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Expression of Natural Antimicrobials by Human Placenta and Fetal Membranes

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Abstract

Preterm birth associated with infection is a major clinical problem. We hypothesized that this condition is associated with altered expression of natural antimicrobial molecules (β-defensins (HBD), elafin). Therefore, we examined expression of these molecules and their regulation by proinflammatory cytokines in placentae and fetal membranes from term pregnancy. HBD1–3 and elafin were localized by immunohistochemistry in fetal membranes and placenta. Real-time quantitative PCR was used to examine mRNA expression in primary trophoblast cells treated with inflammatory molecules. HBD1–3 and elafin were immunolocalized to placental and chorion trophoblast layers of fetal membranes and placenta. Immunoreactivity was also observed in amnion epithelium and decidua. No differences were noted between samples from women who were not in labour compared to those in active labour. In in vitro cultures of primary trophoblast cells, HBD2 and elafin mRNA expression was upregulated by the proinflammatory cytokine, IL-1β. These results suggest that the chorion and placental trophoblast layers may be key barriers to the progression of infection in the pregnant uterus. Natural antimicrobial expression may be altered in response to inflammatory mediator expression associated with the onset of labour and/or uterine infection, providing increased protection when the uterus may be particularly susceptible to infection.

Keywords: Natural antimicrobials; Placenta; Fetal membranes; Labour; Infection

1. Introduction

Successful pregnancy and subsequent delivery of a healthy infant at term is dependent on the presence of optimal uterine conditions, and particularly on the prevention of upper genital tract infection. Preterm labour is the major cause of neonatal morbidity and mortality and around 30% of preterm births are associated with uterine infection [1].

Natural antimicrobials are key molecules of the innate immune system and have anti-bacterial, anti-viral and anti-fungal actions [2]. Human β-defensins (HBD) are a major group of antimicrobials that are expressed at mucosal surfaces by epithelial cells and leukocytes. HBDs are either constitutively expressed (e.g. HBD1) [3] or inducible in response to the presence of proinflammatory cytokines or bacterial products (e.g. HBD2) [4]. Further to their antimicrobial actions, HBDs have been found to have chemoattractant properties suggesting an interaction between the innate and adaptive immune systems [5].

Whey acidic peptide (WAP) motif containing proteins are a second group of molecules which have been shown to have antimicrobial properties [6,7]. This group includes secretory leukocyte protease inhibitor (SLPI) and elafin (skin derived antileukoproteinase; proteinase 3). Both of these molecules

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have anti-protease activity against serine proteases such as neutrophil elastase [8,9]. The anti-protease actions of SLPI and elafin are believed to be involved in the control of the inflammatory response at mucosal surfaces such as the lung [10]. In addition to this, their microbicidal actions suggest a role in innate immune defense.

Natural antimicrobials are expressed throughout the non-pregnant female reproductive tract and are present in vagina, cervix, endometrium and fallopian tube [11]. Peak endometrial mRNA expression of defensins and the WAP proteins has been shown to coincide with implantation and menstruation suggesting a role of the innate immune system in the prevention of upper genital tract infection during key reproductive events [12–14].

In pregnancy, mRNA expression of defensins has been reported in placenta [15,16] while protein expression of HBD3 has been localized to both amnion and chorio-decidua [17]. HBD concentrations in amniotic fluid have not been detailed but concentrations of the related α-defensins, human neutrophil peptides (HNP) 1–3, have been reported. HNP1–3 levels are increased in amniotic fluid in association with parturition at term, preterm labour, preterm premature rupture of membranes and intra-amniotic infection [18]. Expression of elafin has been documented in amnion epithelium, chorion trophoblast and decidua [19]. SLPI is present in both amnion epithelium and decidua [20,21] with high levels also present in the cervical mucus plug at term pregnancy [22]. Reduced expression of both SLPI and elafin is associated with premature rupture of membranes [19,23]. There is little information regarding regulation of natural antimicrobial molecules in placenta and fetal membranes although HBD3 mRNA has been reported to increase in the amnion FL cell line in response to the bacterial products, lipopolysaccharide and peptidoglycan [17].

The expression and regulation of the beta-defensins and elafin in human placenta and fetal membranes have not been fully described. The current study evaluated the localization of HBD1–3 and elafin in these tissues at term pregnancy and examined their regulation in primary chorion and placental trophoblast cells.

2. Materials and methods

2.1. Tissue collection and processing

Placentae and fetal membranes were collected from healthy women attending Mt. Sinai Hospital, Toronto, Ontario, Canada. All subjects provided ethical consent to the collection and use of their tissues, according to the guidelines of the Canadian Institutes for Health Research (Tricouncil Policy). This project was approved by the Mount Sinai Hospital, Toronto, Canada Review Board for Research involving Human Subjects. Tissues were obtained either following spontaneous vaginal delivery (in spontaneous labour, SL) or after elective Caesarean section (not in labour, NIL). All pregnancies had reached term (>37 weeks) and were without complications.

