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The effects of Thrombin and Cytokines upon the Biomechanics and Remodeling of Isolated Amnion Membrane, *in vitro*

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Abstract

Abruption-induced thrombin generation and inflammation/infection induced cytokine production have both been associated with fetal membrane (FM) weakening and preterm premature rupture of the fetal membranes (PPROM). Using our *in vitro* model system we have demonstrated that thrombin, and separately the cytokines, tumor necrosis factor- α (TNF α) and interleukin-1- β (IL-1 β), remodel and weaken full thickness FM. Additionally, we have reported that the anti-oxidant and NF κ B inhibitor, alpha-lipoic acid (LA), blocks these thrombin and cytokine induced effects. The purpose of these studies was to determine whether thrombin and cytokines directly weaken the amnion membrane (AM), the major load-bearing component of FM. Isolated AM or full thickness FM fragments from unlabored Cesarean deliveries were incubated with thrombin, TNF α , or IL-1 β , for 48h. Rupture strength (breaking force) of each fragment was thereafter determined using our published methodology. Biochemical evidence of remodeling and apoptosis; immunoreactive Matrix Metalloproteinase 9 (MMP9), Tissue Inhibitor of Matrix Metalloproteinase 3 (TIMP3) and cleaved poly (ADP-ribose) polymerase (C-PARP) levels in tissue extracts, were determined by western blot and densitometry. Thrombin induced a dose-dependent weakening of isolated AM ($P < 0.001$) coupled with dose-dependent increases in PARP cleavage, and reciprocal increases and decreases, respectively, in MMP9 and TIMP3 protein (all $P < 0.01$). Thrombin receptor activating peptide-6 (TRAP) also weakened isolated AM. Neither TNF α nor IL-1 β weakened isolated AM. However, both cytokines weakened AM when it was incubated together with the choriodecidua as part of full thickness FM ($P < 0.001$). Cytokine conditioned choriodecidua medium also weakened isolated AM ($P < 0.001$). Under conditions in which cytokines weakened the AM, the changes in MMP9, TIMP3 and PARP cleavage were consistent with those seen after thrombin incubation. LA blocked the FM weakening and remodeling effects. In summary, thrombin weakens AM directly whereas cytokines weaken AM indirectly by causing the release of soluble intermediates from the choriodecidua.

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Introduction

Preterm premature rupture of the fetal membranes (PPROM) is the initiating event in approximately 40% of preterm births [1–3]. The mechanisms by which fetal membranes (FM) weaken and rupture at term or preterm gestation have not been fully elucidated, but inflammation with associated cytokine release, and abruption with resultant thrombin production, are both highly associated with PPRM [4–7]. Inflammation in response to bacteria ascending from the lower female genital tract is generally believed to play a key role in the etiology of PPRM, with up to 55 percent of such patients exhibiting culture or PCR evidence of infection [8]. Inflammatory processes at sites remote from the female genital tract (e.g., periodontal infections) may also increase proinflammatory cytokines at the maternal-fetal interface [9,10]. Strong evidence also links PPRM with decidual hemorrhage and placental abruption [11,12]. In the absence of frank bleeding decidual and extraplacental membrane hemosiderin deposition is more prevalent in patients with PPRM [13]. Decidual cells express abundant tissue factor (TF), a transmembrane 45 kDa glycoprotein. Vascular injury initiates clotting when plasma-derived factor VII binds to the extracellular domain of perivascular cell membrane-bound TF. The resulting TF/VIIa complex cleaves pro-thrombin to thrombin [14,15]. Patients with PPRM have higher median plasma concentrations of TF and lower median plasma concentrations of TF pathway inhibitor (TFPI) than women with normal pregnancies [16]. Arguably, thrombin is produced in larger amounts in PPRM. Conversion of fibrinogen to fibrin enables thrombin to act as the primary initiator of hemostasis. Consistent with this mechanism, fibrinogen consumption is a standard method of assessing abruption severity. Moreover, elevated circulating thrombin-antithrombin complex levels are associated with the subsequent occurrence of preterm delivery due to preterm labor and/or PPRM [17–19].

