Inflammation, but not infection, induces EMT in human amnion epithelial cells

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Abstract

A non-reversible state of epithelial to mesenchymal transition (EMT) at term accumulates proinflammatory mesenchymal cells and predisposes fetal membrane to weakening prior to delivery at term. We investigated the induction of EMT in amnion epithelial cells (AEC) in response to inflammation and infection associated with spontaneous preterm birth (SPTB). For this, membranes from SPTB were screened for EMT markers. Primary AEC in culture were treated with TNF- α (10 and 50 ng/mL) and LPS (50 and 100 ng/mL) for 72 h. Cell shape index (SI) was determined based on morphological shift (microscopy followed by ImageJ software analysis). Immunocytochemistry and Western blot assessed changes in epithelial markers (cytokeratin-18 and E-cadherin) and mesenchymal markers (vimentin and N-cadherin). Involvement of transforming growth factor beta (TGF- β) in EMT induction and EMT associated inflammation was tested using specific markers (Western blot) and by measuring MMP9 (ELISA), respectively. We report that PTB is associated with fetal membrane EMT. TNF- α produced dose- and time-dependent induction of EMT; within 24 h by 50 ng/mL and after 72 h by 10 ng/mL. AEC showed mesenchymal morphology, lower E-cadherin, higher vimentin and N-cadherin and higher MMP9 compared to control. TNF- α -induced EMT was not associated with canonical TGF- β pathway. LPS, regardless of dose or time, did not induce EMT in AEC. We conclude that PTB with intact membranes is associated with EMT. Our data suggest that inflammation, but not infection, is associated with non-canonical activation of EMT and inflammation that can predispose membrane to undergo weakening.

Reproduction (2020) **160** 627–638

Introduction

Preterm birth (PTB, birth before the 37th week of gestation) is the leading cause of neonatal morbidity and mortality (Beck *et al.* 2010). Spontaneous preterm birth (SPTB), either with intact membranes or with preterm prelabor rupture of the fetal membranes (pPROM), represents 65–70% of all cases (Andrews *et al.* 2000, Menon & Fortunato 2007, Romero *et al.* 2014a). PTB etiology is not completely understood; however, infection and the infection-induced host inflammatory response are major factors considered as triggers of preterm labor (Srinivas & Macones 2005, Bastek *et al.* 2011, Romero *et al.* 2014a, Behnia *et al.* 2016). PTB is also associated with non-infectious (sterile) inflammation due to various other maternal risk exposures (Romero *et al.* 2014b, Behnia *et al.* 2016, Keelan 2018, Menon 2019). Although risk exposure is often at the maternal level, the fetal inflammatory response along with maternal inflammation is a major effector of the preterm labor pathway (Goepfert *et al.* 2004, Agrawal & Hirsch 2012,

Menon & Taylor 2019). The precise mechanisms of fetal inflammatory response leading to preterm labor are still being investigated. Our laboratory has been investigating the role of mechanisms leading to the fetal inflammatory response using fetal membranes (amniochorionic membranes) as a model system (Menon & Moore 2020).

Human fetal membranes are the innermost lining of the intraamniotic cavity (Menon *et al.* 2019). These membranes protect the fetus during pregnancy by functioning as both a mechanical and immune barrier (Kumar *et al.* 2016, Richardson *et al.* 2020a). The membranes undergo a systematic rebuilding process, where both cells and extracellular matrix (ECM) are constantly remodeled to strengthen the tissue (Lei *et al.* 1995, Athayde *et al.* 1999, Fortunato *et al.* 1998, 2003, Strauss 2013). Membranes are composed of single layer of cuboidal amnion epithelial cells (AEC) and multiple layers of chorion trophoblast cells that are connected by a collagen-rich ECM containing disbursed mesenchymal cells (Menon *et al.* 2019). The amnion layer is highly elastic and is able to withstand constant stretching due

to the increasing volume of pregnancy and constant changes in the biochemical environment of the amniotic fluid. AEC are shed throughout pregnancy and the membranes develop biologic microfractures characterized by the loss of cells and degradation of the basement membrane that connects amnion cells to the ECM as well as ECM collagen (Richardson *et al.* 2017). However, these microfractures do not persist as they are remodeled to maintain the integrity of the amnion layer (Richardson *et al.* 2017). At term, amnion membranes undergo an inflammatory transition due to two key factors: (1) senescence (a mechanism of aging) of the amnion cells and the release of senescence-associated secretory phenotype (SASP) and senescence-associated inflammation (Behnia *et al.* 2015, Jin *et al.* 2018, Lavu *et al.* 2019, Menon 2019) and (2) epithelial–mesenchymal transition (EMT) of AEC, with the accumulation of highly inflammatory mesenchymal cells in the membrane matrix, causing localized production of proinflammatory mediators (Richardson *et al.* 2020b). These proinflammatory mediators further degrade and weaken the membranes, resulting in the loss of mechanical and structural function and integrity. Membrane inflammation is propagated to feto-maternal interface tissues by extracellular vesicles that can further enhance the overall inflammatory load of the uterine tissues, priming them for parturition (Menon 2019). The generation of a proinflammatory state in fetal membranes can be considered as one of the fetal signals that initiate parturition.

