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Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected *in vitro* by human cell-based angiogenesis tests

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ABSTRACT

Objective: To explore *in vitro* angiogenic properties of maternal and umbilical cord blood sera from women with symptomatic pre-eclampsia in comparison with sera from women with normotensive pregnancies.

Study design: Maternal and umbilical blood serum samples were collected from eleven primiparous women with pre-eclampsia and ten healthy gestational-age-matched primiparous controls. The samples were tested for tubule formation in two different types of *in vitro* angiogenesis tests. The first test (fibroblast-HUVEC) showed effects on angiogenesis and the second test (hASC-HUVEC), in addition to angiogenesis, also showed effects on vasculogenesis. The pro-angiogenic and inhibitory properties of the samples were microscopically quantified after immunostaining tubular structures, using markers for von Willebrand factor (vWf) and collagen IV.

Results: Serum samples from pre-eclamptic women inhibited tubule formation in both models, while those from normal pregnancy didn't. Umbilical blood samples were inhibitory both after pre-eclampsia and normal pregnancy. In the fibroblast-HUVEC model the inhibition was stronger after preeclampsia pregnancy, and the difference between groups was statistically significant. In the pre-eclampsia group a correlation between the inhibitory effect of umbilical blood and birth weight adjusted to gestational age was found. No clear correlation between sera from pregnant women and corresponding umbilical sera was found.

Conclusion: The strong inhibitory effect of maternal serum samples on tubule formation reflects the anti-angiogenic state that is present in pre-eclampsia.

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1. Introduction

Although the exact pathogenesis of pre-eclampsia remains unclear, there is incremental data indicating that angiogenic factors play a major role in the development of this disease [1–6]. An imbalance between pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) has been detected in numerous studies [1–4,7]. Changes in the concentrations of circulating angiogenic and anti-angiogenic proteins may occur weeks before the clinical recognition of pre-eclampsia [8,9].

The angiogenic properties of umbilical blood after pre-eclamptic pregnancy have been studied to a lesser extent. It is known that sFlt-1 levels are high in umbilical blood after pre-eclampsia [5,10]. According to the results of recent studies there are also other factors that affect the vasculogenic properties of umbilical blood. In a study by Xia et al. (2007) venous cord blood samples were collected during labour from pre-eclamptic mothers and normotensive controls in order to investigate endothelial progenitor cells (EPCs). It was noticed that the level of EPCs was significantly lower after pre-eclamptic pregnancies when compared with normal ones. Endothelial progenitor cells mediate neovascularization in uterine

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endometrium and it has been hypothesized that they might be involved in neovascularization of the placental vasculature [9].

Vasculogenesis (formation of blood vessels from EPCs) and angiogenesis (formation of new blood vessels from existing ones) are crucial for placentation and embryonic development [11]. Later in human life angiogenesis is involved in physiological processes such as wound healing and the menstrual cycle as well as in pathological processes such as tumour development [12]. Angiogenesis is a multi-step process. The key stages of angiogenesis are endothelial cell proliferation, migration, differentiation and tubule formation. Each stage is regulated by different growth factors and inhibitors [13]. Evaluation of the factors that affect angiogenesis would optimally be studied *in vivo*, but animal models have several disadvantages such as variability and animal-specificity. However, a standardized *in vitro* angiogenesis assay is reliable for testing the modulators of angiogenesis [14]. The human primary cell-based *in vitro* assay, which was used in the present study, mimics the effects in humans well [14,15]. As yet only a few models of angiogenesis using cell-culture techniques are available to explore the effects of pre-eclampsia. Maynard et al. measured endothelial tubule formation in an *in vitro* model of angiogenesis using human umbilical vein endothelial cells (HUVECs) and proved that sera from women with pre-eclampsia inhibit tubule formation [16].

Our aim was to utilize two different *in vitro* angiogenesis models to study whether sera from women with pre-eclampsia exhibit different angiogenic effects compared with sera from healthy controls. Furthermore, we wanted to explore the angiogenic properties of umbilical blood sera from newborns after pre-eclamptic and normotensive pregnancies.

2. Material and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit number R11088). All patients gave written informed consent. The study conforms to the principles outlined in the Declaration of Helsinki. The use of human adipose stromal cells (hASCs) and human umbilical cord endothelial cells (HUVECs) was separately approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit numbers R03058 and R08028, respectively).

