Concentration of plasminogen activators and inhibitor in the human endometrium at different phases of the menstrual cycle

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Summary. The concentrations of tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA) and plasminogen activator inhibitor (PAI-1) have been determined in endometrial curettings obtained from 46 subfertile women during proliferative, early or late secretory phases of the menstrual cycle. t-PA activity and antigen concentrations was significantly higher ($P < 0.001$) in late secretory endometrium than in proliferative or early secretory endometrium. Higher concentrations of PAI-1 antigen ($P < 0.05$) were also noted in late secretory phase than in proliferative and early secretory endometrium. However, u-PA concentration was not significantly different and no PAI activity could be demonstrated in the menstrual phases studied. Zymography studies confirmed the presence of both t-PA and u-PA in the endometrium. Ovarian hormonal patterns may therefore influence the activity of plasminogen activators especially of t-PA in the endometrium during various phases of the menstrual cycle.

Keywords: plasminogen activators; urokinase; plasminogen activator inhibitor; endometrium; human

Introduction

The plasminogen activators (PA) play an important role in biological processes involving cell migration, tissue degradation and remodelling (Martin & Arias, 1982). It is suggested that the concentrations of PA in the endometrium are regulated by sex hormones (Mullins *et al.*, 1980; Kneifel *et al.*, 1982; Peltz *et al.*, 1983). Oestrogen stimulates endometrial growth and synthesis or release of PA, whereas progesterone is involved in secretory changes reducing the concentration of PA by inhibiting the conversion of the proactivator or synthesis of PA or inhibiting both (Liedholm, 1975; Casslen & Astedt, 1983a, b; Kjaelgaard *et al.*, 1988). Two forms of PA, urokinase (u-PA) and tissue activator (t-PA), are reported to be present in the endometrial tissues and uterine fluids (Casslen & Astedt, 1983a, b; Koh *et al.*, 1988). However, the physiological role of these two forms of endometrial PA is not clear. They are immunologically different but both of them cleave plasminogen in the same way. t-PA is involved in thrombolysis, whereas u-PA is, among other functions, involved in the tissue degradation in normal as well as in pathological conditions including cancer (Astedt *et al.*, 1981; Dano *et al.*, 1985). PA may be released into the uterine cavity to remove fibrin deposits that might occur, and may aid in the migration of spermatozoa and implantation of the blastocyst (Casslen & Astedt, 1981).

Wide variations in endometrial PA concentrations have been reported throughout the menstrual cycle (Koh *et al.*, 1988). The present study was undertaken to investigate whether the hormonal changes during the menstrual cycle have any effects on the concentrations of t-PA, u-PA and PAI-1 in the endometrium.

Materials and Methods

Endometrium

Endometrial curettings ($n = 45$) were obtained from 45 subfertile subjects. The subjects had been trying unsuccessfully for conception for more than 12 months and attending our Infertility Clinic for their routine infertility check-up. The age and menstrual patterns of the subjects are shown (Table 1). The subjects underwent routine laparoscopy, dilatation and curettage. Half of the curettings were immediately rinsed with cold $0.15 \text{ mol NaCl l}^{-1}$ to remove blood, blotted dry, wrapped in parafilm and stored at -70°C until homogenization. They were classified into either proliferative ($n = 20$), early ($n = 12$, corresponding to day 14–18) or late ($n = 14$, day 24–28 of the menstrual cycle) secretory by histological dating.

Table 1. Age and menstrual patterns of subfertile subjects

	Age (years)	Menstrual patterns		
		(<21 days)	(21–35 days)	(unknown)
<i>n</i>	45	1	34	10
Mean (SD)	31.9 (3.9)	15	28.4 (1.8)	—
Range	(25–40)	15	(23–33)	—

Although endometrial tissues used were from subfertile females, all of them were histologically normal and had normal menstrual patterns of 28 ± 7 days cycle.

