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Plasminogen Activators Activities in Oviductal Epithelial Cells during Estrus Cycle in the Pig

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ABSTRACT

The present study was undertaken to identify changes of plasminogen activators (PAs) in porcine oviductal epithelial cells (POECs) during the estrous cycle classified with post-ovulatory stages (Post-Ov), early to mid-luteal stages (Early-mid L) and pre-ovulatory (Pre-Ov) stages. The urokinase-type plasminogen activator (uPA) was only observed on day 5 and day 7 of culture in the POECs on all the estrous cycles and gradually increased according to increasing culture times, but not Early-mid L. In POECs-conditioned medium, uPA, tissue-type (tPA) and tPA-PA inhibitor (tPA-PAI) activity were observed at all culture times during estrous cycles. The uPA activity of POECs-conditioned medium on Post-Ov stage were significantly (p<0.05) decreased during prolonged cultures. On the other hand, the tPA activity of POECs-conditioned medium at Post-Ov stage was significantly (p<0.05) higher on day 5 than compared to the other days. Although was higher on day 1 at Post-Ov stage, the tPA-PAI activity of POECs-conditioned medium was significantly (p<0.05) higher on day 7 at all stage than that of day 5 of the culture. Taken together, these results suggest that uPA, tPA and tPA-PAI are produced by POECs, and the variations of the PAs activity are regulated in the different stages of the estrous cycle.

(Key words : Plasminogen activators, Oviduct, Estrous cycle, Epithelial cells, Pig)

INTRODUCTION

Plasminogen, a ubiquitous zymogen, is abundant in blood plasma and in many other extracellular fluids, including, uterine fluid (Bruse *et al.*, 1998; Finlay *et al.*, 1983), ovarian follicular fluid (Beers 1975; Colgin *et al.*, 1997; Reinthaller *et al.*, 1990), and seminal plasma (Kobayashi *et al.*, 1992; Liu *et al.*, 1996). Plasminogen activators (PAs) are specific proteolytic enzymes which convert the inactive proenzyme plasminogen to the active protease plasmin. There are at least two types of plasminogen activators, tissue (tPA) and urokinase types (uPA) (Danø *et al.*, 1985; Rajput *et al.*, 1985). PAs/plasmin systems can degrade directly or indirectly, through the activator of metalloproteinse zymogens, which are all components of the extracellular matrix (Danø *et al.*, 1985; Robbins *et al.*, 1967; Smokovitis *et al.*, 1989). Several observations suggest that the PAs/plasmin system might also play a role in multiple phases of mammalian fertilization, including the acrosome reaction of capacitated sperm (Taitzoglou *et al.*, 2004; Taitzoglou *et al.*, 2003) and alteration of zona pellucida (Cannon *et al.*, 1998). Although these PAs are believed to be important in extracellular tissue remodeling in many physiological processes, including fibrinolysis, ovulation, mammary involution, implantation (Danø *et al.*, 1985), fertilization (Huarte *et al.*, 1993), and pathological process, including cardiovascular diseases and tumor metastasis (Schmitt *et al.*, 1997), the unrestrained generation of plasmin from plasminogen by the action of PAs is potentially hazardous to cells (Irigoyen *et al.*, 1999).

The porcine oviduct provides an important microenvironment for final maturation of gametes, fertilization, and early cleavage-stage embryonic development. In part to provide an effective environment for these reproductive processes, numerous proteins derived from

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serum as a transudation or from oviductal epithelium contribute to the composition of oviductal luminal fluid (Buhi *et al.*, 1997; Feigelson *et al.*, 1972; Sutton *et al.*, 1984). Specially, the epithelial cell of the porcine oviduct provides a suitable microenvironment for maturation of gametes, successful fertilization and early development of the zygote. In the previous researcher reported that the expression of PA isozymes by human mammary epithelial cells is subject to modulation by the extracellular matrix (Yang *et al.*, 1983). PAs activity has been demonstrated in embryos, oocytes and sperm from rat, pig and bovine, but reports of PAs in uterus and oviduct are limited.

Therefore, the present study was carried out 1) to identify the PAs activity in oviduct epithelial cells during the estrous cycle and 2) to examine the changes of the PAs activity by culture period-dependent oviduct epithelial cells during the estrous cycle in pigs.

