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Short Communication

Fetal Growth Restriction is Associated with Accelerated Telomere Shortening and Increased Expression of Cell Senescence Markers in the Placenta

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ABSTRACT

A hallmark of fetal growth restriction (FGR) is restricted placental development and insufficient nutrient supply to the fetus. It has previously been shown that activity levels of telomerase, the enzyme responsible for completing replication of telomeric DNA during cell division, is suppressed in FGR placenta samples as compared to control placenta samples from donors of the same gestational age. Here we examine whether telomere length maintenance is also compromised in FGR placenta samples. Southern analysis of telomere length for placenta and cord blood samples from 32 FGR and 36 control donors, ranging in gestational age from 37 to 40 weeks, revealed significantly shorter telomeres ($P \leq 0.001$) in FGR placenta samples, but not cord blood samples. Furthermore, analysis of telomerase extracts, RNA and DNA placental samples from donors with and without idiopathic FGR confirmed a direct association between suppression of telomerase activity and reduced telomere length in FGR placenta. In addition, expression levels of markers of telomere-induced senescence, p21, p16 and EF-1α, were significantly elevated in FGR placenta samples ($P \leq 0.01$). These observations support a direct affect of reduced telomerase activity levels on the placental pathology associated with FGR.

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1. Introduction

Fetal growth restriction (FGR) is a relatively common, pleiotropic complication of pregnancy, affecting ~5–10% of newborns [1,2]. It is associated with substantially increased infant mortality as well as childhood and adulthood morbidity, including increased risk for cardiovascular disease, obesity and diabetes [2]. While the etiology is poorly defined, it is associated with a utero-placental insufficiency, with attenuated placental development and restricted nutrient supply to the fetus. The majority of instances of FGR (~60%) appear to be caused by other known pregnancy complications, including pre-eclampsia, congenital abnormalities and intrauterine viral infections [3,4]. The idiopathic instances of FGR (~40%) are generally characterized by asymmetric growth [4].

Previous studies have shown that FGR is associated with reduced levels of telomerase activity in the placenta [5]. Telomerase is an enzymatic complex that functions to complete the replication of telomeres, genetic elements that cap and protect the ends of chromosomes [6]. In normal human cells, the absence of telomerase leads to gradual reduction in telomere length with each cell division, ultimately compromising telomere function and signaling cell senescence [7–9]. Therefore in the present study, we sought to examine whether FGR is also associated with accelerated telomere loss and aberrant expression of genes associated with telomere-induced senescence in the placenta.

2. Materials and methods

2.1. Study groups and sample collection

All donors for this study were recruited, with informed consent and Institutional Review Board approval, at the Kapiolani Medical Center for Women and Children (KMCWC). All placental samples were obtained thru the Clinical Center for Research Excellence phenotyping core at the John A Burns School of Medicine. For all DNA placental samples used in the initial analysis of telomere length (Fig. 1), FGR is defined as any newborn having a birth weight of ≤5th percentile for Filipino newborns at a given gestational age (percentile cut-off was calculated from recent birth weight records from 2004 thru 2006 for KMCWC hospital). The percentile cut-off was calculated from birth weight data for Filipino newborns, since newborns of this ethnic background have the smallest average birth weight, and therefore false positive FGR donors (i.e. small for gestational age donors) should be minimal. The control cohort is defined as any newborn donor having a birth weight of ±1 standard deviation of the mean birth weight for all newborns at a given gestational age delivered at KMCWC hospital (2004–2006).

For the fresh placental samples obtained from idiopathic FGR donors and gestational age-matched controls (Fig. 2), the fresh placental biopsies were obtained

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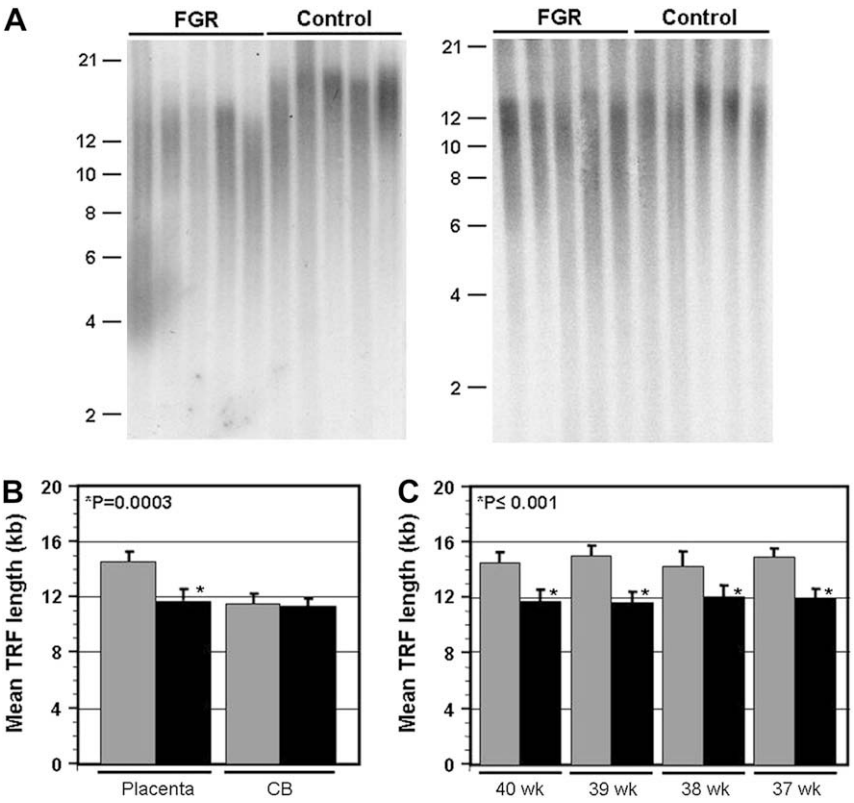

