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Proinflammatory Cytokines Found in Amniotic Fluid Induce Collagen Remodeling, Apoptosis, and Biophysical Weakening of Cultured Human Fetal Membranes[1]

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ABSTRACT

The mechanisms by which fetal membranes (FM) rupture during the birth process are unknown. We have recently reported that FM weaken, at least in part, because of a developmental process of extracellular matrix remodeling and apoptosis. We now hypothesize that cytokines that normally increase in amniotic fluid at term induce FM collagen remodeling and apoptosis with concomitant weakening. Full-thickness FM fragments were cultured with (0–100ng/ml) or without tumor necrosis factor (TNF) or interleukin 1, beta (IL1B). Physical properties were then examined with specially adapted industrial rupture strength testing equipment. Cultured FM were also evaluated for evidence of collagen remodeling and apoptosis. Cytokine-treated FM exhibited a dose-dependent decrease in strength and work to rupture. Compared with controls, the highest TNF dose caused maximal decrease in FM rupture strength (13.2 ± 1.2 N versus 3.8 ± 1.5 N; $P = 0.0003$) and work to rupture (0.035 ± 0.005 J versus 0.005 ± 0.002 J; $P < 0.0001$). The highest IL1B dose also decreased rupture strength (12.9 ± 3.2 versus 4.6 ± 1.1 N; $P = 0.0027$) and work to rupture (0.018 ± 0.005 J versus 0.005 ± 0.002 J; $P < 0.01$). Matrix metalloproteinase 9 (MMP9) protein increased, tissue inhibitor of matrix metalloproteinase 3 (TIMP3) protein decreased, and poly (ADP-ribose) polymerase (PARP1) cleavage increased with increasing TNF or IL1B doses (all $P < 0.05$), suggesting collagen remodeling and apoptosis. TNF and IL1B cause significant weakening of cultured FM. Both cytokines induce biochemical markers in the FM in a manner characteristic of the weak zone of FM overlying the cervix. TNF and or IL1B may be involved in the development of the weak zone of the FM.

apoptosis, cytokines, parturition, placenta

INTRODUCTION

Fetal membrane (FM) rupture is an essential part of the delivery process and usually follows the onset of uterine contractions. Rupture of membranes (ROM) precedes the initiation of uterine contractions in at least 10% of term and nearly 30% of premature births. We have postulated that the FM weaken during gestation as a result of a combination of two processes: a programmed biochemical set of events leading to collagen remodeling and apoptosis, and superimposed physical stretch forces leading directly to tissue damage [1]. The physiological mediators that normally initiate the processes leading to weakening and rupture of FM are unknown.

Cytokines and prostaglandins (PGs) have been implicated in tissue remodeling and apoptosis in many tissues. The amniotic fluid concentrations of cytokines such as TNF and IL1B increase during labor [2, 3]. Upregulation of matrix metalloproteinases during labor and FM rupture [4, 5] has been speculated to be cytokine mediated. TNF and IL1B, specifically, have been shown to induce apoptosis and increase PGE_2 production in chorioamnion and cultured primary amnion cells [6–10]. Apoptosis, noted in animal [11] and human FM studies [12, 13], has been speculated to be a significant contributor toward membrane weakening before rupture.

We have previously shown that several physiological agents and reactive oxygen species (hydrogen peroxide) induce apoptosis with concomitant PGE_2 release in amnion derived WISH cells, primary amnion cells, and intact amnion [14, 15]. More recently, we have identified the development of a weak zone in the FM region overlying the cervix before labor. This weak zone exhibits distinct histological characteristics, evidence of collagen remodeling, and increased apoptosis [1].

The mechanisms leading to the development of this weak zone with its associated biochemical findings are unknown. We hypothesized that cytokines that cause apoptosis and inflammatory reactions in cells derived from FM (TNF and IL1B) will cause physical and biochemical changes that we observed in the weak zone of FM.