There are no animal models for the study of human FM weakening and rupture. We have developed FM strength testing equipment and methodology that allow us to systematically measure and map human FM biomechanical properties over the entire FM surface and then correlate these observations with local biochemical properties [5]. We have used these methods in conjunction with an adaptation of the explant culture system developed by Menon and Fortunato [20] to produce a model system with which to study the process of human FM weakening. Full thickness FM fragments are cut from regions of the FM distant from the weak zone [6] of Cesarean delivered FM without labor. The explants are then incubated for 2–4 days with agents postulated to affect the weakening process. Biomechanical testing is then performed and correlated with biochemical changes [21–24].

Using our model system we initially demonstrated that inflammatory cytokines (TNF α and IL-1 β) weaken full thickness FM explants in a dose dependent manner. Concomitant with weakening, these cytokines produced biochemical changes in FM explants that mimicked the biochemical signature of the natural para-cervical FM weak zone. Specifically, TNF α and IL-1 β increased MMP9, decreased TIMP3, and increased PARP cleavage suggesting collagen remodeling and apoptosis [6,7,21]. Recently, we have shown that thrombin also weakens full thickness FM explants in a dose dependent manner with concomitant induction of MMP9 and MMP3 (23), and that LA (α -lipoic acid) pre-incubation inhibits both cytokine and thrombin induced FM weakening and this remodeling [22–23].

Thrombin is presumably introduced into the FM by bleeding in the choriodecidua. The choriodecidua is cellularly dense, relative to the amnion, and the literature is robust with studies of cytokine action upon choriodecidua and its constituent cells [25,26]. The amnion membrane (AM), however, is the load-bearing component of the FM and must undergo extensive remodeling and weakening as part of any process that substantially weakens the

full thickness FM. The purpose of this study was to determine whether thrombin and cytokines are capable of directly weakening the AM, or cause AM weakening indirectly through interaction with the choriodecidua. A positive finding would also allow the use of the isolated AM, in the place of full thickness FM, in our FM weakening model system, thereby greatly simplifying the model system and facilitating studies of the mechanism of FM rupture.

Methods

Materials

All reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) unless specified otherwise. TRAP-6 was obtained from BACHEM Americas INC (Torrance, CA).

Biological Samples

The study protocol was approved by the Institutional Review Board of the MetroHealth Medical Center, Case Western Reserve University (Cleveland, OH). FM were collected from patients undergoing prelabor, repeat Cesarean section at term (37–39 weeks gestation) with no complications of pregnancy. Placentas from 18 such patients were used in these studies; the specific numbers used in each of the experiments is noted in the figure legends. After delivery of the fetus, the FM overlying the cervix were marked by the obstetrician using a sterile swab with gentian violet. Membranes were discarded if they were meconium stained, if infection was suspected from clinical history, or if chorioamnionitis was detected in pathology review. They were also not utilized if there was more than 5% separation of the AM and choriodecidua [27–29]. Briefly, immediately following Cesarean delivery of the placenta and marking of membranes, FM were cut from the placental disc avoiding the gentian violet marked region and 1cm perimeters adjacent to the disc and adjacent to the tear line. Fragments from the para-cervical weak zone were not utilized. Each FM was sectioned into multiple 3cm × 3cm pieces using our previously reported methodology [6].

For culture of isolated AM, it was separated from the choriodecidua of intact fragments by gentle traction. AM from the region overlying the placental disk was not utilized as there have been numerous reports that major biochemical and histological differences exist between the placental and reflected AM [30–32]. Fragments were washed briefly in Hanks balanced salt solution (HBSS, pH 7.4) and then incubated with or without cytokines, thrombin, or LA as described below [21–23]. Samples from all FM were submitted for pathology review.

Explant Culture

AM fragments measuring 3cm × 3cm (and in some experiments, isolated choriodecidua or full thickness FM) were cultured according to previously described protocols [21–23]. Tissue fragments were bluntly dissected and placed in 100mm² culture dishes containing 10 ml of Minimum Essential Medium, alpha modification (MEM), antibiotic antimycotic solution, 50 mg/L gentamicin sulfate, and 0.2% lactalbumin hydrolysate (EMEM-L). Culture dishes were rocked gently in an atmosphere containing 5% CO₂, air and 100% relative humidity at 37°C. After 24 h of equilibration in EMEM-L, medium was removed and replaced.

