Preliminary studies on the role of plasminogen activator in seminal plasma of human and rhesus monkey

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Two types of plasminogen activators (PA), tissue type (tPA) and urokinase type (uPA), were identified in the seminal plasma of both the human and the rhesus monkey. We studied the possible relationship between PA activities in the seminal plasma and the sperm counts and motility and demonstrated that: (i) PA activity in human seminal plasma from infertile patients was associated with immotile spermatozoa; (ii) the treatment of fertile men with testosterone enanthate (TE) to induce azoospermia was accompanied by an increase in seminal PA activity; (iii) when monomer T4 (isolated from multiglycosides of Tripterygium wilfordii) was administered to fertile male rhesus monkeys to induce azoospermia, PA activities in seminal plasma increased considerably; and (iv) immunocytochemistry studies showed that both uPA and PAI-1 antigens were localized on the surface of human spermatozoa, indicating that human spermatozoa were capable of binding uPA and PAI-1 through their receptors or forming a complex. These data demonstrate that seminal PA activity may be related to azoospermia, and possibly, to the fertilizing capability of spermatozoa in primates.

Key words: human/plasminogen activator/rhesus monkey/seminal plasma

Introduction

Extracellular proteolysis mediated by plasminogen activators (PA) is associated with many physiological processes (Dano et al., 1985; Saksea and Rifkin 1988; Vassalli et al., 1991), including some reproductive events such as ovulation (Beers, 1975; Strickland and Beers, 1976; Reich et al., 1985; Liu et al., 1987, 1991; Peng et al., 1993), luteolysis (Feng et al., 1993; Liu et al., 1995d), spermatogenesis (Lacroix et al., 1977; Vihko et al., 1984; Liu et al., 1995c) and embryo implantation (Strickland et al., 1976; Sappino et al., 1989, Liu and Feng, 1992). Several lines of evidence have shown that 10- to 100-fold higher levels of PA activities are secreted during stages VII and VIII of spermatogenesis in comparison with other stages by rat (Lacroix et al., 1977; Vihko et al., 1984; Toppari et al., 1986) and mouse (Liu et al., 1995c) seminiferous tubule segments. This coincides both with the initiation of the complex restructuring process leading to the passage of spermatagonia from the basal to the adluminal compartment and spermiation in the Sertoli cells (Clerment, 1972; Russell, 1977). Recent studies have demonstrated that vas deferens and seminal vesicles of mouse (Huarte et al., 1987) and rhesus monkey (H.-M. Zhou, T. Zhang and Y.-X. Liu, unpublished data) were also capable of secreting PA, which bind to ejaculated spermatoza (Huarte et al., 1987; Rekkas et al., 1991; Smokovitis et al., 1992) and may be involved in the process of fertilization (Lison et al., 1993; Huarte et al., 1993). However, most of these studies centered on non-primate animals and no direct evidence showed the relationship between seminal PA activities and the fertilizing capacity of spermatozoa. In this study we provide evidence to show the possible relationship between seminal PA activities and sperm counts/fertilizing ability in man. Experiments were also performed to study the changes of seminal PA activities during hormone- and drug-induced infertility with azoospermia in the human and the rhesus monkey.

Materials and methods

Hormones and reagents

Testosterone enanthate (TE) and monomer T4 (isolated from multiglycosides of Tripterygium wilfordii) were provided by the National Research Institute for Family Planning, Beijing, China. Acrylamide, N,N-methylene-bis-acrylamide, sodium dodecyl sulphate, tetra-methylene diamine (TEMED), ammonium persulphate and Coomassie Brilliant Blue were from Bio-Rad Laboratories (Santa Clara, USA). Human fibrinogen was obtained from KabiVitrum (Stockholm, Sweden). Human plasminogen, goat anti-human tissue-type tPA and PAI-1 polyclonal antibodies were obtained from BioPool Company (Umeå, Sweden). Thrombin and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO, USA). BCA protein assay reagents A and B were purchased from PIERCE Company (Illinois, USA). The vectastain ABC Kit was obtained from Vector Laboratories (Burlingame, CA, USA).

