Measurement of Protein C Inhibitor in Seminal Plasma Is Useful for Detecting Agenesis of Seminal Vesicles or the Vas Deferens

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ABSTRACT: Protein C inhibitor (PCI), a plasma serine protease inhibitor of activated protein C, is present at high concentrations in the seminal plasma of normal subjects and is decreased in some infertile patients. We measured the concentrations of PCI, prostate-specific antigen, and fructose in the seminal plasma of infertile patients (n = 125) and of normal subjects (n = 13). We also measured time-dependent changes in the concentrations of PCI and fructose in seminal plasma after ejaculation. A weak correlation was found between the levels of PCI and fructose ($r = 0.268$, $P = 0.016$). The PCI level in seminal plasma of patients with seminal vesicle and/or vasal agenesis was significantly lower ($P < .01$) than in normal subjects. The level of fructose in seminal plasma decreased in vitro in a time-dependent manner after ejaculation, whereas the concentration of PCI was stable at 48 hours after ejaculation. These data suggest that PCI in seminal plasma, as well as fructose, may become one of the markers for agenesis of seminal vesicles and/or the vas deferens.

Key words: Protein C inhibitor, fructose, prostate-specific antigen, male infertility, seminal vesicle, seminal plasma.

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Protein C inhibitor (PCI), a member of the plasma serine protease inhibitor (SERPIN) family, is the major plasma inhibitor of activated protein C (APC). APC is the major proteolytic enzyme of the protein C anticoagulant pathway (Esmon, 1987, 1989). PCI also inhibits thrombin (Suzuki et al, 1984, 1990), thrombin–thrombomodulin complex (Rezaie et al, 1995), factor Xa (Suzuki et al, 1984, 1990), factor XIa (Meijers et al, 1988; Suzuki et al, 1990), urokinase (u-PA) (Stief et al, 1987; Heeb et al, 1987), tissue plasminogen activator (t-PA) (Stief et al, 1987; Heeb et al, 1987), plasma kallikrein (Meijers et al, 1988; Suzuki et al, 1990), trypsin-like human prostatic kallikrein (THPK) (Deperthes et al, 1996), and several other plasma serine proteases. Plasma PCI seems to be produced mainly in the liver (Morito et al, 1985).

It was reported that PCI is present at a high concentration (160–220 μg/mL) in seminal plasma and that it partly forms complexes with prostate-specific antigen (PSA) (España et al, 1991; Christensson and Lilja, 1994; Ahlgren et al, 1995), u-PA (España et al, 1993a), and with tissue kallikrein (España et al, 1993b). Interestingly, the level of seminal plasma PCI was reported to be decreased in some infertile patients (España et al, 1991; Laurell et al, 1992). PCI inhibits acrosin, the serine protease located in the acrosome of spermatozoa (Hermans et al, 1994: Zheng et al, 1994), and also inhibits the binding of spermatozoa to oocytes (Moore et al, 1993). Previously, we reported that PCI is trapped by seminal proteins (semenogelin I and II) in seminal vesicles, and that after ejaculation, PCI is released from the gel in parallel to the PSA-catalyzed gel liquefaction in seminal plasma (Kise et al, 1996). These findings suggest a potential role for seminal plasma PCI in the regulation of fertilization.

Ejaculated human semen consists of seminal fluid secreted by the seminal vesicles, prostatic fluid produced by the prostate, and epididymal fluid-containing sperm. The seminal vesicles play important roles in the formation of seminal coagula, modification of sperm function (motility and capacitation), and in immunosuppression (Aumüller and Riva, 1992). Transrectal ultrasonograghy is effective for the diagnosis of seminal vesicle agenesis, although it is slightly invasive. The concentration of seminal plasma fructose has been used as a basis for the diagnosis of seminal vesicle agenesis, absence of the vas deferens, and/or obstruction of the ejaculatory duct. However, the concentration of fructose diminishes soon after ejaculation due to its rapid metabolism and is not correlated with that of seminal PCI (Ahlgren et al, 1995).

In the present study, we measured the level of PCI, PSA, and fructose in seminal plasma of infertile patients as well as the time-dependent changes in concentrations of PCI and fructose in seminal plasma after ejaculation. We found that PCI in seminal plasma, as well as fructose, is a useful marker for agenesis of seminal vesicles and/or the vas deferens.

