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Melatonin prevents preeclamptic sera and antiphospholipid antibodies inducing the production of reactive nitrogen species and extrusion of toxic trophoblastic debris from first trimester placentae

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ABSTRACT

Background: The exact cause of preeclampsia is unknown. However a "toxin" from the placenta triggers the condition via activation of the maternal endothelium. Extracellular vesicles (EVs) from the syncytiotrophoblast, may be an endothelial-activating toxin. Antiphospholipid antibodies (aPL) and preeclamptic sera both induce the production of endothelial cell-activating EVs by mechanisms which may produce excess free-radicals in the placenta. Melatonin is produced by the human placenta and has both direct and indirect anti-free-radical properties and may therefore counter the effects of aPL and preeclamptic sera.

Methods: First trimester placental explants were exposed to preeclamptic sera or aPL in the presence or absence of melatonin. Nitrosylative damage was assessed in the explants by immunohistochemistry and the effect of EVs from these explants on endothelial cell activation determined by ICAM-1. Release of nitrosylated proteins from the explants was also measured.

Results: Placental explants showed reduced secretion of melatonin after treatment with preeclamptic sera. Nitrosylated proteins were more abundant in placentae that had been treated with aPL or preeclamptic sera and EVs from such placentae induced endothelial cell activation. Adding melatonin to the aPL or preeclamptic sera reversed the protein nitrosylation and production of endothelial-activating EVs.

Discussion: Our data are consistent with reports that the levels of circulating melatonin are reduced in preeclampsia and suggest that aPL and factors in preeclamptic sera induce free-radical-mediated damage in the placenta leading to the production of endothelial-activating EVs. Melatonin reversing production of endothelial-activating EVs indicates that melatonin may have therapeutic benefits in women with preeclampsia and/or aPL.

1. Introduction

Preeclampsia, a human pregnancy-specific hypertensive disease, is a leading cause of maternal and perinatal mortality and morbidity globally [1]. Preeclampsia is characterised by maternal endothelial cell activation which may be detected in women destined to develop preeclampsia several weeks before the presentation of the clinical signs and symptoms of the disease [1]. Although the exact causes of preeclampsia are still unclear, it is thought that there is a cascade of events starting with an increase in oxidative stress/free radicals in the placenta. This increase in oxidative stress results in the production, and release, of one or more factors which are toxic (or dangerous) to the maternal endothelium and that the maternal endothelium is activated in response to that toxic/dangerous factor [2]. Finally the activation of the maternal endothelium prevents the normal adaptation the maternal vasculature to pregnancy with consequent hypertension and the other signs and symptoms of preeclampsia. Despite extensive investigations over many years, the nature of the toxic/dangerous factor(s) that is released from the placenta in

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preeclampsia, is unknown but several factors such as inflammatory cytokines and antiangiogenic factors (including soluble FLt-1) have been proposed [3,4]. In addition, for more than a century it has been known that fragments of the syncytiotrophoblast, which today are referred to as extracellular vesicles or trophoblastic debris may be one of the toxic/dangerous factors released from the placenta [5–7].

The surface of the human placenta is covered by a single multinucleated cell, the syncytiotrophoblast, which is bathed in maternal blood. During pregnancy, a large number of extracellular vesicles (EVs) are extruded from syncytiotrophoblast into the maternal circulation as early as six weeks of gestation [8]. The extracellular vesicles extruded from the syncytiotrophoblast include, multinucleated syncytial nuclear aggregates (which range in size from 20 − several 100s of um in size), trophoblastic ghosts, and mononuclear trophoblasts which together may be referred to as trophoblastic debris, as well as, much smaller microvesicles and nanovesicles [9]. Trophoblastic debris is deported from the placenta by the maternal blood and due to its large size, trophoblastic debris becomes trapped in the microvasculature of the maternal lungs which are the first small vessels the debris encounters after leaving the placenta [5,10]. These extracellular vesicles interact with maternal endothelial cells (and other maternal cells) and we, and others, have shown that in normal pregnancy placental extracellular vesicles/trophoblastic debris protect endothelial cells from activation [11]. In contrast trophoblastic debris from preeclamptic placentae has been shown to activate endothelial cells and thus may be considered as toxic or dangerous to the maternal endothelium [12–14].

Antiphospholipid antibodies (aPL) are autoantibodies that increase the risk of women developing preeclampsia up to 10 fold [15,16]. We have shown that aPL are internalised into the syncytiotrophoblast where they bind to and disrupt mitochondria leading to the production of toxic/dangerous trophoblastic debris which activated endothelial cells [16,17]. Likewise, treating normal placentae with preeclamptic sera also produced toxic/dangerous trophoblastic debris [18]. These studies suggest that aPL and other unknown factors contained in preeclamptic serum trigger mitochondrial dysfunction in the syncytiotrophoblast.