Subjects and semen sample collection

Human semen from patients undergoing investigation for involuntary infertility or from voluntary donors was obtained from the National
Research Institute for Family Planning, Beijing, China (Zhang et al., 1992). All samples were collected after 3–7 days abstinence. Liquefied samples from patients were centrifuged to separate the spermatozoa from seminal plasma. Sperm analysis was performed using the World Health Organization (WHO, 1987) criteria. If not used immediately, the seminal plasma for PA measurement was stored at −20°C.

To estimate the PA activity changes in human seminal plasma during TE-induced azoospermia, 16 voluntary fertile young fathers (25–30 years old, with at least one child) received a weekly 1 ml injection of 200 mg TE for 3 months based on a WHO Research Program (WHO Task Force Method for the Regulation of Male Fertility, 1990). Semen samples were collected and processed (WHO, 1987) before and after the TE injection.

**Rhesus monkey treatment**

To study the effect of monomer T4 isolated from multiglycosides of *Tripterygium wilfordii* (Zheng, 1985) on PA activities in rhesus monkey (*Macaca mulatta*) seminal plasma, three fertile male monkeys (5–6 years old) were fed with 10 μg/kg of T4 in the diet daily for 6 weeks. Semen samples were taken weekly by an electrical-stimulating method (Pu et al., 1994) and processed (WHO, 1987).

**SDS-PAGE and fibrin overlay**

PA in the seminal plasma was fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method described by Laemmli (1970). The total protein concentration of each semen sample was determined using the BCA protein assay reagent. Electrophoresis samples containing 20 μg of total protein were diluted with phosphate-buffered saline (PBS, pH 7.0) and adjusted to 2.5% (v/v) SDS. Electrophoresis was performed at 50 V overnight (~16 h) until the tracker dye reached the bottom of the gel. PA activity was then determined as follows: a fibrin/agar indicator gel was prepared as described by Granelli-Piperno and Reich (1978), containing plasminogen (50 μg/ml), fibrinogen (2.4 mg/ml) and thrombin (0.5 IU/ml). After electrophoresis, each SDS–polyacrylamide gel was rinsed twice (45 min each) with 2.5% (v/v) Triton X-100 to remove the SDS in the gel, followed by a rinse with distilled water, then laid carefully onto the fibrin/agar indicator gel and incubated at 37°C in a humid chamber until the lysis zone became visible. Quantification of the lysis area was performed by densitometry, control samples being ascribed a value of 1.

**Immunolocalization of uPA and PAI-1 antigens on human spermatozoa**

Human spermatozoa were collected by centrifugation of freshly liquefied semen from healthy donors. After washing three times by resuspension/centrifugation in PBS, the spermatozoa were smeared on slides by centrifugation in a cytopsin (5 min at 400 g) and fixed in 4% paraformaldehyde (in PBS) for 3 min at room temperature followed by five rinses in PBS. Polyclonal antibodies against human uPA, PAI-1 or tPA were applied to the slides and incubated for 2 h at room temperature, with normal rat serum added to slides as a control. After rinsing in PBS, fluorescein isothiocyanate (FITC)-protein A (20 μg/ml) was applied and incubated for 30 min at room temperature, followed by one wash in PBS. Slides were then mounted with glycerol and photographed under fluorescence microscopy. Antigens were located by the presence of fluorescent areas on the slide.

**Data analysis**

All experiments measuring PA activities were repeated at least three times. The relative mean values of PA activities (mean ± SEM) obtained by densitometry from fertile and infertile patients were statistically analysed using Student’s t-test. Some data were analysed by one-way analysis of variance. Differences among groups were detected by Tukey’s Multiple Comparison Test (Sokal et al., 1981). Differences between or among points were considered as significant when *P* < 0.05.

