Differential localization and expression of urokinase plasminogen activator (uPA), its receptor (uPAR), and its inhibitor (PAI-1) mRNA and protein in endometrial tissue during the menstrual cycle


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Normal endometrium is a highly dynamic tissue, which responds to ovarian steroids with cyclic proliferation, differentiation (secretion), and degradation (menstruation). The urokinase plasminogen activator (uPA)-dependent proteolytic cascade as well as ligand activation of the uPA receptor (uPAR) is critically involved in physiological as well as pathophysiological aspects of tissue expansion and remodelling. Cyclic variation and distribution of uPA, uPAR and plasminogen activator inhibitor 1 (PAI-1) mRNA were examined by in situ hybridization, real-time PCR and northern blot in normal endometrium. Their corresponding proteins were localized with immunohistochemistry. uPA mRNA is exclusively expressed by stromal cells, whereas uPA protein is present in both epithelial and stromal cells. Immunostaining for uPA protein is reduced or undetectable at mid-cycle, thus coinciding with peak concentration of uPA in the uterine fluid. uPAR mRNA is expressed by epithelial cells in the proliferative phase and by stromal cells in the secretory phase. However, epithelial cells stain for uPAR protein throughout the cycle, suggesting that uPAR may detach from stromal cells and then bind to epithelial cells in the secretory phase. PAI-1 mRNA is located in vessel walls. The late secretory phase has greatly increased expression of all three mRNA and their proteins, mainly in pre-decidual cells in the superficial stroma. Discordant localization of the mRNA and proteins suggest that uPA is produced by stromal cells, released and bound to epithelial cells in both the proliferative and secretory phases, whereas uPAR is released from the stroma and bound to epithelial cells in the secretory phase. Also, the present data together with earlier reports suggest that uPA is released from the epithelial cells to the uterine fluid.

Key words: endometrium/epithelial cells/menstrual cycle/uPA/uPAR

Introduction

Plasmin is a key enzyme in pericellular proteolysis that is derived from the pro-enzyme plasminogen through proteolytic cleavage on the cell surface by urokinase plasminogen activator (uPA) (Andreasen et al., 2000). Binding of the pro-form of uPA to a specific, high affinity cell surface receptor (uPAR) allows this interaction with plasminogen. In addition, ligand binding to uPAR initiates interactions at the cell surface with integrin receptors and subsequently assembly of focal adhesion sites. This is followed by intracellular phosphorylation on tyrosine, and subsequently cell migration. Thus, binding of pro-uPA to uPAR is a key event, which initiates both pericellular proteolysis and cell migration, two processes which together form the basis for invasive cell phenotypes. Consequently, the uPA system is critically involved in both normal and pathological aspects of tissue remodelling. A third function of uPAR is endocytosis of complexed or free uPA for lysosomal degradation (Nykjaer et al., 1992), and this function is operative in endometrial stromal cells (Cassléń et al., 1995, 1998). In addition to degradation, the catalytic activity of uPA is regulated at multiple levels, i.e. expression of the receptor, of uPA itself, and also of the inhibitors (Andreasen et al., 1997). Two specific inhibitors of uPA have been identified in humans, plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2). They are members of the serpin family and are present in the human endometrium (Cassléń et al., 1992b; Nordengren et al., 1996; Sandberg et al., 1997).

The endometrium undergoes extensive cyclic variations each month throughout the reproductive life of women. It proliferates in response to estradiol in the proliferative phase, and differentiates in response to progesterone in the secretory phase. Hormone withdrawal results in tissue disintegration and menstruation. The endometrial release of PA activity in uterine fluid increases during the proliferative phase to a maximum at midcycle, is low in the secretory phase, and increases again pre-menstrually (Cassléń and Åstedt, 1981; Cassléń and Ohlsson, 1981). This pattern is mirrored by the release of uPA from endometrial tissue both in vivo and in vitro (Cassléń et al., 1981; Cassléń and Åstedt, 1983), and by the pattern of plasminogen activation in the uterine fluid (Cassléń and Ohlsson, 1981). Progesterone treatment of tissue explants from proliferative phase endometria reduced the release of uPA (Cassléń et al., 1986). Similarly, progesterone treatment of stromal cells, but not epithelial cells, reduced the release of uPA (Cassléń et al., 1992a). Progesterone treatment increased complex formation with
PAI-1 together with increased surface expression of uPAR, which resulted in increased lysosomal degradation of uPA (Casslén et al., 1986, 1995, 1998).

