Progesterone Inhibits Apoptosis in Fetal Membranes by Altering Expression of Both Pro- and Antiapoptotic Proteins

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Abstract
Objective: Progesterone supplementation prevents preterm birth (PTB) in some high-risk women, but its mechanism of action is unknown. One-third of PTB is associated with preterm premature rupture of membranes (PPROMs). We have previously shown that progesterone inhibits basal and Tumor Necrosis Factor (TNF) α-induced apoptosis in an explant model of human fetal membranes. This study investigates the molecular mechanisms responsible for progesterone-mediated inhibition of apoptosis in fetal membranes. Methods: Human fetal membranes were collected at elective cesarean at term (no labor, no infection; n = 6), washed, and pretreated with/without progesterone (125 ng/mL) for 24 hours. Thereafter, membranes were treated with/without TNFα (50 ng/mL) and/or progesterone for 48 hours, harvested, and homogenized. Apoptosis was determined by evaluating caspase-3, -8, and -9 activities. Expression of pro- BH3 interacting domain death against, Bc1-2 associated X protein (BID, BAX) and antiapoptotic proteins (X-linked inhibitor of apoptosis protein [XIAP], Bcl-2, FLICE inhibitory protein [FLIP]) were measured by Western blot.

Results: TNFα increased apoptosis (measured by caspase-3, -8, and -9 activities) in fetal membranes, and this effect was abrogated by progesterone. Under basal conditions, progesterone suppressed expression of the proapoptotic protein, BID, by 0.45 (0.14)-fold, and increased expression of the antiapoptotic proteins, Bcl-2 and XIAP; no change was seen in BAX or FLIP. In contrast, TNFα increased BID expression by 5.15 (2.92)-fold, which was prevented by pretreatment with progesterone.

Conclusions: Progesterone inhibits apoptosis in fetal membranes by suppressing expression of the proapoptotic protein, BID (for both basal and TNFα-induced apoptosis), and upregulating expression of the antiapoptotic proteins, XIAP and Bcl-2 (under basal conditions only). These data provide a mechanism by which progesterone supplementation may prevent PPROM and PTB in some women at high risk.

Keywords
apoptosis, progesterone, fetal membranes, PPROM, preterm birth

Introduction
Preterm birth (PTB) complicates around 10% of all live births in the United States4 and is a major cause of perinatal morbidity and mortality.2,3 One-third of PTB is associated with preterm premature rupture of membranes (PPROMs),2,3 defined as rupture of the membranes prior to 37 weeks’ gestation. Preterm premature rupture of membranes is a serious obstetric complication that occurs in up to 3% to 4% of all pregnancies and has a high recurrence risk.4,5 The fetal membranes act as a structural and functional barrier to ascending infection/inflammation. Once ruptured, the membranes rarely reseal and efforts to repair the membranes have thus far been unsuccessful. A better understanding of the molecular mechanisms underlying PPROM may allow for the development of interventions to prevent this complication and subsequent PPROM-related PTB.
Although the precise etiology of PPROM cannot be established in most cases, accumulating evidence suggests that the primary pathways leading to weakening of the fetal membranes include intrauterine inflammation (with cytokine, matrix metalloproteinase, and prostaglandin activation), oxidative stress, and programmed cell death (apoptosis). These biological processes can be triggered by multiple factors including intrauterine infection, decidual bleeding (placental abruption), and uterine overdistention.

Clinical trials have shown that progesterone supplementation can prevent PTB in some women at high risk by virtue of a prior unexplained spontaneous PTB or a short cervix. In February 2011, the food drug administration (FDA) approved the use of progesterone supplementation for the prevention of recurrent PTB in women at high risk due to a prior PTB, although its mechanism of action is not known. We hypothesize that progesterone supplementation may prevent PTB, in part, by preventing PPROM. To this end, we have previously shown that progesterone inhibits basal and TNFα-induced apoptosis in fetal membranes. This study investigates the molecular mechanisms responsible for this progesterone-mediated inhibition of apoptosis in the fetal membranes.