**Results**

**Plasminogen activator activities in human seminal plasma**

To examine the possible relationship between PA activity and sperm count in human semen, >60 semen samples were collected from healthy voluntary donors or from patients undergoing investigation for involuntary infertility (21–45 years old). PA activities in seminal plasma and sperm concentration (motile plus immotile) in the semen samples were compared. As shown in Figure 1, seminal plasma from six infertile patients (A–F) contained high levels of both tPA and uPA activities. The sperm concentration in the semen samples A–F was 0, 1.8, 2.0, 3.0, <20, <20×10^6 spermatozoa/ml respectively. The specimens obtained from four healthy men (G–J) contained much less tPA and uPA activity. The sperm concentration was 24, 33, 40, and 112×10^6 spermatozoa/ml respectively.

To obtain more information about the relationship between seminal PA activity and the number of immotile spermatozoa, the seminal plasma from 30 infertile patients were analysed. Representative samples are shown in Figure 2A, in which the numbers of immotile spermatozoa are indicated on the top of each lane of the figure. A higher number of immotile spermatozoa in the semen was associated with higher PA activities. An aliquot of serum (K) with a protein content equivalent to that of the seminal plasma showed no measurable amount of PA activity. The relationship between seminal PA activities and numbers of immotile spermatozoa in semen is also shown in Figure 2B in which the results from 30 infertile semen samples (including samples A–J in Figure 2A) were analysed. Semen samples from patients were centrifuged to separate the spermatozoa from seminal plasma. Sperm analysis was performed using the World Health Organization (WHO, 1987) criteria. If not used immediately, the seminal plasma for PA measurement was stored at −20°C.
Figure 2. Seminal plasma samples from infertile men were collected and handled as described in the Materials and Methods section. (A) PA activities shown on fibrin-agarose indicator gel. Lanes A–J are arranged in order according to the number of immotile spermatozoa (as indicated at the top of each lane) in the seminal plasma. Lane K shows PA activities in equivalent amount of serum. (B) Relative mean values of PA activities (mean ± SEM) from three groups of semen samples (obtained from 30 infertile men including lanes A–J in Figure 2A) based on the lysis area on the gel by setting the PA activity in a suitable semen sample as 1.0. Column A, semen samples whose immotile sperm concentration were >50×10^6 spermatozoa/ml semen; Column B, semen samples whose immotile sperm concentration are 10–49×10^6 spermatozoa/ml semen; Column C, semen samples whose immotile sperm concentration <10×10^6 spermatozoa/ml semen. Significant differences were found between groups A and B (P <0.01), B and C (P < 0.05) and A and C (P < 0.05).

samples were divided into three groups: group A (n = 10), samples in which the number of immotile spermatozoa was >50×10^6 spermatozoa/ml; group B (n = 10), samples in which the number of immotile spermatozoa was 10–49×10^6 spermatozoa/ml; group C (n = 10), samples in which the number of immotile spermatozoa was <10×10^6 spermatozoa/ml semen. The relative mean values of PA activities (mean ± SEM) from the three groups were calculated by densitometric measurement of the lysis area, the control sample being set at 1. It is apparent that, in infertile men, higher seminal PA activities are associated with greater numbers of immotile spermatozoa in corresponding semen samples.

**Effect of testosterone enanthate on PA activities in human seminal plasma**

The azoospermia in fertile men induced by weekly injection of TE has been well documented (WHO, 1990). Following a 4 month period of treatment with 200 mg TE weekly, all healthy fertile men were found to have azoospermia as assessed by semen analyses with conventional methods (Zhang, et al., 1992). Before and after the 4 month injection period, seminal plasma was collected from 16 volunteers and subjected to PA measurement. Representative samples are shown in Figure 3A. Both tPA and uPA activities in the semen after 4 months' treatment with TE (T) increased in comparison with that before injection (C), indicating that the increase in PA activities in seminal plasma occurred in parallel with the decrease in sperm number and azoospermia. Figure 3B shows the relative mean value of PA activities (mean ± SEM) from samples of all the 16 donors based on the lysis area on the gel; the PA activity before TE treatment (C) being ascribed a value of 1.0.

