[Frontiers in Bioscience 9, 3356-3373, September 1, 2004]

PLASMINOGEN ACTIVATOR / PLASMINOGEN ACTIVATOR INHIBITORS IN OVARIAN PHYSIOLOGY

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1. ABSTRACT

The target extracellular matrix (ECM) degradation generated by plasminogen activator (PA) and regulated by plasminogen activator inhibitor (PAI) is an event that affects a wide variety of physiological and pathological processes in the ovary. Studies carried out over the past 25 years in a number of laboratories have elucidated some of the biochemical events related to the function and regulation of the PA system in the ovary. Hormone-induced coordinated expression of tissue-type PA (tPA) produced mainly by granulosa cells and its inhibitor PAI-1 secreted by theca cells in the preovulatory follicles is responsible for a controlled and directed proteolysis leading to the rupture of selected follicles in the rat, monkey and other mammals. Increase in tPA and PAI-1 expression in corpus luteum (CL) of rat and monkey at a later stage is well correlated with a sharp decrease in CL progesterone production, indicating its important role in the initiation of luteal regression. In contrast, the urokinase-type PA (uPA) may play an essential role in the early growing follicles during cell proliferation and migration, and in the early CL formation related to ECM degradation and angiogenesis. Ovarian function is also modulated by endogenously-produced local factors that regulate expression of the PA activator and inhibitor, and the MMP system. Thus, the next challenge is to identify the interrelationship between multiple paracrine and autocrine factors and the PA system, and to know how they regulate the protease and the protease inhibitor in the ovary.

2. INTRODUCTION

There are two major classes of proteases that have been implicated in ECM degradation: the plasminogen activator (PA) system and the zinc-ion-containing matrix metalloproteinases (MMPs) (1-3). Proteolytic activity generated by the PA system has been associated with many physiological and pathological processes that involve remodeling of the extracellular matrix, such as angiogenesis, wound healing, inflammation, embryo implantation, tumor invasion and metastasis, and ovulation.

The PA system is a versatile, temporally controlled enzymatic system where plasminogen is activated to the proteolytic enzyme plasmin by either of the two physiological PAs, the tissue type PA (tPA) or the urokinase PA (uPA). The PA system, a multi-component,

contains not only the proteolytic enzymes, but also the regulatory components including inhibitors, cofactors, cell surface receptors and binding proteins. The proteases of the PA system are characterized by their reactive pocket which contains the catalytic triad of histidine (His), asparagine (Asp) and serine (Ser) residues (4). The primary enzyme of the PA system is plasmin, a protease with a broad trypsin-like activity. The precursor of plasmin, plasminogen, is a single-chain glycoprotein containing 790 amino acids with a molecular weight of approximately 92 kda. Native plasminogen is referred to as Glu-plasminogen since it has a glutamic acid at its aminoterminus (N-terminus). The Glu-plasminogen is cleaved by plasmin at Lys76-Lys77 to produce Lys-plasminogen (5). Both the Glu- and the Lys-plasminogen are activated to plasmin by a single PA-catalyzed cleavage at the Arg560-Val561 bond, which results in a two-chain protein held by two disulfide bonds (6). Plasmin catalyses the hydrolysis of argentine-x and lysine-x bonds in fibrin. In addition, there are several other proteins of ECM that are degraded by plasmin, including gelatin, fibrinogen, type IV collagen, fibrinectin, laminin, elastin and proteoglycans (7-9).

The PA system in cells is regulated by hormones, growth factors, and cytokines at various levels of its synthesis and secretion (1, 10), and modulated by specific PA inhibitors, which are released in cells in response to stimulatory signals (1, 11, 12). At least four immunologically distinct molecules with PA inhibitor activity have been identified. Kinetic analysis suggests that PA inhibitor type-1 (PAI-1) and type-2 (PAI-2) are the only physiologically relevant PA inhibitors (13, 14). PAI-1 and PAI-2 are the serine protease inhibitor superfamily (serpins). Regulation of PA and PA inhibitors occurs at several levels, including the rate of synthesis and the complex interactions between the activator and inhibitor. Many other components are also involved in determining the specificity of the proteolysis carried out by the PA system.

In the ovary, a controlled and targeted extracellular proteolytic activity mainly by the PA system is required in follicular development, differentiation and angiogenesis. Ovary produces and releases mature oocytes with subsequent CL formation. These processes are repeated during each reproductive cycle and involve a series of sequential steps including cell proliferation, differentiation or apoptosis, oocyte maturation, detachment of cumulus-oocyte complexes, ovulation, and subsequent CL formation and regression. In each of these diverse physiological processes, extracellular proteolysis must be conducted with a great specificity in order to maintain the integrity of the ovary. Accommodation of such structural changes in the ovary demands flexibility of surrounding ovarian matrix and stroma tissues. This flexibility mainly depends on degradation of matrix substances by the extracellular proteolytic system. Such specificity is achieved by a strict regulation of the biosynthesis of the molecules and complex interactions involving the catalytic enzymes, zymogens, specific inhibitors as well as receptors and binding protein components which serve to localize the proteolytic activity for the overall control of the PA system (10). The precise multi-level regulation seems to fine-tune the expression of the PA system and provides a controlled proteolytic activity (15-18).

3. TISSUE TYPE PLASMINOGEN ACTIVATOR (tPA): GENE EXPRESSION AND REGULATION IN GRANULOSA CELLS

3.1. Hormonal regulation of tPA gene expression

GCs are well-responsive cell types that contain receptors for various hormones and growth factors. Rat GCs express tPA in response to treatments with FSH, LH (15, 19-21), GnRH (19), VIP (22, 23) and other compounds (24). tPA is also induced by various growth factors (25-28). Different hormones and growth factors induce tPA mRNA in GCs in a transient fashion and in a time-dependent manner (29, 30), implying that the expression of tPA may be mediated via different intracellular pathways. FSH induces the most rapid response, resulting in maximal levels of tPA mRNA after just 3-6 h (29), in accordance with the known stimulatory effect FSH on intracellular cAMP levels in the GCs (30). tPA mRNA levels were enhanced in the presence of the phosphodiesterase inhibitor, MIX, suggesting that FSH activates the tPA gene through the cAMP-dependent protein kinase A pathway, leading to phosphorylation of the cAMP-responsive element binding protein (CREB) and activation of the promoter containing a cAMP-responsive element (CRE) (30). GnRH is known to stimulate phosphatidylinositol metabolism in GCs (31), and is likely to stimulate tPA mRNA levels through the protein kinase C pathway. Further studies have shown that growth factors, EGF and TGF-alpha induce tPA mRNA and activity in GCs through a pathway independent of protein kinase A and C. Co-treatment of a saturating dose of EGF with FSH or GnRH resulted in an additive increase in both tPA enzyme activity and mRNA levels (28). The induction of tPA mRNA by GnRH and EGF was blocked in the presence of protein synthesis inhibitor, cycloheximide, suggesting that protein synthesis is required for the induction of tPA mRNA in GCs by these hormones (21, 28). Surprisingly, when GCs were treated with FSH in the presence of cycloheximide, a superinduction of tPA mRNA was observed (21). This effect may be due to either an increased rate of transcription of the tPA gene or an increase in the stability of the produced tPA messenger, prolonging the transcription of tPA gene induced by FSH.

