Research Article

Dexamethasone induces primary amnion epithelial cell senescence through telomere-P21 associated pathway[†]

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Abstract

Dexamethasone (Dex), a corticosteroid hormone, is used during the perinatal period to help fetal lung and other organ development. Conversely, Dex-induced cell proliferation has been associated with accelerated aging. Using primary amnion epithelial cells (AECs) from term, not in labor, fetal membranes, we tested the effects of Dex on cell proliferation, senescence, and inflammation. Primary AECs treated with Dex (100 and 200 nM) for 48 h were tested for cell viability (crystal violet dye exclusion), cell cycle progression and/or type of cell death (flow cytometry), expression patterns of steroid receptors (glucocorticoid receptor, progesterone receptor membrane component 1&2), inflammatory mediators (IL-6 and IL-8), and telomere length (quantitative RT-PCR). Mechanistic mediators of senescence (p38MAPK and p21) were determined by western blot analysis. Dex treatment did not induce AEC proliferation, cell cycle, influence viability, or morphology. However, Dex caused dependent telomere length reduction and p38MAPK-independent but p21-dependent (confirmed by treatment with p21 inhibitor UC2288). Senescence was not associated with an increase in inflammatory mediators, which is often associated with senescence. Co-treatment with RU486 produced DNA damage, cell cycle arrest, and cellular necrosis with an increase in inflammatory mediators. The effect of Dex was devoid of changes to steroid receptors, whereas RU486 increased GR expression. Dex treatment of AECs produced nonreplicative and noninflammatory senescence. Extensive use of Dex during the perinatal period may lead to cellular senescence, contributing to cellular aging associated pathologies during the perinatal and neonatal periods.

Summary Sentence

Dexamethasone induces telomere-dependent p21 mediated non-replicated and non-inflammatory senescence in fetal amnion epithelial cells.

Key words: steroids, preterm birth, lung maturity, aging, RU486, inflammation.

Introduction

Preterm birth (PTB; birth before 37 weeks' gestation) is a major complication of pregnancy that affects 15 million births and accounts for 1 million neonatal deaths globally per year [1]. Survivors of PTB experience various morbidities including bronchopulmonary dysplasia, asthma, intraventricular hemorrhage, cerebral palsy, delayed development, learning disabilities, and hearing loss [2]. The risks of complications are inversely proportional to the gestational age at which labor occurs. Therefore, women and their fetuses who experience preterm labor are specifically intervened to rapidly enhance fetal organ maturation [3]. The outcome of babies born preterm has improved drastically during the past 40 years; however, recent data suggest that several morbid conditions in preterm babies are likely due to impaired fetal programming impacted either by PTB or due to interventions for preterm labor [4, 5].

Several endocrine factors produced by fetomaternal tissues maintain pregnancy and help fetal growth and organ maturation [6]. Among these factors, glucocorticosteroid hormones have long been thought to control the timing of parturition as well as contribute to organ maturation and fetal growth [7, 8]. Endogenous glucocorticoids provide a critical developmental trigger later in gestation, which is essential for normal maturation of the fetal lung by inducing the production of pulmonary surfactant [3], a significant determinant of lung function. In most mammalian species, increased concentrations of glucocorticoids are evident in the maternal and fetal circulations and amniotic fluid before the onset of labor. Intrauterine fetal tissues, fetal membranes, and placentae are the major sites of native glucocorticoid production [9]. These tissues produce corticotropin releasing hormone (CRH) and synthesize fewer CRH-binding proteins that increase free CRH availability at term [10]. Elevated CRH levels regulate the production of ACTH and cortisol, which in turn promotes the expression of the placental CRH gene, creating a positive feedback loop and the output of glucocorticoids [11]. Besides that, when applied at a precise stage of fetal maturation, glucocorticoids are critical for the development of many other organ systems, including the thyroid, kidney, brain, and pituitary [2].

Lower birth weight, primarily due to underdeveloped fetal organ systems, in preterm neonates is attributed to delivery before glucocorticoid production in utero. Maternal glucocorticoid administration has been shown to reduce the risk of PTB-associated mortality and neonatal morbidity. Standard glucocorticoid injection procedure calls for four doses of 6 mg every 12 h for up to 48 h [1, 12]. Currently, pure cortisol, the native glucocorticoid in utero, is not given as an injection to pregnant women due to its conversion to an inactive form as it crosses the placenta. Instead, dexamethasone (Dex) and betamethasone, both synthetic glucocorticoids, are used depending on availability, cost, and standard clinical practice to induce organ maturation, as they are not inactivated by endogenous systems.