Pre-menstrual and menstrual endometrial tissues contain and release increasing amounts of PA activity, which is comprised of both tPA and uPA (Rybo, 1966; Casslén and Åstedt, 1981; Casslén et al., 1981; Koh et al., 1992; Gleeson, 1994). This study examines the tissue content and distribution of uPA, uPAR and PAI-1 mRNA and their proteins in normal endometrial tissue over the menstrual cycle.

Materials and methods

Tissue sampling and processing

Endometrial tissue samples were obtained from patients (n = 116) undergoing hysterectomy or curettage for benign non-endometrial conditions (fibromyomas, uterine prolapsus, adenomyosis, cervical dysplasia), all with normal endometrial histology. Sampling was approved by the Review Board for studies in Humans at the University Hospital in Lund. Each endometrial sample was classified as belonging to the early, mid or late proliferative, the early, mid or late secretory, or the menstrual phase (Noyes et al., 1950; Hendrickson and Kempson, 1980).

Tissue samples to be assayed for mRNA quantification were cut into 4 × 4 × 4 mm pieces, snap-frozen on dry ice, and stored at −80°C until northern blot analysis (n = 45) or real-time PCR (n = 39). Tissue distribution of the mRNA species was studied with in situ hybridization. Serial 12 µm thick cryostat sections (n = 9) were collected onto sialinated glass slides in groups of ten adjacent sections. For comparison, formalin-fixed samples (n = 5) were also processed for in situ hybridization. Serial 4–5 µm thick sections were prepared from the paraffin blocks, collected on glass slides which were processed for routine histology and immunohistochemistry (n = 18).

mRNA quantification

mRNA extraction

Frozen tissue samples were weighed and homogenized with a micro-dismembrator. Total RNA was extracted according to the manufacturer’s instructions, using RNeasy total RNA purification kit™ (Qiagen, Germany) for northern blotting, and Trizol Reagent™ (Life Technologies, Sweden) for real-time PCR.

cDNA synthesis

RNA was reverse-transcribed according to protocols from Applied Biosystems. In a 50 µl reaction containing: 0.5 µg total RNA, and at final concentrations of 1 × TaqMan RT buffer, 5.5 mmol/l MgCl2, 50 µmol/l dNTP, 2.5 µmol/l random hexamers, 0.4 IU/µl RNase inhibitor, and 1.25 IU/µl MultiScribe Reverse Transcriptase. The reactions were incubated at 25°C for 10 min, at 48°C for 30 min and then 5 min of inactivation at 95°C. The samples were stored at −20°C until further use.

Real-time PCR amplification

Primers and probes

Gene transcripts were quantified using real-time PCR on ABI Prism™7000 sequence detection system (Applied Biosystems). Primers and probes were ordered from Assays on-Demand™ (Applied Biosystems). Each primer pair was located on different exons of the investigated gene in order to avoid genomic DNA contamination (Table I). Oligonucleotide probes labelled with fluorogenic dye, 6-carboxyfluorescein (Fam) and quenched with 6-carboxy-tetramethylrhodamine were used (Tamra) (Table I).

Amplification

PCR reactions were carried out in a 25 µl final volume containing final concentrations: 1 × Universal PCR Master Mix (Applied Biosystems), 0.5 µmol/l TaqMan probe, 0.9 µmol/l of forward and reverse primers respectively, and 1 µl of 10 ng/µl of a DNA aliquot. For transcripts analysed with pre-manufactured probes the reactions were carried out in a 25 µl final volume containing final concentrations: 1 × Universal PCR Master Mix (Applied Biosystems), 1 × Assaymix (Applied Biosystems), 0.25 µmol/l probe, 0.9 µmol/l of forward and reverse primers respectively, and 1 µl of 10 ng/µl of a DNA aliquot. The thermal cycling conditions were initiated by UNG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s, annealing at 60°C for 1 min. Two negative controls, without template, were included in every amplification. RNA samples were tested for genomic DNA contamination prior to further investigation. For each reaction, triplicate or duplicate assay was carried out. Transcript of β-actin, as a house-keeping gene, was quantified as endogenous RNA of reference to normalize each sample. Quantification was achieved through a calibration curve obtained by serial 10-fold dilutions of the template DNA (0.08–80 ng). Results are expressed as relative values.