Materials and Methods

Tissue Source and Culture

Human fetal membranes were collected and cultured as previously described. Briefly, fetal membranes were collected from women with uncomplicated pregnancies who had an elective repeat cesarean at term prior to the onset of labor ($n = 6$). Samples were excluded if the women had any medical or obstetrical complications or had received any medications in the preceding 24 hours. Infection was excluded using standard clinical criteria (absence of maternal fever, fundal tenderness, maternal and/or fetal tachycardia, foul vaginal discharge) and laboratory test (absence of elevated white cell count). Membranes were harvested under sterile conditions within 30 minutes of delivery, placed in sterile PBS containing 100 U/mL heparin, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone, and transported to the laboratory. Membranes were washed 3 times with Hank balanced salt solution and adherent blood clots and decidual tissues removed. Membrane discs were cut using a 5-mm biopsy punch (Premier Medical Products, King of Prussia, Pennsylvania) and placed in 0.4-µm cell culture inserts (Becton Dickinson Labware, Franklin Lakes, New Jersey) which, in turn, were placed in 24-well tissue culture plates (Becton Dickinson Labware). Membrane discs were cultured in Dulbecco Modified Eagle Medium (DMEM) with Ham F-12 nutrient mixture (1:1; Invitrogen, Carlsbad, California) and continued in fresh Opti-MEM (Invitrogen, Carlsbad, California) and continued in fresh Opti-MEM without antibiotics for subsequent stimulation studies. After culture in DMEM/Ham’s F-12 medium for 48 hours, membrane discs were washed twice with Opti-MEM medium (Invitrogen, Carlsbad, California) and continued in fresh Opti-MEM without antibiotics for subsequent stimulation studies. Based on prior dose- and time-course experiments, membrane discs were pretreated with/progesterone (125 ng/mL [Yale-New Haven Hospital Pharmacy, New Haven, Connecticut]) for 24 hours. Thereafter, medium was changed to fresh Opti-MEM (no antibiotics) containing progesterone alone or progesterone plus human recombinant TNFα (50 ng/mL [R&D Systems, Minneapolis, Minnesota]) for 48 hours. After stimulation, membrane discs and conditioned media were harvested, snap-frozen, and stored at $-80^\circ$C until further analysis. Experiments were carried out in triplicate and repeated a minimum of 3 times.

Protein Extraction and Caspase Activity Measurements

Total protein from amniochorionic tissues were isolated by homogenization with 20 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and protease inhibitors (Roche, Indianapolis, Indiana). Homogenization was followed by centrifugation at 12 000 rpm to precipitate tissue debris. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Benicia, California). Caspase activity was determined using the Caspase-Glo assay (Promega, Madison, Wisconsin) as previously described. Briefly, 10 µg of the tissue lysates were incubated in the dark at room temperature with either caspase-3, -8, or -9 specific substrates. Following 1-hour incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, California) and specific caspase activity reported in relative light units (RLU). All samples were assayed in triplicate.

Western Blot

Explants of human fetal membranes were isolated, stimulated, and total protein extracted and quantified as described. Western blot analysis was conducted using standard protocols as previously described. Briefly, equal amounts of protein lysate (35 µg) were separated on 4% to 15% Tris-HCl linear gradient gel (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Life Sciences, Boston, Massachusetts). After blocking, membranes were incubated overnight at 4°C with primary antibodies followed by 1-hour treatment at room temperature with the appropriate HRP-conjugated secondary antibody. Primary antibodies used included rabbit polyclonal anti-human BID (1:1000 dilution; Cell Signaling Technology, Beverly, Massachusetts), mouse monoclonal anti-human BAX (1:1000 dilution; BD Biosciences, San Jose, California), mouse monoclonal anti-human X-linked inhibitor of apoptosis protein (XIAP; 1:1000 dilution; BD Biosciences), rabbit monoclonal anti-human Bcl-2 (1:1000 dilution; Cell Signaling
and rabbit polyclonal antihuman FLICE inhibitory protein (FLIP, 1:1000 dilution; Cell Signaling Technology). Rabbit polyclonal antihuman heat shock protein 90 (HSP-90, 1:1000 dilution; Cell Signaling Technology) and/or rabbit polyclonal antihuman α-tubulin (1:1000 dilution; Cell Signaling Technology) were used to confirm equal loading of protein. The blots were developed and the intensity of the bands analyzed using the enhanced chemiluminescence system according to the instructions of the manufacturer (PerkinElmer Life Sciences, Boston, Massachusetts). The density of the resulting bands was measured using NIH Image J software, version 1.51t, and the relative signals were quantified as the ratio normalized RLU (corrected for HSP-90 band intensity). As negative control, membranes were incubated with secondary antibody alone to validate the specificity of the signal.

