JCI The Journal of Clinical Investigation

Protein C inhibitor in human body fluids. Seminal plasma is rich in inhibitor antigen deriving from cells throughout the male reproductive system.

M Laurell, … , J Stenflo, H Lilja

J Clin Invest. 1992;89(4):1094-1101. https://doi.org/10.1172/JCI115689.

Research Article

An assay was developed for the measurement of human protein C inhibitor antigen (PCI) in blood plasma and other biological fluids. Both native PCI, modified inhibitor, and complexes of inhibitor with activated protein C or plasma kallikrein could be measured with the assay. Inhibitor antigen concentrations were found to be very high in seminal plasma (greater than 200 mg/liter), more than 40 times the concentration of PCI found in blood plasma. The inhibitor in seminal plasma was unable to form complexes with activated protein C. Gel filtration and immunoblotting findings indicated that the inhibitor in seminal plasma is present in a high molecular mass complex or cleaved to its modified form. As PCI antigen was absent from seminal plasma of patients with dysfunctional seminal vesicles, the seminal vesicle glands would appear to be the major source of seminal plasma PCI, a conclusion supported by immunohistochemical demonstration of the presence of PCI epitopes in the secretory epithelium of the seminal vesicles. Specific PCI immunoreactivity was also shown to be present in the testes, the epididymis glands, and the prostate, suggesting the inhibitor to have a complex or multiple function in the male reproductive system. Conclusive evidence of a local synthesis of PCI in the four male sex glands was provided by Northern blot analysis of RNA from these organs.

[…]

Find the latest version:

https://jci.me/115689/pdf

Protein C Inhibitor in Human Body Fluids

Seminal Plasma Is Rich in Inhibitor Antigen Deriving from Cells throughout the Male Reproductive System

Martin Laurell, Anders Christensson, Per-Anders Abrahamsson,* Johan Stenflo, and Hans Lilja
*Departments of Clinical Chemistry and *Urology, University of Lund, Malmö General Hospital, S-21401 Malmö, Sweden*

Abstract

An assay was developed for the measurement of human protein C inhibitor antigen (PCI) in blood plasma and other biological fluids. Both native PCI, modified inhibitor, and complexes of inhibitor with activated protein C or plasma kallikrein could be measured with the assay. Inhibitor antigen concentrations were found to be very high in seminal plasma (> 200 mg/liter), more than 40 times the concentration of PCI found in blood plasma. The inhibitor in seminal plasma was unable to form complexes with activated protein C. Gel filtration and immunoblotting findings indicated that the inhibitor in seminal plasma is present in a high molecular mass complex or cleaved to its modified form. As PCI antigen was absent from seminal plasma of patients with dysfunctional seminal vesicles, the seminal vesicle glands would appear to be the major source of seminal plasma PCI, a conclusion supported by immunohistochemical demonstration of the presence of PCI epitopes in the secretory epithelium of the seminal vesicles. Specific PCI immunoreactivity was also shown to be present in the testes, the epididymis glands, and the prostate, suggesting the inhibitor to have a complex or multiple function in the male reproductive system. Conclusive evidence of a local synthesis of PCI in the four male sex glands was provided by Northern blot analysis of RNA from these organs. (*J. Clin. Invest.* 1992. 89:1094–1101.) Key words: plasminogen activator inhibitor • prostate-specific antigen • glandular kallikreins • seminal vesicles • serine proteinase inhibitor

Introduction

Protein C is a vitamin K–dependent zymogen of a serine protease and an important regulator of blood coagulation. Activated protein C (APC)[1] has a high degree of specificity for the active forms of two factors, Factor Va and Factor VIIIa, whereas the precursor forms of the cofactors are resistant to APC. Thus, APC functions in vivo as a very specific circulating anticoagulant (1, 2). An inhibitor of APC (PCI) has been identified in human plasma and found to be a 57-kD, single-chain glycoprotein containing one cysteine residue (3, 4). Sequence analysis of a cDNA derived from human liver has shown PCI to be a member of the serine protease inhibitor (serpin) superfamily, homologous to α_1-antitrypsin and α_1-antichymotrypsin (5–7), the sequence identities being 41.6% and 42.3%, respectively (5). The mechanism by which PCI inhibits serine proteases appears to be identical to that of the other serpins (8, 9). Plasma PCI is a remarkably slow inhibitor of APC. The reaction is accelerated 20–200-fold in the presence of heparin and other sulfated polysaccharides, but is still slow compared to that of the other serpins, e.g., the inhibition of thrombin by antithrombin III (3, 4, 10–14). The slow inhibition of APC by PCI is reflected in the long half-life of APC in plasma, which is ~ 20 min in vivo (15, 16).

PCI is identical to a plasminogen activator inhibitor (PAI-3) that has been isolated from urine both in complex with urokinase and in a degraded form (17–20). The relative importance of PCI as an inhibitor of APC and urokinase in vivo is unknown. Recently it was demonstrated that APC is inhibited by α_1-antitrypsin, although at a rate that is even slower than the inhibition of APC by PCI. However, considering the high concentration of α_1-antitrypsin in plasma, it remains possible that it is a physiologically important inhibitor of APC (16).

Several proteases involved in blood coagulation and fibrinolysis have been shown to be inhibited slowly by PCI (14). Both in date-expired blood plasma and in fresh plasma, complex formation between PCI and plasma kallikrein has been shown to occur after activation of the contact system (12, 21). This suggested that PCI might be involved in systems other than blood coagulation and fibrinolysis.

In this paper we describe a sensitive assay for PCI and the results obtained when using it to measure the inhibitor antigen in biological fluids. Seminal plasma was found to be rich in PCI immunoreactivity, and PCI antigenic epitopes were identified immunohistochemically throughout the male reproductive system. Northern blotting results suggested the inhibitor to be synthesized locally, indicating that PCI functions as an inhibitor of proteolytic activity in the male reproductive system.

Address correspondence to Martin Laurell, M.D., Department of Clinical Chemistry, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden.

Received for publication 11 February 1991 and in revised form 15 November 1991.