Thrombin Experiments—The effect of thrombin on isolated AM was examined in dose response experiments. Four study conditions were used: control, and three doses of thrombin (1 u/ml, 10 u/ml, 100 u/ml). TRAP-6 (1 μM), a specific activating peptide of PAR 1 (Protease Activated Receptor #1) was also utilized to confirm thrombin involvement. After

the incubations were complete, AM fragments were then subjected to rupture strength and Western blot testing as described below.

Cytokine Experiments—Dose response experiments were not performed with cytokines because of the failure of even high doses in pilot experiments to weaken the isolated AM. Control medium and high doses of the cytokines (50 ng/ml TNF α , 50 ng/ml IL-1 β) were utilized in the protocol diagrammed in Figure 4. Three conditions were used: 1. Full thickness FM fragments were incubated with cytokines for 72 h, followed by separation and testing of the AM; 2. Isolated AM fragments were incubated with cytokines for 72 h and then tested; and, 3. Isolated choriodecidua (CD) fragments were incubated with or without cytokines for 24 h. Then, medium from control or cytokine treated CD cultures was separately pooled, clarified by centrifugation at 15000xg/30m/10°C, and then added to triplicate AM explant cultures prepared as above. Incubations of the clarified media with the AM explants were carried out for an additional 72 h. After the incubations were complete, AM fragments were then subjected to rupture strength and Western blot testing as described below.

Lipoic Acid Experiments—LA pre-incubation studies were only done with thrombin. Studies with cytokines were not performed because cytokines did not weaken isolated AM, and because we have previously reported that LA inhibits cytokine induced weakening of full thickness FM [21]. To determine the effect of LA pre-incubation on thrombin induced weakening and remodeling of isolated AM, four study conditions were used: 1. Control; 2. LA pretreatment alone; 3. 10 units/ml thrombin alone; and 4. LA pretreatment followed by 10 u/ml thrombin. Cultures were first pretreated with 0.1% DMSO (vehicle) or 0.25 mM LA for 6 h, and then the 48 h experimental incubation initiated with or without addition of 10 u/ml thrombin. Following incubation, AM fragments were subjected to biomechanical and biochemical testing as outlined below.

FM Biomechanical Testing

Isolated AM was removed from culture, washed twice in 20 ml of MEM, maintained in MEM, and kept moist for the entirety of biomechanical testing. In the case that incubation of full thickness FM was carried out, the AM was separated from the choriodecidua prior to biomechanical testing. AM physical properties were determined using our previously reported methodology [6,27]. Briefly, biomechanical testing was performed using modified industrial rupture testing equipment (Com-Ten Industries, St Petersburg, FL) by the American Society for Testing and Materials standards. A mechanically driven, 1-cm diameter, rounded plunger was forced at a speed of 8.4 cm/min through membrane pieces supported on a fixture with a 2.5-cm diameter orifice. Force (applied to the membrane component) and resultant displacement data were collected continuously and analyzed by data reduction software. Rupture strength and work to rupture were determined from generated force/displacement curves. After biomechanical testing, samples of each AM fragment of equal surface area (1 cm $\times$ 1 cm) were punched, for histological analysis. The remaining specimen was snap frozen in liquid nitrogen and stored in liquid nitrogen at -70°C until biochemical testing.

Sample Homogenization

Thawed AM samples (equal 1cm $\times$ 1cm surface areas) were normalized by volume prior to homogenization by combining each sample with 5ml ice-cold 2X RIPA Buffer (Santa Cruz Biotechnology, Santa Cruz, CA). All samples were maintained on ice throughout subsequent processing. Samples were homogenized twice for 30s (with a 30s rest on ice) using a Tekmar Tissuemizer (Cincinnati, OH) fitted with a 85mm $\times$ 10mm diameter probe at 100%. After homogenization, samples were allowed to extract on ice for 30m and then frozen at

–70°C overnight. After thawing on ice, this homogenization protocol was again repeated. Thawed samples were strained through 2mm × 2mm stainless wire mesh and eluates were clarified by centrifugation at 12000 × g for 15m at 10°C. Supernatants were stored at –70°C prior to Western Blot analysis.