Northern blot hybridization

Probe

Human uPA cDNA nt 1–1340, was subcloned into the PstI site of Bluescript SK (Stratagene, USA).

Hybridization

RNA aliquots (15–20 µg) were size-separated on 1% agarose gels containing 2.2 mol/l formaldehyde. The RNA was then transferred to Gene Screen Plus nylon filters (Dupont). Radiolabelled probes were labelled with [32P]dCTP using Megaprime DNA-labelling system (Amersham Pharmacia Biotech). The filters were hybridized in 0.25 mol/l sodium phosphate, 7% sodium dodecyl sulphate (SDS), 1 mmol/l EDTA at 65°C for 12 h and then washed in 0.02 mol/l sodium phosphate, 1% SDS for 30 min at 65°C. Autoradiography was performed for 1–12 h, and signal intensities were measured by densitometry on a Bio Image (USA) computer system. The integrated optical density (IOD) value was related to the IOD value of β-actin. Differences between filters were normalized by introducing an internal standard, i.e. in each filter the median of the samples was set to 1.0 and all samples were expressed in relation to this level.

Localization studies

In situ hybridization

Probes. The probes consisted of human cDNA fragments with the following nucleotide (nt) sequences: uPA (nt 1–1340), uPAR (nt 497–1081) and PAI-1 (nt 1–2876), previously described in Verde et al. (1984), Ny et al. (1986) and Roldan et al. (1990).

DNA templates for generating antisense cRNA probes were prepared by linearizing the plasmid containing the uPA insert with Bsal digestion, the uPAR plasmid with EcoR I, and PAI-1 with Bgl II. DNA templates for sense control cRNA probes were prepared in a corresponding manner, by analogue linearizing with Sal I, Bsal and Sal I respectively. The products were confirmed by agarose gel electrophoresis.

cRNA probes were transcribed from DNA templates using Ambion Maxi Script™ and 800 U/ml [35S]UTP (Du Pont NEN, USA), specific activity 800 Ci/mmol.

Hybridization. Prior to hybridization, tissue sections were fixed, dehydrated, delipidated and air-dried (Young, 1990). Hybridization was performed as previously described (Bradley et al., 1992). Briefly, sections were hybridized with the 35S-labelled probe for 20–24 h at 55°C. Following

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<th>Table I. Specifications of primers and probes used for real-time PCR amplification</th>
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656
hybridization, the slides were washed to remove excess probe, and then coated with undiluted nuclear track emulsion. After 3–5 weeks exposure at 4°C, the slides were developed, fixed and counterstained with 0.2% Toluidine Blue. Every second experiment included sections incubated with the labelled sense probe as a control for unspecific signal.

**Immunohistochemistry**

Sections were dried at 60°C for 60 min, deparaffinized, and gradually rehydrated. For antigen retrieval purposes, sections were microwaved (750 W) for 15 min in Tris–EDTA buffer 0.01 mol/l, pH 9.0 (Shi et al., 1991). The staining procedure was performed in an automated immunostainer TechMate™ (Ventana Biotek, USA) using ChemMate EnVision™ detection kit (Dako, Denmark). The primary antibody was applied for 1 h. Two monoclonal antibodies to uPA were used, 3689 (American Diagnostica, USA) and clone 16 (Monozyme, Denmark). A monoclonal antibody to uPAR, R2, which detects the free as well as the occupied receptor, was kindly provided by Dr G.Høyer-Hansen, Copenhagen, Denmark. Two monoclonal antibodies to PAI-1 were used, clone 1 (Monozyme, Denmark) and clone 6, kindly provided by Dr P.Andreasen, Århus, Denmark. A monoclonal antibody to an irrelevant antigen (*Aspergillus niger* glucose oxidase) was used as control.