1. *Abbreviations used in this paper:* APC, activated protein C; CV, coefficient of variation; IRMA, immunoradiometric assay; PCI, protein C inhibitor; serpin, serine protease inhibitor; TBS, Tris-buffered saline.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/92/04/1094/08 $2.00
Volume 89, April 1992, 1094–1101

Methods

Materials. Glutaraldehyde (25% in H_2O, electron microscopy quality, product number 36080) was from BDH Chemicals Ltd., Dagenham, Essex, UK, and polyethylene glycol (mean M_r = 6,000) from Kebo Lab, Stockholm, Sweden. Enzymobeads® were from Bio-Rad Laboratories, Richmond, CA, and microtiter plates (M1798A Removastrip®) were a product of Dynatech Labs, Inc., Chantilly, VA. Gel filtration standards and PD-10 columns were from Pharmacia, Uppsala, Sweden. Immobilone polyvinylidene difluoride membranes were from Millipore Corp., Bedford, MA, and advanced protein purification system (650E) were purchased from Waters Associates, Millipore Corp., and Hybond N filters for Northern blots were from Amersham International, Amersham, Bucks, UK.

Oligonucleotide synthesis. Three oligonucleotides with sequences complementary to nucleotides 1054-1077 (PI-5), 1186-1207 (PCI-4),

and 734-752 (PCI-2) of the known cDNA sequence for PCI (5, 22) were synthesized with a nucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. The oligonucleotides were labelled to a specific activity of ~ 0.2 mCi/μg with $\gamma[^{32}/P]$ ATP (3,000 Ci/mmol).

Patient material and controls. Healthy volunteers (n = 83) without history of thromboembolic disorders, 38 males and 45 females (mean age 37 yr, range: 16–68) were used as controls. Pregnant women, users of oral contraceptives, and all individuals on medication or with suspected infection were excluded from the control group.

Venipuncture blood samples were collected in Vacutainer® tubes (Becton-Dickinson, UK), with 1:10 vol of 129 mM trisodium citrate or 1:83 vol of 0.34 M K_3EDTA. Platelet-poor plasma was obtained by centrifugation (5,000 g for 5 min at room temperature). Samples were stored at −70°C until analyzed.

Biological fluids. Samples of Graaf follicle fluids were generously provided by Prof. B. Åstedt of the Department of Obstetrics and Gynaecology, University Hospital, Lund, Sweden. The samples were collected at laparoscopy from women undergoing operation for in vitro fertilization. Synovial fluids were obtained from patients with rheumatoid arthritis. Saliva, tears, and urine were obtained from healthy laboratory personnel; amniotic fluid was obtained at parturition; breast milk from healthy lactating females was obtained later than four weeks after delivery, and cerebrospinal fluid from patients with minor neurologic complaints but with total protein concentrations and cell counts within normal ranges was obtained from the clinical laboratory. Seminal plasma samples were from a pool derived from 70 donors, or collected from individual patients undergoing investigation for involuntary infertility. Azoospermic ejaculates were obtained from two patients with nonfunctional seminal vesicles and aplasia of the deferent ducts (23) and from another three patients with defective function of the seminal vesicles as judged by the electrophoretic pattern of the seminal plasma (24). PCI was also measured in the azoospermic ejaculates from 10 vasectomized patients. All samples from patients were made available to us after the completion of diagnostic studies and had been coded so that individual patients could not be identified.

Proteins. PCI was purified from fresh human plasma by immunoaffinity chromatography using an immobilized monoclonal antibody (25), followed by heparin-Sepharose chromatography with NaCl gradient elution (21). Modified PCI and PCI–kallikrein complexes were derived from date-expired plasma and purified as previously described (21).

Antibodies. A polyclonal antiserum against native PCI was produced by subcutaneous injection of rabbits with 0.1 mg antigen emulsified in Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI), the injection being repeated after three weeks and the rabbits bled every second week. The antiserum was tested with immunoelectrophoresis of human plasma and the purified native inhibitor. The antibody fraction was prepared by ammonium sulfate precipitation of the antisera followed by DEAE Sepharose chromatography (26). The production of the monoclonal antibodies against PCI (M11-5 and M1-12) has been described previously (25). Rabbit antibodies against mouse immunoglobulins and alkaline phosphatase conjugated rabbit immunoglobulins against mouse immunoglobulins were from DAKOPATTS, Copenhagen, Denmark. Rabbit antibodies against human albumin were available at the laboratory.

Radiolabelling of proteins with ^{125}I was performed with the immobilized lactoperoxidase/glucose oxidase system (Enzymobeads®; Bio-Rad) as described by the manufacturer. The iodinated proteins were separated from unincorporated iodide by chromatography on Sepharose G-25 columns (PD-10).

Immunoradiometric assay of PCI. An immunoradiometric assay (IRMA) was developed for the measurement of PCI in biological fluids, using 96-well, separable microtiter plates. Polyclonal anti-PCI was used as the catcher antibody and ^{125}I-labelled monoclonal anti-PCI (M11-5) as the tracer antibody for the bound PCI. To increase the binding capacity of the plastic wells, the catching antibody was coupled covalently to the plastic (27). To this end, the plates were prepared with 2.5% glutaraldehyde in 0.1 M $NaHCO_3$, pH 9.5, for 2 h at room temperature. The plates were then washed three times with distilled water and filled with anti-PCI (200 μl, 2 μg/well), in 0.1 M sodium phosphate buffer, pH 7.5. After overnight coating at 4°C and subsequent aspiration of excess antibodies, unsaturated free binding sites were blocked for at least 2 h with quenching buffer (50 mM Tris, 0.1 M NaCl, pH 7.5, containing 1% BSA, 0.05% Tween 20 and 0.05% sodium azide). Plates treated this way could be stored filled with the buffer in a moist chamber at 4°C for at least 3 wk. Immediately before use, the plates were washed five times with washing buffer (10 mM Tris, pH 7.5, 0.1 M NaCl containing 0.05% Tween 20 and 0.05% sodium azide). Samples to be analyzed were diluted appropriately with assay buffer (50 mM Tris, pH 7.5, 0.1 M NaCl, 5 mM EDTA containing 0.05% Tween 20 and 0.25% BSA) and incubated (100 μl/well) for 4 h at room temperature or at 4°C overnight. The plates were then washed three times in the assay buffer and incubated overnight at 4°C with ^{125}I-M11-5 (100 μl/well, ≈ 10,000 cpm). Finally, after five washing cycles to remove unbound radioactivity, each separate well was placed in a polystyrene tube and measured in a gamma counter. A standard curve was constructed from dilutions of pooled plasma in assay buffer to give concentrations ranging from 0 to 105 μg/liter. Control samples, high (5.26 mg/liter) and low (1.8 mg/liter), were made by the addition of purified PCI to PCI depleted plasma. The concentration of purified PCI was estimated from the absorbance at 280 nm, assuming an extinction coefficient of 14.1 (4). Standard and control samples were prepared in batches and stored in aliquots at −70°C to avoid repeated thawing. A standard curve was run for each plate. Controls were run at the beginning and end of each plate. All samples were run in duplicate. Calculations were made with the computer program RIACALC (LKB Instruments Inc., Bromma, Sweden). The intraassay coefficient of variation (CV) was determined by analyzing two different samples 18 times in one assay run. The interassay CV was determined by analyzing frozen aliquots of two different samples in 32 different assay runs.