Our studies have shown that both senescence and EMT in amnion membranes share similar mechanisms. Membranes from term deliveries, *in vivo* animal models and *in vitro* primary AEC models have shown that increased oxidative stress (OS) can accelerate senescence and increase EMT (Richardson *et al.* 2020b). OS experienced at term in the uterine cavity increases the stress signal p38 mitogen-activated kinase (MAPK) (Menon & Papaconstantinou 2016) and endogenous production of transforming growth factor- β (TGF- β) (Richardson *et al.* 2020b), an inducer of EMT. Regulation of OS and/or reducing p38MAPK activation and TGF- β production can minimize both senescence and EMT (Richardson *et al.* 2020b). There is evidence that these processes are linked, but it is still unclear whether these mechanisms are interdependent. Our studies have shown that cyclic EMT and MET (mesenchymal to epithelial transition) are essential for the maintenance of amnion membrane integrity. A terminal state of EMT (or lack of MET) due to increasing OS in the uterine cavity compromises membrane homeostasis at term. Both these mechanisms are considered as normal and physiological processes required to generate parturition associated with fetal inflammatory signals (Richardson *et al.* 2020b).

Although the contributions of senescence and SASP have been reported in PTB (Menon *et al.* 2014, Poletini

et al. 2015, Dutta *et al.* 2016), the contributions of EMT to the PTB pathway is unclear. OS and inflammation are inseparable components of a cascade of events, resulting either in tissue remodeling or tissue destruction during physiological or pathological processes, respectively. Balanced changes in the redox state and inflammation are required for feto-placental growth during pregnancy. Therefore, it is critical to know how an imbalance in inflammation, either through infectious or sterile risk exposure, can arise prematurely and how this contributes to amnion membrane pathology and PTB. We hypothesize that balanced cellular transition maintains fetal membrane homeostasis during pregnancy, but overwhelming OS at term or an infectious (LPS) or inflammation (TNF- α) stimuli at preterm can cause EMT and localized inflammation that can promote membrane dysfunctions. Since senescence and SASP have been reported previously in term and PTB membranes, the objectives of this study were (1) to determine an association between EMT and PTB and (2) to determine if proxies for infection (lipopolysaccharide (LPS)) and inflammation (TNF- α) can induce EMT in AECs and the mechanistic pathway of EMT development.

Materials and methods

Institutional review board approval

Placentas for this study were collected from John Sealy Hospital (University of Texas Medical Branch (UTMB) in Galveston, Texas), according to the inclusion and exclusion criteria defined by our laboratory (see below). As discarded placentas were used after delivery for the study, subject recruitment or consent was not required. The Institutional Review Board (IRB) at UTMB approved the study protocol, and placentas were collected according to the regulations of the IRB as an exempt protocol that allowed the use of discarded placentas (UTMB 11-251). Under the same protocol, fetal membrane samples were also collected from cases of spontaneous preterm birth (SPTB) and preterm prelabor rupture of the membranes (pPROM). According to UTMB OB&GYN clinical practice standards, placentas collected from such cases are not subjected to histopathologic evaluation (e.g. chorioamnionitis or funisitis) and are therefore considered to be discarded samples with no identifiers. Studies using these samples were limited to determining receptor expression changes as the clinical data collected were minimal. Correlative analyses between receptor expression and subject clinical status were not performed, as these samples were de-identified. The inclusion criteria were normal term birth from women with term labor (TL) and delivery from elective cesarean delivery (between 37 and 41 weeks of gestation) prior to the onset of labor. Subjects with multiple gestations, placenta previa, fetal anomalies and/or medical or surgical interventions for clinical conditions not linked to pregnancy during pregnancy were excluded. Severe cases of preeclampsia or persistent symptoms (headache, vision changes, right upper quadrant pain), abnormal laboratory findings (thrombocytopenia, repeated abnormal liver function tests, creatinine doubling

or >1.2, or HELLP syndrome) or clinical findings (pulmonary edema or eclampsia) were excluded. Subjects who had any surgical procedure during pregnancy or who were treated for hypertension, preterm labor or for suspected clinical chorioamnionitis (reports of foul-smelling vaginal discharge, fetal tachycardia), positive Group B *Streptococcus* (GBS) screening or diagnosis of bacterial vaginosis, behavioral issues (cigarette smoking, drug or alcohol abuse). For fetal membranes samples from preterm deliveries, subjects who delivered at term following preterm labor were excluded from the control group.

