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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BACKGROUND: Current treatment modalities for preventing preterm premature rupture of membranes are limited, but progestins may play a role. Tumor necrosis factor α (TNFα) 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 TNFα-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 TNFα-induced MMP-9 activity and gene expression in primary chorion and amnion cells harvested from the fetal membranes of term nonlaboring patients.

METHODS: Primary amnion and chorion cells were isolated from fetal membranes obtained from term uncomplicated nonlaboring patients following elective cesarean delivery (n = 11). Confluent primary amnion and chorion cell cultures were both pretreated with vehicle (control), progesterone (P4), 17α-hydroxyprogesterone caproate (17P), or medroxyprogesterone acetate (MPA) at 10−6 M concentration for 6 hours followed by stimulation with TNFα at 10 ng/mL for an additional 24 hours. Cell cultures pretreated with the vehicle only served as the unstimulated control and the vehicle stimulated with TNFα 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 real-time quantitative PCR. Basal MMP-9 activity and gene expression data were normalized to the unstimulated control. TNFα-stimulated MMP-9 activity and gene expression were normalized to the stimulated control. The primary outcome was the effect of progestins on TNFα-induced MMP-9 enzymatic activity in term human amnion and chorion cells in vitro. Secondary outcomes included the effect of progestin therapy on TNFα-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 with the unstimulated control, TNFα increased MMP-9 activity (P = 0.005 versus control in primary amnion cells and P < 0.001 versus control in primary chorion cells) and MMP-9 gene expression (P = 0.030 versus control in primary amnion cells, P < 0.001 versus 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 TNFα-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 TNFα-induced MMP-9 activity and gene expression in primary chorion cells.

CONCLUSIONS: The inhibitory effect of MPA on both basal and TNFα-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 preterm premature rupture of membranes. (Anesth Analg 2015;120:1085–94)
deliveries result from preterm premature rupture of membranes (PPROM)—rupture of membranes prior to the onset of labor and before 37 weeks of gestation. Infants born as a result of PPROM are at a greater risk of developing complications related to prematurity compared with those resulting from spontaneous preterm delivery from other etiologies. The resulting emotional impact on parents and the significant health care costs incurred make PPROM a major public health problem.

Mechanisms leading to PPROM are distinctly different from those that result in other causes of preterm delivery. Inflammation from localized or systemic infections, uterine overdistension or increased genetic susceptibility, stimulate the release of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), which, in turn, stimulate a localized inflammatory response in fetal membranes. 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. These matrix proteins provide the tensile strength for fetal membranes, particularly in the amnion layer. They also form the basement membrane for cell adherence. 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. The clinical importance of MMP-9 in the pathophysiology of PPROM 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 PPROM 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 PPROM; and (3) TNFα-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.

Clinically, progestin therapy has been used for the prevention of recurrent preterm birth, but its role in the prevention of PPROM remains unclear. Progestins such as medroxyprogesterone acetate (MPA) have been shown to inhibit cytokine-induced MMP-1 and MMP-3 expression and activity in term decidua cells in vitro. Progestins also inhibit basal and TNFα-induced apoptosis in fetal membranes. We have also demonstrated that pretreatment with MPA inhibits TNFα-induced MMP-9 activity in a human cytotrophoblast cell line, highlighting a possible mechanism by which progestins may prevent PPROM. 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 TNFα-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 TNFα-induced MMP-9 enzymatic activity in human primary amnion and chorion cells in vitro. Secondary outcomes included the effect of progestin pretreatment on TNFα-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 × 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 antimitotics (streptomycin and penicillin and amphotericin B). Fetal membranes were washed in culture media to remove blood and debris and then cut into 5 × 5 cm squares. The amnion layer was peeled off from the choriodecidual 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. 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 to 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 resuspended 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 antimitotics, cultured in 12-well cell culture plates and grown to 80% to 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.

**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 hours. Using our previously described methodology, the 2-cell culture wells were each pretreated with ethanol 0.01% as vehicle (Decon Labs, King of Prussia, PA), progesterone (P4) (Sigma-Aldrich), medroxyprogesterone 17-aceate (MPA) (Sigma-Aldrich), or 17α-hydroxyprogesterone caproate (17P) (Steraloids, Newport, RI) at 10⁻⁶ M for 6 hours in serum-containing media. The concentrations of progestins used were based on placental levels measured in pregnancy. 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. 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 TNFα 10 ng/mL for an additional 24 hours in serum-free media. The vehicle without TNFα served as the unstimulated control for basal MMP-9 activity and gene expression. Cell cultures pretreated with vehicle and progestins followed by TNFα stimulation were used for TNFα induced MMP-9 activity and gene expression. P4 = progesterone; 17P = 17α hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate; RT-qPCR = real-time quantitative polymerase chain reaction.

