ORIGINAL ARTICLE

Infertility

PC6 levels in uterine lavage are closely associated with uterine receptivity and significantly lower in a subgroup of women with unexplained infertility

Sophea Heng¹, Natalie J. Hannan¹, Luk J.F. Rombauts², Lois A. Salamonsen¹, and Guiying Nie¹,*

¹Prince Henry’s Institute of Medical Research, PO Box 5152, 246 Clayton Rd, Clayton, VIC 3168, Australia ²Department of Obstetrics and Gynecology, Monash University, Clayton, VIC 3168, Australia

*Correspondence address. Tel: +61-3-9594-4380; Fax: +61-3-9594-6125; E-mail: guiying.nie@princehenrys.org

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BACKGROUND: Embryo implantation requires a healthy embryo and a receptive uterus. Uterine incompetence contributes significantly to implantation failure and infertility. To date, there are no reliable biochemical methods that can determine whether the uterus is receptive. Proprotein convertase 5/6 (PC6) is tightly regulated in the uterus and critical for receptivity and implantation; its secretory nature predicts PC6 to be secreted into the uterine cavity. The present study examines whether PC6 is detectable in uterine lavage and whether there is any correlation between secreted PC6 levels and uterine receptivity.

METHODS: Western blotting determined the presence of PC6 protein in uterine lavage. A sensitive and high-throughput activity assay was established and validated. This assay was applied to 103 lavages collected from different phases of the menstrual cycle from women with proven fertility or unexplained infertility.

RESULTS: Uterine lavage contained PC6 protein with levels paralleling enzymatic activity. PC6 levels were significantly higher in the receptive than in the non-receptive phase in fertile women, and the putative receptive phase levels in a subgroup of women with unexplained infertility were significantly lower than in the fertile counterparts.

CONCLUSIONS: PC6 levels in uterine lavage are significantly elevated in the luteal phase of fertile women and markedly reduced in a subgroup of women with unexplained infertility. Uterine fluid is a valuable source of material to evaluate uterine function. Detection of PC6 in uterine fluid may lead to the development of a rapid and relatively non-surgical assessment of uterine receptivity.

Key words: PC5/6 / endometrial receptivity / embryo implantation / infertility / unexplained infertility

Introduction

Embryo implantation is a key event in mammalian reproduction, requiring a healthy embryo and a receptive uterus. Implantation failure is a major cause of infertility in otherwise healthy women (Wilcox et al., 1988), including those undergoing in vitro fertilization therapy where up to ~70% of healthy embryos fail to implant (Norwitz et al., 2001; Margalioth et al., 2006). Uterine incompetence is estimated to be responsible for a large proportion of implantation failure (Lééée-Baëlle et al., 2002). However, to date, there are no reliable biochemical methods that can effectively distinguish between a receptive and non-receptive uterus.

In women, the endometrium undergoes morphological and functional changes in each menstrual cycle, becoming receptive for embryo implantation only during the mid-secretory phase (Sharkey and Smith, 2003). Establishment of receptivity is highly regulated, involving substantial changes initially in the endometrial epithelium and subsequently in the stroma (Salamonsen et al., 2009). One important feature is that the endometrial epithelial cells (predominantly glandular but also luminal) become highly secretory (Smith et al., 1989; Salamonsen et al., 2009), releasing substances including growth factors, cytokines, hormones, proteases, glucose, ions and nutrient transport proteins into the uterine cavity, that likely regulate the uterus and/or embryo (Kane et al., 1997; Hempstock et al., 2004; Dominguez et al., 2010). Recent proteomic studies revealed that uterine fluid contains more than 800 proteins with complex and diverse functions, with changes depending on the phase of the menstrual cycle (Casado-Vela et al., 2009; Scotchie et al., 2009; Hannan et al., 2010). Because of the close association between
uterine function and the secretion profile, uterine fluid is envisaged as a valuable and non-invasive biological material for analysis (Amelzaurra et al., 2009; Boomsma et al., 2009).

We have established that proprotein convertase 5/6 (PC6), a serine protease and a member of the proprotein convertase (PC) family, is a critical uterine regulator for implantation (Nie et al., 2003, 2005; Okada et al., 2005; Dimitriadis et al., 2010). PC6 is up-regulated in the uterus specifically at the time of epithelial receptivity (human and monkey) and stromal cell decidualization (human, monkey and mouse) (Nie et al., 2003, 2005). Knockdown of PC6 in the uterus in mice results in implantation failure (Nie et al., 2005). In both rhesus monkeys and women, PC6 is expressed in the epithelium across the menstrual cycle peaking during the receptive phase (Nie et al., 2005). PC6 is the only PC family member that is tightly regulated in the human uterus in this manner (Freyer et al., 2007), making PC6 the only PC relevant to endometrial receptivity and implantation. Although the exact function of PC6 in the human endometrial epithelium remains to be determined, PC6 is predicted to be secreted and act as a critical regulatory molecule controlling the conversion of a range of functionally important proteins from precursors into mature bioactive forms (Nie et al., 2005; Seidah et al., 2008).

