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Pre-labour Fetal Membranes Overlying the Cervix Display Alterations in Inflammation and NF-κB Signalling Pathways

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ABSTRACT

To generate new insights into the process of fetal membrane rupture, we have developed a technique whereby fetal membranes overlying the cervix (i.e. supracervical site, SCS) are tagged in women undergoing term elective Caesarean section. The specific aim is to determine the effect of supracervical apposition on the release of inflammatory mediators and NF-κB signalling in pre-labour fetal membranes. Fetal membranes were collected from both the SCS and from a distal site (DS). The level of NF-κB proteins and its transcriptional co-activator protein CBP and p300 was determined by Western blotting and/or immunohistochemistry (IHC), and cytokine and prostaglandin release was quantified by enzyme immunoassay. Tissues obtained before labour at term possess an area of the fetal membranes (i.e. supracervical site) that exhibit decreased release of IL-1β, IL-6, IL-8, TNF-α and PGE_2. IHC revealed that NF-κB signalling proteins, CBP and p300 were significantly increased in SC fetal membranes compared to distal membranes. In summary, data from this study confirm that supracervical fetal membranes display altered structural and biochemical characteristics.

1. Introduction

Preterm birth is a major cause of perinatal morbidity and mortality accounting for 85% of early infant deaths [1]. Rupture of membranes (ROM) precedes the initiation of uterine contractions in at least 10% of term and nearly 40% of premature births [2]. The physiological mediators that normally initiate the processes leading to weakening and rupture of fetal membranes are unknown, although it has been attributed to cellular apoptosis, extracellular matrix (ECM) remodelling, and stretch-induced physical weakening of fetal membranes [3–6]. Investigations into the ROM have shown that there is a region of the fetal membranes that is characterised by increased thickness or swelling of the connective tissue layer, reduction in thickness of both the cytotrophoblast and decidual layers compared to the rest of the membrane and reduced overall thickness of supracervical membrane [7–16]. Further, this site also exhibits increased ECM remodelling and apoptosis [9,10,16]. It was concluded that this site most likely represents the 'rupture site' (i.e. supracervical site, SCS) in the fetal membranes. However, to develop clinically useful interventions to prevent premature rupture of the membranes, it is necessary to gain a better understanding of the physiological mechanisms responsible for ROM.

Recent evidence suggests that pro-inflammatory cytokines, prostaglandins, NF-κB may play an important role in the mechanisms of human fetal membrane rupture. TNF-α and IL-1β can cause significant fetal membrane weakening through a process of collagen remodelling and apoptosis [17]. It has been shown that PGE_2 decreases synthesis of fetal membranes collagen and increases production of MMP-9 in cultures of human fetal membranes [18]. In amnion cells, stretch activates NF-κB which induces COX-2 [19]. Activated NF-κB may further regulate genes involved in the degradation and weakening of the fetal membranes including pro-inflammatory cytokines and ECM remodelling enzymes [20–23]. The transcriptional activity of NF-κB is regulated through their interaction with by transcription co-activators such as p300 and CBP (CREB binding protein) which is believed to link enhancer-bound transcription factors [24]. CBP/p300 are versatile transcriptional co-activators that participate in many physiological processes, including cell cycle control, differentiation and apoptosis [24,25]. p300/CBP possess an intrinsic histone acetyltransferase (HAT) activity that regulates gene expression, in part, through

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acetylation of the N-terminal tails of histones. Acetylated histones are associated with transcriptionally active segments of chromatin, whereas deacetylated histones accumulate in transcriptionally repressed regions. Moreover, they act as protein bridges connecting specific transcription factors to the basal transcription machinery and provide a scaffold to integrate multiple transcription cofactors.

In recent studies, we have used transcervical application of Bonney's blue dye, before the onset of term labour to identify the supracervical membranes for analysis after elective Caesarean section delivery. Given the importance of pro-inflammatory cytokines, prostaglandins, and NF-κB signalling in the physiological mechanisms of fetal membrane rupture, the specific aims of this study are to (i) compare the release of pro-inflammatory cytokines and prostaglandins from supracervical fetal membranes to a distal site; and (ii) characterise NF-κB signalling proteins and its transcriptional co-activator proteins p300 and CBP in supracervical and distal fetal membranes from pre-labour fetal membranes.

2. Materials and methods

2.1. Reagents

The IL-1β, IL-6, IL-8 and TNF-α kits were supplied by Biosource International (Camarillo, CA, USA). The PGE_2 and $PGF_{2\alpha}$ EIA kits were supplied from Cayman Chemical Company (Ann Arbor, MI, USA). The TransAM NF-κB p50 and p65 transcription factor assay kit were purchased from Active Motif (Carlsbad, CA, USA). Rabbit polyclonal anti-NF-κB p65, rabbit polyclonal anti-IκB-α, mouse monoclonal anti-phosphorylated IκB-α, rabbit polyclonal anti-CBP, rabbit polyclonal anti-p300, goat polyclonal anti-β-actin, horseradish peroxidase conjugated goat anti-rabbit IgG and mouse anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Sample collection

Fetal membranes (total of 12 women) were collected from women with uncomplicated pregnancies at term (37–42 weeks' gestation) undergoing elective caesarean section. The Research Ethics Committee of Mercy Health and Aged Care approved this study. Written, informed consent was obtained from participating women. Identification of the supracervical site was performed as we have previously detailed [16]. The placenta and fetal membranes were collected within 30 min of application of Bonney's Blue and transferred within 10 min of delivery to the laboratory within the hospital. The fetal membranes were washed briefly in PBS and three membrane samples were taken from the identified SCS (Bonney's Blue staining) and three membrane samples from a distal site (DS, no Bonney's Blue staining). Fetal membrane samples from both sites were (I) fixed and paraffin embedded for immunohistochemical analysis; (II) snap-frozen in liquid nitrogen for analysis of antioxidant enzyme activity and NF-κB signalling; (III) prepared for tissue explants and the release of pro-inflammatory cytokines and prostaglandins quantified by enzyme immunoassay. For parts II and III, due to the difficulties inherent in separating chorion and decidua from distal tissues, these were examined as a single entity, the choriodecidua. Similarly, the amniontic epithelium and its connective tissue were also examined as a single entity, the amnion. The composition of the choriodecidua and the amnion was confirmed by histologic analysis.