MATERIALS AND METHODS

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 (37–42 wk) following a written informed consent. Membranes were discarded if they were meconium stained, if infection was suspected from clinical history, or if chorioamnionitis was detected in pathological review. Each FM was sectioned into multiple pieces using our previously reported methodology (see the following discussion). Full-thickness fragments were washed briefly in Hanks balanced salt solution (HBSS, pH 7.4) and then incubated with or without TNF or IL1B as described here. Samples from all specimens were submitted for pathological review.

Materials

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless specified otherwise.

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Membrane Cutting and Topographical Mapping

Relatively homogeneous FM specimens were required to carry out the experiments in this report. Because FMs are heterogeneous in histological, biochemical, and physical characteristics over their surface—and most particularly because there is a weak zone in the FM overlying the cervix—we employed our previously published methodology to enable us to identify homogeneous regions of the FM (1). Specifically, a circular area 10 cm in diameter centered on the weakest point along the tear line was used to define the weak zone [1] of each FM. Membrane pieces from within the weak zone and within 1 cm of the placental rim were excluded from further analysis.

Tissue Culture

FM fragments were cultured as per previously described protocols [14, 16]. FM fragments were bluntly dissected (8×8 cm) and placed in 150-mm^2 culture dishes containing 20 ml Earles Minimum Essential Medium, alpha modification, antibiotic antimycotic solution, 50 μg/ml gentamicin sulfate, and 10% fetal calf serum (FBS). Culture dishes were rocked gently in an atmosphere containing 5% CO_2, air, and 100% relative humidity at 37°C. After 24 h equilibration, medium was removed and replaced with 20 ml serum-free medium without (controls) or with increasing (0–100 ng/ml) concentrations of TNF or IL1B for a period of 72 h.

Physical Testing

After various time periods, control and treated FM fragments were removed from culture, washed twice in 20 ml HBSS, maintained in 50 ml HBSS, and kept moist for the entirety of strength testing. FM physical properties were determined using our previously reported methodology [1]. Briefly, the biophysical testing was performed using modified industrial rupture testing equipment (Com-Ten industries) by ASTM (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 FM) and resultant FM displacement data were collected continuously and analyzed by data reduction software. Physical properties (rupture strength and work to rupture) were determined from generated force/displacement curves.

Western Blotting

Sample homogenization and gel electrophoresis were performed as per previously described methodology [1]. MMP9, TIMP3, and PARP1 cleaved fragments were primarily investigated because they have been found to be characteristically different in the identified physiological weak zone of fetal membranes overlying the cervix [1]. Antibodies used were as follows: MMP9 (Oncogene Research Products, San Diego, CA; 1:100), TIMP3 (CalBiochem, San Diego, CA; 1:25), or PARP1 (Chemicon, Temecula, CA; 1:100). Standards for MMP9 and TIMP3 were from CalBiochem; no reliable SDS-PAGE standard is available for cleaved PARP1. Gel lanes were loaded based on protein and assessed by blotting for tenascin C, an extracellular matrix protein that we found did not change with TNF or IL1B.

Statistical Analyses

Differences in physical and biochemical properties between control and treated FM were compared using paired and unpaired Student *t*-test or ANOVA as appropriate, with two tailed P value < 0.05 considered statistically significant. All experiments were performed at least three times.

To quantitate Western blotting results for MMP9, TIMP3, and PARP1 cleavage response to TNF and IL1B, densitometric analysis was undertaken. Quantitative values for Western blots were obtained by densitometry using Image J version 1.29x software (W. Rasband, National Institutes of Health, Bethesda, MD). The MMP9 antibody recognizes both the pro form at 92 kDa and the intermediate form at 83 kDa. The 83-kDa band was analyzed. The TIMP3 antibody recognizes the glycosylated form at 30 kDa and the unglycosylated form at 24 kDa. The 24-kDa band was analyzed. The cleaved PARP1 antibody recognizes the 85-kDa cleaved fragment, which was analyzed. Normalization was done to compare results between blots. Each blot was performed with tissue from a single fetal membrane; thus, different pieces of the same patient's fetal membrane were exposed to different cytokine concentrations and run on the same blot. The densitometer readings for controls (zero cytokine exposure) from different patients varied significantly, likely because of baseline differences in tissue from patient to patient and film exposure differences. To compensate for these, we divided the densitometer readings obtained for the cytokine exposed lanes in a given blot by the densitometer reading of the control lane (no cytokine) for that same blot. Data analysis between blots was then done using these normalized values.