Dex is a long-acting synthetic corticosteroid hormone that ranks among the most widely used prescribed drugs, and it is increasingly employed during the perinatal and neonatal periods. However, it has been claimed that Dex exposure during pregnancy can affect the developing embryo, although this remains highly controversial [2–5, 7, 8, 13, 14]. The short-term beneficial effects of glucocorticoids have outweighed their long-term risks of complication throughout life, leading to its routine administration in women experiencing preterm labor. Administration of antenatal glucocorticoids is recommended in pregnancies likely to deliver before 34 weeks, as it

reduces the incidence of respiratory distress syndrome in the newborn and improves neonatal survival [15]. Besides that, premature infants exposed to this therapy are also less likely to experience an intraventricular hemorrhage or necrotizing enterocolitis than unexposed preterm infants [8]. However, at the price of accelerated lung maturity, the fetus might experience an increased risk of neuronal loss, neurodevelopmental disability, intrauterine growth restriction, programming of postnatal hypertension, and postnatal activity in the hypothalamic–pituitary–adrenal (HPA) axis [8]. It has been postulated that exposure of the developing fetus or neonate to these powerful glucocorticoid signals at an incorrect stage or in excess might lead to substantial alterations in normal developmental trajectories, resulting in altered physiological function experienced throughout life [2]. Additionally, the latest follow-up studies in children suggest that exposure of the fetus to glucocorticoids has profound long-term effects on HPA function, which is known to be associated with the development of chronic diseases [2]. A better understanding of the consequences of antenatal glucocorticoid exposure is essential prior to proceeding with such therapy.

Dex administration in preterm laboring subjects is expected to accelerate the proliferation of cells in fetal organs to mature them faster. We tested the hypothesis that Dex-induced rapid proliferation of fetal cells can also promote telomere-dependent cellular senescence (a mechanism of aging), a natural consequence of cell division leading to biologic aging. Thus, Dex may benefit fetal organ maturation but program them to age in utero contributing to adult-onset diseases during childhood. To test this hypothesis, we used fetal membrane-derived amnion epithelial cells (AECs) and treated them with Dex and determined Dex-induced effects on cellular changes (proliferation, telomere attrition, senescence, and inflammation). Fetal membrane cells were used based on our prior report that these cells can function as a proxy for epigenetic programming changes in the fetus [16]. Additionally, at term, pluripotent AECs are viable, proliferate, transition to different phenotypes, migrate, and demonstrate changes often seen in the fetus at parturition. We report that Dex treatment induces telomere reduction and p21-mediated senescence in AECs, even in the absence of rapid proliferation of cells. RU486, a progesterone receptor antagonist, used as a control in this experiment led to AEC necrosis and release of inflammatory mediators. The physiologic dose of Dex can accelerate fetal cell senescence without senescence-associated inflammation.

Materials and methods

Fetal membranes for this study were obtained from subjects who delivered at John Sealy Hospital, The University of Texas Medical Branch (UTMB), Galveston, TX. The UTMB Institutional Review Board approved this study (protocol number 11–251, original placental collection protocol amended and approved in 2017 to collect samples for this study) as an exempt protocol, where informed written consent from subjects was not required as only unidentified and discarded placental samples were used. Stringent inclusion and exclusion criteria were used to avoid any confounding variables to interfere with interpretation of data. Exclusion criteria included history of preterm labor in current pregnancy or prior pregnancies, treatment with steroid or any other drugs for conditions related to pregnancy, Group B *Streptococcus* colonization, bacterial vaginosis, behavioral risks such as cigarette smoking, alcohol, or other illicit drug use, and surgery history or other complications during pregnancy.

Clinical samples and cell culture

Primary AECs were isolated from the amnion layer obtained from fetal membranes from term, not in labor, Cesarean sections. Approximately 10 g of amnion, peeled from the chorion layer, was dispersed by successive treatments with 0.125% collagenase and 1.2% trypsin. All cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). Details of AEC isolation protocols can be found in our previous reports [17]. Briefly, the dispersed cells were plated in a 1:1 mixture of Ham's F12/DMEM, supplemented with 10% heat-inactivated fetal bovine serum, 10 ng/mL epidermal growth factor (EGF), 2 mM L-glutamine, 100 U/mL penicillin G, and 100 mg/mL streptomycin at a density of 3–5 million cells per T75 flask and incubated at 37 °C with 5% CO₂ until 80–90% confluence was achieved.

Cell culture treatments

AECs were treated with 200 nM of Dex (Sigma-Aldrich) in a complete DMEM medium for over 48 h based on prior reports that showed similar doses of Dex producing labor-associated changes in AECs as well as a reported dose of Dex crossing the placental barrier in animal models [18–21]. RU486 (0.4 μ M; Mifepristone, APEXBio, Houston, TX), a known antagonist to the glucocorticoid receptor (GR), was used alongside Dex to test Dex-induced signaling. The dose of RU486 was chosen based on previously published reports [22–24] as well as a titration in AECs treated with RU486 and RU486+Dex for over 48 h (Supplemental Figure S1). The p21 inhibitor UC2288 (20 μ M) (Sigma-Aldrich) was used to block p21 at the transcription and post-transcription levels. Once cells reached 70–80% confluence, each flask was rinsed with sterile 1x PBS, serum starved for 1 h, followed by treatment with control media (Dex) or Dex+RU486-containing media and incubated at 37 °C, 5% CO₂, and 95% air humidity for over 48 h. After 48 h, bright-field microscopy was performed to determine cell morphology, after which cells were collected for quantitative RT-PCR, western blots, or flow cytometry assays.