Melatonin, originally shown to be a marker of circadian rhythms, is a lipid soluble hormone released mostly by the pineal gland. More recently melatonin has been found to have direct free radical scavenging and indirect antioxidant activities in both mother and fetus [19–22]. Melatonin is also produced by several other cells and tissues including the placenta during pregnancy. Thus when melatonin levels peak in pregnancy (80–100 pg/mL), they are at least double that in men or non-pregnant women [23–27]. However, women with preeclampsia have decreased serum levels of melatonin [23,24]. Given the action of melatonin in modulating free radicals/oxidative stress, we undertook this study to investigate whether melatonin could reverse the effects of preeclamptic sera or aPL on the production of toxic endothelial cell-activating trophoblastic debris.

2. Material and methods

This investigation conforms to the principles outlined in the Declaration of Helsinki. This study was approved by the Auckland Regional Health and Disabilities Ethics Committee, New Zealand (NTX/12/06/057/AM03). All patient-derived tissues and blood samples were obtained with written informed consent.

2.1. Collection of placentae and blood

First trimester placentae, ranging from 8 to 12 weeks of gestation were collected from elective surgical terminations of pregnancies (n = 25). In addition, blood samples from six women presenting with preeclampsia and six gestation-matched normotensive pregnant women were collected by venepuncture into plain vaccutainer tubes. The blood was then allowed to clot, centrifuged at 2500 × g and the serum was aspirated and stored in aliquots at −80 °C.

Preeclampsia was defined as a maternal systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg measured on two occasions separated by at least 6 h, and proteinuria >300 mg in a 24 h period or qualitative, >1+, or impaired liver function, after 20 weeks of gestation in accordance with the guidelines of the American College of Obstetricians and Gynaecologists [28]. The patient characteristics are summarised in Table 1.

2.2. Antiphospholipid antibodies (aPL)

The murine monoclonal aPL, ID2 [29] was produced in our laboratory and generated by hybridoma culture and then purified on HiTrap Protein G columns (GE Healthcare). This monoclonal aPL has been extensively characterised and has, anticardiolipin, anti β_2GPI, and lupus anticoagulant activities [30]. In addition, a murine IgG1 antibody (Life Technologies, Auckland) was used as an isotype-matched treatment control antibody in experiments involving ID2.

2.3. Culture of placental explants and preparation of placental EVs

Placental macro EVs/trophoblastic debris was collected from elective surgical abortion first trimester placentae that had been treated with aPL, ID2 or IgG, or 10% sera, from women with preeclampsia (n = 6) or gestation-matched normotensive controls (n = 6), as described previously [12,31,32]. Briefly, approximately 400 mg placental explants were dissected from first trimester placentae (8–12 weeks of gestation). The explants were then cultured in Netwell™ culture inserts, suspended in 12 well culture plates, for 24 h at 37 °C in 3 ml DMEM/F12 containing 10% fetal bovine serum in an ambient oxygen atmosphere containing 5% CO_2 in the presence of ID2 (20 μg/ml) or an isotype-matched control antibody (20 μg/ml) with or without melatonin (1 μM or 10 μM) (Sigma-Aldrich, Auckland). In some experiments, explants were then cultured in Netwell™ culture inserts, suspended in 12 well culture plates, for 24 h at 37 °C in 3 ml DMEM/F12 in an ambient oxygen atmosphere containing 5% CO_2 in the presence of 10% sera, from women with preeclampsia or gestation-matched normotensive controls with or without melatonin (1 μM and 10 μM). In these experiments the media did not contain fetal bovine serum. The Netwell™ inserts (containing the explants) were then removed from the culture wells and the trophoblastic debris shed from the explants, which passes through the Netwell™ inserts, was aspirated from the culture wells in total of 3 ml and centrifuged at 300 g for 10 min. The supernatant was discarded and the pellets containing trophoblastic debris were washed with PBS, and re-suspended in 1 mL of PBS then depleted of contaminating $CD45^+$ leukocytes using magnetic beads (Dynal, Invitrogen, Auckland) according to the manufacturer's instructions. Contaminating red blood cells were removed by incubation in nine mLs of MilliQ water for 1 min, then one mL of 10X PBS was added to immediately return the trophoblastic debris to isotonic conditions. The trophoblastic debris were then suspended in MCB1 medium (endothelial cell culture medium) and exposed to endothelial cells. These procedures resulted in trophoblastic debris (confirmed by cytokeratin 7 and vimentin immunostaining) essentially free from contaminating non-trophoblast cells as described previously [13,31,33].

The trophoblastic debris from approximately 1200 mg of