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Ovine trophoblasts express cathelicidin host defence peptide in response to infection

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ABSTRACT

Cationic host defence peptides (CHDP; also known as antimicrobial peptides) are key components of the immune response in the female reproductive tract. The role of the placental trophoblast in ovine host defence remains poorly understood. This study characterises expression of genes for cathelicidin and defensin peptides in primary ovine placental tissues, the ovine trophoblast cell line (AH-1) and in response to the TLR-4 ligand LPS, the abortifacient organism *Waddlia chondrophila* and 1α,25-dihydroxyvitamin D_3.

Using RT-PCR, expression of the CHDP SMAP-29, sBD-1 and sBD-2 was assessed in the AH-1 cell line in response to LPS, 1α,25-dihydroxyvitamin D_3 exposure (a known stimulator of cathelicidin gene expression), or *W. chondrophila* infection. Expression of cathelicidin in the trophoblast compartment of the ovine placenta and in the ovine trophoblast cell line (AH-1) was also established. AH-1 cells did not upregulate expression of CHDP in response to LPS, but sBD-1 and sBD-2 expression was significantly increased in response to *W. chondrophila* infection. SMAP-29 expression was not altered by *in vitro* exposure to 1α,25-dihydroxyvitamin D_3.

This study demonstrates that the ovine trophoblast expresses cathelicidins, but does not upregulate expression of CHDP in response to LPS. Ovine trophoblasts are shown to differentially regulate expression of CHDP and lack a demonstrable vitamin D-mediated cathelicidin response.

1. Introduction

Cationic host defence peptides (CHDP) are a diverse group of evolutionary conserved peptides with a broad range of antimicrobial and immunomodulatory functions (Brogden et al., 2003; Zasloff, 2002; Barlow et al., 2014). Increased expression and release of CHDP is an important part of the innate immune response following detection of infection *via* recognition of pathogen-associated molecular patterns (PAMP) through pattern recognition receptors (PRR) such as the Toll-like receptors (TLR) and nucleotide-binding oligomerisation domain (NOD) receptors (NOD-like receptors or NLRs) (Ganguly et al., 2009; Lande et al., 2007; Rietdijk et al., 2008). In humans, there are two main families of CHDP, cathelicidins, and defensins (Peschel and Sahl, 2006) and these peptides have been identified in several cell types, including neutrophils, macrophages, lymphocytes, eosinophils, epithelial cells and placental trophoblasts (Bowdish et al., 2006).

The expression of CHDP within the human female reproductive tract is well characterised (Horne et al., 2008; King et al., 2003, 2007a). During pregnancy, the human placenta expresses several β-defensins, secretory leukocyte protease inhibitor (SLPI), and the sole human cathelicidin hCAP-18. Notably, it has been demonstrated that human placental trophoblasts lack expression of Toll Like Receptor-4 (TLR4) translating to a lack of inducible secretion of CHDP in this cell type in response to lipopolysaccharide stimulation (King et al., 2003; Klaffenbach et al., 2011). In contrast, it was demonstrated that ovine trophoblasts do possess intact TLR4 signalling pathways and can respond to LPS stimulation with a pro-inflammatory response characterised by TNF-α and IL-8 (CXCL8) secretion (Wheelhouse et al., 2009). However, unlike well-characterised cells and tissues such as the gut and lung (Skerlavaj et al., 1999), it is not yet known if ovine trophoblasts are capable

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of expressing cathelicidin peptides. Indeed, the potential for the TLR-4 signalling pathway to stimulate CHDP production in ovine trophoblast cells is not yet known.

In humans the expression of CHDP can be stimulated by 1α,25-dihydroxyvitamin D3, signalling *via* the vitamin D receptor (Gombart et al., 2005; Wang et al., 2004), and peptide concentrations can be increased rapidly at sites of infection and inflammation. The use of vitamin D_3 as a potential therapeutic is of significant clinical interest and several studies have shown that vitamin D attributed antimicrobial activity is solely mediated by the expression of cathelicidin (Liu et al., 2007). However, while cathelicidin and defensin expression in humans in response to vitamin D is well characterised, CHDP gene expression and release in key immune cells of ovine species, and indeed the role that cathelicidins and defensins can play in host defence against infections in these species, is poorly understood.

Within livestock species, *Chlamydia* and *Chlamydia*-related pathogens such as *Chlamydia abortus* and *Waddlia chondrophila* are associated with abortion in ruminant species (Koschwanez et al., 2012; Rurangirwa et al., 1999; Longbottom et al., 2013; Sammin et al., 2006). Understanding the upregulation of expression of host defence molecules such as cathelicidins and defensins in reproductive tissues would provide key insights into host defence in these areas, particularly since the ovine cathelicidin SMAP-29, ortholog of the human cathelicidin LL-37, has been shown to have potent antimicrobial activity (Entrican et al., 2010; Longbottom et al., 2013; Brogden et al., 2001).

This study characterises the expression of CHDP within the ovine placenta, using both primary ovine tissue and the ovine trophoblast AH-1 cell line. We characterise the expression of genes associated with CHDP production in trophoblasts following stimulation with the TLR4 ligand, LPS, and during infection of AH-1 cells with the invasive clinically relevant *Chlamydia*-related pathogen of emerging pathological significance *Waddlia chondrophila*.

