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Human extravillous trophoblasts bind but do not internalize antiphospholipid antibodies

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ABSTRACT

Introduction: Obstetric morbidity in women with antiphospholipid antibodies (aPLs) may reflect the adverse effects of aPLs on placental cells such as extravillous trophoblasts and the syncytiotrophoblast. Antiphospholipid antibodies may affect the syncytiotrophoblast after being internalised by members of the Low-density lipoprotein receptor (LDLR) family and the antigen of aPLs, β_2 glycoprotein I.
Aim: This study aimed to determine whether aPL internalization was a mechanism by which aPLs adversely affect extravillous trophoblasts.
Method: of Study: Fluorescently-labelled monoclonal aPLs IIC5 or ID2 were incubated with first trimester extravillous trophoblast outgrowths and visualized by microscopy. The subcellular expression of β_2 glycoprotein I and LDLR family members was investigated by live/permeabilised immunocytochemistry.
Results: Unlike the syncytiotrophoblast of anchoring villi, monoclonal aPLs were not internalised by extravillous trophoblasts, which expressed LDLR family members intracellularly. The aPL IIC5 bound to the surface of extravillous trophoblasts in a pattern similar to the extracellular expression of β_2 glycoprotein I.
Conclusions: The mechanisms of action of aPLs are different in extravillous trophoblasts and the syncytiotrophoblast. The interaction of aPLs with the extravillous trophoblast surface, which may involve β_2 glycoprotein I, is consistent with reports that aPLs trigger intracellular signaling cascades through cell-surface receptors.

1. Introduction

Antiphospholipid antibodies (aPLs) are a diverse group of autoantibodies that are associated with adverse pregnancy outcomes [1–4]. Women who experience obstetric morbidity and have clinically significant levels of at least one of three aPL subtypes (anticardiolipin antibodies, anti-β_2 glycoprotein I antibodies and the lupus anticoagulant) are described as having the antiphospholipid antibody syndrome (APS) [5]. Although not fully understood, it is hypothesised that obstetric morbidity in women with the APS may reflect the ability of aPLs to perturb the function of the human placenta.

The human placenta is composed of finger-like villi that float in the maternal blood-filled intervillous space. Some placental villi are anchored to the uterine decidua by specialized epithelial cells called extravillous trophoblasts [6]. In the decidua, extravillous trophoblasts become invasive and migrate through the stroma into the uterine spiral arteries. Initially, plugs of extravillous trophoblasts occlude the spiral arteries, but by the end of the first trimester, extravillous trophoblasts replace the endothelial lining of the spiral arteries and remodel the vessels into large-bore non-vasoactive conduits that maximize maternal blood supply to the placenta [6,7]. These extravillous trophoblast-induced changes to spiral arteries are termed the 'physiological changes of pregnancy', and are critical to the success of pregnancy [8].

Antiphospholipid antibodies may affect the process of placentation, and ultimately the success of pregnancy, by causing dysfunction and death of extravillous trophoblasts. Experimental work performed *in vitro* with the extravillous trophoblast-like

Abreviations: aPLs, Antiphospholipid antibodies; APS, Antiphospholipid antibody syndrome; LDLR, Low-density lipoprotein receptor; RAP, Receptor associated protein; TLR4, Toll-like receptor 4.

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choriocarcinoma JEG and the immortalized extravillous trophoblast HTR-8/SVneo have shown that aPL increase the rate of apoptosis [9], decrease the rate and extent of proliferation, invasion and migration [9–15] and promote the secretion of pro-inflammatory cytokines from these extravillous trophoblast cell lines [9]. Detrimental effects on extravillous trophoblasts are also evident *in vivo*, as demonstrated by impaired spiral artery remodeling and decidual inflammation in the placentae of women with aPLs [16–19].

The mechanism by which aPLs affect extravillous trophoblasts is unknown, although interactions with surface receptors such as toll-like receptor 4 (TLR4) have been implicated [9]. We have recently shown that aPLs are internalised by the syncytiotrophoblast, a multinucleated cell that covers the surface of the villous placenta [20]. The internalization of aPLs by the syncytiotrophoblast may involve their antigen, β_2 glycoprotein I, as well as members of the low-density lipoprotein receptor (LDLR) family, and results in altered syncytiotrophoblast death [20]. Whether extravillous trophoblasts also internalise aPLs is unknown. Therefore, this study was conducted to determine whether extravillous trophoblasts internalise aPLs in order to further understand how aPLs exert detrimental effects on extravillous trophoblasts, and ultimately on pregnancy.

2. Materials and methods

2.1. Ethical approval

Placentae were collected with written informed consent from Epsom Day Unit, Greenlane Hospital and The Auckland Medical Aid Centre, Mt Eden, Auckland following surgical terminations of pregnancy between 6.0 and 8.3 weeks gestation as determined by ultrasound dating. Protocols for the collection and use of placentae and blood were approved by the Northern X Regional Ethics Committee.

2.2. Antiphospholipid and control antibodies

Hybridoma cell lines producing the monoclonal aPLs IIC5 and ID2 were previously generated by fusing NS1 murine myeloma cells with splenocytes from a mouse that had been immunised with human β_2 glycoprotein I [21]. IIC5 and ID2 were purified from hybridoma culture supernatants by Protein G chromatography as described [20]. These antibodies are of the IgG_1 isotype and react with β_2 glycoprotein I when it is immobilised on cardiolipin or an irradiated polystyrene plate, and are therefore classified as anti-cardiolipin and anti-β_2 glycoprotein I antibodies [22]. In addition, IIC5 and ID2 share epitopes on β_2 glycoprotein I with some human polyclonal aPL, and are thus good models of human aPL [20].

