JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 5, 2023

EDITORIAL COMMENT

Extracellular Vesicles as Bridges Between Host Immune Cells and Graft Organ During Cellular Rejection*

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The enrichment of cellular secreted extracellular vesicles (EVs) from body fluids, including small EVs of endosomal origin (namely exosomes) and microvesicles budding from cell membranes, has emerged as a powerful tool to improve the sensitivity and speed of downstream multiomics analysis including proteomics, transcriptomics, lipidomics, and the like. Such "ExosOMICS" on isolated EVs is being explored by researchers in a range of diseases for sensing clinically relevant biomolecules at low concentrations, thus enabling the detection of new biomarkers and the study of protein-protein interactions. As in conventional immunoprecipitation experiments to enrich specific proteins, biocomplexes are captured by means of isolating nanosized vesicles with different methods (eg, ultracentrifugation, size exclusion chromatography, immunocapture) and are subsequently digested into monomers (peptides, microRNA, lipids) that are analyzed and referenced to a larger database. For EV-enclosed biomolecules, this allows us to break the limit-of-detection barrier, reducing the presence of contaminants that can be nonspecifically retrieved from the biological sample.

By following this approach and using proximity extension assays, which allow the targeting of 92 immune-related proteins as a downstream analysis, in this issue of *JACC: Basic to Translational Science,* Celick et al[1] have found that tumor necrosis factor-related weak inducer of apoptosis (TWEAK) was significantly elevated in circulating blood-derived EVs of patients undergoing acute cellular rejection (ACR) following cardiac transplantation. Interestingly, this finding was masked when analyzing general serum levels of circulating TWEAK, and no correlation between serum and EV levels of TWEAK could be detected.[1] Their paper clearly showed that EV-TWEAK has a role in the progression of ACR. They found that EV-associated TWEAK may induce canonical nuclear factor κ-light chain enhancer of activated B-cell signaling via the fibroblast growth factor-inducible 14 (Fn14) receptor, which is mainly expressed on the surface of cardiac fibroblasts of the graft organ. Moreover, EV-associated TWEAK can induce gene expression in endothelial and smooth muscle cells, potentially leading to increased leukocyte adhesion and chemotaxis, as well as the release of important soluble factors (MIF and SERPINE1), that have been linked to ACR progression.

The hypothesis of intercellular communication via secreted vesicles had its infancy in the seminal work by Raposo et al,[2] who first described that B-cell lymphocytes secrete EVs carrying membrane-bound molecules essential for the adaptive immune response.[2] Another report also showed that dendritic cells secrete EVs, which carry functional immune agents promoting antitumor responses in mice.[3] Together, these results formed the basis for unveiling EV-based cell-free mechanisms of cell-cell communication and interaction. The EV-mediated cell-cell signaling described by Celick et al[1] reveals typical "juxtacrine" signaling that involves close membrane-bound protein contact with the binding

*Editorials published in *JACC: Basic to Translational Science* reflect the views of the authors and do not necessarily represent the views of *JACC: Basic to Translational Science* or the American College of Cardiology.

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The author attests they are in compliance with human studies committees and animal welfare regulations of the author's institution and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2023.01.015

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 8, NO. 5, 2023
MAY 2023:457–459

of a ligand on one surface to a receptor on another adjacent surface. Indeed EVs have a topology similar to cells that can help such an interaction, with extravesicle domains that can act as receptors and/or ligands on the outside and enclosed proteins and as nucleic acids in the luminal compartment.[4] The potential role of EVs in mediating such pathways has been studied as a new mechanism for Notch signaling, which is an evolutionary, well-conserved cell-cell contact mechanism involved in a variety of developmental processes and angiogenesis. Notch receptors and their ligands can be carried by EVs as plasma membrane proteins and induce Notch-specific gene expression in target cells at a distance.[5,6] However, the mechanism underlying these effects implies that proteins are transferred into target cells (eg, endothelial cells) and incorporated into their cell membrane via EVs by endocytosis or direct fusion, which differs from the extracellular ligand-receptor interaction occurring in canonical Notch signaling. Celick et al[1] indirectly showed that the EV-enclosed isoform of TWEAK contains the tumor necrosis factor homology domain that binds to the Fn14 receptor and the effect was reverted when the Fn14 blocking mouse monoclonal antibody ITEM-4 was used. Although the mechanism behind such a specific mechanism needs further investigation, the close interaction between the 2 transmembrane proteins (TWEAK/Fn14) seems crucial for eliciting the downstream intracellular effect. This highlights once again that EVs are unique intercellular delivery vehicles. The signaling that they can mediate is multifaced and complex, including not only the release of their contents into recipient cells, but also EV binding to the cell surface, EV-plasma membrane fusion, and uptake by endocytosis, as well as the release of their contents into the extracellular space.[4] Notably, membrane-spanning TWEAK acts differently from its soluble isoform. Indeed, vesicular TWEAK was more potent than soluble TWEAK in inducing MIF release, whereas only soluble TWEAK induced CXCL1 release.[1] The notion that both TWEAK isoforms, the membrane-anchored form and the smaller secreted form that is probably generated by proteolytic processing, are biologically active was already known. The EV-mediated TWEAK interaction with its specific receptor might represent a third, less-explored way of action caused by differences in ligand-receptor interactions, which may bring new insights into the role of this controller factor on several cellular responses, including angiogenesis, proliferation, and induction of inflammatory cytokines. It remains to be clarified in more detail whether vesicles can contain the 249 amino acid transmembrane protein or whether this full-length protein undergoes enzymatic cleavage during its intracellular sorting within EV membranes.

One aspect that requires further assessment is the potential role of EV-TWEAK as a biomarker of ACR in heart transplantation; the study by Celick et al[1] was not designed to assess the diagnostic value of this marker. The number of subjects included was not suitable to discriminate ACR samples from non-ACR samples. Moreover, vesicular and plasma TWEAK have limited specificity for the rejection process, as shown by receiver-operating characteristic curve analyses. However, the fact that EV-TWEAK and not the total amount of circulating TWEAK was significantly increased during ACR highlights the potential role of EV-associated markers as a liquid biopsy to increase the specificity and sensitivity of the clinical diagnosis. Given that TWEAK$^+$ EVs are released by T cells (CD3$^+$) and monocytes (CD16$^+$), which infiltrate the graft during ACR, as well as by endothelial cells (CD31$^+$), one would assume that a combination of these markers on the surface of circulating EVs may result in a specific signature that could increase their relevance in monitoring organ rejection following transplant. Interestingly, among other EV-surface markers, CD3 has been identified as a discriminator between control and ACR subjects,[7] and CD31 was directly and independently associated with the risk of fatal cardiovascular events and further increased during an acute cardiovascular event.[8] Finally, EV-specific tetraspanins are slightly expressed in patient-derived TWEAK$^+$ EVs (7%-8% were also CD9$^+$ and CD63$^+$). It follows that TWEAK-bearing EVs represent a particular subfraction of vesicles that should be better classified and characterized. The fact that vesicles were isolated from different starting materials, including serum, plasma, and conditioned cell medium, increases the uncertainty as to which type of vesicle is responsible for the effect. However, this weakness does not diminish the role of TWEAK$^+$ EVs as a potential pathophysiological culprit and therapeutic target in ACR pathogenesis and progression.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

Prof Barile was supported by research grants from Fondazione Leonardo, Lugano (FScBioMed_2020) and by the Swiss National Science Foundation (grant CRSII5_202302), Switzerland.

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KEY WORDS acute cellular rejection, extracellular vesicles, heart transplantation, TWEAK