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Bacterial Modulation of Human Fetal Membrane Toll-like Receptor Expression

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Abstract

Problem—Preterm premature rupture of fetal membranes (pPROM) occurs in 30–40% of spontaneous preterm births (PTB) and is associated with intra-amniotic infection and inflammation. The membranes may sense and respond to microbes via Toll-like receptors (TLRs), however, little is known about their expression and regulation in this tissue. The objective of this study was to evaluate the expression of TLR1–10 in fetal membranes after exposure to pathogens associated with intra-amniotic infection and PTB.

Method of study—Normal human term fetal membrane explants were exposed to various bacteria. After 24hrs RNA was extracted and quantitative RT-PCR performed for TLRs 1–10.

Results—Treatment of fetal membranes with *Mycoplasma hominis* increased expression of TLR4, TLR6, and TLR8 mRNA. *Ureaplasma parvum* upregulated TLR8 mRNA, and *Porphyromonas gingivalis* significantly increased fetal membrane TLR7 expression. In contrast, treatment with Gram-negative *Escherichia coli* (and its cell wall component LPS) downregulated TLR10 mRNA. No effect was detected for *Ureaplasma urealyticum*, *Gardnerella vaginalis*, or Group B Streptococcus.

Conclusions—These findings demonstrate that different types of bacteria have distinct effects on fetal membrane TLR expression patterns. Moreover, these findings highlight the disparity of fetal membrane responses to infection and thus, suggest heterogeneity in the mechanisms by which infection-associated pregnancy complications, such as pPROM and PTB, arise.

Keywords

Bacteria; Chorioamnion; Infection; Preterm birth; Toll-like Receptor

INTRODUCTION

Preterm delivery (< 37 weeks gestation) affects 12.3% of live births in the US¹, the highest rate in the developed world, and is a major cause of neonatal morbidity and mortality. Approximately 60% of these deliveries are spontaneous where the causality is as yet,

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unclear. However, inflammation of the chorioamniotic membranes (chorioamnionitis) is a strong indicator of pathology and is common in spontaneous preterm births (PTB), particularly where there is preterm premature rupture of membranes (pPROM). pPROM occurs in 30 – 40% of all PTB^{2–4}. There is an established clinical association between infection and preterm labor^{5,6}, and an intrauterine bacterial infection poses a significant threat to pregnancy by gaining access to gestational tissues, such as the decidua, the placenta, and the fetal membranes⁷. The fetal membranes are likely the first tissue colonized by an ascending infection⁸, and while most normal term deliveries have evidence of bacteria in the fetal membranes, it is the association with inflammation that correlates with pathology and adverse pregnancy outcome⁴.

Normal term fetal membranes stimulated *in vitro* with various bacteria or bacterial components produce elevated levels of pro-inflammatory cytokines, such as IL-1 β , TNF α , IL-6, and IL-8^{8–12}, an inflammatory profile also found clinically in preterm fetal membranes¹³. Furthermore, IL-1 β and TNF α are known to be damaging to the fetal membranes by upregulating degradative matrix metalloproteinases (MMPs)^{14–16} and by inducing apoptosis^{17–19}. This leads to weakening of the membranes, predisposing them to pPROM^{2,13}. The way in which an infection can lead to inflammation and PTB is thought to be mediated by innate immune pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs)²⁰. To date, 10 human TLR genes have been identified to encode protein, and each sense distinct infectious products. TLR4 recognizes Gram-negative bacterial lipopolysaccharide (LPS)^{21,22}. TLR2 recognizes bacterial lipoproteins, Gram-positive bacterial peptidoglycan (PDG) and lipoteichoic acid, and fungal zymosan^{23–25}; and forms heterodimers with either TLR1, TLR6, or TLR10^{26–28}. TLR3 senses viral dsRNA, TLR5 senses bacterial flagellin, TLR8 senses viral ssRNA, and TLR9 senses bacterial CpG DNA^{29–31}. While the natural ligand for human TLR7 is unknown, in the mouse, TLR7 recognizes the human TLR8 ligand, viral ssRNA²⁹.