Western Blotting

Tissue lysates (20ul) were combined with an equal volume of Laemmli Buffer(BIO-RAD, Hercules, CA) containing 5% β-Mercaptoethanol, and samples were incubated in a boiling water bath for 5 min. Denatured samples were electrophoresed on 4%–15% gels/Tris-HCl, and resolved proteins were semi-dry blotted for 90m at 14V to polyvinylidene fluoride membrane (GE Healthcare, Piscataway, NJ) according to Bio-Rad protocols. Transfer efficiency (100%) was routinely monitored by Coomassie Blue (BIO-RAD, Hercules, CA) gel staining. Membranes were blocked in 2%ECL *Advance*TM blocking agent (GE Healthcare, Buckinghamshire, UK)/[Tris-buffered saline, 0.5% Tween-20 (TBST)], (BTBST) overnight at 10°C then incubated with MMP9(6-6B) TIMP3(Z188) or cleaved PARP(h215) antibody (1:10000; Santa Cruz Biotechnology, Santa Cruz, CA)/BTBST for 1h at room temperature. Membranes were washed three times in TBST(15m), incubated with horseradish peroxidase-conjugated secondary antibody (1:10000) in BTBST for 30 min, and then again washed three times in TBST. Chemiluminescent detection was performed using Lumigen TMA-6 according to the manufacturer's protocol (GE Healthcare), and blots were exposed against Super RX film (Fuji, Tokyo, Japan) for 5–10s for densitometry, or 15–30s for illustrative purposes.

Quantization and Statistical Analysis

Developed films and stained gels were scanned using an Epson Perfection 1200U scanner. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD). The value for an equivalent scanned blank region within a “dye-only” lane of each blot was subtracted from the value for each lane containing protein. Data points for cumulative Western blots represent the mean ±SD for AM obtained from four different patients unless otherwise noted. All experiments were performed in triplicate. Data (biomechanical, biochemical) were analyzed by ANOVA followed by post-hoc pair-wise comparisons using Sigmaplot (Systat Software, Inc, Chicago, IL). Data and results are termed significant when $P < 0.05$.

Results

Effect of Thrombin on Isolated AM

Mechanics—Thrombin weakened isolated AM in a dose-dependent manner (Figure 1). Increasing doses (1 – 100 u/ml) of thrombin caused a stepwise decrease in the strength (breaking force) and work (data not shown) required to rupture the AM. Thus, compared with controls, maximal induction of AM weakness, as evidenced by rupture strength (8.58 ± 1.79 N vs. 3.38 ± 2.10 N, $P < 0.001$) was observed with the highest thrombin dose (100 u/ml) used (Figure 1). A significant weakening effect was seen at the minimum dose of 1 u/ml ($P = 0.01$), however. The maximal effect of thrombin treatment on AM strength occurred by 48 h; no effect of thrombin (100 u/ml) on AM strength was demonstrable at 24 h (data not shown). TRAP (1 μM), the specific receptor agonist for the thrombin receptor PAR1 (protease activated receptor 1) also weakened isolated AM ($P < 0.01$; Figure 2).

Remodeling—We have previously reported that full thickness FM incubated with cytokines demonstrate increased MMP9 and decreased TIMP3 protein expression, as well as increased PARP cleavage, suggestive of remodeling and apoptosis [21]. We therefore looked specifically for these changes in the experiments with thrombin upon isolated AM.

Following 48 hour incubation with thrombin (0–100 u/ml), AM fragments exhibited increased MMP9 protein and decreased TIMP3 protein in a dose dependent manner (Figure 3). Both the pro-MMP9 (92 kD) and active MMP9 (78 kD) proteins showed stepwise increases with increasing thrombin dose. The basal (24 kD) and glycosylated (27 kD) forms of TIMP3 both showed decreases. AM fragments incubated with thrombin (0–100 u/ml) for 48 hours also exhibited dose dependent increases in cleavage of PARP suggesting a dose dependent increase in amnion cellular apoptosis (Figure 3).

