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The Effect of Progestins on Tumor Necrosis Factor α -Induced Matrix Metalloproteinase 9 Activity and Gene Expression in Human Primary Amnion and Chorion Cells *In Vitro*

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Abstract

Background—Current treatment modalities for preventing preterm premature rupture of membranes (PPROM) are limited, but progestins may play a role. Tumor necrosis factor α (TNF α) enhances matrix metalloproteinase 9 (*MMP-9*) gene expression and activity in fetal membranes, contributing to membrane weakening and rupture. We previously demonstrated that progestins attenuate TNF α -induced *MMP-9* activity in a cytotrophoblast cell line. However, whether they have a similar effect in primary amnion and chorion cells of fetal membranes is unknown. In this study we evaluated the effect of progestins on basal and TNF α -induced *MMP-9* activity and gene expression in primary chorion and amnion cells harvested from the fetal membranes of term non-laboring patients.

Methods—Primary amnion and chorion cells were isolated from fetal membranes obtained from term uncomplicated non-laboring patients following elective cesarean delivery (n=11). Confluent primary amnion and chorion cell cultures were both pretreated with vehicle (control), progesterone (P4), 17 α -hydroxyprogesterone caproate (17P) or medroxyprogesterone acetate (MPA) at 10⁻⁶ M concentration for 6 h followed by stimulation with TNF α at 10 ng/mL for an additional 24 h. Cell cultures pretreated with the vehicle only served as the unstimulated control and the vehicle stimulated with TNF α served as the stimulated control. Both controls were assigned a value of 100 units. Cell culture medium was harvested for *MMP-9* enzymatic activity quantification using gelatin zymography. Total RNA was extracted for quantifying *MMP-9* gene expression using RT-qPCR. Basal *MMP-9* activity and gene expression data were normalized to the unstimulated control. TNF α -stimulated *MMP-9* activity and gene expression were normalized to the stimulated control. The primary outcome was the effect of progestins on TNF α -induced *MMP-9* enzymatic activity in term human primary amnion and chorion cells in vitro. Secondary outcomes included the effect of progestin therapy on TNF α -induced *MMP-9* gene expression and on basal *MMP-9* activity and gene expression in primary amnion and chorion cells in vitro.

Results—Primary cells were harvested from 11 patients. Compared to the unstimulated control, TNF α increased *MMP-9* activity (p=0.005 vs control in primary amnion cells and p<0.001 vs control in primary chorion cells) and *MMP-9* gene expression (p=0.030 vs. control in primary amnion cells, p<0.001 vs. control in primary chorion cells). Compared with the unstimulated controls MPA, but not P4 or 17P, reduced basal *MMP-9* activity [mean difference (95% CI) -49.6 (-81.9, -17.3) units, p=0.001] and gene expression [mean difference (95% CI) -53.4 (-105.9, -0.9) units, p=0.045] in primary amnion cells. Compared with the stimulated control, MPA also reduced TNF α -induced *MMP-9* activity [mean difference (95% CI) -69.0 (-91.8, -46.3) units, p<0.001] and gene expression [mean difference (95% CI) -86.0 (-120.7, -51.3) units, p<0.001] in primary amnion cells. Progestin pretreatment had no significant effect on basal or TNF α -induced *MMP-9* activity and gene expression in primary chorion cells.

Conclusion—The inhibitory effect of MPA on both basal and TNF α -induced *MMP-9* activity and gene expression in primary amnion cells demonstrate a possible mechanism by which progestins may prevent fetal membrane weakening leading to PPRM.

Introduction

The rate of preterm delivery in the United States is 11.4%.¹, which is the highest preterm delivery rate of all industrialized nations. Up to 30% of preterm deliveries result from preterm premature rupture of membranes (PPROM)--rupture of membranes prior to the onset of labor and before 37 weeks of gestation.² Infants born as a result of PPRM are at a greater risk of developing complications related to prematurity compared with those resulting from spontaneous preterm delivery from other etiologies.^{3,4} The resulting emotional impact on parents and the significant healthcare costs incurred make PPRM a major public health problem.⁴