3.2. Rat tPA gene

The first 621 nucleotides of the 5'-flanking region of the rat tPA gene was found to be sufficient to confer both the basal and the FSH-induced promoter activity to a reporter gene construct when transfected into the primary cultures of GCs (32-34). The rat tPA promoter contains a consent as CRE (TGACGTCA) at position -178 to -185, a nuclar factor 1 (NF 1), SP1 binding sites, and a GC box binding factor. All of these factors seem to play a role in constitutive expression as well as cAMP activation of the tPA gene. Preliminary results have shown that the regulatory elements for GnRH response are located between positions -621 and 2,300 bp of the 5'-flanking sequence (33). These data suggest that FSH and GnRH induce tPA gene activation by different intracellular mechanisms that do not converge at the gene level (32-34).

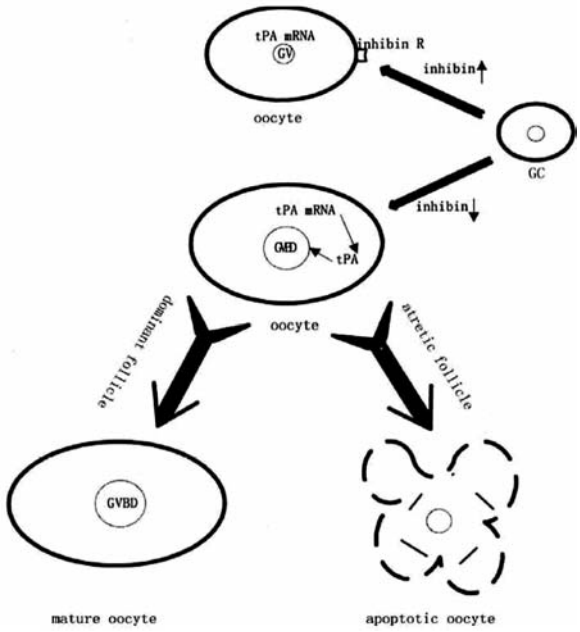

Figure 1. Effect of inhibin emanated from the GC on tPA mRNA translation in the oocyte. Inhibin originated from the GCs inhibits the oocyte maturation by inhibiting tPA mRNA translation in the oocyte. Once expression of inhibin in the GCs decreases, the oocyte tPA mRNA starts to translate into its active protein, the subsequently increased tPA activity induces the oocyte GVBD in the dominant follicle leading to the oocyte maturation and ovulation; On the other hand, decreases in the GC inhibin expression in the developing follicle, the oocyte tPA mRNA would be triggered to translate tPA protein activity and induce its certain morphological changes similar to GVBD in the unmatured follicle, subsequently leading to the oocyte and / or the follicle apoptosis (42).

4. TISSUE PLASMINOGEN ACTIVATOR IN OOCYTE AND ITS REGULATION

4.1. Oocyte tPA activity is controlled by inhibin produced in GC

Denuded oocytes collected from ovaries of hypophysectomized estrogen-treated immature rats contained only tPA. Cellular localization of tPA was observed in the cytoplasm of the oocyte, but not in the germinal vesicle or zona pellucida (35). Further studies confirm that the haploid oocytes from rat, mouse, rhesus monkey and human possess an ability to synthesize tPA which is regulated by gonadotropins and other hormones (35-40).

Rat or mouse oocytes express measurable amount of tPA activity (35, 36, 40); when they underwent spontaneous meiotic maturation in the culture, the oocyte tPA activity increased progressively and reached a plateau level at the time of germinal vesicle break down (GVBD). Addition of GVBD inhibitor to the culture prevented the increase in oocyte tPA activity, indicating that GVBD is required for tPA accumulation (36). Further evidence in a well-controlled in vivo study indicated that only tPA mRNA, but not its protein activity was detected in the rat primary oocytes of developing follicles. However, the oocyte tPA activity dramatically expressed in the follicles undergoing atresia (41). Production of the inhibin subunits in GCs was demonstrated to be negatively correlated with the expression of the oocyte tPA protein activity. The oocyte in a follicle does not express tPA protein activity if its surrounding granulosa cells express normal inhibin subunits. In contrast, the follicle lacking inhibin expression in the GCs contains high levels of tPA protein stain in its oocyte. Therefore, we suggested that a relationship between the inhibin expression in GC and tPA mRNA translation in the oocyte is present, and may play an essential regulatory role in deciding the fate of the follicular development (41). We propose that inhibin may be constantly expressed in the GCs during the normal follicular development, and translation of the oocyte tPA mRNA might be constantly suppressed until the follicle approaches to the ovulatory stage, when the inhibin expression in GCs may sharply decrease and the oocyte tPA mRNAs start to translate into its protein, and the subsequently increased tPA activity might be responsible for the oocyte maturation and the cumulus cell expansion / dispersion which are required for the morphological changes of ovulation (42). In the developmental follicles, on the other hand, the inhibin emanated from GC inhibits its oocyte maturation by inhibiting oocyte tPA mRNA translation. Once inhibin production in GC decreases, the increased tPA activity in the oocyte may induce certain morphological changes similar to GVBD leading to the oocyte apoptosis (Figure 1). This hypothesis is supported by the fact that the rat oocytes in atretic follicles contain high level of tPA activity (41).

4.2. Hormonal regulation of oocyte tPA activity

Plasminogen activator activity was analyzed in the cumulus-oocyte complexes. Only tPA activity was detected in the freshly obtained cumulus-oocyte complexes (37, 43). FSH, GnRH and vasoactive intestinal peptide (VIP) are capable of stimulating tPA activity in the cumulus-oocyte complexes, but not in the denuded oocytes (19, 22, 35), suggesting that the oocyte tPA activity was regulated by the hormones via cumulus cells. In vivo experiments indicated that tPA activity in the oocytes was dramatically elevated immediately before ovulation, and well correlated with the morphological changes of cumulus expansion and oocyte maturation (37). The cumulus-oocyte complexes from the follicles in the rat before and after PMSG treatment contained low amount of tPA, but no uPA activity. After hCG treatment tPA activity showed a time-dependent increase, reaching a maximum at 24 h after hCG injection. Morphological analysis indicated that the increase in oocyte tPA activity was correlated with the extent of cumulus cell expansion and dispersion (Figure 2). The appearance of the high molecular weight lysis zones was also detected, suggesting the formation of tPA-PAI-1 complexes during interaction of the oocyte and its surrounding cumulus cells. PAI-1 activity was detected only in the cumulus cells, not in the denuted oocyte (37).

To examine hormonal responsiveness, the cumulus-oocyte complexes obtained from the follicles of the PMSG-treated rats were incubated with FSH and hCG for 24 h, the total (medium plus cell lysate) tPA activity in

