Histological Evidence of Oxidative Stress and Premature Senescence in Preterm Premature Rupture of the Human Fetal Membranes Recapitulated in Vitro


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Preterm prelabor rupture of the membranes (pPROM) may lead to preterm births (PTBs). We investigated premature senescence of fetal membranes in women with pPROM and spontaneous PTB with intact membranes (<34 weeks) and the inducibility fetal membrane senescence phenotype by oxidative stress in vitro. IHC was performed for p53, p21, and phospho (p)-p38 mitogen-activated protein kinase (MAPK) as markers of senescence phenotype in pPROM, PTBs, and term births. Term fetal membranes were exposed to cigarette smoke extract to induce oxidative stress. Western blots documented p-p53 and p-p38 MAPK. Transmission electron microscopy assessed cellular morphologic features in clinical and cigarette smoke extract treated membranes. A total of 80% of pPROM cells and >60% of term cells were positive for all three senescence phenotype markers, and concentrations were higher than in PTBs (P < 0.05). p53 staining was comparable in membranes from PTB and term birth pregnancies, whereas only <30% and <45% of cells were positive for p21 and p38 MAPK, respectively. In vitro cigarette smoke extract exposure increased p-p38 MAPK without any detectable change in p-p53 MAPK. Enlargement of organelles consistent with senescence phenotype was evident in pPROM and term membranes in vivo and after cigarette smoke extract treatment in vitro but was less apparent in PTBs. Histologic and biochemical resemblance of pPROM and term membranes suggests premature senescence of the membranes is a mechanistic feature in pPROM, and this can be phenocopied in an in vitro model. (Am J Pathol 2014, 184: 1740–1751; http://dx.doi.org/10.1016/j.ajpath.2014.02.011)
stress–induced lipid peroxidation product F2-isoprostane were present in term amniotic fluid samples compared with gestational age–matched PTB amniotic fluid, and our unpublished observations indicate that pPROM amniotic fluid samples have F2-isoprostane concentrations similar to those seen in term birth samples. The data suggest that increased oxidative stress is present more in pPROM than in PTB with intact membranes. Reduction of fetal leukocyte and fetal membrane telomere lengths, an indicator of oxidative stress and senescence, was noted in pPROM pregnancies compared with gestational age–matched PTBs. Fetal leukocyte telomeres in pPROM cases at ≤34 weeks of gestation were as short as those of normal term births, supporting an accelerated aging process in pPROM. At term, oxidative stress and fetal membrane senescence are likely normal physiologic responses that lead to labor and delivery. Similar findings in pPROM suggest that exposures, such as infection, cigarette smoking, high body mass index, psychosocial stress, and antioxidant nutrient deficiencies, cause reactive oxygen species (ROS) to accumulate, activating premature fetal membrane senescence. One characteristic of the senescence phenotype (SP) involves the phenomenon of irreversible arrest of cell growth. Unlike apoptosis, these cells persist, alter their function, and change the tissue environment, inducing a unique signature of inflammatory markers similar to that seen in pPROM.

Three key mediators of cell senescence are p53, p21, and p38 mitogen-activated protein kinase (MAPK). The association between senescence and PTB in mice is suggested by the observation that a functional p53 mutation in decidua results in PTB. Further analysis of these decidua tissues documented a reduction in the antioxidant system that is hypothesized to be the mechanism that results in PTB. We selected p53 and its effector p21 for this study given their potent inhibition of cyclin-dependent kinase complexes and interference with cell cycle progression, resulting in senescence. p38 MAPK activation by phosphorylation of its catalytic site residues, threonine-180 and tyrosine-182, also promotes cell cycle arrest and cellular senescence by targeting the expression of proteins of SP.

On the basis of our observation of telomere length reduction and accelerated senescence in pPROM, the primary purpose of this study was to provide histologic evidence of markers for senescence in human fetal membranes from normal term births and in clinical settings of pPROM and PTB. We examined the levels of expression of p53, p21, and p38 MAPK by immunohistochemistry (IHC). In addition, morphologic changes of senescence were observed through transmission electron microscopy (TEM) in fetal membranes from early pPROM and PTB with intact membranes pregnancies (both <34 weeks) compared with membranes from normal term vaginal deliveries. We also examined the inducibility of ROS-associated senescence markers in normal term, not in labor, fetal membranes in vitro. Water soluble cigarette smoke extract (CSE) was used to induce ROS in fetal membranes. Activation of p-p53, p-p38 MAPK was documented by Western blot analysis, and morphologic changes were documented by TEM to confirm senescence.