RESULTS

Fourteen FM were obtained from patients undergoing elective repeat cesarean delivery without labor at term. Of the 14 specimens, five were discarded because the membranes were excessively torn or there was evidence of meconium staining.

Effect of TNF on FM Strength

TNF induced FM weakness in a dose- (Fig. 1) and time- (Fig. 2) dependent manner. Increasing doses (0.1–100 ng/ml) of TNF caused a stepwise decrease in both strength and work required to rupture the FM. Compared to controls, maximal induced FM weakness, as evidenced by rupture strength (13.2 ± 1.2 N versus 3.8 ± 1.5 N; $P = 0.0003$) and work to rupture (0.035 ± 0.005 J versus 0.005 ± 0.002 J; $P < 0.0001$), was observed with the highest TNF dose (100 ng/ml) used (Fig. 1). No effect of TNF (50ng/ml) on FM strength was demonstrable at 24 h. The maximal effect of TNF treatment on FM strength occurred by 48 h (Fig. 2).

TNF Induced Markers of Collagen Remodeling and Apoptosis

TNF induced collagen degradation/remodeling in the FM, as evidenced by induction of MMP9 protein and parallel suppression of TIMP3 protein. Following 72 h incubation with TNF (0–100 ng/ml), FM explants exhibited increased

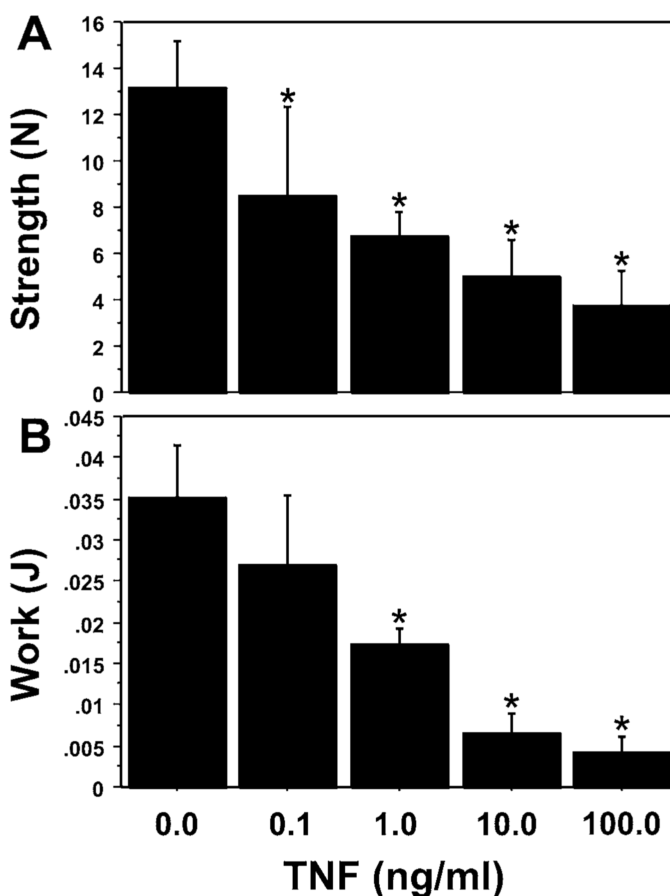

FIG. 1. TNF dose response. TNF-induced dose-dependent decreases in FM strength (**A**) and work (**B**) to rupture. All incubations were for 72 h. The data shown represent triplicate FM cultures for one representative experiment repeated three times using three different placentas (data are presented as mean ± SD, * $P < 0.01$).

