Placenta 63 (2018) 26–31

Contents lists available at ScienceDirect

Placenta

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

Oxidative stress induces senescence and sterile inflammation in murine amniotic cavity

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ARTICLE INFO

Article history:
Received 2 November 2017
Received in revised form
10 January 2018
Accepted 14 January 2018

Keywords:
Fetal membranes
Protein damage
p38MAPK
Labor
Inflammation
Murine model
Cigarette smoke

ABSTRACT

Objective: A physiologic increase of reactive oxygen species (ROS) is observed through pregnancy. ROS-induced damage to major cellular elements, specifically protein peroxidation, can lead to fetal and placental tissue senescence and inflammation often associated with normal parturition. The purpose of this study was to examine the effects of oxidative stress (OS) in inducing changes in proteins, senescence, and sterile inflammation in pregnant mice.

Methods: CD-1 mice (n = 5/group) on day 14 of gestation were subjected to minilaparotomy and the uterine horn between gestational sacs was injected with the following: saline (control), cigarette smoke extract (CSE) CSE diluted in saline and CSE + SB 203580 (SB) (a p38 mitogen-activated protein kinase (MAPK) inhibitor). Mice were sacrificed on day 18, and amniotic sacs, placentas and amniotic fluid (AF) were collected. Protein damage was evaluated by immunostaining for 3-Nitrotyrosine modified proteins (3-NT). Activation of prosenescence p38MAPK was evaluated by western blot. Senescence features, β-galactosidase (SA-β–Gal) and AF inflammatory cytokines were analyzed by immunostaining and multiplex luminex-based immunoassays, respectively. The data were analyzed by ANOVA and Tukey's test, $p < .05$ was used for significance.

Results: Amniotic sac from CSE-treated animals showed significant protein peroxidation compared to control as indicated by 3-NT staining. CSE activated p38MAPK phosphorylation in amniotic sac but not in placenta. Membrane p38MAPK activation was reduced after treatment with SB. CSE increased fetal membrane senescence (staining for SA-β–Gal) and increased AF concentrations of all evaluated cytokines. High inflammation correlated with pup loss and a decrease in placental weight. Treatment with p38MAPK inhibitor (SB) minimized damages, senescence and sterile inflammation.

Conclusion: OS induction by cigarette smoke extract cause fetal tissue protein damage, p38MAPK activation, senescence and sterile inflammation in the amniotic cavity of mouse. Prevention of p38MAPK activation can be a novel approach to prevention of adverse pregnancy outcomes related to OS induced premature senescence.

1. Introduction

Oxidative stress and generation of reactive oxygen species (ROS) in the fetal compartments may contribute to a variety of a physiological processes during pregnancy [1]. ROS generated in gestational tissues is required for fetal, placental, and fetal membrane growth and tissue remodeling required to maintain normal pregnancy. ROS in tandem with inflammatory mediators such as cytokines matrix metalloproteinases, and prostaglandins [2,3] are one of the major components that maintain pregnancy homeostasis. At term, ROS substantially increases in fetal compartments mainly due to increased metabolic demands, reduction in substrate supply, depletion of antioxidant reserves and elevated tissue oxygen requirements. Increase in ROS causes fetal tissue damage beyond repair and enhances inflammation at term, which promotes labor-associated changes in maternal compartments [4–6].

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https://doi.org/10.1016/j.placenta.2018.01.009
0143-4004/

Inflammation, the main labor-inducer [7], is commonly associated with infection, however, non-infection factors such as maternal bleeding, deteriorating of the feto-maternal interface, physical stretch and the presence of Damage-associated molecular patterns (DAMPs) can also lead to intrauterine inflammation. In especial highlight, increases in ROS seen to induce a cycle of events that propels delivery [8], once in vitro studies have shown that treatment of chorioamnion explants with antioxidant reduces the release of prostaglandins, cytokines and metalloproteinases activity induced by lipopolysaccharide [9]. However, these in vitro data do not explain specific fetal signals that generate such an inflammatory overload at term in the absence of microbial invasion and intrauterine infection, a condition associated with term births and majority of preterm births [10].

Multiple enzymatic and non-enzymatic processes can generate ROS in fetal cells [10], and the exposition of intrauterine cavity to abnormal environment and chemicals substances can generate ROS [11,12]. As mentioned above, the main concern about the excessive ROS is the toxic effects in damaging cellular components such as proteins, lipids, carbohydrates and DNA [13–15]. Cellular damage-associated processes can induce the arrest of cellular growth resulting in senescence, a mechanism associated with aging, defined as the irreversible loss of replicative capacity in somatic cells [16]. Although senescence is a normal biological process, it can be either beneficial or deleterious, since contributes for tumor suppression and is also linked to the aging related diseases [17]. Senescent cells acquire many changes in expressions, altered mRNA and proteins, including a wide range of growth factors, proteases, chemokines and cytokines [18]. This is referred as senescence-associated secretory phenotype (SASP), contributing to 'sterile inflammation' associated with term parturition [17,18]. The regulation of the SASP complex is still not well established; however, its main consequence is the influence on tissue microenvironment through the induction of a local proinflammatory response [19]. Senescent cells and SASP were recently highlighted in human fetal membranes at term labor [4]. At term, ROS contributes to senescence of fetal cells and sterile inflammation; however, the mechanism by which senescent cells could lead to labor is still unclear.