Clinical samples

Fetal membranes were collected (amniochorion full thickness membranes) from the following categories of patients: preterm prelabor rupture of membranes (pPROM; $n=16$) with a mean gestational age of 32 weeks resulting in preterm delivery, the samples were gestationally grouped as before ($n=8$) or after 34 weeks ($n=8$), spontaneous preterm labor leading to preterm birth (SPTB; $n=17$) with a mean gestational age of 34 weeks, and elective cesarean delivery (between 37 and 41 weeks of gestation) prior to the onset of labor ($n=5$). SPTB samples were gestationally grouped as before ($n=9$) or after 34 weeks ($n=8$).

Isolation and culture of human amnion epithelial cells (AECs)

The amniotic membrane was obtained and processed as described previously (Richardson & Menon 2018). Briefly, fetal membrane was cut from the placenta midzone portion and the amnion membrane was manually peeled from the chorion, rinsed in saline and transferred to a Petri dish containing Hank's Balanced Salt Solution (HBSS; Mediatech Inc., Manassas, VA). All the reagents and media used were warmed to 37°C prior to use. After cutting the amnion into 2 cm² pieces, they were digested twice in 0.25% trypsin and 0.125% collagenase A (Sigma-Aldrich) in HBSS for 35 min at 37°C. After each digestion, the tissue was filtered through a 70 µm cell strainer (Thermo Fisher Scientific) and trypsin was inactivated using complete DMEM: Nutrient Mixture F-12 media (DMEM/F12; Mediatech Inc.) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich), 10% penicillin/streptomycin (Mediatech Inc.) and 100 µg/mL EGF (EGF; Sigma-Aldrich). The collected filtrate was centrifuged for 10 min at 31693 g and the pellet was resuspended in 3 mL of complete DMEM/F12. Once cells were counted, approximately 3 million cells per flask were cultured in T75 flasks containing complete DMEM/F12 media at 37°C, 5% CO₂ and 95% air humidity to 70–80% confluence.

Stimulation of AEC with TNF-α and LPS

Once cells reached 70–80% confluence, the cells were passaged. Approximately 30,000 cells were cultured on 8-well coverslips for immunocytochemistry and 400,000 cells were plated in 60 mm dishes for 24 h and then treated with TNF-α or LPS. Recombinant TNF-α expressed in *E. coli* (Sigma-Aldrich #T6674) was reconstituted at a concentration of 0.1 mg/mL and

further diluted to 10 and 50 ng/mL in culture media prior to use. This concentration was chosen based on previously reported in vitro experiments on AECs and fetal membranes (Romero *et al.* 1989a, Fortunato *et al.* 2001). LPS from *E. coli* 055:B5 (Sigma-Aldrich #L2880) was reconstituted at a concentration of 1 mg/mL, further diluted to 50 and 100 ng/mL in media prior to use. These concentrations were based on endotoxin concentrations seen in the amniotic fluid of women with documented intraamniotic infection with Gram-negative pathogens (Romero *et al.* 1987, 1989b). Treated cells were incubated at 37°C, 5% CO₂ and 95% air humidity for 24, 48 and 72 h.

Crystal violet assay

AECs were seeded on an eight-well slide (30,000 cells per well) and treated with TNF-α (10 and 50 ng/mL), LPS (50 and 100 ng/mL) and cigarette smoke extract (CSE), a validated and reproducible oxidative stress inducer used as a positive control for cell death (Richardson *et al.* 2018). After each time point (24, 48 and 72 h), cells were washed with 1x PBS, fixed with 4% paraformaldehyde for 20 min, washed with PBS and stained with 0.1% crystal violet for 20 min. After 20 min, cells were washed, allowed to dry, and 10% acetic acid was added to each well. A 1:4 dilution of the colored supernatant was measured at an absorbance wavelength of 590 nm.