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 (TNFα) were used for basal matrix metalloproteinase-9 (MMP-9) activity and gene expression. Cell cultures pretreated with vehicle and progestins followed by TNFα stimulation were used for TNFα induced MMP-9 activity and gene expression. P4 = progesterone; 17P = 17α hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate; RT-qPCR = real-time quantitative polymerase chain reaction.

Gelatin Zymography
Gelatin zymography was used to quantify MMP-9 enzymatic activity in vitro using the manufacturer’s protocol. Briefly, harvested cell culture media was incubated in a 1:1 ratio with a Novex® (Life Technologies) tris-glycine sample buffer sodium dodecyl sulfate for 10 minutes at room temperature. Samples were loaded onto a 10% Novex gelatin zymogram gel and electrophoresed for 90 minutes. The gels were run in duplicates and were incubated in Novex renaturing buffer for 30 minutes, followed by Novex developing buffer for a further 30 minutes to allow the enzymes to renature. After incubation with fresh developing buffer for a further 16 to 18 hours, the gels were washed with deionized water and stained with Novex Simplyblue Safestain for 1 hour. The gel was destained by washing with deionized water for 2 hours at room temperature and MMP-9 activity was quantified by analyzing band densities at 88 and 92 kDa using ImageJ® 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 RNAeasy® minikit (Qiagen, Valencia, CA). RNA concentration was measured using the Nanodrop® spectrophotometer (Thermo Scientific, Wilmington, DE) and 1μg 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 (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). RTqPCR 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 glyceraldehyde-3-phosphate dehydrogenase 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 TNFα-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. Because of 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 TNFα-induced MMP-9 activity and gene expression data, progestin-pretreated groups stimulated with TNFα 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 TNFα-induced MMP-9 activity and gene expression, the stimulated control was compared with the groups pretreated with progestins and stimulated with TNFα. Both comparisons were analyzed using two-way ANOVA to compare progestin treatments within cell types with post hoc Dunnett 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 TNFα for TNFα-induced MMP-9 activity and gene expression. To determine whether the distribution of the residuals approximated a normal distribution, quantile-quantile 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 were summarized as mean ± SEM, mean differences, and 95% confidence intervals, and was analyzed using GraphPad prism (version 6.0 for Mac OS X, GraphPad Software, La Jolla, CA, www.graphpad.com) and SAS Enterprise Guide (Version 5.1, SAS Institute Inc., Cary, NC).

**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 TNFα on MMP-9 Activity and Gene Expression**

Compared with the unstimulated control TNFα increased MMP-9 activity and MMP-9 gene expression in both primary amnion (Fig. 2) and chorion cells (Fig. 3). The predominant band that was observed by zymography was the 92 kD pro MMP-9 band (Fig. 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] (Fig. 4A, Table 1). 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] (Fig. 4B, Table 2). 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 (Fig. 4, A and B, Tables 1 and 2).

In chorion cells, no significant differences in basal MMP-9 activity between the unstimulated control and the progestin treated groups were observed (Fig. 4A, Table 1). Similarly no differences in basal MMP-9 gene expression were observed between the unstimulated control and the progestin-treated groups (Fig. 4B, Table 2).

**The Effect of Progestins on TNFα-Induced MMP-9 Activity and Gene Expression**

Pretreatment with MPA significantly reduced TNFα-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] (Fig. 5A, Table 3).
Pretreatment with MPA also significantly reduced TNFα-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, \( P < 0.001 \)] (Fig. 5B, Table 4). Pretreatment with P4 and 17P did not significantly reduce TNFα-induced MMP-9 activity and gene expression compared with the stimulated control in primary amnion cells (Fig. 5, A and B, Tables 3 and 4).

No significant differences in TNFα-induced MMP-9 activity were observed between the progestin-treated groups stimulated with TNFα and the stimulated control in primary chorion cells (Fig. 5A, Table 3). Similarly, no significant differences in TNFα-induced MMP-9 gene expression were observed between the progestin-treated groups stimulated with TNFα and the stimulated control in primary chorion cells (Fig. 5B, Table 4).

