PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 182, 419–424 (1986)

Hormonal Regulation of the Release of Plasminogen Activators and of a Specific Activator Inhibitor from Endometrial Tissue in Culture (42360)

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Abstract. Two types of plasminogen activator (PA), t-PA (tissue type) and u-PA (urokinase type), are released from endometrial tissue in organ culture, as judged by immunological identification and molecular weight. Addition of estradiol to the medium greatly enhanced the release of u-PA, whereas that of t-PA was not low. Addition of progesterone, on the other hand, after priming of the endometrial tissue with estradiol, resulted in a much lower release of both types of PA. This pattern of PA release in response to hormonal stimulation *in vitro* agrees with previous observations of the PA activity of endometrial secretion *in vivo.* Endometrial tissue also released a PA inhibitor with molecular weight of approximately 50,000, which complexed both t-PA and u-PA. In cultures stimulated with estradiol the amount of free u-PA increased gradually during incubation and minor amounts of free t-PA appeared after 4–6 days culture. The amount of complexes, and thus the amount of PA inhibitor also increased under influence of estradiol. In cultures stimulated with progesterone, on the other hand, only minor amounts of free u-PA and no free t-PA was detected. The inhibitor might be of either the endothelial or the placental type, or both. © 1986 Society for Experimental Biology and Medicine.

It has been known since 1956 that the human uterus is a rich source of plasminogen activators (PA) (1). It was subsequently recognized that two main types of PA are present in the human organism—i.e., t-PA (tissue plasminogen activator) and u-PA (urokinase, urine plasminogen activator). Large amounts of t-PA can be obtained from myometrial tissues (2, 3), while the endometrium contains both t-PA and u-PA (4, 5). Furthermore, the endometrium releases PAs variably during the menstrual cycle, both *in vitro* and *in vivo* (6). During endometrial proliferation in the follicular phase, u-PA is released in greater quantities than after ovulation in the luteal phase.

Since, however, PA activity is the result of a balance between PAs and PA inhibitors, the study both of inhibitors and of activators becomes necessary if this balance is to be properly understood. In recent years, sensitive assays have been developed for measuring u-PA and t-PA (7, 8), and the development of a zymographic technique for detecting PAs in gels after electrophoresis has made it possible to demonstrate the inhibitors in complex with PAs (9).

The aim of this investigation was to study hormonal control of the release of PAs and PA inhibitors from endometrial tissue in culture.

Materials and Methods. Endometrial tissue was obtained by currettage from patients undergoing operation for preinvasive carcinoma of the uterine cervix. Informed consent was given. None of the patients had any endometrial pathology, or were using oral or intrauterine contraceptives; all were in the proliferative phase according to histological dating of the endometrium, and had serum progesterone <8 nmole/liter. Only 50–100 mg wet weight of endometrial tissue from each patient was available for use in the study, the remainder of the samples being required for histopathological diagnosis. After sampling, the tissue specimens were immediately used in the culture procedure and were handled sterilely throughout.

Tissue culture: (a) Hormonally treated. Endometrial tissue was obtained from seven patients, and cut in 1-mm^3 pieces. Twenty such explants were incubated in each of two dishes, A and B, both containing 5 ml Dulbecco's modification of Eagles' medium (Flow Laboratories, Inc.). For hormonal exposure of the cultures, estradiol 17 and progesterone (Sigma, St. Louis, Mo.) were used. Hormones

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were dissolved in ethanol to a final concentration of 10 g/ml progesterone and 270 ng/ml estradiol. Fifty microliters of either solution was added to 5 ml culture medium in the following manner: Dish A had medium containing estradiol Days 1 to 6; dish B had medium containing estradiol Days 1 and 2, and progesterone Days 3–6.

(b) Untreated. Endometrial tissue from five patients was equally cut in 1-mm^3 pieces. Twenty explants from each patient were incubated with 5 ml medium without added hormones (C).

The medium was changed daily in all dishes and after centrifugation it was stored at −20°C until analyzed. On Day 6, the tissue explants were fixed in Formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin for histologic evaluation of tissue survival.

PA assays. u-PA was measured with a radioimmunoassay (7). t-PA was measured with an immunoradiometric assay (8), in which polystyrene tubes were coated with IgG antibodies to t-PA (melanoma cell PA), incubated with either culture medium or standard dilutions, and finally incubated with ^{125}I-labeled IgG antibodies to t-PA. After careful washing, the tubes were counted in a gamma scintillator. Both free and complexed PAs reacted in the assays. For t-PA, we used the WHO standard 83/517 (1 IU corresponds to 2 ng in our assay). Plasminogen activator activity was measured on plasminogen-rich fibrin plates (10), which were incubated for 18 hr at 37°C. The activity was measured as the product of two perpendicular diameters of the lytic area.

