Expression and regulation of plasminogen activator and plasminogen activator inhibitor type-1 in rat epididymis

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EPIDIDYMIS is the site where spermatozoa released from testis undergo maturation and become

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capable of fertilizing ova. The epididymal secretions including enzymes, nutrients and hormones are believed to be essential for sperm maturation[1, 2]. Limited proteolysis contributes to loss or modification of surface molecules of spermatozoa, which is an important aspect of sperm maturation[3, 4]. Plasminogen activator (PA) is a highly selective trypsin-like serine protease that catalyzes the conversion of plasminogen into plasmin, a protease of broad specificity[5]. PA activity is neutralized by its specific inhibitor PAI. To study the regulation of protease activity in epididymis, we investigated the expression of PA and PAI-1 in rat epididymis. Activities of both tissue- and urokinase-type PA were detected in the medium of cultured rat epididymis and regulated by hormones in culture. mRNAs for tPA and PAI-1 were detected in epididymal epithelium by *in situ* hybridization. These results suggest that PA and PAI-1 may be involved in the fine regulation of proteolysis in epididymis.

1 Materials and methods

1.1 Animals and reagents

Adult Sprague-Dawley (SD) male rats were obtained from Animal Breeding Department, Institute of Zoology. hCG (CR-127) was provided by NIDDK; Δ_4-androstene-3, 17-dione (Δ_4) and McCoy's 5a medium (modified) were from Sigma Biochemical Company (St. Louis, USA); GnRH agonist (GnRHa) was provided by Dr. Ling (Salk Institute, CA, USA). cDNAs for rat tPA and PAI-1 were kindly provided by Prof. Ny (Umeå University, Sweden). DIG-RNA Labeling and Detection Kit was purchased from Boehringer Mannheim (Beijing); RNA polymerases T7 and SP6 were from Promega (Beijing); restriction endonucleases *Hind* Ⅲ, *Pvu* Ⅱ and poly-lysine were from SABC (Beijing). Paraformaldehyde was from Merck (Germany).

1.2 Tissue culture

Epididymides were dissected free of fat tissues. The caput and cauda epididymides were minced respectively into pieces (1 mm^3) in McCoy's 5a medium. Gelatin-sponge (Spongia Gelatini Absorbents, 3rd Medicine-Manufacturing Plant, Nanjing) was pre-saturated in 1 mL medium with or without hormones (hCG: 100 ng/mL, GnRH: 1×10^{-6} mol/L, Δ_4: 1×10^{-6} mol/L) and used as matrix for tissue culture in a 24-well plate. The minced tissues were washed vigorously by repeating resuspension/sedimentation 5 times to remove the secretions and spermatozoa, seeded on gelatin-sponges in a 24-well plate and cultured at 37℃ in atmosphere of 95% air with 5% CO_2 for 48 h. Culture medium was collected by discarding pellet after centrifugation (4℃, 2 000g, 10 min). Protein concentration in medium was determined using protein assay dye reagent (Bio-Rad Laboratories) reading at 595 nm and the content was adjusted to 100 μg/mL. After addition of Tween-80 and bovine serum albumin (0.1% for each), the medium was stored at −80℃ until analysis.

1.3 Fibrin overlay assay

PA activity in culture media was analyzed by fibrin overlay assay as described previously[6]. Briefly, 15 μg proteins was fractionated by SDS-PAGE. Following electrophoresis, the gel was washed twice in 2.5% (v/v) Triton X-100 with gentle agitation for 45 min to remove SDS, and then overlaid on the indicator gel and incubated in humid chamber at 37℃. PA activities were indicated by the appearance of fibrin lysis in the gel. The lysis zones were recorded using darkfield photography.