MATERIALS AND METHODS

Preparation of Porcine Oviduct by Estrous Cycle

Porcine oviducts during the estrous cycle were collected at a local slaughterhouse and kept in 0.85% saline at 4℃. The stage of the estrous cycle was defined by careful examination of the ovaries (follicles and corpora lutea). The diameter of follicles and corpora lutea was measured with calipers, with accuracy to the nearest 0.1 mm. The criteria used for the classification of the oviducts (Foxcroft *et al.*, 1985; Hunter *et al.*, 1975; Ricke *et al.*, 1999) into three groups were the following (Fig. 1.): (1) Post-ovulatory stage(days 1 ~2): presence of corpus hemorrhagicum, absence of $medium_{1,2}$ and $large_{1,2}$ follicles (m_1: 3.0~4.9 mm and m_2: 5.0~6.9 mm, and l_1: 7.0~8.9 mm, l_2:> 9 mm in diameter, respectively). (2) Early to mid-luteal stage (days 3~12): presence of corpus luteum, absence of $medium_{1,2}$ and $large_{1,2}$ follicles (m_1: 3.0~4.9 mm and m_2: 5.0~6.9 mm, and l_1: 7.0~8.9 mm, l_2:> 9 mm in diameter, respectively). (3) Pre-ovulatory stage (days 17~20): regressed corpus luteum, presence of $medium_{1,2}$ and $large_{1,2}$ follicles (m_1: 3.0~4.9 mm and m_2: 5.0~6.9 mm, and l_1: 7.0~8.9 mm, l_2:> 9 mm in diameter, respectively).

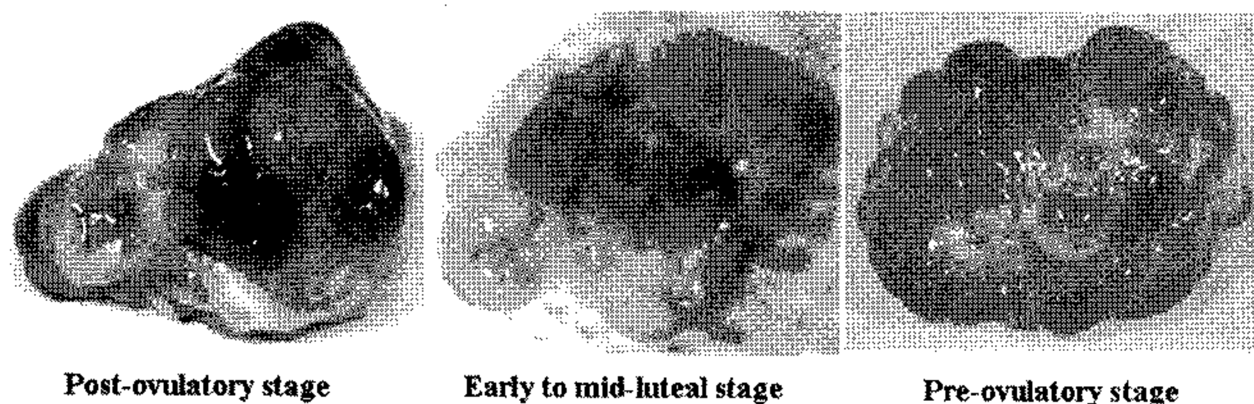

Fig. 1. The classification of the porcince ovary by estrous cycles. The estrous cycles were distinguished by diameter of follicles and corpora lutea of ovary. The POECs were isolated from porcine oviduct of ovary classified the by estrous cycles, and cultured in culture medium.

