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Functionally inactive protein C inhibitor in seminal plasma may be associated with infertility

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Protein C inhibitor (PCI) has been found in seminal plasma and is considered to protect intact surrounding cells and seminal plasma proteins from possible proteolytic damage. In the present study, we showed that although the antigenic levels of PCI in two seminal plasma samples from patients with infertility were normal or slightly elevated, their inhibitory activities toward urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) were absent. In contrast, uPA and tPA proteolytic activities in these two samples were 20–60-fold higher than that from normal volunteers. A time-course analysis of PCI–uPA complex formation showed that >80% of the complex had been formed within 15 min in normal seminal plasma in the presence of heparin, compared with the total complex formed after 150 min incubation, whereas no response to heparin stimulation was observed in the assays with the two patient samples. Similarly, >90% of PCI–tPA complex was formed after 30 min of heparin stimulation in normal seminal plasma but no response was observed in the two patient samples. Kinetic assays of PCI inhibitory function in the presence of activated protein C (APC) showed that PCI inhibitory activity in the two patient samples was absent and not stimulated by heparin. Western blotting also showed that most of the intact PCI molecules, in normal samples, formed complexes with either uPA or tPA but there was no complex formed in one of the two patient samples and very little complex was observed in the other, suggesting that PCI in the two patient samples is inactive. These results suggest that the presence of functionally inactive PCI in seminal plasma may be associated with infertility.

Key words: infertility/protein C inhibitor/seminal plasma/urokinase plasminogen activator

Introduction

Protein C inhibitor (PCI) is a single polypeptide chain glycoprotein of 387 residues, as predicted from cDNA sequence (Suzuki *et al*., 1987), with five potential glycosylation sites at Thr20, Ser39, Ser232, Thr245 and Ser321. PCI belongs to the superfamily of serine protease inhibitors (serpins) and inactivates a wide range of serine proteases including tissue kallikrein (Espana *et al*., 1995), thrombin (Rezaie *et al*., 1995), urokinase plasminogen activator (uPA), tissue-type plasminogen activator (tPA), factor XIa, factor Xa (Espana *et al*., 1989), and activated protein C (APC) (Marlar and Griffin, 1980). PCI forms sodium dodecyl sulphate (SDS)-stable complexes with a very wide range of serine proteases and plays an important role in the regulation of coagulation and fibrinolysis. PCI is synthesized principally in hepatocytes.

Recently, PCI was found to be synthesized in seminal vesicles where it undergoes glycosylation and is then secreted into seminal plasma, where the function of the serpin is not yet clear in relation to reproduction. PCI is present at high concentrations (3–4 μM) in seminal fluid in both a high molecular mass and low molecular mass form (Christensson and Lilja, 1994). PCI–uPA and PCI–tPA complexes have been identified by sandwich enzyme-linked immunosorbent assay (ELISA) and have been shown to occur in large amounts in normal seminal plasma (Espana *et al*., 1991). Apart from the complexes formed between PCI and uPA/tPA in seminal plasma, PCI was also found to inhibit prostate-specific antigen (PSA)-catalysed degradation of insoluble semenogelin I + II by forming a PSA–PCI complex (Kise *et al*., 1996). It was suggested by the same authors (Kise *et al*., 1996), that PCI binds to semenogelin II and regulates the PSA-catalysed degradation of semenogelin in seminal plasma. Moreover, tissue kallikrein (tKK) in seminal plasma occurs as a complex with PCI (Espana *et al*., 1995), confirming that PCI is a major regulator of serine proteases in seminal plasma. However, the major physiological role of PCI in seminal plasma has never been fully addressed, especially in the studies of human reproduction.

PCI is found in human urinary system as well as blood and seminal plasma. Although it appears to be only a weak inhibitor of uPA when tested *in vitro* with the purified proteins, large amounts of PCI–uPA complex were found in normal human urine (Giegar *et al*., 1991). Results from in-situ hybridization experiments have shown that PCI is synthesized in the kidney, where it might function as a regulator of uPA and tPA by forming PCI–uPA/tPA complexes which may be cleared by secretion into urine.

