The Effect of Progesterone on Tumor Necrosis Factor-α Induced Matrix Metalloproteinase-9 in Human Choriodecidual Membranes

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Objective: Progesterone is used to prevent recurrent preterm delivery, however the molecular mechanisms of its effect are incompletely understood. The objective of this study was to determine the effect of progesterone on tumor necrosis factor (TNF)-α-induced matrix metalloproteinase (MMP)-9 activity in human choriodecidual (CD) membranes.

Methods: We collected CD membranes from women with uncomplicated term pregnancies who were scheduled for elective cesarean delivery (n=10). CD membranes (1×1 cm) were incubated in tissue culture media at 37°C. We pre-treated the CD membranes with progesterone (P4), 17α-hydroxyprogesterone caproate (17P), promegestone (R5020), or vehicle (ethanol) for 24 hours. The CD membranes were subsequently treated with TNF-α (with continued progesterone treatment) for 48 hours, then media was harvested for measuring MMP-9 activity by zymography and total protein was isolated from CD membrane tissues for MMP-9 expression by western blot analysis.

Results: P4, 17P, and R5020 significantly reduced TNF-α-induced MMP-9 activity in fetal membrane tissue samples (*P*=0.0078, *P*=0.0156, and *P*=0.0391, respectively) by zymography. Western blot analysis also showed decreased expression of MMP-9 in progesterone pretreated groups (*P*=0.0313).

Conclusion: Progesterone reduces TNF-α-induced MMP-9 activity in human CD membranes. These findings may provide further support for the role of progesterone in preventing preterm birth.

Key Words: Chorion, Matrix metalloproteinase 9, Preterm labor, Progesterone, Tumor necrosis factor-alpha

Introduction

Preterm birth is a major cause of perinatal morbidity and mortality. Worldwide, preterm birth was estimated to be 9.6% of all births in 2005.[1] Spontaneous preterm delivery, caused by preterm labor or preterm premature rupture of membranes (PPROM) accounts for approximately 75% of all preterm birth.[2] Although the mechanisms of preterm parturition have not been elucidated precisely, inflammation is thought to play a major role leading to preterm birth. Tumor necrosis factor alpha (TNF-α) is elevated in the amniotic fluid of women with preterm labor especially when associated with infection.[3] TNF-α increases matrix metalloproteinase (MMP)-9 and prostaglandin production in fetal membranes, leading to PPROM and/or preterm labor.[4] Specifically, Mackenzie et al.[5] have demonstrated a significant decrease in MMP activity when decidual cells were pretreated with progesterone followed by thrombin, a known mediator inflammation and proteases. Further, evidence for progesterone's protective effect was demonstrated in chorion cells exposed to TNF-α. Importantly, this effect was mediated by a novel progesterone receptor, progesterone re-

ceptor membrane component 1.[6]

Several randomized controlled trials have demonstrated a protective effect with either 17α-hydroxy-progesterone caproate (17P) or progesterone (P4) for prevention of preterm birth.[7,8] Therefore, we hypothesized that progesterone inhibits TNF-α induced MMP-9 activity in fetal membranes. We tested this hypothesis using an *in vitro* tissue culture model in which normal choriodecidual (CD) membranes were treated with TNF-α and MMP-9 activity analyzed.

Methods

1. Collection of fetal membranes

Fetal membranes were collected from women who underwent planned cesarean delivery at term, before labor and without rupture of membranes after written informed consent (n=10). This study protocol was approved by Institutional Review Board for Research Ethics at Yonsei University Wonju College of Medicine. Fetal membranes were cut from placenta and placed in sterile phosphate buffered saline containing antibiotic/antimycotics (penicillin G, 100 U/mL; streptomycin sulfate, 100 mg/mL; amphotericin B, 1.0 mg/mL) under sterile conditions. The membranes were transported to the laboratory and amnion was seperated from the CD. CD membranes were washed 3 times with Hank Balanced Salt Solution, and adherent blood clots were removed manually. A 1×1 cm full thickness CD tissue sample was placed in 12 well tissue culture plates. The CD membranes were cultured in Dulbecco Modified Eagle Medium (DMEM) with Ham F-12 nutrient mixture supplemented with 15% heat-inactivated fetal bovine serum, penicillin G 100 U/mL, streptomycin sulfate 100 mg/mL, amphotericin B 1.0 mg/mL. Cultures were carried out at 37℃ in an atmorphere containing 95% air/5% CO_2.