The chorioamniotic membranes may sense and respond to microbes via TLRs; however, little is known about their expression, regulation or function in this tissue. TLR2 and TLR4 are expressed by normal fetal membranes^{32–34}, and their expression is increased in chorioamnionitis³³. This suggests that inflammation is associated with altered fetal membrane TLR expression, and perhaps changes in pathogen sensing by this tissue. Different fetal immune responses to different intra-amniotic and cervico-vaginal pathogens associated with PTB, suggests that each pathogen may elicit a unique response through specific TLR mediated signaling mechanisms¹². Therefore, the objective of this current study was to assess the expression levels of fetal membrane TLRs 1–10 after exposure to various bacteria associated with PTB. In this study we examined 7 different bacteria belonging to the following categories: genital mycoplasmas (*Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum*); Gram-negative urogenital tract infectious agents (*Escherichia coli* and *Gardnerella vaginalis*), a Gram-negative periodontal pathogen (*Porphyromonas gingivalis*); and a Gram-positive genital tract colonizer (Group B Streptococci), all of which are associated with PTB and pPROM.

MATERIALS AND METHODS

Patients and collection of fetal membranes

The study was approved by Western Institutional Review Board, Seattle, WA (for The Perinatal Research Center) and IRB at The University of Texas Medical Branch at Galveston. Placentas were obtained from elective repeat Cesarean sections at term (³ 37 weeks of gestation) prior to onset of labor (n=11). Maternal ages were between 20 and 32 years. Fetal membranes were dissected from placenta under sterile conditions. Decidua and adhering clot clots were removed from the membranes using saline and sterile cotton gauze.

These tissues were placed in Hanks balanced salt solution (HBSS, Sigma Chemical Co., St. Louis, MO) containing penicillin/streptomycin (10 μ l/ml) and 1 μ l/ml amphotericin B (Sigma). Women with a prior history of pPROM or PTB were excluded, as were patients with obstetrical and medical complications, cervico-vaginal Group B streptococcus colonization, infections (sub-clinical infections as indicated by high CRP levels, foul smelling vaginal discharge, fever, or those undergoing antibiotic treatment). A sample of fetal membranes from each patient was examined by light microscopy (H&E staining of paraffin-embedded sections) to rule out subclinical chorioamnionitis (> 5 PMNs/high power field).

Tissue-culture of normal term fetal membranes

Fetal membranes were washed twice with HBSS as described above and cut into 6 mm-circles using a biopsy punch and washed in HBSS before placing into a tissue culture system. The details of this method can be found elsewhere^{35,36}. Briefly, tissue biopsies were placed in a Falcon cell culture insert (Becton-Dickinson Labware, Franklin Lakes, NJ) containing 200 μ l Dulbecco's Modified Eagle's Medium: F12 Ham's mixture. These inserts were placed in a Falcon cell culture plate with 500 μ l medium. Media contained 15% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) glutamine solution, 1% (v) penicillin/streptomycin solution and 1 μ l/ml amphotericin B (all from Sigma Chemical Co., St. Louis, MO). Cultures were incubated at 37 °C, 5% CO for 48 hours. Culture media was changed every 24 hours.

Preparation of bacteria

Stock cultures of *Ureaplasma urealyticum* (ATCC #27816; *U. urealyticum*, UU), *Ureaplasma parvum* (ATCC #27813; *U. parvum*, UP), *Mycoplasma hominis* (ATCC #14027; *M. hominis*, MH), *Gardnerella vaginalis* (ATCC #49145; *G. vaginalis*, GV), *Polyporhorans gingivalis* (ATCC# BAA-1703; *P. gingivalis*, PG), Group B Streptococci (ATCC#BAA-25; *Streptococcus agalactiae*, GBS), *Escherichia coli* (ATCC #33908; *E. coli*, EC) were purchased from the American Type Culture Collection (Manassas, VA). *U. urealyticum* and *U. parvum* were cultivated in Beef Heart Infusion Broth supplemented with 20% (v/v) Horse Serum + 10% (w/v) Yeast extract + 0.2% (w/v) Urea + 1000U/ml Penicillin G + 40 mg/ml Phenol Red (pH = 6.0). *M. hominis* was cultivated in PPLO broth supplemented with 20% (v/v) Horse Serum + 10% (w/v) Yeast extract + 0.2% Arginine (w/v) + 1000U/ml Penicillin G. *G. vaginalis* was cultivated in Casman's Broth + 5% (v/v) defibrinated sheep blood. *S. agalactiae* was grown in Brain Heart Infusion Broth supplemented with 5% (v/v) defibrinated sheep blood. *E. coli* was cultured in nutrient broth and *P. gingivalis* was cultivated anaerobically in Chopped Meat Broth. To prepare bacteria for the studies below, organisms were cultured to late log-phase, concentrated by centrifugation and re-suspended in RPMI 1640. Quantitative cultures were then established for color changing unit (CCU) or CFU determination by serial dilution and plating on appropriate agar. Bacteria were then heat-killed by heating at 80 °C for 1 h. Stocks of heat-killed bacteria were then stored at -80 °C until use.

