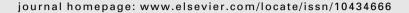
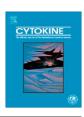


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Cytokine





Effect of hypoxia and exogenous IL-10 on the pro-inflammatory cytokine TNF- α and the anti-angiogenic molecule soluble Flt-1 in placental villous explants

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ABSTRACT

Introduction: The placenta plays a pivotal role in the pathophysiology of preeclampsia. Insufficient trophoblast invasion within the placenta can cause focal regions of ischaemia/hypoxia that, in turn, may stimulate the production of inflammatory cytokines. These cytokines are thought to cause endothelial cell activation and dysfunction, resulting in the clinical signs of preeclampsia. In addition to insufficient trophoblast invasion, the presence of inadequate maternal vasculature remodelling by trophoblasts also leads to changes in angiogenesis that may result from variations in the inflammatory cytokine profile. Aims: This study examined changes in the protein levels of IL-10 (immunoregulatory), TNF- α (pro-inflammatory) and sFlt-1 (anti-angiogenic) in normal term placentas under different oxygen tensions. The second aim was to determine if the link between varying levels of the cytokine, IL-10, and the expression/ release of TNF- α was oxygen dependent, and whether there was a concurrent change in sFlt-1. Methods: Normal term placentas (n = 6) were cultured at three different oxygen tensions (2%, 8% or 21%) in the presence or absence of exogenous IL-10. Protein (TNF-α and sFlt-1) secretion was measured using commercial ELISA kits, and qRT-PCR was used to examine gene expression. Results: Placental IL-10 release was significantly reduced at 2% oxygen when compared to 8% (p = 0.045) and 21% (p = 0.013). Expression of TNF- α and sFlt-1 was not significantly different. Exogenous IL-10 significantly reduced TNF- α protein levels only when explants were cultured in 2% oxygen (p = 0.05). Soluble Flt-1 protein secretion was unaffected by the addition of IL-10 at any of the oxygen tensions tested. Conclusion: TNF- α release can be inhibited in vitro by IL-10 under hypoxic conditions. However, IL-10 has no affect on sFlt-1 in term placentas, suggesting that these molecules act either via different pathways, or if linked, may be so at different stages of placental development.

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1. Introduction

Preeclampsia is a common condition affecting approximately 3% of pregnant women [1], and is one of the leading causes of both perinatal and maternal morbidity and mortality. It normally manifests itself in the third trimester of pregnancy. The main signs include hypertension, proteinuria and oedema, as well as generalised vasoconstriction resulting in end-organ hypoperfusion, and a coagulopathy due to platelet activation.

The pathogenesis of preeclampsia is thought to have three main components: abnormal remodelling of the placental vasculature [2], utero-placental ischaemia [3] and endothelial cell dysfunction [4]. One hypothesis of inadequate trophoblast invasion leading to focal regions of placental ischaemia/hypoxia correlates a localised

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overproduction of pro-inflammatory cytokines and anti-angiogenic molecules with a corresponding loss of immunoregulation by interleukin-10 (IL-10) [5–7].

Several studies have demonstrated a relationship between maternal blood pressure and placental secretion of IL-10. It is known that women with higher levels of IL-10 tend to have lower blood pressures during pregnancy [8]. Furthermore, anti-IL-10 antibodies administered to pregnant baboons significantly increased their blood pressure [9], and hypertensive pregnancies are thought to be associated with a deficiency of IL-10 [10,11]. Moreover, IL-10 is a known inhibitor of pro-inflammatory Th1 cytokines, such as tumour necrosis factor-alpha (TNF- α), in normal (non-pregnant) immune cells [12]. TNF- α is known to cause endothelial dysfunction, and a study conducted by Alexander et al. [13] found that administration of TNF- α to pregnant rats resulted in a significant rise in blood pressure, one of the main clinical signs of preeclampsia.

Elevated serum soluble fms-like tyrosine kinase 1 (sFlt-1), an anti-angiogenic molecule, has been reported in preeclamptic wo-

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men and it is thought to act via antagonism of vascular endothelial growth factor (VEGF) [7,14]. Animal models have been used to demonstrate that an up-regulation of sFlt-1 can result in hypertension, proteinuria and endothelial damage [3,7]. It has also been shown that both TNF- α and VEGF induce a concentration-dependent release of sFlt-1 from placental explants [14]. Thus, elevated release of TNF- α and VEGF by the placenta may result in the endothelial dysfunction of preeclampsia via a pathway involving sFlt-1.

The aim of this investigation was to determine if IL-10 could inhibit the expression and release of TNF- α and sFlt-1 from placental explants when cultured at oxygen tensions relevant to the uteroplacental interface. This will examine the hypothesis that control of trophoblast inflammatory cytokine release by IL-10 operates in hypoxic conditions.

2. Materials and methods

2.1. Patient selection

Normal term placentas were obtained, with written consent, from patients at our local institution and with Institutional Ethics Committee approval (Table 1). Placentas from caesarean sections were used as labour has been reported to alter cytokine profiles (n = 6) [15]. Therefore patients were selected who had elective, pre-labour caesarean sections.