1.4 *In situ* hybridization

Epididymides of adult SD rat were dissected, embedded in Tissue-Tek (Miles, Elkhart, ZN, USA), snap-frozen in liquid nitrogen and stored at −80℃ until use. The frozen tissues were equilibrated at −20℃, and 15-μm thick sections were prepared by using a cryostat microtome, mounted on poly-lysine coated slides, air-dried and fixed in 4% paraformaldehyde (Merck, Germany) in PBS. Following fixation, the sections were digested with 0.2 N HCl for 25 min at 37℃, incubated in PBS containing 0.3% Triton X-100 for 15 min, rinsed in PBS and post-fixed for 5 min. After being washed 3 times in PBS, the sections were incubated in 0.1 mol/L triethanolamine/0.25% acetic anhydride for 15 min to reduce background and then prehybridized (2 × SSC/50% deionized formamide, 20 × SSC: 3 mol/L NaCl/0.3 mol/L Na_3 Citrate) for 2 h at 40℃. The sections were hybridized with dig-labeled antisense or sense cRNA probes in hybridization solution (2 × SSC/50% formamide/0.5% SDS/250 μg/mL yeast tRNA/1 × Denhardt's/10 mmol/L Tris, pH 7.5). Hybridization was performed in a humidified box for 20 h at 45℃. The sections were washed at 37℃ in 2 × SSC for 2 h; 1 × SSC, 2 × 30 min and at 40℃ in 0.1 × SSC for 2 × 15 min. The hybridized probes were detected using alkaline phosphatase-coupled anti-dig Fab fragment. Color reaction was developed by incubation with mixture of NBT and BCIP in colour development buffer (100 mmol/L Tris/150 mmol/L NaCl/50 mmol/L $MgCl_2$, pH 9.5). The reactions were terminated by immersing the slides in 0.1 mol/L EDTA. Following rinse in distilled water, the sections were mounted in 95% glycerol and photographed using brightfield illumination. The darkness indicates positive staining.

2 Results and discussion

2.1 PA activities in culture medium of caput and cauda epididymis

Figure 1 is the zymography of PA activity. Both tPA and uPA activities of high level were present in medium of cultured caput (CAP) epididymis, whereas the PA activities in the medium of cultured cauda (CAUD) epididymis were primarily of tPA; detectable uPA activity was present only in the presence of hCG. PA activities in both caput and cauda epididymis were significantly stimulated by hCG. GnRH and Δ_4 also regulate PA activities of cultured caput epididymis; GnRH inhibits whereas Δ_4 stimulates PA activities in the medium.

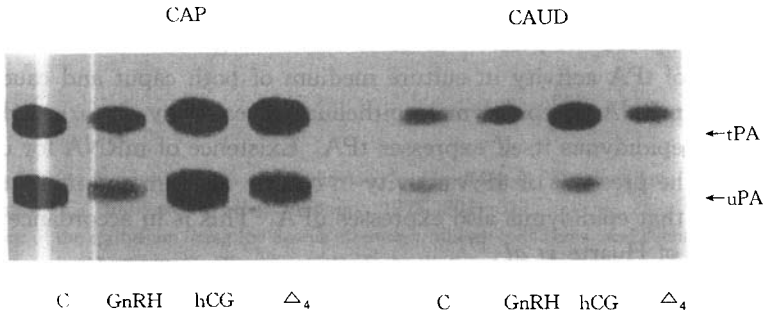

Fig. 1. Zymography of PA activity in culture medium of caput (CAP) and cauda (CAUD) epididymis of adult rat. PA activities were resolved by SDS-PAGE and fibrin overlay assay. Both tPA and uPA activities were present in medium of cultured caput (CAP) epididymis, whereas the PA activities in the medium of cultured cauda (CAUD) epididymis were primarily of tPA. PA activities in both caput and cauda epididymis were significantly stimulated by hCG (100 ng/mL). GnRH (1×10^{-6} mol/L) inhibits whereas Δ_4 (1×10^{-6} mol/L) stimulates PA activities in the culture medium of caput epididymis. They have little effect on PA activities of cauda epididymis.