Materials and Methods

This study was approved by the Western Institutional Review Board (Seattle, WA) and the institutional review board at the University of Texas Medical Branch (Galveston, TX; protocol 11-251 UTMB). Placental tissues from pPROM, PTBs, and normal-term births were obtained from individuals after obtaining written consent.

Fetal Membrane Collection for in Situ Analysis

Fetal membranes were collected from participants with normal birth after vaginal delivery at term after spontaneous onset of labor and artificial rupture of the membranes (between 39 and 41 weeks with no prior history of PTB or pPROM), PTB with intact membranes, or pPROM (both <34 weeks) (n = 8 in each group). Preterm labor was defined as the presence of ≥2 regular uterine contractions every 10 minutes accompanied by documented cervical change. pPROM was confirmed by pooling, funneling, or AmniSure test. Membranes were dissected from the placenta, washed three times in normal saline, and cleansed of decidua and blood clots using cotton gauze. Sections were cut from the midzone of the reflected membranes, avoiding the regions overlaying the cervix. Although this area may contribute to pPROM, our objective was to document evidence of senescence as a generalized phenomenon distributed throughout the membrane and not uniquely localized at the rupture site. Specimens were stored in 3.7% buffered formaldehyde.

Tissue Processing for IHC

Tissue sections were cut at 3 to 5μm, mounted on positively charged slides, and dried in a slide oven at 60°C for 30 minutes to ensure adherence to the slides. Sections were deparaffinized in four changes of xylene for 5 minutes each and then rehydrated through a series of graded alcohols with a final rinse in distilled water. Endogenous peroxides were quenched by soaking sections in two changes of 0.3% H2O2 in methanol.

Antigen Retrieval and Immunostaining for p53, p21, and p38

Before IHC, some sections were treated with antigen retrieval solution to facilitate antibody binding. Briefly, slides were incubated in a Black and Decker Vegetable Steamer for 20 minutes in Target Retrieval Solution (catalogue no. S1699; Dako Corporation, Carpinteria, CA) preheated to 99°C. They were removed from the steamer and placed on an open counter to cool for 20 minutes in the solution. The slides were then rinsed in three changes of distilled water and placed into a container of Tris-buffered
saline with Tween 20 (catalogue no. 2380; Signet Pathology Systems, Inc, Dedham, MA) for 5 minutes to decrease the surface tension of the slides and facilitate coating of the IHC reagents as described below.

**Primary Antibody Reaction**

The following antibodies and dilutions were used for immunostaining: mouse monoclonal p53 antibody (1:200; catalog no. ab1101; Abcam, Cambridge, MA), which recognizes a 6-aminoacid 20-25 epitope at the N-terminus; rabbit polyclonal antibody to p21 (1:800; catalog no. ab18209; Abcam); and rabbit polyclonal antibody to p38 MAPK (1:200; catalog no. ab7952; Abcam), which recognizes both the native and phosphorylated p38z isoform. Treatments with primary antibodies were performed for 1 hour. Human colon tissue was used as a positive control and PBS as a negative control for IHC experiments.

