Immunobiology 221 (2016) 753–760

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

High levels of circulating extracellular vesicles with altered expression and function during pregnancy

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ARTICLE INFO

Article history:
Received 22 December 2015
Accepted 11 March 2016
Available online 15 March 2016

Keywords:
Pregnancy
Extracellular vesicles
TGFβ-1
IL-10
NK cells
Caspase-3 activity

ABSTRACT

Extracellular vesicles (EVs) are widely considered important modulators of cell–cell communication and may interact with target cells locally and on a systemic level. Several studies had shown that circulating EVs' levels are increased during pregnancy. However, EVs characteristics, composition and biological functions in pregnancy still need to be clarified. This study aims to determine if circulating EVs during pregnancy are modified regarding levels, markers and cytokine profile as well as their reactivity towards peripheral blood cells. 26 pregnant women (PW) being in the second gestational trimester and 59 non-pregnant women (NPW) were investigated. EVs enrichment was performed by ExoQuick™ or ultracentrifugation; nanoparticle tracking analysis, SDS-PAGE followed by Western Blotting and densitometry, and IFN-γ, IL-10 and TGF-β1 ELISA for EVs characterization; imaging flow cytometry to analyze EVs' uptake by peripheral blood cells and flow cytometry were performed to analyze EVs function regarding induction of caspase-3 activity. Circulating EVs' levels were increased during pregnancy [26.9×10^6 EVs/ml (range: 6.4–46.3); $p = 0.003$] *vs* NPW [18.9×10^6 EVs/ml (range: 2.5–61.3)]. Importantly, the immunosuppressive TGF-β1 and IL-10 cytokine cargo were increased in EVs of PW even after normalization to 1 million EVs [TGF-β1: 0.25 pg/10^6 EVs (range: 0.0–2.0); $p < 0.0001$] and [IL-10: 0.21 pg/10^6 EVs (range: 0.0–16.8); $p = 0.006$] *vs* NPW. Although EVs derived from non-pregnant and pregnant women were taken up by NK cells, the latter exclusively enhanced the caspase-3 activity in $CD56^{dim}$ NK cells (8.2 ± 0.9; $p = 0.02$). The qualitative and quantitative pregnancy-related alterations of circulating EVs provide first hints for an immune modulating role of circulating EVs during pregnancy.

1. Introduction

Extracellular vesicles (EVs) are secreted membrane vesicles released by most cell types (Ludwig and Giebel, 2012). EVs are widely considered to be important modulators of cell–cell communication and may interact with target cells either locally or in a systemic level. EVs have been detected in a wide range of fluids, including amniotic fluids, urine (Keller et al., 2007) and peripheral blood (Hunter et al., 2008). They are heterogeneous with regard to their size and molecular composition. According to their size EVs can be stratified into apoptotic bodies (100–600 nm), microvesicles (0.1–2 μm) and exosomes (30–160 nm). They carry a conserved set of proteins including tetraspanins (CD9, CD81, CD63, CD82), heat shock proteins (Hsp70, Hsp90), adhesion molecules (ICAM-I), cytoskeletal proteins (tubulin, actin) and members of endosomal sorting complexes required for transport (ESCRT) like TSG101 (for overview see (Ludwig and Giebel, 2012; Mincheva-Nilsson and Baranov, 2014; Sokolova et al., 2011; Tannetta et al., 2014; Toth et al., 2007)). In addition, EVs bear several types of antigens, cell surface expressed receptors or ligands. Furthermore, EVs can serve as transport cassettes or disseminated storage pools of mRNA, microRNA (Liu and Lu, 2015) or bioactive effector molecules e.g. cytokines transcription factors or onco-proteins (Sohda et al., 2015;

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http://dx.doi.org/10.1016/j.imbio.2016.03.001
0171-2985/

Balaj et al., 2011). In this way EVs can stimulate target cells directly via a ligand-receptor-mediated interaction or may transfer genetic information/bioactive molecules to target cells. Consequently, EVs have been reported to be involved in the modulation of immune response, in the cell migration, angiogenesis and cell proliferation (for overview see (Kharaziha et al., 2012; van Dommelen et al., 2012; Vlassov et al., 2012; Zhang et al., 2012)). It is important to note that their size, shape, content and mode of generation mostly reflect the state and the composition of the cellular sources and these features probably influence their function.

Regarding immune modulation, EVs like exosomes can act as immunoactivating vesicles by presentation of MHC-peptide complexes to T cells and/or to dendritic cells (DC) resulting in the stimulation of these effector cells (Admyre et al., 2006; Thery et al., 2002). On the other hand, they can act as immunosuppressive vesicles, introducing homeostasis or immune tolerance by the induction of T cell apoptosis, impairment of DC maturation or preventing NK and T cell cytotoxicity "(for overview see (Southcombe et al., 2011; Thery et al., 2009)). For all those reasons, EVs are discussed to be operative in pathophysiological processes of autoimmunity, cancer, transplantation or pregnancy. Recently, EVs like exosomes have been used in a clinical setting for the treatment of therapy-refractory graft-versus-host disease (Kordelas et al., 2014).

