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PREGNANCY: ORIGINAL ARTICLE

Impact of Progesterone on Molecular Mechanisms of Preterm Premature Rupture of Membranes

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Abstract

The role and mechanisms of progesterone in preterm premature rupture of membranes (PPROM) remains unclear. This study aims to investigate the molecular mechanisms of action of progesterone in pre-labor full-term fetal amniotic membrane cells with and without stimulation by microbial, pro-inflammatory, or thrombogenic agents. Fetal amniotic membranes were collected from 30 women with a normal singleton pregnancy undergoing elective cesarean section at term prior to the onset of labor. The human amniotic epithelial cells isolated were pretreated with and without medroxyprogesterone acetate for 24 h. Then, cells were treated with and without TLR/NLR agonists, pro-inflammatory cytokines, or thrombin for 48 h. Semi-quantitative RT-PCR, Western blot, and caspase-3 activity measurement were performed. Progesterone stimulation decreased the expression of TLR2, TLR5, and Nod2 genes (alone and/or in combination with TLR/NLR agonists) and decreased the expression of IL-1β and IL-8 genes increased by stimulation with specific agonists for TLR2, TLR4, TLR5, Nod1, and Nod2. Moreover, progesterone decreased thrombin-induced IL-8 gene expression. Progesterone also decreased expression of Bax and Bid proteins (pro-apoptotic factors) increased by stimulation with pro-inflammatory cytokines (TNF-α, NGAL, IL-18, and IL-1β) and thrombin. Progesterone stimulation alone as well as co-stimulation with TNF-α, NGAL, IL-18, IL-1β, or thrombin with progesterone either increased, decreased, or did not change the expression of Bcl-2, Bcl-XL, or XIAP genes (anti-apoptotic factors). These data suggest progesterone plays protective roles against PPROM through anti-microbial, anti-inflammatory, and anti-thrombogenic actions on human-term fetal amniotic membrane cells. Progesterone alters pro-inflammatory cytokine- and thrombin-induced apoptosis by controlling the expression of pro-apoptotic and anti-apoptotic factors.

Keywords Amnion · Apoptosis · Preterm premature rupture of membrane · Progesterone · Pro-inflammatory cytokine · Toll-like receptors/Nod-like receptors

Introduction

Preterm premature rupture of membranes (PPROM) is defined as the rupture of fetal membranes at less than 37 completed weeks of gestation and leads to one-third of preterm births (PTBs) [1, 2]. PPROM affects 3–4% of pregnancies [1, 3].

Although the etiology and mechanism of fetal membrane weakening and PPROM remain unclear, intrauterine inflammation, decidual bleeding/abruption, and programmed cell death (apoptosis) have been suggested to play a role [4–14]. Infection of fetal membranes may trigger secretion of pro-inflammatory cytokines through innate immune capacities [15–21]. Pattern recognition receptors (PRRs) including trans-membrane Toll-like receptors (TLRs) and cytoplasmic-based nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are expressed by the cells of the innate immune system and identify pathogen-associated

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molecular patterns associated with microbial pathogens [22]. Previous studies have reported that PRRs are expressed in fetal membranes, decidua, and trophoblasts (whole period of gestation, preterm labor, term labor, chorioamnionitis, and PPROM), mediate the release of pro-inflammatory cytokines, and induce cell apoptosis [15–21, 23–25]. Decidual bleeding/abruption-induced thrombin has also been found to increase the expression of pro-inflammatory cytokines in human-term decidual cells [13, 14, 26]. Moreover, some studies have reported that in human-term fetal membranes, pro-inflammatory cytokines and decidual bleeding/abruption-induced thrombin are associated with fetal membrane weakening and PPROM via collagen remodeling, apoptosis, and biophysical weakening of membranes [6, 11–14, 27, 28].

Progesterone supplementation is used to prevent recurrent PTB in women at high risk due to a prior PTB [29, 30]. However, the mechanism of action of progesterone supplementation is unclear. In a retrospective study (n =1183), patients given progesterone supplementation due to a prior PTB with PPROM had a lower rate of recurrent PTB compared to those who received progesterone supplementation due to a prior spontaneous PTB [31]. Some researchers have reported that progesterone inhibits TLR expression, pro-inflammatory cytokine secretion, apoptosis, and fetal membrane weakening in human-term fetal membranes [27, 28, 32–35]. The potentially protective role of progesterone, however, and its mechanisms in fetal membrane weakening and PPROM remain unclear and need to be further evaluated.

We hypothesize that in human-term fetal membranes, progesterone will prevent inflammation induced by activation of TLRs/NLRs or pro-thrombotic stimuli and will prevent apoptosis induced by pro-inflammatory cytokines or pro-thrombotic stimuli. This study aims to investigate the molecular mechanisms by which progesterone may play a role in the pre-labor full-term fetal amniotic membrane utilizing human epithelial cells (hAECs) with and without stimulation by microbial, pro-inflammatory, and thrombogenic agents.