Mechanisms leading to PPRM are distinctly different from those that result in other causes of preterm delivery.⁵⁻⁷ Inflammation from localized or systemic infections, uterine over-distension or increased genetic susceptibility, stimulate the release of inflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin -1 β (IL-1 β), which, in turn, stimulate a localized inflammatory response in fetal membranes.^{2,6-11} Inflammatory cytokines stimulate the release of matrix metalloproteinases (MMPs) from the amnion and chorion layer of fetal membranes. Matrix metalloproteinases, specifically matrix metalloproteinase-9 (MMP-9), degrade the extracellular matrix proteins, such as types I, III and IV collagen, fibronectin and laminin.¹² These matrix proteins provide the tensile strength for fetal membranes, particularly in the amnion layer. They also form the basement membrane for cell adhesion. Increased MMP-9 expression and activity leads to matrix protein degradation, resulting in cell detachment from the basement membrane, apoptotic cell death and a reduction in tissue tensile strength.¹² The clinical importance of MMP-9 in the pathophysiology of PPRM is highlighted by the following: 1) Increased MMP-9 protein levels in amniotic fluid and enhanced MMP-9 gene expression in fetal membranes have been associated with PPRM compared with other causes of preterm delivery, 2) A polymorphism in the promoter region for the *MMP-9* gene in human amnion epithelial cells is associated with an increased risk of PPRM, and 3) TNF α -induced MMP-9 expression results in a significant decrease in the force and work needed to rupture human term fetal membranes in in-vitro studies.^{6,12,13}

Clinically, progestin therapy has been used for the prevention of recurrent preterm birth, but its role in the prevention of PPRM remains unclear.^{14,15} Progestins such as medroxyprogesterone acetate (MPA) have been shown to inhibit cytokine-induced MMP-1 and -3 expression and activity in term decidua cells in vitro.¹⁶ Progestins also inhibit basal and TNF α induced apoptosis in fetal membranes.¹⁷ We have also demonstrated that pre-treatment with MPA inhibits TNF α -induced MMP-9 activity in a human cytotrophoblast cell line, highlighting a possible mechanism by which progestins may prevent PPRM.¹⁸ However, the effect of progestins on cytokine-induced MMP-9 activity and gene expression in human primary chorion and amnion cells has not been described. The underlying hypothesis for this study is that progestins attenuate TNF α -induced MMP-9 activity and gene expression in human primary amnion and chorion cells. This could indicate a possible mechanism by which progestins may prevent inflammation-induced fetal membrane rupture. The primary outcome of this study was to evaluate the effect of progestin pretreatment on TNF α -induced MMP-9 enzymatic activity in term human primary amnion and chorion cells

in vitro. Secondary outcomes included the effect of progestin pretreatment on TNF α -induced *MMP-9* gene expression, basal *MMP-9* enzymatic activity and basal *MMP-9* gene expression.

Methods

Fetal membrane collection and primary cell harvesting

This study was approved by the Duke Medicine Institutional Review Board with a waiver of consent for fetal membrane collection. Full thickness fetal membrane samples were collected from term healthy patients at elective cesarean delivery without prior rupture of membranes or labor. To anonymize sample collection, both the identification of suitable patients and the collection of fetal membranes were performed by authorized research personnel who had no further involvement in subsequent experiments. Samples were collected when these research personnel were available between September 2013 and July 2014. Immediately following delivery of the placenta, pieces of fetal membranes (approximately 10 $\times$ 10 cm) were cut from the placenta and transported in Dulbecco Modified Eagle Media – Ham's F12 (DIMEM/F12) (Gibco®, Life Technologies, Carlsbad, CA) culture media with 10% fetal bovine serum (FBS) and antibiotics and antimycotics (streptomycin and penicillin and amphotericin B). Fetal membranes were washed in culture media to remove blood and debris and then cut into 5 $\times$ 5 cm squares. The amnion layer was peeled off from the choriondecidua with forceps, and the chorion then bluntly dissected from the decidua with a scalpel blade. Using a previously described protocol, primary amnion and chorion epithelial cells were harvested.¹⁹ Briefly, both the amnion and chorion were diced using two scalpel blades and then digested in 0.125% trypsin with 0.2% collagenase for 60 – 90 minutes at 37°C with intermittent shaking to promote tissue digestion. The cells were then filtered through 4 layers of sterile gauze and centrifuged at 2000 rpm for 5 minutes. The resulting pellet was re-suspended in serum-free media and layered on a cell separation gradient prepared with an Optiprep™ Density Gradient Medium (Sigma-Aldrich, St. Louis, MO) column (4%:6%:8%:10%:20%:30%:40%) and centrifuged at 2000 rpm for 30 minutes. Harvested cells were washed in DIMEM/F12 media with 10% FBS and antibiotics and antimycotics, cultured in 12-well cell culture plates and grown to 80-90% confluence in 21% Oxygen, 5% CO₂ at 37°C. Immunocytochemistry staining was performed using cytokeratin and vimentin to determine the purity of the cell population cultured.²⁰

