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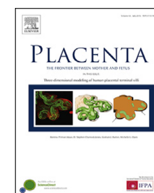

Differential senescence in feto-maternal tissues during mouse pregnancy

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ABSTRACT

Background: Human studies show that fetal membranes have a limited lifespan and undergo telomere-dependent cellular senescence that is augmented by oxidative stress and mediated by p38 mitogen activated protein kinase (MAPK). Further, these studies suggest that fetal membranes are anatomically and physiologically positioned to transmit senescence signals that may initiate parturition at term.
Methods: Longitudinal evaluation of feto-maternal tissues from mouse pregnancies was undertaken to determine the molecular progression of senescence during normal pregnancy. On days 10–18 of gestation, C57BL/6 mice were euthanized. Fetal membranes, placenta, and decidua/uterus were collected. Tissues were examined for Telomere length (TL) and the presence of Phosphorylated (P) p38MAPK and p53, p21 and senescence associated β-Galactosidase (SA- β-Gal).
Findings: Linear regression modeling of observed telomere length as a function of gestational age revealed that beta (β), the slope of the linear regression was negative and significantly different from zero for each tissue (fetal membranes, $\beta = -0.1901 \pm 0.03125$, $p < 0.0001$; placenta $\beta = -0.09000 \pm 0.03474$, $p = 0.0135$; decidua/uterus $\beta = -0.1317 \pm 0.03264$, $p = 0.0003$). Progressive activation p38MAPK was observed in all tissues from days 10 to day18, with the highest activation observed in fetal membranes. Activation of p53 was progressive in fetal membranes. In contrast, active p53 was constitutive in placenta and decidua/uterus throughout gestation. Detection of p21 indicated that pro-senescent change was higher in all compartments on day 18 as compared to other days. The number of SA-β-Gal positive cells increased in fetal membranes as gestation progressed. However, in placenta and uterus and decidua/uterus SA-β-Gal was seen only in days 15 and 18.
Conclusions: Telomere dependent p38 and p53 mediated senescence progressed in mouse fetal membranes as gestation advanced. Although senescence is evident, telomere dependent events were not dominant in placenta or decidua/uterus. Fetal membrane senescence may significantly contribute to mechanisms of parturition at term.

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1. Introduction

The length of gestation and parturition in humans and other mammals is determined by gene-by-environment interactions, which ultimately lead to catastrophic failure in functioning of the fetal membranes, leading to uterine contractions, cervical ripening and dilation, and expulsion of the fetus [1]. This is likely a physiologic process designed to promote parturition. Although debate continues, it is thought that the signals leading to parturition may be of both fetal and maternal origin [2]. It is generally thought that a fetal signal occurs when the fetus achieves a certain developmental stage, at which time it no longer requires the protection and support of in utero life, specifically the support provided by placenta and fetal membranes for fetal growth, development and protection [1]. The exact source of the "fetal signal" is unknown, but may include surfactant protein [3] cell free DNA [4], and other hormonal [5], mechanical [6] and immune [7] signals.

We have shown that human fetal membranes (amniochorion)

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undergo a developmental program that involves the generation of a group of "fetal" signals that initiate or augment mechanisms leading to parturition [8,9]. This developmental program is senescence [10], a mechanism associated with aging of the intrauterine tissues determining their longevity of in utero life. The signals generated from senescent feto-maternal tissues include a large group of senescence associated factors collectively called as senescence associated secretory phenotype (SASP) [11].

Senescence is the process of cellular aging by which cells change morphologically (size, shape, and organelle structure [12,13]), in gene (p16Ink4 [14], and p21 [10]) and protein (beta gal [15]) expression, and in the activation of key signaling constituents, such as p38 [16] and p53 [17,18] that can determine the fate of a tissue. Although it has been postulated that cellular apoptosis transmits an inflammatory signal that promotes parturition [4], senescent cells may be resistant to apoptosis [19], and still transmit both inflammatory and senescence-promoting signals. Thus senescence may comprise a broader array of cell signals relevant to induction of both term and preterm parturition. We have demonstrated senescence associated changes to fetal tissues and SASP signature in human parturition [8]. One of the mediators of senescence associated inflammation is oxidative stress experienced at term due to increased metabolic demand of the growing fetus, depletion of antioxidants or reduced maternal supply of substrates required for redox balance [20]. It is likely that other factors such as increased stretch [21], endocrine changes and fetal stressors can also contribute to this process. Therefore, we posit that oxidative stress at term can perturb homeostatic balances of the intrauterine cavity, promoting senescence associated inflammation that can trigger parturition. Several issues remain in testing this hypothesis and in understanding the potential underlying mechanisms. One is a clear sense of the kinetics of senescence development and propagation of inflammatory signals and another is the relative impact of senescence in other fetal and maternal tissues. Yet another is the exact delineation of mediators/modifiers of this process. These are difficult to study in humans due to the impracticality of longitudinal sampling and therefore 'cause/effect' will remain to be answered. Although in vitro studies have recapitulated many of these findings and addressed many of the mechanistic events associated with senescence associated parturition signals, in vitro models are limited due to their inability to accommodate multitudes of factors associated with the in vivo environment. To further address these issues, we have elected to perform a study in normal mouse fetal membranes, placenta, and decidua/uterus. By several parameters we find increased senescence in fetal membranes from mid gestation onward.