Like anti-thrombin and heparin cofactor, PCI binds to heparin, which greatly stimulates its inhibitory activities. Binding of heparin to PCI at two regions, A-helix (residues 1–11) and H-helix (residues 264–280), has been identified by site-directed mutagenesis (Elisen *et al*., 1996). The effectiveness of heparin stimulation of PCI depends on the formation of a ternary complex of inhibitor, protease and glycosaminoglycan (Pratt and Church, 1992; Pratt *et al*., 1992).

In the present study, we report two cases of dysfunctional PCI found in seminal plasma of two infertile patients. These dysfunctional PCIs do not form complexes with either uPA or tPA, nor do they respond to heparin stimulation. Normal or slightly elevated PCI antigenic values were found in the seminal plasma of the patients, but no inhibitory activities toward the serine proteases, uPA and tPA, could be found. Moreover, immunoblotting showed that all the PCIs in the two samples were present in functionally inactive forms. These findings suggest a physiological role for PCI in reproduction.

Materials and methods

Reagents

uPA, tPA, bovine serum albumin (BSA), Tween 20, *o*-phenylenediamine (OPD) and heparin were obtained from Sigma Chemical Co (Poole, Dorset, UK). Goat anti-uPA [purified immunoglobulin (Ig)G form], tPA (purified IgG form) and rabbit anti-goat Ig conjugated with horseradish peroxidase (HRP) were purchased from Incstar (Workingham, Berkshire, UK). Spectrozyme UK, spectrozyme tPA, goat anti-human protein C (purified IgG form), activated protein C (APC) and PCI deficiency plasma were obtained from Alpha Lab (Eastleigh, Hampshire, UK).

PCI purification

Purification of human PCI from citrated plasma was carried out as previously described (Suzuki *et al*., 1983). Briefly, to human plasma containing benzamidine chloride (10 mM), di-isopropyl fluorophosphate (1 mM), phenylmethylsulphonyl fluoride (PMSF, 1 mM) and soybean trypsin inhibitor (50 μg/ml), $BaCl_2$ was added to a final concentration of 20 mM to precipitate protein C. This was followed by a 2-step polyethylene glycol (PEG) precipitation [6% (w/v)]. PCI contained in the precipitate was then purified chromatographically [diethylaminoethy(DEAE)–sepharose CL-6B, Dextran sulphate–agarose, ultrogel ACA-44 and DEAE–sephacel]. After the last DEAE–sephacel chromatography, fractions containing PCI activity were pooled and concentrated for subsequent experiments. Protein concentration was determined using a protein assay kit (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

Sample collection

Sperm samples from 10 patients with infertility and 10 from normal volunteers were obtained from the Third Hospital, Beijing Medical University, Beijing, China and treated as described previously (Zhang *et al*., 1992). All samples were collected after 4.5–7 days abstinence and clinical diagnostic assays were carried out to determine semen volume, liquefaction time, sperm concentration (sperm count) and sperm motility. The liquefied semen samples were spun by a centrifugation at 13 000 rpm for 15 min at 4°C to separate spermatozoa from seminal plasma. Sperm analysis was performed using the World Health Organization (WHO, 1987) criteria. Clinical tests showed that the liquefaction time of all patient samples was <40 min, sperm concentration was $>17\times10^6$, but sperm motility (12–40%) was far below the normal controls (50–75%), e.g. in sample 5 sperm motility was 13% and in sample 7 only 12%. Spermatozoa and seminal plasma were stored separately at –80°C until use.

uPA, tPA and PCI assays

uPA activity was determined using commercially available uPA (assayed by active site titration) as a standard sample and the activities of uPA in seminal plasma samples were assayed using Spectrozyme UK (Jankun *et al*., 1997). Briefly, aliquots of stock uPA (1 μg/ml) with decreasing amounts of the enzyme was incubated in a 96-well microplate with 50 μl of 2.5mM Spectrozyme UK [carbobenzyl-L-(γ)-Glu-(α-tBuO)-Gly-Arg-*p*-nitroanilide·$2C_2H_5OH$] for 10 min. Absorbance, which is inversely proportional to the uPA inhibitory activity, was measured at 405 nm in a microplate reader. uPA activity of seminal plasma was determined using a standard curve assayed from the known activity of the standard sample which was assayed along with the increasingly diluted samples. tPA proteolytic activity was assayed in a similar way using Spectrozyme tPA.

