Placenta 47 (2016) 63–72

Contents lists available at ScienceDirect

Placenta

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

ATF3 is a negative regulator of inflammation in human fetal membranes

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ARTICLE INFO

Article history:
Received 18 July 2016
Received in revised form
7 September 2016
Accepted 13 September 2016

Keywords:
ATF3
Human labour
Inflammation
Infection
Fetal membranes

ABSTRACT

Introduction: Infection and inflammation stimulate pro-inflammatory cytokines, prostaglandins and matrix metalloproteinase (MMP)-9, which play a central role in myometrial contractions and rupture of fetal membranes. In human and mouse immune cells, activating transcription factor 3 (ATF3) is a negative regulator of inflammation. No studies have examined the role of ATF3 in human labour.
Methods: Primary amnion cells were used to determine the effect of interleukin (IL)-1β and the bacterial product fibroblast-stimulating lipopeptide (fsl-1) on ATF3 expression, and the effect of ATF3 siRNA on pro-labour mediators. ATF3 expression was assessed in fetal membranes from non-labouring and labouring women at term and preterm, and after preterm pre-labour rupture of membranes (PPROM).
Results: IL-1β and fsl-1 significantly increased ATF3 expression. Silencing ATF3 significantly increased IL-1β- or fsl-1-induced expression of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6) and chemokines (IL-8 and monocyte chemoattractant protein-1 (MCP-1)); cyclooxygenase-2 (COX-2) mRNA expression and prostaglandin $PGF_{2\alpha}$ release; and MMP-9 expression. ATF3 expression was decreased in fetal membranes with term labour. There was no effect of preterm labour or PPROM on ATF3 expression.
Discussion: ATF3 is a negative regulator of inflammation in human fetal membranes; in primary amnion cells, ATF3 expression is induced by IL-1β and fsl-1, and ATF3 silencing further exacerbates the inflammatory response when stimulated with these factors. Subsequently, ATF3 expression is decreased in fetal membranes after term labour and with preterm chorioamnionitis, conditions closely associated with inflammation and infection. Our data suggest that ATF3 may play a role in the terminal processes of human labour and delivery.

1. Introduction

Preterm birth is the leading cause of infant morbidity and mortality [1]. For survivors, long-term risks associated with preterm birth include cerebral palsy, learning difficulties and respiratory illnesses [2]. Approximately 70% of preterm births occur spontaneously as a result of idiopathic preterm labour or preterm prelabour rupture of membranes (PPROM); PPROM is associated with higher rates of neonatal mortality and morbidity [3]. Alongside the impact preterm birth has on the child and family, data from the USA indicates the annual costs associated with preterm birth exceeds US$26 billion per year [4]. The costs imposed upon families and communities stem from the absence of effective therapeutics that can stop preterm labour.

The processes involved in parturition are incompletely understood which has prevented the development of effective strategies to prevent spontaneous preterm birth. What we do know, however, is that the majority of spontaneous preterm births are due to pathological activation of the normal labour processes [3,5]. The bacterial product fibroblast-stimulating lipopeptide (fsl-1), while not a pathogenic micro-organism, is able to model the effects of such microbes [6], by stimulating the production of pro-labour mediators including pro-inflammatory cytokines, chemokines, cyclooxygenase (COX)-2 and subsequent prostaglandins, and

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http://dx.doi.org/10.1016/j.placenta.2016.09.006
0143-4004/

metalloproteinase (MMP)-9 in fetal membranes [7,8]. These pro-labour mediators participate in the uterine components of this common pathway of parturition i.e. myometrial contractility, cervical ripening and rupture of fetal membranes. Notably, and even in the absence of detected pathogens, most cases of spontaneous preterm birth have histological evidence of inflammation in the uteroplacental unit [9]. Pro-inflammatory cytokines such as IL-1β can activate the terminal processes of human labour and delivery [10–13] and induce preterm birth in mice and monkeys [14–16].

Recent studies in non-gestational tissues have demonstrated a role for activating transcription factor 3 (ATF3) as a negative regulator of inflammation [17–19]. ATF3 is a member of the activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of transcription factors [20]. ATF3 functions as a transcriptional repressor [21]; while ATF3 expression is maintained at low levels in quiescent cells, ATF3 gene expression can be induced by a variety of stress signals [22], such as Toll-like receptor (TLR) agonist bacterial lipopolysaccharide (LPS). In non-gestational tissues, the role of ATF3 as a negative regulator of inflammation is demonstrated whereby ATF3 is induced by TLRs, such as TLR2, 3, 4 and 9 [23], but that ATF3 silencing leads to an exacerbated inflammatory response [17–19,23–25].