Fibrin overlay technique. Culture media were analyzed with SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), using 6% resolving gels (2.5 × 82 mm) (11). The gels were subsequently incubated with a fibrin agar overlay to detect PA activity (9). The gels were stained with Coomassie blue. In addition to the culture media, normal serum, u-PA (7), t-PA (8), and human plasmin (Kabi, Sweden) were also studied for comparison. In a further experiment, the fibrin agar had been supplemented with IgG (0.2 mg IgG/ml fibrin agar), obtained from goat antiserum to u-PA (mol wt 30,000) or t-PA, or from goat normal serum. IgG was prepared by ammonium sulfate precipitation and DEAE–Sephadex chromatography (Pharmacia, Sweden). In another experiment, the fibrin agar film was first heated to 85°C for 45 min.

Inhibitor assay. The presence of uncomplexed reactive PA inhibitor in the culture media was analyzed by incubating 100 liters culture medium with 100 liters u-PA solution (20 Ploug u/ml) for one-half hour at 37°C. Residual PA activity was measured on fibrin plates as described above.

Results. Under continuous influence of estradiol (A), PA activity of the medium as measured on fibrin plates increased rapidly from Day 2 on (Table I). The release of u-PA increased parallel with the increase in PA activity, whereas the release of t-PA remained at a low level (Fig. 1). The medium in the B dishes (i.e., those in which estradiol was replaced by progesterone from Day 3 on), had much lower concentrations of PAs, and no activity was detected on fibrin plates in most of the dishes. A weak band of free u-PA activity was, however, detected with SDS–PAGE, indicating either a higher sensitivity of SDS–PAGE, or a partial degradation of the PA-inhibitor complex by SDS (12).

Figure 2 depicts SDS–PAGE results for the culture media from one patient, being repre-

TABLE I. PA ACTIVITY OF THE CULTURE MEDIA OBTAINED AFTER 1 TO 6 DAYS INCUBATION OF ENDOMETRIAL TISSUE IN THE PRESENCE OF ESTRADIOL (A) AND PROGESTERONE (B) AND IN THE ABSENCE OF HORMONES (C)

Day of culture	PA activity with A	B	C
2	0	0	0
3	54 (25–97)	0	124 (43–133)
4	225 (90–320)	12 (0–25)	180 (173–202)
5	310 (182–420)	30 (0–64)	175 (165–253)
6	350 (208–480)	16 (0–25)	120 (113–187)

Note. The media were harvested daily, and each curve represents the median and range of results from seven patients. Activity was expressed as mm^2 of lytic zones on fibrin plates.

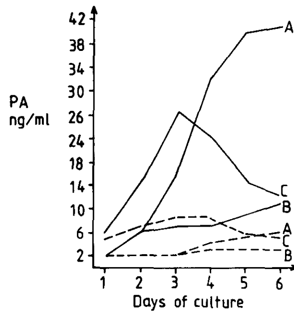

FIG. 1. Concentrations of u-PA (—) and t-PA (---) in the media obtained from endometrial tissue cultures treated with estradiol (A), progesterone (B), and untreated (C). The media were harvested daily. Each curve represents the median concentrations of results from seven patients (A, B) and from five patients (C).

sentative for all other patients included in the study. Lane 1 shows the normal serum PA activity band, which is considered to represent a complex between an inhibitor and t-PA. Lane 2 has the same band together with another two bands which appeared when u-PA (mol wt 54,000 and 31,000) was added to the serum sample. These latter two bands were believed to represent complexes between the serum inhibitor and u-PA 31,000 and u-PA 54,000. The band with lowest molecular weight was not present when only u-PA 54,000 was added (not shown in figure). The band, which is caused by u-PA 31,000, is very similar in molecular weight to plasmin (lane 3). Initially during culture all PA activity is present as a band (lane 4) of similarly high molecular weight to the PA activity band of normal serum (lane 1). As the estradiol effect continues (A), PA activity of the same molecular weight as u-PA (54,000) appears (lanes 5–9). A second high molecular weight band (lanes 6–9) also appears, similar in molecular weight to that of u-PA (54,000) in normal serum. Finally, a band with the molecular weight of free t-PA appears (lane 9). This band of free t-PA appeared on the fourth day in three of the cultures, on the fifth day in one culture, and on the sixth day in three cultures. Under the influence of progesterone (B), only two bands are visible, one similar in molecular weight to the band in normal serum, and one with the molecular weight of u-PA (54,000).

