Syncytiotrophoblast Extracellular Microvesicles in Preeclampsia

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Abstract

Preeclampsia, a placental disease, is typically characterized by hypertension and proteinuria in pregnant mothers. There is a need for improved noninvasive detection and diagnosis of this condition. Extracellular microvesicles (EVs), including exosomes, are tissue specific nanoparticles released by many tissue types including the placenta into peripheral circulation. Tissue specific EVs have potential to serve as biomarkers, and their cargoes are dynamic and may reflect functional activity of their tissue counterparts. Several groups have reported an increase in whole plasma EVs in healthy pregnant women in comparison to healthy nonpregnant women, leading to the question of, how does the preeclampsia EV profile differ? In our analysis of this condition, there was no difference in total EV quantity between samples from healthy pregnant females versus those with preeclampsia. However, when we assessed for syncytiotrophoblast EVs (STEVs) using syncytin-1 as the expressed EV surface marker for placental tissue specificity, preeclampsia subjects had significantly lower circulating STEV signal, suggesting that STEV profiles have potential to serve as a noninvasive diagnostic of preeclampsia. STEVs may also play a role in the underlying preeclampsia mechanism, where STEV protein and microRNA cargoes mediate intercellular communication in maternal tissue resulting in the onset of the disease. Coordinated, detailed investigations of STEV cargoes during early pregnancy will need to be performed to understand their potential functional roles. Overall, STEV cargo profiles provide a promising insight into the diagnosis of placental injury, particularly preeclampsia, with an opportunity to noninvasively understand their functional implications.

Keywords: Preeclampsia, Extracellular vesicles, Exosomes, Biomarkers, Syncytin-1

Introduction

This short communication is a commentary on Levine and Habertheuer’s paper titled, “Syncytiotrophoblast extracellular microvesicle profiles in maternal circulation for noninvasive diagnosis of preeclampsia” published in: Scientific Reports – Nature [1].

Preeclampsia is a major cause of mortality and morbidity in expecting mothers and is one of the most common causes of pregnancy complications and premature birth [2]. Along with eclampsia, it is directly associated with 10-15% of maternal deaths [2]. Currently, it is diagnosed during the second or third trimester after clinical symptoms are displayed, but studies have attempted to understand the pathophysiologic mechanism underlying preeclampsia, noting its beginnings in the first trimester. Although there is no defined cause nor is there a cure for the disease, it is widely agreed that preeclampsia stems from the placenta and is related to a decrease in uteroplacental blood flow and hypoxia [3].

Attempts at predicting and diagnosing preeclampsia has so far been unsuccessful, with most studies investigating circulating free proteins or microRNAs. There is a need for improved noninvasive diagnosis and monitoring for preeclampsia outside the current clinical parameters. Such a biomarker would allow for preventative treatments to keep both the mother and fetus safe. It is well known that placental syncytiotrophoblasts at the maternal-fetal
interface release nanoparticles, including extracellular microvesicles (EVs), including exosomes, into the maternal blood during pregnancy [1]. Maternal EVs may provide a window for development of noninvasive biomarker platform in aiding in diagnosis and earlier surveillance of preeclampsia [4-16].

Although the exact definition of the size of microvesicles remains variable, it is well established that exosomes are nanovesicles derived from the multivesicular body ranging in size from 30 to 150 nm. They are released by most cell types and are believed to participate in cell-to-cell communication [17,18]. The first discovery of exosomes was made in the mid-1980s, where membrane-associated elements were selectively released in multivesicular body-derived circulating vesicles in maturing mammalian reticulocytes [19-21]. The first report of exosomes carrying RNAs was made in 2007, and it is now known that exosomes represent stable and tissue-specific proteomic and RNA signature profiles, including proteins, lipids, mRNAs, and microRNAs, that can reflect the conditional state of their tissue of origin [22]. Exosomes are vesicles released by many tissue types and can be found in most bodily fluids including blood, urine, bronchoalveolar secretions, saliva, and amniotic fluid [22-27]. Although exosomes were initially thought to serve as cell debris to dispose of unwanted components, increasingly investigations have demonstrated its influence on physiological and pathological processes as well as its association with diseases and treatments, implying their potential as a diagnostic tool [17].

