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Infection-Induced Thrombin Production: A Potential Novel Mechanism for Preterm Premature Rupture of Membranes (PPROM)

Liping Feng[1,3], **Terrence K. Allen**[2], **William P. Marinello**[1], and **Amy P. Murtha**[1]

[1]Department of Obstetrics and Gynecology, Duke University, Durham, North Carolina

[2]Department of Anesthesiology, Duke University, Durham, North Carolina

[3]Ministry of Education-Shanghai Key Laboratory of Children's Environmental Health, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Abstract

Background—Preterm premature rupture of membranes (PPROM) is a leading contributor to maternal and neonatal morbidity and mortality. Epidemiologic and experimental studies have demonstrated that thrombin causes fetal membrane weakening and subsequently PPROM. Although blood is suspected as the likely source of thrombin in fetal membranes and amniotic fluid of patients with PPROM, this has not been proven. *Ureaplasma Parvum (U. parvum)* is emerging as a pathogen involved in prematurity, including PPROM, but until now, prothrombin production directly induced by bacteria in fetal membranes has not been described.

Objectives—This study was designed to investigate whether *U. parvum* exposure can induce prothrombin production in fetal membranes cells.

Study Design—Primary fetal membrane cells (amnion epithelial, chorion trophoblast, and decidua stromal) or full-thickness fetal membrane tissue explants from elective, term, uncomplicated cesarean deliveries were harvested. Cells or tissue explants were infected with live *U. parvum* (1×10^5, 1×10^6, or 1×10^7 colony forming units (cfu)/ml) or lipopolysaccharide (*Escherichia coli* J5, L-5014, Sigma, 100 ng/ml or 1000 ng/ml) for 24 hours. Tissue explants were fixed for immunohistochemistry staining of thrombin/prothrombin. Fetal membrane cells were fixed for confocal immunofluorescent staining of the biomarkers of fetal membrane cell types and thrombin/prothrombin. Protein and mRNA were harvested from the cells and tissue explants for Western blot or qRT-PCR to quantify thrombin/prothrombin protein or mRNA production,

Corresponding author: Liping Feng, MD; Department of Obstetrics and Gynecology, Division of Maternal Fetal Medicine, Box 103208, Duke University Medical Center, Durham, NC 27710. Phone: 919-613-1459. Fax: 919-681-9938. liping.feng@duke.edu.

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Author Contributions

Liping Feng designed the study, performed experiments, analyzed and interpreted the data, and prepared the manuscript. William Marinello performed experiments. Terrence Allen provided the primary amnion cells and contributed to the manuscript preparation. Amy Murtha contributed to study design, discussion, and manuscript preparation.

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respectively. Data are presented as mean values ± standard errors of mean. Data were analyzed using one-way ANOVA with post hoc Dunnett's test.

Results—Prothrombin production and localization was confirmed by Western blot and immunostainings in all primary fetal membrane cells and tissue explants. Immunofluorescence observations revealed a perinuclear localization of prothrombin in amnion epithelial cells. Localization of prothrombin in chorion and decidua cells was perinuclear and cytoplasmic. Prothrombin mRNA and protein expression in fetal membranes was significantly increased by *U. parvum*, but not lipopolysaccharide, treatments in a dose-dependent manner. Specifically, *U. parvum* at a dose of 1×10^7 cfu/ml significantly increased both prothrombin mRNA (fold changes in amnion: 4.1±1.9; chorion: 5.7±4.2; decidua: 10.0±5.4; FM: 9.2±3.0) and protein expression (fold changes in amnion: 138.0±44.0; chorion: 139.6±15.1; decidua: 56.9±29.1; fetal membrane: 133.1±40.0) compared to untreated controls. *U. parvum* at a dose of 1×10^6 cfu/ml significantly upregulated prothrombin protein expression in chorion cells (fold change: 54.9±5.3) and prothrombin mRNA expression in decidua cells (fold change: 4.4±1.9).

Conclusions—Our results demonstrate that prothrombin can be directly produced by fetal membrane amnion, chorion, and decidua cells. Further, prothrombin production can be stimulated by *U. parvum* exposure in fetal membranes. These findings represent a potential novel underlying mechanism of *U. parvum*-induced rupture of fetal membranes.

Keywords

preterm birth; amnion; chorion; decidua; endotoxin; lipopolysaccharide; *Ureaplasma parvum*; prothrombin

Introduction

Preterm birth (PTB) remains a major public health problem and complicates approximately 10% of deliveries in the US[1]. Preterm premature rupture of membranes (PPROM) accounts for approximately one third of all PTB[2]. Prevention of pregnancy complications through enhanced understanding and appropriate intervention is of critical importance to improve both maternal and neonatal outcomes. The causes of PPROM are multifactorial and include: pathologic anatomical remodeling[3, 4], altered membrane morphology[5–10], complications from invasive procedures[11], inflammation[12–14], and genetic factors[15–17]. Although the mechanisms of PPROM are not well-understood, infection in the fetal membranes has been implicated as an early event in the pathogenesis of PPROM and/or preterm labor[18–25]. Histological chorioamnionitis complicates almost half of all PPROM cases[26]. The propagation of bacteria is an important contributing factor not only in PPROM, but also in adverse neonatal and maternal complications following PPROM.