Quantification of tPA and uPA was carried out with sandwich ELISA (Espana *et al*., 1991). Briefly, rabbit anti-human uPA antibodies diluted (1:1000) in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6) were added to the wells of an ELISA plate (Falcon; Becton Dickinson Labware Company, Oxnard, USA) and incubated overnight at 4°C. Non-specific binding sites were blocked by the addition of 1% (w/v) BSA in PBS–0.05% (v/v) Tween 20 with incubation at 37°C for 1 h. Serial dilutions (1/100, 1/1000, 1/10 000, 1/100 000 and 1/1 000 000) of seminal plasma were added to the wells which had been coated with capture antibodies and incubated at 37°C for 1 h more. A standard curve was constructed for known concentrations of uPA. Goat anti-human uPA (1:1500) was added to bind to the antigens, followed by a further incubation for 1 h at 37°C. Mouse anti-goat Ig conjugated with HRP (1:2000) was then added for 1 h at 37 °C. Three washes were carried out in PBS–0.05% (v/v) Tween 20 after each step. Finally the plates were developed with OPD and read at 490 nm in a plate reader. The detection limit of this assay was ~1 ng/ml. tPA and PCI antigen values were determined in a similar way to uPA.

PCI–uPA and PCI–tPA complex assay

Quantification of PCI–uPA complexes was carried out in a similar manner to that described above. The primary antibodies (or capture antibody) used in the assay were rabbit anti-human PCI, which was used to coat ELISA plate wells at 1:1000 dilution in coating buffer. Serially diluted seminal plasma samples were added after non-specific binding sites were blocked with BSA. The secondary antibody was goat anti-human uPA. This was followed by mouse anti-goat Ig conjugated with HRP. The plates were developed using OPD as described above.

Quantification of PCI–tPA complexes was carried out similarly to that for the detection of PCI–uPA complex except that the secondary antibody used in the assay was goat anti-human tPA. Standard curves of purified plasma PCI were included in each assay. Detection limit was ~1 ng/ml which was similar to the uPA/tPA assays.

Heparin stimulation of PCI activity

Heparin stimulation of PCI was carried out to determine the kinetic response to heparin of PCI in seminal plasma from two patients (numbers 5 and 7). In the stimulation experiment, heparin was added to a final concentration of 10 IU/ml. Normal seminal plasma and PCI-deficient plasma were used as controls. Samples were assayed with or without addition of heparin. Samples were incubated at 37°C and aliquots were withdrawn at intervals and the amount of complex formed was determined using the sandwich ELISA.

In another set of experiments, heparin stimulation kinetics were determined with different concentrations of heparin (Radtke *et al*., 1996). Seminal plasma (fixed concentration) was incubated with different concentrations of heparin and then human APC (fixed concentration) was added. Incubation was carried out for 20 min at 37°C. At intervals, aliquots were withdrawn from the reaction mixture and transferred to 1% casein solution. The final quantities of PCI, APC and casein were 15 ng, 0.25 ng and 0.9% (w/v) respectively, in a total reaction volume of 150 µl. The time required for 50% APC inactivation ($t_{1/2}$) was calculated and converted into relative first order rate constants using the equation $k_1 = \ln 2/t_{1/2}$.

Detection of PCI–uPA/tPA complex by Western blotting

Detection of PCI–uPA or PCI–tPA complexes by Western blotting was carried out as described previously for the detection of C1-inh/C1s complex (He and Lin 1998). Briefly, seminal plasma proteins were separated by 7.5% polyacrylamide gel electrophoresis (PAGE). Following the electrophoresis, protein bands were transferred onto a nitrocellulose sheet (Hybond-Super; Amersham International plc, Little Chalford, UK). A primary antibody (goat anti-human PCI) was used to bind PCI antigen so that both PCI–uPA and PCI–tPA complexes could be detected. The secondary antibody used in the detection was anti-goat Ig conjugated with HRP. The membrane was then exposed to high performance chemiluminescence detection film (Hyperfilm ECL; Amersham International plc).

