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Assessment of potential biomarkers of pre-receptive and receptive endometrium in uterine fluid and a functional evaluation of the potential role of CSF3 in fertility

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ABSTRACT

The endometrium lines a women's uterus becoming receptive, and allowing embryo implantation to occur, for just a few days during the post-ovulatory mid-secretory phase of each menstrual cycle. We investigated whether concentrations of proposed receptivity biomarkers (VEGF, IL8, FGF2, CSF3 sFlt-1, sGP130 and PlGF) secreted by the endometrium into the uterine cavity and forming the microenvironment for embryo implantation is altered among a population of age-matched women with unexplained (idiopathic) infertility compared to fertile women during the receptive mid-secretory phase (n = 16 fertile, 18 infertile) and the prior pre-receptive early secretory phase (n = 19 fertile, 18 infertile) of their cycle.

In the mid-secretory cohort significantly elevated concentrations of five biomarkers; PlGF ($p = 0.001$), IL8 ($p = 0.004$), sGP130 ($p = 0.009$), sFlt-1 ($p = 0.021$), and CSF3 ($p = 0.029$) was present in uterine fluid of infertile women during the mid-secretory phase, but only CSF3 was significantly elevated in the pre-receptive early secretory phase ($p = 0.006$). In vitro studies of glycosylated and non-glycosylated forms of CSF3 at representative fertile (20 ng/mL) and infertile (70 ng/mL) effects on endometrium and embryo behaviour were performed.

Non-glycosylated CSF3 at fertile concentrations significantly ($p < 0.001$) elevated endometrial epithelial cell proliferation however chronic treatment or elevated (infertile) concentrations of CSF3 in glycosylated form abrogated the positive effects. Both forms of CSF3 increased trophoblast cell invasion ($p < 0.001$) regardless of concentration. Mouse embryo outgrowth was significantly ($p < 0.01$) increased at fertile but not at infertile concentrations.

The study confirmed potential utility of five biomarkers of endometrial receptivity for future application in the mid-secretory phase while highlighting CSF3 is elevated in the earlier pre-receptive phase. Our data provides evidence that CSF3 acts on both human endometrium and embryo in a manner that is concentration and glycosylation dependent.

1. Introduction

Synchronous development of blastocyst and endometrium is essential for successful establishment of pregnancy. The endometrium becomes receptive to embryo implantation for only a brief period of time during the mid-secretory phase of each menstrual cycle. Failure to develop a receptive endometrium results in infertility. A number of laboratories have sought to identify biomarkers of endometrial receptivity to identify women suffering endometrial based infertility. Studies have used genomic [1] and proteomic [2–5] approaches to characterise tissue and secretions, in addition to single marker studies [6–12]. The majority of studies have focussed on the receptive mid-secretory phase encompassing the 'window-of-implantation', of a natural menstrual cycle to provide diagnosis. Receptivity markers could

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greatly improve the success of Assisted Reproductive Technology (ART) cycles by providing a predictive test to inform patient and clinician of the likelihood of successful embryo implantation. In this context a mid-secretory marker, present 4–5 days post-ovulation induction with human chorionic gonadotrophin (hCG), provides little scope due to time constraints for testing prior to an embryo transfer at hCG + 5. Additionally in the case of endometrial tissue collection the potential of endometrial tissue damage compromising embryo success.

This study evaluates proposed mid-secretory phase biomarkers for applicability to both the early and mid-secretory cycle phases. The biomarkers examined were interleukin-8 (CXCL8/IL8) [13,14], colony stimulating factor-3 (CSF3/g-CSF) [12], basic fibroblast growth factor (FGF-2/b-FGF) [11], vascular endothelial growth factor (VEGF) [9,10,15,16], soluble fms-like tyrosine kinase-1 (sFlt-1/VEGFR1) [7], soluble glycoprotein 130 (sGP130, IL6ST) [8] and placental growth factor (PlGF) [16,17]. To date these biomarkers have mostly been examined on small cohorts (< 6 women). This study for the first time examines these biomarkers on a single cohort. The initial data highlighted relevance of CSF3 concentration in uterine fluid of infertile women prompting further study of its impact on aspects of endometrial and embryo development.

2. Materials and methods

2.1. Ethics statement

Clinical samples were collected with informed written consent in accordance with the guidelines of the National Health and Medical Research Council (Australia) using protocol reviewed and approved by the Human Research Ethics Committees of Monash Surgical Private Hospital (approval #06066) and Southern Health (approval #03066B). Mouse embryo studies were approved by Monash Medical Centre Animal Ethics Committee of Monash Health (MMCA/2015/57).