**Streptavidin-Biotin—Based Detection**

Slides were loaded horizontally onto the Dako Autostainer, where each of the following steps was automated. All incubations occurred at room temperature, and sections were coated with 200 µL of each reagent unless otherwise specified. Tris-buffered saline was used to rinse the sections between each of the IHC steps. Both streptavidin and biotin (catalog no. SP2001; Avidin Biotin blocking kit; Vector Laboratories, Burlingame, CA) were diluted in antibody diluent (Dako) at a ratio of 1 mL of avidin and biotin to 5 mL of diluent. Diluted avidin was applied to sections and incubated for 7 minutes. The primary antibody was diluted to specified concentrations in the biotin solution and applied for 1 hour. Sections were then incubated in universal secondary antibody (catalog no. K0675; LSAB2 kit; Dako) for 15 minutes followed by label from the same kit for 15 minutes. Finally, the chromogen liquid diaminobenzidine (Dako, Carpinteria, CA) was applied for 5 minutes. The following 15 minutes followed by label from the same kit for 15 minutes. Finally, the chromogen liquid diaminobenzidine (Dako, Carpinteria, CA) was applied for 5 minutes. The slides were manually rinsed in distilled water, counterstained with Harris hematoxylin (Fisher Scientific, Hampton, NH) for 1 minute, rinsed in distilled water followed by 0.25% ammonia water to blue, and then rinsed in distilled water. The slides were dehydrated through a graded series of alcohols, cleared in four changes of xylene, and coverslipped with Permount mounting medium.

**Assessment of Immunostaining**

Eight sections each for p53, p21, and p38 MAPK were examined independently by two laboratory staff members, one of whom was blinded to the case/control status. For each antigen, staining was assessed by evaluating 10 randomly selected high-power fields (40× objective). The number of positively stained cells per field was determined, and the mean number of antigen positive cells per 100 cells was calculated.
3-nitrotyrosine (3-NT) based on the principle that ROS produce highly reactive peroxynitrite radicals that react with exposed tyrosine residues on proteins, resulting in a stable polypeptide-bound 3-NT.\textsuperscript{22–27} IHC was performed using the anti–3-NT antibody (39B6; catalog no. ab61392; Abcam) at a dilution of 1:200. The color was developed as described above in the IHC section.

Ultrastructural Morphology of Membranes from Clinical Conditions and after CSE Exposure

Fetal membranes from term birth, PTB, and pPROM pregnancies (n = 3 in each group) and fetal membrane disks from normal-term pregnancies with or without CSE exposure (n = 3 each) were fixed, stained, and embedded in PolyBed 812. Initial fixation was for 24 hours at 4°C in a fixative with 2.5% paraformaldehyde, 0.2% glutaraldehyde, and 0.03% picric acid in 0.05 mol/L cacodylate buffer. After fixation, samples were rinsed three times with cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer. Osmicated tissue was then dehydrated by a series of ethanol-water solutions (50%, 75%, 95%, and 100% ethanol for three exchanges). Dehydrated tissue was infiltrated with two exchanges of propylene oxide, then with propylene oxide for three exchanges. Dehydrated tissue was then dehydrated by a series of ethanol-water solutions (50%, 75%, 95%, and 100% ethanol for three exchanges). Dehydrated tissue was infiltrated with two exchanges of propylene oxide, then with propylene oxide–diluted PolyBed resin at 1:1 ratio and 1:2 ratio, and then twice with pure PolyBed 812. Finally, the samples were embedded in PolyBed 812 and cured overnight at 60°C. The samples were then dehydrated by a series of ethanol-water solutions (50%, 75%, 95%, and 100% ethanol for three exchanges). Dehydrated tissue was infiltrated with two exchanges of propylene oxide, then with propylene oxide–diluted PolyBed resin at 1:1 ratio and 1:2 ratio, and then twice with pure PolyBed 812. Finally, the samples were embedded in PolyBed 812 and cured overnight at 60°C. Because precise tissue orientation could not be maintained during curing of the resin, the first resin blocks were cut to give a wide flat face the desired sectioning plane, replaced into new embedding molds, and cured again. Samples were cut as 90-nm sections, placed on Formvar-coated slotted grids, and poststained for 3 minutes with a solution of Reynold’s lead citrate. Images were taken with a JEM 1400 electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis

Western blot experiments were replicated a minimum of 6 times. Densitometric units of p-p38 MAPK and total p38 MAPK were compared using the Kruskal-Wallis test followed by Dunn’s multiple comparisons test. Data are expressed as arithmetic means ± SEM. For the quantitative analysis of IHC data, we used analysis of variance followed by Tukey’s multiple comparisons test to correct for pairwise treatment effects or t-test, as appropriate. All data were analyzed using GraphPad Prism software version 6 (GraphPad Software, San Diego, CA). P < 0.05 was considered significant.