Immunohistochemical techniques. Tissue specimens were cut in thin slices and fixed by immersion in Bouin's fluid for 18–24 h. After rinsing in tap water and 70% ethanol to remove excess picric acid, the specimens were dehydrated, cleared, and embedded in paraffin. The avidin-biotin peroxidase complex (ABC) procedure (28) was applied, using diaminobenzidine as a chromophore to detect biotinylated horseradish peroxidase. As the primary antibody, we used either affinity purified polyclonal antibodies (3 μg/ml) or monoclonal antibodies against PCI (6 μg/ml). The control experiments done to verify the specificity of the staining were: (*a*) elimination of primary antibody; (*b*) substitution of primary antibody with purified normal rabbit or mouse IgG; and (*c*) pretreatment of the primary antibody for 18 h with an excess of purified PCI derived from plasma before incubation with the tissue sections. Structures that were stained using the ABC procedure as stated above, but remained unstained during the control experiments, were considered to be specific (29).

Gel filtration of seminal plasma. Pooled seminal plasma (40 μl) was diluted four times in 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl and applied to a 0.8 × 30 cm column (Protein PAK Glass 200 SW; Waters Associates) equilibrated in the same buffer. The column was developed with a flow rate of 0.6 ml/min and fractions (0.2 ml) were collected and analyzed for PCI. The column was calibrated by determining the elution volumes for: IgG (150 kD); APC-PCI complexes (122 kD); and PCI (57 kD).

SDS-PAGE and immunoblotting. SDS-PAGE was performed in 10% slab gels with a 5% stacking gel in a Bio-Rad Mini Protean apparatus using a Tris-glycine buffer, pH 8.7, as recommended by the manufacturer. Before separation in the gels, samples were prepared by incubation for 60 min at 37°C with sample buffer. Protein transfer to Immobilone membrane was performed in a semi-dry electroblotting apparatus (Ancos, Copenhagen, Denmark), according to the manufacturer's instructions. Lanes with transferred molecular weight standards were excised from the membranes and visualized by staining with Coomassie brilliant blue (30). The rest of the membranes with the transferred samples were immunostained according to the method of Bur-

nette (31) with the following modifications. Membranes were blocked in a solution of Tris-buffered saline (TBS = 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl) containing 3% nonfat dried milk (32). Monoclonal anti-PCI antibodies (M1-12), in TBS buffer containing 5% BSA were incubated with the membranes for 2 h and washed for three 10-min intervals in TBS with 0.1% Tween-20. Alkaline phosphatase-conjugated monospecific anti-mouse IgG antibodies were used to visualize the immunoblots using 5-bromo 4-chloroindoxylphosphate and nitrobluetetrazolium to detect the precipitated indoxyl group (33).

Isolation of RNA and Northern blot analysis. RNA was prepared from human tissue specimens obtained at surgery or at autopsy (34, 35). The polyadenylated mRNA fraction was recovered by two passages of the total RNA on oligo(dT) cellulose as described (35). After electrophoresis in denaturating buffer RNA was transferred to Hybond N membranes (Amersham, London, UK) and hybridized with ^{32}P-labelled oligonucleotides in 6 × standard saline citrate (SSC), 5 × Denhardt's solution, 0.5% SDS at 37°C. The filters were washed in several changes of 6 × SSC, 0.5% SDS at ambient temperature. High stringency conditions with the PCI-4 probe were accomplished by a final wash in 2 × SSC, 0.5% SDS for 60 min at 64°C, whereas membranes probed with PCI-2 and PI-5 probes were washed for 60 min in 2 × SSC, 0.5% SDS at 55°C and 64°C, respectively.

Other methods. Complexes of APC-PCI were prepared and assayed as previously reported (25). PCI-depleted plasma was prepared by passing aliquots of fresh plasma over an immunoaffinity column with immobilized monoclonal anti-PCI antibodies (M11-5). Albumin concentrations were determined by electroimmunoassay, using a rabbit antiserum available at the laboratory (36).

Results

Immunoassay of PCI in biological fluids. The antibodies used as catcher and tracer in the assay were first tested for their reactivity against various molecular forms of PCI, i.e., native, cleaved, and complexed inhibitor. The polyclonal anti-PCI showed a single precipitin line with fresh and date-expired plasma when analyzed with immunoelectrophoresis (not shown). This indicated that the antiserum reacted with the native, cleaved, and complexed forms of PCI since the inhibitor in date-expired plasma is cleaved to its modified form or complexed with plasma kallikrein (21).