In Vitro Culture of Porcine Oviduct Epithelial Cells

Porcine oviduct epithelial cells (POECs) were separated using a modification of a procedure previously described (Wijayagunawardane *et al.*, 1996; Wijayagunawardane *et al.*, 1998; Zhang *et al.*, 1991). Basically, the surrounding connective tissues were trimmed from the oviduct and washed with Hanks' balanced salt solution (HBSS). The oviductal lumen was flushed with 5 ml HBSS, and the POECs were dislodged by scraping the oviduct with a sterile glass slide while flushing the lumen with an additional 15 ml HBSS. The collected POECs were washed once with HBSS by centrifuging at 1,500 rpm for 10 min at 4℃. Then the cell pellet was washed once with Tris-NH_4 buffer and then washed twice with Dulbecco's Modified Eagles Medium Hepes Modification (DMEM; Sigma) by centrifuging at 1,500 rpm for 10 min at 4℃. The POECs were resuspended with Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM/Ham's F-12 ; Sigma) supplemented with 10 % BSA, 2 μg/ml amphotericin B (Sigma) plated in 4-well culture dishes at a density of 1×10^6 cell/ml and cultured under the conditions of a humidified atmosphere of 5 % CO_2 in air at 37.5℃. The culture media were changed every 48 h with fresh medium during the culture for 7 days. After culture, the POECs-Condiationed medium was separated into microtubes. Each microtube was centrifuged at 300 ×g for 10 min. The POECs-Condiationed medium were supplemented into microtubes containing sample buffer without β-macaptoethanol and conserved at −20℃ until they were used for zymographic analysis.

Zymographic Assay

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and zymography were performed using procedures described by Dyk and Menino (1991) with a slight modification. Frozen samples were thawed and homogenized with a sonicator. As a standard of tPA, stock solution of 0.5 ng/ml tPA from human melanoma cell culture (Sigma) was prepared in a sample buffer. Each homogenized sample (20 μl) being compared in each experiment, a stock solution of human tPA (10 μl), and molecular mass markers (10 μl; Bio-Rad Lab., Hercules, CA) were placed in a castellated well in a 4.5% stacking gel with a 12.5% separating gel. Electrophoresis was conducted at 20 mA for 2 h. After electrophoresis, the gels were gently shaken in 2.5 % Triton X-100 for 45 min at room temperature to remove the sodium dodecyl sulfate and

incubated for 30 min at 38.5℃ in phosphate-buffered saline (PBS). Each gel was carefully laid on a casein-agar gel (zymogram) containing purified human plasminogen supported in a plastic chamber. For preparing zymograms, 0.4 g of nonfat dry milk was dissolved in 10 ml of buffer containing 0.0013 M $CaCl_2 \cdot 2H_2O$, 0.1 M glycine, 0.038 M Tris, and 0.005 M sodium azide. The nonfat dry milk mixtures were heated to 55℃ and combined with 9.9 ml of 1% (v/v) melted agarose maintained at 55℃. Purified human plasminogen stock solution (100 μl) was added to 19.9 ml of the warmed mixture to yield a final plasminogen concentration of 50 μg/ml and 10 ml of this mixture was cast into a warmed dish and allowed to cool. Polyacrylamide gels and zymograms were incubated at 38.5 ℃ for 24~48 h to allow the development of lytic bands. After the distance from the edge of the separating gel to the center of the clear lytic bands in each lane was measured, incubation of the zymograms was terminated by separating the gels. All experiments measuring PA activity were repeated at least three times. Proteolytic enzymes patterns were analyzed by Multi Gauge V 3.0 (FUJIFILM).

Statistical Analysis

Data were analyzed by GLM using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). When F-test results were significant in GLM, individual data were further tested by Duncan's multiple-range test. Differences with values of $p<0.05$ were considered to be statistically significant.

RESULTS

Porcine oviduct epithelial cells (POECs) at different estrous cycle stages were cultured in culture medium for 7 days, and each cell during the culture period analyzed the PAs activity by zymography assay. The results revealed that the uPA was observed on days 5 and 7 in POECs during the estrous cycle stage (Fig. 2 (A)). However, both tPA and tPA-PAI activity were not observed in POECs during the estrous cycle stage. uPA activities in POECs significantly ($p<0.05$) increased from days 5 to 7 at the Post-Ov stage, but uPA activities in POECs significantly ($p<0.05$) decreased from days 5 to 7 at Early to mid-L (Fig. 2 (B)).