Murine myeloma IgG_1 monoclonal antibody (control mAb) (Life Technologies, New Zealand) was used as an isotype-matched control for experiments involving IIC5 or ID2. Control mAb, IIC5 and ID2 were fluorescently labelled with AlexaFluor 568 using hydrazide chemistry as described [20].

2.3. Kaolin clotting time assay

Blood from three healthy donors was collected into sodium citrate-buffered vacutainers (BD Biosciences, New Zealand) and centrifuged for 10 min at 3000 rpm. Plasma was collected and centrifuged for a further 10 min at 3000 rpm before collecting the overlying twice-spun platelet-poor plasma (2 × PPP). The 2 × PPP from three donors was pooled and used immediately in a kaolin clotting time assay.

The Kaolin clotting time assay is a coagulation test usually used to determine the presence of lupus anticoagulant in plasma [23]. To test whether the monoclonal aPL ID2 and IIC5 possessed lupus anticoagulant activity, ten microliters of monoclonal aPLs diluted to 100, 50, 25, 12.5 or 0 μg/ml in 0.2 M Tris pH 7.35 were added to clear polycarbonate test tubes containing 200 μl pooled 2 × PPP. Test tubes were agitated in a 37 °C waterbath for 20 s before addition of 100 μl kaolin suspension (2% kaolin in 0.2 M Tris pH 7.35). After three minutes, clotting was initiated by addition of 200 μl 25 mM $CaCl_2$ at 37 °C. Formation of a clot was timed and recorded for each test tube as the kaolin clotting time.

2.4. Culture and visualization of extravillous trophoblast outgrowths

Extravillous trophoblast outgrowths were generated from anchoring villi of first trimester placental explants as described [24] with modifications. Specifically, wells of a μClear 96-well tissue culture plate (Raylab, New Zealand) were incubated with 50 μl of Matrigel (BD Biosciences, New Zealand) diluted 1:10 in DMEM/F12 medium (Life Technologies, New Zealand) for 30 min at 37 °C before removing excess Matrigel from each well. Villous tips were excised from first trimester placentae and placed into the middle of each Matrigel-coated well. Two hundred microliters of outgrowth medium (DMEM/F12, 15% conditioned medium from previous outgrowth cultures, 10% fetal bovine serum, 1% penicillin/streptomycin, 5 ng/ml epidermal growth factor, 5 μg/ml insulin, 10 μg/ml transferrin, 20 nM sodium selenite, 400 U/L human chorionic gonadotropin) were added to each well before centrifuging the plate at 200 g for 1 min. Villous tips were incubated for 96 h at 37 °C in a humidified ambient oxygen atmosphere with 5% CO_2.

To investigate the internalization of aPLs by extravillous trophoblasts, anchoring villi and their extravillous trophoblast outgrowths were incubated for 1 h at 37 °C in outgrowth medium containing 50 μg/ml AlexaFluor 568-labelled ID2, IIC5 or control mAb. Wells containing anchoring villi and extravillous trophoblast outgrowths were then rinsed three times with 100 μl phosphate buffered saline (PBS: 120 mM NaCl, 2.7 mM KCl, 1.5 mM Na_2HPO_4, 8 mM KH_2PO_4, pH 7.3) and visualized while live on a Nikon Eclipse Ti inverted microscope (Nikon, Japan).

To investigate the interaction between aPLs and extravillous trophoblasts by confocal microscopy, anchoring villi and their extravillous trophoblast outgrowths were incubated for 1 h at 37 °C in outgrowth medium containing 10 μM CellTracker Red CMTPX (Life Technologies, New Zealand) or 50 μg/ml AlexaFluor 568-labelled ID2, IIC5 or control mAb. Following this, anchoring villi and their extravillous trophoblast outgrowths were stained with 2.5 μg/ml CellMask Deep Red Plasma Membrane Stain (Life Technologies, New Zealand) and 10 μg/ml Hoechst 33342 nuclear stain (Sigma-Aldrich, Auckland) in PBS for 10 min at 37 °C. Wells containing anchoring villi and extravillous trophoblast outgrowths were then rinsed in PBS at 4 °C and visualized while live on a Zeiss LSM710 inverted confocal microscope (Zeiss, Germany).

2.5. Dual live/permeabilised immunocytochemistry

To examine the subcellular expression of LDLR family members, β_2 glycoprotein I and receptor associated protein (RAP), extravillous trophoblast outgrowths were incubated with primary antibodies while live, or following permeabilisation.

For live staining, extravillous trophoblast outgrowths were chilled on ice and blocked of non-specific binding with block buffer (1% bovine serum albumin in PBS) for 10 min at 4 °C. Primary antibodies to cytokeratin 7 (catalog no. M701801; Dako, Denmark), CD55 (the kind gift of Professor PM Johnson, University of Liverpool), β_2 glycoprotein I (Rabbit polyclonal antiserum, this laboratory) [21], LRP1 (catalog no. SC57353; SantaCruz, USA), LRP2