Crystal violet proliferation assay

AECs were seeded in a 12-well plate (90 000 per well) and treated with either Dex, RU486, or Dex+RU486 media for over 48 h. All experiments were performed in triplicate. After each time point, cells were washed with 1x PBS, fixed with 4% paraformaldehyde for 15 min, washed with 1x water, 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 of 590 nm. Untreated cells maintained in standard cell culture conditions were used as controls.

Flow cytometry assays

Proliferation rate and cell cycle

To determine the pattern of the cell cycle in control and treated AECs, cell cycle analysis was performed using the flow cytometer, as described previously [25] using the Coulter DNA Prep Reagents Kit (Beckman Coulter, Indianapolis, IN). Briefly, 50 μ L of DNA Prep LPR was added to each sample and vortexed. Then, 1.0 mL DNA Prep Stain was added to the tubes, vortexed, and run immediately on the Cytoflex flow cytometer (Beckman Coulter). After selecting for single cells, gating was set for the control cells and applied to histograms for the Dex and Dex+RU486-treated AECs using Cytexpert (Beckman Coulter). Cycle analysis by measuring DNA content was

used to distinguish between different phases of the cell cycles. Fluorescence intensity that directly correlated with the amount of DNA contained in a cell was measured. Concurrent parameter measurements made it possible to discriminate between S, G₂, and mitotic cells. As the DNA content doubles during the S phase, the intensity of the fluorescence increases, making it possible to ascertain the action of Dex on cell proliferation.

RNA extraction and quantitative RT-PCR

Treated AECs were spun down at 3000 g for 10 min, and cells were collected for RNA extraction and quantitative RT-PCR analysis. Quantitative RT-PCR was used to determine changes in GRs, membrane progesterone receptors (PGRMC1 and 2), IL-6 and IL-8, and gene expression. RNA was extracted using the Direct-zol RNA Miniprep Kit (Zymo-Research, CA). RNA samples (0.1 mg/mL) were subjected to reverse transcription by the High-Capacity cDNA Archive Kit (Applied Biosystems, CA). Real-time PCR using SYBR green was performed using an ABI 7500 Fast RealTime PCR System (Applied Biosystems). Predesigned human PGRMC1, PGRMC2, GR, IL-6, and IL-8 forward and reverse primers were obtained from Integrated DNA Technology (San Diego, CA). Primer specificities were tested by RT-PCR and confirmed by melting (dissociation) curve analysis. GAPDH was used as an internal control. Amplification was performed under the following conditions: initial denaturation for 30 s at 95 °C was followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing/extension for 30 min at 60 °C. All reactions were performed in duplicate, and template controls were included in each run. The comparative $\Delta\Delta$ Ct method was used to calculate relative quantification of gene expression.

Telomere length

Quantitative RT-PCR was used to determine changes in average telomere length of treated (Dex and Dex+RU486) and untreated AECs based on ScienCell's Absolute Human Telomere Length Quantification qPCR Assay Kit (#8918). The telomere primer set recognizes and amplifies telomere length by comparing samples to reference genomic DNA containing a 100 base pair (bp) telomere sequence located on human chromosome 17. Treated AECs were spun down at 3000 g for 10 min, and cells were collected for DNA extraction and quantitative RT-PCR analysis. DNA was extracted utilizing buffers and spin columns following the DNeasy Blood and Tissue Kit instructions provided by Qiagen (Qiagen # 69506, Germany). Each PCR reaction contained genomic DNA sample (0.01 μ g/ μ L), telomere primer, 2x qPCR master mix, and nuclease-free water. Primer-probe real-time PCR was performed using BioRad's CFX96 Real-Time System (BioRad, Hercules, CA). Reference genomic DNA was used as an internal control. All reactions were performed in duplicate, and template controls were included in each run. Amplification was performed under the following conditions: denaturation for 10 min at 95 °C followed by 32 cycles of denaturation for 20 s at 95 °C, annealing for 20 s at 52 °C, and extension for 45 s at 72 °C. The average telomere length was calculated by following the manufacturer's instructions.

Flow cytometry assays

Senescence-associated β -galactosidase activity

Senescence was assessed with the commonly used biomarker senescence-associated β -galactosidase (SA- β -Gal) activity, adapted for flow cytometry in our laboratory as previously described [26, 27]. Briefly, cells were incubated for 1 h in complete DMEM growth

medium supplemented with 100 nM bafilomycin A1 (baf A1) for 1 h at 37°C. Without changing media, 5-dodecanoylaminofluorescein di- β -D-galactopyranoside (C₁₂FDG) was added (final concentration of 6 μ M) and incubated at 37°C for 1 h. Cells were harvested by trypsinization and centrifugation at 3000 g for 10 min at 4°C. The cell pellet was resuspended in 500 μ L Coulter DNA Prep Stain (Beckman Coulter, Indianapolis, IN), which contains propidium iodide (PI) to indicate viable and nonviable cells, and run immediately on the CytoFlex flow cytometer (Beckman Coulter). Unstained, control AECs were used as negative controls for gating. Data were analyzed using Cytexpert software (Beckman Coulter), and cells positive for C₁₂FDG and negative for PI (viable) were considered for analysis.