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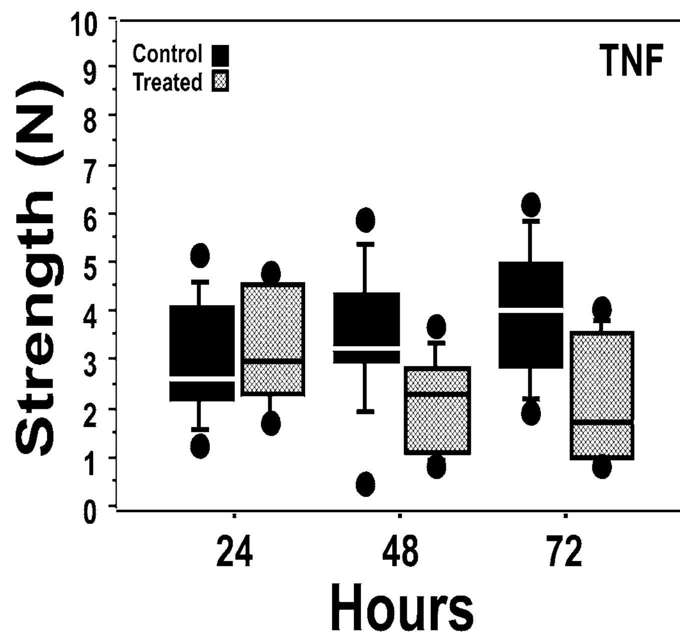

FIG. 2. TNF time course. TNF (50 ng/ml) treatment of FM explants induced weakness over 48 h but further continued incubation (to 72 h) demonstrated no additional effect. Three-day incubation of control FM explant cultures did not affect FM rupture strength. The data shown represent triplicate FM cultures for one representative experiment repeated three times using three different placentas. The data are presented in standard box plot form in which the interior horizontal line represents the median; the upper and lower box edges represent the 75th and 25th percentiles, respectively; and the upper and lower error bars represent the 90th and 10th percentiles, respectively. Outliers above and below the latter are represented as dots.

MMP9 protein and decreased TIMP3 protein in a dose-dependent manner (Fig. 3). FM explants incubated with TNF (0–100 ng/ml) for 72 h also exhibited dose-dependent increase in cleavage of PARP1, suggesting dose-dependent increase in apoptosis (Fig. 3).

Effect of IL1B on FM Strength

IL1B induced FM weakness in a dose- (Fig. 4) and time- (Fig. 5) dependent manner. Although increasing doses (0–100 ng/ml) of IL1B caused a decrease in strength and work required to rupture the FM, a minimal dose of 10 ng/ml was required to induce an effect. IL1B concentrations of 1 ng/ml had no effect on FM strength and work to rupture. Compared to controls, induced FM weakness, as evidenced by rupture strength (12.9 $\pm$ 3.2 versus 4.6 $\pm$ 1.1 N; $P = 0.0027$) and work to rupture (0.018 $\pm$ 0.005 J versus 0.005 $\pm$ 0.002 J; $P < 0.01$), was demonstrated with the highest IL1B dose (100 ng/ml) used (Fig. 4). IL1B (50 ng/ml) had no effect on FM strength after 24 h of incubation. The maximal effect of IL1B treatment on FM rupture occurred by 48 h (Fig. 5).

IL1B Induced Collagen Remodeling and Apoptosis

IL1B induced collagen degradation/remodeling in the FM, as evidenced by induction of MMP9 and an associated suppression of TIMP3. Following 72 h incubation with IL1B (0–100 ng/ml), FM explants exhibited increased MMP9 protein and decreased TIMP3 protein in a dose-dependent manner (Fig. 6). FM explants incubated with IL1B (0–100 ng/ml) for 72 h exhibited a dose-dependent increase in cleavage of PARP1, suggesting dose-dependent apoptosis (Fig. 6).