Experimental conditions

The experimental conditions are highlighted in Figure 1. For each fetal membrane sample collected, unpassaged cultured primary amnion and chorion cells were switched to serum-free media for 24 h. Using our previously described methodology, the two cell culture wells were each pretreated with ethanol 0.01% as vehicle (Decon Labs, King of Prussia, PA), progesterone (P4) (Sigma-Aldrich, St. Louis, MO), medroxyprogesterone 17-acetate (MPA) (Sigma-Aldrich), or 17 α -hydroxyprogesterone caproate (17P) (Steraloids, Newport, RI) at 10⁻⁶ M for 6 h in serum containing media.¹⁸ The concentrations of progestins used were based on placental levels measured in pregnancy.²¹ Prior experiments performed in our lab and previously published data have established that concentrations of progestins in the micromolar range have therapeutic effects in vitro.^{17,18,22,23} The cell cultures were switched

to serum-free media supplemented with the vehicle and progestins. One cell culture well for each treatment group was incubated with TNF α 10 ng/mL for an additional 24 h in serum-free media.¹⁸ The vehicle without TNF α served as the unstimulated control for basal MMP-9 activity and gene expression. The vehicle control with TNF α served as the stimulated control for the progestin pretreated groups, stimulated with TNF α . Following 24 h of TNF α stimulation, cell culture medium for MMP-9 activity quantification by gelatin zymography was harvested, centrifuged at 15000 rpm for 5 min and the supernatant was immediately frozen at -80°C. Cell lysates were harvested with TRIzol® reagent (Life Technologies, Carlsbad, CA) and frozen at -80°C for RNA extraction and real-time quantitative PCR (RT-qPCR).

Gelatin zymography

Gelatin zymography was used to quantify MMP-9 enzymatic activity in-vitro using the manufacturer's protocol.¹⁸ Briefly, harvested cell culture media was incubated in a 1:1 ratio with a Novex® (Life Technologies) tris-glycine sample buffer sodium dodecyl sulfate (SDS) for 10 min at room temperature. Samples were loaded onto a 10% Novex® gelatin zymogram gel and electrophoresed for 90 min. The gels were run in duplicates and were incubated in Novex® renaturing buffer for 30 min, followed by Novex® developing buffer for a further 30 min to allow the enzymes to renature. After incubation with fresh developing buffer for a further 16-18 h, the gels were washed with deionized water and stained with Novex® Simplyblue Safestain for 1 h. The gel was de-stained by washing with deionized water for 2 h at room temperature and MMP-9 activity was quantified by analyzing band densities at 88 and 92 kDa using Image J® densitometry software (NIH, Bethesda, MD). The duplicate densitometry readings were averaged for each treatment group.

Real-time quantitative reverse transcription (RT)-qPCR

Total RNA was extracted from TRIzol lysates using the RNeasy® minikit (Qiagen, Valencia, CA). RNA concentration was measured using the Nanodrop® spectrophotometer (Thermo Scientific, Wilmington, DE) and 1 μ g RNA was reverse transcribed into complementary DNA (cDNA) using the Superscript III® first strand synthesis system (Life Technologies). cDNA (25 – 50 ng) was used for RT-qPCR with prevalidated Taqman® gene expression probes (Life Technologies) targeted against MMP-9 (assay ID: Hs00234579_m1) in both amnion and chorion samples. The housekeeping gene, glyceraldehyde -3- phosphate dehydrogenase (*GAPDH*) (assay ID:Hs03929097_g1), was used in amnion samples (Life Technologies). For chorion samples, β -actin was used as the housekeeping gene with the SYBR® green detection method (Bio-Rad, Hercules, CA) and primer pairs for β -actin (RealTimePrimers.com accession ID: NM_001101.2). Real-Time qPCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad). Samples were run in duplicates and the mean cycle thresholds (C_T) were normalized to average GAPDH C_T values for amnion samples and β -actin for chorion samples, respectively. A control sample without reverse transcriptase was included with each RT-qPCR run.