Stimulation of fetal membranes with heat inactivated bacteria and LPS

Tissue cultures were stimulated with either 10 CFU/CCU of heat-inactivated bacterial suspension or 100ng/ml LPS (*E. coli* O55:B5). Dilutions of the bacterial suspensions and LPS were made in tissue culture media. Tissue cultures were stimulated for 24 hours. Culture and stimulation conditions are chosen based on our prior reports¹² and also based on other published studies. Tissue samples from both stimulated and unstimulated controls (hereafter referred as controls) were harvested and stored at -80 °C for further analysis.

Quantitative Real-Time RT-PCR

Fetal membrane tissues were homogenized in 1ml of Trizol (Invitrogen, Grand Island, NY), and RNA was extracted following the manufacturer's instructions using chloroform and phenol (Invitrogen). Extracted RNA was quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE), after which the RNA was treated with amplification grade DNase I from Invitrogen. Complementary DNA (cDNA) was synthesized from 1µg of RNA using the First-Strand cDNA Synthesis SuperScript II reverse transcriptase kit (Invitrogen). Relative mRNA expression levels of TLRs 1–10 and the housekeeping gene, GAPDH, were measured using SYBR-Green based quantitative real-time PCR using the KAPA SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA). PCR amplification was performed on the BioRad CFX Connect Real Time System (BioRad, Hercules, CA) for 50 cycles using the following cycling conditions: 95 C for 3 min, followed by 50 cycles of 95 C for 10 s and 58 C for 45 s. The primer pairs obtained from Real Time Primers (Elkins Park, PA) were as follows: TLR1, FWD (5'-ATT CCG CAG TAC TCC ATT CC-3') and REV (5'-TTT GCT TGC TCT GTC AGC TT-3'); TLR2 FWD (5'-ATG CCT ACT GGG TGG AGA AC-3') and REV (5'-TGC ACC ACT CAG TCT TCA CA-3'); TLR3, FWD (5'-GAA AGG CTA GCA GTC ATC CA-3') and REV (5'-CAT CGG GTA CCT GAG TCA CA-3'); TLR4, FWD (5'-CAG CTC TTG GTG GAA GTT GA-3') and REV, (5'-GCA AGA AGC ATC AGG TGA AA-3'); TLR5, FWD (5'-GGA ACC AGC TCC TAG CTC CT-3') and REV (5'-AAG AGG GAA ACC CCA GAG AA-3'); TLR6, FWD (5'-CCT CCC AGG ATC AAG GTA CT-3') and REV (5'-GGA TTG TCC CCT GCT TTT AT-3'); TLR8, FWD (5'-GGG CAT TGC ATT TAA GAG GT-3') and REV (5'-TCC GGA TAT GAC GTT GAA AA-3'); TLR10, FWD (5'-ACT TTG CCC ACC ACA ATC TC-3') and REV (5'-CCC AGA AAA GCC CAC ATT TA-3'). Primer pairs ordered from Yale Keck facility were as follows: TLR7, FWD (5'-TTT ACC TGG ATG GAA ACC AGC TA-3') and REV (5'-TCA AGG CCT GAG AAG CTG TAA GCT A-3'); TLR9, FWD (5'-GGA CCT CTG GTA CTG CTT CCA-3') and REV (5'-AAG CTC GTT GTA CAC CCA GTC T-3')³⁷; and GAPDH, FWD (5'-GGG GAA GGT GAA GGT CGG AGT-3') and REV (5'-GGA GGG ATC TCG CTC CTG GAA-3'). The lack of DNA contamination was confirmed by amplifying mock controls of RNA that was not reverse-transcribed. Amplification (Ct) for each reaction was estimated using the instrument's software (BioRad CFX Manager 2.1, BioRad) and exported to Statistical Analysis Software (SAS) for analysis.