2. Materials and methods

2.1. Mice

C57BL/6 mice (aged 3–5 months) were obtained from Jackson Laboratory and housed under specific pathogen free and American Association for Laboratory Animal Care-accredited conditions. Animal use was under protocol approved by the Institutional Animal Care and Use Committee at the University of Vermont. Females and males were timed-mated as previously described [22]. Males and seasoned females were housed together overnight (16 h) and the males removed the next morning (designated as day 0 of gestation).

2.2. Collection of gestational tissues from different gestational age groups [23,24]

Pregnant uteri were removed and placed in sterile Iscove's Modified Dulbecco's Medium (Invitrogen). The uteri were cut longitudinally on the side opposite the implantation sites. Individual fetal-placenta units with associated amniotic sacs and underlying uterus were isolated, and the membranes grasped at the edge of the placenta with a fine forceps and peeled away. Then the placenta was removed from the uterus. If there was a well-defined decidua basalis cap [23], then we attempted to remove it entirely and this was used as the "decidua". If this was not possible, we used a sample of uterus just below the implantation site after peeling off the placenta. Thus the decidual samples are labeled "decidua/uterus" and, despite careful dissection, these samples have possibly been contaminated with either trophoblast or non-decidual uterine tissue, including myometrium.

2.3. Telomere length measurement

Fetal membranes, placenta and decidua/uterus collected on gestational day 9, 10, 12, 15, 17 and 18 of mouse pregnancies were subjected to a semiquantitative telomere length measurement [25]. DNA isolation was performed using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Germantown, MD) following the manufacturer's recommendations. The quality and concentration of extracted DNA were determined by 260/280 nm absorbance ratio (Gen5, Epoch, Bio Tek, Winooski, VT, USA), and the relative concentration of telomere fragments was analyzed using quantitative real-time PCR (qPCR). References for relative number of telomere fragments were generated by performing serial dilutions from a reference DNA sample to produce concentrations of DNA ranging from 30 to 0.315 ng/μL triplicate (for standard curves) and duplicate (for samples) PCR reactions using 5 ng DNA for each sample were carried out in a 20 μL volume using 2× DNA Master SYBR Green kit (Applied Biosystems (ABI), Foster City, CA, USA) on an ABI 7500 real-time PCR machine with SDS software, version 1.3.1. Primers for telomere (*tel1b*, 5′-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3'; and *tel2b*, 5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3′) were added to the final concentrations of 0.2 μM. The thermal cycling profiles were as follows: 95 °C for 10 min, followed by 20 cycles of 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 60 s. No template controls were included in all plate reactions. The relative number of telomere fragments in each specimen was normalized to the reference sample [$2^{-(\Delta Ct(sample) - \Delta Ct(control)} = 2^{-\Delta\Delta Ct}$].

2.4. Western blot analysis

Fetal membranes, placenta and decidua/uterus from various gestational days were homogenized in Radio immunoprecipitation assay buffer (RIPA) buffer with protease inhibitors using a bullet blender (Next Advance, Averill Park, NY). Protein quantification was done using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples containing 45 μg of protein were separated by SDS-gel electrophoresis (Bio-Rad, Hercules, CA) according to manufacturer's suggestions and proteins were transferred to a Polyvinylidene fluoride membrane using the iBlot dry blotting system (Life Technologies, Grand Island, NY). The membranes were blocked for 2 h in 5% milk in TBS-Tween-20. The blots were then incubated with primary antibody to total p38 (Cell Signaling #9212, Danvers, MA), P-p38 (Cell Signaling #9211, Danvers, MA) or P-p53 (ab1431, Abcam, Cambridge, MA), and total p53 (ab31333, Abcam) overnight at dilutions of 1:1000, 1:400, 1:200 and 1:400, respectively. Blots were then washed and incubated with secondary antibody for 1 h and exposed using Luminata Forte Western HRP substrate (Cat# WBLUF0100) from Millipore (Billerica, MA). In order to avoid inter-assay variability between blots, samples from the same experiments were run on the same gel for a given marker. The blots were all reprobed with antibodies to β-actin (Sigma, St. Louis,

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