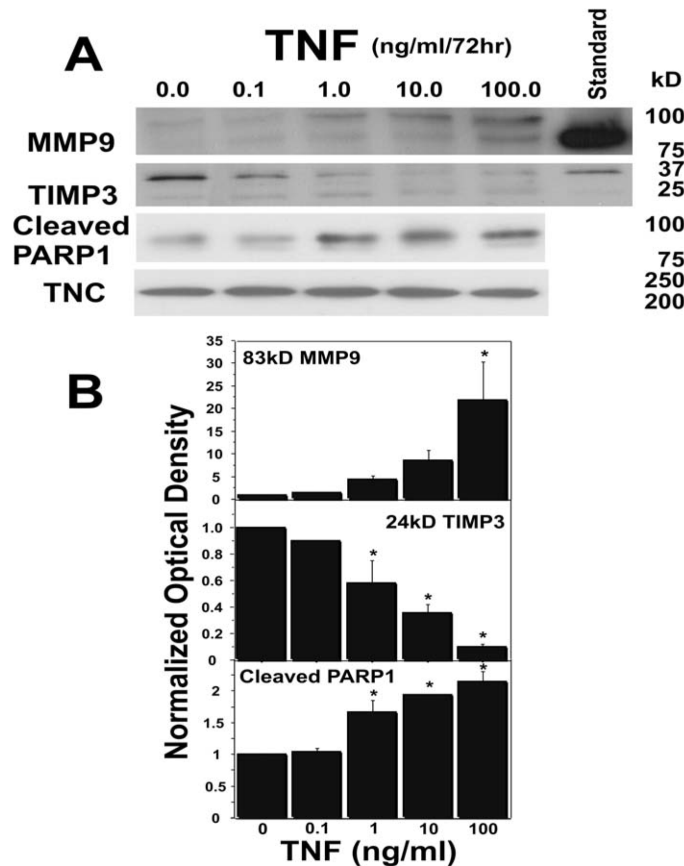

FIG. 3. **A**) TNF (0–100 ng/ml for 72 h) induced increases in MMP9 protein (upper panel) and decreased TIMP3 protein (middle panel) in FM explants in a dose-dependent manner. TNF (0–100 ng/ml for 72 h) induced apoptosis in FM explants as evidenced by cleavage of PARP1 (113 kD) into 85 kDa (and 24 kDa; not shown) (lower panel) fragments. The upper section (**A**) of the figure is a representative illustration of three replicate experiments. To confirm the significance of qualitative results, blots of FM of three different patients were scanned and subjected to densitometric analysis (**B**). The specific band subjected to densitometic analysis is indicated in the figure. Tenascin C (TNC) was used as a loading control. Each blot was normalized to the zero-cytokine controls as indicated in Methods (data are presented as mean $\pm$ SD, * $P < 0.05$).

DISCUSSION

Our data clearly demonstrate that two proinflammatory cytokines, TNF and IL1B, that physiologically increase toward end of pregnancy and during labor cause significant FM weakening. Furthermore, TNF and IL1B cause FM weakness through a process involving collagen remodeling and apoptosis. Significantly, the pattern of FM weakness associated with collagen remodeling and apoptosis demonstrated with TNF and IL1B exposure is similar to that which we have previously reported regarding the weak zone of FM overlying the cervix before labor at term [1].

Proinflammatory cytokines such as TNF and IL1B normally increase in the amniotic fluid with advancing gestation, and particularly with onset of labor, presumably as a result of increased diffusion from FMs where higher tissue production locally increases tissue cytokine concentration. During normal pregnancy, these cytokines have an important impact on placental growth and development [17]. Cytokine elevations during normal-term pregnancy are modest compared to those observed with infection-associated preterm deliveries, however. Excessive amounts of cytokine production resulting in high FM tissue cytokine levels during labor and premature delivery