Materials and Methods

Reagents

Biotinyl-*N*-hydroxysuccinimidoester was obtained from E-Y Laboratories (San Mateo, Canada). Streptavidin–peroxidase conjugate was from Amersham International plc, (Buckinghamshire, UK). Tetramethylbenzidine microwell peroxidase substrate system was from Kirkegaard & Perry Laboratories (KPL; Gaithersburg, Md). All other chemicals were of the highest grade commercially available.

Proteins and Antibodies

PCI was purified from freshly frozen human plasma, as previously described (Suzuki et al, 1983). PSA was purified from freshly frozen human seminal plasma, as described by Christensson et al (1990). Anti-human PCI rabbit IgG was prepared as described previously (Suzuki et al, 1984). Anti-human PSA rabbit IgG was obtained from DAKO (Denmark).

Semen Samples

Human semen was obtained from healthy male volunteers (n = 13) and from subjects with azoospermia (n = 34), subjects with asthenozoospermia (n = 31), subjects with oligozoospermia (n = 10), subjects with oligoasthenoteratozoospermia (n = 46), and subjects with seminal vesicle and/or vasal agenesis (n = 4) by masturbation after 5 days of sexual abstinence. Semen analysis was performed according to WHO criteria (WHO, 1992), and then the seminal plasma was separated by centrifugation at 15 000 rpm for 5 min and stored at −80°C until use.

Preparation of Seminal Plasma Samples from Ejaculated Semen

Freshly ejaculated semen of 4 healthy volunteers was incubated at room temperature and at various times (15, 60 minutes, 10, 24, and 48 hours). An aliquot of the semen (200 μL) was withdrawn and diluted with 10 mL of Tris-buffered saline (TBS: 50 mM Tris, pH 7.5, 150 mM NaCl) containing 10 mM diisopropylphosphate (DFP) and 10 mM benzamidine. Each sample was centrifuged at 3000 rpm at 4°C for 5 minutes, and the supernatant was used for measuring PCI, PSA, and fructose.

Measurement of PCI and PSA Concentrations in Seminal Plasma

Seminal plasma PCI and PSA concentrations were measured by an enzyme-linked immunosorbent assay (ELISA). Anti-human PCI rabbit IgG was used for measuring PCI, and anti-human PSA rabbit IgG was used for measuring PSA. Biotinylated rabbit

IgG was prepared using biotinyl-*N*-hydroxysuccinimidoester according to the manufacturer's instructions. Seminal plasma samples were diluted to 1/30 000 for measuring PCI and to 1/100 000 for PSA with an ELISA buffer (TBS containing 0.1% bovine serum albumin [BSA], 0.05% Tween-20, and 0.02% thimerosal) containing 5 mM 4-amidino-phenylmethyl-sulfonyl fluoride (aPMSF) and 10 mM DFP. The assay procedure was as follows: anti-PCI or PSA IgG (5 μg/mL) in 50 μL of 100 mM NaHCO$_3$ buffer, pH 9.3, was coated onto the wells of a MaxiSorp microtiter plate (Nunc, Roskilde, Denmark) at 4°C overnight. The wells were blocked with TBS containing 3% BSA at 4°C overnight, then washed 3 times with the ELISA buffer. Thereafter, 50 μL of diluted seminal plasma samples was added to the wells in duplicate and incubated at room temperature for 3 hours. After washing the wells 3 times with the ELISA buffer, 50 μL of biotinylated anti-PCI or PSA IgG (2 μg/mL) in the ELISA buffer was added to the wells followed by incubation for 2 hours. After washing the wells with the ELISA buffer, 50 μL of streptavidin–peroxidase conjugate diluted to 1/500 with the ELISA buffer was added to the wells followed by incubation for 1 hour. Finally, after washing with the ELISA buffer, each well was filled with 50 μl of tetramethylbenzidine (TMB) peroxidase substrate. The reaction was terminated by adding 50 μL of 2.25 M H$_2$SO$_4$, and absorbance at 450 nm was measured using an EAR 340-microplate reader (TECAN Austria GmbH, Salzburg). The calibration curve was prepared using purified human plasma PCI.

Measurement of Fructose Concentration in Seminal Plasma

The concentration of fructose in seminal plasma samples was measured by the UV method using a D-glucose/D-fructose kit (Boehringer Mannheim, Mannheim, Germany) following the manufacturer's instructions.