Data Analysis

Our sample size was determined by the availability of eligible patients during the enrollment period of the study. Relative MMP-9 gene expression was quantified using the comparative C_T method after normalization to the relevant housekeeping genes for primary chorion and amnion cell samples.^{24,25} To demonstrate that TNF α induced MMP-9 activity and gene expression the stimulated control was normalized to the unstimulated and both were compared using the paired t-test for both amnion and chorion primary cell cultures. Due to the variability in MMP-9 activity and gene expression among individual patient samples, both MMP-9 activity and gene expression for the progestin treated groups were also normalized to the unstimulated control for the basal data for each sample. For TNF α -induced MMP-9 activity and gene expression data, progestin pretreated groups stimulated with TNF α were normalized to the stimulated control. In both cases the stimulated and unstimulated controls were assigned a value of 100 units. For basal MMP-9 activity and gene expression, the progestin treated groups were compared with the unstimulated control. For TNF α induced MMP-9 activity and gene expression the stimulated control was compared with the groups pretreated with progestins and stimulated with TNF α . Both comparisons were analyzed using two-way ANOVA to compare progestin treatments within cell types with post hoc Dunnett's test for multiple comparisons. Post hoc comparisons were limited to those between the unstimulated control and progestin treated groups for basal data and the stimulated control and progestin pretreated groups stimulated with TNF α for TNF α -induced MMP-9 activity and gene expression. To determine if the distribution of the residuals approximated a normal distribution, quantile-quantile (Q-Q) plots generated for each two-way ANOVA model were assessed by visual inspection. A family-wise $P < 0.05$ adjusted for multiple comparisons was considered significant. Data was summarized as mean $\pm$ SEM, mean differences and 95% confidence intervals, and was analyzed using GraphPad prism (version 6.0 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com) and SAS Enterprise Guide (Version 5.1, SAS Institute Inc, Cary, NC, USA).

Results

Fetal membranes were harvested from 11 subjects. Primary amnion cells were harvested from the fetal membranes of 11 subjects and primary chorion cells were harvested from 10 subjects. In 1 patient we were unable to quantify MMP-9 gene expression in the primary amnion samples. In 2 patients, the basal MMP-9 activity in the primary amnion cells was too low to allow adequate quantification by zymography. These data were excluded from the subsequent analysis. Immunocytochemistry staining revealed that our harvested primary cells were >90% epithelial cells in both cultures.

The effect of TNF α on MMP-9 activity and gene expression

Compared with the unstimulated control TNF α increased MMP-9 activity and *MMP-9* gene expression in both primary amnion (Figure 2) and chorion cells (Figure 3). The predominant band that was observed by zymography was the 92 kD pro MMP-9 band (Figure 2A and 3A, top panel).

The effect of progestins on basal MMP-9 activity and gene expression

Pretreatment with MPA significantly reduced basal MMP-9 activity in primary amnion cells compared with the unstimulated control [mean difference (95% CI)= -49.6 (-81.9, -17.3) units, $p=0.001$] (Figure 4A, Table 1A). Pretreatment with MPA also significantly reduced basal *MMP-9* gene expression in primary amnion cells compared with the unstimulated control [mean difference (95% CI)= -53.4 (-105.9, -0.9) units, $p=0.045$] (Figure 4B, Table 1B). Pretreatment with P4 and 17P did not significantly reduce basal MMP-9 activity and gene expression when compared with the unstimulated control in primary amnion cells (Figure 4 A&B, Table 1A&B).

In chorion cells, no significant differences in basal MMP-9 activity between the unstimulated control and the progestin treated groups were observed (Figure 4A, Table 1A). Similarly no differences in basal *MMP-9* gene expression were observed between the unstimulated control and the progestin treated groups (Figure 4B, Table 1B).

