Placenta 68 (2018) 15–22

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Evidence of oxidative stress-induced senescence in mature, post-mature and pathological human placentas

Tereza Cindrova-Davies[a,*], Norah M.E. Fogarty[a,b], Carolyn J.P. Jones[c], John Kingdom[d], Graham J. Burton[a]

[a] Centre for Trophoblast Research, University of Cambridge, UK
[b] Francis Crick Institute, London, UK
[c] Maternal and Fetal Health Research Centre Division of Developmental Biology & Medicine School of Medical Sciences Faculty of Biology, Medicine and Health University of Manchester, Manchester Academic Health Science Centre, St Mary's Hospital Oxford Road Manchester M13 9 WL, UK
[d] Mount Sinai Hospital, University of Toronto, Canada

ARTICLE INFO

Keywords:
Senescence
Syncytiotrophoblast
Oxidative stress

ABSTRACT

Introduction: Premature ageing has been implicated in placental dysfunction. Senescence can be activated by oxidative stress, a key intermediary in the pathophysiology of pre-eclampsia. We examined senescence markers across normal gestation, and in pathological and post-mature pregnancies. Inducers of oxidative stress were used to mimic senescence changes in term explants.

Methods: Placental samples were collected with ethical approval and informed consent: first and second trimester samples from surgical terminations; term and pre-term controls, and early-onset pre-eclampsia samples from caesarean deliveries. Paraffin and EM blocks of post-mature placentas were from an archival collection. Term explants were subjected to hypoxia-reoxygenation (HR) or hydrogen peroxide (H_2O_2).

Results: p21 was increased significantly in term homogenates compared to first and second trimester samples, and was significantly higher in PE compared to term controls. Immunostaining revealed nuclear localisation of p21 and phosphorylated histone γH2AX in syncytiotrophoblast, with abundant foci in pathological and post-mature placentas. Abnormal nuclear appearances were observed in post-mature placentas. Sudan-Black-B staining demonstrated abundant lipofuscin, an aggregate of oxidised proteins, lipids and metals, in post-mature and pathological placentas. The percentage of nuclei positive for 8-hydroxy-2′-deoxy-guanosine, a marker of oxidised DNA/RNA, was increased in pathological placentas compared to age-matched controls. These changes could be mimicked by challenge with HR or H_2O_2.

Discussion: Senescence markers increase in normal placentas with gestational age, and are exaggerated in post-mature and pathological cases. Oxidative stress triggers equivalent changes in explants, and may precipitate senescence *in vivo*. The consequent pro-inflammatory senescence-associated secretory phenotype may contribute to the pathophysiology of pre-eclampsia.

Introduction

Senescence is a critical feature of mammalian cells, and can be both beneficial and detrimental. Thus, oncogene-triggered senescence suppresses tumour growth [1], whereas loss of tissue homeostasis during ageing [2,3] contributes to atherosclerosis [4] and neurodegeneration [5]. Senescence has been implicated in cell fusion [6], and so is relevant to the syncytiotrophoblast of the human placenta. Transfection of normal and malignant non-trophoblast cells with syncytin 1 causes cellular fusion and formation of syncytia, which exhibit features of senescence. Replicative senescence is a permanent cell cycle arrest resulting from high levels of the cyclin kinase inhibitors p21/or p16. Cells remain metabolically active and adopt characteristic phenotypic changes [7]. Cell senescence can also be activated by a variety of intrinsic and extrinsic stresses, including oxidative stress, DNA damage, nucleolar stress, epigenetic stress, telomere damage, chronic mitogen signaling, and oncogene activation/inactivation. For example, reactive oxygen species activate DNA damage response (DDR) by perturbing gene transcription and DNA replication, and by inducing telomere shortening. Senescence stressors ultimately activate the p53 and/or $p16^{Ink4a}$ pathways; p53 activation inducing p21 and cell cycle arrest via cyclin E-Cdk2, while $p16^{Ink4a}$ targets cyclin D-Cdk4 and D-Cdk6

* Corresponding author. Centre for Trophoblast Research, Physiological Laboratory, Downing Street, Cambridge CB2 3EG, UK.
E-mail address: tc269@cam.ac.uk (T. Cindrova-Davies).

https://doi.org/10.1016/j.placenta.2018.06.307
Received 5 January 2018; Received in revised form 15 June 2018; Accepted 18 June 2018

complexes [8]. These actions prevent inactivation of RB (retinoblastoma protein), leading to continued repression of E2F target genes required for S-phase onset. Some cells form regions of highly condensed chromatin, called senescence-associated heterochromatin foci, that sequester genes facilitating cell-cycle control. These foci contain chromatin modifications, such as activated H2AX (γH2AX) and H3K9me, and reinforce senescence-associated growth arrest [9,10]. One emerging feature of senescent cells is inflammation. Senescent cells secrete a unique cocktail of factors, collectively known as senescence-associated secretory phenotype (SASP), which includes pro-inflammatory cytokines and chemokines, and various growth factors and proteases that together alter the tissue microenvironment [11,12]. Damaged human cells develop persistent chromatin lesions bearing hallmarks of DNA double-strand breaks, which initiate increased secretion of inflammatory cytokines. Evidence suggests that a feedback loop involving mitochondrial dysfunction and ROS production might be important in various physiologically relevant forms of cell senescence [13]. Senescent cells have distinct phenotypic features, including flattened and enlarged morphology with marked actin stress fibres, stability in culture, and increased senescence-associated β-galactosidase (SA-β-gal) activity [14]. SA-β-gal is the most reliable senescence biomarker. However, the SA-β-gal activity can be detected only in snap-frozen tissues, not in archival paraffin-embedded sections. A recent study validated the histochemical Sudan-Black-B (SBB) specific stain of lipofuscin, an aggregate of oxidised proteins, lipids and metals known to accumulate in aged tissues, as an additional reliable approach to detect senescent cells independently of sample preparation [15]. In addition, cytoplasmic chromatin fragments can pinch off from intact nuclei of primary cells during senescence. Recent evidence shows that the presence of DNA in the cytoplasm can initiate a SASP response by activating the innate immunity cytosolic DNA-sensing cyclic GMP-AMP synthase (cGAS)-STING pathway [16,17]. The enzyme cyclic GMP-AMP synthase (cGAS) acts as a first responder as it binds to cytoplasmic DNA, and catalyses production of the molecule cGAMP and triggers a pro-inflammatory response [16,17].