Western blot analysis

AEC grown under normal cell culture conditions (control), and with TNF-α and LPS were lysed in RIPA lysis buffer with freshly added protease, phosphatase inhibitor cocktail and phenylmethylsulphonyl fluoride. The lysate was collected after scraping the culture plate. The insoluble material was removed by centrifugation at 11,200 g for 20 min at 4°C and the supernatant was collected and protein concentration in each lysate was determined using the BCA protein assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific). The protein samples were separated using SDS-PAGE on a gradient (4–20%) Mini-PROTEAN TGX Precast Gels (Bio-Rad) and transferred to a membrane using an iBlot Gel Transfer Device (Thermo Fisher Scientific). Membranes were blocked in 5% non-fat milk in 1x TBS-Tween 20 for a minimum of 2 h at room temperature and then probed (or reprobed) with primary antibody overnight at 4°C. The membrane was incubated with the appropriate secondary antibody for 1 h at room temperature and immunoreactive proteins were developed using chemiluminescence reagents (ECL Western Blotting Detection System) and visualized using Luminata Forte Western HRP substrate (Millipore). The following anti-human/mouse antibodies were used for Western blot: N-cadherin (1:600, Abcam, ab98952), E-cadherin (1:500, Abcam, ab15148), TGF-β activated kinase 1 binding protein 1 (TAB1) (1:1000, R&D, AF3578), phospho-TAB1 (1:600, Millipore, 06-1334) and β-actin (1:10,000, Sigma-Aldrich, A5441).

Immunochemical localization

Immunocytochemical staining for vimentin (1:300, ab92547; Abcam) and cytokeratin-18 (CK-18) (1:500, ab668; Abcam)

were performed on AEC after treatment with either TNF- α or LPS for 24, 48 and 72 h. After treatment at each time point in culture, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X, and blocked with 3% BSA in PBS before incubation with primary antibodies overnight at 4°C. After washing with PBS, slides were incubated with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Life Technologies) diluted 1:400 in PBS for 1 h in the dark. Slides were washed with PBS, treated with NucBlue Fixed Cell ReadyProbes Reagent (containing DAPI; Thermo Fisher Scientific) and then mounted using Mowiol (Sigma-Aldrich).

Microscopy and image analysis

Brightfield microscopy images were captured using a Nikon Eclipse TS100 microscope (20 $\times$) (Nikon). The cell shape index (SI) was determined for 2D AEC culture by evaluating one frame per treatment from each sample. The ImageJ software version 1.51J (NIH; <http://imagej.nih.gov/ij>) was used to determine the cell circularity and the SI was calculated using the formula: $SI = 4\pi \times \text{Area}/\text{Perimeter}$ (Richardson *et al.* 2020b). Confocal microscopy images were captured using a Zeiss 880 confocal microscope ($\times 40$) (Zeiss). Five random regions of interest per field were used to determine red (CK-18) and green (vimentin) fluorescence intensity. Uniform laser settings, brightness, contrast and collection settings were matched for all images. Images were not modified (brightness, contrast and smoothing) for intensity analysis. ImageJ was used to measure vimentin and CK-18 staining intensity from two focal planes of three different regions per treatment condition at each time point.

Matrix metalloproteinase 9 (MMP9) assay

AEC-conditioned media were collected from 72 h samples. An active MMP-9 fluorescent assay (R&D Systems, F9M00) was conducted following the manufacturer's instructions. Standard curves were developed with *p*-aminophenylmercuric acetate (APMA) samples of known quantities. Sample concentrations were determined by correlating sample absorbance to the standard curve by linear regression analysis. This assay detected active MMP9 free of inhibitors.

TGF- β assay

AEC-conditioned media were collected from control and treated cells at each time point. A human/mouse TGF- β MILLIPLEX MAP kit (EMD Millipore) was conducted following the manufacturer's instructions. Standard curves were developed with recombinant protein samples of known quantities. Sample concentrations were determined by correlating sample absorbance to the standard curve by linear regression analysis.

Statistical analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc.). One-way ANOVA and *t*-tests were used for

statistical analysis as appropriate and $P \leq 0.05$ was considered significant. Graphs are represented as mean $\pm$ S.E.M.

Results

Characterization of epithelial-to-mesenchymal transition (EMT) markers in human fetal membranes from preterm deliveries

