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HLA-DR is aberrantly expressed at feto-maternal interface in pre-eclampsia

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ABSTRACT

In normal pregnancy, villous cytotrophoblast and syncytiotrophoblast do not express HLA Class I and Class II molecules, while invasive extravillous trophoblast only express class I HLA-C and the atypical class Ib antigens, HLA-G, -E and -F. Inadequate maternal tolerance of invasive trophoblast has been proposed as a possible immunologic trigger of poor trophoblast invasion and subsequent occurrence of pre-eclampsia. This study aimed to investigate possible aberrant expression of class II HLA-DR on placentae and syncytiotrophoblast-derived extracellular vesicles (STEVs), obtained by dual placental perfusion, from pre-eclampsia (n = 23) and normal pregnant (n = 14) women. Here we demonstrate that HLA-DR can be detected in syncytiotrophoblast from a significant proportion of pre-eclampsia but not control placentae. HLA-DR was also observed, by flow cytometry, on STEVs and associated with placental alkaline phosphatase to validate their placental origin. HLA-DR positive syncytiotrophoblast was detected in placental biopsies from pre-eclampsia but not normal control cases, using immunohistochemistry. The HLA may be fetal or maternal origin. In the latter case a possible mechanism of acquisition is trogocytosis.

1. Introduction

Pre-eclampsia, complicating 3–5% of all pregnancies, is still a major cause of severe maternal and newborn morbidity and mortality worldwide (Khan et al., 2006; Abalos et al., 2014). The placenta plays a central role in its pathogenesis (Redman and Sargent, 2005). According to the "two stage model" of the development of pre-eclampsia, in the first half of pregnancy, inadequate trophoblast invasion into the placental bed occurs, causing insufficient utero-placental spiral arteries remodelling. The first stage is termed poor placentation. Inadequate placental development may become clinically relevant at different gestational age according to its severity, leading, in the second stage of the disorder, to insufficient maternal blood supply of the fetal-placental unit. Then, an oxidatively stressed and dysfunctional placenta releases factors (like Flt-1 ad s-Endoglin) into the maternal circulation, that cause the clinical features of the syndrome, including hypertension and proteinuria as well as clotting and liver dysfunction. These cause a generalized systemic inflammatory response, of which endothelial dysfunction is a prominent component.

Semi-allogeneic trophoblast forms several interfaces with the maternal immune system (Sargent et al., 2006). Interface 1 is between invasive, extra-villous trophoblast (EVT) and decidual immune cells, which is where the success of placentation is determined. It is most active during the first half of pregnancy. Interface 2 lies between the syncytiotrophoblast and maternal blood. Syncytiotrophoblast forms the epithelial covering of the chorionic villous and presents a large surface area in contact with the maternal circulating immune cells blood, which is most active in the second half of pregnancy. Thus, syncytiotrophoblast-derived extracellular vesicles (STEVs) shed into maternal circulation represent and extension of interface 2. Interface 3 is that between the decidua parietalis and the amniochorion.

Tight control of human leukocyte antigen (HLA) class I and class II expression in chorionic and EVT is essential for successful pregnancy outcome (Moffett et al., 2017). At maternal interface 1, invasive EVT express polymorphic Class I HLA-C and the atypical oligomorphic class Ib antigens, HLA-G, HLA-E and HLA-F, which stimulate immunoregulation between EVT and maternal decidual natural killer (NK) and T cells, that promote placentation in the first half of pregnancy (Parham and Guethlein, 2010; Parham et al., 2012). The lack of HLA-A, HLA-B and general lack of HLA class II (-DP, -DQ and -DR) expression on trophoblasts prevents maternal T cell allo-immune responses against paternal-derived major histocompatibility complex (MHC) antigens. Then, proper immune interaction between decidual NK and T cells and HLA Class I antigens on EVT, as well as, complete suppression of HLA class II molecules expression in trophoblast tissues, is a necessary condition for successful pregnancy (Dragovic et al., 2015). Inadequate

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maternal tolerance of invasive EVT, which is closely associated with spiral artery remodelling at the beginning of the second trimester has been proposed as a possible immunologic trigger of insufficient trophoblast invasion and subsequent pre-eclampsia (Saito et al., 2007).