Materials and Methods

Tissue Source

Fetal amniotic membranes were obtained from 30 women with a normal singleton pregnancy undergoing uncomplicated elective cesarean section at term prior to the onset of labor. Women who had any medical or obstetric complications or medications within 24 h were excluded [35]. Women exhibiting signs of infection were excluded based on standard clinical infection criteria (maternal fever, fundal tenderness, maternal and/or fetal tachycardia, foul vaginal discharge) and laboratory tests (elevated white cell count) [35]. This study was approved by the Institutional Review Board of The Catholic Medical Center at the Catholic University of Korea (No. UC19TOSI0123).

Reagents and Chemicals

Dulbecco's modified Eagle medium/F12 (DMEM/F12) and antibiotics-penicillin/streptomycin solution were purchased from Welgene (Seoul, Korea). Peptidoglycan (PDG), lipopolysaccharide (LPS), flagellin, thrombin, tumor necrosis factor (TNF)-α, and medroxyprogesterone acetate (MPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-gamma-Glu-mDAP (iE-DAP), and muramyl dipeptide (MDP) were purchased from Invivogen (San Diego, CA, USA). Neutrophil gelatinase-associated lipocalin (NGAL), interleukin (IL)-18, and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA).

Isolation, Culture, and Stimulation of hAECs

Preparation and isolation of hAECs were performed as described previously [36]. Briefly, the amniotic membrane was manually stripped from the chorion layer. The membrane was washed several times with ice-cold phosphate-buffered saline (PBS, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) (pH 7.2) to remove blood clots. The blood-free amniotic membrane was cut into pieces 5–7 cm long and then transferred with sterile forceps to a new 250-ml polystyrene bottle (Nalgene, Rochester, NY, USA). To this bottle, 50 ml pre-digestion buffer (0.05% trypsin/EDTA) was added, and incubated for 15 min at 37 °C in a humidified 95% air/5% CO_2 incubator. Then, the amnion pieces were transferred into a new polystyrene bottle and incubated for 1 h at 37 °C with DMEM-F12 containing pre-warmed 0.05% trypsin/EDTA; this step was repeated twice. The cell suspension digest was vigorously shaken and then filtered through 100-μM nylon gauze (Falcon, Corning, NY, USA). The isolated hAECs were suspended in DMEM/F12 supplemented with 15% fetal bovine serum (FBS, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin and then cultured in 150-mm culture dishes at 37 °C in a humidified 95% air/5% CO_2 incubator. Cell culture medium was changed after 3 days of culture. Upon reaching confluence, adhered hAECs were trypsinized and reseeded at a density of 4×10^5 cells per well in six-well plates. hAECs were pretreated with and without medroxyprogesterone acetate (MPA, 10 and 50 μM) for 24 h. Then, the cells were treated with and without TLR/NLR agonist for 48 h with one of the following agonists: TLR2 agonist, Gram-positive bacterial PDG from *Staphylococcus aureus* at 10 μg/ml; TLR4 agonist, Gram-negative LPS from *Escherichia coli* O111:B4 at 100 ng/ml; TLR5 agonist, Gram-negative bacterial flagellin from *Salmonella typhimurium* at 1 μg/ml; Nod1 agonist, Gram-positive/negative bacterial iE-DAP at 100 μg/ml; Nod2

agonist, Gram-positive/negative bacterial MDP at 10 μg/ml. In the apoptosis assay, hAECs were pretreated with and without MPA (10 and 50 μM) for 24 h and then treated with and without pro-inflammatory cytokines/thrombin for 24 h with one of the following agents: TNF-α (50 ng/ml), NGAL (1 ug/ml), IL-18 (100 ng/ml), IL-1β (100 ng/ml), or thrombin (2 U/ml). Cells not treated with any agents were used as the negative control.

Semi-quantitative RT-PCR

Total RNA was extracted from confluent amniotic epithelial cells using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. RNA concentration and purity were measured using a NanoDrop Onec spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 μg) was reverse-transcribed using the AccuPower RT premix (Bioneer, Daejeon, Korea) and oligo (dT) primers (Bioneer, Daejeon, Korea). The PCR products were generated using AccurPower PCR Mix (Bioneer, Daejeon, Korea). The resulting products were separated using 2.0% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under UV light. Band intensity was quantified using National Institutes of Health ImageJ software, version 1.52v, and gene expression was normalized to that of the housekeeping gene β-actin [37]. The oligonucleotide primers are presented in Table 1.