Flow cytometry assays

Apoptosis and necrosis staining

To determine the population of cells undergoing apoptosis and/or necrosis, cells were stained using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & PI (Life Technologies, Carlsbad, CA). Briefly, cells were harvested by trypsinization and centrifuged for 5 min at 2000 g. Cell pellets were washed with cold 1x PBS and centrifuged at 2000 g for 5 min. Pellets were resuspended in 100 μ L 1x annexin binding buffer supplemented with 5 μ L Alexa Fluor 488 Annexin V and 1 μ L 100 μ g/mL PI. After a 15-min incubation, 400 μ L annexin binding buffer was added, and samples were run immediately on the CytoFlex flow cytometer (Beckman Coulter). Unstained control AECs were used as negative controls for gating. Data were analyzed using Cytexpert software (Beckman Coulter).

Protein extraction and immunoblot assay

Cells were lysed with RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, and 1.0 mM EDTA pH 8.0, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail and phenylmethylsulfonyl fluoride. The lysate was collected after scraping the culture plate, and the insoluble material was removed by centrifugation at 9000 g for 20 min at 4°C. The supernatant was collected, and protein concentrations in each lysate were determined using Bicinchoninic Acid Protein assay kits (Pierce BCA Protein Assay Kit, Thermo Scientific, Waltham, MA). The protein samples were separated using SDS-PAGE on a gradient (4–15%) with Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA) and transferred to the membrane using the iBlot Gel Transfer Device (Thermo Fisher Scientific). The membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% skim milk for 2 h at room temperature. Blots were incubated separately with antibodies against total p38MAPK (Cell Signaling, Danvers, MA, #9212, 1:1000), phosphorylated (P)-p38MAPK (Cell Signaling, #9211, 1:300), total p21 (Santa Cruz, SC-397, 1:1000), phosphorylated p21 (Santa Cruz, SC-20220, Thr 145, 1:300), and ATG16L β (Cell Signaling, 1:600) at 4°C and shaken overnight. The membrane was incubated with appropriate peroxidase-conjugated IgG secondary antibody for 1 h at room temperature. All blots were developed using chemiluminescence reagents ECL Western Blotting Detection System (Amersham, Piscataway, NJ), in accordance with the manufacturer's recommendations. The stripping protocol followed the instructions of the Restore Western Blot Stripping Buffer (Thermo Fisher). No blots were used more than three times. Densitometry was performed to normalize the data for statistical analysis.

Statistical analyses

All experiments were conducted in triplicate. Data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., LaJolla, CA). One-way ANOVA followed by Tukey comparison and independent sample *t*-tests were used as appropriate. A *P* < 0.05 was considered significant.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

Results

Dexamethasone does not induce rapid proliferation of amnion epithelial cells in vitro

We studied the effect of Dex on AEC proliferation compared to standard cell culture conditions. Co-treatment with RU486, an antagonist to glucocorticoid function, was used as a control [28]. Cytotoxicity, as determined by crystal violet dye exclusion studies, determined the viability of AECs under normal cell culture conditions and after treatments. After 48 h in culture, viability was 100% in control cells, 92% in Dex-treated, 30% in Dex+RU486-treated cells, and 50% in RU486-treated cells (Figure 1a and b) (Supplemental Figure S1a). Co-treatment of RU486 with Dex significantly decreased cell viability after 48 h (*P* = .04) (Figure 1b). Flow cytometry was used to determine cell proliferation by measuring changes in the DNA content as a proxy for increasing number of cells. Dex did not induce rapid AEC proliferation as determined by similar levels of DNA in S phase after 48 h (Figure 1c and d) compared to control cultures. However, RU486 co-treatment produced significant DNA damage (*P* = 0.02) after 48 h (Figure 1c). These data suggest that Dex does not induce rapid cell proliferation of AECs in vitro. As RU486 treatment alone over a period of 24–48 h produced cytotoxicity (Supplemental Figure S1a) (shown by tissue necrosis—Supplemental Figure S2b) similar to that of Dex+RU486 treatment, data from Dex+RU486 treatment are only presented in this manuscript.

Dex treatment does not increase glucocorticoid or membrane progesterone expression

As Dex is known to exert its functions through GRs as well as PGRMCs [29], we tested changes in the expression of these receptors (PGRMC1 and 2). Quantitative RT-PCR was used to determine the ability of Dex to induce its native GRs and PGRMCs in AECs. Dex treatment did not induce either GRs or PGRMCs expression, whereas Dex+RU486 increased the expression of all steroid receptors (Figure 2a). As AECs do not express genomic progesterone receptors [30], we did not examine them in this report.