The effect of progestins on TNF α -induced MMP-9 activity and gene expression

Pretreatment with MPA significantly reduced TNF α -induced MMP-9 activity in primary amnion cells compared with the stimulated control [mean difference (95% CI)= -69.0 (-91.8, -46.3) units, $p<0.001$] (Figure 5A, Table 2A). Pretreatment with MPA also significantly reduced TNF α -induced *MMP-9* gene expression in primary amnion cells compared with the stimulated control [mean difference (95% CI)= -86.0 (-120.7, -51.3) units, $p<0.001$] (Figure 5B, Table 2B). Pretreatment with P4 and 17P did not significantly reduce TNF α -induced MMP-9 activity and gene expression compared with the stimulated control in primary amnion cells (Figure 5 A&B, Table 2 A&B).

No significant differences in TNF α -induced MMP-9 activity were observed between the progestin treated groups stimulated with TNF α and the stimulated control in primary chorion cells (Figure 5A, Table 2A). Similarly no significant differences in TNF α -induced *MMP-9* gene expression were observed between the progestin treated groups stimulated with TNF α and the stimulated control in primary chorion cells (Figure 5B, Table 2B).

Discussion

Our results demonstrate that MPA pretreatment significantly inhibited both basal and TNF α -induced MMP-9 activity and gene expression in primary amnion cells. Interestingly, P4 and 17P did not inhibit basal and TNF α -induced MMP-9 activity and gene expression in primary amnion cell cultures. Progestin pretreatment also did not significantly inhibit basal and TNF α -induced MMP 9 activity and gene expression in primary chorion cells.

Our findings suggest that the amnion layer maybe a site of action for progestin therapy in preventing inflammation-induced fetal membrane tissue remodeling. The amnion layer is responsible for most of the tensile strength of fetal membranes and ultimately has to rupture for PPRM to occur.²⁶ In-vitro studies have demonstrated that the sequence of events that leads to fetal membrane rupture includes fetal membrane distension, separation of the amnion and choriodecidua, choriodecidual rupture, further nonelastic amnion distension followed ultimately by rupture of the amnion.²⁶ MMP-9 significantly contributes to the

initiation of membrane rupture by degrading collagen fibers in the spongy layer of the amnion, leading to dissociation of the amnion and chorion.²⁷ This is in addition to degrading collagen and other extracellular matrix proteins in the compact and fibroblast layer along with the basement membrane of the amnion, which also contributes to the reduction in tensile strength. A reduction in fetal membrane tensile strength is directly correlated with MMP-9 protein expression in fetal membranes in term laboring patients.²⁸ TNF α -induced MMP-9 protein expression and the resulting collagen remodeling have also been correlated with fetal membrane weakness in-vitro.¹³ Additionally, inhibition of TNF α -induced fetal membrane weakening by alpha-lipoic acid, has been associated with a concomitant reduction in TNF α -induced MMP-9 expression in full thickness fetal membranes and primary amnion epithelial cells.²⁹ We hypothesize, based on the profound suppression by MPA of MMP-9 activity and gene expression in primary unstimulated and TNF α stimulated amnion cells, that this is a mechanism by which progestins may act to maintain fetal membrane integrity in the setting of inflammation and other idiopathic causes of PPRM. However, tissue biomechanical studies are now needed to confirm this hypothesis.

Our findings suggest that the reductions in basal and TNF α -induced MMP-9 activity in primary amnion cells by MPA are mainly a result of effects on gene expression. In most tissues, *MMP-9* gene expression is low but can be induced by inflammatory cytokines. Transcriptional activation of *MMP-9* gene expression by inflammatory cytokines occurs primarily through the transcription factors nuclear factor κ B (NF- κ B) and activator protein 1 (AP-1) pathways in fetal membranes.^{30,31} Additionally, there are transcription factors that negatively regulate MMP-9 gene expression, such as kisspetin (KISS-1) and metastasis associated gene 1 (MTA-1).³²⁻³⁴ These repressors inhibit MMP-9 gene expression either by inhibiting NF- κ B activation or by forming a repressor complex that binds to the MMP-9 promoter site and maintaining it in a silenced state.^{32,33} Inhibition of positive regulators, or induction of negative regulators of MMP-9 gene expression, maybe mechanisms by which MPA inhibits both basal and TNF α -induced MMP-9 mRNA expression in primary amnion cells.