On the other hand, uPA, tPA, and tPA-PAI activity were observed at days 1, 3, 5 and 7 of culture in the POECs-conditioned medium of different stages of the estrous cycle by zymography assay (Fig. 3 (A)). uPA activity of the POECs-conditioned medium at the Post-Ov stage was gradually decreased during the prolonged culture and was significantly ($p<0.05$) higher on

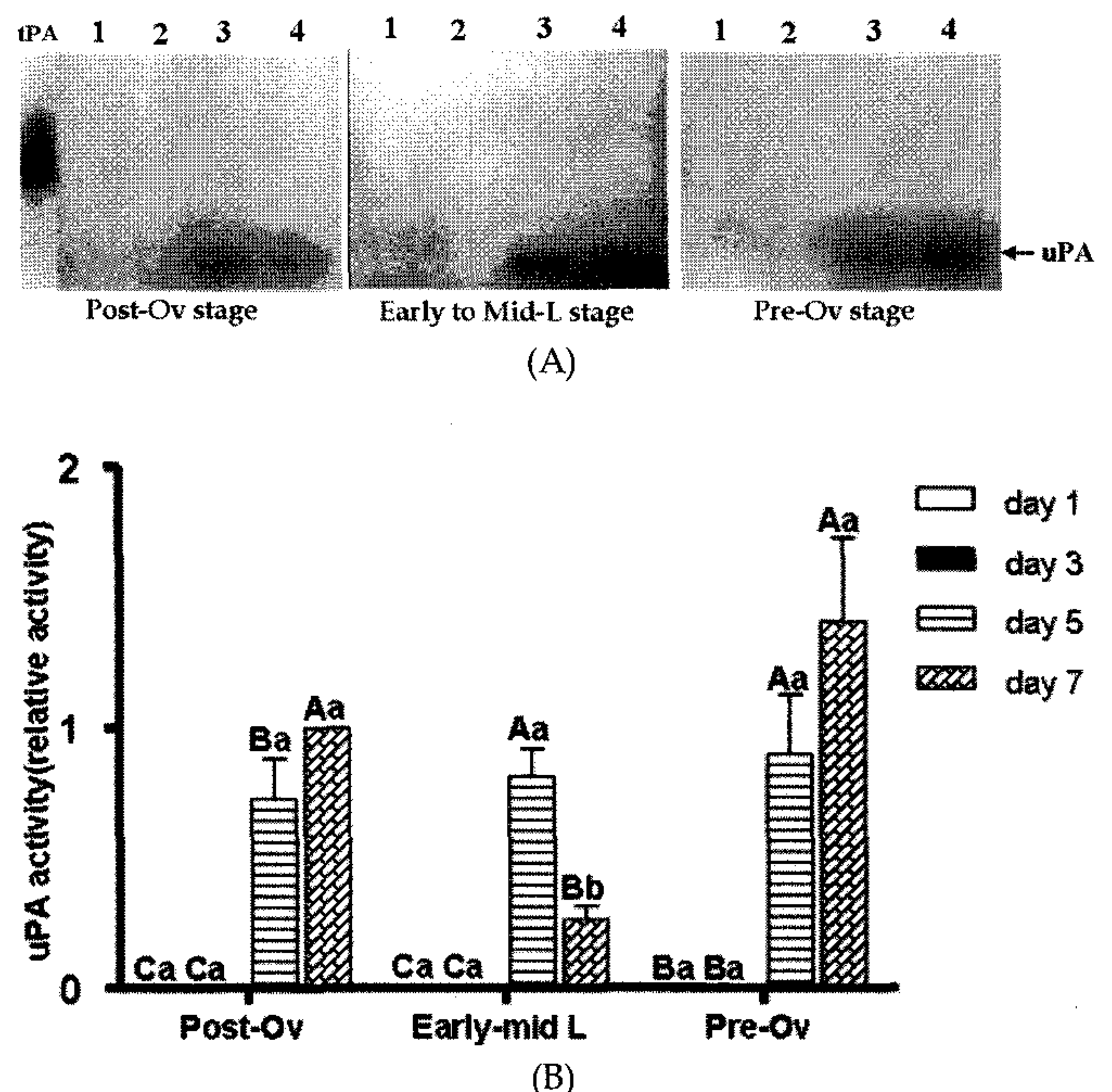

Fig. 2. Culture period-dependent changes of PAs activity by zymography in POECs at different stages of the estrous cycle. A) Substrate gel zymography for the detection of Pas by days of POECs culture at different stages of the estrous cycles. Lane 1, 2, 3, and 4 were day 1, 3, 5, and 7 of POECs culture, respectively. B) The relative band intensities of the PAs activity to PAs activity of day 7 of culture were analyzed densitometrically. Different letters indicate significant differences ($p<0.05$) during culture period of POECs at different stages of the estrous cycle (A, B, and C) and ammong Post-Ov, Early to Mid-L and Pre-Ov (a, b, and c).