Fig. 1. Reduced telomere length in placenta samples from FGR newborns. A. Telomere length was assessed by Southern analysis of terminal restriction fragment (TRF) length for placental and cord blood (CB) DNA samples for both FGR and control newborns. Shown are sample blots for placental samples (left panel) and corresponding cord blood samples (right panel) for 5 FGR and control donors at 40 weeks gestational age. Size of molecular weight markers (Kb) is shown on the side. B. Calculation of the average mean TRF length for placenta and cord blood samples for all donors at 40 weeks gestational age ($n \geq 8$ for both groups). Control samples are represented by the grey bars, and FGR samples by the black bars. Error bars represent standard deviation from 4 measurements of mean TRF length. The *P* value (student's *t*-test) is shown. C. Comparison of mean TRF length for placenta samples for FGR (40 weeks, $n = 7$; 39 weeks $n = 9$; 38 weeks $n = 8$; 37 weeks $n = 8$) and control ($n = 9$ for all 4 cohorts) newborns at different gestational ages.

within 1 h of birth, and were taken near the umbilical cord on the fetal side. All samples were immediately frozen in liquid nitrogen for future work-up (see below). Inclusion criteria for all samples were singleton births delivered at KMCWC for non-incarcerated mothers ranging in age from 18 to 45. The exclusion criteria for all donors were pre-eclampsia, maternal substance abuse, congenital abnormalities, intrauterine viral infections, and chromosomal abnormalities. The idiopathic FGR cohort was additionally defined as having a birth weight of $\leq$5th percentile for Filipino newborns, and ponderal index $\leq$10% percentile. It is important to note that there are instances of FGR with birth weights above the 5th percentile that have not been examined in this study, and which may be of interest to assess in future studies. Furthermore, we would like to acknowledge the possibility of the inadvertent inclusion of some small for gestational age donors in this study, despite the tight birth weight percentile cut-off.

2.2. Preparation of telomerase extracts, RNA and DNA

Frozen placenta samples were crushed into a fine powder using a cooled stainless steel press, and extracts were divided into 3 parts for extraction of telomerase, RNA, or DNA. Telomerase extracts were prepared using CHAPS lysis buffer according to manufacturers' instructions (Chemicon), and included 20 units of RNasin per sample. RNA was extracted using Trizol according to manufacturers; instructions (Sigma), and DNA was extracted using Phenol:chloroform as previously described [8].

2.3. Telomere length analysis

For all DNA samples, telomere length was measured by Southern analysis of terminal restriction fragment length, as previously described [10].

2.4. Analysis of telomerase activity

Telomerase activity was assessed for all extracts using the TRAP assay [11] according to manufacturers' protocol (Chemicon), with the following exceptions. The TRAP assay was performed on 0.5 μg protein per sample extract. Twenty-five cycles of PCR (94/min, 60/1 min) was performed.

2.5. Real time RT-PCR analysis of gene expression

Reverse transcription (RT) was performed in a 20 μl reaction with an oligo(dT) primer and SuperScript III (Invitrogen) at 50 °C for 60 min using 1.5 mg total RNA that had been pre-treated with RNase-free DNase to remove any contaminating genomic DNA. For all samples, quality of RNA was confirmed beforehand by gel analysis. For all genes analyzed in this study, primers were designed to be 22–24 nucleotides in length, and spanned the most 3′ intron. Each primer set was verified to yield a single amplicon of 100–120 bp in length. Quantitative real time PCR was performed on a MyiQ system using iQ SYBR Green Supermix (BioRad). The reaction mix contained 500 nM of each primer, 200 μM dNTPs, 2 mM $MgCl_2$. All PCR reactions were performed for 40 cycles (95 °C, 15 s; 66 °C, 45 s) followed by continuous melt curve analysis to ensure product accuracy. For each primer set, standard curves (Cp plotted against the log of relative cDNA concentration) generated by serial dilutions of first-strand control cDNA were linear through a range spanning at least one log greater and less than the amount of cDNA used in the test reactions.

3. Results and discussion

To assess telomere length in FGR placental samples, we performed southern analysis of telomere length for 32 FGR donors and 36 gestational age-matched control donors by Southern analysis of terminal restriction fragment (TRF) length [7,10]. As shown in Fig. 1A&B, the TRF lengths for FGR donors at 40 weeks gestational age are noticeably shorter, in general, as compared to gestational age-matched control donors. Quantitative analysis of mean TRF length for all donors at 40 weeks gestational age confirmed that the average telomere length is significantly shorter ($P = 0.0003$) in the 40-week FGR cohort (Fig. 1B). We have previously shown that telomere length is substantially longer in placenta as compared to cord blood (CB) samples from the same donor [10]. In the present

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