Dex-induced telomere attrition leads to AEC aging

Although Dex treatment did not induce rapid proliferation in AECs, Dex induced telomere length reduction (Figure 3a). After 48 h, telomere lengths were 5.7 ± 0.8 and 2.6 ± 1.2 kb in 100 and 200 ng/mL Dex-treated cells, respectively, compared to control (8.5 ± 3.1 kb; *P* = 0.0015). Although Dex treatment did not induce cell proliferation, it produced a shortening of telomere length. As cells were showing necrotic phenotypes after Dex+RU486 treatment, telomere lengths were not attempted in those cells.

Figure 1. Dexamethasone (Dex) treatment does not affect viability or induce rapid proliferation in amnion epithelial cells (AECs). (a) Morphology of primary AECs under normal cell culture conditions (control cultures), Dex, and Dex+RU486 conditions. Cells were contoured or puckered after Dex+RU486 and RU486 alone, indicating some form of cell stress. Crystal violet staining was used to show AEC viability. The numbers of crystal violet positive cells after 48 h of Dex+RU486 and RU486 alone were less compared to confluent cells after Dex or control treatments. Images were taken with $\times 10$ magnification. (b) Dex did not affect the viability of AECs as determined by intensity of crystal violet dye excluded from cells. Dye exclusion was not different after Dex treatment regardless of the time point compared with control cells. Dex+RU486 treatment significantly decreased viability after 48 h (6 h control 0.13 ± 0.03 RIU, Dex 0.13 ± 0.03 RIU, Dex + RU486 0.11 ± 0.03 RIU) (24 h control 0.18 ± 0.04 RIU, Dex 0.17 ± 0.05 RIU, Dex + RU486 0.12 ± 0.013 RIU) (48 h control 0.26 ± 0.08 RIU, Dex 0.24 ± 0.08 RIU, Dex + RU486 0.08 ± 0.00 RIU) ($P = 0.04$) ($N = 3$). RIU = random intensity unit. (c, d) Flow cytometry analysis of the cell cycle shows no differences in cell cycle status between Dex and control samples after 48 h. There is a significant increase in DNA damage in Dex+RU486-treated cells compared to control- or Dex-treated cells (control 3.0 ± 1.3 RIU, Dex 4.6 ± 1.9 RIU, Dex + RU486 12.8 ± 5.1 RIU) ($P = 0.02$) ($N = 3$). RIU = random intensity unit.

Figure 2. Dexamethasone (Dex) treatment does not affect receptor expression in AECs. (a) Quantitative RT-PCR data determined that Dex treatment did not change either glucocorticoid receptors (GRs) or nongenomic progesterone receptors (PGRMC1 and PGRMC2) expression, whereas Dex+RU486 produced increased expression of all steroid receptors. (PGRMC1: control 1.2-fold; Dex 0.6-fold; Dex + RU486 2.5-fold) (PGRMC2: control 1.2-fold; Dex 1.1-fold; Dex + RU486 2.2-fold) ($P > 0.05$) (GRs: control 1.2-fold; Dex 0.7-fold; Dex + RU486 3.9-fold) ($N = 5$).

Cell fate after Dex treatment

Flow cytometry analysis of the fate of AECs after Dex treatment showed cellular senescence, as determined by the increased presence of SA- β -Gal activity assays (control: 13.53 ± 4.26 RFI, Dex: 37.69 ± 10.83 RFI, Dex + RU486: 17.76 ± 10.77 RFI) ($P = 0.001$) (RFI: random fluorescent intensity) (Figure 3b and c). Interestingly, Dex treatment did not cause autophagy (Figure 4a) or necrosis (Figure 4b and c), while Dex+RU486 predominantly induced necrosis ($P = 0.0003$) (Figure 4b and c). Base levels of apoptosis (22.5%) were observed in cells after all treatments (Figure 4b and c).

Dex induces p21 activation but not p38MAPK

As noted above, senescence was seen in AECs after Dex treatment. Previous reports have shown that oxidative stress-induced telomere loss and generation of cell-free fetal telomere fragments can induce p38MAPK-mediated cellular senescence. Additionally, p38MAPK-mediated senescence also involves its downstream effector p21 in AECs [17, 31, 32]. To explore signaling mediators causing senescence after Dex treatment in AECs, activation of p38MAPK and p21 (phosphorylation) was analyzed by western blot. Contrary to our expectations, 48-h treatment with Dex suppressed p38MAPK activation compared to control group ($P < 0.05$) and with co-treatment of RU486 ($P < 0.05$) (Figure 5a). However, Dex treatment induced p21 activation when compared with control ($P < 0.05$) and Dex+RU486 ($P < 0.05$) groups (Figure 5b). Unlike many other stimulants (lipooligosaccharides, tumor necrosis factor- α , and cigarette smoke) that caused p38MAPK activation and senescence [27], AEC senescence after Dex treatment was not mediated via with p38MAPK activation. These data are suggestive of a unique pathway between Dex-induced telomere reduction and activation of cell cycle inhibitor p21.