**Statistical Analysis**

All data were subjected to normality testing using the Kolmogorov-Smirnov method and reported as mean (standard deviation; for normally distributed data) or median and range (for nonparametric data). Comparisons between the groups were performed using analysis of variance and $\chi^2$ tests. For Western blot quantification, statistical analysis was performed using MINITAB statistical software (Minitab, State College, Pennsylvania). Significance of differences for protein expression was determined using paired 2-tailed $t$ tests. For all studies, $P < .05$ was used to define statistical significance.

**Results**

**TNFα Induces Apoptosis in Fetal Membranes, Which Can Be Inhibited by Progesterone**

Consistent with our prior publication, we confirmed that TNFα-induced apoptosis in fetal membranes in vitro as measured by an increase in caspase-3, -8, and -9 activities (data not shown). We further confirmed that progesterone pretreatment inhibited both basal and TNFα-induced caspase-3 activity (data not shown).

**Effect of Progesterone on Mediators of Apoptosis in Human Fetal Membranes**

A number of proteins are known to regulate the apoptotic cascade within cells. Some of these proteins are known to promote apoptosis (BID, BAX), whereas others suppress programmed cell death (XIAP, Bcl-2, FLIP). Under basal conditions in our experimental model, progesterone significantly decreased expression of the proapoptotic protein, BID, by 0.45 (0.14)-fold ($P = .001$; Figure 1A). Progesterone also increased the expression of the antiapoptotic proteins, XIAP by 4.14 (2.06)-fold ($P = .028$; Figure 1D) and Bcl-2 by 5.32 (2.94)-fold (although this failed to reach statistical significance with $P = .061$; Figure 1C). Progesterone stimulation had no effect on the expression of BAX (Figure 1B) or FLIP (Figure 1E). These findings are summarized in Figure 2.

**Effect of TNFα With/Without Progesterone on Mediators of Apoptosis in Fetal Membranes**

In contrast to progesterone, TNFα significantly increased expression of the proapoptotic protein, BID, in fetal membrane explants by 5.15 (2.92)-fold ($P = .034$; Figure 1A). Interestingly, all of the other TNFα-mediated effects were similar to that seen with progesterone stimulation, including increased expression of the antiapoptotic proteins, Bcl-2 by 3.40 (0.89)-fold ($P = .013$; Figure 1C) and XIAP by 5.85 (4.52)-fold ($P = .019$; Figure 1D). Similarly, TNFα stimulation had no effect on the expression of BAX (Figure 1B) or FLIP (Figure 1E).

When looking at the effect of progesterone pretreatment, it is notable that progesterone completely abrogated the TNFα-mediated induction of the proapoptotic protein, BID ($P = .031$), although not below the level of expression seen with progesterone treatment alone ($P = .655$; Figure 1A). Progesterone pretreatment did not alter the response of BAX (Figure 1B), XIAP (Figure 1D), or FLIP (Figure 1E) to TNFα stimulation; it was neither additive nor synergistic. Interestingly, although progesterone alone increased the expression of Bcl-2, progesterone pretreatment blocked the increased expression of Bcl-2 induced by TNFα stimulation (Figure 1C).