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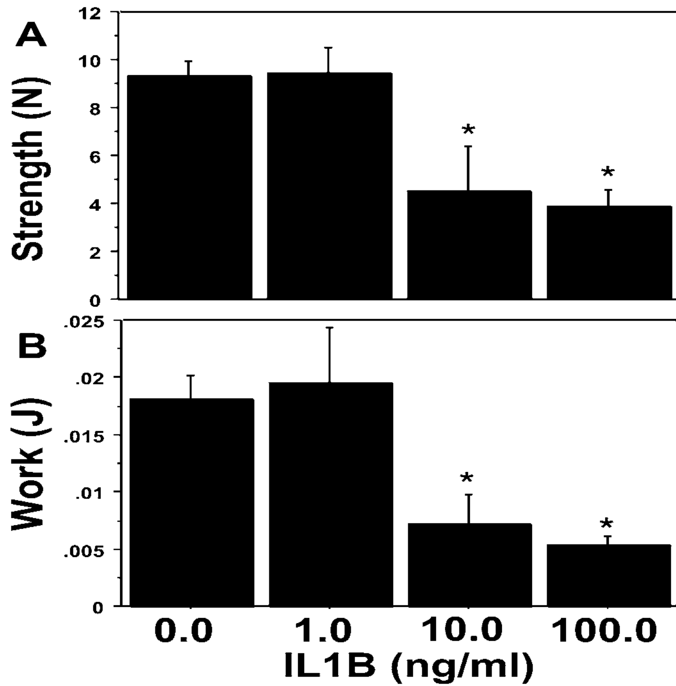

FIG. 4. IL1B dose response: IL1B-induced dose-dependent decreases in FM strength (**A**) and work (**B**) to rupture. All incubations were for 72 h. The data shown represent triplicate FM cultures for one representative experiment repeated three times using three different placentas (data are presented as mean ± SD, * $P < 0.01$).

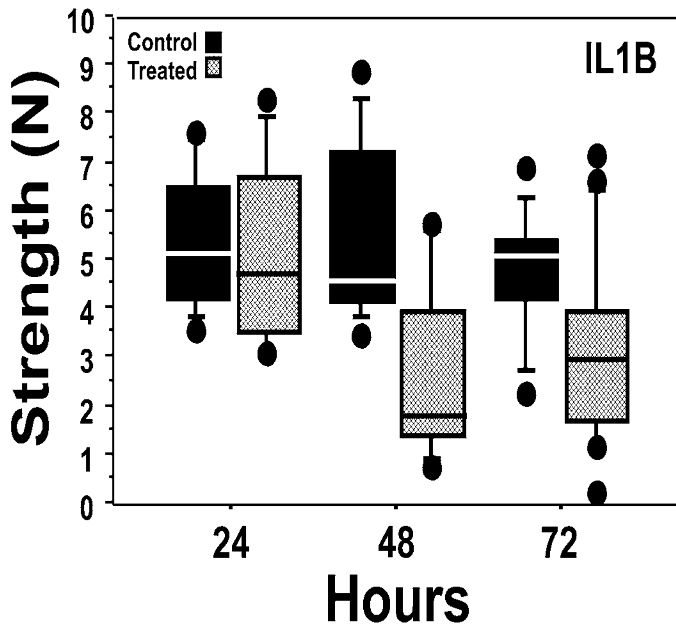

FIG. 5. IL1B time course: IL1B (50ng/ml) treatment of FM explants induced weakness over 48 h but further continued incubation (to 72 h) demonstrated no additional effect. Three-day incubation of control FM explant cultures did not affect FM rupture strength. The data shown represent triplicate FM cultures for one representative experiment repeated three times using three different placentas. The data are presented in standard box plot form in which the interior horizontal line represents the median; the upper and lower box edges represent the 75th and 25th percentiles, respectively; and the upper and lower error bars represent the 90th and 10th percentiles, respectively. Outliers above and below the latter are represented as dots.