Previously, our group investigated tissue specific EVs as a noninvasive diagnostic for early rejection in models of islet, heart, and lung transplantation [28-31]. We also recently developed methodologies for enrichment of a subpopulation of pancreatic islet β exosomes, where we can reliably track an insulin specific signal in this exosome subset [data not published]. Here, we hypothesized that syncytiotrophoblast EVs (STEVs) can serve as a biomarker for predicting the onset of placental injuries such as preeclampsia. Other groups have investigated EVs and STEVs in this context, and they have shown that healthy maternal plasma has an increase in total EVs compared to plasma from nonpregnant female. Reports have also shown that expression levels of placenta specific EVs, those expressing placental proteins syncytin-1, syncytin-2, and/or PLAP, are altered in pregnant patients experiencing placental disorders in comparison to healthy pregnancies (Table 1) [32-40].

STEVs are specifically released by the fetal-derived syncytiotrophoblast layer at the maternal-fetal interface. Therefore, profiling their quantitative and intraexosomal

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**Table 1: Analyses of preeclampsia EVs in human samples.**

| Reference number | Author                  | Year Published | Exosome Analysis Type | Methodology                                                                 | Protein Marker Used                      | Number of subjects studied |
|------------------|-------------------------|----------------|----------------------|----------------------------------------------------------------------------|------------------------------------------|----------------------------|
| 1                | Levine, et al.          | 2020           | Tissue specific      | Western Blot and Nanoparticle detector                                   | Syncytin-1                                | 44                         |
| 4                | Bersinger, et al.       | 2003           | Tissue specific      | ELISA                                                                    | PAPP-A                                    | 38                         |
| 34               | Pillay, et al.          | 2016           | Tissue specific      | Nanoparticle detector                                                   | PLAP                                      | 60                         |
| 35               | Salomon, et al.         | 2017           | Tissue specific      | Nanoparticle detector and RNA analysis                                   | PLAP                                      | 47                         |
| 37               | Tan, et al.             | 2014           | Whole plasma         | ELISA, Antibody array, and Mass spectrometry                            | CD9, VEGFR1, BNP, ANP, PLGF               | 22                         |
| 39               | Vargas, et al.          | 2011           | Tissue specific      | Cell Fusion Assay, Real-Time RT-PCR, and Western Blot                   | Syncytin-1, Syncytin-2                    | 24                         |
| 40               | Vargas, et al.          | 2014           | Tissue specific      | Western Blot, RT-PCR, ELISA, and Flow Cytometry                          | Syncytin-1, Syncytin-2                    | 22                         |
| 48               | Dragovic, et al.        | 2015           | Tissue specific      | Nanoparticle detector, Western Blot, and Flow Cytometry                  | PLAP                                      | 8                          |
| 51               | Dragovic, et al.        | 2013           | Tissue specific      | Flow Cytometry, Nanoparticle detector                                   | PLAP                                      | 10                         |

**References:**

1. Levine, et al. 2020
2. Bersinger, et al. 2003
3. Pillay, et al. 2016
4. Salomon, et al. 2017
5. Tan, et al. 2014
6. Vargas, et al. 2011
7. Vargas, et al. 2014
8. Dragovic, et al. 2015
9. Dragovic, et al. 2013
cargoes may reflect conditional stress imposed on the placenta, and possibly on the fetus. In this context, several placental specific proteins have been studied, especially placental alkaline phosphatase (PLAP), syncytin-1, and syncytin-2 [1, 41-47]. These STEVs are released directly into the maternal circulation, where they may influence the surrounding tissue or may have systemic effects. These protein markers have provided some interesting insights into how STEV profiles may be altered under condition of preeclampsia compared to healthy pregnancy; but at this point, their role as a noninvasive diagnostic is promising and yet to be carefully investigated.