2. *In vitro* treatment conditions

After 24 hours of stabilization in DMEM/F-12, medium was changed to fresh Opti-MEM serum free media without antibiotics. CD membranes were pretreated with either progesterone (P4, 10-6 mol/L; Sigma, St Louis, MO, USA), 17α-hydroxy-progesterone caproate (17P, 10-6 mol/L; Tokyo Chemical Industry, Tokyo, Japan), promegestone (R5020, 10-6 mol/L;

Pelkin Elmer, Waltham, MA, USA), or an ethanol (vehicle) for 24 hours. The CD membranes were subsequently treated with 0.1 μg/mL TNF-α (R&D Systems, Minneapolis, MN, USA) with or without continued progesterone treatment for 48 hours. TNF-α treated control also included equivilant exposure to the progestin vehicle ethanol. The media was changed every 24 hours. CD membranes and conditioned media were harvested, snap frozen, and stored at -80℃ until analysis.

3. Protein extraction

Total protein from CD membrane tissues were isolated with the use of a homogenization buffer consisting of 25 mmol/L Tris-hydrochloric acid (pH 7.6), 150 mmol/L NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5 mmol/L ethylenediaminetetraacetic acid, and protease inhibitors (Pierce Biotechnology, Rockford, IL, USA). Samples were homogenized for 3 minutes using a tissue lyser II (Qiagen, Hilden, Germany) at 30 Hz. Homogenization was followed by centrifugation at 15,000 rpm for 10 minutes at 4℃ and harvested supernatants were stored at -80℃ prior to Western blotting. Protein concentrations were determined using a Qubit 2.0 Fluorometer (Life technologies, Carlsbad, CA, USA).
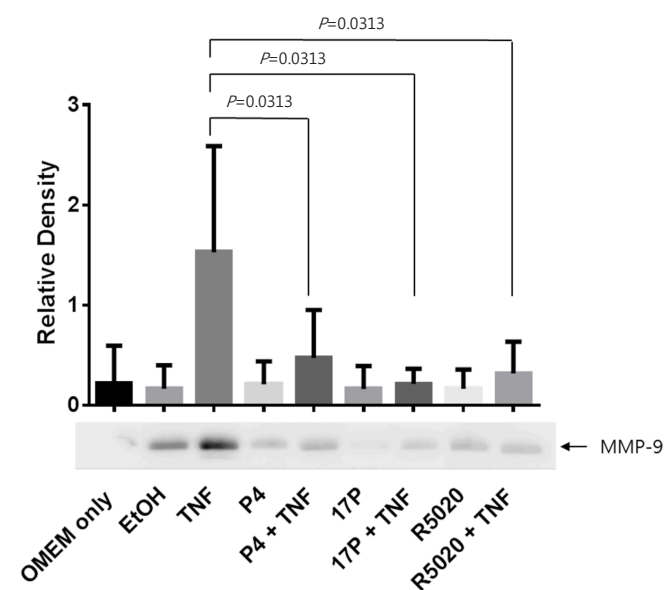
4. Western blotting

Protein samples (40 μg/lane) were combined with sample buffer, heated at 70℃ for 10 minutes, and separated on a 10% Bis-Tris gel at 200 V. The proteins were transferrred to polyvinyl difluoride membranes (Pierce Biotechnology) in a XCell II blot module (Invitrogen, Carlsbad, CA, USA) at a constant 30 V for 60 minutes. Membranes were blocked in 5% non-fat dry milk (Santa cruz biotechnology, Santa cruz, CA, USA), Tris-buffered saline, 0.3% Tween 20 for 60 minutes. Immunoblotting was performed with MMP-9 (GE-213) antibody (1:100 dilution, Santa cruz biotechnology) for 60 minutes. Membranes were washed three times in Tris-buffered saline with Tween 20 (TBST), and incubated with an anti-mouse horseradish peroxidase-linked secondary antibody (Santa cruz biotechnology) for 60 minutes, and then again washed three times in TBST. Proteins were detected using an supersignal west pico chemiluminescent substrate (Pierce Biotechnology). Beta actin monoclonal antibody (MA1-91399 AC-15; Pierce Biotechnology) was used as internal control in

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the Western blots.