Normal syncytiotrophoblast at interface 2 expresses no HLA and, in particular, HLA class II (Faulk and Temple, 1976; Sunderland et al., 1981; Sutton et al., 1983; Apps et al., 2009). Furthermore, although trophoblasts have been demonstrated to contain the IFN-γ receptor and the necessary transduction mechanism, they do not constitutively express MHC class II antigens, even after IFN-γ exposure (Murphy and Tomasi, 1998).

The present study is based on our unpublished proteomic studies that show the presence of HLA-DR in STEVs obtained by dual placental perfusion of placentae donated by women with pre-eclampsia (Tannetta, unpublished data). STEVs are released into the maternal circulation at higher concentrations in pre-eclampsia than in normal pregnancy (Redman and Sargent, 2008). HLA-DR was not detected on STEVs from normal placentas in the proteomic data.

2. Materials and methods

2.1. Patients and samples

This study has been designed and conducted according to the principles of the Declaration of Helsinki. The Central Oxford Research Ethics Committee (07/H060744) and the Ethics Committee of the Catholic University of Rome approved this study (P/285/CE/2010). Written informed consent was obtained from all recruited individuals.

For recruitment of cases, pre-eclampsia was defined as new onset hypertension and proteinuria that appeared after 20 weeks of gestational age in a previously normotensive woman. Depending on the gestational age of occurrence we distinguished early onset pre-eclampsia (EOP; ≤34th week gestation) from late onset pre-eclampsia (LOP; < 34th week gestation).

Term placentae were obtained by elective caesarean sections from the delivery suite of the John Radcliffe Hospital, Oxford, UK, and from the Gemelli Hospital of Rome, Italy, from healthy women with uncomplicated pregnancies and pre-eclamptic women. Placentae were immediately processed for dual placental perfusion to collect STEVs. Placental biopsies were also taken from the same placentae, washed and then fixed in parafolmadehyde for 12 h before being stored in ethanol at R/T until subsequent embedding in paraffin.

2.2. Immunohistochemical staining of placenta sections

Placental sections were deparaffinised by successive washings (1 min each) in Histo clear 1 and Histo clear 2 solutions (National Diagnostics, Atlanta, GA, USA) before being rehydrated in a series of washes in 100% (×2), 95%, 90%, 80%, 70% and 50% alcohol solutions. Slides were dipped in sodium citrate buffer (10 mM, pH 6.0) (VWR, UK) for 10 min in a microwave oven to bring the temperature up to close to boiling, cooled at room temperature (R/T) for 30 min and washed in PBS before being incubated in a blocking solution (10% v/v FCS in PBS) for 1 h at R/T. To investigate abnormal syncytiotrophoblast expression of HLA-DR in pre-eclampsia, sections were incubated overnight (O/N) at 4 °C in PBS containing 1% FCS and 20 μg/ml of HLA-DR (clone L243-Abcam, Cambridge, UK), or non-immune IgG (Dako, Denmark) primary monoclonal antibody. To exclude a concomitant aberrant expression of HLA-G or -C in the syncytiotrophoblast of pre-eclampsia cases, sections were also incubated with anti-HLA-C (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) or anti-HLA-G (Abcam) primary monoclonal antibody. Then, sections were washed 3 times 10 min in 0.01% PBS-Tween and stained with biotinylated secondary antibody (diluted 1:250; Vector Laboratories) for 45 min, washed and incubated with streptavidin-peroxidase (diluted 1:400; Immunotech, Beckman-Coulter). Bound antibody was visualized by incubation with diaminobenzidine/H2O2. Slides were counterstained with hematoxylin, mounted and analyzed by Zeiss AxioPlan 2 optical microscope. Images were taken with a Hamamatsu Orca digital camera with Simple PCI software.

