ROLE OF ENDOTHELIAL AND ENDOMETRIAL FIBROBLASTIC CELLS IN PLACENTAL FIBRINOLYSIS.
— HORMONAL REGULATION OF t-PA AND PAI PRODUCTION IN THE CELL CULTURE

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SUMMARY

Tissue type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) in the conditioned medium of endothelial cells and endometrial fibroblastic cells cultured in the presence of several hormones were assayed.

PA activity in the endometrial fibroblastic cells culture was enhanced by estrogen with increasing the t-PA production, and suppressed by promoting the PAI-1 synthesis stimulated by progesterone, estradiol+progesterone, testosterone, dexamethasone and indomethacin. On the other hand, no hormonal effect was observed on the t-PA and PAI-1 production by endothelial cells.

The fibrinolytic system for placental blood circulation in the intervillous space might be controlled by the PA and PAI production in endometrial fibroblastic cells rather than those in endothelial cells under hormonal regulation, and the balance of fibrinolysis and hemostasis in the intervillous spaces appeared to be important for placentation.

KEY WORDS: tissue type plasminogen activator, plasminogen activator inhibitor-1, endometrial fibroblastic cell, endothelial cell, cell culture

I. INTRODUCTION

The invasion of the endometrium by trophoblasts is required for placentation until a system to supply abundant maternal blood is established. During this process, it is important to keep the balance between fibrinolytic and inhibitory activity for the maintenance of blood flow in the villous spaces.

To investigate the control mechanism for homeostasis in the fibrinolytic system during placental formation, human endothelial cells (HUVEC) and
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endometrial fibroblastic cells (EF) were cultured in the presence of several hormonal stimulators, and plasminogen activator (PA), PA inhibitor (PAI) and PA activity in the conditioned medium were assayed. The morphology of the cell growth on fibrin substrate in the same medium was also checked.

II. MATERIAL AND METHODS
1. Endometrial fibroblastic cell (EF): The endometrial tissue was obtained from the operated uterus of benign tumorous disease. It was washed in Dulbecco's phosphate buffered saline free of magnesium and calcium pH7.2 (PBS (-)) to eliminated the blood and mucus. It was cut into small pieces, placed in 0.25% trypsin solution (Difco 1:250 in PBS (-)) and incubated for 20 minutes while stirring continuously. The cells were collected by centrifugation for 5 minutes at 800 rpm. The supernatant was removed and the pellet was suspended in Basal Medium Eagle Modified with Earle's Salts pH7.2 (BME: FLOW Lab. Co.) supplemented with 10% fetal calf serum (FCS: GIBCO) and 100 units/ml of penicillin and kanamycin, and transferred to a culture flask (25 cm² growth area, Corning) by the closed-stationary-monolayer method. In the primary culture, a few contaminated epithelial cells forming colonies were easily separated by discarding the trypsin solution after treatment for a short time before detachment of fibroblastic cells for the passage.

2. Human endothelial cell (HUVEC): Human umbilical cords were obtained under aseptic conditions at the time of vaginal delivery or Cesarean section. The umbilical vein was cannulated at both ends with blunt needles and held by clamping the cord over the needle with cord clamps and silk sutures. The vein was perfused with 100-150 ml of PBS (-) to wash out the blood. Then the vein was filled with 0.25% trypsin and 0.05% EDTA dissolved in PBS (-) until moderate dilatation occurred. The umbilical cord was incubated for 5 minutes at 37°C. The vein was flushed with 20ml of PBS (-) and the perfusate was collected in tubes containing 10% FCS. After centrifugation for 5 minutes at 800 rpm the pellet was resuspended in 10ml growth medium (M199: Medium 199, Nissui) supplemented with 20% FCS and 100 μg/ml CR-Endothelial Cell Growth Supplement (ECGS: Biomedical Products Division), and transferred to collagen coated dishes (Corning). The cultures were incubated at 37°C, 5% CO₂ in air for 7-10 days. The medium was changed twice weekly. It was then removed and replaced by PBS (-). After gentle titling, PBS (-) was removed and a few
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drops of 0.025% trypsin was added over the cell layer which was then incubated at 37°C for 2-3 minutes. The loose cells were resuspended in the fresh growth medium and transferred to new dishes for subculture.

The cells in the monolayer were confirmed to be HUVEC by immunofluorescent staining with an anti-factor VIII serum. HUVEC was used at the second passage for the experiments.