Ureaplasma spp. are among the organisms frequently implicated in prematurity-linked conditions and chorioamnionitis[27–34]. The two species of *Ureaplasma* known to colonize humans are *U. urealyticum* and *U. parvum*. Of these, *U. parvum* is the most common species isolated from the genital tract of men and women[29, 35, 36]. Increasing evidence suggests that *U. parvum* is an significant pathogen in pregnancy and is associated with PPROM, PTB, and chorioamnionitis[37]. Specifically, *U. parvum* is the most frequently isolated pathogen in the

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amniotic fluid of women who deliver preterm[22, 31, 32, 35] and its colonization in neonates is inversely related to gestational age at delivery[28, 38]. A recent study of the human microbiota during pregnancy indicated that elevated vaginal *Ureaplasma,* when combined with poor vaginal colony state, was associated with PTB[39]. The presence of *U. parvum,* but not *U. urealyticum,* in vaginal fluid during pregnancy was significantly associated with higher risk of spontaneous PTB in an Australian cohort of pregnant women[40]. Another study demonstrated that human placenta infection with *Ureaplasma* is associated with histological chorioamnionitis and adverse outcomes in moderate- and late-preterm infants[41]. The strongest evidence linking *U. parvum* to preterm labor is from experiments in animal models. Intra-amniotic inoculation of *U. parvum* resulted in chorioamnionitis in Rhesus macaques[42, 43] and sheep[40] and also promoted preterm delivery in the former[42]. However, to date, the pathogenicity of *U. parvum* in preterm birth is poorly understood[44]. Using our *in vitro* fetal membrane infection model, we have demonstrated that *U. parvum* adheres to fetal membrane cells and induces inflammation[45, 46].

Using the same model, we harvested the condition media to identify the proteins stimulated by *U. parvum* infection using a proteomics-based approach. Prothrombin was detected in *U. parvum*-treated condition media but not control condition media. This preliminary finding indicated that *U. parvum* has the potential to induce prothrombin production in fetal membrane cells. We postulate that infection-induced prothrombin production (and subsequent conversion to thrombin) might be a novel mechanism of *U. parvum* pathogenicity in preterm birth[47–54]. Thrombin generation has been associated with fetal membrane weakening and PPROM[50, 54], and treatment of amnion explants with thrombin results in increased levels of matrix metallopeptidase 9 (MMP9) and mechanical weakening[55]. However, thrombin generation in fetal membranes and the source of thrombin in amniotic fluid is not well described[47, 56]. Decidual hemorrhage associated with either intrauterine infection[57] or bleeding in pregnancy[58] is thought to be a primary source of thrombin. The ability of fetal membrane cells (amnion, chorion and decidua) to produce prothrombin and respond to infection through regulating prothrombin production is not established.

In this study, we hypothesize that *U. parvum* exposure stimulates prothrombin production in fetal membrane amnion, chorion, and decidua cells. Our objectives were two-fold: **1)** Confirm the prothrombin localization in fetal membrane cells with immunostaining in *ex vivo* fetal membrane tissues and primary cultured fetal membrane cells and **2)** Quantify prothrombin expression with Western blot analysis and PCR in fetal membrane tissues and cells with/without *U. parvum* exposure. In addition, each pathogen may elicit a unique response through specific signaling mechanisms. In this study, Gram-negative bacterial lipopolysaccharide (LPS) was used as a comparison. Gram-negative bacteria are also frequently associated with intra-amniotic infections, and LPS is often used to induce preterm birth in animal models.

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Materials and Methods

This study used human fetal membrane tissues collected following planned, uncomplicated cesarean delivery at term without rupture of membranes or labor and a genital isolate *U. parvum* strain as described previously[45].

Fetal membrane tissue explant and primary cell cultures

For tissue explant culture, fetal membranes were collected following planned, uncomplicated cesarean delivery at term (39 weeks of gestation) without rupture of membranes or labor. All subjects had no previous preterm birth and were not on progesterone therapy. All subjects had no pregnancy or medical complications. Membranes were washed in warm media, and blood clots were removed. Full-thickness fetal membrane tissue was cut into 1 × 1 cm pieces and cultured in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS) in 6-well plates. The fetal membrane fragments were collected away from the area near the disk or the rupture site.

For primary cell culture, fetal membranes were cut into 2 × 2 inch pieces. The reflected amnion layer was removed manually. Amnion epithelial cells were harvested using a modification of a technique which was previously described[45, 59]. Briefly, the amnion tissue was minced and digested in DMEM/F12 containing 0.2% trypsin (Sigma) at 37°C for 30 min with periodic agitation. The mixture was then filtered using a tissue strainer to separate the dispersed amnion epithelial cells from the tissue fragments. This process was repeated three times, and the dispersed epithelial cells were combined and counted. The yield was 8–12 million amnion epithelial cells/g of amnion tissue; viability was 90% (assessed by trypan blue dye exclusion).

Separation of the decidua and chorion involved blunt dissection with forceps and scalpel. Separated chorion and decidua layers were minced and digested in DMEM/F-12 containing 0.125% trypsin and 0.2% collagenase (Sigma) at 37°C for 30–90 min with periodic agitation. Cells were filtered through 70 μm nylon cell filters (Falcon). A cell-separation gradient was prepared to purify the cells as described previously [60]. Cells were plated in the same culture conditions as amnion cells for 48 h. Purity of primary cells was confirmed using immunofluorescence staining for cytokeratin and vimentin to distinguish amnion/chorion from decidua cells.

All cell types or tissue explants were confirmed to be free of *Mycoplasma* and *Ureaplasma* contamination using a chemiluminescent-labelled single-stranded DNA probe hybridization method (MTC-NI kit, Millipore).

Tissue explants (0.5 cm × 0.5 cm) and primary cells (2×10^6 cells/well in 6-well plates) were treated with and without live *U. parvum* (1×10^5, 1×10^6, or 1×10^7 cfu/ml) or lipopolysaccharide (LPS, 100ng/ml or 1000ng/ml, *Escherichia coli* J5, L-5014, Cat# L5014, Sigma). After 24h, culture media was collected for ELISA and cell or tissue lysates were harvested for Western blot. Total RNA was extracted from the cells or tissue explants using the RNeasy Mini Kit (Qiagen) for PCR.

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Immunohistochemistry (IHC) staining

To determine basal prothrombin protein localization in fetal membranes, freshly harvested, full-thickness fetal membranes without *U. parvum* infection were fixed in 10% formalin for IHC. Paraffin sections were de-waxed and rehydrated. After heat-induced antigen retrieval, the sections were stained using the anti-mouse HRP-DAB Cell & Tissue Staining Kit following manufacturer's instruction (R&D Systems). Mouse anti-human thrombin antibody (Abcam) was used at a 1:500 dilution in PBS with 1% BSA and 5% goat serum. Placenta tissue was used as positive control. For each batch of staining, one slide was incubated with normal mouse IgG (Abcam) instead of the primary antibody as a negative control. Images were taken using a Zeiss Axio Observer (20X). Three rounds of IHC staining were performed using fetal membrane tissues collected from three subjects.

