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Inflammatory processes are specifically enhanced in endothelial cells by placental-derived TNF-α: Implications in preeclampsia (PE)☆

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ABSTRACT

Introduction: There is a consensus that factors released by the placenta to maternal circulation, including TNF-α, play a key role in activating the maternal endothelium in pregnancies with preeclampsia (PE). Dual perfusion preserves the structural organization of the placenta to a greater degree than other *in vitro* systems and has been used by our group and others to examine placental pathophysiology associated with PE. The objective of this study was to use the dual perfusion model to test whether TNF-α released by the placenta to maternal perfusate affects pro-inflammatory cytokine secretion by, and activation of, endothelial cells, thereby furthering our understanding of placental and endothelial dysfunction in PE.

Method: We used maternal perfusate, two endothelial cell lines (HUVECs and HEECs), and a TNF-α blocking antibody to test whether placental-derived TNF-α plays a significant role in altering the expression and secretion of pro-inflammatory cytokines in endothelial cells as well as the expression of activation markers in this cell type.

Results: The presence of maternal perfusate significantly enhanced IL-6, IL-8, and MCP-1 secretion, levels of their mRNA, as well as mRNA levels of markers of endothelial activation (E-selectin, ICAM-1, and VCAM-1). The addition of a TNF-α blocking antibody significantly inhibited the maternal perfusate-mediated enhancement of cytokine secretion by, and expression of activation markers, in both HUVECs and HEECs.

Discussion: These results demonstrate that TNF-α significantly contributed to endothelial cell pro-inflammatory cytokine secretion and activation suggesting that blocking TNF-α action may mitigate the effects of maternal endothelial dysfunction in PE.

1. Introduction

Preeclampsia (PE) is a disease characterized by pregnancy induced-hypertension, and is a leading cause of maternal morbidity and adverse neonatal outcomes [1]. PE affects approximately 3–8% of all pregnancies worldwide, and principally appears during the late second trimester of pregnancy until term [1–3]. Delivery generally resolves symptomology, suggesting that the placenta plays a major role in the pathophysiology of PE [4]. There is now a consensus that activation of the maternal endothelium is a hallmark of preeclampsia [2,5–7]. Deficient trophoblast invasion and failure of conversion of maternal uterine spiral arteries have been proposed as a source of placental hypoxia/ischemia in PE leading to placental damage and enhanced syncytial release of factors to maternal blood which activate/dysregulate maternal endothelial function [1,8–10]. These factors include cytokines [8,11,12], syncytiotrophoblast microparticles (STBMs) [13,14], prostanoids and lipoperoxides [15–17], free fetal hemoglobin [18,19], and the anti-angiogenic factors soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (Eng) [20–24].

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Several *in vitro* human placental model systems have been used to study placental pathophysiology in PE including primary cultures of syncytiotrophoblasts [25], explants of whole placental tissue [9,26], and dual (maternal + fetal) perfusion of a single placental cotyledon [27–30]. Dual perfusion preserves the structural organization of the placenta to a greater degree than other *in vitro* systems [31–33]. Dual perfusion of normal human term placenta is initiated by cannulating fetal vessels supplying a single cotyledon of the placenta to establish the "fetal" perfusion [32,33]. The "maternal" perfusion is established through the superficial insertion of cannulae under the decidua basalis layer of the placenta so that the intervillous space is bathed by perfusion buffer. In this way, products released by the syncytiotrophoblast are collected in the maternal perfusate thereby mimicking the process whereby placental factors are released to maternal blood in the intervillous space during pregnancy and then enter the systemic circulation through the endometrial venous system [32,33]. During perfusion, despite relatively high oxygenation levels [34], local hypoxia/reperfusion does develop to some degree, mimicking processes observed during PE [27,28]. This hypoxic state (compared to *in vivo*) promoted a time-dependent release of plasminogen activator inhibitor-1 (PAI-1), an anti-fibrinolytic factor, to maternal perfusate, which is similar to the observed increase in PAI-1 levels in serum of women with PE [28,35]. It was demonstrated that hypoxic treatment induced release of inflammatory cytokines to maternal perfusate, including TNF-α, using a novel dual perfusion system [29]. Levels of cell-free fetal hemoglobin (Hb), an inducer of oxidative stress, were found to be higher in placental tissue and maternal serum in pregnancies complicated by PE [18,19]. Perfusion of placenta with cell-free Hb hemoglobin promoted oxidative stress and placental damage characteristically observed in PE [36], and also elicited the release of STBMs containing Hb and altered micro-miRNA (miRNA) content, suggesting they carry Hb and miRNA to the maternal circulation [37]. Of note, STBMs shed to maternal perfusate contained high levels of Eng and PAIs, suggesting a role in altering the fibrinolytic/angiogenic balance in PE [38]. Taken together, these results indicate that dual perfusion of the human placenta is uniquely suited to dissect placental pathophysiology associated with PE.