The present study aimed to examine whether endometrial PC6 is detectable in uterine lavage and whether secreted PC6 levels correlate with uterine receptivity.

Materials and Methods

Uterine lavage collection and patient details

Ethical approval was obtained from appropriate Institutional Ethics Committees for all sample collections, and written informed consent was provided by all subjects. Uterine lavages (n = 103) were obtained from women with proven fertility during the mid-late proliferative (Prolif, Days 7–13, n = 22), early secretory (E-Sec, Days 14–18, n = 14) and mid-secretory (M-Sec; Days 19–23, n = 26) phases of the menstrual cycle, or from women with unexplained infertility during the Prolif (n = 8) and M-Sec (n = 33) phases. The fertile women all had proven parity and were presenting for tubal ligation or assessment for reversal of tubal ligation. All infertile women were screened for non-endometrial causes of infertility, including ovarian dysfunction, tubal patency, endometriosis and male factor. For uterine lavage, 5 ml of sterile saline was gently infused transcervically into the uterine cavity through a fine flexible catheter; the fluid was recovered by aspiration, centrifuged (235g/5 min) to remove cellular debris and aliquots immediately stored at −80°C. Self-reported menstrual cycle stage was confirmed by routine histological dating of the tissue.

Western blot analysis of PC6 protein in uterine lavage

PC6 levels were determined by western blotting using an affinity purified polyclonal PC6 antibody (Nie et al., 2005) following SDS–PAGE of 30 µl lavages. All samples for comparison were loaded on the same gel and relative PC6 levels were determined densitometrically based on equal volume of sample loading.

Determination of PC6 activity in uterine lavage

PC6 activity was determined by the cleavage of a fluorogenic peptide substrate pERTKR-AMC (Bachem, Torrance, CA, USA) as published (Fugere et al., 2007). Purified active recombinant human PC6 (1–5 U) (Phenswitch Biosciences Inc., Quebec, Canada) was incubated at 37°C with the peptide substrate (final concentration 100 µM) in DMEM/F12 in 96-well plates (total volume 100 µl), and the real-time kinetic progression of substrate hydrolysis (release of fluorescent AMC) was monitored every 5 min at excitation/emission of 355/460 nm (Wallac, Victor 2 spectrophotometer, PerkinElmer, MA, USA). To determine PC activity in uterine lavage, 98 µl of lavage was incubated with 2 µl peptide substrate (final concentration 100 µM) as for the purified PC6 and substrate hydrolysis was monitored. To confirm specificity, the activity was assayed without or with nana-b-arginine peptides (Poly R) (Mimotopes, Clayton, Australia), a previously published potent PC6 inhibitor (Fugere et al., 2007; Heng et al., 2010). To compare PC6 activity among lavages, the rate of substrate hydrolysis (AMC increase/min) was calculated from the linear phase of each kinetic progression curve and used as the activity unit.

Statistical analysis

Comparisons were made using one-way analysis of variance (for three groups) or unpaired Student t-test (for two groups) after confirming normal distribution. P < 0.05 and <0.01 were considered significant (*) and highly significant (**), respectively.

Results

Detection of PC6 protein in uterine lavage

We first determined whether PC6 protein was detectable in uterine lavage by western blotting. Analysis of 11 lavages representing Prolif (n = 3) and M-Sec (n = 3) phases of fertile and M-Sec of infertile women with proven fertility during the mid-late proliferative (Prolif, n = 3) and mid-secretory (M-Sec, n = 3) phases of fertile and mid-secretory phase of infertile (M-Sec-Infert, n = 4) women. (B) Densitometric analysis of band intensity of (A).
(-Infert, putative receptive) women showed that PC6 was indeed detectable in lavages (Fig. 1A). Densitometric analysis showed that in fertile women, the mean levels were higher in the Mid-Sec (receptive) than in the Prolif (non-receptive) phase (Fig. 1B). Further, the Mid-Sec levels in fertile women were significantly higher ($P = 0.01$) than in the infertile women (Fig. 1B).