**DISCUSSION**

Our results demonstrate that MPA pretreatment significantly inhibited both basal and TNFα-induced MMP-9 activity and gene expression in primary amnion cells. Interestingly, P4 and 17P did not inhibit basal and TNFα-induced MMP-9 activity and gene expression in primary amnion cell cultures. Progestin pretreatment also did not significantly inhibit basal and TNFα-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 PPROM to occur. 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 chorioamnion, choriodecidual rupture, further nonelastic amnion distension followed ultimately by rupture of the amnion. 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. 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. TNFα-induced MMP-9 protein expression and the resulting collagen remodeling have also been correlated with fetal membrane weakening in vitro. Additionally, inhibition of TNFα-induced fetal membrane weakening by alpha-lipoic acid has been associated with a concomitant reduction in TNFα-induced MMP-9 expression in full thickness fetal membranes and primary amnion epithelial cells. We hypothesize, based on the profound suppression by MPA of MMP-9 activity and gene expression in primary unstimulated and TNFα-stimulated amnion cells, that this is a mechanism by which progestins may act to maintain fetal membrane integrity in the setting of inflammation and
Progestins and TNFα-Induced MMP-9 Activity and Expression

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other idiopathic causes of PPROM. However, tissue biomechanical studies are now needed to confirm this hypothesis.

Our findings suggest that the reductions in basal and TNFα-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 κB and activator protein 1 pathways in fetal membranes.30,31 Additionally, there are transcription factors that negatively regulate MMP-9 gene expression, such as kisspeptin and metastasis associated gene 1.32–34 These repressors inhibit MMP-9 gene expression either by inhibiting nuclear factor-κB and activator protein 1 pathways in fetal membranes.30,31

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, 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 progesterone membrane component 1 mediates the inhibitory effect of MPA on TNFα-induced MMP-9 activity.18 Recently, the glucocorticoid receptor has also been implicated as possible receptor mediating the suppression of IL-1β 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 TNFα-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 TNFα-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

![Figure 3. Tumor necrosis factor α (TNFα)-induced matrix metalloproteinase-9 (MMP-9) activity and gene expression in primary chorion cells. Stimulation with TNFα 10 ng/mL increased MMP-9 enzymatic activity (P = 0.001 versus unstimulated control, n = 10 patients) (A) and gene expression (P < 0.001 versus 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 (TNFα only group) using a paired t test. Data are mean with the error bars representing the SEM.](image-url)
patients have recurrent preterm births while on treatment.\textsuperscript{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\textsuperscript{-3} to 10\textsuperscript{-5} M) suppress both spontaneous myometrial contractility and cytokine-induced cyclooxygenase 2 expression.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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

### Table 1. The Effect of Progestins on Basal MMP-9 Enzymatic Activity

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<th>Comparison (N)</th>
<th>Mean difference (normalized units)</th>
<th>95% CI (normalized units)</th>
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<td>Amnion</td>
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<td>−49.9 to 11.3</td>
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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.

The significant values, P < 0.05 are in bold.

MMP-9 = matrix metalloproteinase-9; P4 = progesterone; 17P = 17α-hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate.
Progestins and TNFα-Induced MMP-9 Activity and Expression

The effect of progestins on basal MMP-9 gene expression is debatable. Another limitation is that MPA was the only progestin which attenuated both basal and TNFα-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. Additionally, it is not in clinical use for the prevention of preterm delivery. However, MPA is commonly used in vitro cell and tissue culture experiments due to its increased stability when compared with other progestins. 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.

In summary, we demonstrated that MPA inhibits both basal and TNFα-induced MMP-9 activity and gene transcription in primary amnion cells harvested from term pregnant women. Our data highlight one of the mechanisms by which progestins may prevent inflammation-induced fetal membrane weakening that may lead to PPROM. The cell

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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. The significant values, P < 0.05 are in bold.

P4 = progesterone; 17P = 17α-hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate; CI = confidence intervals; N = number of patients; MMP-9 = matrix metalloproteinase-9.

Figure 5. The effect of progestins on tumor necrosis factor α (TNFα)-induced matrix metalloproteinase-9 (MMP-9) activity and gene expression. Compared with the stimulated control (TNFα) group, MPA inhibited TNFα 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 TNFα-induced MMP-9 activity and gene expression were observed between the other progestin-pretreated groups plus TNFα and the stimulated control in primary amnion cells. No significant differences in TNFα-induced MMP-9 activity and gene expression were observed between the progestin-treated groups plus TNFα 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 SEM. P4 = progesterone; 17P = 17α-hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate.