day 1 than those of days 3, 5 and 7 of culture (Fig. 3 (B)). However, there was no significant difference on uPA activity in POECs-conditioned medium at Early-mid L and Pre-Ov stage during the culture period.

Culture period-dependent changes of tPA activity in the POECs-conditioned medium of different stages of the estrous cycle also showed (Fig. 3 (A)). tPA activity was significantly ($p<0.05$) higher in the POECs-conditioned medium of Post-Ov stage on days 5 and 7 than that of days 1 and 3 of culture (Fig. 3 (C)). tPA activities increased until day 5 of culture at all estrous stages, and it subsequently decreased on day 7 of culture in the POECs-conditioned medium.

Furthermore, the culture period-dependent changes of tPA-PAI activity in the POECs-conditioned medium of different stages of the estrous cycle was shown (Fig. 3 (D)). tPA-PAI activity was significantly ($p<0.05$) higher on day 7 than that of day 5 of culture in POECs-conditioned medium at Pre-Ov stage and Early-mid L. And tPA-PAI activity was significantly ($p<0.05$) higher in the POECs-conditioned medium on days 1, 3 and 7 of culture at Post-Ov stage than those of Pre-Ov and Early-Mid L. In addition, tPA-PAI activity was not detected in the POECs-conditioned medium on day 1 and 3 of culture at Early-Mid L and Pre-Ov.