2. Materials and methods

2.1. Cell culture

The SV40 large T antigen transformed ovine trophoblast cell line AH-1, developed as previously described (Haldorson et al., 2006) (a kind gift from Professor T. Baszler, Washington State University, USA), was grown and maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% heat inactivated fetal bovine serum (PAA Laboratories Ltd., Yeovil, Somerset, UK) in a heated, humidified incubator at 37 °C and 5% CO_2. Cells were passaged at sub-confluency with 1× Trypsin-EDTA.

2.2. Propagation of W. chondrophila

Waddlia chondrophila strain ATCC VR-1470 was grown at 37 °C in McCoy cells with RPMI-1640 medium that was supplemented with 2% heat inactivated fetal bovine serum (PAA Laboratories Ltd., Yeovil, Somerset, UK). After 72 h growth, cell monolayers were disrupted with glass beads, and medium containing cell debris aspirated then centrifuged at 50*g* for 5 min at 4 °C to remove intact cells. Supernatant was aspirated and subsequently centrifuged at 20,000*g* using a J-LITE JLA-16.250 rotor (Beckman Coulter Ltd. High Wycombe, UK). The pellet was resuspended in ice-cold sucrose-phosphate-glutamic acid (SPG) buffer (10 mM sodium phosphate [8 mM Na_2HPO_4–2 mM NaH_2PO_4], 220 mM sucrose, 0.5 mM L-glutamic acid pH7.4), aliquoted into microcentrifuge tubes and stored at −80 °C. To quantify viable organisms, aliquots were thawed at room temperature and titrated on McCoy cells. Serial dilutions of the inoculum were added to confluent cell monolayers in 8-well chamber slides (BD Falcon, Becton Dickinson, Bedford, UK). After 24 h, medium was removed, cells were fixed in ice-cold acetone, air-dried, and slides were frozen at −20 °C prior to analysis by fluorescent immunocytochemistry as previously described (Wheelhouse et al., 2014) (polyclonal sera kindly supplied by Prof Gilbert Greub, University of Lausanne).

2.3. Expression of cationic host defence peptides by AH-1 ovine trophoblast cells

To assess CHDP expression in response to infection, an *in vitro* ovine placental model was employed. AH-1 trophoblast cells were grown to 80% confluency overnight in a 24 well plate, and subsequently exposed to LPS or infected with live *W. chondrophila*. For LPS exposures, AH-1 cells were treated in duplicate with LPS (500 ng/mL, lipopolysaccharide from *E.coli* O111:B4, Sigma, L2630–10 mg) which was dissolved in ultrapure water and stored at −80 °C (n = 4). AH-1 cells were also infected with *W. chondrophila* at a multiplicity of infection (MOI) of 0.1, 1 and 10 (equivalent of 1 inclusion forming unit (IFU) per 10 cells, 1 IFU per cell and 10 IFU per cell, respectively) or exposed to UV irradiated organisms at MOI 10 (n = 3). Cells were maintained in a heated, humidified incubator at 37 °C with 5% CO_2 for 48 h during treatment. For Vitamin D_3 exposures, 1α,25-dihydroxyvitamin D_3 (calcitriol, Enzo Life Sciences, Exeter, United Kingdom) was dissolved in absolute alcohol at a stock concentration of 1 mM and stored at −80 °C. AH-1 cells were exposed to Vitamin D_3 in duplicate at concentrations of 1 nM, 10 nM and 100 nM for 24 h and in all treatments, a vehicle control was performed (1 μl/mL EtOH) (n = 3).

2.4. Expression of cationic host defence peptides in the ovine placenta

All animal studies were approved by the UK Home Office (conducted under approved Project Licence PL 60/3744) after review by the University of Edinburgh Animal Research Ethics Committee. Scottish Greyface ewes were fed to achieve comparable body condition prior to estrous cycle synchronization. Estrous cycles were synchronised *via* intravaginal Chronogest sponge pessaries (20 flugestone acetate, synthetic progesterone analogue) (Intervet UK Ltd.). Sponges were removed after 12 days, and 0.5 ml Prostaglandin estrumate injected intramuscularly to terminate luteal phase prior to mating with Texel rams 48 h later. On day 90 of gestation (term is 147 days), ewes were euthanized by barbiturate overdose and a placentome was collected from the ewe (as described in (Rae et al., 2013)). Lung samples were also obtained from the foetus as a positive tissue control. All tissues were snap frozen and stored at −80 °C for mRNA expression analysis.

2.5. RNA extraction, DNase treatment and cDNA synthesis

For all RNA extractions, RLT buffer from RNeasy® mini kits with 2-mercaptoenthanol, 1% v/v (Qiagen, Crawley, UK) were used for lysis of *in vitro* and *ex vivo* samples. For frozen tissues, 30 mg tissue was weighed and lysed in RLT buffer using magnetic beads and a Qiagen TissueLyser. *In vitro* samples were lysed *in situ* with RLT buffer and collected into sterile DNase/RNase free tubes and stored at −80 °C until extraction. Once samples were lysed, RNA was extracted from the lysates using the RNeasy® mini kit system following manufacturers protocols. All samples were analysed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) to ascertain RNA quantity, and RNA quality was determined using an Agilent Bioanalyser (Agilent, UK), where RIN ≥ 8 was used as a quality filter for further downstream analyses. Genomic DNA (gDNA) was removed from extracted RNA using PrimerDesign Precision DNase Kits (PrimerDesign, Southampton,