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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-alpha (TNFα) and interleukin-1-beta (IL-1β), remodel and weaken full thickness FM. Additionally, we have reported that the anti-oxidant and NFκB 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, TNFα, or IL-1β, for 48 h. 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 TNFα nor IL-1β 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.

1. 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 PPROM [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 PPROM, 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 PPROM 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 PPROM [13]. Decidual cells express abundant tissue factor (TF), a transmembrane 45 kDa glycoprotein. Vascular injury initiates

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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 PPROM 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 PPROM. 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 PPROM [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 (TNFα and IL-1β) 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, TNFα and IL-1β 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.

2. Methods

2.1. 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).

2.2. 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 pre-labor, 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 1 cm 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 3 cm × 3 cm 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.

2.3. Explant culture

AM fragments measuring 3 cm × 3 cm (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 100 mm^2 culture dishes containing 10 ml of Minimum Essential Medium, alpha modification (MEM), antibiotic antimycotic solution, 50 mg/L gentamicin sulfate, and 0.2% lact-albumin hydrolysate (EMEML). Culture dishes were rocked gently in an atmosphere containing 5% CO_2, air and 100% relative humidity at 37 °C. After 24 h of equilibration in EMEML, medium was removed and replaced.

2.3.1. 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.

2.3.2. 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 TNFα, 50 ng/ml IL-1β) were utilized in the protocol diagrammed in Fig. 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 15000 × g/30 m/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.

2.3.3. 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 pre-treatment alone; 3. 10 u/ml thrombin alone; and 4. LA pre-treatment 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.

2.4. 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