The receptors by which MPA initiates these effects in the amnion are unclear. Amnion epithelial cells do not express the PR-A and PR-B subtypes of the progesterone receptor, which is thought to mediate many of the biological effects of progestins.³⁵ Our previous work has demonstrated the presence of a novel progesterone receptor, which may play a role in mediating the effects of progestins in fetal membranes.^{18,36} This receptor, progesterone membrane component 1 (PGRMC1), is expressed in all layers of the fetal membranes and in the HTR8/SVneo cytotrophoblast cell line. In the HTR8/SVneo cell line we demonstrated that PGRMC1 mediate the inhibitory effect of MPA on TNF α -induced MMP-9 activity.¹⁸ Recently the glucocorticoid receptor has also been implicated as possible receptor mediating the suppression of IL-1 β induced increases in COX-2 expression by MPA and P4 in human myometrial cells.³⁷ Both receptor pathways are being investigated in our laboratory.

Progestin therapy did not attenuate basal or TNF α -induced MMP-9 mRNA expression or activity in primary chorion cells. Although the chorion layer is thicker than the amnion, it does not possess its tensile strength. The role of the chorion in inflammation-induced membrane rupture is still unclear, but it does appear to contribute indirectly to the

inflammatory process through its production of soluble factors that may weaken the amnion.³⁸ The inflammatory response of the chorion layer and its response to progestin therapy may also be modulated by both the decidua and amnion layer through paracrine effects. This may lead to different responses in cell cultures experiments when compared with tissue culture and in vivo treatment conditions.²² We were also unable to demonstrate a response to P4 or 17P on basal or TNF α induced MMP-9 activity and expression in primary amnion cells. Genetic variations in receptor types and the metabolic enzymes involved in progestin mediated mechanisms and metabolic pathways may partially explain these results.^{39,40} For example, we know that the clinical response to 17P therapy is quite variable; up to two thirds of patients have recurrent preterm births while on treatment.^{39,40} A lack of response to P4 may also be related to the concentrations of P4 used in our experiments. Myometrial studies have established that higher concentrations of progesterone (10^{-3} to 10^{-5} M) suppress both spontaneous myometrial contractility and cytokine- induced cyclooxygenase 2 expression.^{37,41} However non-specific steroid effects due to changes in cell membrane fluidity can occur with doses of progesterone in excess of micromolar concentrations.^{37,41} Another possibility for the lack of response in primary amnion cells is that both P4 and 17P may exert their effects on mechanisms that prevent PPROM independent of MMP-9 activity or gene expression in fetal membranes.

One limitation of our study is that experiments were performed using cells isolated from the fetal membranes of term pregnant patients, and not preterm or PPROM patients. We opted not to use fetal membranes from PPROM patients due to their increased bacterial colonization, and because the fetal membranes of these patients have already undergone the pathological changes, such as MMP activation and apoptosis, that result in membrane rupture.^{42,43} There is also limited data on the biomechanical properties of preterm fetal membranes. But preterm fetal membranes may have greater tensile strength and less regional variation in biomechanical and histological properties than term fetal membranes harvested from patients who did not labor.^{44,45} Whether primary amnion and chorion cells isolated from preterm fetal membranes would respond to progestin treatment and cytokine stimulation in a similar way to those harvested from term fetal membranes is debatable. Another limitation is that MPA was the only progestin which attenuated both basal and TNF α -induced MMP-9 activity and gene expression in primary amnion cells. MPA bears a category X classification from the Food and Drug Administration and is contraindicated in pregnancy.^a Additionally, it is not in clinical use for the prevention of preterm delivery. However, MPA is commonly used in in-vitro cell and tissue culture experiments due to its increased stability when compared with other progestins.⁴⁶ Fetal membranes also express enzymes that metabolize progesterone, and although systematic studies comparing the metabolism of natural and synthetic progestins by the amniochorion have not as yet been conducted, it is possible that P4 may be more effectively metabolized when compared with MPA or 17P in vitro.⁴⁷

In summary, we demonstrated that MPA inhibits both basal and TNF α -induced MMP-9 activity and gene transcription in primary amnion cells harvested from term pregnant

^aDrugs@FDA. Available at http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/011839s0711b1.pdf accessed January 15, 2015.

women. Our data highlight one of the mechanisms by which progestins may prevent inflammation-induced fetal membrane weakening which may lead to PPRM. The cell signaling pathways by which MPA decreases MMP-9 activity and gene expression require further investigation in light of the absence of the classic nuclear progesterone receptor in primary amnion cells.