Table 3. The Effect of Progestins on TNFα-Induced MMP-9 Enzymatic Activity

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<td>P4 TNFα versus stimulated control (11)</td>
<td>8.8</td>
<td>−13.9 to 31.5</td>
<td>0.68</td>
</tr>
<tr>
<td>17P TNFα versus stimulated control (11)</td>
<td>−3.4</td>
<td>−26.2 to 19.3</td>
<td>0.97</td>
</tr>
<tr>
<td>MPA TNFα versus stimulated control (11)</td>
<td>−69.0</td>
<td>−91.8 to −46.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chorion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4 TNFα versus stimulated control (10)</td>
<td>−5.4</td>
<td>−29.2 to 18.5</td>
<td>0.91</td>
</tr>
<tr>
<td>17P TNFα versus stimulated control (10)</td>
<td>−4.8</td>
<td>−28.6 to 19.0</td>
<td>0.93</td>
</tr>
<tr>
<td>MPA TNFα versus stimulated control (10)</td>
<td>−21.0</td>
<td>−44.8 to 2.9</td>
<td>0.10</td>
</tr>
</tbody>
</table>

MMP-9 enzymatic activity for the progestin plus TNFα-treated groups was normalized to the stimulated control (TNFα only group), with the stimulated control assigned a value of 100 units. The progestin plus TNFα-treated groups were compared with the stimulated control using two-way ANOVA. The distribution of the residuals for the model approximated a normal distribution. The significant values, P < 0.05, are in bold.

P4 = progesterone; 17P = 17α-hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate; TNFα = tumor necrosis factor α.

Table 4. The Effect of Progestins on TNFα-Induced MMP-9 Gene Expression

<table>
<thead>
<tr>
<th>Comparison (N)</th>
<th>Mean difference (normalized units)</th>
<th>95% CI (normalized units)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amnion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4 TNFα versus stimulated control (10)</td>
<td>2.7</td>
<td>−31.9 to 37.4</td>
<td>0.995</td>
</tr>
<tr>
<td>17P TNFα versus stimulated control (10)</td>
<td>−0.2</td>
<td>−34.8 to 34.5</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>MPA TNFα versus stimulated control (10)</td>
<td>−86.0</td>
<td>−120.7 to −51.3</td>
<td>=0.001</td>
</tr>
<tr>
<td>Chorion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4 TNFα versus stimulated control (10)</td>
<td>1.5</td>
<td>−33.2 to 36.2</td>
<td>0.999</td>
</tr>
<tr>
<td>17P TNFα versus stimulated control (10)</td>
<td>1.7</td>
<td>−17.7 to 51.6</td>
<td>0.51</td>
</tr>
<tr>
<td>MPA TNFα versus stimulated control (10)</td>
<td>−9.9</td>
<td>−44.6 to 24.8</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Gene expression for the progestin plus TNFα-treated groups was normalized to the stimulated control (TNFα only group), with the stimulated control assigned a value of 100 units. The progestin plus TNFα-treated groups were compared with the stimulated control using two-way ANOVA. The distribution of the residuals for the model approximated a normal distribution. The significant values, P < 0.05, are in bold.

P4 = progesterone; 17P = 17α-hydroxyprogesterone acetate; MPA = medroxyprogesterone acetate; CI = confidence intervals; N = number of patients; MMP-9 = matrix metalloproteinase-9; TNFα = tumor necrosis factor α.

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.

DISCLOSURES

Name: Terrence K. Allen, MBBS, FRCA.

Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.

Attestation: Terrence K. Allen has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.

Name: Liping Feng, MD.

Contribution: This author helped design the study, conduct the study, and write the manuscript.

Attestation: Liping Feng has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

Name: Matthew Nazzal, BS.

Contribution: This author helped conduct the study and write the manuscript.

Attestation: Matthew Nazzal approved the final manuscript.

Name: Chad A. Grotegut, MD, MHS.

Contribution: This author helped analyze the data and write the manuscript.

Attestation: Chad A. Grotegut reviewed the analysis of the data and approved the final manuscript.

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Contribution: This author helped design the study and write the manuscript.

Attestation: Irina A. Buhimschi approved the final manuscript.

Name: Amy P. Murtha, MD.

Contribution: This author helped design the study and write the manuscript.

Attestation: Amy P. Murtha approved the final manuscript.

REFERENCES

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Progestins and TNFα-Induced MMP-9 Activity and Expression