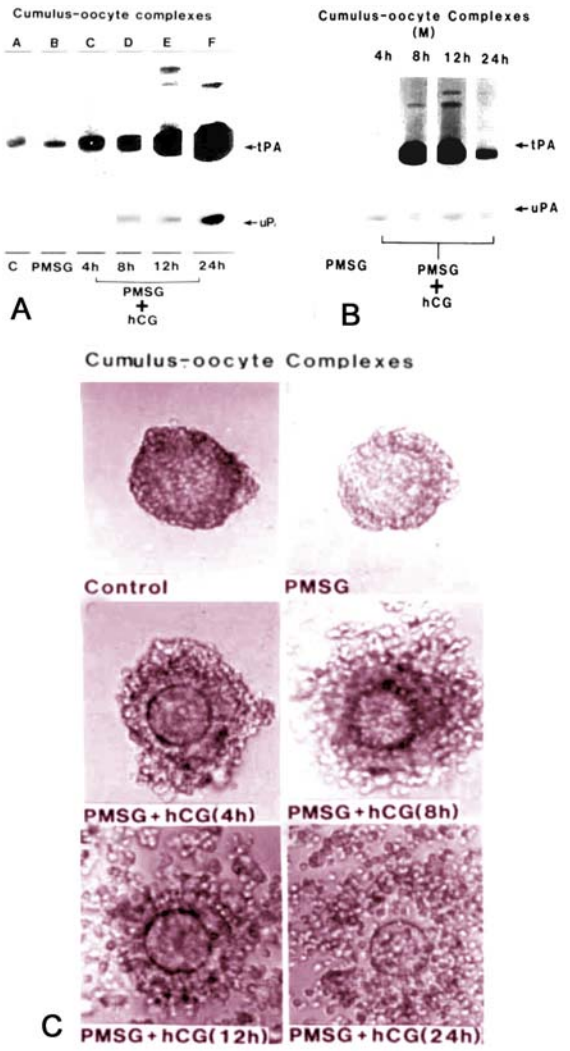

Figure 2. Content (A), secretion (B) of tPA activity, and light micrographs (C) of rat cumulus-oocyte complexes during the periovulatory period. The cumulus-oocyte complexes were obtained from PMSG / hCG treated immature rat ovaries at the time points as indicated. For the measurement of PA activities samples (100 ul) containing 50 cumulus-oocyte complexes for each group were extracted with 2.5% SDS and fractionated by SDS-polyacrylamide gel electrophoresis, the gels were analyzed for PA activities by fibrin-overlay technique. To examine the secretory ability of tPA, 50 cumulus-oocyte complexes in each group in 0.1 ml medium were incubated for 24 h, the media were then collected and the PA activity was measured by fibrin-overlay technique. For the light micrographs, only one represent cumulus-oocyte was shown. C, untreated control; PMSG, 48 h after PMSG injection; 4h, 8h, 12h and 24 h, the time after hCG injection (37).

the complexes was determined. The tPA activity was markedly increased by the addion of both FSH and hCG. After the *in vivo* hCG treatment, however, the elevated tPA activity measured in the complexes was not further affected by the gonadotropins in the 24 h incubation. Thus, the gonadotropin stimulation of ovulation is associated with the increase in a limited amount of the cellular content and the secretion of tPA activity by the cumulus-oocyte complexes. The oocyte tPA activity may be involved in the process of detachment of the cumulus oophorus from the stratum granulosum prior to ovulation (35, 37, 40). A similar temporal pattern of PA expression and matrix degradation was demonstrated in the mouse cumulus-oocyte complexes treated with the gonadotropins (43). Plasminogen activators in the bovine cumulus-oocyte complexes during the *in vitro* maturation were also examined by Park *et al.* who demonstrated that tPA-mediated degradation of the matrix and the cumulus-oocyte complex dissociation is required in the complexes before ovulation (44). The oocytes from amphioxus and frog also have ability to synthesize tPA [unpublished data], indicating that the presence of tPA activity in the oocyte is evolutionarily conserved.

The fertilized ovum in rat and mouse still contained high level of tPA activity (38, 45). The enzyme activity completely disappeared only after implantation (38), indicating that the oocyte carried tPA activity may play a role in uterus during process of implantation. This suggestion is also supported by the evidence that embryos from implantation-defective mice secreted less tPA activity than embryos from normal mice (46).

5. PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 IN THE OVARY

The secretory ability of PAI-1 activity has been comparatively examined in the cultured unstimulated GCs, TCs, cumulus cells, luteal cells and oocytes isolated from rat ovary (47). In the rat oocyte does not secretes PAI-1, GCs synthesize negligible amount of PAI-1 activity, while both cumulus cells and luteal cells secrete considerable amount of PAI-1 activity. The majority of PAI-1 activity in the ovary is produced by the TCs (16, 17, 48). Further experiments with PMSG/hCG treated immature rats demonstrated that GCs do not produce PAI-1 until 12 h after hCG injection (17, 48). In contrast, TCs secrete the inhibitor throughout the periovulatory period, and the inhibitor activity is stimulated by the gonadotropins in a time-dependent manner (16, 17, 49). Incubation of the isolated follicles obtained from the PMSG/hCG treated immature rats indicated that no measurable tPA, but uPA activity, and a huge amount of PAI-1 activity were detected in the conditioned media at all the time points after the gonadotropin treatment (16), indicating that tPA activity by GCs may be completely neutralized by the presence of PAI-1 secreted by the follicular wall (TC). The occurrence of a high molecular weight lysis zones on the indicator gel suggests the formation of complexes between the plasminogen activator and the inhibitor.

To analyze the interactions between tPA produced by GCs and PAI-1 sythesized by TCs in rat, two types of the purified cells obtained from the ovaries at various time points after the PMSG/hCG treatment were incubated alone or in combination. The interactions between the plasminogen activator and the inhibitor in the conditioned media of the two cell combined cultures completely inhibited the tPA activity produced mainly by

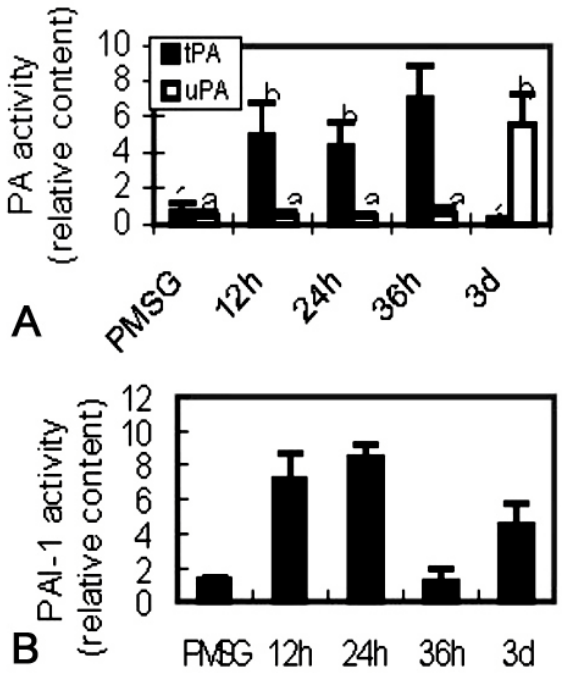

Figure 3. Gonadotropin regulation of tPA and PAI-1 activities in monkey ovaries during the periovulatory period. The monkeys were treated with PMSG and/or hCG. The ovaries were removed at various times after gonadotropin treatment and the GCs and TCs were prepared. GCs or TCs (5×10^5 cells/time point) were incubated at 37°C in 0.5 ml of McCoy's 5A medium for 24 h, The PA activities in the conditioned media were measured by fibrin overlay technique, and PAI-1 antigen levels were measured by western blot**.** The relative activities of tPA and uPA (mean ± SD), and PAI-1 (mean ± SD) were estimated by densitometric scanning three photographs at least and qualitatively analyzed by ANOVA. Different letters indicate significant difference between the points ($p< 0.05$) PMSG: 12 days after PMSG treatment; 12h, 24h, 36 h, 3d: time points after hCG treatment (49).

GCs before 8 h after hCG injection. However, after 12 h of hCG injection, despite the presence of high level of PAI-1 activity in the conditioned media produced by both TC and GC, the total tPA activity in the culture reached a maximum level, that may be important leading to the follicle rupture (16).

We have demonstrated just recently that the profile and regulation of PAI-1 expression in TCs of the PMSG/hCG treated monkeys (49) exactly follow the same rule as we detected in the rat (17).

Taken together**,** it is suggested that the interaction and regulation of the PA activator and inhibitor in mammal follicles may play a very important role in maintaining normal ovarian function and mechanism of ovulation.