IRMA of PCI. The assay response in the PCI IRMA was compared for dilutions of fresh plasma, purified native PCI, inactive PCI, and PCI complexes from date-expired plasma. The dilution curves were parallel, indicating comparable affinity between the antibody (M11-5) and the different forms of PCI. From a Scatchard plot of the binding data for purified PCI, the association constant for binding of PCI to M11-5 was calculated to be 5×10^8 M^{-1}. Covalent coupling of the catcher antibody (polyclonal anti-PCI) to the microtiter wells resulted in a twofold increase in the capacity of the plates while minimizing background noise, as compared with the conventional coupling method used in ELISAs (37). The grade of glutaraldehyde was critical, and not all grades worked for reasons that are unclear (27). The intraassay variation (CV) for two samples with concentrations (mean±1 SD) of 1.59±0.06 and 4.47±0.18 μg/ml was estimated to be 3.8% and 4.0%, respectively, and for two samples with concentrations (mean±1 SD) 2.68±0.33 and 5.15±0.26 μg/ml the interassay variation (CV) was estimated to be 12.2% and 5.1%, respectively. Recovery in the assay was 100% and 96% when purified PCI (27 μg) was added to 1 ml of buffer and PCI-depleted plasma, respectively. The concentration of PCI in 83 plasma controls was measured to be 5.32±1.76 mg/liter (mean, ±2 SD) with a range of 3.58–8.38 mg/liter. No difference was observed between the 38 males and 45 females tested.

Table I. Concentration of PCI in Biological Fluids

Fluid	n	PCI*	Albumin
		(mg/liter)	*(g/liter)*
Plasma	83	5.3±1.7	40
Urine	10	0.13±0.04	<1
CSF	13	0.05±0.03	<1
Saliva	8	0.03±0.01	<1
Tears	4	0.1±0.09	<1
Synovial fluid	12	6.2±2.2	ND
Graaf follicle fluid	24	8.0±1.6	36
Amniotic fluid	9	0.88±0.71	2
Breast milk	10	0.61±0.5	ND
Seminal plasma	70‡	220	<1

* Mean±2 SD. ‡ Pool from 70 donors.

Determination of PCI antigen in body fluids. The concentration of PCI in synovial fluid and Graaf follicle fluid was similar to that in plasma (Table I). In contrast, a very high concentration of PCI, 220 mg/liter (i.e., more than 40 times the plasma concentration) was measured in the seminal plasma pool. The concentration of PCI in breast milk and in amniotic fluids was about one-tenth that in plasma. Still lower concentrations were found in urine, cerebrospinal fluids, saliva, and tears. The albumin concentrations were also measured in the fluids and are listed in Table I. The results suggest that there is no significant plasma admixture to the fluids except for the Graaf follicle fluid.

Characterization of the PCI antigen in seminal plasma. Dilutions of plasma and seminal plasma gave parallel dose-response curves in the IRMA, indicating immunological identity between PCI in blood and the antigen in seminal plasma. This was further supported by the demonstration of complete antigenic identity for the PCI-antigen in seminal plasma and that in blood plasma by Ouchterlony immunodiffusion (not shown). This prompted a further investigation of the secretory origin of seminal PCI. To this end, samples of azoospermic seminal fluid from patients with nonfunctional seminal vesicles and samples from vasectomized patients were analyzed. The results are summarized in Table II. The normal concentrations of PCI in the vasectomized patients indicate that the testes and epididymal glands do not significantly contribute to the seminal antigen. However, the concentrations of PCI were much lower in the patients with nonfunctional seminal vesicles, which implies that the predominant secretory origin of seminal PCI is the seminal vesicles.

The functional activity of seminal PCI was investigated in the experiment outlined in Fig. 1. Seminal plasma and fresh blood plasma were incubated with APC in the presence of heparin, and at the indicated intervals, aliquots were drawn from the mixtures and analyzed for the amount of APC–PCI complexes that had formed. Contrary to the finding in blood plasma, no APC–PCI complexes were formed in the seminal plasma, indicating the form of PCI present in the ejaculate to be inactive.

The molecular size of seminal PCI was investigated by means of gel filtration of seminal plasma on a Protein PAK

Table II. Concentration of PCI in Patient Seminal Plasma

Patient	PCI*
	(mg/liter)
Vasectomized (*n* = 13)	234±158
1‡	12.5
2‡	13.1
3‡	12.6
4‡	5.6
5‡	36.3

* Mean±2 SD. ‡ Patients with nonfunctional seminal vesicles.

Glass 200 SW column (Waters Associates), the eluted fractions being subsequently separated by SDS-PAGE and immunoblotted (Fig. 2). The PCI immunoreactivity eluted in volumes corresponding to molecular masses between 100 and 55 kD. Immunoblotting demonstrated two major molecular forms of PCI, one migrating with an apparent molecular mass of 80–90 kD, and the other of 50–60 kD (Fig. 2). The mobility of the 50–60-kD species corresponds more closely to that of cleaved PCI, found in date-expired blood plasma than to that of native PCI, normally found in fresh plasma, both of which have close mobility on SDS gels (21). This suggested that PCI antigen in seminal plasma occurs as two distinct molecular entities: the 50–60 kD form which is probably identical with cleaved, inactivated PCI, and the 80–90-kD form which may represent a complex with an unidentified target enzyme.

Demonstration of antigenic epitopes of PCI in the testes, epididymis, seminal vesicles, and prostate. The occurrence of a very high concentration of PCI immunoreactivity in the seminal plasma prompted an investigation of the tissue distribution of the antigen in the male sex glands. To this end, monoclonal anti-PCI or affinity purified polyclonal antibodies from an antiserum raised against plasma PCI were used to probe tissue specimens for the presence of the corresponding antigenic epitopes. Examples of positive immunohistochemical reactions that were identified are shown in Fig. 3. The testes yielded a positive PCI stain, evenly distributed through the germinal cell layer. The interstitial Leydig cells also stained positively, whereas the stroma and the Sertoli cell layer were both negative. In the epididymal glands, the secretory epithelium and secretory content in the glandular lumina stained positively. In the seminal vesicles, the apical secretory epithelium of the gland stained most intensely, in the prostate, the PCI-immunoreactivity was found in the basal cell layer, adjacent to the parenchymal stroma cells. The luminal content of the seminal vesicles stained positively, whereas no PCI immunoreactivity was seen in the intraluminal secretions of the prostate.