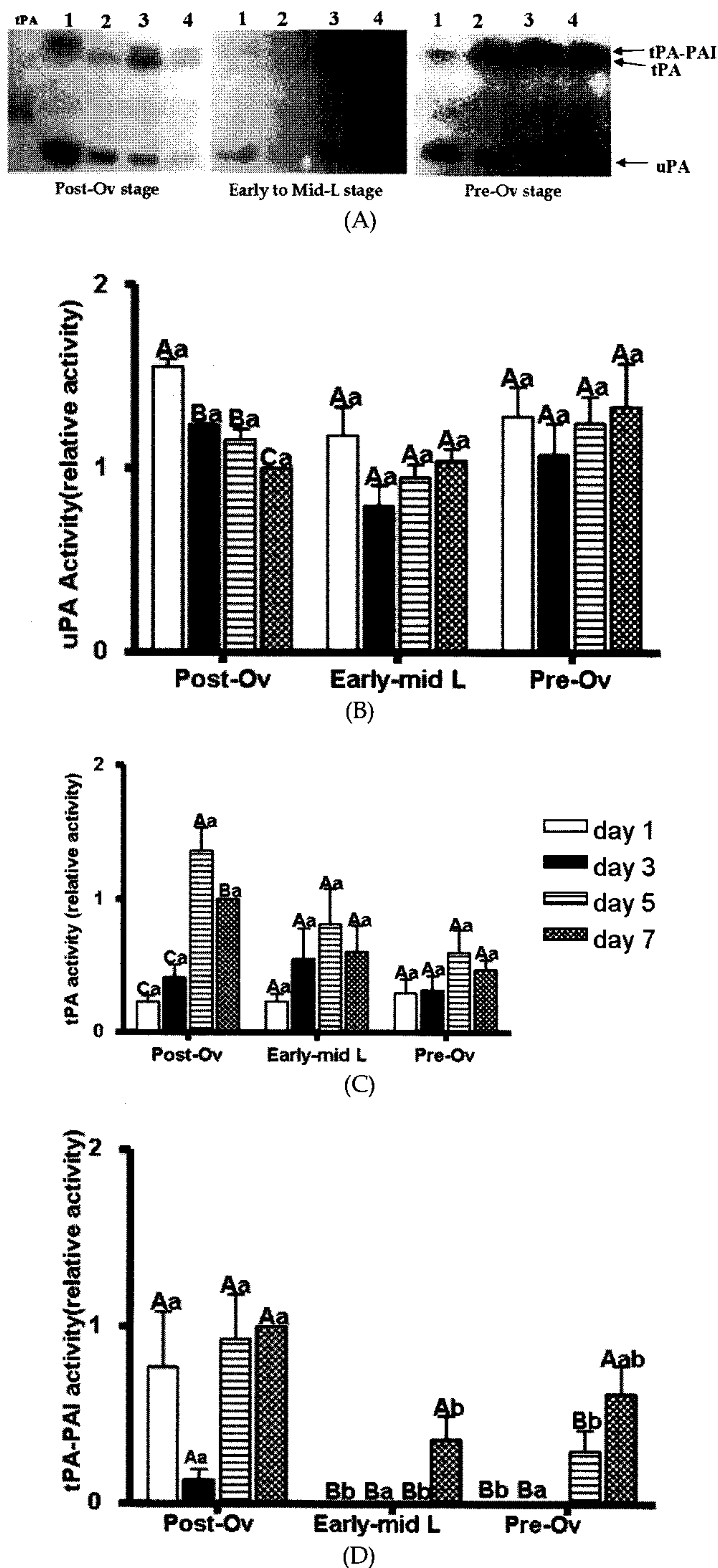

Fig. 3. Culture period-dependent changes of PAs activity by zymography in POECs-condition medium at different stages of the estrous cycle. (A) Substrate gel zymography for the detection of Pas by days of POECs-condition medium at different stages of the estrous cycles. Lane 1, 2, 3, and 4 were day 1, 3, 5, and 7 of POECs-condition medium, respectively. (B, C and D) The relative band intensities of the PAs activity to PAs activity of day 7 of culture were analyzed densitometrically. Different letters indicate significant differences ($p<0.05$) during culture period of POECs-condition medium at different stages of the estrous cycle (A, B, and C) and ammong Post-Ov, Early to Mid-L and Pre-Ov (a, b, and c).

DISCUSSION

The mammalian oviduct secretes various proteins into the oviductal lumen that influence gamete interaction, fertilization process and early cleavage stages of embryonic development. Varying proteins in mammalian oviducts are produced owing to oviductal region and stages of estrous cycle. PAs affect physiological and physiopathological processes in epithelium lining the oviduct. Therefore, the present study was undertaken to identify the PAs activity in POECs during the estrous cycle and to examine the changes of the PAs activities by culture period-dependent POECs during the estrous cycle.

Hart and Rehemtulla (1988) reported that uPA is secreted as an inactive single-chain molecule of 31~54 kDa and tPA is secreted in an active form with a molecular weight of around 70 kDa. Both uPA and tPA can form SDS-stable complexes with PAI, a family of specific inhibitors for PA, yielding PA-inhibitor complexes detectable by zymography (Hart *et al.*, 1988; Rehemtulla *et al.*, 1990). tPA and PAI form complex with a molecular weight of approximate 110 kDa (Kruithof *et al.*, 1984; Thorsen *et al.*, 1984; Wagner *et al.*, 1986). This study found three plasminogen-depententent proteases in the POECs-conditioned medium on different stages of the estrous cycle (Fig. 3 (A)). The resistance to amiloride treatment in zymographic analysis and molecular mass suggested that the 58.5±3.5 kDa, 79.0±3.0 kDa, and 113.5±6.5 kDa species that were detected in the POECs-conditioned medium are the uPA, tPA, and tPA-PAI complex, respectively. Although both tPA and tPA-PAI activity were not detected, uPA activity was detected on days 5 and 7 in the POECs during culture (Fig. 2). uPA activity was observed in the POECs-conditioned medium throughout the estrous cycle. Specially, uPA activity was significantly higher in the POECs-conditioned medium of Post-Ov stages (Fig. 3 (B)). Although the participation of u-PA in the mechanism of fertilization or early embryo development has not been studied yet, it is possible that u-PA may be involved in these processes in a specific and finely regulated proteolytic cascade. It has been reported that u-PA released into the bovine oviductal lumen occurs in an estrous-cycle-dependent manner and that oviductal epithelial cells may contain the u-PA receptor to bind u-PA to the cell surface (Gabler *et al.*, 2001).