**Effect of monomer T4 on PA activities in monkey seminal plasma**

Monomer T4 isolated from multiglycosides of *Tripterygium wilfordii* is known to be capable of inducing oligozoospermia or azoospermia by damaging spermatozoa in mammals (Zheng, 1985). To examine whether the administration of T4 changes...
Figure 4. Three fertile male monkeys were fed with T4 (isolated from the multiglycosides of Trpaeusium wilfordii) daily with a dose of 10 μg/kg body weight. Seminal plasma samples were collected every week after the feeding (from Week 1 to Week 6; W = week) as described in the Materials and Methods section. PA activities in the seminal plasma were measured by fibrin-overlay method W1, the first week after T4 feeding; W6, 6 weeks after T4 feeding when sperm number decreased to zero in the semen. (A) PA activities shown on fibrin-agarose indicator gel. (B) relative mean value of PA activities (mean ± SEM) from seminal plasma of three monkeys based on the lysis area on the gel by setting the PA activity in the control (W1) as 1.0 (P < 0.05).

Figure 5. Human spermatozoa were collected and treated as described in the Materials and Methods section. Briefly, spermatozoa were first washed 3 times in PBS and then smeared on slides. After fixing in 4% paraformaldehyde and rinsing with phosphate-buffered saline (PBS), polyclonal antibodies anti-human uPA or PAI-1 were applied on the slides for 2 h under room temperature. For the control slide (C), normal rat serum instead of antibodies was used to determine non-specific binding. Slides were then rinsed with PBS. Fluorescein isothiocyanate (FITC)-protein A (20 μg/ml) was then applied and incubated for 30 min at room temperature. After washing with PBS, slides were mounted with glycerol and pictures were taken under a fluorescence microscopy. Antigens were located from the presence of fluorescent areas on the slide.

Immunolocalization of uPA and PAI-1 antigens on human spermatozoa

To study the localization of tPA, uPA and PAI-1 on the sperm surface, immunocytochemical staining was performed using human spermatozoa obtained from fertile healthy donors. As shown in Figure 5, the positive staining for uPA (A) and PAI-1 (B) was localized on the sperm head, midpiece and tail, while no positive staining for tPA was observed (not shown).

Discussion

In this preliminary study we have demonstrated that human seminal plasminogen activator activities may be related to the immotile sperm counts and azoospermia. This result is further confirmed by the fact that treatment of fertile men with TE to
induce azoospermia was accompanied by an increase in the seminal PA activities. Furthermore, during administration of monomer T4 to fertile male rhesus monkeys to induce azoospermia, PA activities in the seminal plasma also increased considerably. These preliminary data suggest that seminal PA activities may be related to the azoospermia in primates. However, the mechanism of the effect of PA on azoospermia is still unclear, as is the type of activator which may be involved. Epithelial cells of epididymis, vas deferens, seminal vesicles and the prostate gland have been reported to be capable of synthesizing and secreting PA and PA-inhibitors (Kirchheimer and Binder 1983; Rennie et al., 1984; Huarte et al., 1987; Reese et al., 1988; Martikainen et al., 1989; Andreasen et al., 1990; Wilson et al., 1990; Zhou and Liu, 1995). However, the spermatozoa present in the epididymis before ejaculation are not in contact with the components of the seminal plasma, which originate from the vas deferens, seminal vesicles and prostate glands. One may speculate that PA activities in epididymis are more likely to be involved in the appearance of azoospermia. Immunocytochemistry and in situ hybridization studies indicate that positive staining of antigens and mRNA of tPA, uPA and PAI-1 are localized on the epithelial cells of the epididymis of both rat and monkey. Staining of uPA and PAI-1 antigens was also observed on the surface of the spermatozoon (Zhou and Liu, 1995). However, it is difficult to elucidate the possible relationship between the PAs/PAI-1 activities and the behaviour of spermatozoa before ejaculation in these experiments (Zhou and Liu, 1995). It is well established that the epididymis is an androgen-dependent organ and androgen receptor mRNAs have been also identified in the epididymis epithelial cells of both rat and monkey (Zhou and Liu, 1995). These data, together with the observation that the administration of TE induces azoospermia with increased PA activities in seminal plasma, raise the question whether PA synthesis in epididymis may require androgen stimulation. This would be consistent with the fact that injection of TE induced oligozoospermia (Wallace et al., 1992; Zhang et al., 1992) and PA/PA-inhibitor activities in spermatozoa and seminal plasma showed a positive correlation with blood testosterone concentrations. (Rekkas et al., 1991).