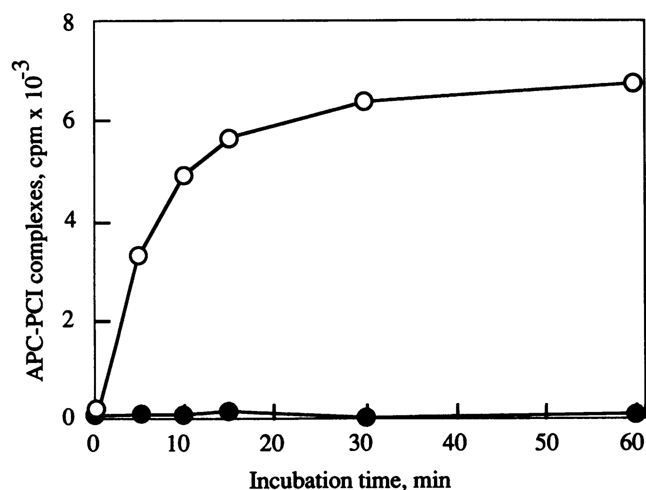


Figure 1. Formation of APC-PCI complexes in plasma and seminal plasma. 1 ml of blood plasma (○) and seminal plasma (●) were incubated at 37°C with 1 ml of APC in TBS, final concentration 5 μg/ml in the presence of heparin (10 U/ml). At the indicated intervals, aliquots (50 μl) were drawn from the incubation mixtures, diluted with an equal volume of buffer containing 50 mM benzamidine, and stored on ice. After 60 min, the samples were analyzed simultaneously for APC-PCI complexes as described (25).

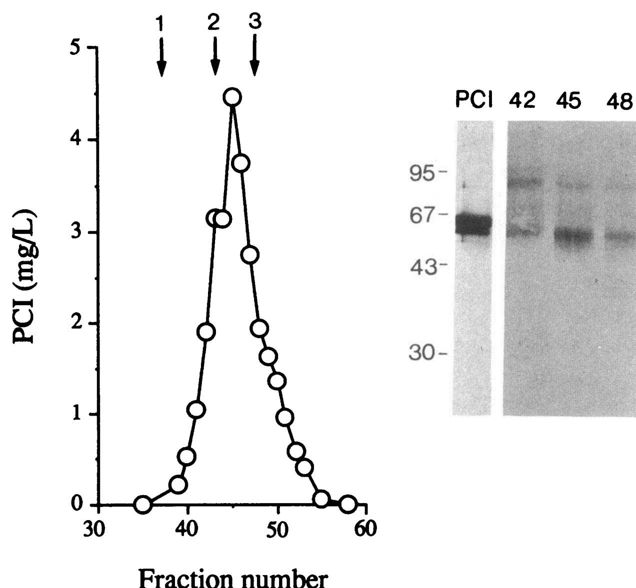


Figure 2. Investigation of PCI antigen in seminal plasma by gel filtration and immunoblotting. Seminal plasma was gel chromatographed on a Protein PAK Glass 200 SW (Waters Associates) and aliquots of the fractions were assayed for PCI antigen. The arrows denote the elution volumes for the calibration proteins: 1, IgG (150 kD); 2, APC-PCI complexes (122 kD); and 3, PCI (57 kD). (*Inset*) Immunoblots of PCI isolated from blood plasma and fractions 42, 45, and 48 detected with monoclonal anti-PCI (M1-12) after separation with reduced SDS-PAGE. Figures to the left denote the molecular weight standards (kD).

To determine whether the PCI in the male sex glands is the result of local synthesis or if it represents an accumulation of circulating plasma PCI, Northern blot analyses of RNA isolated from these tissues were performed. Using three different γ^{32}P-labelled probes representing translated parts in the cDNA reported by Suzuki et al (5), and stringent hybridizing conditions, each of the radioactive probes PCI-4, PCI-2, and PI-5 hybridized to one major 2.1-kb RNA species present in the prostate, the testes, the seminal vesicles and the epididymis as well as to the identical sized RNA species from human liver (Fig. 4). Identically sized hybridization signals were also detected by probing the ^{32}P-labelled PCI-4 with RNA from several other organs like the pancreas, small intestines, stomach, parathyroid glands, kidney, brain, adrenal gland. A negative control was provided with RNA from transverse colon (not

