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Isolation of human trophoblastic extracellular vesicles and characterization of their cargo and antiviral activity

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ABSTRACT

Introduction: Primary human trophoblasts release a repertoire of extracellular vesicles (EVs). Among them are nano-sized exosomes, which we found to suppress the replication of a wide range of diverse viruses. These exosomes contain trophoblastic microRNAs (miRNAs) that are expressed from the chromosome 19 miRNA cluster and exhibit antiviral properties. Here, we report our investigation of the cargo of placental EVs, focusing on the composition and the antiviral properties of exosomes, microvesicles, and apoptotic blebs.

Methods: We isolated EVs using ultracentrifugation and defined their purity using immunoblotting, electron microscopy, and nanoparticle tracking. We used liquid chromatography-electrospray ionization-mass spectrometry, protein mass spectrometry, and miRNA TaqMan card PCR to examine the phospholipids, proteins, and miRNA cargo of trophoblastic EVs and an *in vitro* viral infection assay to assess the antiviral properties of EVs.

Results: We found that all three EV types contain a comparable repertoire of miRNA. Interestingly, trophoblastic exosomes harbor a protein and phospholipid profile that is distinct from that of microvesicles or apoptotic blebs. Functionally, trophoblastic exosomes exhibit the highest antiviral activity among the EVs. Consistently, plasma exosomes derived from pregnant women recapitulate the antiviral effect of trophoblastic exosomes derived from *in vitro* cultures of primary human trophoblasts.

Discussion: When compared to other trophoblastic EVs, exosomes exhibit a unique repertoire of proteins and phospholipids, but not miRNAs, and a potent viral activity. Our work suggests that human trophoblastic EVs may play a key role in maternal-placental-fetal communication.

1. Introduction

The placenta plays a central role not only in maternal-fetal exchange functions and immunological defense, but also in communicating maternal-fetal signals that are essential for pregnancy health. Accordingly, the human villous trophoblast layer, which includes the syncytiotrophoblast that is directly bathed in maternal blood and the subjacent progenitor cytotrophoblast, regulates the release of these communication messages into the maternal circulation. In contrast, the transmission of trophoblastic biological signals into the fetal circulation may require trafficking through the villous basal membrane and fetal endothelial cells before entering the fetal circulation. In addition to hormones, growth factors, and other signaling proteins and akin to other epithelial cells, trophoblasts release a diverse repertoire of lipid-encapsulated extracellular vesicles (EVs) into the maternal blood, including apoptotic cell-derived EVs (ABs), microvesicles (MVs),

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and exosomes (EXs) [1–4]. The size of ABs ranges between 1 and 5 μm, and are produced by deportation of trophoblastic fragments during apoptosis [5]. MVs display a relatively smaller size (100 nm - 1 μm) and are released to the extracellular environment by budding off from the cell membrane [4]. In contrast, EXs (30–200 nm) are generated within multivesicular bodies, which fuse with plasma membrane to release the EXs' cargo into the intercellular space or maternal blood [6,7]. Human placental EXs derived from trophoblasts and placental non-trophoblastic cells such as placental mesenchymal progenitor cells are involved in cellular adaptation during the course of normal pregnancy, including immunomodulation and tolerance [8–13], endothelial cell migration [14–16], and proliferation and invasion of extravillous trophoblasts [17]. We have previsouly shown that EXs derived from primary human trophoblast (PHT) cells confer resistance to a broad panel of viruses to non-placental cells that are normally permissive to viral replication [7,18].

In light of their presence in the maternal circulation and their diverse functions at the feto-maternal interface, placental EVs, particularly EXs, have been assessed as potential biomarkers for diagnosis and prognosis of pregnancy disorders such as preterm birth or preeclampsia [3,4]. Indeed, when compared to normal pregnancies, trophoblastic EVs are elevated in the circulation of women with preeclampsia [19–22]. Interestingly, several proteins, including sFlt-1, endoglin, tissue factor, and PAI, all presumed to play a role in the angiogenic and coagulation imbalance of preeclampsia, are present within trophoblastic EVs [23,24], raising the possibility that EVs may contribute to the pathogenesis of preeclampsia [21,25].

Recent data indicate that microRNAs (miRNAs) may have important gene regulatory functions not only in cells that produce them, but also in neighboring or distant cells [26,27]. This unprecedented finding of cell-to-cell communication, mediated by extracellular miRNAs, suggests that extracellular miRNAs play a role in tissue physiology, homeostasis, and disease [7,28,29]. In general, extracellular miRNAs can be found within EVs or in non-vesicular form, bound by proteins such as argounate2 (Ago2), high-density lipoproteins, and nucleophosmin 1 [30–33]. Among the circulating miRNAs associated with pregnancy [34–36], one family of miRNAs, which are expressed from the chromosome 19 miRNA cluster (C19MC), is placenta-specific and highly expressed in the maternal blood throughout pregnancy and sharply diminishes after delivery [37–39]. Moreover, we discovered that C19MC miRNAs are packaged into trophoblastic EXs and, when delivered to non-placental cells, can suppress replication by a wide range of DNA and RNA viruses [7,18].