Protein Extraction and Western Blot

Amniotic epithelial cells at 70–80% confluency were washed twice with ice-cold PBS (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and lysed on ice for 30 min in 1× RIPA buffer (#9806; Cell Signaling Technology, Danvers, MA, USA) containing 1× protease inhibitor cocktail reagent (Promega, Madison, WI, USA) and 1× phosphatase inhibitors (EMD Millipore Corp, Saint Louis, MO, USA). Lysates were immediately frozen and stored at − 80 °C until use. Protein concentrations were determined by the Bradford method using BSA as the standard. Equal amounts of proteins (20 μg) were separated on a 4–12% NuPAGE gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a nitrocellulose membrane (0.45-μm pore size, Invitrogen, Carlsbad, CA, USA). Nonspecific binding sites were blocked by 5% BSA in 1× TBST buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween 20 at pH 7.5). The membranes were incubated overnight at 4 °C in 1× TBST buffer/5% BSA with rabbit polyclonal anti-Bid (1:1000; #2002; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-Bax (1:1000; #5023; Cell Signaling Technology), and mouse monoclonal anti-β-actin antibody (1:10000; A5441; Sigma-Aldrich, St. Louis, MO, USA). Then, membranes were rinsed three times using washing buffer (1× TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies (goat polyclonal anti-rabbit IgG; 1:2000; #1706515; BioRad, Hercules, CA, USA) (Amersham ECL sheep anti-mouse IgG; 1:5000; NA931; Cyvita, Marlborough, MA, USA) in 1× TBST for 1 h at room temperature. Target proteins were detected with ECL western blotting substrate reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Gangnam-stain™ prestained protein Ladder (Intron Biotechnology, Seongnam, Gyeonggi-do, Korea) was used for molecular weight sizing. Band intensity was quantified using National Institutes of Health ImageJ software, version 1.52v [37].

Caspase 3 Activity Measurement

Fluorogenic caspase 3 substrate N-Ac-DEVD-AFC (BioAssay Systems, Hayward, CA, USA) was used to identify caspase 3 activation. Briefly, hAECs were trypsinized and reseeded at a density of 3 × 103 cells per well in a sterile black clear-bottom 96-well plate (Falcon, Corning, NY, USA) and then were pretreated with and without MPA (10 and 50 μM) for 48 h. Then, the medium was changed to fresh DMEM/F12 with and without pro-inflammatory cytokines/thrombin and cells were further cultured for 24 h. Cells not treated with any agents were used as the negative control. After stimulation, caspase 3 activity was detected using a QuantiFluo™ Caspase-3 Assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. The fluorescence intensity at λ exc/em = 400/490nm, which is proportional to caspase-3 activity, was determined using the SpectraMax i3X (Molecular Devices). Each sample was tested in triplicate.

Statistical Analyses

Statistical analyses were performed using SPSS version 26 (IBM Corporation, Armonk, NY, USA). Statistical differences in the expression of genes or caspase-3 activity between groups were calculated using a Mann-Whitney rank sum test. $P < 0.05$ was considered statistically significant.

Results

Expression of TLRs/NLRs and Their Effects on Pro-inflammatory Cytokine Expression in hAECs With and Without Progesterone Treatment

The expression of TLR2, TLR5, and Nod2 genes increased in cells treated with their respective agonists ($P < 0.05$), whereas the expression of TLR4 and Nod1 genes was unchanged. Co-stimulation with each specific agonist and progesterone

Table 1 Primers used in PCR and PCR conditions

Gene (accession no.)	Primer sequences[a]	Annealing temperature (°C)	Amplicon (bp)	Cycle no.
TLR2 (NM_001318787)	F: 5′-GCCAAAGTCTTGATTGATTG-3′	58	347	40
	R: 5′-TTGAAGTTCTCCAGCTCCTG-3′			
TLR4 (NM_138554)	F: 5′-TGGATACGTTTCCTTATAAG-3′	60	507	40
	R: 5′-GAAATGGAGGCACCCCTTC-3′			
TLR5 (XM_005273242)	F: 5′-ATTGCCAATATCCAGGATGC-3′	58	153	40
	R: 5′-CACCACCATGATGAGAGCAC-3′			
TLR8 (NM_138636)	F: 5′-TCAACAAATCCGCACTTGAA-3′	59	168	40
	R: 5′-CAGGACTGGCACAAATGACA-3′			
Nod1 (NM_006092)	F: 5′-CCACTTCACAGCTGGAGACA-3′	60	200	40
	R: 5′-TGAGTGGAAGCAGCATTTTG-3′			
Nod2 (NM_022162)	F: 5′-GAATGTTGGGCACCTCAAGT-3′	58	317	40
	R: 5′-CAAGGAGCTTAGCCATGGAG-3′			
IL-1β (NM_000576)	F: 5′-ACAGATGAAGTGCTCCTTCCA-3′	62	73	40
	R: 5′-GTCGGAGATTCGTAGCTGGAT-3′			
IL-8 (BT007067)	F: 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′	62	289	40
	R: 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′			
Bcl-2 (NM_000657)	F: 5′-TGCCACCTGTGGTCCACCTGACCCT-3′	58	364	25
	R: 5′-AACAGAGGCCGCATGCTGGGGCCGT-3′			
Bcl-XL (NM_001191)	F: 5′-CATGGCAGCAGTAAAGCAAG-3′	58	351	25
	R: 5′-GCATTGTTCCCATAGAGTTCC-3′			
XIAP (NM_001167)	F: 5′-TGGCAGATTATGAAGCACGGATC-3′	56	140	25
	R: 5′-AGTTAGCCCTCCTCCACAGTGA-3′			
β-actin (NM_001101)	F: 5′-AGCGAGCATCCCCCAAAGTT-3′	58	285	25
	R: 5′-GGGCACGAAGGCTCATCATT-3′			