One important feature of ROS-induced senescence is the telomere shortening that leads to loss of cell functions when it reaches critical lengths. Cell recognizes this change as a signal to exit the cell cycle and activates apoptotic or senescent pathways. We have previously demonstrated human amnion cells treated with ROS inducers (cigarette smoking extract [CSE]) show reduction of telomere length, senescence morphology and biochemical changes mediated through p38 mitogen activated protein kinase (p38MAPK) pathway. Inflammation in these cells were reduced by p38MAPK inhibitor confirming stress signal associated development of inflammation in the absence of infection [6,20].

ROS generated by CSE includes hydrogen peroxide superoxide, hydroxyl radical, nitric oxide, which are potent oxidizing and nitrating compound that have been linked to a variety of pathological conditions [21]. To further expand our knowledge and test the validity of our in vitro model of ROS induced senescence and sterile inflammation [6,22], we tested the effects of CSE induced OS on pregnant mice. Specifically, how OS effects the amniotic sac (fetal membranes, placental membranes, amniochorion), placenta, and pregnancy outcomes.

2. Material and methods

2.1. Water soluble cigarette smoking extract (CSE) stimulation

Water soluble cigarette smoking extract (CSE) preparation was adapted as previously reported [6,23], by bubbling smoke that is drawn from a single lit commercial cigarette that represented high tar (unfiltered Camel; R.J.Reynolds Tobacco Co, Winston-Salem, NC) through 25 mL of saline. The treated saline was sterilized by using a filter 0,2 μm (Corning, New York, USA). Our previous results showed amnion cells are not viable under concentrate CSE treatment [6], therefore, the 1:10 dilution was used for injecting into intrauterine cavity.

2.2. Animals

The Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch at Galveston approved the study protocol. CD-1 pregnant mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were shipped on day 10 of gestation and acclimated in a temperature- and humidity-controlled facility with automatically controlled 12:12 h light and dark cycles. Mice were allowed to consume regular chow and drinking solution ad libitum.

At day 14th of pregnancy, the pregnant CD-1 mice ($n = 5$/group) were weighed and subjected to minilaparotomy in the lower abdomen and injection of 150 μL of the treatment into uteri in between 2 and 3 gestational sacs according to the following experimental groups: 1) cigarette smoke extract (CSE) diluted in saline; 2) CSE in combination with SB203580 (p38MAPK inhibitor) and 3) saline (control). CSE concentration was validated in an amnion cells model and *in vivo* prior to these experiments to rule out toxicity [24,25]. After sacrificing the animals by using carbon dioxide inhalation according to the IACUC and American Veterinary Medical Association guidelines on day 18, maternal, fetal, and placental weight were documented and pup loss/reabsorption was counted. Amniotic sacs and placentae were collected in either formalin 10%or fresh frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Immunohistochemistry

Amniotic sac tissue sections were fixed in 10% formalin for 48 h and embedded in paraffin. Sections were cut at 5-μm thickness and adhered to a positively charged slide and attached by keeping them at 57 °C for 45 min. Slides were deparaffinized using Xylene and rehydrated with 100% alcohol, 95% alcohol, and normal saline (pH 7.4) and stained using oxidative stress marker 3-Nitrotyrosine modified proteins (3-NT). Staining of 3-NT reveals oxidative stress-induced damage at the protein level. Five images for each category were taken at 40× magnification. Images were processed with ImageJ and staining intensity was measured in a uniform manor.

2.4. Western blot

Amniotic sac and placenta samples were lysed in a RIPA lysis buffer with freshly added protease and phosphatase inhibitors (0.01%). The insoluble material was removed by centrifugation at 10,000 rpm for 20 min at 4 °C. The concentration of protein in each tissue lysate was determined by using the BCA protein assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific). The same amount of protein (30 μg) from each sample was loaded onto a 10% SDS-PAGE gel and electrophoresed at 120 V. The resolved proteins were transferred to a PVDF membrane using the iBlot transfer apparatus (Bio-Rad Laboratories). 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 total p38MAPK (Cell Signaling, Danvers, MA, USA, #9212), phosphorylated (P)-p38MAPK (Cell Signaling, #9211S), or β-actin (Sigma-Aldrich, #A5441) specific primary antibody at 4 °C