Cytokine induced weakening of AM is Indirect

Mechanics—High doses of cytokines (50 ng/ml TNF α or 50 ng/ml IL-1 β) did not significantly weaken the isolated AM (control 7.78 ± 2.02 N, TNF α 7.69 ± 0.81 N, IL-1 β 7.41 ± 2.52 N; n = 12, P = 0.91, Figure 4B). However, both cytokines weakened AM, when the same cytokine doses were used on full thickness FM, after which the AM was stripped from the choriodecidua and tested (control 8.68 ± 2.67 N, TNF α 3.74 ± 1.83 N, IL-1 β 3.03 ± 0.89 N; n = 12, P < 0.001 control vs both treatments; Figure 4B). Also, isolated AM was weakened by the medium produced as a result of cytokine (same doses) treatment of isolated choriodecidua (control 7.99 ± 3.51 N, TNF α 3.29 ± 2.27 N, IL-1 β 3.46 ± 1.84 N; n = 12, P < 0.001 control vs both treatments; Figure 4). Thus TNF α and IL-1 β must produce a soluble factor from the choriodecidua which is required for AM weakening.

Remodeling—TNF α (50 ng/ml) or IL-1 β (50 ng/ml) increased MMP9 protein and PARP cleavage, while decreasing TIMP3 protein in AM fragments that were incubated as part of full thickness FM, then separated for analysis. These changes were not seen if isolated AM was incubated with the cytokines. Similar changes were seen, in parallel with weakening, when isolated AM was treated with cytokine conditioned choriodecidua medium (Figure 4B).

LA Inhibits Thrombin Induced FM Weakness

Consistent with the dose response experiments, thrombin (10 u/ml) significantly weakened isolated AM relative to the control (3.12 ± 0.79 (thrombin alone) versus 9.11 ± 2.57 N (control); P < 0.001). However, pre-incubation with LA completely inhibited thrombin-induced AM weakening (3.12 ± 0.79 N [thrombin] versus 11.40 ± 5.2 N [LA pretreatment followed by thrombin]; P < 0.01; Fig. 4). Pre-incubation with LA without subsequent thrombin addition had no effect on the basal FM rupture strength (Figure 5). In parallel with the inhibition of thrombin-induced weakening of the isolated AM, LA inhibited the thrombin-induced increase in MMP9 (both pro and active forms), the decrease in TIMP3 (both 24 kD and 27 kD forms), and the increase in PARP cleavage (Figure 6).

Discussion

The major findings of these studies were: 1) Thrombin weakens isolated AM *in vitro*. Concomitant with the weakening, thrombin increases MMP9, decreases TIMP3, and increases PARP cleavage in the isolated AM, consistent with increased remodeling and apoptosis; 2) Cytokines do not weaken isolated AM, but do weaken the AM if it is exposed to the cytokines as a component of full thickness FM. Medium produced by cytokine-conditioned choriodecidua also weakens AM. Under conditions in which cytokines mediate AM weakening, there are concomitant increases in MMP9 and cleaved PARP, and decreases in TIMP3; 3) The antioxidant and NF κ B inhibitor, LA, blocks both thrombin-induced AM weakening as well as its remodeling effect: it inhibits the increased MMP9 as well as decreased TIMP3 expression, and inhibits enhanced PARP cleavage.

The mechanisms by which cytokines and thrombin weaken AM are different. Thrombin directly weakens isolated AM, to the same degree, and with the same concentration-dependence, that it weakens intact full thickness FM. This weakening effect is reproduced by the thrombin receptor (PAR1) agonist, TRAP6. Cytokines, on the other hand, do not weaken the isolated AM, but dramatically weaken AM when it is exposed to cytokines while adherent to the choriodecidua in intact full thickness FM. Thus cytokines must induce soluble factor(s) from the choriodecidua which are critical in mediating cytokine induced AM weakening. This conclusion is supported by the weakening effect of the cytokine conditioned choriodecidua medium upon the isolated AM.