[a] *F*, forward; *R*, reverse

The date of accession for each primer was May 11, 2020

decreased the expression of TLR2, TLR5, and Nod2 genes ($P < 0.05$), whereas it did not change the expression of TLR4 and Nod1 genes. Stimulation of specific agonists for TLR2, TLR4, TLR5, Nod1, and Nod2 increased the expression of IL-1β and IL-8 genes ($P < 0.05$). Co-stimulation with each specific agonist and progesterone decreased the expression of IL-1β and IL-8 genes ($P < 0.05$). However, in co-stimulation with iE-DAP and progesterone, MPA 50 μM decreased the

expression of the IL-1β gene ($P = 0.061$) and MPA 10 μM decreased the expression of the IL-8 gene ($P < 0.05$). The expression of TLR2 and TLR5 genes decreased by progesterone stimulation compared with basal conditions ($P < 0.05$), whereas the expression of TLR4, Nod1, and Nod2 genes was not altered. The expression of IL-1β and IL-8 genes was not changed by progesterone stimulation compared with basal conditions (Fig. 1).

Effects of Thrombin on Pro-inflammatory Cytokine Expression in hAECs With and Without Progesterone Treatment

Thrombin stimulation did not change the expression of IL-1β and IL-8 genes. Co-stimulation with thrombin and progesterone did not alter the expression of the IL-1β gene, whereas it decreased the expression of the IL-8 gene in MPA 50 μM ($P < 0.05$). The expression of the IL-1β gene was not affected by progesterone stimulation compared with basal condition, whereas the expression of the IL-8 gene decreased by MPA 10 μM ($P = 0.065$) (Fig. 2).

Effects of Pro-inflammatory Cytokines and Thrombin on Mediators of Apoptosis in hAECs With and Without Progesterone Treatment

The expression of Bcl-2-associated X (Bax) and BH3 interacting domain death agonist (Bid) proteins were evaluated as pro-apoptotic factors. Stimulation with each pro-inflammatory cytokine (TNF-α, NGAL, IL-18, and IL-1β) and thrombin increased the expression of Bax and Bid proteins. Co-stimulation of each pro-inflammatory cytokine or thrombin with progesterone decreased the expression of Bax and Bid proteins (Fig. 3).

The expression of B-cell lymphoma (Bcl)-2, Bcl-extra large (XL), and X-linked inhibitor of apoptosis protein (XIAP) genes was evaluated as anti-apoptotic factors. Stimulation with TNF-α increased the expression of Bcl-XL and XIAP genes ($P < 0.05$). However, co-stimulation with