Immunofluorescence staining

To determine the localization of thrombin/prothrombin protein in fetal membrane cells, primary cell cultures without *U. parvum* infection were fixed with cold methanol at −20°C for 10 min. The cells were permeabilized and blocked with 1% BSA, 5% normal goat serum, and 0.1% Tween-20 in PBS for 1h. After blocking, the slides were incubated with primary antibodies overnight at 4°C. Primary mouse anti-cytokeratin and anti-vimentin monoclonal antibodies (Dako) were used at 1:200 and rabbit anti-thrombin polyclonal antibody (Abcam) was used at 1:100. Anti-mouse and rabbit IgG antibodies were used as negative controls (R&D Systems). Goat anti-mouse secondary antibody Alexa Fluor 488 conjugate and goat anti-rabbit secondary antibody Alexa Fluor 594 (Life Technologies) were used at 1:500. Slides were mounted using mounting medium for fluorescence with DAPI (Vector Laboratories). Three rounds of immunofluorescence staining were performed using fetal membrane tissues collected from three subjects.

Real-time quantitative reverse transcription q(RT)-PCR

Total RNA (1 μg) was reverse-transcribed into cDNA using SuperScript III and Oligo dT (Life Technologies). Pre-validated thrombin Taqman® gene expression probes (Life Technologies, assay ID: Hs01011988_m1 F2) were used. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured by the SYBR® Green detection method (Bio-Rad). IQ supermix and IQ SYBR® Green supermix cocktail (Bio-Rad) were used for these assays, respectively. Primers used for GAPDH were forward (CATGAGAAGTATGACAACAGCCT) and reverse (AGTCCTTCCACGATACCAAAGT). Samples were run in duplicate and fold changes were calculated using ΔΔCt method after normalization. Six replicates were performed using fetal membrane tissues or primary fetal membrane cells collected from six subjects.

Western blot

Cell lysates were obtained using radioimmunoprecipitation assay (RIPA) buffer (Sigma) with the complete mini-protease inhibitor cocktail (Roche). For frozen tissue explants and placenta (positive control for Western blots), tissues (~500 mg) were pulverized then homogenized in 1.0 ml of RIPA buffer containing protease inhibitor. Purified plasma prothrombin (Enzyme Research Laboratories) was used as the positive control. Western blot

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analysis was performed using a standard procedure. Rabbit anti-human GAPDH antibody (1:20,000, Cell Signaling Technology), mouse anti-human thrombin antibody (1:2000, Abcam), and the secondary antibodies (1:2500, Cell Signaling Technology) were used. The primary human thrombin antibody detects both prothrombin and thrombin. Film development was optimized to maintain bands within the linear range. Band intensity was measured using ImageJ analysis software (National Institute of Health [NIH], Bethesda, MD). The densitometry of the protein bands were normalized to GAPDH and then compared and presented as ratios. Five replicates were performed using fetal membrane tissues or primary fetal membrane cells collected from five subjects.

Enzyme-Linked Immunosorbent Assay (ELISA)

The conditioned media were analyzed for prothrombin/thrombin protein concentrations by Human Thrombin ELISA Kit (Factor II;[61] Abcam). Samples without dilution were run in duplicate with serial dilutions of recombinant human thrombin as standards. The sensitivity of this Kit is approximately 0.3 ng/ml. The intra- and inter-assay coefficients of variation are 4.7% and 7.2%, respectively. The absorbance was measured at an optical density (OD) of 450 nm with correction at an OD of 540 nm. Eight replicates were performed using fetal membrane tissues or primary cells collected from eight subjects.

Data analysis

Experiments performed using tissue explant or primary cell culture from one subject was counted as one repeat. All experiments were repeated more than five times ($N > 5$). Data are presented as means ± SEMs. The one-way ANOVA with the post-hoc Dunnett's test was used for multiple comparisons. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 6.0 (La Jolla, CA).

Results

Prothrombin/thrombin protein detected in fetal membrane cells

To demonstrate the localization of thrombin/prothrombin in the fetal membrane cells, we performed IHC in full-thickness fetal membrane tissues and immunofluorescence staining in primary cultured fetal membrane cells. Positive prothrombin/thrombin staining was consistently observed in the amnion and chorion layers of fetal membranes (Figure 1). Prothrombin/thrombin staining in the decidua layer of fetal membranes was inconsistent, with negative staining observed in the fetal membranes of some subjects and positive staining observed in others. Even within the same sample, there were inconsistencies among stained cells in the decidual layer. Additionally, using the same staining conditions (same batch), the intensity of prothrombin/thrombin staining in all three layers of fetal membranes varied between subjects (Figure 1A, weak staining in Subject 1 *vs* strong staining in Subject 2). Intracellular staining indicated the presence of prothrombin, but extracellular prothrombin/thrombin staining was also observed.

Using immunofluorescence, we further verified prothrombin/thrombin localization in primary cultured fetal membrane cells (Figure 2). In amnion cells, prothrombin/thrombin

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staining was predominately perinuclear whereas in chorion and decidua cells, the staining was diffuse in the cytoplasm (Figure 2).

Prothrombin mRNA and protein expression was upregulated following *U. parvum* exposure in fetal membrane explants and cells

While not statistically significant at all doses, both prothrombin transcripts and protein expression were upregulated following *U. parvum* exposure in a dose-dependent manner. The lack of statistical significance for some of the lower doses may result from individual subject variability and the low virulence of *U. parvum* at low dose in fetal membranes.