Dex induces p21-dependent senescence in AECs

To determine the ability of Dex to induce p21-dependent senescence in AECs in culture, flow cytometry measuring SA- β -Gal was performed with and without a p21 inhibitor, UC2288. Co-treatment with Dex and UC2288 after 48 h significantly reduced cellular senescence in AECs ($P = 0.006$) (Figure 5c and d). These results indicate that Dex induces p21-dependent senescence in AECs.

Dex-induced senescence is not associated with changes in inflammatory markers

As expected, Dex, a reported anti-inflammatory steroid, did not induce the expression of proinflammatory cytokines IL-6 and IL-8, whereas Dex+RU486 significantly increased the expression of IL-6 ($P = 0.002$) and IL-8 ($P = 0.0008$) (Figure 6a).

Discussion

Clinically, obstetricians are faced with a recurring issue regarding treatment of fetuses of women undergoing preterm labor. The question to therapeutically intervene at specific developmental stages during adverse pregnancies is an issue most clinicians face without fully understanding or considering the fetal and maternal risks. One such example is a clinician's choice to administer synthetic glucocorticosteroids to women undergoing preterm labor to enhance fetal organ development that is expected to improve birth weight and reduce the risk of neonatal mortality and morbidity. However, synthetic glucocorticosteroid injection has also been linked to maternal and fetal adverse side effects [2, 4, 5, 13, 33]. Maternal short-term effects include an increased risk of infection and a higher incidence of endometritis [5]. On the fetal side, synthetic glucocorticosteroids have been reported to impair programming of the fetal HPA axis, contributing to impaired brain growth, altered behavior, and changes in cardiovascular and metabolic function [2, 4, 8]. Although the consequences are unclear, glucocorticosteroids affect the fetal epigenome by regulating DNA methylation and histone acetylation that can be passed down to future generations [4].

We hypothesized that Dex, similar to other steroids, would promote rapid fetal lung cell replication in utero to promote lung maturation, and during this process, it will accelerate cellular aging. AECs that are fetal in origin were used as a proxy to test this hypothesis. We report that a physiologic dose of Dex may cause fetal cell maturation, not necessarily through rapid proliferation, however, could accelerate senescence and induce adverse effects, such as telomere attrition and aging-associated changes, even in the absence of any other phenotypic changes (Figure 7a). This finding is consistent with other reports documenting Dex's ability to induce surfactant-A production in lung epithelial cells without extensive cellular proliferation [34].

Figure 3. Dexamethasone (Dex)-induced telomere attrition leading to AEC aging. (a) Quantitative RT-PCR for telomere length showed Dex produced time (48 h) and reductions of telomere length in AECs (control 8.5 ± 3.1 kb; Dex 100: 5.7 ± 0.8 kb; Dex 200: 2.6 ± 1.2 kb) ($P = 0.0015$). (N = 5). (b, c) Flow cytometry analysis of senescence-associated β -galactosidase (SA- β -Gal) showed Dex treatment increased senescence in AECs when compared with control ($P < 0.05$) or cells treated with Dex + RU468 (control 13.53 ± 4.2 RFI, Dex 37.69 ± 10.83 RFI, Dex + RU468 17.76 ± 10.77 RFI). ($P = 0.001$, $P = 0.005$) (N = 5). RFI = random fluorescence intensity.

One of the limitations of this study is that we examined proliferation and not differentiation of AECs. It is likely that Dex can cause lung cell differentiation into a distinct mature cell phenotype that can help lung maturation. This phenomenon is not tested in this study and limited to Dex-induced proliferation alone. In addition, we examined Dex-induced transition of AEC into mesenchymal phenotype. As shown in Supplemental Figure S3, we do not see cell transition. It is likely that Dex's role in lung maturation may not involve steroid-induced proliferation, instead signaling to produce lung lubrication critical for breathing ex utero. Although not tested in this study, the effect of Dex may mediate cellular hypertrophy that contributes to increased size and not necessarily proliferative activities to enhance size. To note, Dex treatment may accelerate telomere attrition of lung epithelial cells and cellular aging. Therefore, fetuses exposed to Dex may have aged lungs beyond their gestational age at birth without any clinical indication such as inflammation. However, this programming effect may contribute to adult-onset lung diseases in these children.

