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Functional activity but not gene expression of toll-like receptors is decreased in the preterm versus term human placenta

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ABSTRACT

Introduction: Toll-like receptor (TLR) activity within gestation-associated tissues might have a role in normal pregnancy progression as well as adverse obstetric outcomes such as preterm birth (PTB).
Methods: The expression and activity of TLRs 1–9 in placentas collected following preterm vaginal delivery after infection-associated preterm labour (IA-PTL) at 25–36 weeks of gestation (preterm-svd, n = 10) were compared with those obtained after normal vaginal delivery at term (term-laboured; n = 17). Placental explants were cultured in the presence of agonists for TLR2, 3, 4, 5, 7, 8 and 9 and cytokine production after 24 h examined. Expression of TLR transcripts was determined using real time quantitative PCR.
Results: Reactivity to all agonists except CpG oligonucleotides was observed indicating that other than TLR9 all of the receptors studied yielded functional responses both term and preterm. Significantly less TNFα and IL-6, but not IL-10, were produced by preterm than term samples in response to all TLR agonists. Changes in TLR mRNA expression did not underlie functional differences in the preterm and term groups; nor does a pre-exposure/tolerance model mimic this finding. While glucocorticoids suppressed cytokine production in an *in vitro* model using term tissue the association between lower gestational age and decreased cytokine outputs suggests a temporally regulated response.
Discussion: Pro-inflammatory cytokine output in response to multiple TLR ligands was decreased in the preterm compared to the term placenta but gene expression for each TLR tended to be similar. Reduced cytokine production by the preterm placenta in response to stimulation of TLRs therefore must be regulated at the post-transcriptional level in a gestational age dependent manner.
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1. Introduction

Cytokines and chemokines have a role in the normal physiological processes of pregnancy including parturition. IL-1β, IL-6, IL-8 and TNFα among others are produced by gestation-associated tissues both constitutively and/or in response to insult [1,2]. This has led to interest in the mechanisms of cytokine production in these tissues, with signalling pathways of the innate immune system that produce a defined cytokine output postulated as central to this [3]. Toll-like receptors (TLRs), a family of pattern recognition receptors (PRRs), link microbial agonists to the production of inflammatory mediators. In humans, ten TLRs (TLRs 1–10) have been identified [4]. TLRs could provide a mechanism of cytokine production at the maternal–fetal interface in not only normal physiological aspects of pregnancy but also in various pathological states of pregnancy such as infection associated preterm labour [3,5].

The placenta has been called a pregnancy-specific component of the innate immune system because it constitutes a physical and immunological barrier against invading infectious agents; the activity of TLRs and other PRRs within cells of the placenta would support such a role. Transcripts for TLRs 1–10 have been demonstrated in both the term and preterm human placenta and isolated cytotrophoblast and syncytiotrophobasts [6,7]. Trophoblastic choriocarcinoma cell lines express TLRs 1–10 and several co-receptors and accessory proteins [8]. Functional activity of TLR2, TLR3 and TLR4 has been reported for first and third trimester trophoblast/placenta [9–12] and choriocarcinoma cell lines are responsive to ligands for TLRs 2, 3, 4 and 9 [8]. Other cell types

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within the term placenta including Hofbauer cells express TLR2, TLR3 and TLR4 mRNA and/or protein and have functionally active TLR3 and TLR4 [13,14]. There is temporal variation in the expression of TLRs in the placenta: both first trimester primary trophoblast and trophoblast cell lines express TLRs 1–4 but not TLR6 which is expressed in third trimester trophoblast [9,15], while TLR4 expression is higher at term than during the first trimester [16].

Globally around 10% or babies are born prematurely. There are three main antecedents of PTB: 30–35% maternal or fetal indications, 40–45% spontaneous preterm labour with intact membranes, and 20–25% preterm premature rupture of the membranes (PPROM): the latter two are grouped as spontaneous PTB [17]. Pathophysiological mechanisms underlying spontaneous PTB are largely unknown but a wealth of evidence indicates that preterm labour is an inflammatory process [18,19]. The induced inflammatory milieu is likely heterogeneous depending on the underlying cause of PTB [18,20], and both gestation-associated tissues themselves and infiltrating leukocytes contribute [21]. However, there is little information about the possible role of PRRs and their ligands in spontaneous PTB especially that associated with infection. The probable role of TLRs in the pathogenesis of infection-associated preterm labour (IA-PTL) and other adverse pregnancy outcomes has been studied mostly with regards to TLR4: inflammatory cells infiltrating preterm placentas with chorioamnionitis express TLR4, and TLR4 expression on villous Hofbauer cells is increased in preterm placentas without chorioamnionitis and term placentas [14]; functional TLR4 has been implicated in preterm labour triggered by administration of heat killed *E.coli* in mice [22]; expression of TLRs 2, 4, 5 and 6 mRNA and TLR4 protein are also increased in the preeclamptic placenta [23,24]; the Asp299Gly TLR4 gene polymorphism associated with impaired TLR4 receptor function and an increased likelihood of Gram-negative sepsis [25] is carried more often by preterm than term infants or by mothers delivering preterm rather than at term [26]. A role for functional TLR3 in preterm labour also has been described [27]. Evidence for a role for TLRs in infection-associated preterm birth also comes from genetic studies.

