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The transcription factor Nrf2 is decreased after spontaneous term labour in human fetal membranes where it exerts anti-inflammatory properties

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ABSTRACT

Introduction: Inflammation plays a central role in the terminal processes of human labour and delivery, including the rupture of fetal membranes. Recent studies show a role for the transcription factor Nrf2 (NF-E2-related factor 2) in regulating inflammation. The aims of this study were to determine the effect of human spontaneous term and preterm labour on Nrf2 expression in fetal membranes; and Nrf2 siRNA knockdown on pro-inflammatory cytokines.
Methods: Fetal membranes, from term and preterm, were obtained from non-labouring and labouring women. Primary amnion cells were used to determine the effect of Nrf2 siRNA knockdown on pro-inflammatory cytokines in the presence of inflammation or infection.
Results: Nrf2 mRNA expression and nuclear protein expression were significantly decreased after spontaneous term labour and delivery. There was, however, no effect of spontaneous preterm labour and delivery on Nrf2 mRNA expression and nuclear protein expression. On the other hand, Nrf2 gene expression was significantly lower in fetal membranes obtained from women at preterm with histologic chorioamnionitis compared to fetal membranes obtained from women at preterm without histologic chorioamnionitis. Nrf2 silencing by siRNA in primary amnion cells was associated with a significant increase in IL-6 and IL-8 mRNA expression and release induced by IL-1β, TNF-α, flagellin and poly(I:C).
Discussion: Nrf2 has an anti-inflammatory effect in human fetal membranes. It is decreased with term labour and preterm chorioamnionitis; and Nrf2 silencing increases inflammation- and infection-induced pro-inflammatory cytokines. Further studies are required to determine if agents that can increase Nrf2 expression may be a potential therapeutic strategy in the treatment and management of infection-induced preterm labour.

1. Introduction

Preterm birth is the major cause of perinatal morbidity and mortality accounting for up to 85% of all early infant deaths [1,2]. Of infants surviving preterm birth, 20–25% will have at least one major disability including long-term neurocognitive deficits, pulmonary dysfunction and ophthalmological disorders [3,4]. Compared to infants delivered at term, there is an increased cost of health care for the preterm infant during the first 10 years of life [5]; for the severely disabled survivors of preterm birth, there are substantial societal costs beyond childhood [6].

A major cause of preterm birth is the premature rupture of fetal membranes (PROM). Rupture of membranes precedes the initiation of uterine contractions in at least 10% of term and nearly 40% of preterm births [7]. The physiology of rupture of membranes is yet to be fully elucidated; however apoptosis and degradation of the extracellular matrix (ECM) are key features [8]. These processes are activated by pro-inflammatory cytokines, which are increased with labour onset as well as ascending infection; leucocyte infiltration can further amplify this inflammatory response, leading to the production of proteolytic enzymes in the fetal membranes, predisposing them to rupture [9,10]. Important in the recognition of pathogenic microorganisms to trigger the inflammatory response

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in these uterine tissues are Toll-like receptors (TLRs) [11]. By elucidating the physiological mechanisms that are involved in fetal membrane rupture, as well as pathological activation at preterm, can we begin to develop clinically useful interventions that could improve neonatal outcomes.

Recently, the transcription factor Nrf2 (NF-E2-related factor 2), has been shown to play a central role in the regulation of inflammation. Under basal conditions, Nrf2 is sequestered inactive in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (KEAP1). It translocates into the nucleus following its activation, for example, by reactive oxygen species where it mediates activation of a variety of genes. A large number of cytoprotective and antioxidant proteins have been identified as Nrf2 targets [12,13]; the genes typically contain an antioxidant response element (ARE), a cis-acting DNA regulatory element with a core sequence of 5′-TGA(C/T)nnnGC(A/G)-3′. Notably, other targets with wide-ranging set of biological functions have also been identified [14,15], including genes involved in controlling inflammation [14]. Hypoxic Nrf2 deficient mice, in addition to reduced antioxidant enzyme levels and enhanced oxidative stress, have increased inflammatory cytokine expression [16]. *In vitro*, silencing of Nrf2 increases TNF-α-induced inflammatory responses in human monocytes [17], LPS-induced pro-inflammatory cytokine gene expression in HepG2 cells [18], oxidative stress-induced pro-inflammatory cytokine gene expression in dermal fibroblast [19], and platelet-derived growth factor (PDGF)-induced inflammation in vascular smooth muscle cell (VSMCs) [20].