Prior to the impact of testing infectious vs inflammatory mediators on EMT, we performed a preliminary screening for evidence of EMT markers in fetal membranes from spontaneous PTB and pPROM samples. Fetal membranes from PTB ($n=17$) and pPROM ($n=16$) were screened for markers of EMT using Western blot followed by densitometric analysis. Preterm samples were gestational age matched for comparisons and grouped by early (<34 weeks gestational age (WGA)) or late (>34 WGA) gestational age at delivery (Fig. 1A). In a stratified screening, early PTB membranes had higher (2-fold) vimentin and N-cadherin (13-fold) and lower E-cadherin (2-fold) expression compared to early pPROM samples (Fig. 1B). Similarly, Western blot analysis also determined strong vimentin expression, higher N-cadherin and lower E-cadherin in late PTB compared to late pPROM, suggesting a similar trend as seen in early PTB vs pPROM as well. In the combined screening, PTB compared to pPROM showed higher expression of the mesenchymal markers vimentin (1.6-fold) and N-cadherin (3.7-fold) and lower expression of the epithelial marker E-cadherin (1.6-fold), suggesting a mesenchymal phenotype consistent with EMT (Fig. 1B). A quantitative analysis and discussion based on this data were not attempted here as the sample sizes were relatively low; the primary purpose of this analysis was to determine any apparent differences between the two groups of adverse pregnancies. Also, full thickness membranes were used for this screening and data could have been confounded by the presence of trophoblast cells that may also undergo transitions as well. Comparing these data with previously reported data (Richardson *et al.* 2020b) shows that PTB is associated with EMT. Our results confirmed that fetal membranes from PTB and pPROM differentially expressed EMT markers; however, the contributing factors that induce EMT in PTB membranes remains unclear.

Neither TNF- α nor LPS induce cytotoxicity in AECs

To determine if proxies for infection (LPS) or inflammation (TNF- α) could contribute to the EMT phenotype associated with PTB, AECs were cultured in varying concentrations of LPS and TNF- α over a three-day period. Prior to conducting additional experiments using AECs in culture, we ruled out stimulant-induced cytotoxicity that may have contributed to any observed changes. As determined by crystal violet staining of

Figure 1 Human fetal membranes from spontaneous preterm birth (SPTB) show evidence of epithelial-to-mesenchymal transition (EMT). (A) Representative images of Western blot analysis of mesenchymal markers (vimentin and N-cadherin) and an epithelial marker (E-cadherin) in full fetal membranes from early (less than <34 weeks gestational age (WGA)) and late (more than >34 WGA) preterm birth deliveries were collected for analysis. (B) When divided into early and late phenotypes, densitometry showed that early PTB had a more mesenchymal phenotype, indicative of EMT, than early pPROM. This was identified by higher expression of the mesenchymal markers vimentin (2-fold) and N-cadherin (13-fold) and lower expression of the epithelial marker E-cadherin (2-fold). Combining gestational ages to look at both overall phenotypes, the same trend was observed (higher expression of vimentin (1.6-fold) and N-cadherin (3.7-fold) and lower expression of E-cadherin (1.6-fold)) (pPROM < 34 WGA: $n=8$, pPROM > 34 WGA: $n=8$, SPTB < 34 WGA: $n=9$, SPTB > 34 WGA: $n=8$).

cells, neither LPS nor TNF- α induced AEC cytotoxicity, and cells viability remained like that of control AECs regardless of time or dose (Fig. 2).

Inflammation but not infection can induce EMT in AECs

TNF- α induces a mesenchymal morphology and low cell SI in AECs

To assess the AEC transition to a mesenchymal phenotype, the first analysis performed was to determine

the SI based on morphological shift (epithelial higher SI; mesenchymal lower SI) and molecular markers of EMT after treatment with TNF- α and LPS. After each treatment, morphological changes in treated and control cells were compared ($n=5$) (Fig. 3A). TNF- α at 10 ng/mL did not cause EMT associated an SI shift after 24 or 48 h, but a significant shift to a mesenchymal phenotype (lower SI) was observed after 72 h compared to controls (Fig. 3B; $P < 0.0001$). However, TNF- α at a higher dose (50 ng/mL) showed a significant shift to a mesenchymal morphology (lower SI) starting at 24 h compared to controls (Fig. 3B; $P < 0.0001$), and this

Figure 2 Crystal violet assay for cytotoxicity. (A) Brightfield microscopy of primary AECs under normal culture conditions and cell treatment. CSE was used as a positive control. Crystal violet staining was localized inside viable AECs and used to determine cytotoxicity. (B) The amount of released crystal violet stain in cells after 72 h of CSE stimulation was significantly less compared to confluent cells after TNF- α , LPS or control treatments ($P < 0.05$). Images were taken at $\times 20$ magnification. Scale bar, 50 μ m ($n = 3$).

morphology persisted throughout the culture period (24 h: $P < 0.0001$; 48 h: $P < 0.0001$; 72 h: $P < 0.0001$). Regardless of the duration of treatment or dose (50 and 100 ng/mL), LPS did not induce SI changes and cells remained in an epithelial state (Fig. 3C).