Tumor necrosis factor (TNF-α) induces pro-inflammatory cytokine secretion in endothelial cells including interleukin (IL)-6, IL-8, and monocyte chemotactic protein (MCP-1), as well enhancing the expression of endothelial cell activation markers including E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 [39–41]. It is known that levels of TNF-α are higher in maternal serum from pregnancies complicated by PE compared to normotensive pregnancies as are the levels of IL-6, IL-8, and MCP-1 [12,42]. Therefore, the primary goal of this study was to use the dual perfusion model to test whether TNF-α released by the placenta to maternal perfusate affects the production of pro-inflammatory cytokine secretion by endothelial cells, as well as activation markers in this cell type, thereby modeling aberrant syncytial and maternal endothelial dysfunction in PE [2,6,35,43,44]. Two endothelial cell lines were used for studies: human umbilical vein endothelial cells (HUVECs), previously employed in the evaluation of the effect of placental factors on the endothelium [45,46], and telomerase-immortalized human endometrial endothelial cells (HEECs) which are microvascular in origin [47], a relevant model for studying systemic maternal endothelial dysfunction in PE.

2. Methods

2.1. Dual (maternal + fetal) in vitro perfusion of human placenta

Dual *in vitro* perfusion of an isolated cotyledon of a human placenta was based on previous publications by our group [27,28,38,48]. To mimic intrauterine conditions, the fetal circulation was equilibrated with 95% nitrogen and 5% carbon dioxide and an atmospheric gas mixture was used for the maternal side. The experiments were terminated if any of the following criteria was observed: fetal perfusion pressure above 50 mmHg, loss of perfusate >4 ml/h, and in case of mismatch of materno-fetal circulation as measured by inadequate oxygen transfer (pO2 maternal side < 100 mmHg, pO2 fetal side < 20 mmHg). These are well established criteria for carrying out placental perfusion [27,28,38,48], but despite efforts to mimic *in vivo* oxygenation, relative hypoxia does result using these protocols [27,28]. For this study, perfusion media was obtained from previously published experiments [36] where medium was recycled for up to six hours in both circuits with samples collected every two hours. The first collection was designed as "phase I"; the second, "phase II"; the third, "phase III" were used in this study. In separate perfusions, the impact of free hemoglobin on placental damage was studied [36]; samples from these perfusions were not used in this study. Maternal perfusates were subjected to ultracentrifugation (150,000×*g*) to remove STBM from maternal perfusate, yielding STBM-free supernatant [49]. The perfusion medium consisted of NTCT 135 tissue culture medium in Earl's buffer (1:3 dilution), 4% bovine serum albumin, 0.2% glucose, 1% dextran 40, 2.5 IU/ml heparin, and 250 μg/ml clamoxyl from previously described sources [36].

2.1.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Yale University's tissue culture core laboratory, and telomerase immortalized human endometrium endothelial cells (HEECs) were obtained as we have reported [47,50]. Cells were seeded at a density of 25,000 cells/0.5 ml of endothelial cell growth medium-2 (EGM-2) per gelatin coated well, and grown at 37 °C with air/5% CO_2. Cell cultures were incubated one day before medium was aspirated, replaced with fresh EGM-2, and incubated for one more day. Either EGM-2 or maternal perfusates diluted with EGM-2 at a 1:4 ratio were introduced to cells (50% confluent), and incubated for 24 h. The 24 h time point was chosen based on previous *in vitro* studies including ours which indicated that a 24 h treatment with TNF-α and other inflammatory factors markedly enhance cytokine secretion by HUVECs [40,51]. We monitored total HUVEC cell protein in cells maintained for 24 h with undiluted EGM-2 culture medium, or with perfusate serially diluted two to 128-fold with EGM-2 medium. We found that the presence of four-fold diluted maternal perfusate was the lowest dilution of perfusate which did not reduce total levels of HUVEC protein level compared to EGM-2 culture medium control. Conversely, the use of undiluted perfusion medium reduced HUVEC cell protein levels by approximately 30%. Media were then saved and cells were washed with phosphate buffered saline (PBS) before lysing with 100 μL of 0.4% SDS and subsequent analysis by Bradford assay for protein levels ($n = 5$). Alternatively, cells were lysed with 1 ml of TriZol (Life Technologies, Grand Island, NY) in preparation for RNA isolation and qRT-PCR ($n = 5$). For experiments involving TNF-α blocking antibody, the diluted maternal perfusate was incubated for 2 h prior to incubation with endothelial cells and 1 μg/ml of mouse anti-human TNF-α blocking antibody or 1 μg/ml of mouse immunoglobulin G1 Isotype Control (R&D Systems) ($n = 5$).

2.2. ELISA and protein assays

Levels of TNF-α, IL-6, IL-8, and MCP-1 were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Inflammatory cytokine concentrations in both