5. Zymography

The activity of MMP-9 in CD membranes were assayed by zymography. Conditioned media from CD tissue culture was centrifuged, and the supernatant was mixed with nonreducing sample buffer (Life technologies), and then electrophoresed on 10% Tris-Glycine gel containing 0.1% gelatin at 125 V for 90 minutes. Gels were incubated with renaturing buffer (Life technologies) for 30 minutes at room temperature. After decanting the renaturing buffer, developing buffer (Life technologies) was added and the gel was equilibrated for 30 minutes at room temperature with gentle agitation. The gels were incubated at 37℃ overnight with fresh developing buffer. Gels were stained with Coomassie G-250 stain (Life technologies) for 1 hour then washed with distilled water. Gelatinase activity was detected as unstained bands representing MMP9 activity.
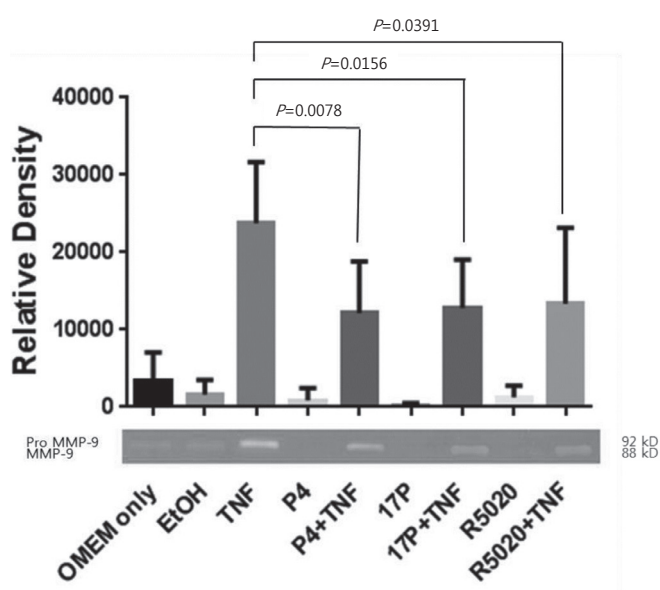
6. Quantitation and statistical analysis

Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Because data were not normally distributed, non-parametric Wilcoxon tests and Friedman tests were used to compare the differences between the groups using GraphPad prism version 6 software (GraphPad, La Jolla, CA, USA) with significance defined as $P<0.05$.

Results

A total of 10 fetal membrane samples were included in this analysis (zymography, n=8; Western blot, n=6). As expected, MMP-9 protein expression was significantly higher in tissue treated with TNF-α when compared with vehicle only, using Western blot analysis (P=0.03) (Fig. 1). A similar pattern of results was obtained by zymographic analysis (Fig. 2). The activity of MMP-9 was higher in TNF-α treated group than

Fig. 1. Effects of progesterone and TNF-α on MMP-9 protein expression in choriodecidual membranes. Choriodecidual membranes were isolated, cultured, and pretreated with P4, 17P, R5020, or an EtOH vehicle for 24 hours, and treated with TNF-α for 48 hours. Densitometric analysis of western blotting of MMP-9/β-actin (top). Representative Western blot (bottom). Shown are mean±standard deviation (n=6). MMP, matrix metalloproteinase; OMEM, Opti-Minimum Essential Media; EtOH, ethanol; TNF, tumor necrosis factor; P4, progesterone; 17P, 17α-hydroxyprogesterone caproate; R5020, promegestone.