To our knowledge, there are no studies on Nrf2 in fetal membranes; however, Nrf2 is expressed in human placenta where its expression is lower with preeclampsia [21] and gestational diabetes [22]. Given the central role of inflammation in the processes of human labour and delivery [23], we hypothesised that Nrf2 would be decreased in fetal membranes after labour, hence permitting increased inflammatory activation in these tissues. Thus, in this study we will determine the effect of spontaneous term and preterm labour, and preterm histologic chorioamnionitis on Nrf2 expression in fetal membranes; the mRNA and nuclear protein expression will be quantified using quantitative real-time-PCR (qRT-PCR) and Western blotting, respectively. To determine if Nrf2 regulates pro-inflammatory cytokines in the presence of inflammation or infection, we will investigate the effect of Nrf2 inhibition in primary amnion cells in the presence of the pro-inflammatory cytokines IL-1β and TNF-α, and TLR ligands, bacterial product flagellin (TLR5) and the viral dsRNA analogue polyinosinic:polycytidilic acid (poly(I:C); TLR3).

2. Materials and methods

2.1. Tissue collection

The Research Ethics Committee of Mercy Hospital for Women approved this study. Written, informed consent was obtained from all participating women. All tissues were obtained from women who delivered singleton infants. All tissues were processed within 15 min of delivery. Women with any underlying medical conditions such as diabetes, asthma, polycystic ovarian syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with multiple pregnancies, obese women, fetuses with chromosomal abnormalities were excluded.

Fetal membranes were obtained from women at (i) term no labour undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section) ($n = 8$ patients) and (ii) term after spontaneous labour, spontaneous membrane rupture, and normal vaginal delivery ($n = 8$ patients). Fetal membranes from the non labouring group, samples were obtained from the supracervical site (SCS). Identification of the SCS was performed as we have previously detailed [24,25]. Briefly, Bonneys blue dye was introduced through the cervix prior to Caesarean section. Upon delivery of the placenta, a blue mark was obvious on the chorion facing membrane where the dye had been applied. In the after labour group, fetal membranes from the site of membrane rupture (SOR) were obtained as we have previously described [25]; amnion and underlying choriodecidua were collected from along the line of fetal membrane rupture. There was no difference in maternal age and body mass index, parity, or gestational age of the patients recruited. In the term no labour group, the average gestational age was 38.8 ± 0.3 weeks. In the term after labour group, the average gestational age was 38.7 ± 0.4 weeks. Furthermore, in the term after labour group none of the patients received any medications to augment or induce labour, and the average length of labour was 6 h 40 min ± 1 h 40 min. Tissue samples were fixed and paraffin embedded for immunohistochemical analysis, or snap frozen in liquid nitrogen and immediately stored at −80 °C for analysis of Nrf2 expression by qRT-PCR and Western blot as detailed below.

Fetal membranes were also obtained from women at (i) preterm no labour undergoing Caesarean section ($n = 10$ patients; mean gestational age of 32.8 ± 0.7 weeks); (ii) after preterm spontaneous labour and normal vaginal delivery ($n = 10$ patients; mean gestational age of 32.5 ± 0.8 weeks); and (iii) after preterm spontaneous labour and normal vaginal delivery with histologically confirmed chorioamnionitis ($n = 8$ patients; mean gestational age of 31.6 ± 4.0 weeks). All placentas collected from the three preterm groups were swabbed for microbiological culture investigations and histopathological examination. Chorioamnionitis was diagnosed pathologically according to standard criteria which included histological evidence of macrophages and neutrophils permeating the chorionic cell layer and often infiltrating the amniotic cell. Histological chorioamnionitis is often accompanied by isolation of a microbiological organism from the fetal membranes.