2.2 *In situ* localization of tPA and PAI-1 mRNA in rat epididymis

In situ hybridization showed that tPA mRNA localized in epithelium of rat epididymis (fig. 2), whereas PAI-1 mRNA localized in both epithelial and mesenchymal cells (fig. 3).

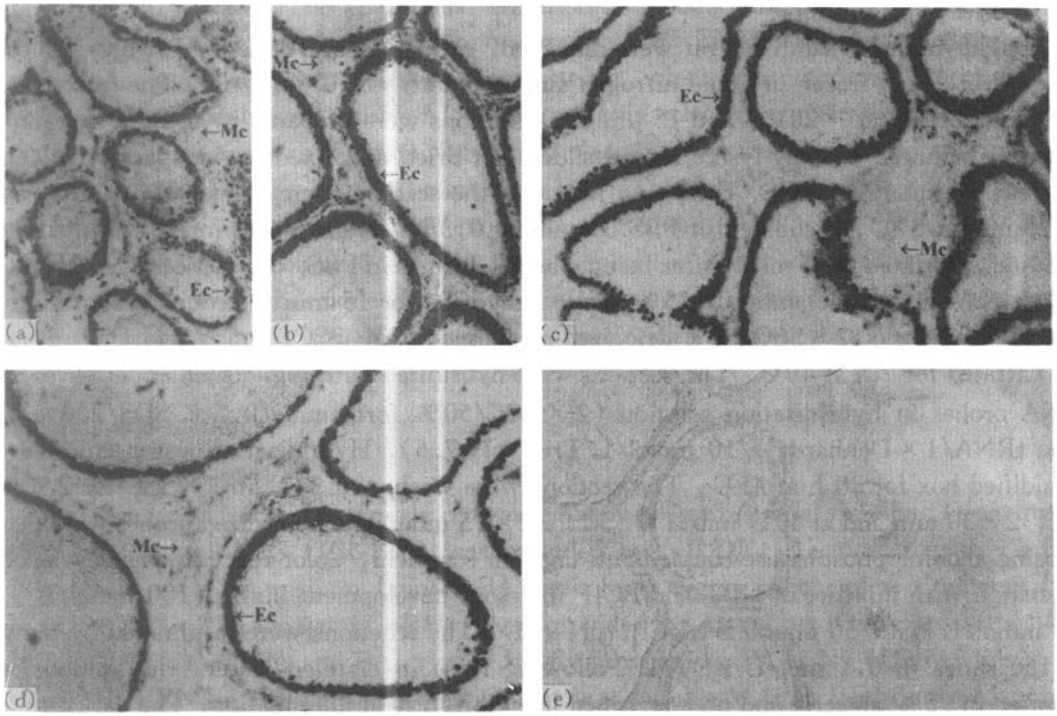

Fig. 2. *In situ* localization of tPA mRNA in rat epididymis using dig-labeled cRNA probes. Positive staining in epithelial cells (Ec) of initial (a), caput (b), corpus (c) and cauda (d) segments was indicated by antisense cRNA probe. No detectable staining in either epithelial cells (Ec) or mesenchymal cells (Mc) was observed when sense cRNA probe was used (e).

These results provide direct evidence that rat epididymis expresses tPA and PAI-1.

Limited proteolysis is an important aspect of epididymal sperm maturation. In this study, we investigated the possible involvement and regulation of PA activities in this process. The observation of tPA activity in culture medium of both caput and cauda epididymis and the existence of tPA mRNA in epididymal epithelium revealed by *in situ* hybridization provide direct evidence that epididymis itself expresses tPA. Existence of mRNA for uPA was not investigated. However, the presence of uPA activity in culture medium and its regulation by hormones suffice to suggest that epididymis also expresses uPA. This is in accordance with the *in situ* hybridization results of Huarte *et al*.[7].

High level of PA activities in caput epididymis may be related to physiological function of the organ. During the process of epididymal maturation, dead and abnormal spermatozoa are selectively absorbed by epididymal epithelium[1]. PA activities may contribute to the clearance of these cells.