**Tissue culture**

Endometrial tissue was obtained from uteri removed because of benign non-endometrial pathology. The endometrium was gently scraped off the upper part of the uterine cavity, and transferred to the laboratory in sterile Hanks buffered salt solution, HBSS. The endometrial tissue was digested with collagenase to separate glands and stromal cells (Casslén et al., 1990). Epithelial cell cultures were established with glands and fragments of glands plated at 300 fragments per well, in 24-well plates. Stromal cells were plated at 50% confluency. Cultures were incubated for 24 h, and conditioned media were kept at 220°C until analysed.

**Assay for suPAR**

The enzyme-linked immunosorbent assay (ELISA) used to quantitate the total amount of suPAR used a monoclonal anti-uPAR antibody R2 for catching, and a rabbit anti-uPAR antibody for detection (Riisbro, 2002). The R2 antibody recognizes all uPAR forms which contain domain 3.

**Statistics**

Real-time PCR results are presented as box-plots. Mann–Whitney U-test was used to evaluate the significance of differences between groups. Progressive variation during the menstrual cycle was evaluated with χ2-test for trend.

**Results**

Real-time PCR quantification of uPA mRNA shows higher levels in the proliferative phase than in the early secretory/mid secretory phase (Figure 1). There is subsequently a steep increase in the late secretory and menstrual phases. A similar cyclic pattern of uPA mRNA is seen in samples analysed by northern blot followed by densitometric scanning (Figure 2). The amount of uPA mRNA is higher in the proliferative phase than in the early secretory/mid secretory phase (Figure 1). This difference may relate to the shift from epithelial to stromal expression (see below). There is a significant increase during the secretory phase to a maximum in the menstrual phase. PAI-1 mRNA levels increase from the late proliferative phase, through the secretory phase, to a maximum in the menstrual phase (Figure 1).

In situ hybridization was performed in 14 tissues altogether, two in each sub-phase of the menstrual cycle. Immunohistochemistry was performed in 18 tissues, three in each sub-phase.

The uPA mRNA localizes diffusely in the stroma of the proliferative and secretory phases (Figure 3). A schematic presentation of the localization is given in Table II. Pre-menstrually, stromal uPA mRNA expression is increased. The uPA mRNA is not present in...
epithelial cells in any part of the cycle. In contrast, weak but gene-
ralized staining for uPA protein is seen in both glandular and luminal
epithelial cells of the proliferative and secretory phases (Figure 6). Intense staining is also seen in some scattered stromal cells through-
out the cycle. Immunostaining for uPA is, like the expression of uPA mRNA, substantially increased pre-menstrually in subluminal parts of the stroma (Table II). Immunostaining with two different mono-
clonal antibodies for uPA gives a similar staining pattern.

uPAR mRNA is expressed by glandular epithelial cells in the pro-
liferative but not in the secretory phase (Figure 4). uPAR mRNA is strongly expressed by stromal cells in the secretory phase and further increased in the pre-menstrual phase, but is not distinguished in the stroma in the proliferative phase. uPAR protein is present in epithelial cells in the proliferative phase, but also in the secretory phase when no uPAR mRNA could be detected in the epithelial cells (Figure 6) (Table II). Also, uPAR protein is present in stromal cells throughout the menstrual cycle with more intense staining and more numerous positive cells in the secretory than in the proliferative phase. Subluminal parts of the stroma stain strongly for uPAR in the late secretory phase. Thus, uPA and uPAR proteins show a similar cyclic pattern and a similar tissue distribution.

PAI-1 mRNA is expressed by endothelial cells in the stroma throughout the menstrual cycle (Figure 5). In the late secretory and

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**Figure 2.** Northern blot analysis of uPA mRNA in endometrial tissue extracts from the early and mid proliferative phase (EP, MP) and early and mid secretory phase (ES, MS).