The finding that measurable thrombin induced AM weakening required more than 24 hours is similar to our finding in two previous reports [21,23]. We assume this is because the major weakening effect of thrombin is indirect (through cell receptors and protein induction) rather than by directly activating MMPs already in the ECF. Significant weakening was demonstrable in full thickness FM explants only after 48 hours of incubation with proinflammatory cytokines (TNF α or IL-1 β) or with thrombin [21,23]. As direct effects should occur more quickly, we presume that any direct effect of thrombin is not sufficient to weaken the membrane. Also, LA completely blocks the weakening effects of both cytokines and thrombin, suggesting that thrombin works via cell receptors and protein induction, as LA is unlikely to inhibit a direct effect [22,23]. Finally, the Protease Activating Receptor Peptide for the thrombin activated PAR #1 (TRAP6) weakens isolated AM. This would clearly be an indirect effect if mediated by thrombin. Nevertheless, in addition to the indirect effects, some direct effect of thrombin on FM is probable based on the literature [33,34]. Thrombin has been shown, *in vitro*, to act directly on the ECM by converting MMPs 1, 2, 3, and 9 from zymogen to active forms [33,34].

The purpose of the studies in this report and many of our earlier studies [5–7,21,23] was to identify conditions under which the FM tissues weaken sufficiently to fail, and use these to develop *in vitro* model systems that would allow elucidation of the mechanisms of FM weakening and identification of points of intervention which could be used clinically to prevent PPRM. Initially, the physiological paracervical weak zone in term unlabored (repeat C-section) and also in labored FM [6,7] was characterized. The weak zone is readily identifiable prior to labor and has spots with rupture strengths that are one-tenth to one-half that of other (non-weak zone) areas of the same FM. In the weak zone, which has also been shown to be the likely spontaneous rupture initiation site, protein levels of MMP9 and cleaved PARP were consistently increased and that of TIMP3 were consistently decreased as assessed by Western blot [6,7]. These three proteins were selected as important markers of tissue remodeling and apoptosis because they were reliably present concomitant with weakened FM, not because they were specifically, or uniquely critical for the weakening process. Although MMP9, TIMP3 and apoptosis are likely involved in FM weakening, many other MMPs especially, collagenases, and other proteases, are also involved [4,35–39]. In addition, although we have used PARP cleavage as an indicator of apoptosis in our model system, a second or third indicator of the apoptotic process is customarily used to confirm involvement of this process.

In vitro model systems were developed using full thickness FM explants (AM with adherent choriodecidua) in which the major phenomenon of interest (FM weakening) could be measured in parallel with its associated biochemical changes in the FM tissue. The ideal model system for investigation of the FM weakening process would incorporate a physiological agent prominently seen in PROM and PPRM deliveries, with which FM weakening was induced *in vitro* with concurrent changes in the same marker proteins (MMP9, TIMP3 and cleaved PARP) that mimic those seen in the physiological weak zone. Two such model systems have been described: cytokine (TNF α or IL-1 β) and thrombin

induced weakening of full thickness FM explants *in vitro* [21–23]. In addition, we have shown that LA acid blocks both the FM weakening and the concomitant changes of the marker proteins in both models [22,23]. This further reinforces the connection between these specific protein markers and the weakening process. Lipoic acid, a common dietary supplement, may prove useful both as a probe to determine pathways of induced FM weakening, as well as a clinically useful agent in the prevention of PPRM related premature births.

The present studies demonstrate that isolated AM can be used as a model for weakening of the AM component of the FM. The AM is the strongest FM component and must be substantially weakened if rupture is to occur. The AM component weakens significantly during both thrombin and cytokine induced *in vitro* weakening of full thickness FM [21,23]. As demonstrated in this report, cytokines and thrombin both ultimately weaken the AM and concomitantly produce the same associated marker protein changes in the AM. Thrombin accomplishes this directly, whereas cytokines require choriodecidua as an intermediate. In addition LA blocks both the weakening and associated marker protein (again connecting these at the AM weakening level) changes. Finally the thrombin receptor agonist, TRAP6, weakens the isolated AM in lieu of thrombin.

There are difficulties and limitations imposed when working with full thickness FM explants large enough for biomechanical testing. The amount of decidua on pieces of full thickness FM is highly variable, tissue conditions near the center of the explants deteriorate in culture over time, and access to central tissue locations with pharmacological agents is difficult (high doses of activators are needed). Many techniques used with cell cultures are not possible in these large, full thickness FM explant studies. Use of isolated AM more closely parallels cell culture conditions (thin, relatively homogeneous membrane, single layer of epithelial cells on surface with embedded mesenchymal cells in the ECM) making more approaches feasible. In addition, the AM contains only two major cell types which can be easily put into primary culture. Activators shown to weaken the AM can be tested in the cells and intracellular pathways elucidated. Positive findings can then be confirmed on the AM. There are also disadvantages to this model. AM weakening clearly does not encompass all of FM weakening; weakening of the chorion also occurs and separation of the AM and choriodecidua contributes to FM weakening. However, these studies suggest that weakening in the AM can be studied separately, in detail, and perhaps form a foundation for detailed studies in more complicated multi-laminar models.