Placental dysfunction is the main cause of many placental-related pathologies. Recent studies indicate that premature placental ageing might be involved in this process. Placental ageing was first described in the 1970s in animal models [18–20] when it was suggested that the rapidly proliferating placental cells start to age as the placenta reaches term. This physiological ageing has been documented in a recent study of human term placentas (39 wk), late term placentas (> 41wk) and placentas from unexplained stillbirth. Increased aldehyde oxidase 1 expression, increased oxidation of DNA/RNA (8-OHdG) and lipid, perinuclear location of lysosomes, and larger autophagosomes were observed in both later term and stillborn placentas, compared to placentas from term deliveries [21]. In addition, the significant increase in the rate of stillbirth commencing at 36–37 wk gestation, suggests that placental ageing might increase the risk of fetal demise [22,23]. Thus the first aim of this study was to examine evidence of senescence across normal gestation, and to evaluate senescent changes in an archival collection of post-mature placentas (delivered 7–20 after the due date).

Strong evidence exists that placental oxidative stress is a key intermediary event in the pathology of preeclampsia (PE) and intrauterine growth restriction (IUGR) [24–26], secondary to deficient conversion of the uterine spiral arteries and malperfusion [27]. Damage to DNA and proteins can result in shortened telomeres in the trophoblast in these pregnancies [28], and induce senescence. In addition, a recent microarray analysis demonstrated increased expression of *p21*, *p53*, *APE1* and *IL-6* in PE and IUGR placentas [29]. Therefore, the second aim of this study was to investigate senescent changes in pathological pre-eclamptic and IUGR pregnancies, and to use inducers of oxidative stress to test whether they are capable of recapitulating senescence changes in healthy term placental explants *in vitro*.

Methods

Tissue collection - first, second trimester and term tissue

All material was collected with informed written patient consent. First and second trimester placental samples were collected from surgical terminations with approval of the Joint UCL/UCLH Committees on the Ethics of Human Research (05/Q0505/82). The samples were collected using a chorionic villus sampling-like (CVS) technique under ultrasound guidance from the central region of the placenta. Gestational age was estimated from the crown rump length of the fetus. Part of the sample was frozen immediately (< 2 min) in liquid nitrogen (time zero, T_0), and part was fixed in 4% paraformaldehyde (PFA) and embedded in paraffin wax for immunohistochemistry (IHC). Frozen samples of first trimester (7–8 wk; N = 5) and second trimester (13–17 wk; N = 6) placentas were analysed in this study. To study changes across gestation, samples were collected from uncomplicated singleton pregnancies at term (N = 5; 39 weeks) with approval from the Cambridge Local Ethics Committee and with informed written patient consent immediately after delivery by elective caesarean section (tissues were harvested within 10 min of delivery). The most common indication for caesarean section was a history of previous section. Samples were transported to the laboratory on ice for further processing or for use as explant cultures (see below).

Tissue collection – post-mature placentas and their term controls

Paraffin and EM blocks of term healthy placentas (N = 5) and post-mature placentas (delivered 7–20 d after due date, N = 6) were from an archival collection from the University of Manchester [30].

Tissue collection – pathological samples and appropriate controls

Pathological placentas and respective term and preterm controls were collected with ethical approval from the Mount Sinai Hospital, Toronto, Canada, following caesarean delivery. Samples included term controls (N = 7, 39 wk), pre-term controls (N = 6, 29 wk), early-onset PE (N = 10, 30 wk) and normotensive IUGR placentas (N = 6, 31.5 wk) from a cohort of samples previously included in our publications [31,32]. The selection criteria used for pre-eclampsia was the onset of new hypertension and proteinuria after 20 weeks of gestation; hypertension being defined as two or more recordings of a diastolic blood pressure of 90 mmHg or more taken at least 4 h apart, proteinuria taken as the excretion of 300 mg protein or more over a 24 h period. All pre-eclampsia cases were early-onset, defined as an onset between 20 and 34 weeks of gestation, and all were associated with IUGR. IUGR was defined according to established criteria [33], i.e. fetal biometry was below the 10th centile for gestational age according to local reference values, and umbilical artery Doppler flow velocity was abnormal (Doppler score class II and III). The pre-term control placentas were from women who had preterm but otherwise uneventful pregnancies, or late terminations of pregnancy for medical reasons. Fetal growth had been normal and birthweight was always above 10th percentile. The umbilical and uterine Doppler measurements were normal. The membranes were never ruptured for longer than 12 h and signs of chorioamnionitis were also excluded by histological studies. The cases included in this study all delivered by a caesarean section, and comprised of 2 late terminations of pregnancy (due to autosomal recessive polycystic kidneys or renal agenesis), 3 cases of early pre-mature rupture of membranes (no chorioamnionitis), and 1 case of early pre-term labour. However, preterm deliveries by definition are not normal controls. We therefore included an additional group of term controls from healthy uneventful pregnancies with normal umbilical and uterine artery Doppler waveforms. Placental tissue samples were snap-frozen immediately and stored at −80 °C, and fixed in 4% PFA and processed for IHC. All placental samples were obtained from 3 to 4 placental regions,