2.2. Placental explant culture

Small sections from the maternal side of normal term placentas were placed in RPMI-1640 medium within 1 h of delivery. The placental tissue was dissected into fragments of chorionic villi ($\sim\!5$ mg wet weight). Visible membranes and blood vessels were removed and the fragments washed in PBS to remove maternal blood. Three to four fragments ($\sim\!20$ mg wet weight) were placed in 24-well plates with 0.5 ml RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin [16]. Concentrations of either recombinant human IL-10 (R&D Systems, MN, USA) or monoclonal anti-human IL-10 antibody (R&D Systems, MN, USA) were added to each well. Each treatment was done in triplicate.

Tissue culture plates were placed into hypoxia chambers on an orbital shaker, and exposed to three oxygen tensions. The cultures were gently shaken as it has been reported that stationary cultures form an unstirred layer that results in reduced pericellular pO_2 [17]. An oxygen tension of 2% was used to simulate a hypoxic preeclamptic environment, given that abnormal placentation is thought to result in regions of focal ischaemia [12]. An oxygen tension of 8% was used to simulate a normoxic placental environment [18], whereas 21% oxygen was used as to study the effect of hyperoxia. The culture plates were maintained at their respective oxygen tensions for 24 h in the above conditions before freezing. Media and placental explants were then collected and snap frozen.

2.3. Glucose assay

Tissue viability was monitored by measuring glucose consumption in the cultured medium in comparison to un-cultured RPMI-

Table 1Ranges of clinical data from six normal pregnancies used for tissue culture.

	Median (IQR)
Age (years)	35 (7.25)
Gestation (weeks)	39 (0.75)
Birth weight (grams)	3605 (722.5)
Maximum systolic blood pressure (mm Hg)	120 (3.75)
Maximum diastolic blood pressure (mm Hg)	80 (7.5)

1640. Glucose concentrations were detected using an Amplex Red glucose assay kit (Molecular Probes, Eugene, OR, USA).

2.4. Cytokine and sFlt-1 detection

Concentrations of IL-10 and TNF- α in the cultured medium were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits (BD Biosciences, CA, USA). sFlt-1 was detected using Bender MedSystems ELISA kits (Vienna, Austria). Results were expressed as pg/mg of total protein for IL-10 and TNF- α , and as ng/mg total protein for sFlt-1. Intra-assay and interassay variability for IL-10, TNF- α and sFlt-1, respectively were 2.0%, 3.0% and 6.5%; and 11%, 11% and 17%, respectively. The sensitivity for IL-10 and TNF- α assays was >4 pg/ml, and the sensitivity for sFlt-1 was 0.06 ng/ml.

2.5. Protein assav

The total protein in the cultured placental explants was measured using the bicinchoninic acid method (Pierce, IL, USA). Total protein was used to calculate the ratio of cytokines/sFlt-1 secreted by placental explants to the total protein content of the explants.

2.6. Ouantitative real time PCR

Total RNA was extracted from placental explants using the QIA-GEN RNeasy Mini kit (Ont., Canada) and 0.25 μ g RNA was reversed into cDNA using random primers (Invitrogen, Vic., Australia) and the Superscript III RNase Reverse Transcriptase protocol. Primers for the genes examined are listed in Table 2.

Data was expressed as the percentage change in gene expression compared to control samples using the $2^{-\Delta \Delta Ct}$ method [21]. Uncultured, and therefore untreated, placentas were used as control samples. Samples were collected and stored in liquid nitrogen at the time of placental collection (within 20 min of delivery). At thawing, samples were treated as outlines above.

2.7. Statistical analysis

Overall significance (p < 0.05) was determined with non-parametric Kruskal–Wallis tests. Mann–Whitney U tests were then performed in order to determine any significant pair wise differences. The exogenous IL-10 experiments were analysed by ANOVA using two factor analyses: IL-10 dose and individual variation. Pair wise comparisons were corrected using a Bonferroni test. Results were expressed as median with IQR for protein, and mean \pm SEM for gene expression.

3. Results

A significant reduction in the level of IL-10 was found with decreased oxygen tension (p < 0.01, using a Kruskal-Wallis test) (Fig. 1). There was a highly significant decline in IL-10 secretion when placental explants were cultured in 2% oxygen (median, IQR: 0.69, 0.95) when compared to 21% oxygen (median, IQR:

Table 2 Primer sequences for the genes of interest.

Gene	Forward sequence	Reverse sequence
TNF-α [10] SDHA [20] ^a	TGGCGTGGAGCTGAGAGATAA GGCGGCATTCCCACCAACTACA	GAGCCCGGGGGTCTCATTATT GATGGCAGAGAGGAGGTTGAC GCACATGCCCGACCAAAGACAA AGTGGGGTGGCTTTTAGGATG

^a Housekeeping gene.

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