Results

uPA and tPA antigen and proteolytic activity

The mean level of uPA in 10 samples from normal volunteer seminal plasma was 25.5 ng/ml (range 22 ± 5.8 to 29 ± 3 ng/ml). uPA was present either as a complex with PCI or in free form. Assays of the 10 samples from patients with infertility showed a similar mean concentration of 28.5 ng/ml (range 22 ± 4 to 34 ± 4 ng/ml) (Figure 1A). There was no significant difference in uPA antigenic values between the two groups. However, in 10 normal samples, uPA proteolytic activity was <0.004 IU/ml (average of 0.00284 IU/ml), whereas the enzymatic activity from many of the patient samples was much higher, up to 0.087 IU/ml. The normal amount of uPA antigen but low enzymatic activity in normal seminal plasma indicated that uPA activity was under appropriate control. Although samples from patients 5 and 7 contained only slightly higher amounts of uPA antigen than the rest of the patient samples (Figure 1A), their enzymatic activities were significantly higher than any others.

tPA antigen quantification in the normal samples showed that tPA antigenic values were ~5–10-fold higher than those of uPA. The mean tPA antigen values in the normal samples were slightly lower than that of patient samples (469.2 ng/ml for normal samples and 398.6 ng/ml for patient samples) (Figure 1B). Assays for proteolytic activity, however, showed that most of the patient samples contained 1.5–6-fold higher activity than the control samples. This is similar to the uPA assays, which showed extraordinarily high uPA activity in samples from patients 5 and 7.

The PCI antigen sandwich ELISA showed that seminal plasma contained an average of 759 ng/ml of PCI from normal samples and 783.9 ng/ml from patient samples (Figure 1C). The reliability of the above measurements was tested by calculating the coefficient of variation; these were <21% in all the assays.