TNF- α induces mesenchymal intermediate filament and adherens junction expression in AECs

TNF- α -induced EMT was further confirmed by staining for the epithelial-specific cytoskeletal marker CK-18 and mesenchymal-specific marker vimentin (Fig. 4). Consistent with our prior studies, control AEC exhibited a 'metastate' with co-staining for both CK-18 and vimentin (Fig. 4A) (Menon & Moore 2020). Low dose of TNF- α (10 ng/mL) did not induce a significant change in vimentin/CK-18 staining. However, vimentin was significantly higher in AEC treated with 50 ng/mL of TNF- α at 72 h (Fig. 4B) ($P = 0.010$). Conversely, LPS (50 and 100 ng/mL) did not change the vimentin/CK-18 ratio compared to controls, regardless of the exposure time.

We also examined the expression of N-cadherin after treatment with TNF- α and LPS. Confirming our prior findings, TNF- α 50 ng/mL increased N-cadherin ($P = 0.003$), whereas a lower dose of TNF- α (10 ng/mL) and all doses of LPS had no significant effect on the expression of N-cadherin (Fig. 5A).

TNF- α induces EMT associated with inflammation in AECs

To examine EMT-induced localized inflammation, we analyzed MMP9, a class of enzyme associated with ECM degradation (Menon *et al.* 2019). TNF- α at 50 ng/mL significantly increased active and inhibitor-free MMP9 (18.81 ± 2.38 ng/mL) compared to control (6.61 ± 0.56 ng/mL) ($P < 0.0001$). LPS did not induce MMP9 activation after LPS treatment (LPS 3.77 ± 0.62 ng/mL; control 6.61 ± 0.56 ng/mL). We examined active MMP9 only after 72 h as the development of EMT was completed at this time point and was likely a prerequisite for the generation of active MMP9 (Fig. 5B).

In summary, TNF- α , but not LPS, induced time- and dose-dependent EMT in human AEC with a mesenchymal morphology, low cell SI, high vimentin/CK-18 ratio, high N-cadherin expression and increased cell inflammation (MMP9).

The TGF- β /TAB1 pathway does not regulate the EMT caused by inflammation in AECs

Since inflammation was correlated with EMT in AECs, we considered whether the canonical TGF- β /TAB1 pathway could be associated with TNF- α -induced EMT. This pathway can promote the autophosphorylation

Figure 3 TNF- α , but not LPS, induces morphological changes in AECs. (A) Brightfield microscopy showing the morphology of human AECs that were untreated (control) or treated with TNF- α or LPS. AECs stimulated with TNF- α (50 ng/mL) demonstrated a morphological change within 24 h. Cells lost their cuboidal shape (epithelial cells) and acquired an elongated morphology (mesenchymal cells). A low TNF- α dose (10 ng/mL) also led to a mesenchymal phenotype after 72 h compared to controls. The SI did not change with LPS treatment (50 and 100 ng/mL). Images are representative of five biological replicates. Scale bar, 10 μ m. (B and C) Dot plot graphs comparing the control group with (B) TNF- α 50 ng/mL treatment ($n=5$) showed a significant increase in mesenchymal morphology in a time-dependent manner (24, 48 and 72 h: $P < 0.0001$). (C) LPS ($n=5$), regardless of duration or dose of treatment, did not induce any cell shape index changes.

Figure 4 Expression of specific markers in human epithelial cells. (A) Immunostaining showing AECs expressing epithelial cytokeratin-18 (CK-18) and mesenchymal markers (vimentin) according to treatment and time point ($n=3$). Scale bar, 10 μ m. (B) The vimentin/CK-18 ratio was significantly increased with the 50 ng/mL TNF- α after 72 h of stimulation ($P=0.010$).

of p38MAPK and consequently induce EMT in AEC at term, as reported previously (Richardson *et al.* 2020b). To verify this, we measured the TGF- β concentration in the culture medium. No increase was observed in the TGF- β concentration in either the TNF- α or LPS treated groups compared to control (Fig. 6A). Next, we performed Western blot analysis for phospho-TAB1 and total TAB1, as they are upstream activators of TGF- β signaling. Regardless of the TNF- α or LPS dose, neither of these proteins exhibited any statistical difference at any time point (Fig. 6B), supporting the notion that the canonical TGF- β /TAB1 pathway is not correlated with TNF- α -induced EMT in human AECs.