It has been suggested that plasminogen activators in spermatozoa play a role in the process of fertilization (Huarte et al., 1987, 1993; Rekkas et al., 1991, Smokovitis et al., 1992; Lison et al., 1993). The positive staining of uPA and PAI-1 antigens observed on the sperm surface in this paper is consistent with the previous reports. Since it is well known that uPA acts by binding to its specific receptors (Blasi et al., 1990), our result implies that uPA receptors may exist on sperm surface. Furthermore, our data clearly show that PAI-1 is also capable of binding to human sperm. PAI-1 is an efficient inhibitor of both tPA and uPA by binding to them to form a PA–PAI-1 complex. It is therefore possible that uPA and PAI-1 on the human sperm surface may function in a counterbalanced and coordinated way. The mechanism by which uPA and PAI-1 cooperate to influence sperm behaviour is interesting, and worthy of further investigation. Smokovitis et al. (1992) identified both tPA and uPA on the plasma membrane and outer acrosomal membrane of human spermatozoa by using a spectrophotometric method with chromogenic substrate S-2215. We did not identify the presence of tPA on the spermatozoa from seminal plasma by using a specific polyclonal antibody against tPA. However we have previously identified tPA antigen on the spermaticids of monkey testis (Liu et al., 1995a).

In-vitro cultured Sertoli cells from immature rat (Lacroix et al., 1977), mouse (Liu et al., 1995c) and rhesus monkey (Liu et al., 1995a) testes have been shown to produce PA, predominately as tPA. Other studies indicated that seminiferous peritubular cells and Leydig cells in culture do not secrete detectable levels of PA (Lacroix et al., 1979; Liu et al., 1995b). Sertoli cells therefore may be the chief source of PA in testes. The tPA activity in Sertoli cells may be associated with restructuring the seminiferous tubules during spermatogenesis. Since tPA secretion is much more sensitive to follicle stimulating hormone (FSH) stimulation in cultured seminiferous segments from rat and mouse at stages IX–XII (Liu et al., 1995c), when residual bodies of released spermatozoon are absorbed into Sertoli cells, activation of the PA system at these stages may also be essential for the process of the residual body degradation. The data provided in this study, together with earlier reports, raise interesting questions, such as whether tPA activity mainly produced by Sertoli cells is responsible for the processes of spermatogenesis and spermiation, while the sperm-bound uPA activity secreted by the male genital tract may be involved in the processes of fertilization. If this is the case, it is important to know the roles of uPA in testis and tPA in seminal plasma; the type of PA responsible for inducing oligozoospermia/azoospermia, and its mode of action on the spermatozoa.

The fact that some semen samples collected from normal fertile voluntary donors also contained relatively high levels of PA activity (such as the sample on the rightest control lane (C) in Figure 3A), while PA activity was almost under detectable level in semen samples of some infertile men (such as lanes I and J in Figure 2A) implies that the relationship between seminal activity and oligo/azoospermia is not clear cut. Further work is required to clarify the situation.

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References


PA in human and monkey seminal plasma


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