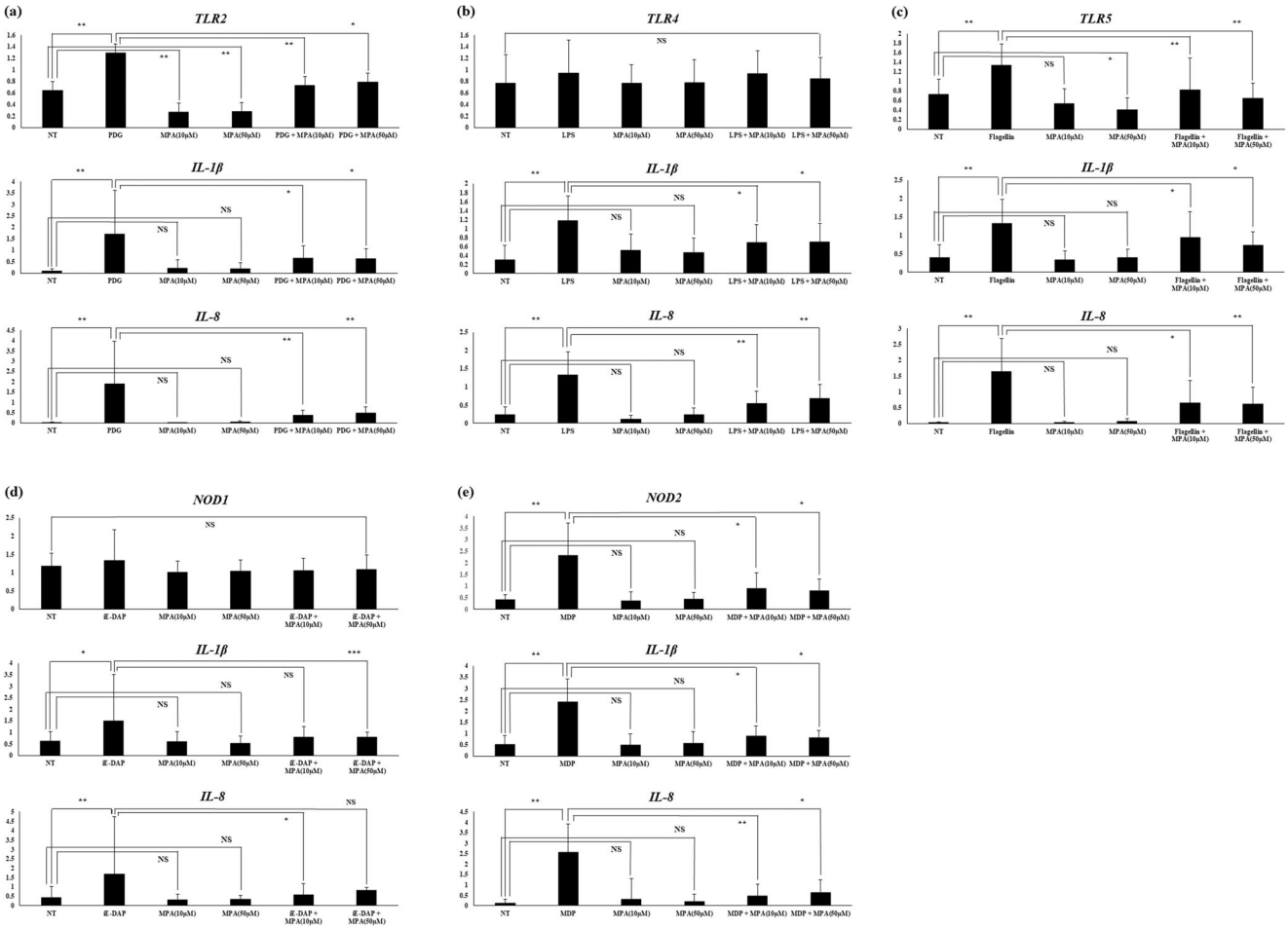


Fig. 1 Expression of TLRs/NLRs and their effects on pro-inflammatory cytokine expression in hAECs with and without progesterone **A** PDG, **B** LPS, **C** flagellin, **D** iE-DAP, and **E** MDP were used as specific agonists. The Y axis represents mRNA expression as determined by RT-PCR and results are expressed as mean ± SD of experiments from 13-19 donors. Asterisks represent statistical differences between groups (* $P < 0.05$, ** $P < 0.001$, *** $P = 0.061$). NS, nonsignificant; NT, no treatment; SD, standard deviation

