Placenta 34 (2013) 1014–1019

Contents lists available at ScienceDirect

Placenta

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

The role of leukemia inhibitory factor in tubal ectopic pregnancy

T. Krishnan [a, b, 1], A. Winship [a, c, 1], S. Sonderegger [a, 2], E. Menkhorst [a], A.W. Horne [d], J. Brown [d], J.-G. Zhang [e], N.A. Nicola [e], S. Tong [f], E. Dimitriadis [a, c, *]

[a] Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168, Australia
[b] Faculty of Medicine, Nursing & Health Sciences, Wellington Road, Monash University, Clayton, Victoria 3800, Australia
[c] Department of Anatomy and Developmental Biology, Wellington Road, Monash University, Clayton, Victoria 3800, Australia
[d] MRC Centre for Reproductive Health, University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom
[e] The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia
[f] Department of Obstetrics and Gynaecology, University of Melbourne, Mercy Hospital for Women, 163 Studley Road, Heidelberg, Victoria 3084, Australia

ARTICLE INFO

Article history:
Accepted 6 September 2013

Keywords:
Leukemia inhibitory factor
Ectopic
Implantation
Placenta
EVT
Pregnancy

ABSTRACT

Introduction: Ectopic pregnancy is unique to humans and a leading cause of maternal morbidity and mortality. The etiology remains unknown however factors regulating embryo implantation likely contribute. Leukemia inhibitory factor (LIF) has roles in extravillous trophoblast adhesion and invasion and is present in ectopic implantation sites. We hypothesised that LIF facilitates blastocyst adhesion/invasion in the Fallopian tube, contributing to ectopic pregnancy.
Methods: We immunolocalised LIF receptor (R) in tubal ectopic pregnancy ($N = 5$). We used an oviduct cell line (OE-E6/E7) to model Fallopian tube epithelial cells and a trophoblast spheroid co-culture model (HTR-8/SVneo cell line formed spheroids) to model blastocyst attachment to the Fallopian tube. We examined LIF signaling pathways in OE-E6/E7 cells by Western blot. The effect of LIF and LIF inhibition (using a novel LIF inhibitor, PEGLA) on first-trimester placental outgrowth was determined.
Results: LIFR localised to villous and extravillous trophoblast and Fallopian tube epithelium in ectopic pregnancy. LIF activated STAT3 but not the ERK pathway in OE-E6/E7 cells. LIF stimulated HTR-8/SVneo spheroid adhesion to OE-E6/E7 cells which was significantly reduced after PEGLA treatment. LIF promoted placental explants outgrowth, while co-treatment with PEGLA blocked outgrowth.
Discussion: Our data suggests LIF facilitates the development of ectopic pregnancy by stimulating blastocyst adhesion and trophoblast outgrowth from placental explants. Ectopic pregnancy is usually diagnosed after 6 weeks of pregnancy, therefore PEGLA may be useful in targeting trophoblast growth/invasion.
Conclusion: LIF may contribute to the development of ectopic pregnancies and that pharmacologically targeting LIF-mediated trophoblast outgrowth may be useful as a treatment for ectopic pregnancy.

1. Introduction

Ectopic pregnancy is the most common cause of maternal mortality in pregnancies during the first trimester, occurring at a rate of 2% of all pregnancies in developed countries [1–3]. Approximately 95% of ectopic pregnancies occur in the Fallopian tube [4]. While the precise etiology of tubal ectopic pregnancy is unknown, factors critical for embryo implantation into the uterus may also contribute to blastocyst implantation in the Fallopian tube [5].

LIF is a member of the interleukin-6 family of cytokines. It is indispensable for uterine blastocyst implantation in mice [6,7] and plays a critical role in implantation in women [8–10]. LIF is a secreted glycoprotein that signals via the gp130/LIFR complex to activate the Janus tyrosine kinases (JAK). This, in turn, can activate downstream signaling pathways, including the signal transducer and activator of transcription (STAT) pathway [11–15], or extracellular signal regulated kinase (ERK) [16]. In the human endometrium and trophoblast, LIF primarily acts via STAT3 [17,18].

* Corresponding author. Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168, Australia. Tel.: +61 3 9594 4392; fax: +61 3 9594 6125.
E-mail address: evdokia.dimitriadis@princehenrys.org (E. Dimitriadis).
[1] Denotes equal contribution.
[2] Faculty of Medicine, Nursing and Health Sciences, Australian Centre for Blood Diseases (ACBD), Monash University, Alfred Centre, 99 Commercial Road, Melbourne, Victoria 3004, Australia.