While ATF2 has been considered to play a role in human myometrium [26,27], to our knowledge the expression or the role of ATF3 has not been investigated in human fetal membranes. Therefore, an aim of this study were to establish the expression of ATF3 in human fetal membranes obtained from labouring and non-labouring women at preterm and term. Furthermore, loss-of-function studies were performed to determine whether ATF3 is involved in the genesis of pro-inflammatory and pro-labour mediators induced by inflammation (IL-1β) or infection (using the bacterial product fsl-1).

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 healthy, singleton infants. All tissues were brought to the research laboratory and processed within 15 min of the Caesarean delivery. Tissues from women with any underlying medical conditions such as diabetes, asthma, polycystic ovarian syndrome, preeclampsia and macrovascular complications were not included. Additionally, tissues from women with multiple pregnancies, obese women, fetuses with chromosomal abnormalities were not included.

2.2. Primary amnion cell culture

Primary amnion cells were used to investigate the effect of pro-inflammatory mediators on ATF3 expression and the effect of ATF3 siRNA-mediated gene silencing on the expression of 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 (37–40 weeks gestation) undergoing elective Caesarean section in the absence of labour. Amnion cells (epithelial and mesenchymal) were prepared as previously described [28]. Briefly, amnion strips were washed in PBS and digested, twice, with 0.125% collagenase A and 0.25% trypsin in serum-free DMEM for 35 min at 37 °C. The cell suspension was filtered through a cell strainer and the eluate was neutralised with 1% FCS. The cell suspensions were centrifuged at 500*g* for 10 min and the cells cultured in DMEM/F-12, 10% FCS and 1% penicillin-streptomycin. The media was replaced after 4 h then every 24–48 h thereafter.

To determine the effect of pro-inflammatory mediators on ATF3 expression, cells at approximately 80% confluence were incubated in the absence or presence of 1 ng/ml IL-1β or 250 ng/ml fsl-1 for 24 h. Cells were collected and stored at −80 °C until assayed for ATF3 protein expression by qRT-PCR or Western blotting as detailed below. Experiments were performed in amnion cells obtained from five patients.

Transfection of primary amnion cells with siRNA was performed as we have previously described [29]. Briefly, cells at approximately 50% confluence were transfected using Lipofectamine 3000 according to manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia). SMARTpool ATF3 siRNA (siATF3) and negative control siRNA (siCONT) were obtained from Dharmacon (GE Healthcare Australia Pty. Ltd; Parramatta, NSW, Australia). Cells were transfected with 200 nM siATF3 or 200 nM siCONT 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 or 250 ng/ml fsl-1, 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, prostaglandin and MMP-9 release as detailed below. Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) proliferation assay as we have previously described [30]. The response to IL-1β and fsl-1 between patients varied greatly, as we have previously reported [29]. Thus, data is presented as fold change in expression relative to the expression level in the IL-1β- or fsl-1-stimulated siCONT transfected cells, which was set at 1. Data could not be normalised to siCONT transfected cells alone as some of the readings were 0. Experiments were performed in amnion cells obtained from six patients.

2.3. RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA extractions and qRT-PCR was performed as previously described [29]. Total RNA was extracted using TRIsure reagent according to manufacturer's instructions (Bioline; Alexandria, NSW, Australia). RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific; Scoresby, Vic, Australia). RNA quality and integrity was determined via the A_{260}/A_{280} ratio. RNA was converted to cDNA using the Tetro cDNA synthesis kit (Bioline; Alexandria, NSW, Australia) according to the manufacturer's instructions. The cDNA was diluted fifty-fold, and 4 μl of this was used to perform RT-PCR using SensiFAST™ SYBR NO-ROX Kit (Bioline; Alexandria, NSW, Australia) and 100 nM of pre-designed and validated QuantiTect primers (Qiagen; Chadstone Centre, Vic, Australia). The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia). Average gene Ct values were normalised to the average β2-Microglobulin (B2M) and 18S ribosomal RNA (rRNA) Ct values of the same cDNA sample. Of note, there was no effect of experimental treatment on B2M or 18S rRNA gene expression. Fold differences were determined using the comparative Ct method.

2.4. Cytokine, chemokine and prostaglandin assays

Assessment of cytokine and chemokine release of IL-6, IL-8, MCP-1 and TNF-α was performed using CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies; Mulgrave, Vic, Australia). The limit of detection of the IL-6, IL-8, MCP-1 and TNF-α assays was 16, 12, 15 and 7 pg/ml, respectively.