In line with our findings in investigating the role of tissue specific exosomes in the transplantation field, we sought to understand whether STEV signal has diagnostic potential in preeclampsia. Given the known markers PLAP, plac-1, syncytin-1, and syncytin-2 reported in the literature, we first performed in vitro studies in BeWo human choriocarcinoma-derived cell line to check for marker expression in their EVs. It was also critical that the commercially available antibodies for these proteins that are designed for cellular protein content showed reliable and reproducible results with EV proteins. By Western blot analysis, we noted the presence of syncytin-1 and PLAP, but not plac-1, as EV surface markers. These results corroborated with nanoparticle tracking analysis, where high levels of syncytin-1 and PLAP were seen on BeWo EVs. Given these promising in vitro data, we performed analysis in maternal plasma samples to assess for STEV differential expression.

Whole plasma EVs were isolated from maternal plasma using size-exclusion column chromatography and ultracentrifugation, with samples from healthy mothers serving as a positive control. Samples were evaluated by Western blot and nanoparticle tracking analysis, along with RT-PCR analysis of EV mRNA. This study was performed with the samples blinded to the staff performing the EV experiments. First, there was no difference between healthy pregnant and preeclampsia samples when assessing for size distribution and EV quantity for whole plasma. This was consistent with our findings in the transplantation models of rejection, where whole plasma EVs were unchanged [28-30]. Western Blot analysis probing for syncytin-1 displayed a markedly higher expression in healthy pregnant EV samples compared to healthy nonpregnant EV samples; however, there was marked decrease in syncytin-1 expression for preeclampsia samples when compared to healthy pregnant samples. This observation was quantified by nanoparticle tracking analysis using anti-syncytin-1 antibody conjugated quantum dots, which strongly validated the above results. PLAP was also detected in both healthy maternal EVs and preeclampsia EVs. However, a small limitation of this study was that PLAP also reacted with non-pregnant female EVs, insinuating that there is some cross-reactivity present [1]. These findings highlighted the importance of enriching/ detecting tissue specific EVs to improve diagnostic accuracy; in this case syncytiotrophoblast EVs, as quantification of whole plasma EVs showed no quantitative, statistically significant difference between healthy pregnant and preeclampsia samples. Collectively, this demonstrated that syncytin-1 expressing EV subpopulation is significantly upregulated during healthy pregnancy. The maternal syncytin-1 EV signal is significantly decreased with preeclampsia, implicating that a tissue specific exosome platform may have diagnostic potential to reflect the placental injury associated with this condition. The finding that preeclampsia results in decreased STEV signal is consistent with our overall findings in other models where disease / injury leads to decreased detection of circulating tissue specific EVs. Similar results were noted in the transplantation setting, where immunologic rejection led to decreased donor tissue EV output.

Studying EVs, particularly STEVs in the maternal circulation, provides a novel perspective on preeclampsia, potentially providing a real time, dynamic analysis of the condition. Therefore, a detailed investigation of syncytin-1 EV expression from early pregnancy time points would provide insights into the biomarker potential of this platform. As preeclampsia is relatively frequent and occurs in 4-5% of pregnant females, a prospective study would be feasible with relative ease. In previous studies, we used tissue specific surface EV markers and successfully purified EV subsets using antibody conjugated bead technology and performed small RNA sequencing of exosomal RNA cargoes [28, 47]. These studies showed differential expression of micro RNAs in tissue specific EVs and provided functional insights into how EV micro RNA cargoes may have mechanistic implications. In this context, syncytin-1 may be utilized as a candidate marker for enrichment of a putative placental EV subpopulation, and analysis of its microRNA cargoes may provide insights into their functional roles and the potential role of STEVs in the pathophysiology of preeclampsia.

Other groups have reported differences using circulating PLAP and syncytin-2 expression, as well as whole plasma EV profiles, and the utilization of these surface markers may also provide insights into understanding putative STEV cargo profiles (Table 1) [38-41]. Our group attempted to understand PLAP specific EVs as potential STEV subpopulation, but using the commercially available antibodies to PLAP we found cross reactivity to other forms of alkaline phosphatases in circulation. According to Tannetta, et al., there is a marked difference in PLAP expression between STEVs of healthy pregnant samples.
and preeclampsia samples by in vitro experiments [41]. PLAP is the most studied placental marker, but our experience with this candidate marker of STEV has been disappointing due to cross reactivity when using commercially tested antibodies.