U. parvum at the highest dose [1×10^7 cfu/ml] consistently and significantly increased prothrombin/thrombin mRNA expression in tissue explants ($P < 0.01$) and primary fetal membrane cells ($P < 0.05$, Figure 3, N = 6). Treatments with LPS (100ng/ml or 1000ng/ml) and lower doses (1×10^5 or 1×10^6 cfu/ml) of *U. parvum* did not significantly change the prothrombin/thrombin mRNA expression in tissue explants and primary fetal membrane cells, with the exception of a dose of *U. parvum* 1×10^6 cfu/ml in decidua cells ($P < 0.05$). The results are summarized in Table 1.

A band at approximately 75 kDa was detected in Western blots for all fetal membrane samples. A single band at approximately 72 kDa was identified for purified plasma prothrombin (Figure 4), which further demonstrated the specificity of the antibody. The slight molecular weight difference might be due to the modifications in prothrombin prior to secretion, such as signal-peptide cleavage[62], complex glycosylation[63], and disulphide-bridge formation[64].

The densitometry analysis of Western blots (N = 5) is consistent with the qRT-PCR findings which demonstrated that *U. parvum* upregulated prothrombin protein expression in a dose-dependent manner. Chorion cells were more susceptible than amnion or decidua cells to *U. parvum* exposure-induced prothrombin protein expression, as demonstrated by significant induction at both high (1×10^7 cfu/ml, $P < 0.001$) and medium (1×10^6 cfu/ml, $P < 0.001$) *U. parvum* doses. In amnion and decidua cells, as well as tissue explants, prothrombin protein expression was significantly induced by *U. parvum* at 1×10^7 cfu/ml ($P < 0.05$), but not at lower doses.

The prothrombin/thrombin protein in conditioning media was not significantly induced by *U. parvum* exposure

Prothrombin/thrombin was detectable in culture supernatants collected from fetal membrane cells and tissue explant cultures at approximately 100 and 2000 pg/ml, respectively (Figure 5). In amnion and chorion cell culture supernatants, *U. parvum* exposure at 1×10^7 cfu/ml resulted in increased prothrombin/thrombin concentration, but results were not statistically significant (N = 8).

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Comment

Principal Findings of the study

In this study, we characterized prothrombin expression in cells of the fetal membranes and demonstrated that *U. parvum* exposure induced prothrombin production by fetal membrane cells. Interestingly, we were not able to demonstrate that LPS-induced prothrombin production either in fetal membranes or in cells derived from fetal membranes.

Clinical and Research Implications

These findings suggest: 1) A connection between microorganisms and thrombin production in cells of the fetal membranes and 2) A possible pathogenicity of *Ureaplasma* which is not completely understood. This novel discovery significantly contributes to our understanding of the pathogenicity of *U. parvum* and *U. parvum* infection-induced PTB and PPROM. Although numerous clinical observational studies regarding *Ureaplasma spp.* infection during pregnancy have been conducted in the past three decades, its clinical significance is still debated[65, 66]. It is a commonly held belief that *Ureaplasma spp.* are not pathogenic because they are common commensal organisms in the female genital tract and often detected in gestational tissues. *Ureaplasma spp.* are considered to be low-virulence bacteria. In fact, studies evaluating the impact on pregnancy of treatment of *Ureaplasma and Mycoplasma spps* have had mixed results[67, 68]. Our findings support a pathogenic mechanism for *Ureaplasma* by which it stimulates prothrombin production in gestational tissues. The previously reported *Ureaplasma* virulence factors include adhesion molecules[69], IgA protease[70], urease[71], phospholipases A and C[72], and production of hydrogen peroxide[73]. However, examination of the genome of multiple serovars of *Ureaplasma* failed to reveal genes encoding these enzymes[74]. The multiple-banded antigen (MBA) of *Ureaplasma* is the predominant antigen recognized during the infection process and may be involved in the stimulation of host inflammatory response[75]. Our findings indicate that there are potential undiscovered *Ureaplasma* virulence factors that regulate host coagulation and hemostasis. Coagulation modifications induced by *Ureaplasma* in fetal membranes indicate that *Ureaplasma* has the potential to act as an opportunistic pathogen during pregnancy.

Our discovery sheds new light on the long-established yet poorly understood link between infection/inflammation and PTB: *U. parvum*-induced thrombin production. Thrombin acts as a serine protease that converts soluble fibrinogen into insoluble strands of fibrin and also catalyzes many other coagulation-related reactions. Beyond its key role in the dynamic process of thrombus formation, thrombin has pronounced pro-inflammatory properties acting via its specific cell membrane protease-activated receptors (PARs)[76–81]. PARs, which control important physiologic and disease-relevant tissue-specific processes, are an emerging therapeutic target for major diseases such as burns and gastric ulcers[82]. The mechanisms of actions of PARs are poorly understood in gestational tissues. Fetal membrane cells express PAR-1[83, 84] and thrombin induces inflammation and weakens fetal membranes[50, 54, 55, 83, 84], and alternative mechanisms of thrombin action have also been demonstrated. For example, one study reported that thrombin weakens the amnion extracellular matrix (ECM) directly rather than through PARs[84]. Protein- and/or peptide-

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based therapies targeted to limit thrombin enzyme activities and ultimately reduce the risk of PPROM should be explored.