2.2. Study population

In this cross-sectional study, we collected maternal blood samples from eleven primiparous women with pre-eclampsia and ten primiparous controls. Corresponding umbilical cord blood samples were obtained from nine women in both groups. Each of the controls had the same gestational age as the women with pre-eclampsia at maternal blood sample collection. The inclusion criteria for healthy controls were: blood pressure <140/90 mmHg, urine dip stick test negative for proteinuria and previously uncomplicated singleton pregnancy. Hypertension later in pregnancy was an exclusion criterion.

The samples were collected during 2011–2014 at the Department of Obstetrics and Gynaecology, Tampere University Central Hospital. In the study group the samples were taken at a maximum of three days before delivery when the patients were already hospitalized because of clinical symptoms of pre-eclampsia. Cord blood samples were taken after delivery of the placenta in both groups. There was no separation between umbilical arterial and venous blood in the cord samples. The serum samples were frozen and conserved at −70 °C until assay. Blood tests for haemoglobin level, platelet count and alanine aminotransferase level were carried out at admission from the women with pre-eclampsia. Baseline demographic details and data on pregnancy outcome were collected from the hospital maternity records. Gestational age was calculated on the basis of the last menstruation and corrected if necessary at first-trimester screening ultrasonography.

Pre-eclampsia was defined according to the guidelines of the International Society for the Study of Hypertension in Pregnancy. Systolic blood pressure should be $\geqslant$140 mmHg or diastolic blood pressure $\geqslant$90 mmHg on at least two occasions 4 h apart after 20 weeks of gestation in previously normotensive women, with proteinuria of 300 mg or more in 24 h [17]. Pre-eclampsia was defined as severe if HELLP syndrome (haemolysis, elevated levels of liver enzymes and low platelet count), eclampsia or exceptionally high blood pressure (>160 mmHg systolic or >110 mmHg diastolic) appeared [18,19].

Deviation from normal growth (mean weight of newborns at the same gestational age) was determined for all newborns according to national weight curves [20]. Small-for gestational-age (SGA) was defined as birth weight more than two standard deviations below the mean.

Pre-eclampsia was divided into early- and late-onset depending on the gestational age when it was diagnosed. The cut-off point between these two groups was 34 weeks [21].

2.3. In vitro angiogenesis/vasculogenesis tests

The tests were performed at the Finnish Centre for Alternative Methods, School of Medicine, University of Tampere. Two different tests were utilized in the study:

1) The fibroblast-HUVEC test (angiogenesis test method), which involves co-culture of human BJ fibroblasts (20,000 cells/cm^2) and primary HUVECs (4000 cells/cm^2) and is used to show basic effects of angiogenesis [14].
2) The hASC-HUVEC test (angiogenesis/vasculogenesis test), based on co-culture of human adipose stem cells (hASCs, 20,000 cells/cm^2) and HUVECs (4000 cells/cm^2) was used to include vasculogenesis [15,22].

Human adipose tissue samples were obtained from waste material from surgical operations and human umbilical cords were received after Caesarean sections (with informed consent) at Tampere University Hospital, Tampere, Finland. The culture medium contained specific exogenous growth factors to induce formation of tubular structures and networks. Two different growth factor concentrations were used to induce either strong vascular formation (to reveal possible inhibitory effects of test samples on vascular formation) or to induce only moderate vascular formation, where stimulation of vascular formation may also take place. In inhibition set-ups the co-cultures were exposed to 1 ng fibroblast growth factor β (FGF-β)/ml and 10 ng VEGF/ml, whereas in stimulation tests the concentrations were 0.25 ng/ml and 2.5 ng/ml, respectively. Table 1 shows assay media components. Serum-free hASC assay medium was modified from our earlier-described medium [14].

During both *in vitro* tests, the co-cultures were exposed to patient serum samples at a dilution of 1:15 and cultured for a further six days (with one replenishment of the growth medium). After exposure, the number of living cells (viability) was evaluated. The purpose was to distinguish possible cytotoxicity (unspecific general toxicity causing cell death) from anti-angiogenetic effects induced by the samples, seen as a reduced amount of formed vascular structures. Viability/cytotoxicity of the samples was evaluated by using a WST-1 assay (Cell Proliferation Reagant, Roche, Basel, Switzerland) which measures mitochondrial activity present only in living cells. After WST-1 assay, the cells were fixed with 70% ethanol and immunostained for vWf (Sigma Aldrich, Manassas, VA, USA) and

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