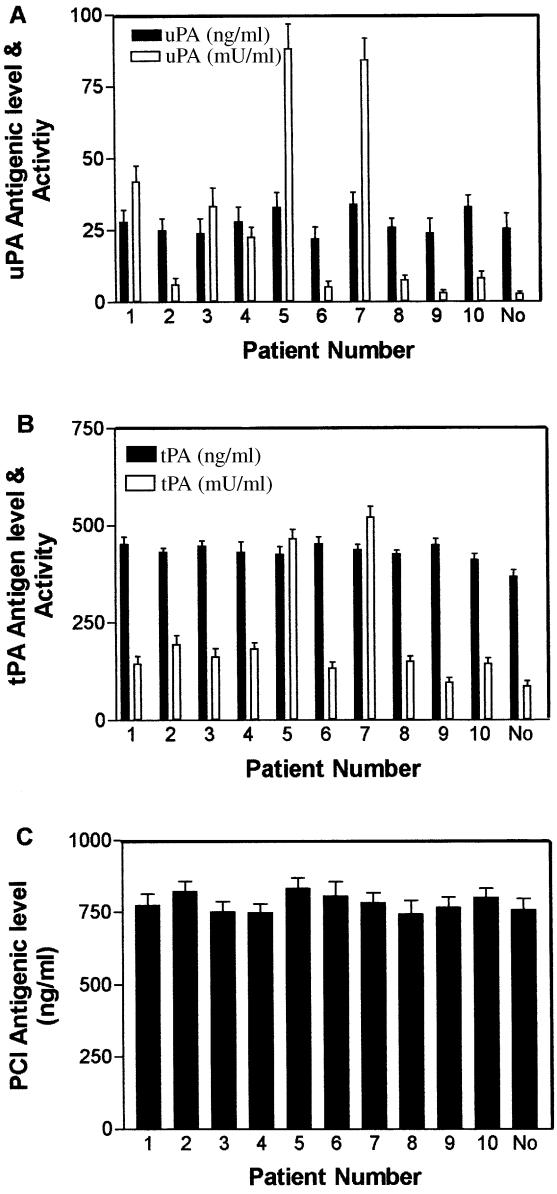
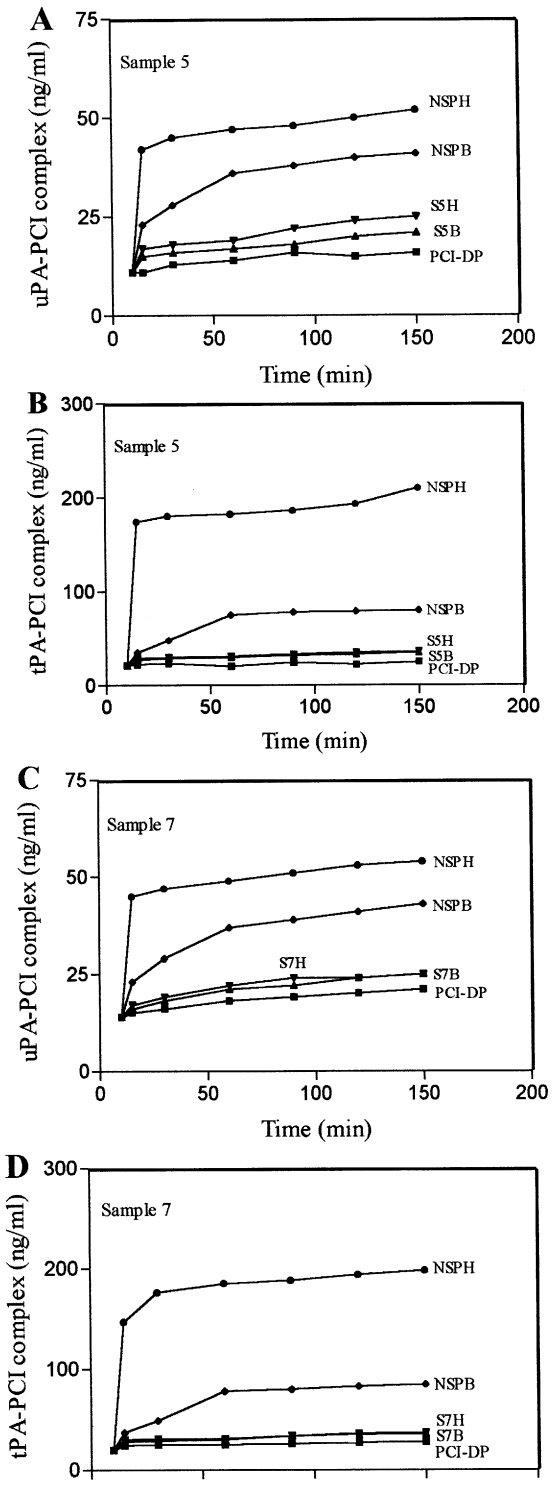


Figure 1. Antigenic concentrations of urokinase plasminogen activator (uPA), tissue-type plasminogen activator (tPA) and protein C inhibitor (PCI) in seminal plasma and the proteolytic activities of the activators. The antigenic concentrations in each bar are averaged from five readings; mean PA activities are averaged from three separate assays. The number under each antigenic level and proteolytic activity bar is the patient number and the normal control (averaged from 10 samples) is expressed as 'No'.
(**A**) uPA antigenic concentration (ng/ml) and activity (mIU/ml).
(**B**) tPA antigenic concentration (ng/ml) and activity (mIU/ml).
(**C**) PCI antigenic concentration (ng/ml).

Time-course of complex formation

The time-course of PCI–uPA and PCI–tPA complex formation from normal seminal plasma (NSP) showed that PCI responds to heparin stimulation (NSPH, Figure 2A). In the presence of 10 IU/ml heparin, 80.7% PCI–uPA complexes were formed in 15 min incubation comparing with the total complex formed in 150 min of incubation time, while only 32.7% PCI–uPA

complexes were formed in buffer without heparin stimulation (NSPB, Figure 2B). No response to heparin stimulation was observed in the assays with PCI-deficient plasma (PCI–DP, Figure 2A), nor in seminal plasma from patient 5 (S5H and S5B, Figure 2A). In the PCI–tPA assay with the same samples, 90.2% of PCI–tPA complexes were formed in 30 min stimulation by heparin in normal seminal plasma (NSPH, Figure 2B) whereas only 24.9% of the complexes were formed within the same time without heparin stimulation. PCI-deficient plasma and sample 5 showed no response to heparin stimulation.