Experiments performed with antisera demonstrated immunological identity of the endometrial activators with both u-PA and t-PA (Fig. 3). Both PAs were present in their complexed as well as in their free forms. Furthermore, as demonstrated with the double antisera technique and heated fibrin agar plates, all fibrinolytic activity was caused by PAs, and not by any other proteolytic enzyme.

The approach used here to assay for PA inhibitory capacity could not demonstrate such in any of the media (data not shown).

Histological examination of the tissue explants after 6 days of culture demonstrated a dense, cell-rich stroma in all explants. In all explants from A dishes, and in half of those from B dishes, glandular tissue was present to varying extents. Epithelial structures were very well preserved in several explants, although their lumen were more or less invaded by

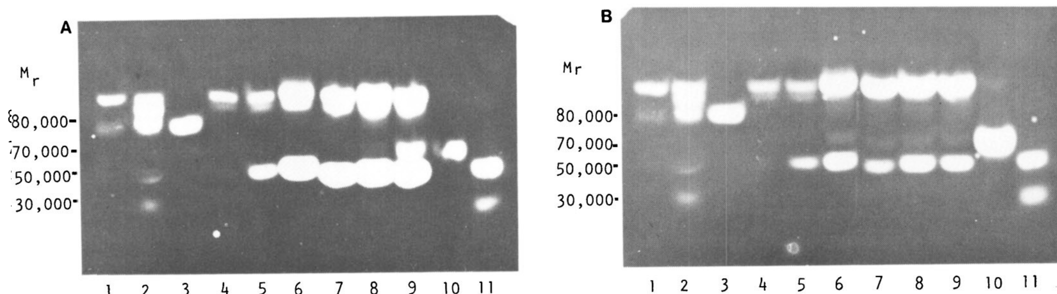

FIG. 2. SDS–PAGE followed by a fibrin agar overlay. PA activity developed as clear zones in the fibrin agar substrate. (1) Normal serum; (2) normal serum + u-PA (M_r 54,000 and 31,000); (3) plasmin (M_r 82,000); (4–9) endometrial culture medium obtained after 1–6 days incubation with estradiol (A) and progesterone (B); (10) t-PA (M_r 70,000); (11) u-PA (M_r 54,000 and 31,000).

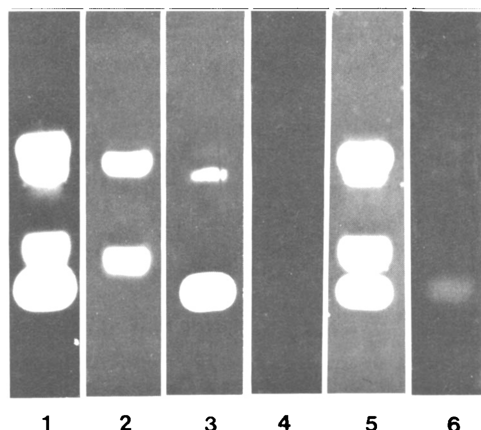

FIG. 3. The medium obtained after 6 days incubation of endometrial tissue with estradiol (Fig. 2A, lane 9) was used in additional experiments with SDS–PAGE, in which the fibrin agar substrate film had been supplemented in the following ways: (1) as in Fig. 2; (2) anti-u-PA IgG; (3) anti-t-PA IgG; (4) anti-u-PA IgG + anti-t-PA IgG; (5) control serum IgG; (6) plasminogen in the fibrin agar film destroyed by heating.

stromal cells. No relation could, however, be detected between histological appearance and biochemical results.

Discussion. This study demonstrates immunological identity and molecular weight conformity of two PAs released from endometrial tissue in culture with u-PA and t-PA. To detect PA activity in gels, we used a conventional fibrin substrate overlay method. To identify the PA in complexes we used a new technique in which specific antibodies to u-PA and t-PA were incorporated in the fibrin agar film to quench the activity of the PAs.

The results also demonstrate hormonal regulation of the PA release. Estradiol promotes the release of u-PA, but not of t-PA. In the presence of progesterone, the relase of both activators was markedly reduced. This endometrial response to hormonal influence accounts for previously observed variations in the PA activity of the endometrial secretion during the menstrual cycle (6, 13). A simlar hormone dependence of uterine PA activity has been demonstrated in the rat (14, 15).