3. Preparation of the fibrin plate: 200μl of 1% fibrin monomer prepared from bovine fibrinogen according to the method described by Donelly11 was poured into the bottom of the 24 well microwell plate (Nunclon, Nunc) previously containing 2ml of 20mM Hepes-3mM CaCl₂-saline pH 7.2 (Hepes-Ca-saline). The fibrin plate was allowed to stand for 3 hours at 37°C. The Hepes-Ca-saline was removed and replaced by activated factor XIII (0.2 vial Fibrogamin: Hoechst Japan + 10IU thrombin: Mochida + 5ml Hepes-Ca-saline) to make the crosslinked fibrin plate. After incubation for 3 hours at 37°C, the fibrin plate was thoroughly washed with Hepes-Ca-Saline. These procedures were all done under aseptic conditions.

4. Reagents: Tranexamate, histamine and indomethacine were dissolved in the culture medium to make 3mM, 1μM and 10μM, respectively, and filtrated through 0.45μm filters. Steroids (estrone, estradiol, estriol, progesterone, estradiol + progesterone, testosterone and dexamethasone) were dissolved in a minimum volume of 50% ethanol and diluted with the culture medium to a final concentration of 0.1μM.

5. Cell culture: 2ml of cell suspension (4×10⁴ living cells) was planted on the plastic surface or on the fibrin plate in the various experimental conditions. The medium was BME supplemented with 10% FCS for EF and M199 supplemented with 20% FCS and 100μg/ml ECGS for HUVEC. 0.025cu/ml plasminogen (Plg) was added in the medium on the fibrin plate. For the assay of PA and PAI secretion, 300μl of cell suspension (4×10⁴ living cells) in the above medium was planted into the 96 well microtiter plate (Nunclon, Nunc) and incubated at 37°C 5% CO₂ in air at first. After 48 hours the medium was removed and the cultured cell layers were washed three times with the medium supplemented with 0.5% bovine serum albumin (BSA: SIGMA) instead of FCS. The cells were incubated with the medium supplemented with 0.25% BSA without FCS in various experimental conditions for 72 hours at 37°C 5% CO₂ in air.
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6. Cell counts in the culture on plastic plate: The sheeted cells in the bottom were dispersed by gentle pipetting with 200 μl of 0.25% trypsin. The single cells were counted in a hemocytometer (modified Neubauer type).

7. Cell counts in the culture on fibrin plate: The cells on the fibrin plate were washed with PBS (−) and replaced by 2 ml of 0.25% trypsin. The cell suspension was transferred into graduated tubes and centrifuged for 5 minutes at 800 rpm. The supernatant was drawn off to leave 0.5 ml on the pellet and 3.5 ml of crystal violet solution (0.1% crystal violet in 0.1M citric acid) was added. The tubes were agitated in a shaking water bath for 1 hour at 37°C and then centrifuged for 15 minutes at 1500 rpm to collect the nuclei. The supernatant was gently sucked off leaving only 0.5 ml of sediment. The suspension of nuclei was made homogenous by pipetting and the count of nuclei was taken as the cell count.
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8. PA and PAI assay: t-PA antigen and PAI-1 antigen in the conditioned medium cultured for 72 hours were determined by EIA (Tint Elize t-PA and Tint Elize PAI-1: Biopool). PA activity was determined by fibrin clot lysis time calibrated with several concentrations of reference sample of t-PA (Mitsubishi Kasei).

III. RESULTS
1. Cell growth on the plastic surface: 4 x 10^4 living cells were seeded on the plastic surface under the conditions shown in Figs. 1 and 2. The reagents used made no difference to the proliferation rate of both types of cells. EF was in the logarithmic proliferation phase during the period from 48 to 72 hours after plantation, and HUVEC from 24 to 48 hours.

2. Cell growth on the fibrin substrate:
   The growth curves of EF are shown in Fig. 3. In the case of the control (in the presence of Plg without reagents), EF began to aggregate from 48 hours and the cell count was 6.0 x 10^4 at 96 hours (Fig. 4). In the absence of Plg or in the presence of Plg with tranexamate or indomethacine, no aggregation was seen and the cells increased to 9.8 x 10^4, 10.2 x 10^4, 10.1 x 10^4 at 96 hours respectively (Fig. 5). Due to histamine fibrinolysis which began gradually after 72 hours incubation the cell count was 8.5 x 10^4 at 96 hours. The cells were well proliferated in the case of dexamethasone, progesterone, estradiol + progesterone and testosterone. Growth at 96 hours was 10.1 x 10^4, 9.2 x 10^4, 9.8 x 10^4, and 9.9 x 10^4 respectively (Fig. 6). Estradiol brought about cell aggregation at 48 hours with remarkable fibrinolysis and cessation of growth at 72 hours (Fig. 7).

Fig. 3 Effect of various reagents on the growth of EF cultured on the fibrin substrate.
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Fig. 4 EF on the fibrin substrate in the presence of Plg with no reagents. Cells were aggregated at 72hr (×200).

Fig. 5 EF proliferated on the fibrin in Plg free control at 120hr (×200). Good growth was observed.