Although Dex did not induce proliferation, it did induce telomere attrition in AECs. Telomeres are structures consisting of repetitive rows of proteins and noncoding DNA that form the ends of the chromosomes. Because of the inability of DNA polymerases to replicate DNA at the very ends of linear chromosomes, telomeres become progressively shortened during successive cell divisions, leading to a permanent cell cycle arrest, also known as replicative senescence [35]. In culture and or in vivo when a cell reaches its Hayflick limit (i.e. when an average cell population can no longer divide), it naturally undergoes "replicative cellular senescence" [36]. Telomere length reduction is one of the indicators of biologic aging. AECs treated with Dex demonstrated a reduction in telomere length of ~ 5 kb, which is slightly lower than the average AEC telomere length at 41 weeks' gestation (22 weeks: ~ 18 kb; 41 weeks: ~ 7 kb) [6]. On average cells lose 50–100 kb per cellular division, suggesting cells treated with Dex have undergone extra 20 cell divisions compared to 41-week fetal cells [6]. Since DNA damage is not seen after Dex treatment, telomere reduction is likely due to human

Figure 4. Dexamethasone (Dex) does not induce autophagy, necrosis, or apoptosis in amnion epithelial cells (AECs). (a) Dex or Dex+RU486 treatment of AECs did not induce autophagy measured by ATG16L β expression when compared with control (control 0.63 ± 0.3 RIU, Dex 0.71 ± 0.5 RIU, Dex + RU486 0.25 ± 0.2 RIU) ($P = 0.36$) ($N = 3$). RIU = random intensity unit. (b,c) Treatments with Dex did not cause AEC necrosis, while treatments with Dex+RU486 increased AEC necrosis when compared with control and Dex treatments (control 4.8 ± 1.3 RFU, Dex 5.9 ± 1.5 RFU, Dex + RU486 45.7 ± 10.2 RFU) ($P = 0.0006$, $P = 0.0008$). Dex treatment of AECs did not induce apoptosis (control 22.0 ± 12.8 RFU, Dex 23.6 ± 12.6 RFU, Dex + RU486 22.5 ± 10.5 RFU) ($P = 0.9$) ($N = 3$). RFU = random fluorescence unit.

Figure 5. Analysis of dexamethasone (Dex)-induced phospho-p21-dependent senescence. Western blot analysis showed induction of p21 phosphorylation in Dex-treated AECs but not p38MAPK. (a) Dex treatment significantly reduced phospho-p38MAPK compared to control or Dex+RU486 treatments (control 1.52 ± 0.62 RFU, Dex 0.94 ± 0.94 RFU, Dex + RU486 1.84 ± 0.75 RFU) ($P < 0.05$) ($N = 3$). RFU = random fluorescence unit. (b) Dex increased P-p21 expression in AECs compared to control treatment and Dex+RU486 co-treatment (control 0.06 ± 0.004 RFU, Dex 0.41 ± 0.3 RFU, Dex + RU486 0.05 ± 0.06 RFU) ($P = 0.02$) ($N = 3$). RFU = random fluorescence unit. (c, d) Flow cytometry analysis showing co-treatment of p21 inhibitor UC2288 with Dex significantly reduced cellular senescence in AECs after 48 h (control 11.19 ± 1.8 RIU, Dex 19.68 ± 4.3 RIU, Dex + UC2288 10.05 ± 5.4 RIU) (control vs Dex: $P = 0.01$) (Dex vs Dex + UC2288: $P = 0.006$) ($N = 3$). RIU = random intensity unit.

Figure 6. RU486, but not dexamethasone (Dex), induced proinflammatory cytokine gene expression. (a) Quantitative RT-PCR data show that treatment with Dex+RU486 significantly increased the gene expression of proinflammatory cytokines IL-6 (control 1-fold, Dex 0.4-fold, Dex + RU486 2.7-fold) ($P = 0.02$, $P = 0.002$) and IL-8 (control 1.1-fold, Dex 1.2-fold, Dex + RU486 114.5-fold) ($P = 0.0008$, $P = 0.0008$) ($N = 5$).

Figure 7. Cartoon representation of proposed dexamethasone (Dex) pathways. Contrary to our hypothesis that Dex will cause rapid proliferation of amnion epithelial cells (AECs), resulting in cellular senescence similar to that seen with lipooligosaccharides, tumor necrosis factor- α , and cigarette smoke extract or environmental pollutants, mediated via telomere attrition and p38MAPK (Proposed pathway), we report that Dex does not stimulate rapid proliferation of AECs, yet reduces telomere length (Observed pathway; left), causing p21-dependent and p38MAPK-independent cellular senescence. This senescence also lacks the senescence-associated inflammatory phenotype (SASP). On the other hand, co-treatment of Dex with RU486 (Observed pathway; right) induces DNA damage, necrosis, and inflammation.

telomerase reverse transcriptase/telomerase inhibition in the absence of rapid cell proliferation [4, 37]. The telomere reduction reported here coincides with p21-dependent senescence, suggesting that in utero synthetic glucocorticoid treatment may prematurely age fetal cells by reducing their overall telomere length without inducing cellular replication. Though these conclusions may be drawn based on our data, it is important to note the key differences between fetal

AECs and fetal lung epithelial cells and how they could respond to Dex treatments differently than what is proposed here. AECs and fetal lung epithelial cells are at different stages of their cellular life at the time of synthetic glucocorticoid administration and both could respond similarly to Dex treatment in utero but diverge to different developmental trajectories due to the removal of Dex after delivery, specifically for lung cells.