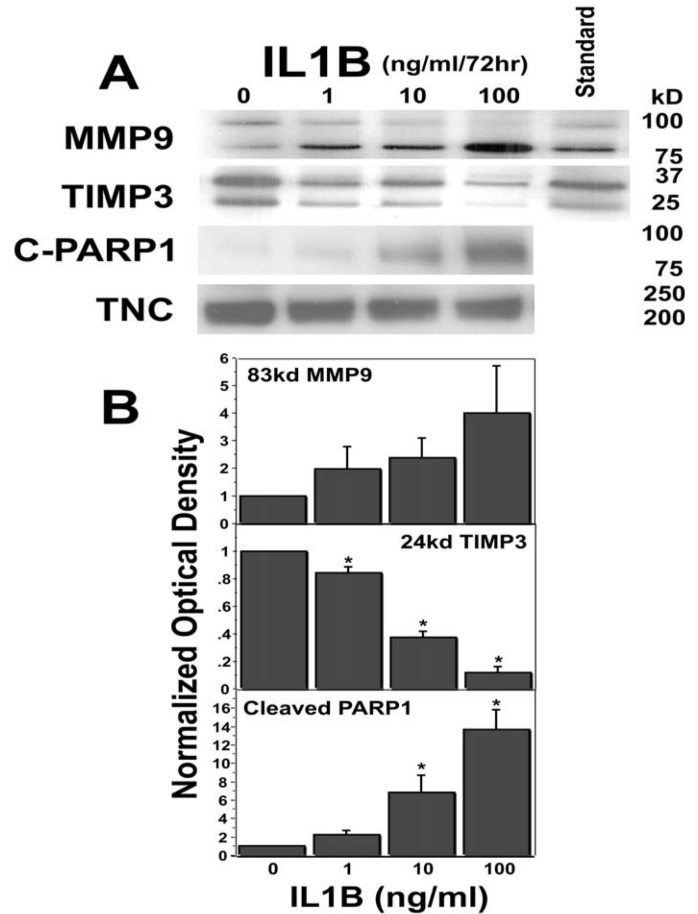

FIG. 6. **A**) IL1B (0–100 ng/ml for 72 h) induced increased MMP9 protein (upper panel) and decreased TIMP3 protein (middle panel) in FM explants in a dose-dependent manner. IL1B (0–100 ng/ml for 72 h) induced apoptosis in FM explants as evidenced by dose-dependent cleavage of PARP1 (113 kDa) into 85 kDa (and 24 kDa; not shown) (lower panel) fragments. The upper section (**A**) of the figure is a representative illustration of three replicate experiments. To confirm the significance of qualitative results, blots of FM of three different patients were scanned and subjected to densitometric analysis (**B**). The specific band subjected to densitometic analysis is indicated in the figure. Tenascin C (TNC) was used as a loading control. Each blot was normalized to the zero-cytokine controls as indicated in Methods (data are presented as mean ± SD, * $P <$ 0.05).

is believed to be, at least in part, responsible for FM weakening and eventual rupture [18, 19]. Although FM cytokine tissue levels were not measured in these experiments and thus a direct in vivo comparison is not possible, cytokine involvement in FM weakening and rupture is supported by our findings that TNF and IL1B cause dose- and time-dependent weakness in FM explants.

Preterm premature rupture of membranes (PPROM) frequently occurs in the setting of infection or inflammation [20, 21]. McGregor et al. demonstrated that both pathogenic and commensal bacteria residing in the genital tract produce proteases and suggested a possible role for genital bacteria in PPROM. They speculated that protease production by female genital tract microorganisms may alter or inactivate a variety of proteins responsible for host defense and structural integrity of collagen-containing chorioamniotic membranes [22]. Bacterial collagenase and collagenase-producing bacteria significantly reduced FM strength and elasticity in vitro [23–25]. Suppression of bacterial growth or collagenase production by antibiotics prevented bacterially induced FM weakening [25].

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Since PPROM can occur following infection with non-collagenase-producing bacteria and despite exposure to effective antibiotic therapy [25], the host inflammatory response following exposure to microorganisms is likely an important mediator of PPROM in some cases [26].