Similarly, complex formation in either assay using seminal plasma from patient 7 could not be stimulated by heparin (Figure 2C and D). Assays carried out for the rest of the patient samples showed that most of the samples could be stimulated by heparin but the stimulation was less than seen with normal seminal plasma.

Kinetics of heparin stimulation of PCI activity

Kinetic assays for heparin stimulation of PCI activity in normal seminal plasma, PCI deficient plasma and patients 5 and 7 showed similar results to those seen with the time-course of complex formation (see above). Under the stimulation of different concentrations of heparin (0, 1, 2, and 5 IU/ml), normal seminal plasma showed a linear response to the stimulation. Samples from patients 5 and 7 showed almost no response to heparin stimulation. The k_1 values from the two samples showed almost no increase in the presence of different concentrations of heparin. That was also the case with PCI-deficient plasma (Figure 3). Assays for other patient samples showed that most of them had different levels of response to heparin stimulation. For instance, samples 1, 3 and 4 showed significantly lower responses than the NSP (Figure 3).

Complex detection in seminal plasma

SDS–PAGE (under reducing conditions) followed by Western blotting was carried out to detect the presence of complexes of PCI–uPA or PCI–tPA. The purified PCI sample from blood contains two forms, the cleaved form and the intact form (Figure 4, lane 1). Normal seminal plasma contains PCI–uPA and/or PCI–tPA complexes (>85 kDa), some intact PCI and a major cleaved form of PCI (~50 kDa) (Figure 4, lanes 2 and 4). It is not clear how the cleaved form is produced, but it may have originated from dissociation of PCI–serine protease complexes after long incubation periods. There is almost no complex formed in the seminal plasma of patient 5 (Figure 4, lane 5). This sample contains some intact PCI and a large quantity of cleaved form. Similarly, very little complex is formed in the sample of patient 7 and almost no intact form was present in this sample (lane 3). Although sample 5 contains a fraction of PCI which appears intact, it does not react with

Figure 2. Time-course of stimulation of protein C inhibitor (PCI)–urokinase plasminogen activator (uPA) or PCI–tissue-type plasminogen activator (tPA) complex formation by 10 IU/ml of heparin. NSPH = normal seminal plasma with heparin stimulation; NSPB = normal seminal plasma without heparin stimulation; S5H = seminal plasma from patient 5 with heparin stimulation; S7H = seminal plasma from patient 7 with heparin stimulation; S5B = seminal plasma from patient 5 without heparin stimulation; S7B = seminal plasma from patient 7 without heparin stimulation; and PCI–DP = PCI deficiency plasma with heparin stimulation. (**A**) PCI–uPA complex formation with different incubation times in the presence or absence of heparin. (**B**) PCI–tPA complex formation with different incubation times in the presence or absence of heparin. (**C**) PCI–uPA complex formation with different incubation times in the presence or absence of heparin. (**D**) PCI–tPA complex formation with different incubation times in the presence or absence of heparin. The results are the average of five separate experiments, each of which contained three replicates.

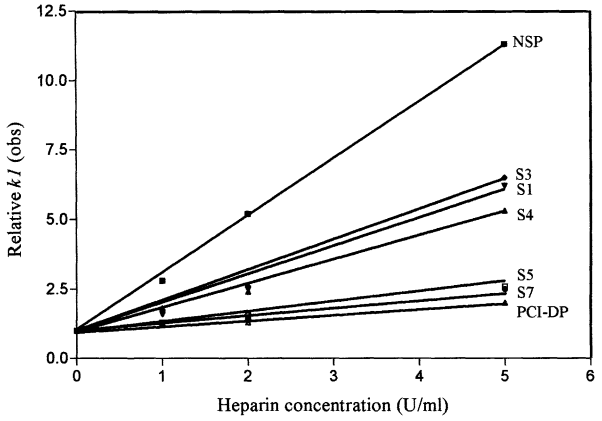


Figure 3. A plot of relative k_1 values versus heparin concentration (IU/ml). NSP = normal seminal plasma; S1, S3, S4, S5 and S7, seminal plasma of patients 1, 3, 4, 5 and 7; PCI–DP = protein C inhibitor (PCI) deficiency plasma.

