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Fibrinogen, an endogenous ligand of Toll-like receptor 4, activates monocytes in pre-eclamptic patients

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ABSTRACT

Pre-eclampsia (PE) remains the leading cause of pregnancy-associated mortality and morbidity, urging the need for a better understanding of its aetiology and pathophysiological progression. A key characteristic of PE is a systemic, exaggerated, inflammatory condition involving abnormal cytokine levels in serum, altered immune cell phenotype and Th1/Th2-type immunological imbalance. However, it is unknown how this heightened inflammatory condition manifests. We previously reported increased expression of the lipopolysaccharide receptor, Toll-like receptor 4 (TLR4), on monocytes from PE patients compared with normotensive, pregnant patients (NP). This upregulation of TLR4 on PE monocytes was accompanied by a hyper-responsiveness to bacterial TLR4 ligands. To determine whether non-microbial, endogenous TLR4 ligands also activate monocytes from PE patients, we investigated the expression of host-derived TLR4 ligands and the response of monocytes to these endogenous ligands. Plasma levels of fibrinogen – but not fibronectin or heparan sulphate – were higher in PE patients than in NP. Exposure to fibrinogen was associated with significantly increased production of inflammatory cytokines by monocytes from PE patients. Interestingly, this effect was not observed with NP monocytes. Our findings suggest that the fibrinogen-TLR4 axis might play an important role in the atypical activation of monocytes observed in PE patients that may contribute to the exaggerated inflammatory condition.

1. Introduction

Pre-eclampsia (PE) is a systemic, pregnancy-associated disorder, characterised by hypertension and abnormally elevated levels of protein in the urine during the second half of pregnancy (Redman, 2011). PE is relatively common in pregnant women (2–7%) and remains a potentially life-threatening disease (Sibai et al., 2005). Although its aetiology is unknown, it is generally accepted that PE arises from a disrupted interaction between the placenta and the maternal uterus causing endothelial cell dysfunction.

Inflammation is another key hallmark of PE. Hypertensive pregnant patients exhibit excessive pro-inflammatory cytokine levels in serum, reduced immunosuppressive cytokines, abnormal immune cell phenotype and activation, as well as Th1-skewed immunity (Greer et al., 1991, 1994; Vince et al., 1995; Kupferminc et al., 1996; Conrad et al., 1998; Saito et al., 1999; Gervasi et al., 2001; Freeman et al., 2004; Jonsson et al., 2006; Luppi and Deloia, 2006;

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Table 1
Patient characteristics.

	Non-pregnant ($n = 11$)	Normal pregnant ($n = 17$)	Pre-eclampsia ($n = 17$)	P value
Age (years)	31.0 ± 5.4	29.3 ± 4.9	31.4 ± 5.6	0.26
Gestational age (weeks)		32.0 ± 3.7	32.9 ± 3.7	0.38
Gravidity		1–4	1–4	
SBP (mm Hg)	110.0 ± 10.0	114.0 ± 11.0	150.4 ± 8.6	0.0001
DBP (mm Hg)	70.0 ± 10.0	70.0 ± 10.0	98.3 ± 4.2	0.0001
24-h urine collection (g/24 h)	0	0	0.84 ± 1.3	
Urine Dipstick Protein Test	0	0	(1+ to 3+)	

Szarka et al., 2010; Xiao et al., 2012). In normal pregnancy, Th1-type immunity is inhibited, owing to the harmful effects on the foetus, and Th2-type immunity is predominant (Wegmann et al., 1993). Like other aspects of PE disease, it is not known why the excessive inflammation occurs. Several epidemiological studies have established an increased risk of PE in patients with infections by specific – not all – pathogens (von Dadelszen et al., 2003; Chen et al., 2012; Minassian et al., 2013), suggesting that bacterial or viral products might cause or contribute to the overactive inflammatory response. However, many PE patients do not exhibit clinical features of infection, indicating that endogenous factors may cause or contribute to the inflammatory features of the condition.

We recently demonstrated that monocytic subpopulations are also dysregulated in PE (Al-ofi et al., 2012). We reported that non-classical $CD14^{low}CD16^{+}$monocytes are significantly increased in women with PE and that their phenotype is profoundly altered compared with monocytes from normal pregnant (NP) women. Expression of the lipopolysaccharide (LPS) receptor, Toll-like receptor 4 (TLR4), was markedly upregulated on monocytes from PE patients, instigating a more thorough, functional analysis of this receptor. *Ex vivo* experiments showed that PE monocytes are hyper-responsive to bacterial TLR4 ligands, releasing pro-inflammatory cytokines several magnitudes greater than NP monocytes. Because PE patients do not often exhibit clinical features of infectious disease, increased cytokine production from monocytes through TLR4 signalling may result from endogenous TLR4 ligand activity rather than being due to the presence of bacterial products. These endogenous ligands may include heat shock proteins, heparan sulphate proteoglycan, fibrinogen, fibronectin, extracellular matrix hyaluronan and high mobility group box 1 protein (HMGB1), which are released from damaged cells (Erridge, 2010). Therefore, we hypothesised that endogenous TLR4 ligands might modulate the behaviour of monocytes in PE patients.

2. Materials and methods

2.1. Ethics statement

Approval for this study was received from South Yorkshire Research Ethics Committee (09/H1310/12).

2.2. Subjects and samples

Women with established pre-eclampsia were diagnosed by the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) (Luppi et al., 2002), and were recruited from the antenatal clinics and obstetric day care unit of the Jessop Maternity Wing of the Royal Hallamshire Hospital, Sheffield. Healthy NP women attending routine antenatal clinics at Jessop Wing were recruited to be a part of the control group. Healthy non-pregnant (Non-P) female volunteers were also studied to determine baseline non-gestational levels (Table 1). Non-P women had normal menstrual cycles and were not on hormonal contraception. All of the participants in the study were negative for systemic infection or urinary tract infection. All participants gave informed written consent for 12 ml of fresh venous blood to be collected into a tube containing EDTA anticoagulant.

2.3. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the plasma levels of fibrinogen (Abnova, Germany), fibronectin (Bender MedSystems, Vienna, Austria) and heparan sulphate (Uscn Life Science Inc. Wuhan, China). The manufacturer's instructions were followed and each sample was tested in triplicate.

2.4. Immunohistochemistry

Immediately following normal vaginal delivery or caesarean section, the placenta was collected from participants (PE or NP) who had previously given consent. Two or three pieces were removed from the placental tissue just around the umbilical cord area and immediately soaked and fixed for 24–48 h in 10% formalin. The samples were wax embedded for immunohistochemistry staining.

Fibrinogen staining (Dako, UK) was optimised according to a previously published study (Karehed et al., 2010). All the placental samples from pregnant and pre-eclamptic women were immunostained at the same time. Paraffin-embedded placental sections (5 μm thick) were de-waxed in two dishes of xylene for five minutes each, and then rehydrated using gradual ethanol. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 30 min. Proteinase K was used for antigen retrieval. Non-specific binding of the antigen and the antibody were blocked by incubating the sections in normal horse serum (Vector Universal Impress anti-mouse/rabbit Ig kit, UK) for 20 min. The diluted primary antibody or the rabbit immunoglobulin fraction (Dako, UK) was added to each slide, and the slides were then incubated in a sealed, humidified container at room temperature for 30 min. The slides were then washed twice in PBS and incubated with a