2.3. Immunohistochemistry

Fetal membrane tissues were rolled up (with amnion innermost) and all rolls were placed in embedding cassettes (Techno-Plas, SA, Australia) fixed in buffered formaldehyde solution (4%) and embedded in paraffin. Serial sections (4 μm thick) were cut and mounted onto superfrost plus slides. Sections were de-paraffined using xylene and a graded series of ethanol and antigen retrieved in citrate buffer (pH 6.0). Endogenous peroxidase activity was removed using 3% H_2O_2 in methanol for 10 min. Sections were transferred to TBS buffer (100 mmol/l, pH 7.6). The sections were incubated for 1 h in antibody diluted in 1% bovine serum albumin (BSA) in TBS. After incubation the binding sites were labelled with Dako Envision + polymer linked secondary reagent and visualised using Dako DAB+ (Dakocytomation). Nuclei were counterstained with Mayer's haematoxylin and the sections were dehydrated and coverslipped using a resinous mounting agent. Negative controls were incubated with diluent only. Positive controls of tissue known to exhibit the antigens in question were included in each run. Sections were assessed microscopically for both intensity and extent of staining. The entire tissue section was scored and the extent of staining was determined on a scale of 0–5 according to the estimated percentage of cells stained: 0 < 10%; 1 = 11–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–90%; 5 > 90%. Staining intensity was assessed on a scale of 0–3: 0 = no staining, negative; 1 = pale brown, weak; 2 = brown, moderate; 3 = dark brown, strong [26].

2.4. Cytokine and prostaglandin assays

Fetal membrane tissue explants were performed as we have previously optimised [27]. Briefly, tissue fragments were also placed in RPMI containing penicillin G (100 U/ml), streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 8% O_2 and 5% CO_2 for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24 well tissue culture plates (200–250 mg wet weight/well). Amnion and choriodecidua explants were incubated, in duplicate, in 2 ml media. After 6 h incubation, tissue and medium were collected separately and stored at −80 °C. The release of IL-1β, IL-6, IL-8 and TNF-α into the explant incubation medium was performed by sandwich ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA, USA). The limit of detection of IL-1β, IL-6, IL-8 and TNF-α assays (defined as 2 SD from the zero standard) was 2.8, 15.6, 15.6 and 7.2 pg/ml, respectively. The release of PGE_2 and $PGF_{2\alpha}$ into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company, Ann Arbor, MI, USA). The limit of detection of the PGE_2 and $PGF_{2\alpha}$ EIA kits was 8.0 and 14.9 pg/ml, respectively. All data were corrected for total protein and expressed as pg per mg protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, USA), using BSA as a reference standard, as previously described [22,23].

2.5. Western blotting

Assessment of NF-κB p65 and IκB-α protein expression was analysed by Western blotting. Cytoplasmic and nuclear protein was prepared as previously detailed [22,23]. Forty micrograms of tissue protein extracts were separated on a 10% polyacrylamide gel and transferred to nitrocellulose as previously described [23]. Protein expression was identified by co-migration with a positive control and by comparison with the mobility of protein standard. Membranes were viewed and analysed using the ChemiDoc system (Bio-Rad). Quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad). Data were corrected for background, and expressed as optical density (OD/mm^2).

2.6. TransAM NF-κB p50 and p65 ELISA

Nuclear NF-κB activity was measured in duplicate using a NF-κB p50 and p65 transcription factor assay kit according to manufacturer's instructions (TransAM; Active Motif; Carlsbad, CA). The colourimetric reaction was quantified by measuring the optical density at 450 nm with a reference wavelength of 655 nm in a Bio-Rad Microplate reader. Data are expressed as pg/ml protein. The limit of detection of the NF-κB p50 and p65 ELISA was 0.5 μg nuclear extract/well.

2.7. Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, Maryland, USA). Normalised values at the SCS were compared with those at the DS and analysed by a paired sample comparison (Student's *t*-test). Statistical significance was ascribed to p value <0.05. Data was expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Cytokine and prostaglandin release

Table 1 is a summary of the comparison of the release of pro-inflammatory cytokines and prostaglandins release from tissue explants from the SCS and DS ($n = 9$). The release of IL-1β, IL-6, IL-8 and TNF-α from distal choriodecidua and amnion was significantly greater compared to the supracervical chorion and amnion, respectively ($p < 0.05$, paired sample comparison). PGE_2 release in chorion, but not amnion, was significantly down regulated at the SCS compared to the DS ($p < 0.05$, paired sample comparison). There was, however, no significant difference in the release of $PGF_2\alpha$ between supracervical and distal fetal membranes (Table 1). To examine the degree of infiltration by inflammatory cells at the SCS and DS, immunohistochemistry was performed, staining for CD3, CD4 and CD8 positive cells. For all 3 markers, staining was only located in the decidual layer of the fetal membranes (Fig. 1, $n = 12$).

3.2. NF-κB signalling pathway

The relative abundance of cytoplasmic IκB-α was not significantly different between the SCS and DS for both choriodecidua and