tPA activities in all estrus cycles were increased until the day 5 and were decreased at day 7 of culture in the POECs-conditioned medium (Fig. 3 (C)). tPA-PAI activity was observed in all estrus stages, and was significantly higher during the culture of the POECs-conditioned medium of Post-Ov compared to those of the other estrus stages (Fig. 3 (D)). These data

may indicate that PAs in oviductal lumen were controlled by female hormones, which is similar to previous report that oviductal epithelium, protein composition of oviductal fluids, and oviductal fluid volume vary according to the hormonal status of the female and appear to be controlled by ovarian estrogen and progesterone (Lippes *et al.*, 1993; Mastroianni L *et al.*, 1979; Verhage HG *et al.*, 1988).

Extracellular proteolysis, linked with PAs / plasmin system activity, is believed to be involved in physiological processes such as cumulus cell expansion and dispersion (Canipari *et al.*, 1995), fertilization (Rekkas *et al.*, 1993; Rekkas *et al.*, 2000), zona reaction (Lee *et al.*, 1994) and embryo implantation (Bray *et al.*, 1975). It has been suggested that fertilization *in vivo* occurs in an environment relatively rich in plasminogen. Plasminogen is abundant in blood plasma and in most extracellular fluids including seminal plasma(Kobayashi *et al.*, 1992; Liu *et al.*, 1996), uterine fluid (Bruse *et al.*, 1998; Finlay *et al.*, 1983), and ovarian follicular fluid (Colgin *et al.*, 1997; Reinthaller *et al.*, 1990). And plasminogen present on the surface of different cells and several proteins found in the extracellular matrix have the capacity to modulate the expression of plasminogen binding sites, a mechanism through which they can regulate local proteolysis and cell migration (Vassalli *et al.*, 1991). Although it has been suggested that the PA activity in the oviductal milieu might play a role in maintaining the fluidity of the medium and facilitate gamete encounters (Vassalli *et al.*, 1991), the involvement of these types of proteases during fertilization or development of the early embryo is not yet clear. The present work demonstrates that activity of plasminogen activators in the porcine oviduct, which agrees with previous studies that reported the presence of components of the plasminogen activation system in hamster and bovine oviduct (Gabler *et al.*, 2001; Jimenez Diaz *et al.*, 2000). A previous study demonstrated the presence of the PA substrate plasminogen, in plasma membrane of porcine oocyte (Roldan-Olarte *et al.*, 2005). The most obvious consequence of oocyte pericellular plasminogen could be the localization of plasmin generation on the oocyte surface and the ECM by means of the oviductal PAs. Plasmin plays a central role in the regulation of the degradation remodeling of the ECM of several cell types. The function of oviductal PAs would be to contribute to the rebuilding of either oocyte or embryo ECM.

The synthesis and release of plasminogen activators and plasminogen activator inhibitors are modulated in several cell types by hormone(Kokolis 1990; Schatz *et al.*, 1993; Tsantarliotou 1997). Reports on PAI-1 regulation in endometrial stromal and decidual cell cultures suggest that this protein is up-regulated by progesterone, while estrogen antagonizes this effect (Kokolis 1990). The oviduct, along with the uterus, is a major target for ovarian steroids. Plasminogen, the natural substrate for u-PA and t-PA, occurs in many extracellular fluids and may be enriched in oviductal fluid at or near the time of fertilization. Estrogens have been shown to stimulate the uptake of plasminogen from plasma by the mouse uterus (Finlay *et al.*, 1983) and a similar function may occur in the oviduct during estrus. Proteases, such as plasmin are present in spermatozoa and genital tract secretions and are thought to be involved in sperm maturation, capacitation and/or acrosome reaction as well as in post-fertilization event (Taitzoglou *et al.*, 2003). However, the enzymatic mechanisms responsible for these events are still not clearly understood.

Taken together, these results suggest that the uPA, tPA and tPA-PAI was produced by POECs, and the variations of the PAs activity was regulated by different stages of the estrous cycle. This requires further study to understand the molecular mechanism of development and cell block of the embryos.

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