As seen in Dragovic, et al. along with many others, nanoparticle flow cytometry can result in improved detection of target proteins specifically on STEVs, allowing for greater tissue specificity determination and better analysis of the EV cargo profiles (Table 1) [48-50]. Moreover, flow cytometry allows for greater accuracy in quantifying whole plasma EVs and STEVs when compared to nanoparticle tracking analysis, given the ability of FACS to better differentiate between background noise and the molecules of interest. But nanoparticle flow cytometry has limitations for smaller sized nanoparticles, especially exosomes, where the sensitivity and specificity of current nanoparticle flow cytometers starts to decrease. However, we anticipate that as this technology improves, nanoparticle flow cytometry would play an important role in this field in the future. In our analysis of STEVs using syncytin-1 as a marker, nanoparticle detector fluorescence and light scatter modes enabled tissue specific exosome subset detection and quantitation. This technique requires repeated sample quality validation and due to the high sensitivity of the nanoparticle detector, protein aggregates or plasma lipoproteins in the size range of exosomes 30 nm to 150 nm can lead to erroneous quantitation. Most likely, the ideal assay for STEV quantitation would be a combination of both the nanoparticle detector tracking analysis and FACS studies [51], which may help improve the overall diagnostic accuracy of STEV quantitative read out. Future studies using these techniques in larger patient cohort and performed at multiples institutions independently would help better understand the diagnostic potential of circulating STEV signal quantitation.

Furthermore, given the unique nature of the placenta as an organ that is naturally removed from the mother after giving birth facilitates performance of ex vivo perfusion experiments to better understand STEV differential profiles using syncytin-1 and PLAP as potential markers [41]. Placenta specific EVs can been isolated directly from the organ, comparing those derived from placenta of healthy subjects versus those with preeclampsia. Additionally, this would help compare and cross validate the in vivo plasma and ex vivo data. As syncytiotrophoblasts are of fetal origin, better understanding of STEV profiles may also provide novel insights into fetal disorders such as intrauterine growth retardation.

These experimental improvements will also help in better understanding the role STEVs and their cargo profiles play in the onset of preeclampsia and determine whether they are more than just a biomarker. In a recent study by Han et al., infused EVs isolated from injured placenta into healthy pregnant mice resulted in preeclampsia symptoms [52]. The mice developed both hypertension and proteinuria, which is an indication that EVs may potentially play a causative role in the development of preeclampsia. Nonpregnant healthy mice that were also infused with these EVs developed the same symptoms, which further highlights the possibility that the STEVs derived from the injured, preeclamptic placenta play a key role in communication throughout the body [52]. These experiments are corroborated by the findings of Kohli et al., as well as a previous study done by Han, et al., where infusion of EVs using mouse models resulted in an inflammatory response [53,54]. Han’s group specifically used low syncytin expression on EV surface as a marker of placental injury, opening up avenues for further study [52]. These findings and methodologies can be extended to developing experimental protocols to understand the mechanisms behind the onset of preeclampsia and the means of inflammatory response in the process, furthering Han’s study [55]. The ability to enrich for a putative circulating STEV population using syncytin-1 opens the door for future studies exploring these concepts.

Conclusion

Levine and Habertheuer’s paper highlights the potential diagnostic utility of STEVs and the importance of tissue specificity when using EVs as a noninvasive biomarker for detection of preeclampsia. With syncytin-1 as a surface marker for EVs derived from fetal-derived syncytiotrophoblasts, our investigation showed that increased expression is a sign of a healthy pregnancy while a decrease in expression can be an indication of preeclampsia. Although currently the development and cause of preeclampsia is somewhat a mystery, recent explorations, including our group’s report, have begun to investigate the utility of STEVs and their diagnostic potential. Understanding STEV cargos may provide insights into the mechanisms underlying preeclampsia onset and progression. With the advancement of technology and research techniques, investigations on STEVs can improve to provide a deeper understanding of preeclampsia and placental pathologies as a whole.

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Author Contributions

CR – original draft preparation, review and editing; RWH – review and editing; LK – review and editing; AH – review and editing; PV – study concept and design, review and editing.
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