Homogenization

After thawing, the endometrial tissues were weighed and then homogenized in $0.15 \text{ mol NaCl l}^{-1}$ (100 mg tissue in 1 ml) using an IKA Turrax homogenizer at $2-4^\circ\text{C}$ for 3–4 min. The homogenate was spun at $27\,000 g$ in an ultracentrifuge (4°C for 5 min) and the supernatant was divided in aliquots in plastic tubes and immediately stored at -70°C until assayed.

Determination of PA and PAI-1

t-PA and PAI-1 activities were determined using the plasmin generation method described by Koh *et al.* (1989a, b). The method used for determining the t-PA and PAI-1 activities in plasma was applied to biological fluids. Briefly, for t-PA assay one volume of endometrial extract was treated with two volumes of $0.1 \text{ mol HCl l}^{-1}$, and after 30 min incubation another two volumes of $0.1 \text{ mol NaOH l}^{-1}$ was added and this acid-treated aliquot was used for assay. A protein solution of homogenized fibrin ($850 \mu\text{g protein ml}^{-1}$) was used as a stimulator in the presence of an excess of plasminogen and the plasmin generated by the t-PA present in the acid-treated sample was measured by the release of para-nitroanilide (pNA) from the chromogenic substrate S-2251 at 492 nm. For the assay of PAI activity $50 \mu\text{l}$ of $50 \text{ iu single chain t-PA ml}^{-1}$ was incubated with $50 \mu\text{l}$ of endometrial extract for 10 min at room temperature. After incubation 0.9 ml Tris buffer was added and the residual t-PA was measured by the t-PA activity assay described. The amount of t-PA remaining after incubation is inversely proportional to the concentration of inhibitor present. The antigen assays for t-PA, u-PA and PAI-1 were determined using ELISA kits purchased from American Diagnostics Inc. (New York, USA). The ELISA kits use a double-antibody principle for t-PA assay and mouse monoclonal antibodies for u-PA and PAI-1 antigen assays. The activity and antigen concentrations are expressed per 100 mg of wet endometrial tissue.

t-PA, u-PA standards and rabbit antibodies

Single chain melanoma cell t-PA 86/67 and two chain u-PA 66/46 were obtained from the National Institute for Biological Standards and Controls (NIBSC), Holly Hill, London. The standards were used for the t-PA and PAI activity assays and for raising antibodies in rabbits for characterization of PAs. Locally bred male rabbits were immunized with t-PA or u-PA, 400 iu, in complete Freund's adjuvant and boosted with the same units every two weeks until the antibody titre was constant. Bleeding was performed from the ear vein and the blood was taken to determine the serum antibody titre in a fibrin plate. Serial dilutions of the antibody were incubated with either t-PA or u-PA (1.0 iu ml^{-1}) for 30 min to determine the inhibition of lysis on the fibrin plate. The highest dilution that gave complete inhibition of lysis is the titre for the antibody. The antibody titre used in this study was 1:64 for t-PA and 1:160 for u-PA. In zymography studies both antibodies gave specific lysis zones for either t-PA or the two chain u-PA markers.

Characterization of PA

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Weber & Osborn, 1969) was performed on stored unreduced endometrial extracts. Endometrial extracts (20 μ l) pre-mixed in solubilizing buffer were used together with single chain t-PA and two chain u-PA as markers. Electrophoresis was performed for 3 h at 35 mA with cooling. After SDS-PAGE, fibrin zymography of PAs was performed as described by Granelli-Piperno & Reich (1978) and Koh *et al.* (1988). Briefly, the fibrin-agar gel was prepared by adding 1 caesin unit of plasminogen (Kabi, Sweden) to 12 ml of 2.30 g human fibrinogen l^{-1} (Kabi) containing 1.0% agarose (Indubiose Agarose A37: Reactifs, IBF France). Bovine thrombin (Roche) was added, 50 ml in 50 iu bovine thrombin ml^{-1} , and the mixture was immediately transferred into a 10 cm $\times$ 10 cm sandwiched glass plate with U-frame of 1.5 mm thickness to form a fibrin gel. After electrophoresis the gel was washed in 1% Triton X-100 for 1 h, laid onto the fibrin-agar gel and incubated in a humid environment at 37°C for about 18 h. After incubation the fibrin-agar gel was washed in distilled water and stained with Coomassie blue. Fibrinolysis appeared as clear zones after staining.