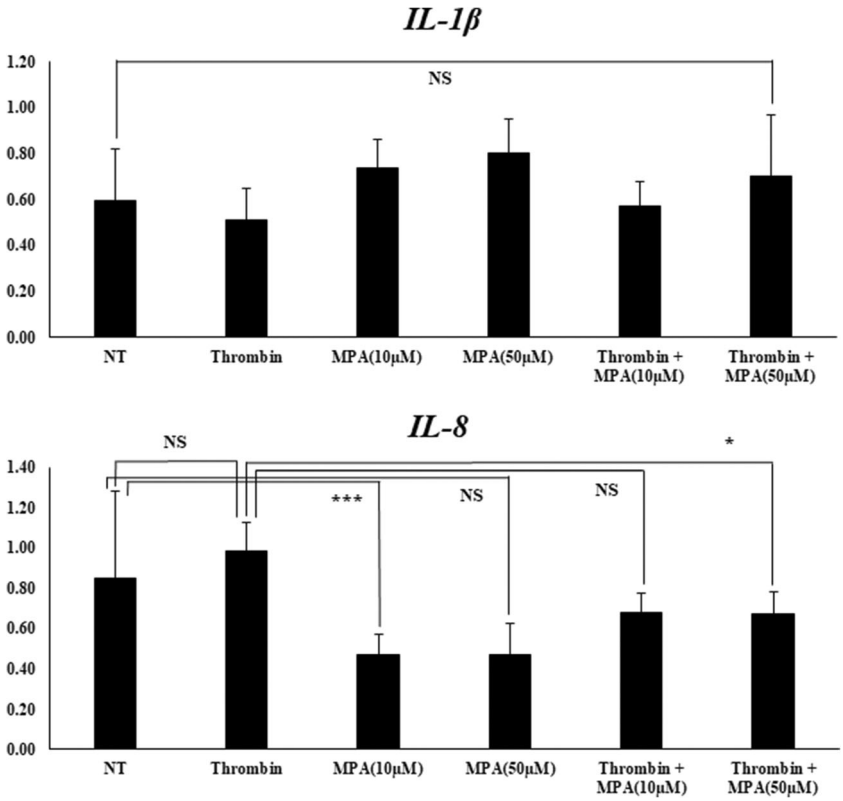


Fig. 2 Effects of thrombin on pro-inflammatory cytokine expression in hAECs with and without progesterone. The Y axis represents mRNA expression as determined by RT-PCR and results are expressed as mean ± SD of experiments from six donors. Asterisks represent statistical differences between groups (* $P < 0.05$, *** $P = 0.065$). NS, nonsignificant; NT, no treatment; SD, standard deviation

TNF-α and progesterone decreased only the expression of the XIAP gene in MPA 10 μM ($P = 0.057$). Co-stimulation with NGAL and progesterone decreased only the expression of the Bcl-2 gene in MPA 10 μM ($P = 0.057$). Co-stimulation with IL-1β and progesterone decreased only the expression of the Bcl-XL gene ($P < 0.05$), whereas co-stimulation with thrombin and progesterone decreased only the expression of the Bcl-2 gene ($P < 0.05$). Progesterone stimulation alone had varied effects on the expression of Bcl-2 (decrease or no change), Bcl-XL (increase or no change), and XIAP (decrease or no change) genes compared with basal conditions ($P < 0.05$, $P = 0.057$, or $P > 0.05$) (Fig. 3).

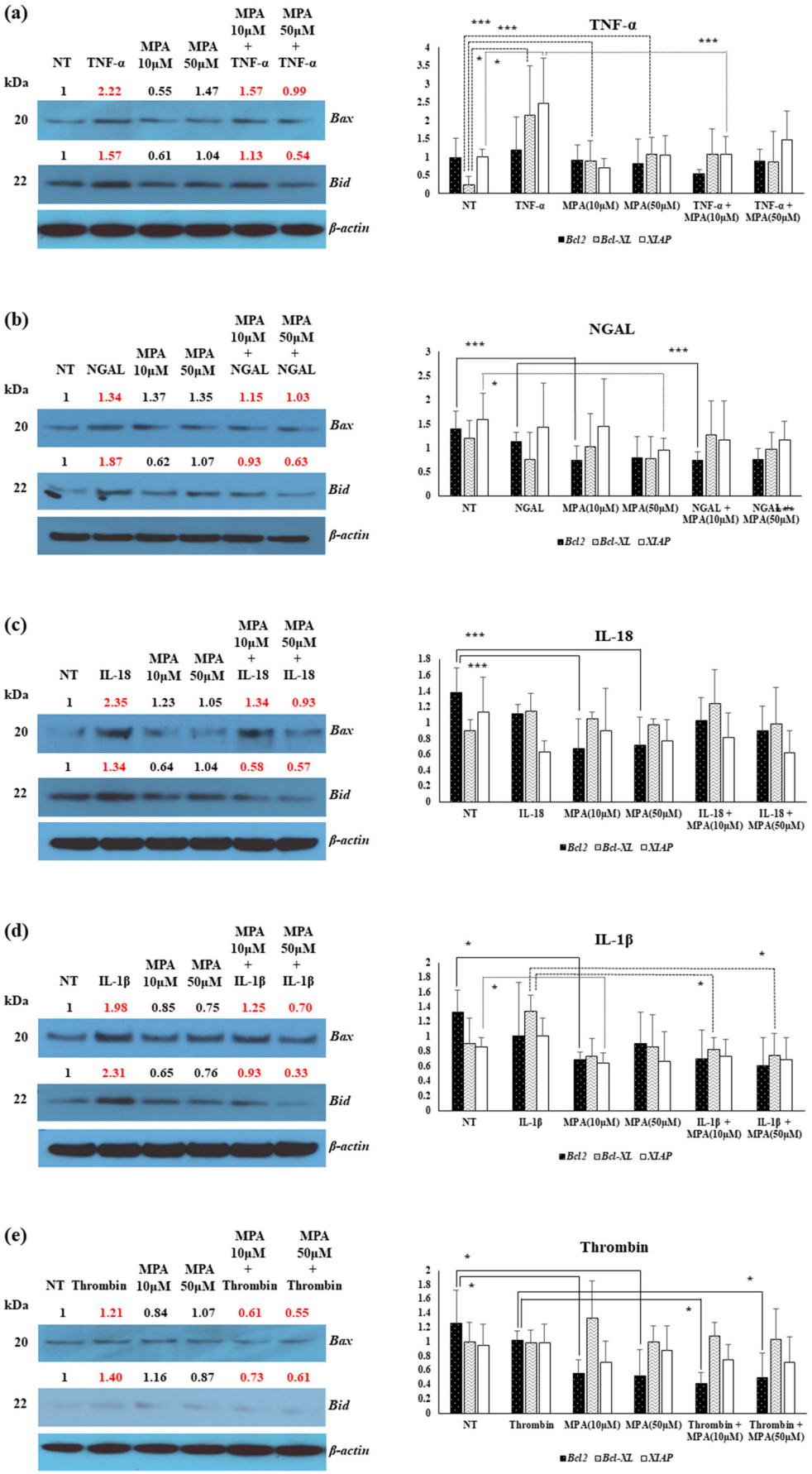
Stimulation with each pro-inflammatory cytokine (TNF-α, NGAL, IL-18, and IL-1β) and thrombin, and co-stimulation of each cytokine or thrombin with progesterone did not change caspase-3 activity (Supplemental Figure S1).