**Discussion**

Progesterone supplementation starting at 16 to 20 weeks and continuing through 36 weeks of gestation can prevent PTB in some high-risk women. This is the first medication that has been shown to decrease the rate of PTB in 30 years, and the first approved by the FDA16 and by the American College of Obstetricians and Gynecologists22 for this indication. Although a number of hypotheses have been put forward, the mechanism by which progesterone exerts this protective effect is not known.

One-third of PTB occurs in the setting of PPROM. While it is clear that initiation of progesterone therapy after PPROM has already occurred does not confer any benefit,25 we hypothesize that progesterone supplementation starting at 16 to 20 weeks may prevent PTB, in part, by preventing PPROM. This hypothesis is consistent with the observations that fetal membranes are biologically active and likely play an important functional role in the timing of labor,26 that many of the clinical trials that demonstrated a protective effect of progesterone supplementation included patients with PPROM,13,15 and that patients on progesterone supplementation because of a history of a prior PTB due to PPROM are less likely to have a recurrent PTB than those on progesterone supplementation because of a prior spontaneous PTB.27 This hypothesis is supported also by a number of experimental studies. We have previously shown that progesterone inhibits both basal and TNFα-induced apoptosis as measured by caspase activities in an explant model of human fetal membranes. Similarly, Kumar et al.28,29 showed that progestogen pretreatment blocked TNFα- and thrombin-induced fetal membrane weakening as
Figure 1. Western blot analysis of pro- (BID, BAX) and antiapoptotic proteins (XIAP, Bcl-2, FLIP) expression in fetal membrane explants. Fetal membrane discs were prepared from term placentas (no labor) and cultured with/without progesterone and/or TNFα as described in the Materials and Methods section above. Total protein was extracted from the membrane discs, purified, and subjected to Western blot using specific antibodies for the proteins of interest. These included the proapoptotic proteins, BID (22 kDa) (A) and BAX (21 kDa) (B). They also include the antiapoptotic proteins, Bcl-2 (26 kDa) (C), XIAP (the antibody detects full-length 57-kDa protein and the degradation product at 38 kDa) (D), and FLIP (58 kDa) (E). Representative Western blot images are shown along with the quantitative analysis. Data are shown as mean (standard deviation) from a minimum of 3 separate experiments. HSP-90 (90 kDa) and α-tubulin (52 kDa) were used as housekeeping proteins. FLIP indicates FLICE inhibitory protein; HSP-90 indicates heat shock protein 90; NS indicates nonsignificant; XIAP, X-linked inhibitor of apoptosis protein.
measured by mechanical stretch and that it did so, in part, by inhibiting both the production and action of their upstream mediator, granulocyte-macrophage colony-stimulating factor. Taken together, these data are consistent with the hypothesis that prophylactic administration of progestogens may prevent PPROM.

The current study investigates the molecular mechanisms responsible for the previously documented progesterone-mediated inhibition of apoptosis in the fetal membranes. In this study, we demonstrate: (1) that progesterone suppresses apoptosis under basal conditions, which appears to be mediated by a decrease in the expression of the proapoptotic factor, BID, and an increase in the expression of the antiapoptotic factors, Bcl-2 and XIAP, and (2) that progesterone blocks TNFα-mediated apoptosis, likely by eliminating the TNFα-induced increase in BID expression. These data suggest a molecular mechanism to explain how progesterone supplementation prevents amniochorion cell apoptosis, PPROM, and PPROM-related PTB.

Two major apoptotic pathways have been implicated in the pathogenesis of PPROM: an “extrinsic” TNFα receptor-Fas-mediated pathway, and an “intrinsic” p53-mediated pathway, which is normally initiated by DNA fragmentation. The TNFα receptor-Fas-mediated pathway initiates signal transduction through various transmembrane death receptors, which in turn activate procaspase-8 to active caspase-8. By contrast, the p53-mediated pathway proceeds via the release of proapoptotic messengers from the mitochondrial membrane (including cytochrome c) and subsequent activation of procaspase-9 to active caspase-9. Both active caspase-8 and -9 can initiate a cascade of downstream caspase activation. Caspase-3, -7, and -6 are activated sequentially, leading to proteolysis of structural proteins, proteins of homeostasis, and several other target proteins resulting in apoptosis (Figure 2).