Statistical analysis

Unpaired Student's *t* test was used for calculation of statistical significance. A *P* value of ≤ 0.05 was regarded as statistically significant.

Results

Late secretory endometrium had significantly higher mean t-PA activity (16.4 ± 12.5 iu in 100 mg), t-PA antigen (24.7 ± 16.1 ng in 100 mg) and PAI-1 antigen (1.25 ± 1.1 ng in 100 mg) than either the proliferative or early secretory endometrium (Table 2). The proliferative endometrium had significantly raised ($P = 0.01$) t-PA antigen (mean 14.0 ± 6.7 ng in 100 mg tissue) compared with the early secretory endometrium (mean 2.9 ± 1.1 ng in 100 mg tissue). The mean t-PA activity of 4.8 ± 4.3 iu in 100 mg tissue in the proliferative phase was higher than the mean of 2.9 ± 1.1 ng in 100 mg of tissue in the early secretory endometrium, but this was not statistically significant. PAI-1 activity could not be detected in all endometrial tissues studied, although low PAI-1 antigen was found in most of the tissues. PAI-1 antigen was significantly higher ($P < 0.05$) in late secretory endometrium (mean 1.25 ± 1.07 ng in 100 mg) than in proliferative (mean 0.52 ± 0.77 ng in 100 mg) and early secretory phase endometrium (mean 0.56 ± 0.32 ng in 100 mg) of tissue. No significant differences in PAI-1 antigen were found between proliferative and early secretory endometrium (Table 2). u-PA antigen concentrations ranged from 0 to 2.6 ng in 100 mg tissue, and were not statistically different in the three phases of the menstrual cycle although late secretory endometrium had a higher amount (0.75 ± 0.78 ng in 100 mg) than proliferative (0.37 ± 0.79 ng in 100 mg) and early secretory endometrium (0.33 ± 0.43 ng in 100 mg tissue). The distribution of t-PA, PAI-1 antigen and u-PA in the endometrium at different phases of the menstrual cycle is shown (Figs 1 and 2).

Characterization of PA

Plasminogen-dependent fibrinolytic activity was seen in the endometrium in the regions corresponding to molecular masses of 31, 54 and 67 kDa (Fig. 3a). When anti-u-PA 1:100 was impregnated into the fibrin-agar gel, free t-PA of 67 kDa molecular mass was seen (Fig. 3b) and one chain u-PA of 54 kDa was observed in the endometrium on the addition of 1:50 anti-t-PA to the fibrin-agar gel (Fig. 3c).

Discussion

The plasminogen activators have been implicated in several reproductive processes including ovulation, trophoblast implantation, menstrual bleeding and mammary gland involution (Kneifel *et al.*, 1982). All of these events share the common feature of tissue remodelling or cell migration, and most of them are under hormonal modulation. Both t-PA and u-PA are found in the

Table 2. Activity and amounts of antigen of plasminogen activators and PAI-1 in 100 mg endometrium from subfertile females at proliferative and late secretory phases of the menstrual cycle compared with early secretory endometrium

	t-PA		PAI-1		u-PA
	Act (IU)	Ag (ng)	Act (IU)	Ag (ng)	Ag (ng)
Proliferative					
<i>n</i>	19	19	19	19	19
Mean	4.8	14.0	nd	0.52	0.37
SD	4.3	6.7	—	0.77	0.68
Range	(0.16–16.9)	(3.6–27.1)		(0–3.5)	(0–2.3)
<i>P</i>	ns	0.001		ns	ns
Early secretory					
<i>n</i>	12	12	12	12	12
Mean	2.9	5.4	nd	0.56	0.33
SD	1.12	3.42	—	0.32	0.43
Range	(1.2–5.4)	(1.0–12.0)		(0.16–1.3)	(0–1.3)
<i>P</i>					
Late secretory					
<i>n</i>	14	14	14	14	14
Mean	16.4	24.7	nd	1.25	0.75
SD	12.48	16.13	—	1.07	0.62
Range	(1.88–50.0)	(4.4–56.0)		(0.25–3.89)	(0–2.6)
<i>P</i>	0.001	<0.001		<0.05	ns

Act: activity; Ag: amount of antigen; ns: not significant; nd: not detected

Fig. 1. Concentration of tissue plasminogen activator (t-PA) (●) activity and antigen (○) in the human endometrium at proliferative ($n = 19$), early ($n = 12$), and late secretory ($n = 14$) phases of the menstrual cycle.