Results

Demographic details of the study participants are provided in Table 1. We examined 24 tissue samples from three different groups (term, PTB, and pPROM). Maternal age, marital status, ethnicity, prevalence of clinical and histologic chorioamnionitis, number of cigarettes smoked during pregnancy, and gestational age were similar between the pPROM and PTB with intact membranes groups. We excluded cigarette smokers from our term group to avoid any confounding effects.

Amnion, chorion, and scattered fibroblast cells embedded in the extracellular matrix of the fetal membranes had positive staining for p53, p21, and p38 MAPK (Figure 1.A–F). p53 staining was seen in 72% and 80% of amnion and chorion cells at term and 58% and 65% in PTB, respectively (Figure 1.A and D). The cells in amnion (79%) and cells in chorion (89%) were positive for p53 in pPROM samples (Figure 1.A). The percentage of p53-positive cells was significantly higher in term and pPROM compared with PTB (both P < 0.05) in amnion cells, but no significant differences were seen between term and pPROM (P = 0.15) (Figure 1D). In chorion, pPROM membranes

### Table 1: Demographic and Gestational Characteristics of Studied Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Term (n = 8)</th>
<th>PTB (n = 7)</th>
<th>pPROM (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)*</td>
<td>32 ± 6</td>
<td>29 ± 5</td>
<td>27 ± 8</td>
<td>0.35</td>
</tr>
<tr>
<td>Marital status†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>3 (37.5)</td>
<td>0</td>
<td>3 (50.0)</td>
<td>0.14</td>
</tr>
<tr>
<td>Married</td>
<td>5 (62.5)</td>
<td>7 (100.0)</td>
<td>5 (62.5)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>5 (62.5)</td>
<td>5 (83.3)</td>
<td>5 (62.5)</td>
<td>0.53</td>
</tr>
<tr>
<td>Black</td>
<td>3 (37.5)</td>
<td>1 (16.7)</td>
<td>3 (37.5)</td>
<td></td>
</tr>
<tr>
<td>Smoked during pregnancy</td>
<td>0</td>
<td>1 (14.3)</td>
<td>0</td>
<td>0.30</td>
</tr>
<tr>
<td>Gestational age at birth</td>
<td>39 ± 1</td>
<td>33 ± 3</td>
<td>32 ± 4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td>0</td>
<td>0</td>
<td>2 (25.0)</td>
<td>0.12</td>
</tr>
<tr>
<td>Gravidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>0.38</td>
</tr>
<tr>
<td>Multiparous</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SD or number (percentage).

*Analysis of variance (Tukey’s multiple comparisons test).
†Fisher’s exact test.
had significantly more p53 staining cells than either PTB or term ($P < 0.05$). For p21, the fractions of positively staining cells in amnion and chorion, respectively, were as follows: term birth, 58% and 62%; PTB, 15% and 24%; and pPROM, 69% and 75% (Figure 1, B and E). Staining was significantly more widespread in term (Figure 1B) and pPROM (Figure 1B) groups compared with the PTB group (Figure 1, B and E) ($P < 0.05$); however, no difference was seen between the term and pPROM groups (Figure 1E). For p38 MAPK, the fractions of cells (amnion and chorion, respectively) that had positive staining were as follows: term birth, 59% and 72%; PTB, 24% and 48%; and pPROM,
80% and 73% (Figure 1, C and F). Both amnion and chorion layers in pPROM membranes had a significantly higher number of p38 MAPK staining cells than term or PTB (both \( P < 0.05 \)), whereas no difference was seen between term and pPROM. In summary, fetal membranes from pPROM pregnancies revealed significantly more numbers of immunopositive cells compared with either term birth or PTB, with PTB cells scoring the lowest for all three SP markers. Chorion cells had the highest staining among all three groups for all markers except p38 MAPK (where amnion cells in pPROM had the strongest reactivity). Two pPROM cases had histologic chorioamnionitis, and three each of our cases in the pPROM and PTB groups had microbial invasion of the amniotic cavity documented by microbial culture. We did not observe any specific differences in immunostaining patterns when data were stratified for infection, although sample size was not sufficient to draw any major conclusion. Analysis of markers based on race was also not significant, but a larger sample size is needed to address this question.