To systematically investigate the properties and function of trophoblastic EVs, we purified the three major EVs (ABs, MVs, and EXs) from *in vitro* cultures of term PHT cells derived form healthy pregnancies and characterized their miRNA profiles and phospholipid and protein content. Moreover, we analyzed their antiviral properties using our previously established viral infection assay. Functionally, we found that trophoblastic EXs displayed the most robust antiviral activity among the three trophoblastic EVs tested and that this antiviral effect was recapitulated using total plasma EXs obtained from pregnant women at term.

2. Methods

2.1. Isolation of trophoblastic EVs from PHT conditioned medium

The collection of placentas used for cell isolation and culture was reviewed and approved by the Institutional Review Board at the University of Pittsburgh. PHT cells were isolated from placentas of uncomplicated pregnancy, labor, and delivery according to our previously published protocol [40]. PHT cells were cultured in 7 × 15-cm plates at 37 °C for up to 72 h in complete DMEM medium that contained 1% antibiotics and 10% of either fetal bovine serum (FBS) that had been depleted of bovine EXs by overnight ultracentrifugation at 100,000 g or purchased bovine-EX-depleted FBS (Thermo Fisher Scientific, Waltham, MA). Conditioned medium samples used for vesicle isolation were collected on the third day of culture, when most PHT cells are syncytialized [40]. Approximately 400 ml of PHT conditioned medium (CM) was collected for EV purification using differential centrifugation and OptiPrep (D1556-250 ml, Sigma-Aldrich, St. Louis, MO) continuous gradient ultracentrifugation. The remaining isolation procedures were performed at 4 °C, unless indicated otherwise. We performed serial centrifugation procedures, first at 500 g for 10 min to pellet cell debris, followed by 2500 g for 20 min to pellet ABs, which were washed three times with 2 ml of PBS and suspended in 50–100 μl of PBS (P5493-1L, RNase-free, Sigma). MVs were pelleted using 12,000 g centrifugation for 30 min, then washed three times with 2 ml of PBS and suspended in 50–100 μl of PBS. To isolate EXs, we filtered AB- and MV-depleted medium through 0.22 μM filter unit (Millipore, Billerica, MA) to exclude any residual particles larger than 200 nm; then the filtrate was concentrated at RT, using a Vivacell 100 filtration unit (100 kDa Mw cut-off, Sartorius, New York, NY). For one Vivacell 100 filtration unit, PHT CM was concentrated to a volume of 5 ml, re-suspended in 9 ml PBS, and centrifuged at 100,000 g overnight. The supernatant was removed, and the pellets were re-suspended in 0.5 ml of PBS and mixed with 1.5 ml of 60% OptiPrep. The mixture (2 ml) was laid at the bottom of the tube and overlaid with 10 ml of 6–40% OptiPrep gradient using a gradient formation chamber and peristaltic pump. After 22 h of OptiPrep gradient ultra-centrifugation at 100,000 g, we withdrew individual fractions from top to bottom and identified the fractions that correspond to trophoblastic EXs by western blotting and NanoSight nanoparticle tracking analysis (NTA) (Malvern Instruments, Westborough, MA). EXs were filtered out of the OptiPrep solution by diluting EXs in PBS and concentrated in a Vivacell 20 filtration unit (100 kDa Mw cut-off, Sartorius). We quantified protein concentration of trophoblastic vesicles, using the Micro BCA method according to the manufacturer's instructions (Thermo Fisher).

2.2. Isolation of EXs from human plasma

The collection of plasma samples, performed during blood testing as a part of routine clinical care, was reviewed and approved by the Institutional Review Board at the University of Pittsburgh. Blood samples (2 ml) were collected in EDTA-treated tubes and centrifuged at 1000 g for 10 min to remove any blood cells. This was followed by centrifugation at 2500 g for 20 min to remove platelets. Either fresh or −80 °C frozen plasma was used, as indicated. For EX isolation, frozen plasma was again centrifuged at 2500 g for 15 min to remove any aggregates. We diluted cleared plasma with PBS (1:1 vol ratio) and filtered the diluted plasma through a 0.2 μM syringe filter (PES membrane, Whatman, Maidstone, UK) to remove particles larger than 0.2 μM. 10 ml of total diluted plasma were loaded into the gravity column that contained 2 ml of gelatin-agarose (G5384, Sigma). We collected the flow-through and repeated loading twice to ensure that fibronectin was bound to the gelatin-agarose [41,42]. We obtained crude EXs by adding PEG6000 (81253, molecular biology grade, Sigma) at the final concentration of 5% (w/v) and incubated the mixture at RT for 10 min. We then centrifuged the mix at 10,000 g at RT for 20 min and suspended pellets in 0.5 ml of PBS. We loaded the crude EX suspension on top of 14 ml of 6%–30% OptiPrep continuous gradient. After overnight 100,000 g OptiPrep gradient ultra-centrifugation, we withdrew