In this study, we identify BID as a putative essential target of progesterone-mediated inhibition of both basal level and TNFα-induced apoptosis. This finding is interesting as BID appears unique in its ability to play important roles in both the “extrinsic” TNFα pathway and the “intrinsic” p53 pathway. In the “extrinsic” pathway, BID interacts with caspase-8 and may be necessary for caspase-8-induced apoptosis. In the “intrinsic” pathway, BID activates BAX, which, in turn, promotes the release of cytochrome c from mitochondria. These data suggest that BID may serve as a keystone protein, common to both apoptotic pathways and responsive to both progesterone and TNFα stimulation (Figure 2).

Both progesterone and TNFα treatments increased the expression of Bcl-2 and XIAP. Whereas these observations are consistent with the facility of progesterone to antagonize apoptosis, the finding that TNFα (an apoptosis-inducing signaling molecule) also increased the expression of these 2 antiapoptotic factors was unexpected. Equally surprising was the fact that coinubcation with both progesterone and TNFα did not result in an additive or synergistic response compared with either progestosterone or TNFα alone. Rather, for XIAP, there was no further increase (Figure 1D), suggesting that these 2 agonists may

![Figure 2. Effect of progesterone on the apoptotic cascade. The effects of progesterone stimulation on the expression of the proapoptotic (BID, BAX) and antiapoptotic proteins (XIAP, Bcl-2, FLIP) in human fetal membranes are shown. ↑ increase; ↓ decrease; → no change. FLIP indicates FLICE inhibitory protein; XIAP, X-linked inhibitor of apoptosis protein.](image-url)
increase protein expression through a shared signal transduction pathway that can be fully saturated. Progesterone pretreatment also appeared to block the increase in Bcl-2 expression induced by stimulation with TNFα (Figure 1C), although this inhibition did not reach statistical significance \( P = .14 \). Both progesterone and TNFα are known to regulate intracellular NFκB signaling, which, in turn, can affect the apoptotic cascade.\(^{32-34}\) It is possible that the NFκB family of transcription factors serve as the common signaling pathway for the regulation of XIAP and Bcl-2 expression by both progesterone and TNFα. While the molecular mechanisms responsible for these observations remains to be fully elucidated, these data suggest that the release of TNFα as part of the intrauterine inflammatory response commonly associated with PTB may contribute to PPROM and PTB by changing the quality of progesterone signaling, which normally acts to promote uterine quiescence and maintain pregnancy.

The observation that progesterone suppresses basal levels of apoptosis in the fetal membranes suggests that this mechanism may be important also for normal labor. In most mammalian viviparous species, the onset of parturition at term is preceded by a decrease in circulating progesterone levels. Although humans do not exhibit a systemic decline in progesterone levels prior to labor, it is likely that a functional withdrawal of progesterone activity within the tissues of the uterus does indeed occur prior to the onset of labor.\(^{24,35,36}\) Whether this is also true within the tissues of the fetal membranes is not known but could explain the progressive weakening of the fetal membranes that occurs in anticipation of labor at term.\(^{37,38}\) Moreover, this weakening of the fetal membranes does not appear to be uniform throughout the uterus. A zone of maximal weakening is typically present directly over the cervix,\(^{37,38}\) the precise location at which ascending inflammation (induced, in part, by TNFα) would be expected to occur in concert with progressive cervical dilatation and loss of the protective mucus plug.

In summary, this study suggests a molecular mechanism involving the differential regulation of pro- and antiapoptotic proteins whereby progesterone is able to inhibit both basal level and TNFα-induced apoptosis in human fetal membranes. A functional withdrawal of progesterone activity at the level of the uterine tissues, which appears to occur in humans prior to the onset of labor at term, would result in increased apoptosis and weakening of the fetal membranes in anticipation of labor. This model also defines a mechanism by which progesterone supplementation may prevent PPROM and PTB in some high-risk women.

**Authors’ Note**

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**Declaration of Conflicting Interests**

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