We examined total and p-p53 in membranes treated with CSE; total p53 was higher after CSE treatment compared with controls (\( P < 0.05 \)) and was decreased after N-acetyl cysteine (NAC) treatment compared with controls (Figure 2A). We did not see p-p53 in any of our membrane preparations, suggesting that CSE does not activate p53 (data not shown). However, in vitro CSE stimulated p-p38 MAPK in normal-term membranes compared with controls. The mean gestational age of the placentas used for this study was 38 weeks. Total p38 produced no difference during a 24-hour period (Figure 2B); however, p-p38 MAPK was increased in CSE-treated fetal membranes after 24-hour stimulation (\( P < 0.05 \)) (Figure 2C). The effect appears to be mediated by ROS because CSE-induced p-p38 MAPK expression was abrogated by NAC (\( P = 0.037 \)) (Figure 2C). In a related study, we examined the kinetics of ROS induction in fetal amnion cells from term pregnancy in culture. ROS production kinetics were documented by the release of 2′,7′-dichlorodihydro-fluorescein diacetate, when amnion cells were exposed to CSE extract. ROS generation was seen as early as 2 minutes after exposure to CSE and was significantly higher at all time points tested compared with unstimulated controls. This effect was also inhibited by NAC treatment (data not shown).

Oxidative stress in fetal membranes from clinical specimens and after in vitro exposure to CSE was confirmed by qualitative IHC for 3-NT (Figure 3, A–G). As shown in Figure 3A, membranes from term and pPROM pregnancies (Figure 3E) had intense 3-NT staining compared with PTB (Figure 3C), confirming that oxidative stress is more dominant in those conditions. Similarly, and as expected, CSE-treated fetal membranes (Figure 3, D and F) had strong 3-NT staining compared with controls, particularly those involving the chorion cells (Figure 3, B and F).

Ultrastructure of Amnion and Chorion in Placental Membranes from Clinical Specimens

To correlate ultrastructural morphologic findings with IHC findings, we performed TEM on three specimens per group from the term birth, PTB, and pPROM groups. A detailed description of morphologic findings, with emphasis on mitochondria and endoplasmic reticulum, which are strong responders of oxidative stress, is provided below, summarized in Table 2, and shown in Figure 4, A–D.

Amnion Epithelium

Normal-Term Birth

The specimens of membranes from placentas delivered at term had the least alteration overall compared with the other two groups (Figure 4A). The amniotic epithelial cells had
short microvilli that often appeared branched. The mitochondria appeared small and rounded with pale matrices. Endoplasmic reticulum was dilated, and cisternae of rough endoplasmic reticulum appeared open with lucent contents. The nuclei generally appeared lucent and distinct single nucleoli were present.

**PTB**

The amniotic epithelium from the PTB with intact membranes had ultrastructural features similar to the term group overall (Figure 4A). Endoplasmic reticulum and mitochondrial profiles were small, round, and appeared pale. The nuclei had mild irregularity of the nuclear contour with more condensation of nuclear chromatin at the inner nuclear envelope than in the term birth group.

**pPROM**

Amniotic epithelial cells appeared finely vacuolated at low magnification, with pale irregular vacuoles standing out in contrast to a moderately electron dense cytoplasm (Figure 4A). This was unique to the pPROM group compared with the term and PTB groups. Unlike PTB, in some cells the nuclear envelope was dilated. Nuclei consistently had irregular nuclear contours with frequent infolding of the envelope into the nucleus, and the chromatin had moderately dense condensation at the inner surface of the nuclear envelope. Similar to that seen in the term birth group, dilated segments of rough endoplasmic reticulum were found in the cytoplasm. The mitochondria appeared enlarged and had pale matrices.

**Chorion**

**Normal-Term Birth**

The nuclei of the cells of the chorion were generally ovoid with slight irregularity and contained a light gray meshwork of chromatin (Figure 3B) with a simple cytoplasm. Cisternae of rough endoplasmic reticulum were numerous and appeared open, with pale contents, and in some cells the rough endoplasmic reticulum was clearly dilated (Figure 3C). Mitochondria were round or swollen with pale matrices (Figure 3D). Both endoplasmic reticulum and mitochondria at term resembled those seen in pPROM membranes (Figure 3, C and D).