6. OVULATION

6.1. Ovulation requires ovarian matrix degradation

Ovulation, triggered by a surge of luteinizing hormone (LH) released from the pituitary and recurring every reproductive cycle in female mammals, is an essential prerequisite for fertilization and subsequent embryonic development (50-53). A mature follicle that is destined to ovulate usually protrudes markedly from the surface of the ovary, and for the ovum to escape from this structure, an extensive breakdown and remodeling of basement membranes and connective tissues that constitute the follicular wall is required (50-54).

Follicle rupture involves a series of tissue-specific and time-coordinated physiological, biochemical and morphological changes in the ovary. In order for the egg to escape from the follicle at the time of ovulation, an opening of the follicle wall and the detachment of cumulus oophorus from the stratum granulosum are required. Factors leading to the occurrence of this very complex process have fascinated biologists for many years. Nearly a century ago, Schochet suggested that proteolysis might be responsible for degradation of the follicle wall during ovulation (55). Supportive evidence for this concept was not obtained until 1975, when a hypothesis for rat ovulation was proposed (56). Beer *et al.* demonstrated that PA-generated plasmin is responsible for disruption of the follicle wall (56-58). Further studies showed that rat granulosa cells (GCs) produce two types of PA, tPA and uPA (15), only tPA is a secretory protein (17, 48, 59), while theca-interstitial cells (TIs) secrete PA inhibitor PAI-1(16, 17). Expression of both tPA, (but not uPA) and PAI-1 in the ovary was stimulated by gonadotropins. tPA activity in the granulosa cells increases following follicular development, reaching a maximum immediately prior to ovulation and declining thereafter (17, 48, 49).

6.2. Coordinated expression of tPA and PAI-1 in ovary induces ovulation in rat

To study whether coordinated regulation of tPA and PAI-1 takes place *in vivo* in response to physiological signals, the changes of the mRNA levels and the protein activities of both tPA and PAI-1 in the ovarian cells were also examined in rat during PMSG/hCG-induced ovulation (17). The mRNA levels and protein activities of both tPA and PAI-1 in the ovary were coordinately regulated by the gonadotropins in a time-dependent and cell-specific manner, such that the maximum level of tPA mRNA and activity in GCs was obtained just prior to ovulation. Both before and after ovulation, PAI-1 mRNA and activity synthesized predominantly by T1s reached the maximum levels ensuring the inhibition of proteolytic activity in the extracellular ovarian compartment. The tissue-specific and time-coordinated expression of tPA and PA-1 genes in the ovary allows a narrow window of periovulatory increase in tPA activity, which may be important for the regulation of the ovulatory process (17, 49). In the rat, ovulation is preceded by a transient and cell-specific expression of tPA and PAI-1, which causes a proteolytic activity localized to the surface of the ovary just prior to ovulation, as shown in Figure 5. Intrabursal injection of α_2-antiplasmin or antibodies against tPA to neutralize the follicular plasmin or tPA activity significantly blocked the gonadotropin-induced ovulation in rats (60), indicating a pivotal role of tPA in ovulation.

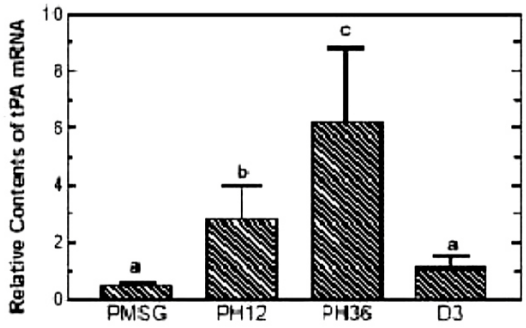

Figure 4. Changes in tPA mRNA levels in monkey granulosa cells treated with PMSG and hCG. Monkeys were treated with PMSG for 12 days and followed by injection of hCG. Total RNA from GCs of each group (5×10^6 cells) was prepared by the Nonidet P-40 method and fractionated by agarose gel electrophoresis before being transferred onto nylon filters and hybridized with ^{32}P-labeled antisense monkey tPA riboprobe. The relative amounts of tPA mRNA (mean ± SD) were estimated by densitometric scanning of the autoradiographs and normalized against the corresponding amounts of GAPDH mRNA from 3 northern blot analyses. Different letters indicate significant difference between the points ($p< 0.05$). PMSG: monkeys treated with PMSG for 12 days ; PH12, PH36 and D3: 12h, 36h and 3 days after hCG injection (49).

6.3. Coordinated expression of tPA and PAI-1 in ovary induces ovulation in rhesus monkey

Although studies have suggested specific crucial role of tPA and PA inhibitor PAI-1 in ovulation in rodents, little is known about whether the PA system participates in the ovulatory process in primate. Answering this question has been somewhat hindered by limited availability of primate ovarian materials that represent the entire periovulatory process.

To clarify the specific role of tPA and PAI-1 in ovulation in primate, we have used a PMSG / hCG-induced, synchronized ovulation model in rhesus monkeys, and systematically investigated the roles of PA system in the ovulatory process (49, 61, 62). At different follicular developmental stages throughout the periovulatory period, the samples of ovaries including GCs, TCs, as well as follicular fluid (FF) were collected, and levels of tPA, uPA and PAI-1 were evaluated. We have demonstrated that in response to an injection of ovulation-triggering hCG which mimics the preovulatory surge of LH in the circulation, the GC-derived tPA was substantially elevated in the preovulatory follicles, and reached its maximum level just prior to ovulation. The TC-derived PAI-1 was also stimulated by the PMSG and hCG treatments, however, the maximum level of PAI-1 appeared 12 h earlier than that of tPA. As shown in figure 3 & 4. when ovulation approached, accompanying the highest tPA level in the preovulatory follicles, the follicular PAI-1 level declined dramatically to its minimum value (49). GCs contained considerable level of uPA activity, which was not regulated by the treatment of gonadotropins before ovulation. However, the uPA activity was dramatically increased and secreted after ovulation in the luteinized GC (49).

These data suggest that in the monkey ovary as the same as in the rat, tPA (but not uPA) and PAI-1 are regulated by the gonadotropins and the coordinated expression of the two genes in the ovary is also responsible for the follicle rupture. Thus, we have demonstrated that in monkey ovaries, GCs are the major source of tPA production, while TCs are the major source of PAI-1.Coordinated expression of tPA and PAI-1 induced the maximum level of tPA activity in the follicle prior to ovulation leading to follicular rupture (49).

6.4. GnRH analogue and FSH stimulate oocyte and GC tPA activity and induce ovulation

To further examine whether hormones or compounds which stimulate tPA expression in oocyte and GC could induce ovulation, GnRH, FSH and vasoactive intestinal peptide (VIP) were tested. GnRH and its agonist (GnRHa) are known to stimulate tPA expression in the cultured rat granulosa cells and cumulus oocyte complexes (19) and to induce ovulation in the hypophysectomized rats by acting directly on the ovary (63). To clarify the specific role of tPA induced by GnRH in ovulation, we have examined the effect of an ovulatory dose of GnRHa on the ovarian tPA and PAI-1 mRNA levels and activities as well as their cellular localization (64-66). GnRHa stimulated the induction of tPA (but not uPA) activity in GCs in a time-dependent manner as hCG does (67), reaching a maximum before ovulation. tPA activity in the cumulus-oocyte complexes also increased before ovulation. Immunohistochemistry indicated substantial increases in tPA staining in GCs and oocytes of the preovulatory follicles (65). GnRH and hCG seem to elicit the similar responses in the ovaries (67). Administration of a GnRH antagonist specifically blocked the GnRHa-induced, but not hCG-induced ovulation, indicating that GnRH and hCG induce ovulation via different receptor pathways (64, 65).