**Figure 3.** *In situ* hybridization of uPA mRNA in endometrial tissue obtained in the early proliferative (EP; \( n = 2 \)), mid secretory (MS; \( n = 2 \)), and late secretory (LS; \( n = 2 \)) phases. Each image is shown in bright field (left) and dark field (right). Scale bar = 100 \( \mu \)m. uPA mRNA is localized in the stroma in all phases of the menstrual cycle. In superficial parts of the stroma, expression is more intense (not shown in the image).
menstrual phases, PAI-1 mRNA signal increases in intensity and is more widely distributed in the stroma, particularly in the subluminal parts. PAI-1 protein is found in single stromal cells in the proliferative and secretory phases (Figure 6). In the late secretory phase, staining extends to pre-decidual cells in subluminal parts of the stroma. The hybridization signal in formalin-fixed samples is equivalent to that obtained in fresh frozen samples.

The conditioned media from endometrial cell cultures contained soluble uPAR, 0.2–1.8 ng/ml in epithelial cell cultures \( \left(n = 4\right) \), and 0.4–4.1 ng/ml in stromal cell cultures \( \left(n = 3\right) \).
Discussion

Our results indicate that the level of uPA mRNA is lower in the early/mid secretory phase than in the proliferative phase, and then clearly increases in the late secretory and menstrual phases. Expression is restricted to the stroma in all stages of the endometrial cycle. Pro-uPA is a secreted protein, which becomes receptor-bound after its release, and binding may involve receptor molecules on adjacent cells. Thus, immunolocalization is likely to reflect both uPA-producing cells and uPA-binding cells. Since uPA is immuno-localized in both stromal and epithelial cells and uPA mRNA is restricted to the stroma, we conclude that uPA, which is produced in the stroma, presumably binds to epithelial cells in a paracrine way.

Notably, both stromal and epithelial immunostaining was weak or absent around midcycle, i.e. the late proliferative and early secretory phases. In some cases we observed immunostaining for uPA in the glandular secretion. The disappearance of uPA immunostaining at mid-cycle coincides with the peaks of PA activity and uPA content in the uterine fluid (Casslén and Åstedt, 1981; Casslén and Ohlsson, 1981; Casslén et al., 1981). Based on these observations, we suggest that uPA is released from the endometrial tissue to the uterine fluid at midcycle. One possible function for uPA in the uterine fluid at this stage of the cycle would be to stimulate sperm migration, since sperm motility is dose-dependently increased by uPA (our own unpublished observation). Solubilized uPA, which is not bound to membranes, may be lost during processing of the tissue sections and will thus not be detected with immunostaining. In contrast, tissue homogenates will include glandular secretion, and an ELISA will detect its content of uPA. Thus, the endometrial tissue content of uPA protein was not different between the proliferative and secretory phases (Koh et al., 1992; Nordengren et al., 1998).

Both our present finding of reduced uPA mRNA in the early and mid cycle coincides with the peaks of PA activity and uPA content in the uterine fluid (Casslén and Åstedt, 1981; Casslén and Ohlsson, 1981; Casslén et al., 1981). Based on these observations, we suggest
Figure 6. Immunohistochemical localization of uPA, uPAR and PAI-1 in endometrial tissue taken in the early proliferative (EP; $n = 3$), early secretory (ES; $n = 3$), mid secretory (MS; $n = 3$) and late secretory (LS; $n = 3$) phases. Staining for uPA is generally weak. Positive staining is seen in epithelial and stromal cells in early and late parts of the cycle, but is faint or absent at midcycle. A pre-menstrual increase is seen in stromal cells in superficial parts. Staining for uPAR is seen both in epithelial and stromal cells throughout the cycle. Stromal staining for uPAR is weak in the proliferative phase, and increases in the secretory phase. PAI-1 staining is present as single positive cells in the stroma. Staining is more extensive and stronger in the late secretory stroma in superficial parts.
the endometrial release of uPA both *in vivo* and *in vitro* (Casslén et al., 1981; Casslén and Åstedt, 1981, 1983). Thus, an increased endometrial synthesis of uPA in the proliferative phase may be masked by increased release to the uterine cavity.