**PTB with Intact Membranes**

Chorionic cells included a mixture of cells that appeared contracted or shrunken (Figure 3, B–D). In the contracted cells, the cytoplasm appeared moderately electron dense, and long, thin, undulating electron dense mitochondria were seen throughout the cytoplasm. The nuclei had irregular nuclear contours with frequent infolding of the nuclear envelope (Figure 3B). Cisternae of the endoplasmic reticulum appeared compact (Figure 3C), with little internal space, and were markedly different from those in the term birth group. Mitochondria had an electron-dense matrix (Figure 3D).

**pPROM**

The chorion cells in the pPROM group were generally swollen and flattened, with only rare condensed cells (Figure 3D). The nuclei had mildly irregular shapes and a degree of nuclear condensation intermediate between that of the term and PTB groups. Cisternae of the rough endoplasmic reticulum generally appeared open or dilated (Figure 3C). Mitochondrial profiles were moderately large, round, and pale and resembled those of term membranes (Figure 3D).

In summary, ultrastructural morphologic features of both amnion and chorion cells from term and pPROM had signs of senescence characterized by overall swelling of cells and endoplasmic reticulum and mitochondria. PTB membranes had more normal-appearing organelles compared with the other two groups; however, irregular nuclear contours were

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Term Amnion</th>
<th>Term Chorion</th>
<th>PTB Amnion</th>
<th>PTB Chorion</th>
<th>pPROM Amnion</th>
<th>pPROM Chorion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvilli</td>
<td>Irregular and branched</td>
<td>Short projected into gaps and attached to desmosomes</td>
<td>Irregular and branched</td>
<td>Short microvilli</td>
<td>Irregular and branched</td>
<td>Short and some branched projected into gaps and attached to desmosomes</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Occasional irregular contours</td>
<td>Predominantly round and some contoured nuclei</td>
<td>Contoured nucleus</td>
<td>Contoured nucleus</td>
<td>Occasional Irregular contours</td>
<td>Mildly irregular and some contoured nuclei</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>Enlarged</td>
<td>Dilated cisternae</td>
<td>Compact cisternae with very little internal space</td>
<td>Compact cisternae with very little internal space</td>
<td>Enlarged</td>
<td>Enlarged and dilated cisternae</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Enlarged with pale matrices</td>
<td>Very large, rounded with pale matrices</td>
<td>Small, round with pale matrices</td>
<td>Small, round and pale matrices</td>
<td>Enlarged with pale matrices</td>
<td>Very large, rounded with pale matrices</td>
</tr>
</tbody>
</table>

Table 2 Summary of TEM Characteristics of Organelles in Term, PTB, and pPROM Pregnancies

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more dominant in both amnion and chorion cells in this group than in the pPROM and term groups.

Changes in Amnion Exposed to Cigarette Smoke

In the CSE amniotic epithelial cells (Figure 3E), the cytosol generally appeared more electron dense than in untreated controls (Figure 3F). The microvilli were elongated, branched, and flattened and had an overall height less than that of controls. Nuclear chromatin appeared more densely granular (Figure 3E).

Amnion in Untreated Controls

The amniotic epithelial cells from untreated membranes were cuboidal with rounded apices and had short, irregular microvilli (Figure 3F). Nuclei were rounded and often contained moderate-sized nucleoli. The cells were joined laterally by multiple desmosomes. Lateral intercellular spaces extend irregularly into adjacent cells.

Chorion Exposed to Cigarette Smoke

In most cells, the nuclear envelope appeared dilated, the cytoplasm appeared pale, and the rough endoplasmic reticulum and nuclear envelope appeared dilated (Figure 3E). Mitochondria appeared swollen and had pale matrices (Figure 3E). Rarely, the mitochondrial matrix contained amorphous dense bodies. In other cells, the mitochondrial matrix appeared more electron dense than normal, and the intermembranous space was enlarged, with a condensed mitochondrial configuration.