Spermatozoal maturation in epididymis is the downstream of spermatogenesis. Therefore, in coordination with spermatogenesis, spermatozoal maturation may also be modulated by the hormones important for spermatogenesis. In the present study, the effects of some hormones were investigated. Only hCG revealed significant regulatory effect on tPA and uPA expression in both caput and cauda epididymis. It is surprising that little effect of androgen has been observed, especially in cauda epididymis. Interpretation for this observation is that the effect of exogenous androgens may be masked by those endogenously synthesized by the organ. The existence of enzymes for androgen synthesis in rat and other mammalian epididymides has been demonstrated[2].

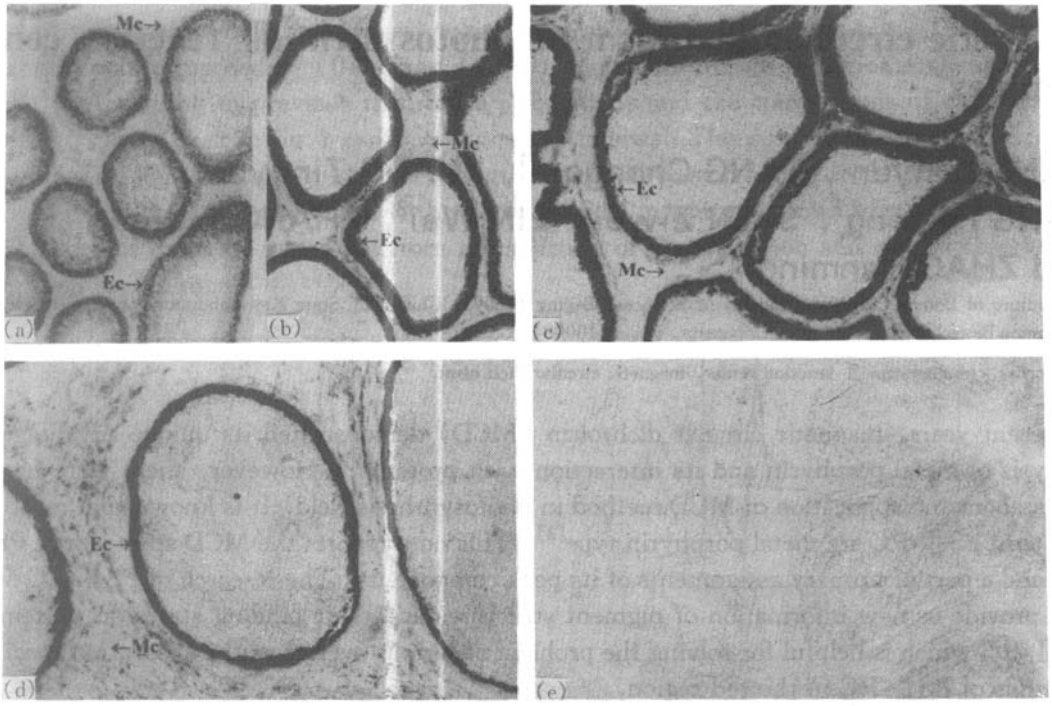

Fig. 3. *In situ* localization of PAI-1 mRNA in rat epididymis using dig-labeled cRNA probes. Positive staining in epithelial cells (Ec) of initial (a), caput (b), corpus (c) and cauda (d) segments was indicated by antisense cRNA probe. Low level of staining was also observed in mesenchymal cells (Mc) of different segments. No detectable staining existed in either epithelial cells (Ec) or mesenchymal cells (Mc) when sense cRNA probe was used (e).

Mechanism of the inhibitory effect of GnRH is not clear at this time.

By *in situ* hybridization, we have demonstrated that rat epididymis is also capable of expressing PAI-1 (fig. 3). Coordinated expression of PA and PAI-1 activities may play an important role in the fine regulation of proteolysis in epididymides.

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