Fortunato and Menon have hypothesized that PPROM is an endogenous autotoxic disease involving activation of extracellular matrix metalloproteinases (MMPs) by the host inflammatory response [27]. They have further hypothesized that the endogenous activation of MMPs leads to extracellular matrix degradation and weakening of membranes, predisposing them to rupture. MMPs are a family of enzymes that hydrolyze and degrade collagen, the main strength-bearing component of extracellular matrix in the FM. The regulation of MMP activity is complex and involves proenzyme activation and suppression by specific tissue inhibitors of metalloproteinases (TIMPs). Several investigators have demonstrated a role for MMPs in ROM in normal labor and PPROM. A dynamic balance between MMPs and TIMPs is thought to facilitate tissue remodeling of the FM to accommodate fetal growth [28, 29]. However, an imbalance in favor of the MMPs over TIMPs is associated with labor and FM rupture [4, 5]. Fortunato and Menon have shown an infection-associated imbalance in the MMP/TIMP ratio increasing bioavailability of MMPs in the amniotic cavity during infection [29]. They have demonstrated a similar imbalance of MMPs over TIMPs in FM in response to bacterial toxins [30]. IL1B and TNF increase MMP and decrease TIMP production from chorion cells in vitro [31]. Proinflammatory cytokines are speculated to be involved in MMP activation during labor and FM rupture. Rupture of FM during labor or PROM has been associated with the expression and activation of MMPs and increased apoptosis. MMP9 expression is believed to be one of the first biochemical events during labor [32, 33]. It is increased in amnion and choriodecidua to levels that correlate with increased interleukin 8 (IL8) concentrations observed during later stages of cervical dilatation [34]. MMP9 activity is increased [35] and TIMP3 is decreased [33] in FM associated with PPROM. IL8, a chemotactic cytokine, is important for processes of cervical ripening and rupture of FM because of its role in neutrophil recruitment-activation and release of matrix remodeling enzymes including MMPs. Mechanical stretching of FM induces increased collagenase activity and IL8 secretion [36, 37]. Both TNF and IL1B are known to increase IL8 production in amnion and choriodecidua [38]. Our data demonstrating TNF- and IL1B-induced increased expression of MMP9 and suppression of TIMP3 in association with FM weakness support these observations.

In addition to MMP9 activation, increased levels of TNF and IL1B during pregnancy may induce FM weakness through the process of apoptosis. TNF and IL1B have been shown to induce apoptosis and increase PGE_2 production in chorioamnion and cultured primary amnion cells. In PPROM-associated FM, expression of proapoptotic genes, bax and p53, is increased, while expression of the antiapoptotic gene, bcl-2, is decreased [39]. TNF is known to induce trophoblast apoptosis in collaboration with interferon gamma [10]. Apoptosis in rat [11] and human FM has also been well documented [12, 13] and has been speculated to be a significant contributor toward membrane weakening before rupture. In our previous report we have identified, in prelabor cesarean deliveries, the presence of a weak zone of FM overlying the cervix. This zone exhibits distinct histological changes, collagen remodeling as evidenced by increased MMP9 and decreased TIMP3, and increased apoptosis as characterized by increased PARP1 cleavage [1]. This is supported by our present data demonstrating that TNF and IL1B induce dose- and time-dependent increases in apoptosis, as evidenced by increased PARP1 cleavage, in FM explants. PARP1 is a conserved nuclear enzyme implicated in DNA repair and in the apoptosis response and is one of the main cleavage targets of caspase3.

We have recently demonstrated that amnion separates from choriodecidua as part of FM rupture during in vitro experiments. The FM becomes significantly weaker as a result of this separation [40]. During the present series of experiments, no spontaneous separation of amnion from choriodecidua was seen in the FM that had been weakened by TNF and IL1B. Hence, separation of amnion from choriodecidua does not appear to be part of cytokine-mediated weakness.

In this report, we demonstrate for the first time that TNF- and IL1B-induced collagen remodeling and apoptosis correlate directly with FM physical weakness. This provides support for the theory that, before term birth, FM undergo a developmental process of remodeling that weakens them in preparation for rupture at the time of labor.

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