Fig. 2. Concentration of (a) urokinase plasminogen activator (u-PA) and (b) plasminogen activator inhibitor (PAI-1) antigen in the human endometrium at proliferative, early and late secretory phases of the menstrual cycle.

endometrium and uterine fluids. Oestrogen stimulates endometrial growth, and synthesis or release of PA. On the other hand, progesterone decreases the content and release of the activators. Inhibitors of PA have been known to reduce the menstrual blood loss (Mullins *et al.*, 1980; Kneifel *et al.*, 1982; Casslen *et al.*, 1983a, b; Peltz *et al.*, 1983).

In this study we observed that t-PA activity and antigen together with PAI-1 antigen concentrations were significantly higher in late secretory endometrium than in either proliferative or early secretory endometrium. This observation of high fibrinolytic activity is similar to that reported by Casslen & Astedt (1983b). t-PA activity and amounts of antigen were significantly higher in the proliferative than in early secretory endometrium. No significant differences were seen in amounts of PAI-1 antigen. PAI-1 activity could not be detected in the endometrium studied although low amounts of PAI-1 antigen were recorded. u-PA antigen concentrations tend to be high in the late secretory phase but the amount was not statistically different from the other two phases studied. Zymography studies demonstrated that both t-PA and u-PA are present in the endometrium at various phases of the menstrual cycle and they have also been detected using ELISA methods.

The physiological significance of PA in the endometrium is not clear. It would appear that in the luteal phase sex hormones could influence t-PA concentration in the endometrium. The increase in t-PA concentration in late secretory endometrium is also accompanied by an increase in amount of PAI-1 antigen when compared with proliferative and early secretory endometrium. No PAI-1 activity could be demonstrated in the phases of the endometrium studied which would suggest that t-PA activity is dominant in the endometrium. The low t-PA activity and amounts of antigen observed during the early secretory period could result from the release of t-PA into the uterine cavity. The low t-PA concentration seen in the early secretory phase may be essential for fertilization and for the attachment of the blastocyst to the endometrial surface for the nutrition

Fig. 3. Zymography studies and characterization of plasminogen activators (PAs) in the human endometrium. (a) Zymography studies. Lane 1: single chain t-PA (1.0 iu ml^{-1}); Lane 2: two chain u-PA (0.25 iu ml^{-1}); and human endometrial extracts. Lanes 3 and 4 at proliferative, 5 and 6 early secretory, 7 and 8 late secretory phases. (b and c) Characterization using rabbit antibodies using the same samples as in (a), (b) anti-u-PA 1:100 dilution and (c) t-PA 1:50 dilution.

and survival of the pre-implantation blastocyst (Casslen & Astedt, 1981) if conception occurs. Significant fibrinolytic changes seen in late secretory endometrium would favour an important biochemical step in the physiology of menstruation.

In this study, significantly increased amounts of t-PA activity and antigen and PAI-1 antigen were found in the late secretory endometrium, whereas PAI-1 activity could not be detected and u-PA remained unchanged throughout the menstrual cycle. Leucocytes and macrophages are known to produce plasminogen activators and to increase in number during the pre-menstrual period (Unkeless *et al.*, 1974; Granelli-Piperno *et al.*, 1977). This factor may have contributed to the increase in t-PA concentrations in the late secretory endometrium. The cyclic variation of the sex hormone concentrations that is known to occur during the proliferative (oestrogenic) and secretory (progestational) stages of the menstrual cycle may also influence t-PA and PAI production.

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