Following bacterial infection, the resultant inflammatory reaction activates a coagulation cascade resulting in the formation of fibrin, which effectively walls off the infection[85, 86]. Mackman et al. postulate that coagulation is an important component of the inflammatory response and necessary to eliminate the infection[87]. The prevailing view is that coagulation factor production is restricted to three compartments: the liver, where most coagulation factor production takes place; the endothelium, which is characterized, for example, by high levels of von Willebrand Factor (vWF); and the platelets, which, despite being devoid of nuclei, are capable of substantial coagulation factor production[88]. However, there is evidence to refute this simplistic model of coagulation factor synthesis. For instance, in the lung, alveolar macrophages express FVIIa[89], epithelial cells express tissue factor (TF)[90], factor VIIa (FVIIa)[91], and factor Xa (FXa)[92], and fibroblasts express tissue factor pathway inhibitor (TFPI)[93]. Our current study also challenges the standard paradigm of coagulation factor production by confirming prothrombin biosynthesis in human fetal membrane cells including amnion epithelial cells, chorion trophoblasts, and decidua stroma cells. Among these cells, there are differences in the distribution of prothrombin and the susceptibility to prothrombin stimulation following *U. parvum* exposure. Our findings suggest a different functional relevance of prothrombin in these cell types. Based on the size of the single band detected by Western blot, the stimulated prothrombin was not activated within the intracellular and pericellular spaces of these cells. Although prothrombin mRNA and protein were consistently upregulated following *U. parvum* exposure, the prothrombin levels in culture supernatants were extremely variable, ranging from below detection to a 5-fold increase in *U. parvum*-treated groups. The individual patient variability is not easily explained, but this may be a result of the complexity of prothrombin secretion and the instability of prothrombin in media. Additionally, as it is impossible to avoid blood contamination in the culture media of full-thickness fetal membranes, this could explain in part the variable results in tissue explants. Our objective in this current study was to demonstrate the ability of gestational tissues to synthesize prothrombin and respond to *U. parvum* exposure. Future studies to understand the individual patient variability and downstream effects of fetal membrane prothrombin biosynthesis and secretion induced by *U. parvum* are warranted.

Strengths and Limitations

A standard bacterial inoculum is critically important for *in vivo* and *in vitro* studies. We have previously demonstrated that the quantity of bacteria present in the fetal membranes is correlated with chorion thinning, suggesting that bacterial presence may incrementally evoke a host response that leads to chorion cell death and tissue degradation[94]. There is now evidence that the dose and variation of the MBA of *U. parvum* might affect the severity of chorioamnionitis in pregnant sheep[95]. Therefore, we optimized the doses of *U. parvum* and treatment duration to avoid cell death as observed in our previous studies[45, 96]. The optimized dose range was chosen because: 1) the consistent and effective induction of interleukin 8 (IL-8), cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2) and MMP9 in these cultures was observed; 2) cell death was not observed for the treatment duration; 3) the

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multiplicity of infection (MOI) of 5 (for example, 10^7 cfu *U. parvum* to 2×10^6 fetal membrane cells) was within the range of positive adherence to fetal membrane cells; and 4) the MOI of *U. parvum* in this study is consistent with other studies in human amniotic epithelial cells[97]. In this study, 10^8 cfu *U. parvum* infected 10^7 amniotic epithelial cells with a MOI of 10[97]. Based on methods previously published, we have established standardized experimental procedures to ensure that the experiments can be directly compared[45]. Overgrowth of *U. parvum* in cell culture is not a concern because of their unique growth conditions: high concentrations of urea, a variety of amino acid supplementation, and acidic conditions of the culture media.

Live *U. parvum* was used in this study for several reasons. The ability of *U. parvum* to have pathogenicity is dependent on its adherence to human cells. The proposed major virulence factor of *U. parvum*, MBA, is a surface protein which is easily altered by heat unlike endotoxin, the major virulence factor of *Escherichia coli (E. coli)*. Heat inactivation can affect the surface of *U. parvum* and therefore attenuate adherence and damage the protein structure of MBA, both of which will result in variations in host-microbe interactions. As expected, our preliminary data suggests that heat inactivation attenuated the ability of *U. parvum* ability to adhere to human cells. A previous study using heat-inactivated Ureaplasma demonstrated a mild inflammatory response in fetal membranes[98].

Specific limitations of our study include the use of LPS instead of live *E. coli* to compare to *U. Parvum*-induced thrombin generation. LPS was selected due to the rapid overgrowth of live *E. coli* in *in vitro* culture models which results in significant cell death and limits the interpretation of findings. LPS, also known as lipoglycans and endotoxins, are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core, and inner core joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria and elicit strong immune responses and coagulopathy including thrombin generation in animals[99–103] and humans[100, 101]. Although we cannot conclude that *E. coli* does not stimulate prothrombin production in fetal membranes, we extrapolated this based on our finding that LPS treatment failed to induce prothrombin production. LPS has, however, been shown to stimulate the production of cytokines (such as IL-1 and TNF) which may be associated with coagulation. The exact relationship between cytokines and coagulation in fetal membranes is poorly understood.

The other limitation of this study is that we did not demonstrate a direct link between prothrombin production and fetal membrane rupture. Due to the complexity of the activation of prothrombin, it is difficult to replicate prothrombin activation *in vitro*. Many coagulation factors necessary for the activation of prothrombin are not present in the cell culture system. Future *in vivo* studies are warranted.

Conclusions

Our findings demonstrate a potential pathway to PPROM and PTB: *U. parvum* exposure-induced prothrombin production in fetal membrane cells. *Ureaplasma spp.* is understudied yet recognized as an important pathogen during pregnancy and in the preterm neonate. Our findings contribute to the field of microbiology and maternal fetal medicine by identifying a

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novel molecular event resulting from the interactions of *U. parvum* and cells of the fetal membranes. Most importantly, the understanding of coagulation regulation as one of the *U. parvum* virulence factors in fetal membranes will likely yield novel therapeutic interventions to limit onset and progression of PTB and PPROM.

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Implications and Contributions

A. Epidemiologic and experimental studies have demonstrated that thrombin causes fetal membrane weakening and PPROM. However, the sources of thrombin in the fetal membranes and amniotic fluid of PPROM patients were previously unknown. This study aimed to investigate whether thrombin production by fetal membrane cells can be induced by *U. parvum* exposure.

B. The key findings are two-fold: 1) Prothrombin can be directly produced by fetal membrane amnion, chorion, and decidua cells and 2) Prothrombin production can be stimulated by *U. parvum* exposure in fetal membranes.