Development and validation of an activity assay to determine PC6 in uterine lavage

The above results prompted us to develop a more sensitive and higher throughput assay that is suitable for determining PC6 in a large number of uterine lavages. One such potential assay is to measure PC6 activity in 96-well plates. The prerequisite is that PC6 activity is detectable in lavage and that the activity reflects the amount of protein. We tested this using an M-Sec lavage from the fertile group that was also analyzed by western blotting. PC6 activity was clearly detectable in the lavage and importantly was dose-dependent (Fig. 2A, left panel); these characteristics mirrored those of purified recombinant active PC6 (Fig. 2A, right panel). We then determined the specificity by inhibiting PC6. The activity in the lavage was completely inhibited by a potent PC6 inhibitor (Fig. 2B, left panel), and this inhibition was similar to that of pure active PC6 (Fig. 2B, right panel). These results established that PC6 activity was detectable in uterine lavage.

Correlation between PC6 activity and protein amount in uterine lavage

We next examined whether PC6 activity correlated with the amount of PC6 protein in uterine lavage. PC6 activity was determined in all 11 lavages analyzed by western blotting (shown in Fig. 1). The activities in fertile women were significantly higher ($P = 0.036$) in the M-Sec than in the Prolif phase, and the M-Sec of fertile women displayed
significantly higher \((P = 0.0065)\) levels than the M-Sec infertile group (Fig. 3). This pattern is highly consistent with the western blotting data (Figs 1 and 3), confirming that PC6 activity reflected its protein concentration in uterine lavage. The above results thus validated the activity assay to assess PC6 levels in the lavage.

**Application of the high-throughput activity assay to a large number of uterine lavages**

The activity assay was then applied to two cohorts of uterine lavages. We first determined PC6 activity in a total of 62 lavages collected from different phases of the menstrual cycle from women with proven fertility. In these women, Prolif \((n = 22)\) was significantly lower than early secretory \(\text{(E-Sec, } n = 14, P = 0.049)\) and highly significantly lower than M-Sec \((n = 26, P = 0.0085; \text{Fig. 4A})\). E-Sec was not found to be significantly different from M-Sec, and the combination of the two \(\text{(Sec, } n = 40)\) was highly significantly greater \((P = 0.0078)\) than the Prolif (Fig. 4B). These results are consistent with the PC6 protein profile in human endometrial tissues across the menstrual cycle.

We next compared PC6 activity in uterine lavages \((n = 103)\) between fertile and infertile women. Lavages collected in the Prolif and Sec phases were evaluated separately. For the Prolif lavages, the fertile \((n = 22)\) and infertile \((n = 8)\) group showed a similar pattern of data distribution and the mean levels were not significantly different between the two groups (Fig. 5A). In contrast, the Sec lavages showed an intriguing pattern (Fig. 5B). While all the lavages from fertile women \((\text{Sec, } n = 40)\) were clustered together, the lavages from the infertile group \(\text{(Sec-Infert, } n = 33)\) naturally separated into two subgroups: 66\% \((n = 22, \text{G1})\) were clustered resembling the fertile group, whereas the remaining 33\% \((n = 11, \text{G2})\) were much scattered.
below the mean value (Fig. 5B). The PC6 activity of G1 (mean = 5390 AMC increase/min) was similar to that of the fertile group (mean = 5798 AMC increase/min), whereas the activity of G2 (mean = 84 AMC increase/min) was significantly lower ($P = 0.025$) than of G1 and highly significantly ($P = 0.0013$) lower than that of the fertile group (Fig. 5B). There was no difference in age range between the two subgroups.

**Discussion**

Uterine lavage is collected by relatively non-invasive means, providing a valuable source of material to evaluate the endometrium. We demonstrated for the first time that uterine lavage contained PC6 protein with levels paralleling enzymatic activity. We established and validated a sensitive, high-throughput assay for PC6 activity in uterine lavage. Its application to a large number of samples enabled us to demonstrate a close association between PC6 levels in uterine lavage and uterine receptivity. PC6 levels were significantly higher in the receptive compared with non-receptive phase of the menstrual cycle in fertile women, and the putative receptive phase levels in a subgroup of women with unexplained infertility were significantly lower than that of the fertile counterparts. Thus, PC6 may represent a promising component to the development of a non-surgical approach for assessment of uterine receptivity. Compared with the western blot method, the activity assay provides greater sensitivity and higher throughput capacity.