Both *in vitro* and *in vivo* studies have indicated that FSH is capable of inducing rat ovarian tPA expression (37). A LH-free recombinant FSH (rcFSH) has been tested for the induction of ovulation in the hypophysectomized immature and adult rats (68). The data showed that rcFSH induced ovulation with associated increases in ovarian tPA, but not uPA gene expression.

6.5. VIP, Forskolin, cAMP, PMA stimulate GC tPA activity and Induce ovulation

VIP, originally considered to be a gut hormone, has recently been found to increase tPA activity in cultured rat GCs and cumulus-oocyte complexes (22, 23). The ovulatory effect of VIP was also studied using *in-vitro* perfused ovaries from immature rats primed with PMSG. VIP-induced ovulation could be observed in all perfused ovaries (69). Furthermore, Forskolin and cAMP (an activator of protein kinase A), and PMA (an activator of protein kinase C) that are all strong inducers of ovarian tPA activity *in vitro* (37), have been demonstrated to be able to induce ovulation in perfused rabbit and rat ovaries (70, 71).

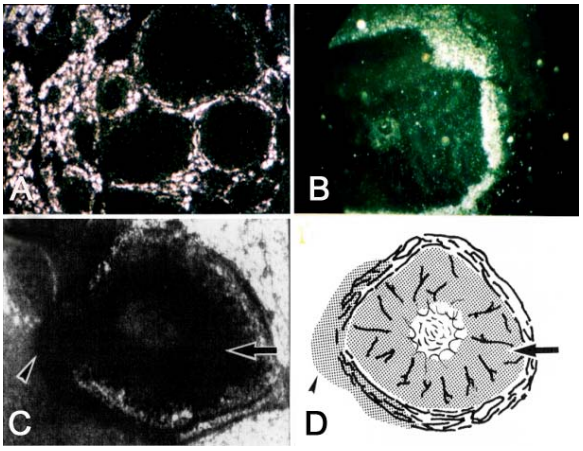

Figure 5. The time-coordinated and cell-specific localization of PAI-1 mRAN (A) in TC and tPA mRNA in GC (B) in the prior ovulatory follicles. Note: the coordinated expression of tPA and PAI-1 in the follicle induces a derected and localized window of proteolytic flow (C, D). The arrows show the direction of the proteolytic activity; the arrowhead indicates the stima area of follicle rupture. The shaded areas indicate the tPA activity. Modified from Liu *et al.* (18, 112)

6.6. Compounds which decrease tPA and/or increase PAI-1 expression inhibit ovulation

To examine whether the inhibitory effect of indomethacin on ovulation is associated with an decrease in ovarian tPA and/or increase in PAI-1 expression in the ovary, the action of indomethacin, a strong inhibitor of ovulation, on the secretion of both tPA and uPA in GCs has been carefully investigated following hCG or GnRH administration in the PMSG-primed hypophysectomized immature rats (72). The results showed that (1) ovulation induced by both hormones was effectively blocked by the concomitant administration of indomethacin (1 mg/rat); (2) indomethacin remarkably suppressed tPA (but not uPA) secretion in GCs obtained at 12 h after hCG or GnRHa injection; (3) this compound also dose-dependently inhibited the GnRHa- and the FSH-stimulated tPA secretion in GCs *in vitro.* The indomethacin inhibition of ovulation and tPA secretion was further confirmed by Tanaka *et al.* (73).

Prolactin (PRL) is a pituitary hormone, mainly involved in stimulating milk production. *In vitro* studies have demonstrated that PRL was capable of inhibiting the LH- and FSH-induced tPA mRNA and activity in the rat granulosa cells (74, 75), and both tPA and uPA activities in the mouse GCs (76) obtained from the PMSG-treated ovaries in a dose-dependent fashion, this hormone was also found to be able to stimulate the ovarian PAI-1 gene expression (74, 75). Using *in-vitro*-perfused rabbit ovaries, it has been shown that PRL causes a decrease in the PA activity of the mature follicles (77, 78). Injection of PRL in the rat inhibited the hCG-induced ovulation in a dose-dependent manner by disturbing the normal coordinated expression of ovarian tPA and PAI-1 leading to ovulation (79). Both tPA mRNA and activity in the GCs induced by the injection of hCG were considerably inhibited after a single co-injection of 200 ug of PRL. The inhibitory effect of PRL on tPA activity was further enhanced by the increase in PAI-1 expression in the ovary, suggesting that the inhibitory effect of PRL on ovulation is due partly to increased PAI-1 production, in addition to the inhibition of tPA expression. A correlation between decreased tPA activity and increased PA-PAI-1 complexes was also observed in the cell-conditioned medium, indicating neutralization of tPA by formation of complexes with PAI-1 (75). Since prolactin also decreased the hCG-induced tPA mRNA and activity, the observed inhibition of ovulation and tPA activity by PRL may be caused by two effects: suppressing tPA expression in GCs, and enhancing PAI-1 synthesis in TIs.

Involvement of regulation of ovarian tPA and PAI-1 gene expression in ovulation is outlined in Figure 6. Because TIs are the major source of PAI-1, whereas GCs produce the most tPA in the ovary, the coordinated expression of tPA in GCs and PAI-1 in TIs should be synchronized in the two types of the cell during the process of ovulation by a tissue-specific and time-programmed reaction to the stimuli and by the interactions between these two cell types (80, 81) to form a directed proteolytic 'window' leading to the rupture of the follicle wall (Figure 4).

6.7. PA System in the ovaries of human, mouse and other Animals

It is well known that species differences exist in the expression of the PA system in ovaries.

Little is known about the roles of the PA system in human ovaries. Human GCs collected from preovulatory follicles were reported to contain little or no tPA or uPA mRNA (82). Nevertheless, in the FF of human preovulatory follicles, low levels of tPA were detected (83). Moreover, relatively abundant amounts of PAI-1 mRNA were reported in human GCs or granulosa-luteal cells (82-84). The difference in the expression of tPA and PAI-1 in GCs and FF of preovulatory human follicles as compared to the results we obtained from the monkey ovaries may have several explanations. There might be species variations between humans and monkeys. However, we have also noticed that the expression of PA and PAI-1 during the periovulatory process in monkeys showed a temporal pattern that changed in a matter of hours. In our experiments, GC or FF samples obtained at different time points during the periovulatory period contained significantly different levels of PA and PAI-1. Therefore, it seems to us that what type of results can be obtained from human GCs and FF may also depend on the stage and condition of the clinical samples. Just recently, Szymanski *et al.* demonstrated that the average tPA level in FF of patients undergoing IVF-ET treatment was significantly lower, whereas PAI-1 significantly higher compared to the control group. The authors suggested that tPA and PAI-1 in human FF may be crucial factors reflecting oocyte maturity (85).