2.3. Dual placental perfusion and STEVs collection

STEVs were prepared using a dual placental perfusion system as described in Dragovic et al. (2011). Briefly, placentae were collected after cesarean section and were processed within 10 min. An individual lobule was isolated and both fetal and maternal sides of the lobe were perfused (open and closed circuits, respectively) with modified M-199 tissue culture medium (Medium 199 with L-glutamine and Earle's salts without NaHCO3, containing 0.8% Dextran 20, 0.5% BSA, 5000 U/L sodium heparin, and 2.75 g/L sodium bicarbonate, pH 7.4) at a controlled perfusion rate of 5 ml/min and 20 ml/min, respectively (perfusion pumps from Watson-Marlow, Manukau City, New Zealand). The perfusion medium was warmed in a 37 °C water bath and oxygenated with 95% O2, 5% CO2, and the oxygen concentration of the maternal side perfusate monitored to ensure the stability of the preparation. After 3 h of placental perfusion, maternal side perfusate was centrifuged twice in a Beckman J6-M centrifuge at 1500*g* for 10 min at 4 °C to remove cellular debris. Supernatants aliquots were spun at 10,000*g* for 30 min at 4 °C in a Beckman L8-80 M ultracentrifuge to obtain the large size vesicles (10 K STEVs) and stored at −80 °C until use (Dragovic et al., 2015). Protein content (mg/ml) was assessed by Bradford assay (BCA protein assay kit, Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Flow cytometry analysis of STEVs

Analysis of large STEVs was carried out by multi-colour flow cytometry, using a BD LSRII Flow Cytometer (BD Biosciences, Oxford UK) equipped with a 488 nm (blue) and 633 nm (red) laser. All data were analysed using FACS DIVA software (Becton Dickinson, Oxford, UK). Firstly, the limits of detection of the flow cytometer were established using standard size calibration beads. 1 μm Fluoresbrite YG microspheres (Polysciences Europe GmbH, Eppelheim, Germany) and NIST polystyrene YG microspheres; 200 nm, 290 nm, 390 nm, 590 nm (Thermo Fisher Scientific) were diluted accordingly in filtered (0.1 μm) PBS and used to determine the optimum side scatter and forward scatter voltages for STEVs detection with minimum interference from background machine noise (Dragovic et al., 2011). 1 μm Fluoresbrite YG microspheres were then used to set a 1 μm limit gate to exclude larger debris. Logarithmic voltages were used for all channels. As STEV sample events were collected over 2 min., BD Trucount tubes were run to monitor flow rate stability. Prior to running STEV samples, filtered PBS was also analysed in triplicate for 2 min to assess the level of background contaminating events.

To confirm placental origin, STEVs were stained with anti-Placental Alkaline Phosphatase (PLAP) NDOG2 mouse monoclonal antibody commercially conjugated to Phycoerythrin (PE) (Biolegend UK Ltd., Cambridge, UK) or its PE-conjugated IgG1 control (Biolegend). PLAP is exclusively expressed in placental tissues and commonly used to distinguish vesicles released by the placenta from those coming from other cell types. The presence of HLA-DR on STEVs was investigated by the binding of fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-HLA-DR antibody (clone L243-Abcam) or IgG1 control (Abcam) antibody. To exclude a possible contamination of isolated STEVs with maternal leukocytes during ultracentrifugation process, or the concomitant abnormal expression of HLA class I molecules, STEVs were also labeled with FITC-conjugated mouse monoclonal anti-HLA-ABC antibody (clone W6/32-Santa Cruz Biotecnology Inc, Dallas, Texas, USA) or anti-HLA-G antibody (clone 4H84-Santa Cruz Biotecnology Inc.), respectively.

Prior to use, all antibodies were filtered through Nanosep 0.2 μm centrifugal devices (Pall Life Sciences) to minimalize interference by