Discussion

The role of inflammation during pregnancy and parturition can be functionally balanced for tissue regenerative or remodeling purposes to maintain pregnancy, or be imbalanced to promote labor and delivery (Peltier 2003, Mor 2008, Mor & Cardenas 2010, Gomez-Lopez *et al.* 2014). Both are physiological processes and involve a variety of activators and mediators. The role of inflammatory cytokines,

chemokines and prostaglandins and their mechanistic contributions to both pregnancy and parturition have been thoroughly reported (Romero *et al.* 2014a, Menon 2019). The generation of biochemical mediators can be considered an effect of tissue/cell level physiological or pathological changes required to promote parturition. The mechanism by which each of the fetomaternal units maintains immune balance locally and how these balances are tilted toward a proinflammatory state to either release mediators or activate immune cells are not well-studied. This is specifically true in human fetal membrane cells. This knowledge will benefit our understanding of how membrane dysfunction can cause either PTB or pPROM, which are associated with increased inflammation.

Our recent success in elucidating the role of EMT in fetal membrane cells and its contribution to term labor led us to test the association between EMT and PTB. In term labor, the terminal state of EMT induced by OS causes (1) the accumulation of highly inflammatory mesenchymal cells (Richardson *et al.* 2020b), (2) the generation of localized inflammatory mediators (cytokines, MMPs, etc.) and (3) the degradation of basement membranes and extracellular matrix (Richardson & Menon 2018).

Figure 5 Western blot for mesenchymal-specific marker N-cad and MMP9 concentration. (A) N-cadherin expression was increased in amnion cells treated with a high concentration of TNF- α ; the analysis showed a two-fold difference in the treated group compared to untreated cells ($P=0.003$) ($n=3$). (B) MMP9 quantification ($n=5$). The inflammatory marker MMP9 showed a significant increase ($P < 0.0001$) after TNF- α treatment. LPS did not cause a change in MMP9 levels.

In this study, we report the following: (1) EMT is associated with PTB, but not pPROM. (2) A dose- and time-dependent activation of EMT was seen in AECs in response to TNF- α treatment. (3) EMT induced by TNF- α was associated with active and inhibitor-free MMP9, suggesting localized tissue damage. (4) EMT induced by TNF- α was not mediated by the canonical TGF- β /TAB pathway; thus, the EMT activation pathway induced by TNF- α is different than the pathway seen in normal term labor membranes under OS conditions. (5) An infectious stimulus, LPS, did not cause EMT, irrespective of dose or duration of treatment. Thus, inflammatory stimuli and not necessarily infection can lead to EMT and further increase localized inflammation. Of note, the absence of EMT in pPROM but the presence in PTB regardless of gestational age further support our earlier conclusions that PTB and pPROM arise from distinct pathways (Menon *et al.* 2012, 2014, Dutta *et al.* 2016). pPROM has a very strong association with senescence (Menon *et al.* 2014, Dutta *et al.* 2016) but not EMT, whereas PTB is associated with EMT but not senescence (Dutta *et al.* 2016). Depending on the type of stimulus, divergent pathologic pathways can lead to either PTB or pPROM. A detailed discussion on this topic is not attempted here as the membranes from cases were insufficient to conduct any in depth or other stratified analysis based on risk profile.

LPS can increase inflammatory mediators such as TNF- α . So, it is logical to think about why

LPS-induced TNF- α did not cause EMT in AEC. A possible explanation can be that AECs are poor producers of TNF- α , and respond to LPS with an increased output of IL-6 (Fortunato *et al.* 1996). The dose of LPS used in this study, a dose often seen in the amniotic fluid during Gram-negative bacterial inflammation (Romero *et al.* 1987), is not capable of producing the level (50 ng/mL) of TNF- α required to induce EMT (Romero *et al.* 1989a). Moreover, in a recent study, we also noticed that IL-6, even at the highest concentration observed during infection-associated PTB in the amniotic fluid, does not induce EMT in AECs (Manuscript in press). Therefore, we conclude that infectious conditions (modeled in this study by LPS) are incapable of inducing a terminal state EMT. The mechanisms of membrane weakening and degradation by infection are unlikely to involve either senescence (as reported previously) (Dixon *et al.* 2018, Jin *et al.* 2018) or EMT.

The TNF- α doses used here have been reported in the amniotic fluid of pPROM and PTB cases with specific infection, and even in cases of non-infectious sterile inflammatory conditions (Romero *et al.* 1989a, 2007, Hayashi *et al.* 2008). However, these doses are not seen in term labor amniotic fluid samples (Hayashi *et al.* 2008). Therefore, TNF- α , even in the absence of infection, can lead to EMT as well as membrane matrix damage and weakening, predisposing to PTB. Prior studies have also reported that TNF- α can induce both senescence and apoptosis in membrane cells

Figure 6 TGF- β /TAB1 pathway is not associated with EMT induction by TNF- α . (A) TGF- β concentrations did not undergo any changes at 24 h, but an overall decrease in concentration was seen at 48 and 72 h regardless of the treatment or dose of the stimulant ($P < 0.05$) compared to control ($n = 5$). (B) Western blot for phospho and total TAB1 ($n = 3$). Downstream mediators of the TGF- β pathway did not change between the treatment groups compared to control, supporting the absence of canonical TGF- β /TAB1 pathway activation during the EMT process in AECs in response to TNF- α .