Canipari *et al*. reported that mouse GCs produced

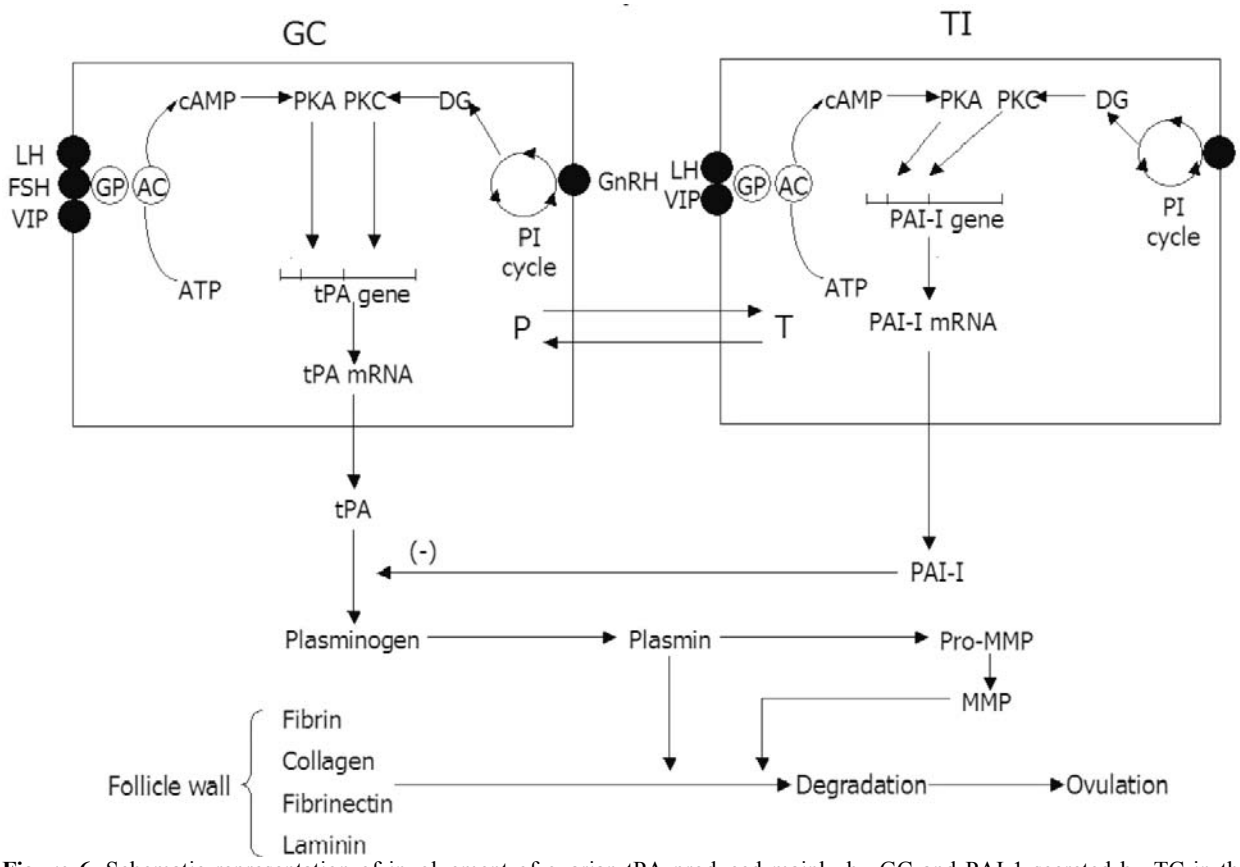

Figure 6. Schematic representation of involvement of ovarian tPA produced mainly by GC and PAI-1 secreted by TC in the process of ovulation. P, Progesterone; T, testosterone; GP, G-protein; AC, adenylate cyclase; DG,1,2-diacylglycerol; PI, phospholipid; PKA, protein kinase A; PKC, protein kinase C. For the explanation of the other abbreviations, see the text. (summarized from references 17, 19, 22, 25, 29, 34, 49, 65, 69, 70, 71, 152).

only uPA activity, suggesting its exclusive role in ovulation (86). We have further carefully reexamined their report by our PMSG/hCG treated mouse model. Two types of PA activities were also identified in mouse oocyte-free GCs, uPA contributed about 70%, while tPA contributed only about 30% of the total ovarian PA activity in this species (87), both uPA and tPA in GC and cumulus-oocyte complexes were regulated by gonadotropin treatments, and both reached the maximum levels just prior to ovulation (87, 88). However, in contrast to rat, no measurable amounts of PAI-1 activity were detected in mouse follicular fluid, but abundant alpha-2 -antiplasmin (a specific inhibitor of plasmin) was found in follicular fluids (89). In addition to uPA, tPA, alpha 2-antiplasmin, mouse ovary also expressed mRNAs for membrane-type (MT)-MMP, stromelysin-3, protease nexin-1 (PN-1) and tissue inhibitor of metalloproteinase type 1 (TIMP-1 (90). Therefore, there may be species-specific differences in proteolytic enzyme-generating systems in the ovaries. It is thus not surprising that ovulation efficiency is greatly reduced, but not completely blocked in mice that lack PA gene function (91). The loss of an individual PA appears to be functionally compensated by the remaining PA or other proteolytic enzymes (92).

To study the mechanism of reduced ovulation efficiency in PA-deficient mice, Ny *et al.* further comparatively examined plasmin activity and regulation in the ovaries of wild-type mice and mice with deficient PA gene function during PMSG- and hCG-induced ovulation. In mice lacking either tPA or PAI-1, the plasmin activity in the ovary prior to ovulation was similar to that of the wild-type mice, whereas the ovarian extract prepared from uPA-deficient mice contained only 10% or less plasmin activity of the normal wild types, indicating that most of the plasmin activity in the mouse ovary is generated by uPA. These data suggest that the amount of plasmin generated by PAs prior to ovulation in wild-type mice greatly exceeds the amount required for efficient ovulation (88, 92).

Using a chicken model, Tilly and Johnson (93) and Jackson *et al.* (94) have found that the dramatic increase in PA activity, specifically in the stigma region, is correlated with the ovulatory process. More specific examination of the changes in the PA system during ovarian follicle development in the domestic hen revealed that PA activity increased while PA inhibitor activity decreased during follicular development and tPA appeared to be the principal PA in the preovulatory follicles (95). Studies have also suggested a role for PA in the ovulatory process of the pig (96). PA activity was highest in the area of rupture of the pig follicle wall at the time of ovulation

Table 1. Inverse relationship between progesterone production/StAR expression and tPA activity in monkey CL at various stages (summarized from references 18, 111, 114, 119 and 123)

Days of CL development	StAR Expression in CL	Levels of progesterone (mean±se)/ng.ml^{-1}	Activity of tPA
5	+++	79.0 ± 5.9	1.0 ± 0.2
10	++	22.4 ± 6.9	1.1 ± 0.1
13	+/-	8.4 ± 1.8 b)	42.2 ± 2.4 b)
15	—	19.7 ± 7.5 a)	10.3 ± 1.2 b)
23	—	7.2 ± 0.9 b)	5.0 ± 1.9 a)

(96) and a net increase in PA activity appeared to be regulated by changes in both PA and PAI content. Dow at al has demonstrated that both PAI-1 and PAI-2 mRNAs are upregulated in preovulatory bovine follicles after the gonadotrophin surge induced by GnRH in a cell-specific manner and suggested that regulation of PAI-1 and PAI-2 may help to control plasminogen activator activity associated with ovulation (97).

We have also examined the ovarian PA activities in other species, such as amphioxus (chordata), rabbit, cat, hamster, and giant panda. Ovaries of these species contained mainly tPA activities, which were regulated by gonadotropins (98). Thus, we suggest that tPA may be the key ovarian PA type which plays roles in the physiological process of ovulation.

6.8. Role of uPA in ovary

Urokinase PA has been suggested to play a role in ovulation in mouse (86). Macchione *at al* demonstrated that uPA secreted from rat follicular cells accumulates on the surface through binding to its receptor. The uPA redistribution in GC and its defferent regulation by gonadotropins has been suggested to have a different function (99). Evidence accumulated from other species indicates that uPA may be important in ECM degradation, cell proliferation and migration during follicular growth at the early stage. Bovine follicles typically increase in size several hundred folds between preantral and preovulatory stages. Studies have shown that uPA is the predominant PA expressed in the small growing follicle in this and various other species. Li *et al.* have provided evidence showing that uPA transcript and protein levels were highest at the earliest stage of follicular growth and decreased dramatically before the expected time of ovulation (100). This finding is consistent with the previous reports by Tilly and Johnson in hen (101), Karakji and Shen *et al.* in rat (102, 103).