Statistical Analysis

The concentrations of PCI, PSA, and fructose in seminal plasma were analyzed by the Mann–Whitney nonparametric test and expressed as means ± SE. The relationship between seminal plasma PCI and fructose levels was determined by Pearson's product-moment.

Results

The concentrations of PCI, PSA, and fructose in seminal plasma of the infertile patients were determined and compared with those of the normal subjects. The concentration of seminal plasma PCI in infertile patients was lower than that in the controls, but the difference was not statistically significant (Table 1). The concentrations of seminal plasma PSA and fructose in infertile patients were only slightly lower than those in the controls (Table 1).

The levels of PCI, PSA, and fructose in seminal plasma of patients with azoospermia, asthenozoospermia, oligozoospermia, oligoasthenoteratozoospermia, or azoospermia due to seminal vesicle and/or vasal agenesis were also determined and compared with those of normal sub-

Table 1. *Concentrations of PCI, PSA, and fructose in seminal plasma of infertile males and normal subjects (means ± SD)*

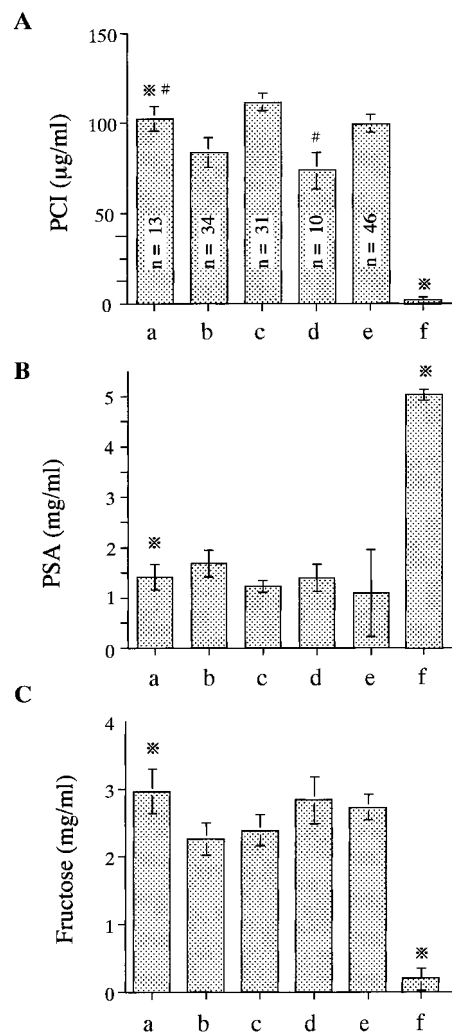|	PCI (μg/mL)	PSA (mg/mL)	Fructose (mg/mL)
Normal subsects (n = 13)	102.56 ± 24.70	1.42 ± 0.92	2.96 ± 1.19
Infertile males (n = 125) (current study)	96.13 ± 37.73	1.32 ± 1.01	2.52 ± 1.31
Normal subjects (n = 6)	160 ± 20	2.5 ± 0.4	
Infertile males (n = 22) (España et al, 1991)	110 ± 35	2.6 ± 0.6	
Normal subjects (pool from 70 donors)	220		
Infertile males (n = 5) (nonfunctional seminal vesicle) (Laurell et al, 1992)	16.02 ± 11.75		
Normal subjects (n = 35)	180 (100–320)	1.5 (0.21–5.0)	2.56 (0.52–4.50)
Infertile males (n = 27) (oligoasthenozoospermia) (Ahlgren et al, 1995)	184 (75–383)	1.0 (0.22–2.9)	2.63 (0.81–5.26)

jects (Figure 1). A significant decrease in seminal plasma PCI was observed in patients with oligozoospermia ($P <$ .05) and seminal vesicle and/or vasal agenesis ($P <$ .01) as compared to normal subjects.

In this study, 4 patients with azoospermia showed an extremely low concentration of seminal plasma PCI ($P <$ .01). Analysis of vesiculography, prostate echo, or MRI of those patients demonstrated that they had agenesis of seminal vesicles and/or absence of the vas deferens. Generally, patients with azoospermia due to seminal vesicle and/or vasal agenesis have a low volume of seminal plasma, acidic pH, and a very low level of fructose. Table 2 shows the characteristics of the semen and laboratory data of the 4 patients. These patients had a low pH of seminal plasma, low semen volume, and low concentration of fructose ($P <$ .01) in their seminal plasma as compared to normal subjects. The concentrations of seminal plasma PSA in these patients were significantly higher than that of normal subjects ($P <$ .01).