Indications for preterm delivery (in the absence of labour) were placenta praevia, placental abruption or antepartum haemorrhage (APH). For these studies, fetal membranes from both the non-labouring and after labour preterm groups were obtained 2 cm from the peri-placental edge. The clinical details of the preterm patients are described elsewhere [26]. Of note, there was no difference in maternal age and body mass index, or parity of the patients recruited. Tissue samples were snap frozen in liquid nitrogen and immediately stored at −80 °C for analysis of Nrf2 expression by qRT-PCR and Western blot as detailed below.

2.2. Immunohistochemistry (IHC)

To determine the localisation of Nrf2 in fetal membranes, IHC was performed on paraffin sections as described previously [27] using the IHC Select® HRP Detection Set (Merck Millipore; Billerica, MA, US). Briefly, sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM Tris, 1 mM EDTA, pH 9.0 for 10 min followed by 20 min incubation) and then endogenous peroxidases were inactivated by adding 3% hydrogen peroxide for 10 min. After blocking (Blocking Reagent: normal goat serum in PBS) for 5 min, sections were incubated with 2 μg/ml rabbit polyclonal Nrf2, #ab62352 (Abcam; Cambridge, MA, USA) in 1% (wt/vol) bovine serum albumin in PBS and incubated in a humidity chamber for 60 min. Binding sites were labelled with biotin conjugated rabbit anti-goat IgG antibody followed by the streptavidin–HRP. Negative control slides, where primary antibody was replaced with rabbit IgG, were also performed.

2.3. Gene silencing of Nrf2 with siRNA

Primary amnion cells were used to investigate the effect of siRNA-mediated gene silencing of Nrf2 on pro-labour mediators. For these studies, fresh amnion was obtained 2 cm from the peri-placental edge from women who delivered healthy, singleton infants at term (37–41 weeks gestation) undergoing elective Caesarean section in the absence of labour. Cells were isolated and cultured as we have previously described previously [28]. Cells at approximately 50% confluence were transfected using SilenceMag reagent according to manufacturer's guidelines (Oz Biosciences; Marseille, France) and as we have previously described [28]. Nrf2 (HSC.RNAI.N001145412.12.1) was obtained from Integrated DNA Technologies (IDT; Coralville, IA, USA) and the negative control (NC) siRNA was obtained from Sigma (Sigma–Aldrich; St. Louis, MO, USA). An initial dose response was performed using 50–200 nM siRNA, with 200 nM giving the greatest knockdown (data not shown); thus, all subsequent experiments were performed using 200 nM siRNA. Cells were transfected with 200 nM Nrf2 or 200 nM NC siRNA in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA) with or without 1 ng/ml IL-1β, 10 ng/ml TNF-α, 1 μg/ml flagellin or 5 μg/ml ploy(I:C), and the cells were incubated at 37 °C for an additional 24 h. Cells were collected and stored at −80 °C until assayed for mRNA expression by qRT-PCR and protein expression by Western blotting as detailed below. Media was collected and stored at −80 °C until assayed for cytokine and prostaglandin release as detailed below. Experiments were performed from amnion obtained from five patients. As we have previously described [29], in the cells studies, there was a very large variability in basal levels of pro-labour mediators between patients. Thus, for the siRNA studies, the data is presented as fold change in expression relative to the expression level in the NC siRNA transfected cells + IL-1β, TNF-α, flagellin or poly(I:C), which was set at 1. Data could not be normalised to NC siRNA transfected cells alone as some target mRNAs and cytokine concentrations were undetectable.

2.4. Western blotting

Extraction of nuclear protein and Western blotting was performed as previously described [28]. Forty micrograms of protein was separated onto 10% polyacrylamide gels (Bio-Rad Laboratories; Gladesville, NSW, Australia) and transferred to PVDF.