It is not known whether the uPA activity in small follicles is regulated by PA inhibitors. Data available showed that PAI-1 and PAI-2 in the developing follicles were low. Interestingly, SERPIN-E2 is highly expressed in the small growing follicles in the rat (104, 105), and in the preantral and growing antral follicles in the cattle (106). It is suggested that SERPIN-E2 may be the major PA inhibtor in GC, which is coordinately expressed with uPA to regulate tissue remodeling during follicular growth and differentiation at the early stage.

7. RORE OF PA SYSTEM IN CORPUS LUTEUM

7.1. CL formation is a dramatic morphological and biochemical process

Corpus luteum (CL), a temporary endocrine organ, is transformed from the residual GCs and TIs of the ovulated follicle. CL mainly secretes progesterone for maintenance of pregnancy by priming the uterus to support implantation and early fetal development (52, 107). CL formation undergoes a dramatic morphological and biochemical processes involving invasion of capillary network from the theca tissue into the granulosa layers and accompanied with the theca and granulosa cell differentiation to transform into luteal cells. Matrix degrading proteases are thought to play important roles in such dynamic processes (108-110), In both rodents and primates, the development of CL is a rapid process with very high cellular turnover (52, 107). However, if fertilization has not occurred, or if the implantation is unsuccessful, the functional phase of the CL is terminated and luteolysis is initiated. This involves a rapid loss of progesterone production (the functional luteolysis), followed by degradation of the luteal tissue (the structural luteolysis) into small fibrous remnants in days (52, 109). Therefore, involvement of proteolytic activities in the above-mentioned processes has been hypothesized to be of importance (108-110).

7.2. Involvement of proteolytic activities in CL

The regulatory and functional roles of the PA and MMP systems in angiogenesis and tissue remodeling in the CL have been widely studied for the last decade (111-118). In the rat, proteolytic activities mediated by tPA and regulated by its inhibitor PAI-1 temporally and spatially are important for the CL formation and regression (112, 113). A fine-tuned tPA activity which is controlled by PAI-1 might be important for the initiation of luteolysis.

It has been demonstrated that luteal level of steroidogenic acute regulatory protein (StAR) is well correlated ith progesterone production (120-122), indicating that StAR is a key regulator of CL function and can be used as a marker of steroidogenesis in rat, monkey and human (116, 119, 123-126). Increase in tPA and PAI-1 activity in rat (116) and monkey CL (119) at later stage is closely related to a dramatic decrease in StAR / progesterone production (table 1), suggesting that the coordinated expression of tPA and PAI-1 in CL may be involved luteal regression. To further confirm this finding in primate we have performed experiments with luteinized ovaries obtained from a PMSG / hCG induced rhesus monkey ovulation model (111, 114). Luteal cells from the rhesus monkeys were able to produce the two types of plasminogen activators (115), tPA and uPA, as well as PAI-1. High levels of uPA and PAI-1 mRNA were simultaneously expressed in the functional monkey CL, indicating that their interplay may participate in the CL development and functional maintenance in the

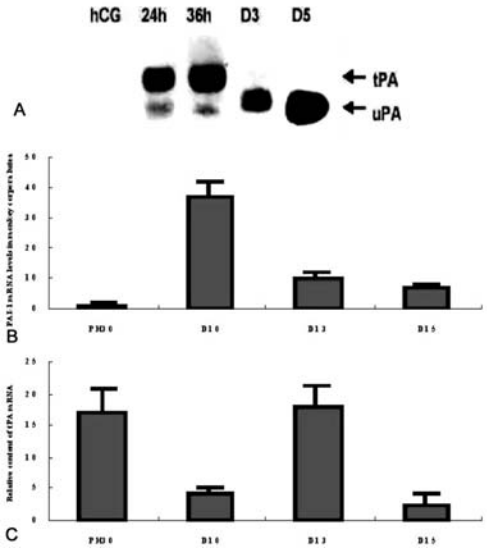

Figure 7. Changes in tPA and uPA activity in the luteinized granulosa cells at early stage (A) and the relative content of tPA (B) and PAI-1(C) mRNAs in the luteal cells at various stages. The monkeys were treated with PMSG and/or hCG. The ovaries were removed at various times after gonadotropin treatment and the luteinized GCs (5×10^5 cells/time point) from 36h to 3 days after hCG injection were prepareds and incubated at 37°C in 0.5 ml of McCoy's 5A medium for 24 h, The PA activities in the conditioned media were measured by fibrin overlay technique. Total RNA of the luteal cells (40μg for each group) for tPA or PAI-1 mRNA analysis was extracted from the corpus luteum on various days and fractionated by agarose gel electrophoresis and transferred to nitrocellulose filter (26). The filter was baked at 80°C for 2h under vaccum, and hybridized with ^{32}P-labeled monkey antisence tPA, PAI-1 and GAPDH RNA probes. The relative amount of tPA or PAI-1 mRNA was estimated by densitometric scanning of the autoradiographs and normalized against the corresponding relative amount of GAPDH mRNA. 24h, 30h, 36h, 3D, D10, D13, D15: the time after hCG injection (from reference 119).

rhesus monkey (111). uPA, but not tPA, is the only active PA species that is identified in the early CL (119), as shown in Figure. 7, suggesting a role for uPA in tissue remodeling and angiogenesis during CL formation. However, during luteolysis when serum progesterone levels and StAR mRNA expression in the CL declined dramatically, a substantial elevation in tPA mRNA and protein was observed. Moreover, tPA and its inhibitor PAI-1 mRNAs and proteins showed a coordinated expression in the monkey CL. Increase in tPA expression in the CL is correlated well with decrease in PAI-1 production (114, 119).

Most interesting of all, just prior to the increment of both tPA mRNA and activity levels in the monkey (119) and rat (113) CL at the time of initiation of luteolysis, a peak level of PAI-1 mRNA was measured. This peak level of PAI-1 mRNA declined dramatically when luteolysis was initiated with an increased tPA level. PAI-1 regulated tPA activity might be important for the initiation of luteolysis in the monkey.

7.3. *In vitro* regulation of luteal cell function by PA activator/inhibitor

To confirm further whether tPA, uPA and PAI-1 or their antibodies directly affect the luteal cells and change the ability of their progesterone secretion, dispersed rat (113) or monkey luteal cells (114, 119) were cultured with or without either tPA, uPA and PAI-1 or their purified antibodies in the presence of plasminogen, a substrate for PA activation. Addition of tPA to the culture significantly decreased progesterone production by cultured rat CL cells**,** in contrast, addition of tPA monoclonal antibodies to neutralize the endogenously produced tPA activity remarkably increased the steroid production by 100% (113). The same effect of tPA and its antibodies was also observed in the primate luteal cells in the culture (114, 119). In contrast, addition of uPA to the culture has no such effect on progesterone production.

Two types of luteal cells have been reported; large luteal cells derived from GCs, and small luteal cells derived from TCs **(**127). GC is known to produce ovarian tPA activity, whereas TC is capable of producing PAI-1 and uPA. Therefore, one may speculate that large luteal cells might be responsible for the tPA production, while small luteal cells might be connected with both the uPA and the PAI-1 activities found in the CL.