Release of PAs seems to be a characteristic feature of cells during migration, and tissues undergoing proliferation and remodeling (16–22). During the follicular phase, estradiol promotes endometrial proliferation which is arrested after ovulation by the ovarian release of progesterone. We found PAs to be released in larger quantities under the influence of estradiol than under that of progesterone and, since u-PA predominated in this response, our results indicate that u-PA has a major role during endometrial proliferation.

The two PAs may be released from different cell types within the endometrium. Release of both PAs from the same cell type has been reported to occur from fibroblasts (21, 22) and endothelial cells (23–25), both of which are present in the endometrial stroma. The biological significance of the release of both u-PA and t-PA from a given tissue is not known. Differentiated functions of the PAs may, however, be related to their different affinities for fibrin.

SDS–PAGE of endometrial culture media revealed PA activity with a mobility similar to that of PA activity in serum, which has been reported to constitute t-PA in complex with an inhibitor (12, 26). We also found a high molecular weight band similar to that appearing when u-PA of mol wt 54,000 is added to serum. Thus, we conclude that endometrial tissue in culture releases a PA inhibitor similar in molecular weight to the inhibitor in serum. Published data on the latter inhibitor give a mol wt of 40–55,000 (12, 27). Figure 2, lanes 2 and 3, shows that u-PA of mol wt 31,000 added to serum results in a complex with a molecular weight close to that of plasmin (mol wt = 82,000 (28)). Although none of the molecules were studied after exposure to reducing agents, we conclude that when complexed, the inhibitors, serum as well as endometrial, have a mol wt of 50,000. The endometrial inhibitor thus forms complexes both with u-PA and t-PA, a quality being shared by the serum inhibitor and the placental inhibitor (29).

No PA inhibitory activity was detected in the conditioned media, indicating that no free PA inhibitor was present. However, u-PA may have been released in the media as a zymogen that was activated by trace amounts of plasmin in the fibrin plate assay (30). Such activation of latent u-PA in the assay could prevent detection of any free PA inhibitor by u-PA-inhibitor complex formation.

The amount of inhibitor released by endometrial tissue seems to be increased to some extent by the influence of estradiol. The amount of complexes is taken to be representative for the amount of inhibitor, since no free inhibitor activity was detected. It thus seems likely that the inhibitor is released in a parallel fashion to the PA. However, PAs were always in excess of the inhibitor, although the trace amounts of free PA, which were present in progesterone-treated media, usually remained undetected with the fibrin plate method. One biological function of the endometrial release of more activators than inhibitors seems to be the removal of fibrin deposits from the walls of the uterine cavity. This may be particularly important at midcycle, in the event of spermatozoa migrating through the uterine cavity. The most prominent release of PAs was evoked by continuous estradiol stimulation, a condition similar to that obtaining in the uterus during ovulation at midcycle, when the highest PA activity is to be observed *in vivo* (6).

Specific inhibitors of PAs have recently been reported to occur not only in plasma (12, 26, 27, 31) but also in endothelial cells (23, 24), thrombocytes (32), mononuclear phagocytic cells (16), fibroblasts (33), and the placenta (29, 34). This study demonstrated that endometrial tissue in culture also releases a PA inhibitor. The inhibitor may originate in endothelial cells, mononuclear phagocytic cells, or fibroblasts, since these cells are present in the endometrial stroma. At present, no method is available for investigating whether the endometrial inhibitor is identical with any of those described. It may even be that two different inhibitors with similar molecular weights, i.e., the endothelial and placental inhibitors, participate in the u-PA inhibitor and t-PA inhibitor complexes. The biological function of the endometrial inhibitor is not known, but its possible involvement during implantation is of special interest, since endometrial release at implantation of an inhibitor serving to balance the PA produced by the invasive trophoblast cells has been reported in the pig (35). The human endometrium has also PA inhibitory activity during early pregnancy (36). In pigs the inhibitor is also released from the normal endometrium under the influence of progesterone. Our results, however, did not indicate that progesterone promotes the release of endometrial PA inhibitor in humans. Endocrine regulation of PA inhibitor release has also been reported in fibroblasts (37) and rat hepatoma cells (38).

This investigation was supported by the Medical Faculty, University of Lund, and the Swedish Medical Research Council (00087 and B85-17X-04523-11B).

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Received March 22, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted February 3, 1986.