The specific markers for remodeling (MMP9 and TIMP3) and apoptosis (PARP cleavage) were chosen for analysis in the current study because they have been identified in our earlier reports as markers of the para-cervical weak zone in term FM of both pre-labor Cesarean patients [6] and term vaginally delivered patients [7]. (The other MMPs and TIMPs were not investigated in this report.) In the pre-labor Cesarean patients' FM, the expression of each of the three proteins was demonstrated to linearly correlate with FM strength in tested fragments [6]. TIMP3 protein expression in the remaining, non-weak zone, FM areas significantly increased in parallel with FM strength. This is particularly interesting as unlike the other TIMPs, TIMP3 inhibits all the gelatinases, inhibits TNF α , and is also stored within the ECM [39]. In contrast, when protein levels of TIMP1, TIMP2 and TIMP4 were evaluated in pre-labor cesarean FM, TIMP1 was found to be barely detectable while TIMP 2 & 4 proteins were detectable in all specimens but did not change with strength [6]. In addition, when full thickness FM were incubated *in vitro* with either TNF or IL-1 β , concomitant with weakening, all three protein markers (MMP9, TIMP3 and cleaved PARP) were altered in a manner similar to the pattern observed in the natural weak zone seen in term FM [21,22]. The experiments in this report now demonstrate that changes in these marker proteins are good indicators of AM weakening, whether due to thrombin or

cytokines. Parallel changes in this protein pattern and AM weakening, induced either directly by thrombin, or indirectly by cytokines, suggest that there is some overlap in the weakening pathways initiated by these agents.

LA blocked thrombin-induced weakening and remodeling of isolated AM. We did not examine the effect of LA upon cytokine action on isolated AM, as cytokines did not directly weaken the isolated AM. We have also previously reported that LA blocks the FM weakening effect of both thrombin and cytokines on full thickness intact FM [22,23]. LA continues to hold promise as both a pharmacological agent to probe the FM weakening pathways and a potential therapeutic agent to prevent PPROM. The mechanism(s) of LA inhibition of FM weakening is not known. LA has a number of antioxidant activities including scavenging reactive oxygen and nitrogen species, regeneration of other antioxidants, metal chelation, and induction of glutathione synthesis [40–46]. LA also modulates important signal transduction pathways including PKB/Akt-dependent signaling and redox-sensitive transcription factors including nuclear factor-kappa B (NF- κ B) and nuclear factor-erythroid2-related factor 2 (Nrf2) [46–48]. Presently it is uncertain which of the mechanism(s) of action of LA is responsible for preventing FM weakening. However, the anti-oxidant properties of LA may not be involved as the anti-oxidant Vitamin C failed to inhibit TNF α induced weakening in our model system [24]. Vitamin C has also proven ineffective in preventing PPROM in several multicenter randomized trials [49–51]. As LA inhibits thrombin induced weakening in the isolated AM, studies to clarify this are in progress.

In summary, thrombin and cytokines both weaken AM in the context of weakening the intact, full thickness FM *in vitro*. Concomitant with weakening the AM, these agents increase remodeling and apoptosis as indicated by increased MMP9, decreased TIMP3, and increased PARP cleavage. Thrombin can accomplish this by acting directly on the AM, whereas TNF α and IL-1 β appear to act indirectly by stimulating the choriodecidua to release soluble intermediate(s). These differential effects suggest that different pathways of FM weakening are initially involved. However, both cytokines and thrombin ultimately modify the same marker proteins of remodeling and apoptosis in AM in the process of their weakening the FM. Also, LA inhibits the weakening and concomitant remodeling effect of these agents. We speculate that there is overlap in the pathways of FM weakening in the weakening of the AM component. Complete characterization of the isolated AM model system presented here should allow identification and confirmation of any overlap. Targeting such an overlap may be clinically exploitable to prevent PPROM.