Prolactin and LH have been reported to exert a synergistic luteotropic effect in the rat. LH alone seems to inhibit tPA activity, and stimulate progesterone production in the cultured monkey luteal cells, while prolactin alone has no such effect (114)]. The two pituitary hormones in combination in the culture, however, synergistically increase luteal progesterone secretion, while completely suppress the medium tPA (but not uPA) activity (114).

Much evidence accumulated in the past several decades clearly reveals that CL is also capable of secreting various paracrine and autocrine luteotropic or luteolytic regulators (105, 128-130). IFN-gama and PGF-alpha 2 were found to stimulate the basal and the hCG-stimulated tPA secretion in the cultured rat luteal cells (113, 130). However, the mechanism by which cytokines decrease CL progesterone, while increase tPA secretion is not known. On the basis of the data provided here, we speculate that the endogenously produced tPA in CL in the control of hormone production may regulate luteal regression through local autocrine or paracrine action. However, questions remain concerning the identity of certain intraluteal factors linking tPA/PAI-1 expression and progesterone production.

8. ROLE OF PA SYSTEM IN OVARIAN ANGIOGENESIS

8.1. Neo-vascularization in growing follicles

Ovary is a highly vascularized organ. During the later stage of follicular development, the follicles undergo rapid growth, ovulation and CL formation / regression or atresia by apoptosis. These structural and morphological

modifications impose a remarkable demand on the formation of new blood vessels and their subsequent regression (131). Shortly after the onset of follicle growth, a rich capillary network near the follicle forms, but it does not penetrate the basement membrane (131, 132). During ovulation and the early stage of luteinization, the most dramatic changes in the capillary network surrounding the basement membrane take place. The degradation of the basement membrane of the vessel is essential since it permits endothelial cells to infiltrate the surrounding stroma. From the endothelium of these vessels, capillary sprouts begin to grow into the GC layer and form a new complex network of sinusoid vessels (131, 133). Endothelial cells produce PAs, MMPs and their specific inhibitors in response to angiogenic factors, which are considered to play a key role in both degradation of the basement membrane and invasion of the stroma by the newly formed capillary sprouts (134, 135). Studies *in vitro* demonstrated that migrating endothelial cells produce both PAs and PAI-1, and that interaction and coordinated expression of PAs and PAI-1 may be important for lumen formation. Natural and synthetic inhibitors of PAs are capable of blocking the capacity of endothelial cells to invade the ECM. Evidence has shown that both uPA and PAI-1 are expressed in the ovary during neo-vascularization of the growing follicle, and in the early stage of CL development (135-137). After ovulation, uPA was dramatically expressed in the developing CL (119) mainly in the capillary sprouts of vessels (136, 141), uPA mRNA was transiently expressed along the route of capillary extension toward growing follicles, terminating at the newly formed capillary sheaths surrounding each growing follicle. Once the formation of the CL was completed, the expression of uPA declined. During CL formation, PAI-1 was also expressed, preferentially in cells in the vicinity of uPA expressing capillary-like structures. These findings suggest a functional interplay between PA and PA inhibitor. The presence of PA inhibitor may protect neovascularized tissues from excessive proteolysis during angiogenesis.

8.2. Mature CL receives the greatest blood supply

A dynamic process of tissue remodeling and angiogenesis involves invasion of a capillary network from the theca tissues into the granulosa cell layers (136). Formation of CL is also accompanied by active cell differentiation when theca and granulosa cells transform into luteal cells. In both rodents and primates, the development of CL is a rapid process with very high cellular turnover (131, 138-144), within hours for the rat and mouse, and within days for monkey and human. The mature CL receives the greatest blood supply per unit tissue of any organ (139, 140). The factors regulating angiogenesis in the ovary are not known. Basic fibroblast growth factor (bFGF)-like activity has been found in developing CLs of various animals (141, 142). This activity was not found in the follicles obtained before ovulation. Using three-dimensional fibrin gels, Mignatti *et al.* (134) demonstrated that bFGF which enhances proteolysis in endothelial cells by increasing the ratio of uPA/PAI-1 was found to induce the formation of tube-like structures, whereas transforming growth factor beta (TGF-bata), a factor that enhances PAI-1 secretion in endothelial cells, is capable of inhibiting the effect of bFGF and causing the formation of a solid endothelial cell cord (132).

9. PERSPECTIVE

In summary, rat and rhesus monkey GCs synthesize the most of follicular tPA activity, whereas their follicular walls (TCs) contribute the most of PAI-1 activity in the ovaries. PAI-1 may therefore serve as a specific barrier to localize the tPA activity within the follicles. As ovulation approaches, the levels of PAI-1 in TCs and GCs dramatically decrease, while tPA activity in the follicles rises to its maximum level. The coordinated expression of tPA and PAI-1 in the follicles may therefore lead to a short pulse of proteolytic activity that could play a role in rupture of the follicles, as shown in figure 5.

The coordinated expression of tPA and PAI-1 in the CLs of the two species also play an important role in the initiating of luteal regression. Urokinase PA, on the other hand, is the predominant PA expressed in the early small growing follicles, and decreases dramatically before the expected time of ovulation, therefore uPA may be important in ECM degradation, cell proliferation and migration during follicular growth at early stage. Because PAI-1 and PAI-2 in the developing follicles are low, while SERPIN-E2 is highly expressed in these small growing follicles, it is suggested that SERPIN-E2 may be the major PA inhibtor in GC, which is coordinately expressed with uPA to regulate tissue remodeling during follicular growth and differentiation at the early stage.

In spite of the importance of tPA in ovulation in both rat and monkey, species differences exist in expression of the PA system in the ovaries. Mouse ovary mainly produces uPA, while rat ovary mainly produces the most tPA activity. Furthermore, studies with tPA and uPA double knock-out mice only have a 26% decreased ovulation rate (88), indicating that plasmin in mouse may be not necessary required for efficient follicular rupture. Other proteases may be also involved in the ovulatory process by other mechanisms in the absence of plasmin in the mouse ovary (53, 89).

Studies of PA and plasminogen-deficint mouse strains have indicated that other families of proteases including the matrix metalloproteinases (MMP) and their inhibitors (TIMPs) may also be involved in ovarian function (53, 145-149). The complicated natures of various protease families have increased the tediousness of such studies. With the help of many strains of gene deficient mice that lack a protease or a combination of proteases, it has been wished that roles of certain proteases would be revealed by corresponding knock out mice, however, due to the complex of both proteases and the ovary itself, it has been even more complicated than anticipated. Further progresses could be made with the recently developed interference RNA (RNAi) technique, by which one may be able to quickly knock down the expression of a protein in cultured defined cells and reveal how important the molecules are in these processes (150,151).

Ovarian physiology is also modulated by endogenously produced local factors that can regulate the expression and localization of PAs and matrix metalloproteinases, thus, it seems to us that the next challenge is to identify how multiple paracrine and autocrine factors regulate proteases and protease inhibitors during the periovulatory period in the rodent and primate.

10. ACKNOWLEDGEMENTS

This work was supported by NSFC (No: 90208025,30370200,0170542,30170452), CAS "Chuangxi" (KSCX-2-SW-201),"973" (G1999055901) and WHO/ Rockefeller Foundation Projects. The author should thank Prof. Hsueh AJW, Ny T, Drs. Liu K, Feng Q, Peng XR, Hu ZY, Zou RJ, Chen YJ, Chen XL, Chen XX and Gao HJ for their contributions to the review data from the Lab.

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Key Words: Plasminogen activator, Plasminogen activator inhibitor type-1, Gene expression, Follicular development, Ovulation, Corpus luteum, Angiogenesis, Review

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