Fig. 6 EF grown on the fibrin substrate in the presence of Plg with dexamethasone at 120hr (×200). Good growth was observed.

Fig. 7 EF cultured on the fibrin in the presence of Plg with estradiol at 72hr (×200). The cells were almost completely aggregated.

The cell count was $2.5 \times 10^4$ at this time. Fig. 8 shows the growth curve of HUVEC on the fibrin plate in various conditions. No fibrinolysis and no cell aggregation were observed in any case (Fig. 9A, 9B).

3. Quantitation of t-PA antigen, PAI-1 antigen and PA activity: Fig. 10 shows t-PA, PAI-1 antigen and PA activity in the conditioned medium from cultured EF. In the case of estrogen, t-PA antigen was found to be higher than that in the control and PA activity was enhanced. PAI-1 antigen was increased and PA activity was suppressed by dexamethasone, progesterone, estradiol+progesterone,
t-PA and PAI-1 production by cultured cells testosterone and indomethacin. Fig. 11 shows those in HUVEC. PAI-1 antigen in the conditioned medium of HUVEC were found to be at an extremely high level in any reagent, especially in dexamethasone, compared with the t-PA level. t-PA activity was therefore inhibited by excess production of PAI-1.

IV. DISCUSSION

The invasive penetration of trophoblasts into endometrium induces the building of a new form of maternal vessels for nutrient supply, since the intervillous space is a kind of vascular space organized by maternal components as well as embryonic cells. It was suggested that the blood flow through the intervillous spaces in the placenta was controlled by trophoblast-derived PA and PAI-1. The variation among the PA and PAI activities might be one of the critical factors in the success or failure of placentation.

Fig. 8 Effect of various reagents on growth of HUVEC cultured on the fibrin substrate.

Fig. 9 HUVEC cultured on the fibrin substrate (×100).
9A: at 48hr, 9B: at 120hr. Cells were well proliferated.
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Fig. 11 t-PA, PAI-1 antigen and PA activity in the conditioned medium of HUVEC.

Fig. 10 t-PA, PAI-1 antigen and PA activity in the conditioned medium of EF.
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The endometrium is a site of high fibrinolytic activity associated with the menstrual cycle and pregnancy. The localization detail of the fibrinolytic activity in the endometrium is not however well known, because no studies based on separated cells instead of whole tissue have been performed in human endometrial tissue. In this respect the fibroblastic cells derived from the endometrium were subcultured to investigate the production of t-PA and PAI-1 under several hormonal conditions. This in vitro study demonstrated that t-PA and PAI-1 were produced in EF and regulated by some hormonal effects in the following manner.

Estrogen enhanced PA activity by promoting the release of t-PA. Normal pig endometrium was reported to release PAI under the influence of progesterone. Cassi
cass reported that it was not necessarily an indication of the promotion of PAI release from human endometrial tissue. In our study, PAI synthesis was markedly increased and PA activity was suppressed in the presence of progesterone or estradiol+progesterone. PA activity in the endometrium might be lowered by the influence of an excess amount of inhibitor in the luteal phase and also during pregnancy, because progesterone is responsible for decidualization. In the case of other reagents (dexamethasone, testosterone and indomethacin), PA activity was suppressed by a high level of PAI-1 production. Dabich et al. reported that an excess amount of exogenous proteinase inhibitor administration in the uterus might impair implantation. Under such abnormal conditions it may be inconvenient to keep the level of the intervillous fibrinolytic system high enough to maintain the physiological blood flow. In this study the PA activities in the EF culture were reflected by the balance between the levels of PA and PAI-1, which could be altered by several hormones.

The intervillous space is organized by several embryonic and maternal components including endothelial cells. The vascular endothelial cells are regarded as playing an important role in fibrinolysis by synthesizing t-PA and PAI-1 regulated by several stimulations. In our study t-PA activity was inhibited by excess production of PAI-1 regardless of any hormonal stimulations. No hormonal effects on HUVEC were observed.

Ikeuchi suggested that the increased fibrinolytic activity of the villous tissue might be an etiology of abortion after the formation of the villous tissue. The villous space is composed trophoblasts, endothelial cells and endometrial fibroblastic cells. The balance of PA and PAI activities in the
t-PA and PAI-1 production by cultured cells embryo and uterine environment is necessary for implantation because placentation requires both lysis and stasis in its growth process. The fibrinolytic systems in the formation of the intervillous space and the maintenance of the blood flow should require fine control of t-PA and PAI-1 production by either EF or vascular endothelial cells as well as trophoblasts. However, hormonal regulation was observed in the fibroblastic cells rather than in the endothelial cells, though EF might not be directly exposed to blood flow.

Reference
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