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Figure 1. Effect of Increasing Thrombin Dose upon AM Mechanical Properties

Thrombin (0–100 u/ml) induced concentration-dependent decreases in FM rupture strength. All incubations were for 48 h. Each data point shown represents the mean $\pm$ SD for four experiments (using different patient FM) with three replicates/experiment (N=12; *P < 0.001).

Figure 2. The Thrombin receptor (PAR1) activating peptide TRAP weakens Isolated AM
TRAP (1.0 μ M) induces significant weakening of cultured AM following 48 h incubation. Each data point shown represents the mean $\pm$ SD for three experiments (using different patient FM) with three replicates/experiment (N=9; *P < 0.001).

Figure 3. Effect of Increasing Thrombin Dose upon AM Remodeling and Apoptosis

Thrombin (0–100 u/ml for 48 h) induced increases in both pro-MMP9 (92 kD) and active MMP9 (78 kD) proteins (upper panel) and decreased TIMP3 (27 kD and 24 kD) proteins (middle panel) in isolated AM fragments in a dose-dependent manner. Thrombin (0–100 u/ml for 48 h) induced apoptosis in AM explants as evidenced by cleavage of PARP (113 kD) into 85 kD (and 24 kD; not shown) (lower panel) fragments. A: Representative western blot. B: To confirm the significance of qualitative results, blots were scanned and subjected to densitometric analysis. Each blot was normalized to the zero-thrombin controls as indicated in Methods. Data points represent normalized data from four experiments each with three replicates; mean $\pm$ SD; N=12, (* P < 0.01).

Figure 4. Cytokines Mediate AM Weakening Through Choriodecidua

A. Study Design: AM strength, MMP9 protein, TIMP3 protein and PARP cleavage were measured after incubation with control medium, 50 ng/ml TNF α , or 50 ng/ml IL-1 β under one of three conditions: 1. Full thickness FM were incubated for 72 h followed by separation and testing of the AM; 2. Isolated AM was incubated for 72 h and then tested; 3. Isolated choriodecidua was incubated for 24 h after which the medium was transferred to isolated AM in culture for an additional 72 h and then tested. B. Results: Upper panel: TNF α or IL-1 β decreased AM rupture strength if the AM was incubated together with choriodecidua directly in intact, full thickness FM (left) or through cytokine-conditioned medium from choriodecidua (right), but not if isolated AM was incubated with the cytokines (data are presented as mean $\pm$ SD; N=12, * P < 0.001). Lower panel: Representative western blots for MMP9, TIMP3 and Cleaved PARP are shown for the same conditions as Rupture Strength data in the upper panel. MMP9 and PARP cleavage increase and TIMP3 decreases in parallel with weakening.

Figure 5. LA inhibits Thrombin-induced Weakening

AM fragments were pre-incubated with or without LA (0.25 mM) for 6h, then incubated with or without the addition of Thrombin (10 u/ml) for 48h. Four groups are displayed: **C**ontrol, **T**hrombin alone, **LA** pre-treatment alone, **LA** pretreatment + **T**hrombin. Rupture Strength was determined as outlined in Methods. Data points represent pooled results of three experiments using three FM fragments per treatment group in each experiment (N=9). Data are presented as mean $\pm$ SD (* $P < 0.001$).

Figure 6. LA inhibits Thrombin induced remodeling in Isolated AM

A. Isolated AM fragments were pre-incubated with or without LA (0.25 mM) for 6h, then incubated with or without the addition of thrombin (10 u/ml) for 48h. Four groups are displayed: **Control**, **Thrombin alone**, **LA** pre-treatment alone, **LA** pretreatment + **Thrombin**. The Western blot shown is representative of three replicate experiments with three different placentas (N=9). Both the pro-MMP9 (92 kD) and the active MMP9 (78 kD) protein, as well as TIMP3 (27 kD and 24 kD) and cleaved PARP are shown. **B.** To confirm the significance of qualitative results, blots were scanned and subjected to densitometric analysis. Each blot was normalized to the zero-thrombin controls as indicated in Methods (Data are presented mean $\pm$ SD; N=9, * P < 0.05).