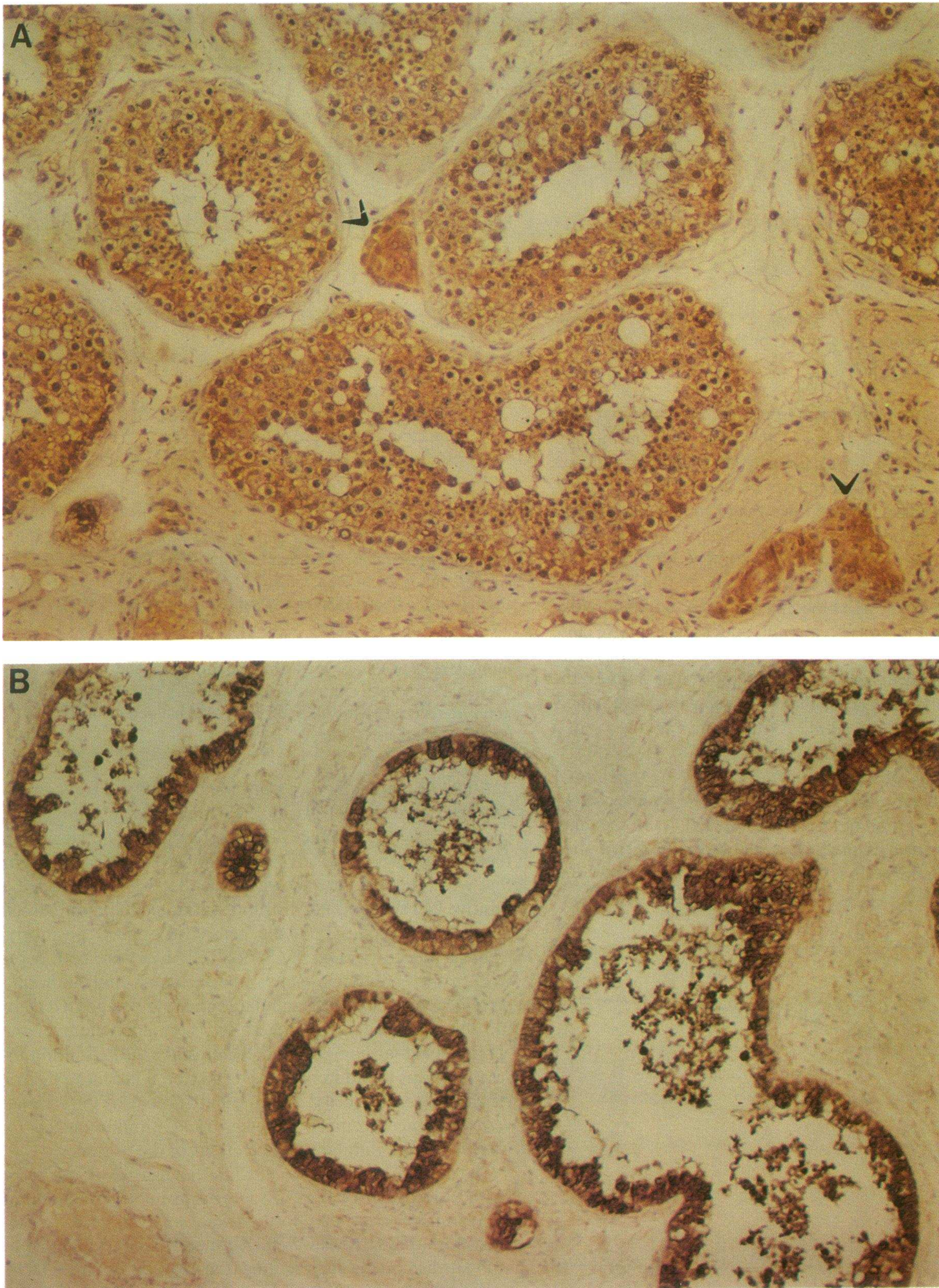
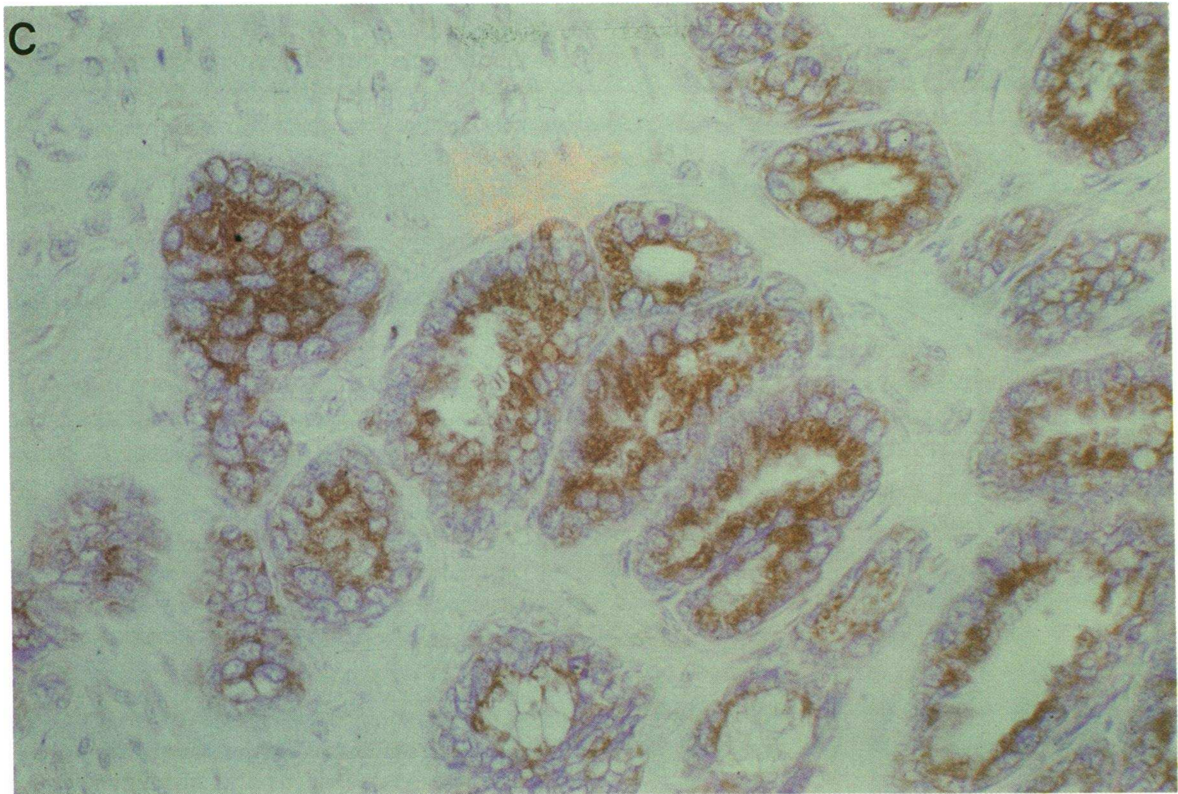


Figure 3. Histochemical visualization of PCI-immunoreactivity in the male sex glands. Examples of positive staining for PCI in tissue specimens. (*A*) Testes, ×450; (*B*) epididymal gland, ×450; (*C*) seminal vesicles, ×450; (*D*) prostate, ×360. The antibodies used were polyclonal anti-PCI for the prostate and monoclonal anti-PCI (M11-5) for the other specimens. The arrow denotes the interstitial Leydig cells in the testes.

shown). This suggests that the PCI-coding transcripts synthesized in the male reproductive glands and other tissues may be identical with the transcript obtained from human liver.

However, the approximate size (2.1 kb) of the detected RNA species in the liver, the male reproductive glands, and other tissues is shorter than expected from the previously characterized cDNA from the liver (5) although this transcript lacked both a polyadenylating signal and a poly(A) tail. To address this issue, total RNA from the liver, testes, and seminal vesicles were fractioned into poly(A)$^+$ and poly(A)$^-$ pools by