2.2. Patient samples

Patient cohorts included fertile women with proven parity undergoing gynaecologic procedures e.g. tubal ligation, mirena insertion, and from idiopathic primary infertile women undergoing dilatation and curettage. Women with diagnoses of male factor infertility, endometriosis, tubal or ovarian abnormalities (e.g. blocked tubes, amenorrhea and polycystic ovarian syndrome) and those using steroidal contraceptives in the prior six months were excluded. Uterine lavage and tissue biopsy were collected as described previously [5]. Collected lavage was mixed with 5 μL of protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany). Following centrifugation at 1000 rpm for 10 min the lavage was aspirated and stored as de-identified aliquots at −80 °C prior to analysis. Tissue biopsies were formalin-fixed and paraffin embedded. Cycle phase dating was performed by accredited independent pathology laboratory based on Noyes' criteria [18]. Only those dated as mid-secretory (n = 33) or early secretory stage (n = 37) were included in the study.

2.3. Luminex analysis of lavage from fertile and infertile women

Lavage diluted 1:1(v:v) in assay buffer were analysed using a Human Cytokine panel assay for VEGF, IL8, FGF2 and CSF3 (Merck KGaA), a Human Soluble Cytokine Receptor panel for sFlt-1 and sGP130 (Merck KGaA), and a singleplex assay for PlGF (R&D systems, Minneapolis, MN, USA). All assays were performed according to manufacturer's instructions. Samples, standards and quality controls were assayed in duplicate with overnight sample incubation at + 4 °C. Assays were read using Bioplex 200 analyser (Biorad, Hercules, California). Mean concentrations for each sample were interpolated from a 5-parameter fit standard curve.

2.4. Cell line culture

ECC-1, an endometrial epithelial cell line, and HTR-8/SVneo (a kind gift from Professor Charles Graham), a trophoblast cell line, were routinely cultured and grown to 80% confluence in appropriate media DMEM/F12 and RPMI 1640 (Invitrogen) respectively containing 10% fetal calf serum and 1% penicillin/streptomycin, before passaging and seeding out for experimental purposes. All cells were genotyped and tested as mycoplasma-free.

2.5. CSF3 preparations

E.coli derived non-glycosylated (CSF3-NG, Peprotech, NJ, USA) and Chinese hamster ovary derived glycosylated (CSF3-G, Abcam, Cambridge, UK) recombinant full-length human CSF3 proteins were utilised in cell culture studies. A 10 ng leading of each CSF3 form was resolved alongside Precision Plus Protein™ Kaleidoscope™ molecular weight markers (Biorad, California, USA) on a 4–20% TGX stain-free electrophoresis gel (Biorad) and transferred to PVDF membrane using Transblot Turbo Transfer system (Biorad). The membrane was blocked with 1%(w/v) skimmed milk protein in Tris buffered saline (TBS), and subsequently incubated with 1 μg/mL overnight at 4 °C with a CSF3 specific antibody (sc-1318, Santa Cruz Biotechnology, Texas, USA) raised in goat. Following extensive washing with TBS containing 0.5%(v/v) Tween 20, bound antibody was detected with 1 μg/mL biotinylated horse anti-goat IgG (Vector Laboratories, California, USA) in combination with Vectastain ABC-horseradish peroxidase (Vector laboratories). Bands were imaged using Clarity™ Western chemiluminescent substrate (Biorad) on a GelDoc XR + ™ Imaging system (Biorad). Molecular weight analysis was conducted using ImageLab™ software (Biorad).

2.6. Real-time cell analysis of CSF3 effects

Real-time experiments were performed using xCelligence RTCA instruments (ACEABiosciences, San Diego, CA, USA) and are based on the principle of electrical impedance. Real-time monitoring of adhesion and proliferation has previously been described [19]. In a second version of the equipment, invasion and migration from upper to lower chambers of a modified Boyden chamber assay, migration and invasion are similarly measured. To examine invasion, matrigel was used as a basement membrane through which cells invade.

2.7. Effect of CSF3 on endometrial epithelial cell (ECC-1) adhesion and proliferation

Endometrial changes to adhesive behaviour and proliferation were investigated in two models; chronic exposure represents the long term exposure to elevated CSF3 throughout proliferative, early and mid-secretory cycle stages, whilst in an acute model CSF3 is applied to mimic exposure only during the secretory phase once progesterone is present.

Two concentrations of each CSF3 preparation (20 ng/mL and 70 ng/mL) were used, representing mean concentrations present in uterine fluid from fertile and infertile women respectively allowing for 20-fold dilution occurring from lavage collection alongside a 0 ng/mL vehicle control.

ECC-1 were primed for 24 h with Estradiol (E) followed by treatment for a further 24 h with Estradiol + Progesterone (E + P) to mimic hormonal changes across the human menstrual cycle before cells (2×10^4 cells/well) were seeded onto fibronectin-coated (5 μg/cm^2, Corning, Corning, NY, USA) xCelligence real time adhesion/proliferation plates (E-plates, ACEA Bioscience). For chronic exposure CSF3 was present throughout both E and E + P treatment phases and the subsequent monitoring phase in xCelligence wells. For acute exposure, CSF3 was added at the time of transfer to xCelligence wells. Prepared plates were placed into the xCelligence RTCA SP instrument and