Discussion

The roles of the TLR/NLR signaling in maintaining a normal pregnancy, labor (preterm and full-term), chorioamnionitis, and PPROM remain unclear. In human-term fetal membranes, TLR2, TLR4, TLR5, Nod1, and Nod2 agonists increase IL-1β, IL-2, IL-6, IL-8, or IL-10 secretion via the nuclear factor-kappa B (NF-κB) signaling pathway [16, 18, 38, 39]. An in vitro study using human-term amniotic epithelium showed that progesterone stimulation inhibited the secretion of TNF-α, IL-6, IL-8, and IL-10 increased by TLR4 agonist [33]. Here we demonstrate that in human-term amniotic epithelial cells, specific agonists for TLR2, TLR4, TLR5, Nod1, and Nod2 increased secretion of IL-1β and IL-8, while progesterone treatment reversed these increase in pro-inflammatory cytokines. Furthermore, progesterone decreased the expression of TLR2, TLR5, and Nod2 that was increased by specific agonists. Because progesterone decreased only TLR2 and TLR5 without influencing other TLRs/NLRs or pro-inflammatory cytokines (IL-1β and IL-8) in pre-labor term amniotic epithelial cells, we presume that TLR2 and TLR5 play roles in pre-labor term fetal membranes.

Previous studies have reported that decidual bleeding/abruption-induced thrombin increases the expression of pro-inflammatory cytokines such as IL-6, IL-8, IL-10, and interferon-γ in human-term decidual cells, and induces fetal membrane weakening and PPROM [11–14, 26]. In human-term decidual cells, thrombin increases the expression of IL-8,

Fig. 3 Effects of pro-inflammatory cytokines and thrombin on mediators of apoptosis in hAECs with and without progesterone **A** TNF-α, **B** NGAL, **C** IL-18, **D** IL-1β, and **E** thrombin were used as experimental cytokines. Pro-apoptotic proteins (Bax and Bid) are presented on Western blot. For anti-apoptotic genes (Bcl-2, Bcl-XL, and XIAP), the Y axis represents mRNA expression as determined by RT-PCR and results are expressed as mean ± SD of experiments from 4 to 7 donors. Asterisks represent statistical differences between groups (* $P < 0.05$, *** $P = 0.057$). NT, no treatment; SD, standard deviation


(a)
NT
TNF-α
MPA 10μM
MPA 50μM
MPA 10μM + TNF-α
MPA 50μM + TNF-α
kDa
1 2.22 0.55 1.47 1.57 0.99
20
Bax
1 1.57 0.61 1.04 1.13 0.54
22
Bid
β-actin
TNF-α
NT
TNF-α
MPA(10μM)
MPA(50μM)
TNF-α + MPA(10μM)
TNF-α + MPA(50μM)
Bcl2 Bcl-XL XIAP
(b)
NT
NGAL
MPA 10μM
MPA 50μM
MPA 10μM + NGAL
MPA 50μM + NGAL
kDa
1 1.34 1.37 1.35 1.15 1.03
20
Bax
1 1.87 0.62 1.07 0.93 0.63
22
Bid
β-actin
NGAL
NT
NGAL
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NGAL + MPA(10μM)
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Bcl2 Bcl-XL XIAP
(c)
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IL-18
MPA 10μM
MPA 50μM
MPA 10μM + IL-18
MPA 50μM + IL-18
kDa
1 2.35 1.23 1.05 1.34 0.93
20
Bax
1 1.34 0.64 1.04 0.58 0.57
22
Bid
β-actin
IL-18
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IL-18 + MPA(10μM)
IL-18 + MPA(50μM)
Bcl2 Bcl-XL XIAP
(d)
NT
IL-1β
MPA 10μM
MPA 50μM
MPA 10μM + IL-1β
MPA 50μM + IL-1β
kDa
1 1.98 0.85 0.75 1.25 0.70
20
Bax
1 2.31 0.65 0.76 0.93 0.33
22
Bid
β-actin
IL-1β
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MPA(10μM)
MPA(50μM)
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(e)
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Thrombin
MPA 10μM
MPA 50μM
MPA 10μM + Thrombin
MPA 50μM + Thrombin
kDa
1 1.21 0.84 1.07 0.61 0.55
20
Bax
1 1.40 1.16 0.87 0.73 0.61
22
Bid
β-actin
Thrombin
NT
Thrombin
MPA(10μM)
MPA(50μM)
Thrombin + MPA(10μM)
Thrombin + MPA(50μM)
Bcl2 Bcl-XL XIAP