0143-4004/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.placenta.2013.09.003

The blastocyst itself expresses LIFR and gp130 on the trophectoderm. We have previously demonstrated that LIF plays a role in blastocyst adhesion to the endometrial epithelial cells [19–21]. Additionally, LIF positively affects the development of the pre-implantation embryo *in vitro* [22,23]. LIF, LIFR and gp130 are expressed in the Fallopian tube surface epithelium and LIF production has been reported to be increased at tubal ectopic implantation sites [24–27]. Together, this indicates a role for LIF signaling in blastocyst attachment to the Fallopian tube epithelium, and the development of ectopic pregnancy.

Placental chorionic villous structures develop in human Fallopian tube ectopic implantation sites, as in uterine implantation [28]. LIF is important for trophoblast adhesion and invasion during normal placental development. LIF and LIFR mRNA are expressed in the chorionic villi and decidua of first-trimester placenta in women [29,30]. LIF activates STAT3 in human primary invasive extravillous trophoblast (EVT) cells and stimulates their adhesion to primary endometrial epithelial cells and extracellular matrix proteins, including fibronectin and collagen IV, produced on the blastocyst surface [18]. LIF has also been shown to mediate EVT cell invasion required for the establishment of the placenta [18]. Thus, it is possible that LIF may facilitate blastocyst adhesion and trophoblast invasion into the Fallopian tube.

Given LIF's role in uterine implantation and trophoblast invasion, and the presence of LIF at tubal ectopic implantation sites, we hypothesised that LIF facilitates blastocyst adhesion and trophoblast invasion in the Fallopian tube to establish ectopic pregnancies. To address our hypothesis, we localised LIFR in human tubal ectopic implantation sites. We examined the effects of LIF and LIF inhibition using a specialised LIF antagonist conjugated to polyethylene glycol (PEGLA) [31,32] on HTR-8/SVneo (trophoblast derived cell line) [33] spheroid adhesion to OE-E6/E7 (oviduct cell line) [34] cells and investigated whether this was mediated by pSTAT3. Using primary first-trimester placentas we also studied the effect of LIF and LIF blockade on placental villous outgrowth.

2. Materials & methods

2.1. Tissue collection

2.1.1. Ectopic pregnancy implantation sites

We collected fallopian tube biopsies from the ectopic implantation site from participants (aged 18–45 years, $N = 5$) undergoing surgery for an ectopic pregnancy at The Royal Infirmary Hospital, Edinburgh, Scotland. None of the women presented acutely with hemodynamic shock, and all required serial serum beta-HCG and ultrasound monitoring prior to diagnosis. None of the patients had a past history of tubal disease or ectopic pregnancy and no fetal hearts were seen on the diagnostic scans. Detailed characteristics of the patients are shown in Table 1. Written and informed consent was obtained from all patients before sample collection. Ethical approval was obtained from Lothian Research Ethics Committee, Edinburgh, United Kingdom (04/S1103/20).

2.1.2. First-trimester placenta

Placental samples were collected from healthy women ($n = 6$) undergoing first-trimester termination of pregnancy (6–10 weeks) for psychosocial reasons. Tissues were washed in 0.9% saline before transfer to DMEM/F12 medium 1:1 (Invitrogen). Informed consent was obtained from all participating patients. Ethics approval was obtained from the Southern Health Human Research and Ethics committee, Victoria, Australia.

Table 1
Clinical information for ectopic pregnancy implantation sites.

Sample	hCG (IU/l)	Serum progesterone (nmol/l)	Gestational age (days)
1	5981	158.1	41
2	453	8.8	47
3	10,285	31.7	46
4	1082	23.9	52
5	508	7.1	44

2.2. Cell lines

The HTR-8/SVneo trophoblast cell line exhibits features of invasive trophoblasts cells, such as HLA-G (EVT marker) and cytokeratin-7 [33]. Cells were maintained in RPMI medium (Sigma–Aldrich) supplemented with 10% FCS at 37 °C, 5% CO_2. Given the difficulty in obtaining primary Fallopian tube epithelial cells, we utilised the only available human oviduct cell line. Oviduct epithelial OE-E6/E7 cells [34] were a kind gift from Professor Calvin Lee (The University of Hong Kong). Cells were cultivated in DMEM/F12 medium containing 10% FCS at 37 °C, 5% CO_2.