Fig. 2. Effects of progesterone and TNF-α on MMP-9 activity in choriodecidual membranes. Choriodecidual membranes were isolated, cultured, and pretreated with P4, 17P, R5020, or an EtOH as a vehicle for 24 hours, and treated with TNF-α for 48 hours. Densitometric analysis of zymography of MMP-9 (top). Representative zymography (bottom). Shown are mean±standard deviation (n=8). MMP, matrix metalloproteinase; OMEM, Opti-Minimum Essential Media; EtOH, ethanol; TNF, tumor necrosis factor; P4, progesterone; 17P, 17α-hydroxyprogesterone caproate; R5020, promegestone.

that of vehicle alone ($P<0.01$).

In this tissue culture study, progesterone did not decrease basal MMP-9 activity. There were no differences in MMP-9 protein expression among P4, 17P or R5020 treated tissue and vehicle only samples ($P=0.56$, $P=0.68$, and $P=0.84$, respectively). Zymography also did not identify any differences in MMP-9 activity in media among progesterone pre-treated and vehicle treated samples ($P=0.64$, $P=0.05$, and $P>0.99$, respectively).

Zymography showed that pre-treatment with P4, 17P, and R5020 significantly diminished TNF-α induced MMP-9 activity when compared to TNF-α alone (51%, 54%, and 56%, respectively) (Fig. 1). In Western blot analysis (n=6), TNF-α induced MMP-9 expression was decreased by P4, 17P, and R5020 pretreatment when compared with the stimulated control (31%, 14%, and 21%, respectively). There were no differences in MMP-9 activity among the P4, 17P, and R5020 pretreated group in Western blot ($P=0.25$), and zymography analysis ($P=0.36$).

Discussion

Progesterone and 17P are currently being used in the clinical setting for the prevention of preterm birth in high risk groups.[7,8] The exact molecular mechanism of progesterones effect has not been clearly described. The current study demonstrated that progestins reduce TNF-α mediated MMP-9 activity and expression in human choriodecidual membranes culture.

Infection and/or inflammation is now widely accepted to play a pivotal role in preterm parturition.[9-11] Intrauterine infection induces secretion of proinflammatory cytokines such as TNF-α.[12] In an LPS-induced murine model, the pre-treatment of anti-TNF-α decreases preterm delivery.[13] TNF-α signaling, mediated through TNF receptor (TNFR)1, forms the TNF-TNFR1 complex and leads to activation of nuclear factor-κB (NF-κB). NF-κB promotes induction of MMP-9 expression and activity, ultimately leading to inflammation and potential weakening of the fetal membranes. The TNF-TNFR2 complex directly interacts with TNF receptor associated factor-2, which results in prostaglandin production and promotes uterine contractions.[4]

Increased activity of MMPs has been documented in fetal membranes from women with PPROM.[14] Fetal membranes are composed of several layers and each layer has a distinct extracellular matrix (ECM) composition.[15] MMPs hydrolyze ECM, resulting in cervical ripening and rupture of the fetal membranes.[16] The fibrillar collagens have different susceptibility to cleavage by MMPs.[17] MMP-9 degrade type IV and V collagen as well as basement membrane components, which maybe important for the maintenance of the amnion epithelial cells. MMP-9 expression has been found to be increased in term rupture as well as PPROM[17-20] and is associated with fetal membrane weakening *in vitro*.[19]

Fetal membranes at the rupture site have been shown to have disruption in connective tissue and thinning of the chorion layer and increased MMP-9 activity including the zone overlying the cervix.[17] In this study we intentionally sampled membranes distant from the edge to avoid the weak zones.

Several putative mechanisms by which progesterone is able to prevent preterm birth have been proposed, including anti-inflammatory action, interfering with cortisol-mediated regulation of placental gene expression, and inhibiting oxytocin binding.[21] Progesterone has also been shown to inhibit apoptosis and secretion of proinflammatory modulators induced by TNF-α in fetal membranes.[22-24] It has also been proposed that binding TNF-α to TNF-R1 in the fetal membranes induces both the apoptotic process and MMP-9 expression.[25] In this study we showed the ability of progesterones to inhibit TNF-α induced MMP-9 expression and this could prevent the associated ECM that lead to membrane weakening.

In summary, the current study shows that progesteone inhibits TNF-α mediated MMP-9 activity in human choriodecidual membranes. These findings may provide further support for the role of progesterone in preventing preterm birth.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Acknowledgments

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