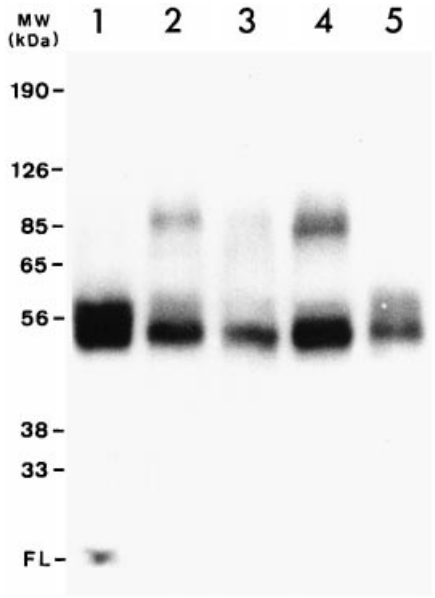


Figure 4. Western blot of seminal plasma showing protein C inhibitor (PCI) and PCI– urokinase plasminogen activator (uPA)/ PCI–tissue-type plasminogen activator (tPA) complexes. Each loading contained 20 μl of seminal plasma (~16 ng of PCI antigen), 20 μl of 50mM Tris–HCl, pH 7.6, and 20 μl of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer [4 M urea, 125 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 0.8% (w/v) SDS, 2% (v/v) β-mercaptoethanol and 0.002% (w/v) Bromophenol Blue]. The band at 55–60 kDa is the intact PCI and that at 50 kDa is the cleaved form of PCI. PCI–uPA and PCI–tPA complexes are at >85 kDa. Lane 1, purified plasma PCI; lanes 2 and 4, normal seminal plasma; lanes 3 and 5, seminal plasma of patients 7 and 5 respectively.

either uPA or tPA in seminal plasma as shown in Figures 2 and 3. Sample 7 contains very little intact protein and therefore almost no PCI–uPA/tPA complexes can be formed as observed in the blot. These results suggest that PCI in sample 5 may be dysfunctional in the intact form which can be cleaved by serine protease(s), such as uPA and tPA, without the formation of serpin–enzyme complexes. In sample 7, however, the dysfunctional PCI may be due to a low level of intact protein which is probably cleaved by proteases at an early stage. In addition, although equal amounts of seminal plasma were loaded in SDS–PAGE, sample 7 seems to have lower PCI antigenic level than other samples and this may be due to self-aggregation of the serpin, such as the formation of inclusion bodies of α1-antitrypsin mutants, Phe51 or 52 deletion (Cox *et al*., 1986), and thus it may have been removed by a brief centrifugation after sample heating before electrophoresis.

Discussion

This is the first report of a deficiency of PCI activity in seminal plasma that results from the production of dysfunctional PCI, a form which can be cleaved by serine protease(s) without the formation of serpin–enzyme complexes, and has no response to heparin stimulation functionally. This may be due to the absence of the inhibitory domain of most PCI molecules and presence of dysfunctional intact PCI in the patient seminal plasma. These observations lead us to suggest that PCI plays a role in maintenance of active sperm cells by regulating proteolytic activities of serine proteases including uPA and tPA. The presence of uPA and tPA in seminal plasma and hydrolysis of structural protein in a zone centred around the sperm head region by plasminogen activators (PA) has been recognized in mouse (Huarte *et al*., 1987) and in humans and rhesus monkeys (Liu *et al*., 1996). Localization of tPA and uPA in the plasma membrane and outer acrosomal membrane of human and boar spermatozoa suggested that the activators might be relevant to fertilization (Smokovitis *et al*., 1992). Furthermore, the activities of PA in spermatozoa had suggested the importance of the activators in the whole process of fertilization (Rekkas *et al*., 1991). Although the precise role of PCI in human seminal plasma has yet to be fully addressed, the presence of large amounts of PCI–uPA and PCI–tPA complexes in normal seminal plasma suggests that PCI may play a role in regulating serine protease activity locally and therefore protects spermatozoa from proteolytic damage by serine proteases, such as uPA and tPA. Our previous study suggested that seminal PA activity might be related to azoospermia and possibly to fertilizing capability of spermatozoa in primates (Liu *et al*., 1996). However the mechanism of the effect of PA on azoospermia is not clear. In the present study, we have suggested that uncontrolled human seminal PA activities are related to the production of infertile spermatozoa: a possible molecular mechanism giving rise to increase of uPA and tPA activities in seminal plasma is a functional deficiency of PCI.