(Menon *et al.* 2002), whereas senescence is always associated with inflammation (SASP) (Behnia *et al.* 2015). Although apoptosis is not an inflammatory state, the release of inflammatory mediators by some apoptotic cells has been reported (Kakarla *et al.* 2020). Thus, cell fate (senescence and EMT) and a localized increase in inflammation driven by pathologic doses of TNF- α can augment the dysfunctional state of membranes. Our findings are further supported by similar findings reported by Janzen *et al.* in human fetal membranes (Janzen *et al.* 2017). Similarly, Manuck *et al.* have shown that PTB, specifically early PTB, is associated with inflammation and this is the sample group where we noticed relative stronger signs of EMT (Manuck *et al.* 2015).

Recently, some mechanistic studies have been performed by our lab to understand the senescence pathway at term and in PTB. OS experienced at term labor induces p38MAPK activation and leads to AEC senescence (Behnia *et al.* 2016). In this model p38MAPK activation occurs through non-canonical mechanisms that involve the activation of p38MAPK by TGF- β , which is also increased in AEC by OS (Richardson *et al.* 2018). TGF- β /TAB1 complex in AECs results in the autophosphorylation of p38MAPK (Richardson *et al.* 2018). Interestingly, TGF- β produced in response to OS is also a major inducer of EMT (Richardson *et al.* 2020b). Thus TGF- β plays two major roles in AECs under OS: (1) it causes senescence under stress conditions and (2) it forces cells to undergo EMT, both of which are a proinflammatory state. When the role of TGF- β was examined in our study, neither LPS nor TNF- α increased TGF- β levels in AECs and its concentration remained like that of control AECs. This suggests that EMT mediated by TNF- α is independent of the TGF- β pathway, which is in contrast to that seen at term labor in response to OS. In colorectal cancer cells, TNF- α induces EMT by increasing the stability of the pro-EMT transcription factor Snail as well as by activating the serine/threonine-specific protein kinase (AKT) pathway (Wang *et al.* 2013). This Snail-AKT pathway subsequently represses GSK-3 β activity (a cell cycle promoter) and decreases the association between Snail with GSK-3 β (Wang *et al.* 2013). We have already reported that TNF- α can increase p38MAPK activation, and this activation in AEC downregulates GSK-3 β (Lavu *et al.* 2019, 2020). Therefore, we speculate that, in PTB, TNF- α -induced p38MAPK activation may downregulate GSK-3 β to facilitate EMT without the involvement of TGF- β . Multiple unknowns still persist, as our study did not examine the impact of TNF- α on GSK-3 β downregulation and how the TNF- α /p38MAPK axis executes EMT.

Understanding how infectious and inflammatory mediators cause cellular level changes is critical in understanding how pathological processes develop at different tissue levels leading to PTB. Using AECs, we report that TNF- α , a powerful pro-labor inflammatory cytokine, can damage fetal membranes by inducing EMT, leading to mesenchymal cell accumulation. Recent study by Kallapur's group successfully tested an anti-TNF- α antibody (adalimumab) that inhibited ~80% of genes induced by inflammation and reduced fetal membrane inflammation and chorioamnionitis and delayed PTB in non-human primate models (Presicce *et al.* 2020). Although we have not yet tested the effect of blocking TNF- α in inhibiting the development of EMT, it is likely that one of the mechanistic events at the cellular level in the primate model study was the inhibition of EMT mediated by TNF- α .

In conclusion, although inflammation may be the ultimate driver of PTB, the cellular mechanisms that generate inflammatory changes at the tissue level

are different. The development of EMT in response to an inflammatory mediator like TNF- α and not by an infectious agent like LPS demonstrates these distinct mechanisms. This knowledge is critical for developing strategies to reduce infection vs inflammation (in the absence of infection) induced preterm deliveries.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study is funded partly by NIH/NICHD (5 R03 HD098469 02) to R Menon and in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 to Mariana de Castro Silva.

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Received 26 May 2020

First decision 23 June 2020

Revised manuscript received 10 July 2020

Accepted 30 July 2020