We measured the concentrations of PCI and fructose in semen after ejaculation. As shown in Figure 2, the concentration of fructose decreased in a time-dependent manner, and the level of seminal plasma fructose after 10 hours was 30% of the level 15 minutes after ejaculation. The decrease in the fructose concentration may be due to its metabolism in seminal plasma. On the other hand, the level of PCI was stable even after complete liquefaction 48 hours after ejaculation.

The correlation between seminal plasma concentrations of PCI and fructose was then analyzed. As shown in Figure 3, only a weak positive correlation between them was found ($r = 0.268$, $P = .016$).

Discussion

Immunohistochemical analysis has revealed the presence of PCI in testes (germinal cell layer and Leydig cells), epididymal glands (secretory epithelium and glandular lumina), prostate (basal cell layer), and seminal vesicles (secretory epithelium). There are some reports describing

Figure 1. Concentrations of **(A)** PCI, **(B)** PSA, and **(C)** fructose in seminal plasma. **(a)** Controls (normozoospermia) (n = 13); **(b)** subjects with azoospermia (n = 34); **(c)** subjects with asthenozoospermia (n = 31); **(d)** subjects with oligozoospermia (n = 10); **(e)** subjects with oligoasthenoteratozoospermia (n = 46); **(f)** subjects with seminal vesicle and/or vasal agenesis (n = 4). The data are shown as means ± SE. ※ : $P <$ .01; #P < .05 (Mann–Whitney nonparametric test).

Table 2. *Characteristics of the semen and laboratory data of patients with seminal vesicle and/or vas deferens agenesis*

Patient no.	1	2	3	4	Normal subjects (mean ± SD)
Diagnosis	SV agenesis and absence of the vas deferens	SV agenesis	Absence of the vas deferens and SV agenesis	SV agenesis	
Semen volume (mL)	1.0	0.8	0.8	0.5	3.62 ± 2.32
pH	6.5	6.5	7.0	6.5	7.2–7.8
PCI (μg/mL)	0.59	1.26	2.0	1.4	102.56 ± 24.7
PSA (mg/mL)	4.25	6.75	6.00	3.45	2.96 ± 1.19
Fructose (mg/mL)	0.01	0.01	0.24	0.09	1.42 ± 0.92

concentrations of seminal PCI of normal subjects and infertile males. España and Laurell reported that PCI level in seminal plasma of infertile males was lower than in normal subjects (España et al, 1991; Laurell et al, 1992). Ahlgren reported that the concentration of patients with oligoathenozoospermia was slightly higher than of normal subjects (Ahgren et al, 1995). Vasectomized patients lacking secretion from the epididymis and the testes had normal concentrations of seminal PCI, but the concentrations of seminal PCI in patients with nonfunctional seminal vesicles were much lower (Laurell et al, 1992).

According to these findings, the predominant secretory origin of seminal PCI is thought to be seminal vesicles. We therefore thought that seminal PCI could be a marker for seminal vesicle agenesis and obstruction of the ejaculation duct. We measured the concentrations of seminal PCI in infertile males by ELISA. This method is simple, and only a small volume of semen (<10 μL) is sufficient for determining the concentration. We found that the level of PCI in seminal plasma of patients with oligozoospermia is significantly lower than that of controls. The level of seminal PCI in patients with asthenozoospermia was

higher than that of controls, but the difference was not significant. There has been no report of influencing the sperm motility. However, the latter data suggest the possibility that PCI or other proteins secreted from seminal vesicles may suppress sperm motility. It was reported that sperm motility is reduced in the seminal vesicle fraction of a split ejaculate (Clavert et al, 1990) and that seminal vesicle secretion contains the sperm motility inhibitor (SPMI; Iwamoto and Gagnon, 1988a,b), suggesting that an excess amount of some proteins in seminal fluid may suppress sperm motility. Although it is unknown whether PSA has a direct influence on sperm motility, it was reported that u-PA and kallikrein stimulate human sperm motility (Schill, 1975; Izzo et al, 1984; Hong et al, 1985). On the other hand, it was reported that PCI inhibits about 75% of the total u-PA (España et al, 1993a) and 28% of total kallikrein in seminal plasma (España et al, 1993b). Thus, an excess of PCI in seminal plasma might lead to PCI–protease complex formation and neutralize the activity of the protease for sperm motility. It may also cause infertility in some patients. We are doing further examinations by increasing samples.

