mean ± standard deviation. P-values less than 0.05 were considered statistically significant in each comparative analysis. The fold changes were calculated after normalization of mean optical density of exosomes containing SAgs with CD-9.

3. Results

3.1. Circulating exosomes isolated from human LTxRs diagnosed with rejection contain the lung SAgs Col-V and Kα1T

To determine whether circulating exosomes were induced in LTxRs diagnosed with chronic rejection (ie, BOS), we isolated exosomes from the sera collected from 10 LTxRs at the time of BOS diagnosis. As shown in Fig. 1A, Western blot analysis demonstrated that all 10 LTxRs who were diagnosed with BOS had significantly increased levels of exosomes containing Col-V ($p = 0.0024$) and Kα1T ($p = 0.0002$) compared with stable LTxRs. CD9 was used as the loading marker for the amount of