C. We discovered a potential novel mechanism by which *U. parvum* infection induce alteration of fetal membranes.

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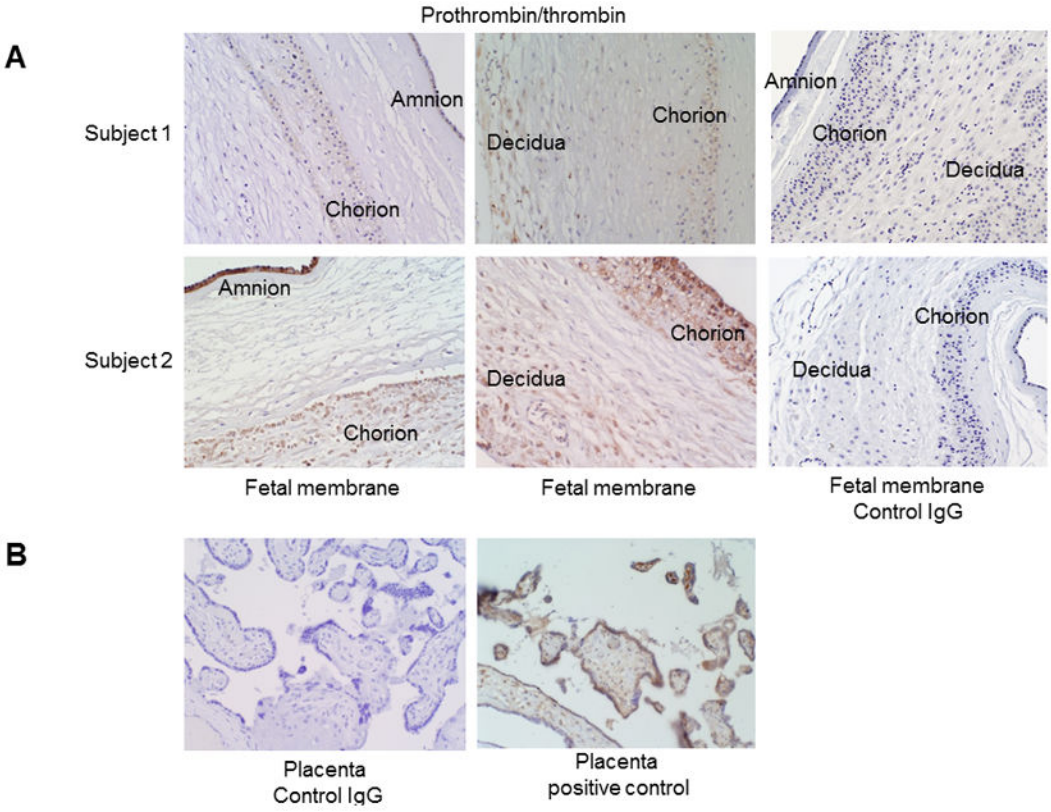

Figure 1. Prothrombin/thrombin staining in fetal membranes

(A) Prothrombin/thrombin protein was present in the amnion, chorion, and decidua layers of the fetal membranes. Prothrombin/thrombin immunostaining intensity in Subject 2 was much higher than in Subject 1, which represented the variable expression of this protein among term fetal membranes. Negative control fetal membranes were stained negative. **(B)** Negative control and positive control of prothrombin/thrombin immunostaining in placenta tissues. Thrombin/prothrombin staining in placentas was in syncytiotrophoblasts and fetal vessels.

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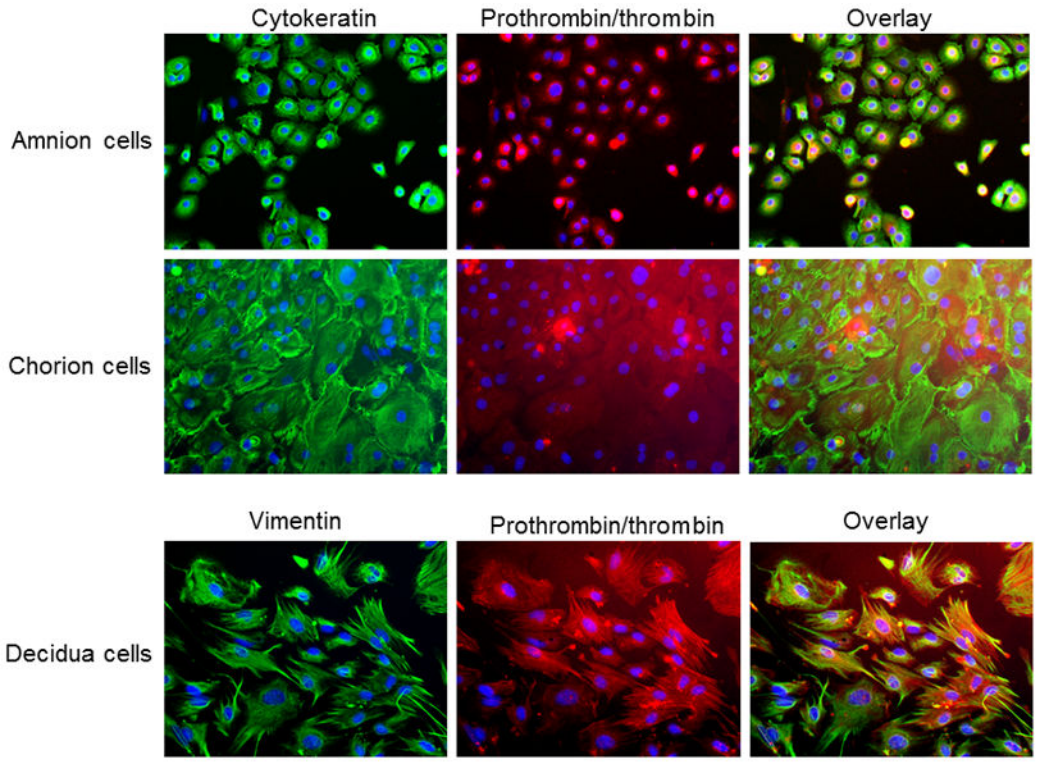

Figure 2. Prothrombin/thrombin expression in primarily cultured fetal membrane cells

Cytokeratin and vimentin stained in green, prothrombin/thrombin stained in red, and DAPI stained in blue.