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Figure 1.

Experimental Design. Fetal membranes were harvested from healthy term pregnant patients who did not labor. Primary amnion and chorion cells were isolated from these fetal membrane samples and both cell types were used for subsequent experiments. Cell cultures pretreated with vehicle and progestins without tumor necrosis factor α (TNF α) were used for basal matrix metalloproteinase 9 (MMP-9) activity and gene expression. Cell cultures pretreated with vehicle and progestins followed by TNF α stimulation were used for TNF α induced MMP-9 activity and gene expression. Progesterone (P4), 17 α hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA), RT-qPCR – real-time quantitative polymerase chain reaction.

Primary Amnion Cells

Figure 2.

Tumor necrosis factor α (TNF α)-induced matrix metalloproteinase 9 (MMP-9) activity and gene expression in primary amnion cells. Stimulation with TNF α 10 ng/mL increased MMP-9 enzymatic activity ($P=0.005$ vs unstimulated control, $n=9$ patients) (A) and gene expression ($p=0.030$ vs unstimulated control, $n=10$ patients) in primary amnion cells (B). The top panel in figure A shows a representative gelatin zymography gel. The unstimulated control (vehicle only) was used as the reference group and compared with the stimulated control (TNF α only group) using a paired t-test. Data are mean with the error bars representing the standard error of the mean (SEM).

Primary Chorion Cells

Figure 3.

Tumor necrosis factor α (TNF α)-induced matrix metalloproteinase 9 (MMP-9) activity and gene expression in primary chorion cells. Stimulation with TNF α 10 ng/mL increased MMP-9 enzymatic activity ($P < 0.001$ vs unstimulated control, $n = 10$ patients) (A) and gene expression ($p < 0.001$ vs unstimulated control, $n = 10$ patients) in primary chorion cells (B). The top panel in figure A shows a representative gelatin zymography gel. The unstimulated control (vehicle only) was used as the reference group and compared with the stimulated control (TNF α only group) using a paired t-test. Data are mean with the error bars representing the standard error of the mean (SEM).

Figure 4.

The effect of progestins on basal matrix metalloproteinase 9 (MMP-9) activity and gene expression. Compared with the unstimulated control MPA inhibited basal MMP-9 activity in primary amnion cells ($P=0.001$, $n=9$ patients) (A). Compared with the unstimulated control MPA also reduced basal *MMP-9* gene expression in primary amnion cells ($P=0.045$, $n=10$ patients) (B). No significant differences in basal MMP-9 activity or gene expression were observed between the unstimulated control and the other progestin treated groups in primary amnion cells. No significant differences in basal MMP-9 activity and gene expression were observed between the unstimulated control and the progestin treated groups in primary chorion cells ($n=10$ patients). The groups were compared using two-way ANOVA. The top panel in figure A shows a representative gelatin zymography. Data are mean with the error bars representing the standard error of the mean (SEM). Progesterone (P4), 17 α hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA).

Figure 5.

The effect of progestins on tumor necrosis factor α (TNF α)-induced matrix metalloproteinase 9 (MMP-9) activity and gene expression. Compared with the stimulated control (TNF α) group, MPA inhibited TNF α induced MMP-9 enzymatic activity ($P < 0.001$, $n = 11$ patients) in primary amnion cells. Compared with the stimulated control MPA also reduced basal *MMP-9* gene expression in primary amnion cells ($P < 0.001$, $n = 10$ patients). No significant differences in TNF α -induced MMP-9 activity and gene expression were observed between the other progestin pre-treated groups plus TNF α and the stimulated control in primary amnion cells. No significant differences in TNF α -induced MMP-9 activity and gene expression were observed between the progestin treated groups plus TNF α and the stimulated control in primary chorion cells ($n = 10$ patients). The groups were compared using two-way ANOVA. Data are mean with the error bars representing the standard error of the mean (SEM). Progesterone (P4), 17 α hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA).

Table 1A

Basal MMP-9 enzymatic activity.