Statistical analysis

TLR1-10 expression was analyzed using a block design where tissues from a given subject were considered the blocking factor. Ct levels were normalized to the housekeeping GAPDH value by calculating fold change to the housekeeping gene expression. Fold change values were used for further analysis. Data were analyzed using PROC MIXED for mixed effects models in Statistical Analysis Software (SAS), version 9.2 (SAS Institute Inc., Cary, NC). Analyses evaluated the effect of bacterial treatment (LPS, EC, GV, PG, GBS, MH, UP, UU) on TLR production for each receptor (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10). Mathematical models evaluated effects due to exposure (bacterial treatment) as a fixed effect and tissue donor as a random effect. Such a model fit a random intercept for each patient to normalize subject-to-subject variability in basal TLR expression. There was no adjustment for multiple comparisons as only a limited number of planned comparisons between control and treatments were conducted³⁸. Data that did not meet the assumptions of normality and/or homoscedasticity of errors were log-transformed and re-checked as appropriate. Differences between individual treatments and the control (no treatment) were evaluated by conducting comparisons of least squares means estimates. Basic descriptive statistics, least squares mean estimates, along with corresponding p-values for least squares mean differences are presented in the tables below. Least squares means

and standard errors of untransformed data are shown for clarity of presentation. Differences were declared statistically significant at $p < 0.05$.

RESULTS

Effect of the treatment of normal human term fetal membrane explants with genital *M. hominis* is shown in Figure 1. *M. hominis* significantly increased expression of TLR4, TLR6, and TLR8 mRNA ($p = 0.01$, 0.03 , and 0.02 , respectively). Treatment with *U. parvum*, significantly upregulated TLR8 mRNA expression ($p = 0.02$) (Figure 1). All other changes in TLR expression after exposure to either *M. hominis* or *U. parvum* were not significant (Figure 1 and supplemental data). Treatment with *U. urealyticum*, had no significant effect on the expression of any of the TLRs (see supplemental data).

P. gingivalis significantly increased fetal membrane TLR7 expression 25-fold compared to controls ($p = 0.01$) (Figure 2). In contrast, treatment of fetal membranes with the Gram-negative bacterial component, LPS, significantly down regulated TLR10 mRNA expression ($p = 0.01$) compared to unstimulated controls, as did the Gram-negative bacterium *E. coli* ($p = 0.02$) (Figure 2). Treatment of fetal membranes with *G. vaginalis* had no significant effect on the expression levels of any of the TLRs (Figure 2 and supplemental data).

Treatment of fetal membranes with the Gram-positive bacterium Group B streptococci had no significant effect on TLR1-10 expression (Figure 3 and supplemental data).

DISCUSSION

Preterm labor and pPROM have been associated with a number of Gram-positive and Gram-negative bacteria from the lower genital tract, as well as pathogenic mycoplasmas and oral cavity microorganisms^{6,39-41}. In this study we show that exposure of normal term human term fetal membranes to a range of heat-inactivated bacteria, associated with PTB, generate distinct TLR expression patterns. While the ability of the fetal membranes to generate an inflammatory response towards bacterial infection is known⁸⁻¹², the precise role of innate immune PRRs, such as the TLRs, is still uncertain. However, that the chorioamnion can express TLRs and their expression can change under pathological conditions³²⁻³⁴, suggests they may play an important role in mediating inflammatory responses towards intrauterine infections, and thus PTB and pPROM. Therefore, TLR stimulation by bacterial infection, may in turn, modulate fetal membrane TLR expression.

Mycoplasma are frequently isolated from the human reproductive tract and are often associated with membrane rupture and PTB⁴¹. *U. parvum* activates TLR2, TLR1 and TLR6⁴², *U. urealyticum*, is sensed by TLR2 and TLR4⁴³ and *M. hominis* is, at present, only known to activate TLR2^{44,45}. Although these three bacterial species can all be sensed by TLR2, they had quite different effects on fetal membrane TLR expression. *M. hominis* significantly increased fetal membrane expression of TLR4, TLR6 and TLR8. *U. parvum*, which has little effect on fetal membrane pro-inflammatory cytokine production¹², also significantly upregulated TLR8 expression. In contrast, *U. urealyticum* had no significant effect on the expression of any of the fetal membrane TLRs. The only other bacterium tested to significantly upregulate fetal membrane TLR expression was the oral Gram-negative bacterium, *P. gingivalis*, which can activate TLR4 and TLR2^{46,47}. *P. gingivalis* can disseminate to the placenta and is associated with preterm labor and chorioamnionitis⁴⁸. Moreover, in pregnant mice *P. gingivalis* increases placental TLR4 expression, reduces fetal weight and reduces fecundity⁴⁹. In our current study we found that exposure of fetal membranes to *P. gingivalis* strikingly and significantly increased TLR7 expression by 25 fold. These findings suggest that different bacteria, all with the capacity to activate TLR2,