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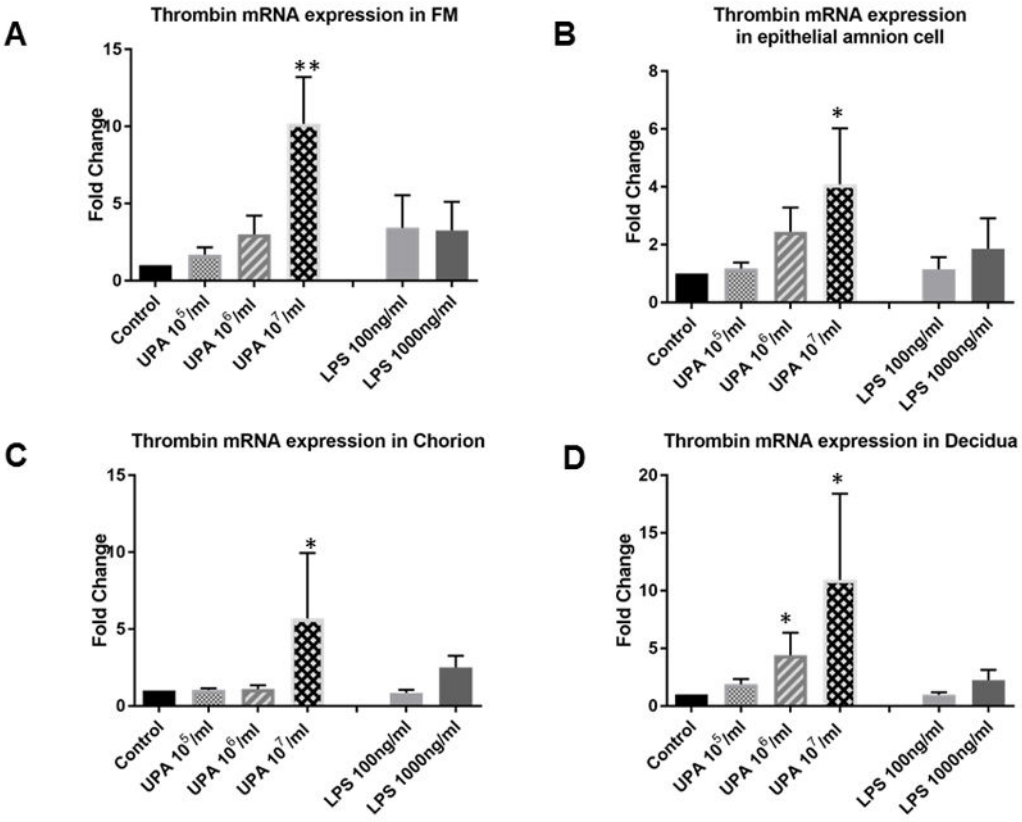

Figure 3. Prothrombin/thrombin transcriptional levels in fetal membrane cells and tissue explants (qRT-PCR analysis)

Control vs. *U. parvum* at 10^7 cfu/ml, P <0.05 in amnion, chorion, decidua cells, and fetal membrane explants. Other comparisons were not statistically significant (N = 6). N represents the number of replicates or subjects. A. FM; B. Amnion; C. Chorion; D. Decidua.

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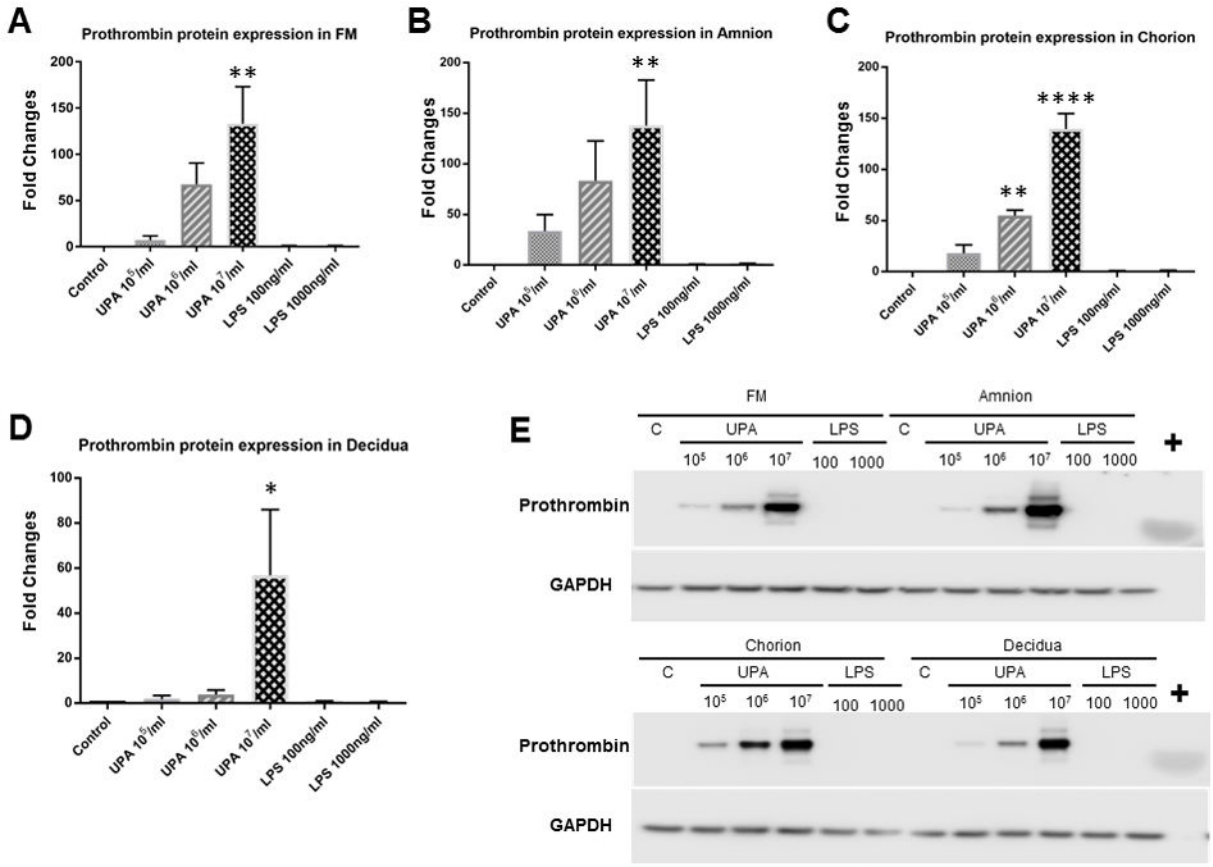