Chorion in Untreated Controls

Electron micrographs revealed a loose collagenous extracellular matrix, and the nuclei of the chorionic epithelial...
cells were oval and contained small nucleoli (Figure 3F). The cytoplasm included rough endoplasmic reticulum, ellipsoid mitochondria, and normal-appearing Golgi apparatus and sometimes contained homogeneous, moderately electron dense material similar to material in the extracellular space (Figure 3F). The structure of organelles appeared normal in untreated controls.

In summary, CSE-treated membranes demonstrated similarities between pPROM and term birth clinical samples, indicating morphologic evidence of senescence.

**Discussion**

In this study, we compared histologic evidence of cellular senescence and related biochemical markers in fetal membranes from term, PTB, and pPROM pregnancies. These data confirm our prior findings of high oxidative stress and accelerated senescence in pPROM and support our postulation that pPROM (especially early pPROM <34 weeks) is a syndrome of premature aging of the membranes. ROS-inducing risk factors can trigger senescence-associated pathways in normal fetal membranes, likely causing inflammation, proteolysis, and rupture. 

Irreversible growth arrest at the G1 phase of the cell cycle is a major characteristic of senescence. The amnionchorionic cells divide at a rapid rate throughout gestation to accommodate the increasing demands of the intrauterine contents. This proliferative activity is a normal process that is expected to continue even at term. Overwhelming oxidative stress forces cells to undergo transformation as either malignant or senescence. Adverse pregnancy environment promotes the senescence of the membrane cells and the activation of an inflammatory condition that we propose to cause pPROM. For example, our data indicate that oxidative stress was increased as indicated by the increased levels of 3-NT staining of clinical specimens, as was its inducibility by CSE in normal membranes. Oxidative stress promotes premature aging and multiple diseases, such as Alzheimer disease, Parkinson disease, cardiovascular diseases, musculoskeletal weaknesses, and bone and muscle loss. Importantly, we also report a tissue-specific difference in that PTB is not independent of senescence, but the extent of senescence and related morphologic changes were higher in pPROM, suggesting that pPROM pathophysiologic features may mostly be related to this precise status of the membranes; hence, pPROM may be considered a disease of the fetal membranes.

We postulate premature senescence of the fetal membrane as a factor associated with pPROM based on the similarities of membranes at molecular, histologic, and morphologic levels seen at term. Most of the risk factors (mainly infection and inflammation) of pPROM and PTB are similar; hence, pregnancy outcome differs where approximately 40% of women experience pPROM before delivery. The 3-NT staining and senescence morphologic features may provide a partial explanation for this phenomenon. We suspect chronic oxidative stress in response to sustained exposure to risk factors as a senescence inducer in pPROM. At term, chronic oxidative stress is a well-documented condition, likely because of natural and physiologic aging of the placenta and membranes and labor-related excessive metabolic activities that promote delivery. Conversely, lack of similar 3-NT staining in PTB may indicate acute oxidative stress. Infection, inflammation, and other risk factors of pPROM and PTB are associated with oxidative stress, but chronic and acute oxidative stress may arise based on the type of risk, its dose, and site of risk exposure. pPROM may be an after effect of senescence induced by chronic oxidative stress similar to that seen at term, whereas PTB is a result of acute oxidative stress that is not sufficient to develop SP. On the basis of the data presented in this report, we also think that senescing signals may initiate labor at term.