Comparison (N)	Mean Difference (normalized units)	95% CI (normalized units)	P value
Amnion			
P4 vs unstimulated control (9)	-1.3	-33.6 to 31.0	0.999
17P vs unstimulated control (9)	-6.8	-39.1 to 25.5	0.92
MPA vs unstimulated control (9)	-49.6	-81.9 to -17.3	0.001
Chorion			
P4 vs unstimulated control (10)	8.4	-22.2 to 39.1	0.85
17P vs unstimulated control (10)	-7.1	-37.8 to 23.5	0.90
MPA vs unstimulated control (10)	-19.3	-49.9 to 11.3	0.31

MMP-9 enzymatic activity for the progestin treated groups was normalized to the unstimulated control (vehicle only), with the unstimulated control assigned a value of 100 units. The progestin treated groups were compared with unstimulated control using two-way ANOVA. The distribution of the residuals for the model approximated a normal distribution. Matrix metalloproteinase 9 (MMP-9); Progesterone (P4), 17 α -hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA)

Table 1BBasal *MMP-9* gene.

Comparison (N)	Mean Difference (normalized units)	95% CI (normalized units)	P value
Amnion			
P4 vs unstimulated control (10)	-6.1	-58.6 to 46.3	0.99
17P vs unstimulated control (10)	12.7	-39.8 to 65.1	0.90
MPA vs unstimulated control (10)	-53.4	-105.9 to -0.9	0.045
Chorion			
P4 vs unstimulated control (10)	-4.9	-57.3 to 47.6	0.99
17P vs unstimulated control (10)	8.0	-44.4 to 60.5	0.97
MPA vs unstimulated control (10)	-11.7	-64.14 to 40.8	0.91

Gene expression for the progestin treated groups was normalized to the unstimulated control (vehicle only), with the unstimulated control assigned a value of 100 units. The progestin treated groups were compared with the unstimulated control using two-way ANOVA. The distribution of the residuals for the model approximated a normal distribution. Progesterone (P4), 17 α -hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA), confidence intervals (CI), number of patients (N), Matrix metalloproteinase 9 (MMP-9).

Table 2A

TNF α - induced MMP-9 enzymatic activity.

Comparison (N)	Mean Difference (normalized units)	95% CI (normalized units)	P value
Amnion			
P4 TNF α vs stimulated control (11)	8.8	-13.9 to 31.5	0.68
17P TNF α vs stimulated control (11)	-3.4	-26.2 to 19.3	0.97
MPA TNF α vs stimulated control (11)	-69.0	-91.8 to -46.3	< 0.001
Chorion			
P4 TNF α vs stimulated control (10)	-5.4	-29.2 to 18.5	0.91
17P TNF α vs stimulated control (10)	-4.8	-28.6 to 19.0	0.93
MPA TNF α vs stimulated control (10)	-21.0	-44.8 to 2.9	0.10

MMP-9 enzymatic activity for the progestin plus TNF α treated groups was normalized to the stimulated control (TNF α only group), with the stimulated control assigned a value of 100 units. The progestin plus TNF α treated groups were compared with the stimulated control using two-way ANOVA. The distribution of the residuals for the model approximated a normal distribution. Matrix metalloproteinase 9 (MMP-9); Progesterone (P4), 17 α -hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA), Tumor necrosis factor α (TNF α).

Table 2B

TNF α - induced *MMP-9* gene expression.

Comparison (N)	Mean Difference (normalized units)	95% CI (normalized units)	P value
Amnion			
P4 TNF α vs stimulated control (10)	2.7	-31.9 to 37.4	0.995
17P TNF α vs stimulated control (10)	-0.2	-34.8 to 34.5	> 0.999
MPA TNF α vs stimulated control (10)	-86.0	-120.7 to -51.3	< 0.001
Chorion			
P4 TNF α vs stimulated control (10)	1.5	-33.2 to 36.2	0.999
17P TNF α vs stimulated control (10)	1.7	-17.7 to 51.6	0.51
MPA TNF α vs stimulated control (10)	-9.9	-44.6 to 24.8	0.83

Gene expression for the progestin plus TNF α treated groups was normalized to the stimulated control (TNF α only group), with the stimulated control assigned a value of 100 units. The progestin plus TNF α treated groups were compared with the stimulated control using two-way ANOVA. The distribution of the residuals for the model approximated a normal distribution. Progesterone (P4), 17 α -hydroxyprogesterone acetate (17P), Medroxyprogesterone acetate (MPA), confidence intervals (CI), number of patients (N), Matrix metalloproteinase 9 (MMP-9), Tumor necrosis factor α (TNF α).