Figure 4. Prothrombin protein levels in fetal membrane cells and tissue explants (Western blotting analysis)

Control vs. *U. parvum* at 10^7 cfu/ml, $P < 0.01$ in amnion cells and fetal membrane tissue explants, $P < 0.001$ in chorion cells, $P < 0.05$ in decidua cells. Control vs. *U. parvum* at 10^6 cfu/ml, $P < 0.01$ in chorion cells. Other comparisons were not statistically significant (N = 5). A. FM bar graph; B. Amnion bar graph; C. Chorion bar graph; D. Decidua bar graph; E. Representative Western blot images. + = purified plasma prothrombin; C = control; N represents the number of replicates or subjects.

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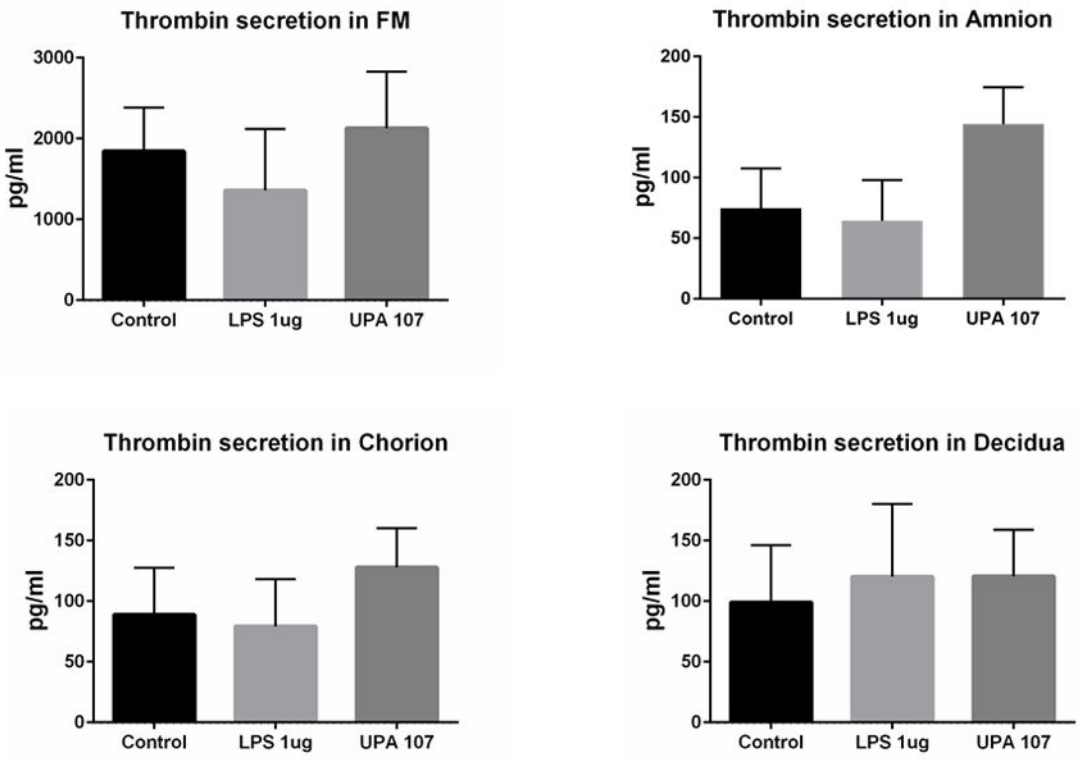

Figure 5. Prothrombin/thrombin protein in conditioned culture media (ELISA analysis)

In fetally derived amnion and chorion cell culture supernatants, *U. parvum* exposure at 10^7 cfu/ml resulted in increased concentrations of prothrombin/thrombin. However, no comparisons were statistically significant (N=8). N represents the number of replicates or subjects.

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Table 1

Prothrombin mRNA and protein expression in fetal membrane cells.

Amnion (fold change)						
Prothrombin	**Control**	**UPA (10^5cfu/ml)**	**UPA (10^6cfu/ml)**	**UPA (10^7cfu/ml)**	**LPS (100ng/ml)**	**LPS (1000ng/ml)**
mRNA	1	1.2±0.2	2.4±0.8	4.1±1.9 *	1.2±0.4	1.9±1.1
Protein	1	33.5±16.4	83.4±39.3	138.0±44.0 *	1.0±0.1	1.4±0.3
Chorion (fold change)						
mRNA	1	1.1±0.1	1.1±0.3	5.7±4.2 *	0.9±0.2	2.5±0.7
Protein	1	18.1±8.2	54.9±5.3 ***	139.6±15.1 ***	0.9±0.2	1.1±0.4
Decidua (fold change)						
mRNA	1	1.9±0.4	4.4±1.9 *	10.0±5.4 *	1.0±0.2	2.3±0.9
Protein	1	1.9±1.6	4.0±1.9	56.9±29.1 *	0.7±0.3	0.5±0.3
FM (fold change)						
mRNA	1	1.7±0.5	3.0±1.2	9.2±3.0 **	3.4±2.1	3.3±1.8
Protein	1	7.1±4.6	67.4±23.1	133.1±40.0 *	1.1±0.2	1.1±0.1

* $P<0.05$;

** $P < 0.01$;

*** $P < 0.001$