the primary neutrophil chemoattractant and activator, and neutrophils release extracellular matrix degrading proteases which can promote PPROM [13, 14]. Some studies on human-term fetal membranes have reported that progesterone inhibited thrombin-induced fetal membrane weakening [27, 28]. Here we demonstrate that in human-term amniotic epithelial cells, progesterone decreased thrombin-induced IL-8 gene expression, although thrombin induced only nonsignificant increase in IL-8 gene expression. Moreover, an in vitro study reported that thrombin increases secretion of pro-inflammatory cytokines such as IL-1β, IL-6, MCP-1, and TNF-α in human adipose cells suggesting that thrombin represents a molecular link between obesity (which is associated with hypercoagulation) and inflammation [40]. However, our results did not show an association between thrombin and IL-1β gene expression in human-term amniotic epithelial cells.

Previous studies have reported that pro-inflammatory cytokines and decidual bleeding/abruption-induced thrombin may induce fetal membrane weakening and PPROM via apoptosis [6, 11, 12]. It has been also reported that progesterone inhibits apoptosis in human-term fetal membranes [32, 35]. In pre-labor term fetal membranes, TNF-α stimulation increases the expression of the pro-apoptotic protein Bid and caspase-3, caspase-8, and caspase-9 activity, while progesterone inhibits TNF-α-induced Bid expression and caspase-3 activity [32, 35]. Moreover, in pre-labor term fetal membranes, progesterone decreases the expression of Bid and increases the expression of anti-apoptotic proteins Bcl-2 and XIAP. However, progesterone and TNF-α stimulation did not influence the expression of pro-apoptotic protein Bax activated by Bid in the intrinsic pathway of apoptosis [35, 41]. It has been reported that thrombin induces activation and mitochondrial translocation of Bid and Bax as a possible apoptotic event in human platelets [42]. Here we demonstrate that in pre-labor term amniotic epithelial cells, pro-inflammatory cytokine (TNF-α, NGAL, IL-18, and IL-1β), and thrombin stimulation increased the expression of Bax and Bid proteins, and progesterone reversed increases of those pro-apoptotic proteins. However, pro-inflammatory cytokines, thrombin, and progesterone stimulation did not influence caspase-3 activity. Moreover, progesterone increased, decreased, or did not change the expression of anti-apoptotic genes Bcl-2, Bcl-XL, and XIAP, and co-stimulation of TNF-α, NGAL, IL-18, IL-1β, or thrombin with progesterone decreased or did not change the expression of Bcl-2, Bcl-XL, and XIAP. This study is the first report to suggest the effects of NGAL, IL-18, and IL-1β on pro-apoptotic and anti-apoptotic factors. These results should be further clarified through additional research.

A previous study reported that in pre-labor term fetal membranes, progesterone decreased the expression of Bcl-2 and XIAP protein induced by TNF-α stimulation [35]. In our study, TNF-α increased the expression of Bcl-XL and XIAP genes and progesterone decreased their expression, although these inhibitions were not statistically significant. It is possible that TNF-α might control the expression of Bcl-2, Bcl-XL, or XIAP through a cell death/survival pathway depending on NF-κB activation, and progesterone might alter those changes [35, 43].

In summary, this study suggests the molecular mechanisms by which progesterone prevents fetal membrane weakening and PPROM using in vitro studies of cells from fetal amniotic membrane, which acts as a barrier to ascending infection/inflammation and a protector of the fetus. Progesterone plays protective roles against fetal membrane weakening and PPROM through anti-microbial, anti-inflammatory, and anti-thrombogenic actions in human-term fetal amniotic membrane cells. Moreover, progesterone alters pro-inflammatory cytokine- and thrombin-induced apoptosis by controlling the expression of pro-apoptotic and anti-apoptotic factors. The role of progesterone and its mechanisms related to fetal membrane weakening and PPROM should be further clarified in additional studies.

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Availability of Data and Material Not applicable

Code Availability Not applicable

Declarations

Ethics Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Institutional Review Board of The Catholic Medical Center at the Catholic University of Korea (No. UC19TOSI0123).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Not applicable

Competing Interests The authors declare no competing interests.

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