PC6 is a critical regulator of implantation both in the mouse and primate including humans (Nie et al., 2005; Okada et al., 2005). Knockdown of PC6 during early pregnancy in vivo in the mouse uterus results in complete failure of implantation (Nie et al., 2005). PC6 is also up-regulated in the human and rhesus monkey uterus specifically in the glandular epithelium and decidual cells at embryo implantation (Nie et al., 2005). It is the only PC family member that is highly regulated in the uterus and relevant for receptivity and implantation (Freyer et al., 2007; Heng et al., 2010). The PCs including PC6 play critical roles in post-translational protein activation by converting precursor proteins into their bioactive forms through limited proteolysis at the general consensus motif (K/R)-(X) n-(K/R) j, where n = 0, 2, 4 or 6 and X is any amino acid (aa) (Seidah et al., 1998, 2008). Thus, PCs are important regulatory molecules required to generate a large number of tissue-specific and functionally important bioactive proteins. These include growth factors, peptide hormones, neuropeptides, extracellular matrix proteins, adhesion molecules, proteolytic enzymes and integral membrane proteins (Seidah and Chretien, 1999). PCs are therefore regarded as critical ‘master switch’ molecules and promising targets for therapeutic applications (Seidah and Chretien, 1999; Rockwell and Thorner, 2004; Scamuffa et al., 2008a,b). PC6 has been reported to process a number of precursor proteins, including Lefty (Ulloa et al., 2001; Tang et al., 2005), MT1-MMP (Yana and Weiss, 2000; Stawowy et al., 2005), platelet-derived growth factor (Siefried et al., 2003, 2005), integrin subunits α4, α5, α6 and αv (Lissitzky et al., 2000; Bergeron et al., 2003), pro-hepcidin (Scamuffa et al., 2008a,b) and Gdf11 (Essalmani et al., 2008), many of which are relevant for implantation. Our recent studies have demonstrated that in the uterus PC6 activates bone morphogenetic protein 2, a critical growth factor for implantation (Heng et al., 2010). Our proteomic search for PC6 substrates also identified that PC6 regulates cytoskeletal proteins such as caldesmon isof orm I during decidualization (Kilpatrick et al., 2009). PC6 was also identified to interact with heparin sulfate proteoglycans (HSPGs), thereby recruiting itself to the cell surface where it activates a number of HSPG-bound substrates at the cell surface or in the extracellular space (Mayer et al., 2008; Seidah et al., 2008).

The detection of a significantly higher level of PC6 in the secretory than the proliferative phase of the menstrual cycle, is in strong agreement with the PC6 protein production profile in the human endometrial epithelium (Nie et al., 2005), confirming that PC6 levels in uterine lavage reflect its in vivo endometrial production. A number of proteins are tightly regulated in the uterine epithelium in a cycle-dependent manner (Sharkey and Smith, 2003; Salamonsen et al., 2009), but their presence in uterine secretions and potential utility for assessing receptivity are unknown. Proteomic studies have revealed the presence of a large number of proteins in uterine secretions (Casado-Vela et al., 2009; Scotchie et al., 2009; Hannan et al., 2010); it remains to be determined whether uterine production/function correlates with secretion of these factors. A very recent comparative proteomic study of uterine lavages revealed differences between receptive and non-receptive states in fertile and infertile women (Hannan et al., 2010). The potential utility of analyzing uterine secretions to assess uterine function is evidenced by a multiplex immunoassay of 17 cytokines in uterine secretions (Boomsma et al., 2009); the ratio of interleukin-1β and tumor necrosis factor-α indicated uterine receptivity. Further support is needed to validate the clinical utility of such assays.

The etiology of unexplained infertility in women with normal hormonal profiles and no organ pathologies or male factor deficiency is not known. Unidentified diverse factors, particularly a disturbance to the uterine establishment of receptivity, may explain the infertility in some of these women. Our analysis identified that a subgroup of women with unexplained infertility contained significantly lower PC6 levels in uterine lavage during the putative receptive phase, directly supporting the above notion. It suggests that an endometrial abnormality at least in PC6 production/secretion during the receptive phase may account for the infertility in these women. Such ‘cohort-specific’ deficiencies for leukemia inhibitory factor, interleukin-11 and their receptors have similarly been demonstrated within endometrial biopsies from infertile women (Dimitriadis et al., 2006). Indeed, it would be unlikely that changes in a single factor are responsible for infertility in all women in whom receptivity is impaired.

In summary, we have demonstrated that PC6 is detectable in uterine lavage and the levels are associated with the status of uterine receptivity. Given the considerable advantage of obtaining/assessing uterine lavage rather than tissue, and the tight uterine regulation of PC6 in association with receptivity and implantation, extended studies are warranted to establish whether assessing PC6 in uterine lavage can contribute to a non-surgical and clinically useful evaluation of uterine receptivity.

**Authors’ roles**

S.H. did all the experiments and analyzed the data, and contributed towards the writing of the manuscript. N.J.H. was responsible for obtaining ethics approval, managing lavage samples and contributed to data interpretation and critical review of the manuscript. L.J.F.R.
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contributed towards lavage collection, data interpretation and critical review of the manuscript. L.A.S. was responsible for recruitment of subjects, contributed towards data interpretation and manuscript writing. G.N. contributed towards study design, result interpretation and manuscript writing.

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