Telomere attrition and senescence has also been documented in AECs under oxidative stress conditions [17, 31]. Here, we show Dex induces nonreplicative cellular senescence and does not cause any other forms of cell deaths (necrosis, apoptosis, or autophagy). Unlike p38MAPK-mediated senescence in AECs under oxidative stress [17], Dex-induced senescence is independent of p38MAPK activation but still involves its downstream effector p21, as confirmed by p21 inhibitor UC2288 treatment that abrogated Dex-induced AEC senescence. Dex-induced p21-mediated senescence is unique in AECs compared to the canonical p38MAPK-mediated process that is persistent and nonreversible and associated with inflammation known as senescence-associated secretory phenotype [38]. Dex is a known anti-inflammatory mediator; therefore, lack of inflammation observed in our study could also be due to lack of p38MAPK activation that can cause activation of NF- κ B, a transcriptional activator. It is also likely that Dex-induced p21-mediated senescence is a “reversible” form of cell cycle arrest. Thus, if fetal lung epithelial cells underwent Dex-induced telomere-p21 nonreplicative senescence, it could enter back into the cell cycle once the stimulant (Dex) is removed from the environment at delivery [39], which will also restore the function of human telomerase reverse transcriptase. However, this restoration likely occurs at the cost of overall telomere length at the time of birth, potentially predisposing the fetus to chronic diseases such as early-onset asthma, chronic obstructive pulmonary disease, and changes in cardiovascular and metabolic function.

Dex injections can reach the fetus to mature the fetal organ systems by crossing the placenta undetected by 11 β -hydroxysteroid dehydrogenase-1 (11 β -HSD1) and by interacting with the GRs and PGRMCs. 11 β -HSD1, also known as cortisone reductase, is NADP-dependent and catalyzes the conversion of cortisol, the native form of glucocorticoids, to cortisone and vice versa, whereas 11 β -HSD2 requires NAD and has only oxidase activity (conversion of hormonally active cortisol to an inactive form of cortisone) [40]. Both isoenzymes were identified in the human placenta and protect the fetus from high glucocorticoid levels throughout gestation. 11 β -HSD1 is more abundant in fetal membrane cells (e.g. AECs) and 11 β -HSD2 in the syncytiotrophoblasts and placental tissue [41]. In our study, Dex did not induce 11 β -HSD1, suggesting that the effect of Dex does not involve endogenous cortisol production in AECs (Supplemental Figure S2A) nor does it induce the overexpression of GRs or PGRMCs. This protects fetal cells from experiencing a positive feedback loop of endogenous cortisol, which could exponentially increase the adverse developmental effects in fetal tissues similar to that shown in this study.

RU486, classically a progesterone receptor antagonist, is known to bind to glucocorticoid and progesterone nuclear and membrane receptors [22]. Clinically, it is prescribed to antagonize progesterone receptors to induce late-stage abortions; however, its mechanism of action is still unclear. We used RU486 as an inhibitor in these experiments with the intention that it would bind to glucocorticoid and progesterone membrane receptors and prevent Dex signaling. However, contrary to the reported mechanisms of RU486 as an antagonist to steroid receptors, it induced DNA damage, necrosis, and increased inflammatory cytokine production. To rule out that this RU486 effect was cell type specific, we conducted similar experiments using myometrial cells. As shown in Supplemental Figure S2B, myometrial cells show similar effects with dose that are reported to be physiologic in in vitro experiments [22–24]. Therefore, we speculate that RU486 did not function as an inhibitor in our study, but instead activates pathways that contribute to tissue necrosis and inflammation often associated with necrosis. The impact induced by RU486

to cause abortions is likely resulting from massive tissue necrosis and not necessarily arising from the withdrawal of endocrine functions that maintain pregnancy or a combination of both functions.

In summary, Dex is a long-acting synthetic corticosteroid hormone that is increasingly employed during the perinatal and neonatal periods to help fetal lung development to reduce neonatal mortality and morbidity. On the contrary, Dex has also been documented to affect the HPA axis and contribute to neurological, cardiovascular, and metabolic anomalies that can pass to future generations on the epigenetic level. This study provides a mechanism by which Dex may cause premature aging of organs during their induced and accelerated organ maturation process. Dex treatment may program fetal cells for telomere-dependent premature aging leading to “over mature” fetal cells at the time of delivery, which could predispose them to adult-onset diseases.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1016/j.biolre.2019.05.001) online.

Supplemental Figure S1. RU486 titration in amnion epithelial cells (AECs).

Supplemental Figure S2. Dex titration in amnion epithelial cells (AECs).

Supplemental Figure S3. Immunohistochemistry analysis of nuclear and membrane progesterone receptors at term in fetal membranes.

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Conflict of interest

The authors declare no competing financial interests.

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