2.2. Isolation of placental and chorion trophoblast cells

Placental ($n = 9$) and chorio-decidual ($n = 10$) tissues were collected from women undergoing Caesarean section (NIL) and trophoblast cells were isolated using a modification of the method described by Kliman et al. [24], and as reported previously [25]. In brief, approximately 60 g of placental cotyledon tissue was collected randomly from the maternal side of the placenta, pooled and placed in a digestion solution (DMEM culture medium containing 0.125% trypsin (Sigma, St. Louis, MO) and 0.02% DNAase (Sigma)). Tissue was incubated with digestion solution for three periods of 30 min. Chorio-decidua was peeled from amnion, chopped and incubated in digestion solution (with the addition of 0.2% collagenase (Sigma)) for three periods of 60 min. At the end of each incubation cells were collected, pooled and subsequently filtered through a 200-μm filter, prior to loading onto a continuous Percoll (Sigma) gradient (5–10%, in 5% steps, 3 ml each). After centrifugation at 1200*g* for 20 min, cells were collected between the markers of 1.049 and 1.062 g/ml. Cells were plated in six well culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a density of 3×10^6 cells/well.

2.3. Placental and chorion trophoblast cell culture

Placental and chorion trophoblast cells were grown in DMEM containing 10% fetal calf serum (Sigma) and 1% antibiotic–antimycotic solution (Sigma; penicillin, streptomycin, amphotericin B) for 72 h prior to treatment. Twenty hours prior to treatment medium was replaced with DMEM without FCS or antibiotics. Cells were treated for 24 h in the absence or presence of IL-1β (0.1, 1 and 10 ng/ml; Sigma), TNFα (1, 10, 20 ng/ml; Sigma) or LPS (0.01, 0.1, 1 μg/ml; Sigma, *Escherichia coli* 055:B5).

2.4. Reverse transcription/quantitative polymerase chain reaction

Cells were harvested in Trizol (Invitrogen, Burlington, Ontario, Canada), RNA was extracted as detailed in the manufacturer's protocol and DNAse treatment (Ambion, Austin, TX) was performed to remove contaminating DNA. Integrity of RNA was assessed on a 1% agarose gel. RNA purity was determined from the OD_{260}:OD_{280} measurements and RNA concentrations were determined from OD_{260} readings. RNA (1 μg) was reverse transcribed using superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). RNAse H (Invitrogen) was used to remove RNA present at the end of the reverse transcriptase reaction.

Real-time quantitative PCR was used to measure expression of HBD1, HBD2 and elafin mRNA. PCR conditions for each primer set were optimized using a gradient PCR machine (DNA Engine DYAD, MJ Research) prior to quantitative PCR. Real-time PCR reaction mixtures contained Platinum Taq (Invitrogen), SYBR green (0.0032% v/v; Molecular Probes, Invitrogen) and specific forward and reverse primers for the gene of interest (0.2 μM; Invitrogen). PCR reactions were performed using the Rotor-Gene SG3000 system. PCR cycles consisted of an initial denaturation step at 95 °C for 5 min, followed by 45 cycles (40 cycles for elafin) of denaturation at 95 °C for 60 s, annealing at 65 °C (63 °C for HBD1) for 45 s and extension at 72 °C for 60 s. At the end of each cycle, a further 15-s step (82 °C, elafin; 83 °C, HBD1; 84 °C, HBD2) was included in order to melt any primer-dimers present and to allow measurement of fluorescence released only by the specific amplicon. Amplification of β-actin was measured in each sample and was used as a housekeeping gene for normalization. Details of forward and reverse primers and product sizes are shown in Table 1.

Messenger RNA expression levels of the genes of interest and β-actin were each determined using relative quantitation by comparison to a standard curve. Standard curves were generated from serial dilutions of a reference sample and were included in each PCR run. Standards and samples were measured in duplicate and a 'no template' control and calibrator sample (pooled cDNA from cultured amnion epithelial and placental trophoblast cells for HBD2, elafin and β-actin; pooled cDNA from cultured placental trophoblast cells for HBD1) were included in all runs. Expression levels of the genes of interest in each sample were normalized to β-actin and are reported relative to the calibrator sample to allow for comparison between separate PCR runs.

2.5. Immunohistochemistry

HBD1–3 and elafin were localized in placenta (NIL, $n = 3$; SL, $n = 3$) and fetal membranes (NIL, $n = 3$; SL, $n = 3$) using standard immunohistochemical procedures. Briefly, tissue sections were dewaxed in xylene and rehydrated in