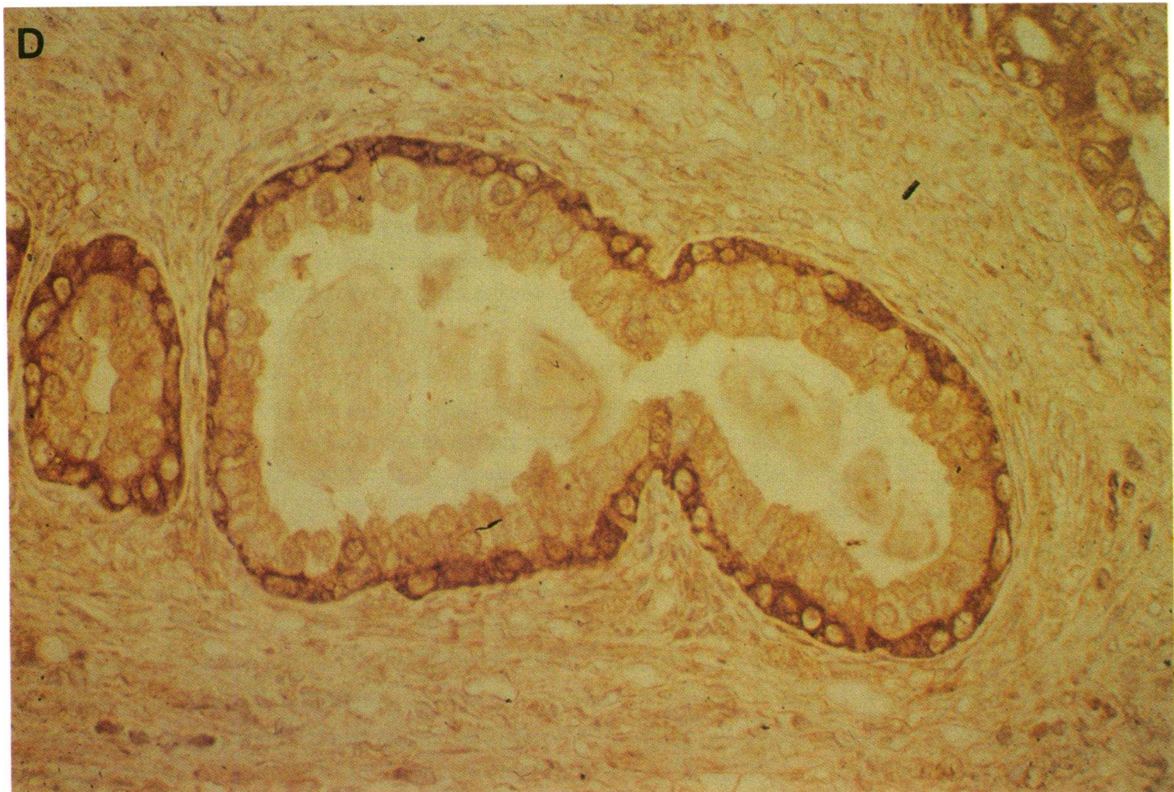


Figure 3 (*Continued*)

chromatography on an oligo(dT)-column. Using either the ^{32}P-labelled PCI-4 or the PI-5 probe, the major 2.1-kb PCI-coding transcript could be detected in the poly(A)$^-$ pools whereas in the poly(A)$^+$ pools only a barely visible 2.3-kb signal could be detected (not shown). A positive control for this set of experiments was provided by the polyadenylated semenogelin related 2.2-kb RNA species in the seminal vesicles (38) probed with an 18-mer complementary to the nucleotide sequence of this transcript (Lilja, H., and Å. Lundwall, manuscript in preparation).

Discussion

The protein C anticoagulant system is an important regulator of hemostasis (1). Since activated protein C was shown to be a

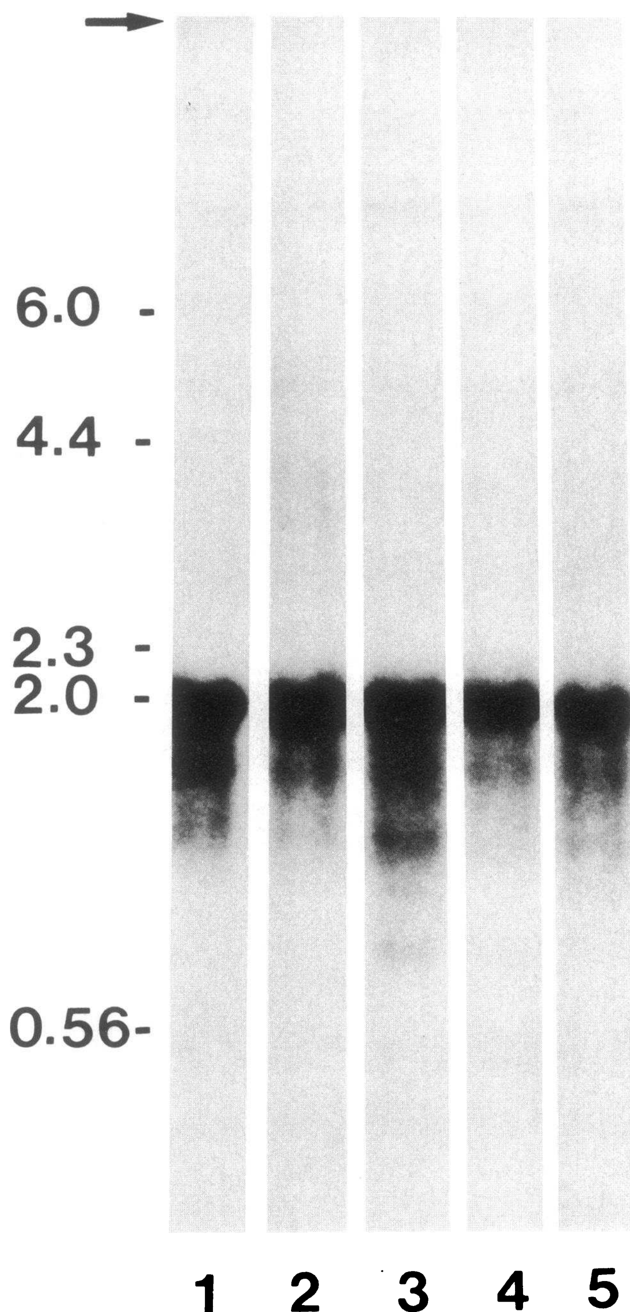


Figure 4. Northern blot analyses of RNA derived from glands of the male genital system. RNA was isolated from the liver and tissues of the male genital system, electrophoresed in denaturating buffer, transferred to Hybond N membranes, and hybridized with γ^{32}P-labelled PCI-oligonucleotide (PCI-4). Lanes *1–5* represent total RNA (5 μg) isolated from the liver, prostate, testes, seminal vesicles, and epididymal glands, respectively. The arrow denotes the application slit and the numerals to the left the molecular weight standards (kb).