A comparison between the morphologic features of membranes from women not in labor used for in vitro studies and membranes from women in labor at term used for clinical data comparisons revealed distinct morphologic features under TEM. Although 3-NT staining was not distinguishable between the two, senescence features are markedly present in membranes from women in labor at term. We predict that the development of SP and the subsequent generation of the senescence-associated secretory phenotype (SASP) are major physiologic changes associated with the initiation of labor besides the disruption of the endocrine clock. In addition, the change from quiescence to active labor is likely to include the development of senescence of fetal membranes and placenta acting as termination signals from the fetus. We further verified that SP could be induced in normal fetal membranes at term, before the onset of labor, using CSE. We report activation of p38 MAPK by CSE in normal fetal membranes. This finding provides further evidence of the physiologic significance of the activation of the SP to adverse challenges. Activation of p-38 MAPK to p-p38 MAPK by CSE could be prevented by antioxidant NAC, confirming the influence of oxidative stress. We have previously reported that the exposure of fetal membrane explants to CSE increases total p53 and apoptotic cell death of other markers. In this study, although total p53 was increased in response to CSE and subsequently down-regulated by antioxidant NAC, p-p53 was not seen, suggesting a lack of p53-mediated mechanisms in fetal membranes in response to CSE. This is a significant difference in the activation of the SP that is unique to fetal membrane response to an exogenous toxin such as cigarette smoke. However, it does not rule out a role for p53 in response to other risk factors that can cause pPROM and PTB. We thus propose that under conditions of oxidative stress, a p-p38 MAPK-mediated senescence pathway seems operational in fetal membranes. Similarly, p21 is a key component of both a p53- and p38 MAPK-mediated senescence pathway. Our CSE-induced ROS model did not produce a reproducible pattern to suggest a definite role for this protein in this in vitro model of senescence. This lack of reproducibility does not rule out a role of p21 because it was seen in in vivo in pPROM and term birth.
Cell senescence is generally accompanied by characteristic morphologic changes, depending on the senescence trigger (e.g., ROS, infection and inflammation, radiation, and stress). Cells enlarge or may become more flat and multinucleated.54 The TEM study found that ROS-related morphologic changes in amniochorionic cells is characterized by enlarged cells (cytoplasm and nucleus), flattened cells, and irregular cells with senescence-associated heterochromatic foci in pPROM and term births. A classic sign of senescence was also indicated by enlargement of organelles, particularly endoplasmic reticulum and mitochondria, which were prominently displayed in membranes from pPROM and term pregnancies55–58 and indeed were reproduced in the CSE-exposed membranes in vitro. These structural changes limit the functional efficiency, resulting in cell cycle arrest and eventual death.39–42

Telomere shortening in fetal leukocytes and placental DNA in pPROM11 is also supportive of premature senescence. New findings indicate that senescence can be triggered by DNA fragmentation, which activates both p53 and p38 MAPK expressions.43–48 with neither of these markers exclusive to apoptosis or senescence.49 Current studies suggest that apoptotic cells are likely to be eliminated from tissues without an inflammatory response, whereas senescent cells can persist in the tissue environment and produce a phenotype of inflammation (ie, a state of chronic inflammation generated by the expression of the SASP that is characterized by inflammatory cytokines, chemokines, growth factors, and matrix metalloproteinases). This inflammatory feature underlies pPROM51,52 and reduces fetal membrane tensile strength, leading to rupture.53,54 Our studies also suggest that both apoptosis and senescence are factors that are associated with pPROM and PTB, and the delineation of pathways very well may depend on the dose of the stimulus and the extent of p53 or p38 MAPK activation.51,55–58 Apoptosis of the fetal membranes as a factor associated with pPROM is well documented.59–63 We propose that these challenges can cause transient growth arrest, and if the conditions surrounding the cells do not improve, it can potentially lead to apoptosis or senescence.64–66 The fate of the cell, either to live and undergo division or to die via apoptosis or senescence, will likely depend on the type and dose of risk factors.

Cigarette smoke contains >7000 recognized chemicals,67 including nicotine, unsaturated aldehydes, heavy metals, and other inducers of ROS and DNA damage.68,69 One of the limitations of this study is the use of crude CSE for stimulation in in vitro cultures. This is not calibrated to the number and duration of cigarettes smoked by a individual. This extract included water soluble toxicants from cigarette smoke, whereas the fetal membranes are likely exposed to metabolites of these toxicants. Another limitation is the use of membranes from women not in labor with full-term pregnancies as controls that are naturally and physiologically expected to be aged. Although natural senescence is expected at term, the effect of CSE was significant in our experiment as indicated by morphologic and biochemical changes justifying the use of these tissues for such studies.

In summary, we found that human fetal membranes from pPROM pregnancies reflect SP in situ and report the indiscernibility of such a phenotype in normal-term membranes in response to ROS. Senescence-associated markers suggest a mechanism for ROS-associated damage to the membranes that may result in premature aging and SASP generation, which is depicted by inflammatory markers normally seen in pPROM. Further characterization of SP and SASP in pPROM is necessary to understand the biomarkers associated with senescence development during adverse pregnancy conditions.

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