Over the past few years there has been a dramatic increase in interest and information about activity mediated by microbial ligands/TLR combinations in various tissues and diseases. As we had previously described that multiple TLRs (with the exception of TLR9) were functional in the term placenta and stimulation with TLR agonists could lead to the production of relevant cytokines and chemokines [6] an investigation of the expression and activity of TLRs in preterm placentas delivered with evidence of intrauterine infection was undertaken.

2. Materials and methods

2.1. Characteristics of preterm samples

Placentas were obtained following preterm labour at varying gestations, ranging from 25 weeks to 36 weeks (n = 15; Table 1). The 10 cases which delivered vaginally after spontaneous onset of labour had no evidence of preeclampsia, intrauterine growth restriction or other obvious materno-fetal reasons but evidence of infection was found in all but one either in the form of histologic chorioamnionitis, positive swabs, urine examination or blood markers of infection (Table 1); all women had received steroid injections as part of their clinical care. None of these women were known to have any autoimmune or other immunological disorders. These cases (n = 10) (IA-PTL) have been included in this study. Gestational age was calculated by ultrasound or by the first day of the last menstrual period. Women were approached either at admission in early labour or soon after delivery in full liaison with the midwives. Term samples were from women who delivered vaginally after spontaneous onset of labour and after 37 completed weeks of gestation. All women delivering preterm had received steroids prior to delivery as part of their clinical therapy for preterm labour; women delivering at term had not. All women gave informed written consent and Wales Research Ethics Committee 6 approved the study.

2.2. Placental explant culture

All placentas were weighed and then explant cultures were prepared as described [23]. Briefly, the overlaying decidua basalis on the maternal side of the placenta was removed and 1 cm^3 pieces of placental tissue were taken from different sites across the placenta and placed into sterile Ca^{++}/Mg^{++} free phosphate buffered saline (PBS; Life Technologies, UK). Care was taken to avoid contamination with chorioamnion. Tissue was washed repeatedly with PBS to remove contaminating blood and was then minced into smaller pieces (approximately 1–2 mm^3). Pieces of minced placental tissue (0.5 g in total) were transferred into each well of a 6-well tissue culture plate (Greiner Bio-one, Germany) containing 2.5 mls of Ultraculture medium (Cambrex, Belgium) supplemented with 2 mM Glutamax (Life Technologies) and 100 U/ml Penicillin G, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B (PSF; Life Technologies). Care was taken to avoid any blood clots or fibrous tissue.

Optimal levels of all agonists were determined for the following final concentrations: peptidoglycan (PGN; TLR2, 3 μg/ml); poly I:C (TLR3, 25 μg/ml); LPS (TLR4, 100 ng/ml); flagellin (TLR5, 100 ng/ml); R848 (TLR7/8, 100 ng/ml); loxoribine (TLR7/8, 100 μM); single stranded polyU/LyoVEC complexes (ssPoly, TLR7/8, 1 μg/ml); ODN2216 CpG (TLR9, 1 μM) and control ODN (all from Invivogen). An unstimulated control was always included. Plates were incubated at 37 °C in 5% CO_2 for 24 h. Cell/tissue free culture supernatants were collected by centrifugation and stored at −20 °C until assayed.

Placental explant cultures prepared from placentas obtained following elective caesarean section at term were: (i) treated with the optimised concentration of each TLR ligand, then after incubation at 37 °C for 24 h, the tissue was washed by centrifugation and re-cultured for 24 h in fresh media containing concentrations of TLR ligands as indicated in the results; (ii) treated with the optimised concentration of each TLR ligand alone and in the presence of 0.4, 4 or 40 ng/ml dexamethasone (Sigma, USA) for 20–24 h. Supernatants were harvested and stored at −20 °C until analysis of IL-6 levels by ELISA.

Extreme care was taken to limit endotoxin contamination during explant preparation. These precautions included the use of disposable plastic-ware and other consumables (e.g. scissors) whenever possible [28]. All media/reagents were tested by the manufacturers and reported as endotoxin free.

2.3. Real time quantitative PCR (qPCR)

Placental biopsies were preserved in RNA*later*® (Sigma, Poole, UK) at −80 °C. Preparation of DNA-free RNA from homogenised tissue and reverse transcription were performed as described in detail previously [6]. Confirmation of genomic DNA-free status and successful reverse transcription was obtained by PCR amplification of the S15 ribosomal protein gene. Real-time PCR for all genes of interest (TLR 1–10) and 3 housekeeping genes was carried out using the iCycler IQ (ver.3.1 Bio-Rad). The house keeping genes, succinate dehydrogenase complex subunit A (SDHA), TATA box binding protein (TBP) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) were selected as they are the most stably expressed in the human

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