2.3. LIFR immunohistochemistry

Formalin fixed, paraffin wax-embedded sections were mounted on Snow Coat X-tra™ charged slides (Surgipath Europe), de-waxed in xylene, rehydrated and subjected to antigen retrieval by pressure cooking for 5 min in 10 mM sodium citrate (pH 6.0), before blocking endogenous peroxidase with 3% hydrogen peroxidase (Sigma). An avidin–biotin block (Vector Laboratories) and protein block (Dako) were performed prior to overnight incubation with 2 μg/ml of rabbit anti-LIF-R antibody (Santa Cruz) or control rabbit IgG (Dako). Sections were incubated with biotinylated secondary antibody and ABC-Elite (Vector Laboratories). Positive immunostaining was visualised using 3,3-diaminobenzidine (Vector Laboratories). Sections were counterstained in Mayer's Hematoxylin and mounted using Pertex (Cellpath PLC).

2.4. LIF and LIFR mRNA expression in OE-E6/E7 cells

Total RNA was isolated from cultured OE-E6/E7 cells using the RNeasy Minikit (QIAGEN) according to the manufacturer's instructions. RNA samples were analysed by spectrophotometry (Nanodrop) using the absorbance ratio of A260/280 nm. cDNA was synthesised from total RNA (500 ng) using Superscript III reverse transcriptase (Invitrogen).

2.5. RT-PCR

PCR reactions were performed using PCR express machine (ThermoFisher Scientific) and GoTaq master mix (Promega) according to the manufacturer's instructions. OE-E6/E7 cDNA was analysed for LIF, LIFR and 18s as previously described [35].

2.6. LIF enzyme-linked immunosorbent assay

A LIF ELISA (ELH-LIF-001; RayBiotech) was performed to quantify LIF secretion from OE-E6/E7 and HTR-8/SVneo cells. OE-E6/E7 and HTR-8/SVneo cells were cultured in 96-well plates in DMEM/F12 or RPMI media respectively, ±10% FCS, before media was collected. Total protein was determined using BCA Protein Assay Kit (Pierce). LIF was detected according to the manufacturer's instructions.

2.7. STAT3 activation in OE-E6/E7 and HTR-8/SVneo cells

OE-E6/E7 and HTR-8/SVneo cells were serum starved for 24 h prior to treatment. HTR-8/SVneo cells were treated for 30 min with recombinant LIF (R&D Systems) diluted in 0.1% bovine serum albumin (BSA) (Sigma) in PBS (100 ng/ml). OE-E6/E7 cells were originally treated for 30 min with 5, 50 or 100 ng/ml LIF. Subsequently, OE-E6/E7 cells were treated for 30 min with either; vehicle control; LIF (100 ng/ml); PEGLA (100 ng/ml); or PEGLA + LIF (cells pre-treated with PEGLA (100 ng/ml) for 1 h before the addition of LIF for 30 min (100 ng/ml)).

2.8. SDS-PAGE and Western blotting

Cells were washed with PBS and lysed in lysis buffer (50 mM Tris base, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF and 25 mM β-glycerophosphate; pH 7.5, 2 ml/ml protease inhibitor cocktail set; Calbiochem). Cellular protein was quantified by the BCA Protein Assay Kit (Pierce). Equal amounts of protein were resolved on 8–10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) gels, transferred to polyvinyl difluoride (PVDF) membranes (GE Healthcare), blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (Bio-Rad) before being probed with polyclonal antibodies against pSTAT3 (1:1000), STAT3 (1:1000), pERK (1:1000) and β-actin (1:1000) (Cell Signaling) overnight at 4 °C, followed by three wash steps. Membranes were incubated for 1 h at room temperature with secondary antibodies (anti-rabbit IgG) conjugated to horse-radish peroxidase (HRP) (1:5000) (DakoCytomation) and signals were developed with enhanced chemiluminescence Western blotting detection system reagent (Pierce).

2.9. Trophoblast spheroid-oviduct cell adhesion co-culture model

To determine the effect of LIF and LIF inhibition on the adhesive properties essential for the attachment of the blastocyst to the Fallopian tube, a co-culture model was established based on published methods [36]. OE-E6/E7 cells were grown to confluence 48-well plate and serum starved for 24 h, and then treated for 24 h with either: vehicle control; LIF (100 ng/ml); PEG (100 ng/ml); PEGLA (100 ng/ml);