Epithelial cells express uPAR mRNA only in the proliferative phase, and this expression is often intense. This is reflected in the RT–PCR results, which show the endometrial content of uPAR mRNA to be higher in the proliferative than in the early and middle secretory phase. However, epithelial immunostaining for uPAR is seen not only in the proliferative but also in the secretory phase. Thus, presence of uPAR protein in epithelial cells in the secretory phase suggests a different cellular origin, i.e. the stroma. In fact, uPAR can be detached from the cell surface through cleavage of its glycosyl phosphatidyl inositol anchor by phosphatidyl inositol-specific phospholipase C or possibly other enzymes. Soluble uPAR is released from vascular endothelial, smooth muscle and monocytic cells (Chavakis et al., 1998), and appears in the blood under normal circumstances, and in other body fluids in tumour patients (Ronne et al., 1995; Wahlberg et al., 1998). Subsequently, soluble uPAR can bind to, and be operative in, other cells in a paracrine way (Chavakis et al., 1998; Mizukami and Todd, 1998). The fact that the late secretory phase increase of epithelial uPAR protein mirrors that of stromal uPAR protein, which in turn is very similar to that of stromal uPAR mRNA, suggests that epithelial uPAR in the secretory phase originates in the stroma. In fact, our tissue culture experiments show that uPAR can be detached from both epithelial and stromal cells, and support the hypothesis that uPAR is released to the extracellular fluid *in vivo*. However, since stromal uPAR in the secretory phase is also involved in internalization and degradation of uPA (Casslén et al., 1995), it is likely that uPAR molecules are in part membrane-bound and in part solubilized. Also, the higher content of uPAR mRNA in the proliferative phase, which is located in the epithelium, is not reflected in the number of functional receptor sites in endometrial tissue membranes (Casslén and Gustavsson, 1991). This observation may further support the idea that uPAR is solubilized and released to the uterine fluid.

We found that stromal expression of uPAR mRNA increases gradually during the secretory phase, and is prominent in the late secretory and menstrual phases, and that this is paralleled by immunodetected uPAR protein in the stroma. Furthermore, this pattern of expression is in agreement with our earlier observation of higher content of receptor sites in membranes prepared from secretory phase than from proliferative phase endometria (Casslén and Gustavsson, 1991). The function of uPAR has been studied in detail in endometrial stromal cells *in vitro* (Casslén et al., 1998). Expression of uPAR is up-regulated together with that of PAI-1 in response to progesterone (Casslén et al., 1992b, 1995). Increased release of PAI-1 will increase complex formation with uPA, resulting in increased elimination of uPA through internalization of the complex. However, this process requires binding to uPAR and increased expression of uPAR (Casslén et al., 1995; Sandberg et al., 1998). Thus, our present observation of increasing uPAR mRNA and protein in the stroma during the secretory phase supports a specific role for uPAR in regulation of uPA.

PAI-1 mRNA is expressed in the walls of small vessels, and the protein is localized in single cells in the stroma, which are at least partly related to small blood vessels. In the late secretory and menstrual phases, PAI-1 mRNA and protein showed congruent increase in stromal cells around the vessels and beneath the luminal epithelium, i.e. in pre-decidual cells. This increase is in agreement with higher tissue levels of PAI-1 in menstrual phase endometria (Gleeson, 1994), as well as the progesterone-induced increase of PAI-1 in endometrial stromal cell cultures (Casslén et al., 1992, 1995; Schatz and Lockwood, 1993; Sandberg et al., 1997). In fact, this increase results from PAI-1 mRNA stabilization rather than increased transcription (Sandberg et al., 1997). uPA bound to uPAR is internalized and degraded in the lysosomes (Cubellis et al., 1990) and internalization in endometrial stromal cells is more efficient for uPA in complex with PAI-1 than free uPA (Casslén et al., 1995, 1998). The observed co-localization of PAI-1 and uPAR in predecidual cells strongly indicates that internalization occurs *in vivo* in these cells. Stromal expression of PAI-1 and subsequently increased elimination of uPA is likely to be a prerequisite for implantation of the blastocyst, promoting consolidation of the extracellular matrix by limiting pericellular proteolysis. This mechanism may be of importance during early pregnancy too, since PA activity is absent in early decidua (Liedholm and Åstedt, 1976).

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