PCI is a heparin binding molecule and its inhibitory activity can be stimulated up to 10-fold with heparin. Heparin binding sites have been studied extensively and residues 1–11 in the A-helix and 264–280 in the H-helix (Elisen *et al*., 1996) have been suggested as the binding sites. The PCI from two patient samples showed no response to heparin stimulation, suggesting that either the heparin binding sites had been destroyed or the inhibitory domain had been lost. A Western blot of PCI–uPA and PCI–tPA complexes and assays for the complexes confirmed the latter possibility that most of the inhibitory domains of PCI molecules in the two samples had been cleaved

off (Figure 4) and, therefore, there was almost no complex formation between PCI and uPA or tPA.

The finding of the functional PCI deficiencies in seminal plasma from two patients with infertility suggests that PCI is an important serpin in seminal plasma to protect spermatozoa from damage by proteolytic activities of serine proteases, especially uPA and tPA since these two enzymes are the major serine proteases in seminal plasma. This suggestion is supported by the observation that a large amount of PCI–uPA and PCI–tPA complexes are normally present in seminal plasma and that uPA and tPA activities are generally low because of the formation of these complexes. In the seminal plasma from two patients (samples 5 and 7) with infertility, however, the proteolytic activity of uPA and tPA was about 30 times as high as the normal sample, indicating that the spermatozoa may be damaged by the proteolytic activity of the two enzymes. The results from clinical tests of the samples also support this deduction with only 10–15% of normal sperm motility observed in these two samples.

A cleaved form of PCI exists in normal seminal plasma and this is also seen in purified PCI from a normal blood sample (Figure 4). It is known that PCI–protease complexes can be dissociated by alkaline treatment so that a lower molecular weight form of PCI can be obtained (Christensson and Lilja, 1994). The cleaved form observed on the western blot could have come partially from the dissociation of PCI–protease complexes. However, most of the cleaved PCI in the two patient samples may have come from mutation(s) in the inhibitory domain so that the serine proteases cleaved the inhibitor without forming inhibitor–enzyme complexes. This suggestion was supported by the observations that almost all intact PCI in normal samples had been used in the formation of PCI–PA complexes whereas there were almost no complexes formed in the patient samples.

However, PCI may not be the only regulator for serine protease activities in seminal plasma. In-situ hybridization showed that *tPA* mRNA was localized in Sertoli cells and plasminogen activator type 1 (*PAI-1*) mRNA was expressed mainly in germ cells except late spermatids (Zhang *et al*., 1997a) in rhesus monkeys and the two mRNAs were also found in adult monkey epididymis (Zhang *et al*., 1997b). These results and the present study have made it clear that the activities of plasminogen activators in seminal plasma must be controlled at certain levels to maintain normal liquefaction (Arnaud *et al*., 1994) and therefore normal sperm activity. In an earlier work we had shown that both uPA and tPA activities were required for stimulation of human sperm motility (Hong *et al*., 1985). However, if the activities of plasminogen activators are not under appropriate control, such as the PCI functional deficiency in patient samples 5 and 7, the spermatozoa may be damaged by the uncontrolled proteolytic activities of the serine proteases.

In conclusion, PCI plays an important role in many physiological processes. It modulates proteolytic activities of many proteases in plasma and uPA activity in the urological system. PCI is a major serine protease inhibitor in seminal plasma and a proportion is found in complexes with uPA and tPA. The mechanism by which this serpin maintains active spermatozoa during spermiogenesis and fertilization may be by preventing hydrolytic damage to the surrounding tissues and glycoproteins and possibly regulating corona radiata or zona pellucida breakdown. It is therefore reasonable to believe that PCI plays a role in the human reproductive system by regulating PA proteolytic activities. The finding of the PCI functional deficiencies in two patients with male infertility further supports the suggestion that PCI is required for maintaining active spermatozoa in the human reproductive system.

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