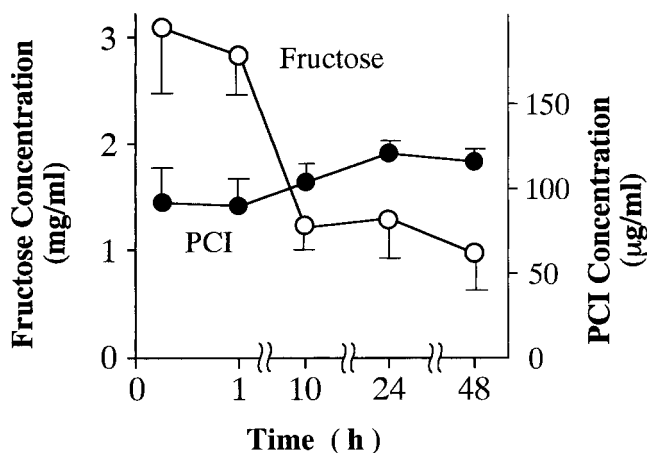Patients with seminal vesicle dysfunction are reported

Figure 2. Time-course changes of PCI and fructose in semen. After ejaculation, aliquots of semen were withdrawn at defined intervals. For measurement of the concentration of PCI (●), semen was diluted with TBS containing 10 mM DFP and 10 mM benzamidine. For measurement of the concentration of fructose (○), semen was frozen immediately after ejaculation. Data are expressed as means ± SD (n = 4).
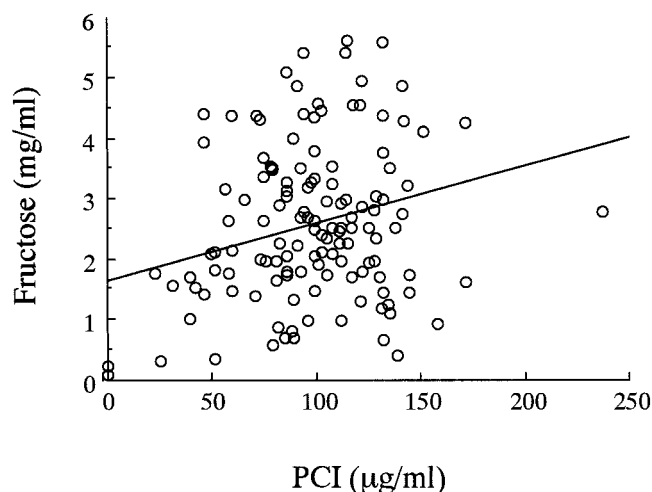

PCI (μg/ml)

Figure 3. Correlation between the levels of PCI and fructose in seminal plasma. The relationship was determined by using Pearson's product-moment ($Y = 7.543X + 77.415$, $r = 0.268$, $P = .016$).

to have very low levels of PCI in seminal plasma (Laurell et al, 1992). We also found that patients with seminal vesicles and/or vasal agenesis had extremely low levels of PCI in their seminal plasma. These patients also had low levels of fructose (<0.24 mg/mL), a low volume of semen, and significantly high levels of PSA in their seminal plasma. The fructose level in seminal plasma is widely used as a basis for the diagnoses of seminal vesicles and/or vasal agenesis and the obstruction of ejaculatory duct. However, the level of fructose in seminal plasma decreases in a time-dependent manner after ejaculation due to its metabolism. On the other hand, it was found that the seminal plasma PCI level increases until semen liquefaction has been completed and remains stable 48 hours after liquefaction. Recently, Tremblay analyzed semen of infertile patients by western blot analysis and found that THPK–PCI complex was not detected in semen of the patients with obstructive azoospermia. They described that the assessment of THPK–PCI complex in semen would add an advantage for identification of seminal vesicle agenesis or obstruction (Tremblay et al, 1998). Due to the stability of the PCI antigen, the simplicity of the method for assaying PCI, and the requirement of only a small volume of material, we suggest that seminal PCI, as well as fructose and THPK–PCI complex, may be useful for determining seminal vesicle and vasal agenesis.

Acknowledgments

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