have distinct effects on fetal membrane TLR expression and thus, the capacity of the chorioamnion to subsequently respond to other types of infection. Through the upregulation of TLR7 and TLR8, *M. hominis*, *U. parvum* and *P. gingivalis* may augment the ability of the fetal membrane to subsequently sense and respond to a viral infection. Indeed, in THP-1 monocytic cells, exposure to Gram-positive *S. aureus* or *E. coli*, which activate TLR2 and TLR4, respectively, strongly increase expression of the viral sensors, TLR7 and TLR8⁵⁰. The opposite has been reported in trophoblast cells, whereby after a viral infection, TLR2 and TLR4 expression is increased⁵¹. Similarly, through the upregulation of TLR4 and the TLR2 co-receptor, TLR6²⁷, *M. hominis* may allow the chorioamnion to generate a heightened response towards Gram-positive and Gram-negative bacterial infections.

The other Gram-negative bacterium tested were *G. vaginalis*, which modestly triggers fetal membrane inflammation, and *E. coli*, which robustly activate fetal membranes to produce inflammatory cytokines and prostaglandins¹². However, *G. vaginalis* had no significant effect on the expression of any TLRs and *E. coli*, instead of upregulating TLR expression, downregulated TLR10 mRNA levels, as did the Gram-negative bacterial component, LPS. Since TLR10 is a co-receptor for TLR2²⁸, exposure of fetal membranes to *E. coli* may limit or alter subsequent responses to bacterium that can stimulate TLR2.

The Gram-positive bacterium, Group B Streptococcus (GBS), similar to *G. vaginalis*, modestly triggers fetal membrane inflammation¹², yet it too had no significant effect on fetal membrane TLR expression levels. Previous studies have demonstrated that while GBS can activate TR2 and TLR6 via a secreted factor, heat-killed GBS is unable to stimulate TLR1, TLR2, TLR4 or TLR6⁵². Thus, it is possible that we might observe different TLR expression patterns and cytokine responses of the fetal membrane after exposure to live bacteria.

While significance was not reached, we did observe additional changes in TLR expression patterns that were greater than 2 fold. The mycoplasmas, *M. hominis*, increased TLR1, TLR2, and TLR5 expression in the fetal membranes, and *U. parvum*, increased TLR1, TLR2, TLR5, TLR6 and TLR7 mRNA levels (Figure 1 and supplemental data). In response to Gram-negative bacterium we observed a non-significant >2 fold increase in: TLR1 and TLR6 by LPS and *P. gingivalis*; TLR2 by LPS, *G. vaginalis* and *P. gingivalis*; and TLR7 by *P. gingivalis* (Figure 2 and supplemental data). Treatment of fetal membranes with the Gram-positive bacterium Group B streptococci increased TLR1, TLR2 and TLR6 expression levels (Figure 3 and supplemental data). The lack of significance for these changes may be due to our sample size not giving us sufficient power. Nevertheless, these observations may indicate a greater ability for various bacterial species to regulate fetal membrane TLR expression.

In summary, we have demonstrated that various genitourinary tract and oral bacteria, associated with PTB, even from the same genus, have distinct effects on fetal membrane TLR expression patterns. These findings highlight the disparity of fetal membrane responses to infection and thus, suggest heterogeneity in the mechanisms by which infection-associated pregnancy complications, such as pPROM and PTB, arise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of mycoplasmas on fetal membrane TLR Expression

Fetal membrane explants were cultured with and without *M. hominis* or *U. parvum*. Bar chart shows TLR1-10 mRNA expression as the least squares mean (LSM) and Standard Error of fold changes relative to the control. Values were graphed using the control (no treatment) value as a baseline.

Figure 2. Effect of Gram-negative bacteria and LPS on fetal membrane TLR Expression

Fetal membrane explants were cultured with and without LPS, *E. coli*, *G. vaginalis* or *P. gingivalis*. Bar charts show fetal membrane TLR1-10 mRNA expression in response to each bacterial treatment as the least squares mean (LSM) and Standard Error of fold changes relative to the control. Values were graphed using the control (no treatment) value as a baseline.

Figure 3. Effect of Gram-positive bacteria on fetal membrane TLR Expression

Fetal membrane explants were cultured with and without Group B Streptococci. Bar charts show fetal membrane TLR1-10 mRNA expression in response to each bacterial treatment as the least squares mean (LSM) and Standard Error of fold changes relative to the control. Values were graphed using the control (no treatment) value as a baseline.