In pregnancy there is a growing body of evidence that the human placenta cells, including syncytiotrophoblast and cytotrophoblast, release EVs carrying signaling molecules and genetic information to specific targets locally at the maternal-fetal interface or systemically through the peripheral blood, contributing to promote intracellular signaling for endometrial receptivity (Mincheva-Nilsson and Baranov, 2014; Redman and Sargent, 2007; Salomon et al., 2014). EVs like exosomes trafficking within the pregnancy scenario are proposed to have a role in modulation of immune cells involved in the maternal-fetal cross-talk e.g. influencing T and NK cell functions or introducing apoptotic activity through CD95 ligand (CD95L or Fas-L) mediated pathway (Abrahams et al., 2004; Frangsmyr et al., 2005; Hedlund et al., 2009; Stenqvist et al., 2013; Taylor et al., 2006) or modulating peripheral immune cells activation and dampening via trophoblast microvesicles (Holder et al., 2012). Furthermore, the maternal immune system has to reshape to an immune-tolerance-inducing status leading to a shift of effectors in the periphery including immune cells, cytokines, growth factors and EVs (Beer et al., 1996; Chernyshov et al., 2014), allowing the viability of the semi-allogeneic conceptus.

In the maternal blood circulation, a mixture of EVs population from distinct sources can be expected to be found, including vesicles derived from syncytiotrophoblasts, leukocytes, endothelial cells and platelets. Moreover, the placenta-derived EVs are a constitutive component in the peripheral blood during normal pregnancy and the placenta seems to release EVs in the maternal circulation of several sizes, sources and morphologies (reviewed by (Mincheva-Nilsson and Baranov, 2014)). Interestingly, a considerable number of recent studies revealed that the concentration of EVs like exosomes or syncytiotrophoblast microparticles present in the maternal circulation increase significantly during normal pregnancy compared to non-pregnant women (Salomon et al., 2014; Taylor et al., 2006; Germain et al., 2007; Sarker et al., 2014). The presence of increased levels of EVs in the blood circulation of pregnant women may directly be associated with the immune mechanisms supporting gestation. Thus, the identification and characterization of bioactive EVs in maternal blood during pregnancy open a new perspective to gain insight into novel aspects contributing to maternal immunomodulation favoring or maintaining pregnancy.

To establish qualitative and quantitative pregnancy-related alterations of EVs circulating in the maternal blood, EVs derived from blood samples of pregnant women in the second gestational trimester were studied in comparison to EVs enriched from blood samples of healthy voluntary non-pregnant women regarding EVs concentration, their markers and cytokine profiles as well as their reactivity towards peripheral blood cells.

2. Material and methods

2.1. Study population and sample collection

26 pregnant women (PW) and 59 non-pregnant women (NPW) were enrolled in this study and analyzed for biologic and clinical characteristics including age, previous conceptions and previous abortions. The blood samples of PW and NPW groups were collected at the Clinical Hospital of Federal University of Paraná, Curitiba and at the Reproductive Immunology Centre, Porto Alegre, Brazil.

The blood samples belonging to the PW group were collected between 12 and 28 gestational weeks. The mean age of the PW group was 31.5 ± 8.3 (mean $\pm$ SD) (range: 20–46) and the mean number of the PW group with previous conceptions (n = 16, 61.5%) was 1.8 ± 2.9 (range: 0–14). The mean age for the NPW group was 37.1 ± 4.1 (range: 29–45) and the mean number of the NPW group with previous conceptions (n = 24, 40.6%) was 0.9 ± 1.1 (range: 0–3). Two hours after blood collection, the samples were centrifuged at 1500 *g* for 20 min and the corresponding serum and plasma were stored at −80 °C for subsequent biological analysis.

2.2. Enrichment of circulating EVs

For purification and precipitation of extracellular vesicles ExoQuick™ solution (System Biosciences, Inc.) was used according to manufacturer's instructions. Briefly, serum samples were centrifuged at 3000 *g* for 15 min to remove cells and cells debris. After centrifugation, 250 μl from samples were mixed with 63 μl of ExoQuick™ solution and incubated overnight at 4 °C followed by a second centrifugation step at 1500 *g* for 30 min. The supernatant was discarded and tubes were centrifuged once more (1500 *g* for 5 min). All traces of fluid were aspirated, and then pellets were filled up to the starting volume (250 μl) with distilled water and stored at −80 °C.

2.3. Identification of nanoparticles by nanoparticle tracking analysis (NTA)

NTA measurements were performed using ZetaView® Particlemetrix (Particle Metrix, GmbH) as previously described (Sokolova et al., 2011). The determination of vesicle concentration and size distribution in liquid suspension were based in the rate of Brownian motion and of nanoparticles in a light scattering system. The samples were diluted (1:10.000) with NaCl 0.9% and injected manually and data acquisition was undertaken at room temperature. Polystyrene Nanobeads Nist Traceable Particle Size of 0.1 μm (Polysciences, Inc.) were used as a size control. Data were analyzed by ZetaView version 8.02.24 software. NTA analysis showed a particle size distribution with an average of 145.0 ± 9.9 (mean $\pm$ SD) (Fig. 1 Supplementary material). The coefficients of variation among different ZetaView measurements were 24.8% for the enumeration and 6.7% for the size.

2.4. SDS-PAGE and western blot analysis of EVs protein expression

The expression of typical EVs proteins were analyzed by Western blot after ExoQuick™ enrichment from serum samples. As recommended (Lotvall et al., 2014), we tested our EVs fractions under reducing conditions for the presence of transmembrane or