serine proteinase, it has been postulated that a specific inhibitor would modulate the activity of the system. An inhibitor for activated protein C was first demonstrated in human plasma (3), and later purified and characterized (4). Progress in our knowledge of the PCI biology was hampered, however, by the difficulty of purifying PCI from blood plasma, owing to the fact that plasma kallikrein is a target enzyme for PCI (12, 21). Thus, extreme care and speed are required to avoid triggering the plasma contact activation system when the inhibitor is purified from blood plasma (11–14). PCI has also been shown to be present in urine, where it occurs as an inhibitor of urokinase and as such has been named PAI-3 (18). Its presence in urine suggests an interesting possibility that PCI participates in the regulation of proteinase activity in the kidney. PCI is a heparin-dependent serpin, but even in the presence of sulfated polysaccharides the reported kinetics of the inhibition of APC by PCI is slow compared to that reported for other specific inhibitor systems, e.g., antithrombin III-thrombin or PAI-1-t-PA (4, 39, 40). This highlights such issues as the physiological function of PCI and which its primary target enzyme may be.

Here we have described a sensitive IRMA designed to assay PCI concentrations in different biological fluids. High concentrations of antigen, more than 40 times the blood plasma concentration, were detected in seminal plasma. This strongly suggested inhibitor contribution by local tissues, which prompted study of the molecular forms, tissue distribution, and secretory origin of the inhibitor in the male genital system.

The monoclonal anti-PCI (M11-5) was previously reported to be unsuitable for Western blot analysis (25), and dilutions in buffer of blood and seminal plasma resulted in parallel curves, when assayed with the PCI IRMA. We thus concluded that the IRMA recognized the same antigenic epitope in the two fluids. The notion of immunologic identity between plasma PCI and the antigen in seminal plasma was further supported by the immunodiffusion experiments and the immunohistochemical findings. The major secretory origin of seminal PCI was the seminal vesicles, as evidenced by the normal levels measured in vasectomized patients lacking the secretory contributions from the epididymis and the testes, the low levels in semen from the patients with nonfunctional seminal vesicles, and the immunohistochemical finding of PCI in the secretory epithelium and its intraluminal fluids. Although PCI immunoreactivity was absent from the intraluminal fluid of the prostate, its presence in cells of the testes and in the nonsecretory basal cell layer of the prostate, suggests the occurrence of other sites of PCI synthesis in the male reproductive system. The question of a local synthesis of PCI within the male sex glands was addressed by Northern blot analysis of RNA derived from the testes, the epididimal glands, the seminal vesicles, and the prostate. This demonstrated that the presence of major RNA species from these glands may be identical to the major PCI-coding RNA species from the liver. The absence of a poly(A) tail in these species may be in accordance with the absence of both a polyadenylation signal and a poly(A) tail in the cDNA previously characterized by Suzuki et al. (5). However, it is more likely that the previously characterized cDNA may correspond to the low abundance polyadenylated 2.3-kb species detectable in some of the RNA preparations.

As no protein C was present in seminal plasma (results not shown), and no APC-PCI complexes were formed when APC was incubated with semen, seminal PCI would seem to be inactive, possibly as a result of complex formation with a proteinase or cleavage to its modified form. This hypothesis is supported by the detection in gel filtration and immunoblotting experiments of a high molecular form of PCI, which may represent an enzyme inhibitor complex, and a low molecular form which may represent the cleaved modified inhibitor. The functional implications of these findings would seem to be that in the male reproductive system PCI regulates some other proteinase than APC. Candidate target enzymes are the prostate-specific antigen, the human glandular kallikrein-1 gene product, acrosin, or some unknown serine proteinase that may be involved in the

regulation of the gelation and lysis of ejaculated semen (41–45). It should, however, be emphasized that the molar concentration of PCI in seminal plasma is not more than 10% of the molar concentration of PSA normally present.

To sum up, protein C inhibitor is a heparin-dependent serpin that was first described as a regulator of APC in blood, but which has also been proposed to be a urokinase inhibitor in urine and to be involved in the contact activation system of plasma. Although no APC inhibitory activity was detected in seminal plasma, our data clearly demonstrate the local production in the male sex glands of a protein apparently identical with PCI. This indicates that the function of this inhibitor may be more diverse than formerly believed.

Acknowledgments

This investigation was supported by The Swedish Medical Research Council (grants B90-13X-7903-4B and B89-13x-04487-15A), the Faculty of Medicine at the University of Lund, the Research and Cancer Research Funds at Malmö General Hospital, the Magnus Bergvall Foundation, the Crafoord Foundation, the John and Augusta Persson Foundation, the Syskonen Svensson Foundation, the Alfred Österlund Foundation, the Fundacion Federico S. A., and Kungliga Fysiografiska Sällskapet.

References

1. Esmon, C. T. 1987. The regulation of natural anticoagulant pathways. *Science (Wash. DC)*. 235:1348–1352.

2. Esmon, C. T. 1989. The roles of protein C and thrombomoduline in the regulation of blood coagulation. *J. Biol. Chem.* 264:4743–4746.

3. Marlar, R. A., and J. H. Griffin. 1980. Deficiency of protein C inhibitor in combined factor V/VIII deficiency disease. *J. Clin. Invest.* 66:1186–1189.

4. Suzuki, K., J. Nishioka, and S. Hashimoto. 1983. Protein C inhibitor. Purification from human plasma and characterization. *J. Biol. Chem.* 258:163–168.

5. Suzuki, K., Y. Deyashiki, J. Nishioka, K. Kurachi, M. Akira, S. Yamamoto, and S. Hashimoto. 1987. Characterization of a cDNA for human protein C inhibitor. A new member of the plasma serine protease inhibitor superfamily. *J. Biol. Chem.* 262:611–616.

6. Hunt, L. T., and M. O. A. Dayoff. 1980. A surprising new protein superfamily containing ovalbumin, antithrombin-III and alpha 1-proteinase inhibitor. *Biochem. Biophys. Res. Commun.* 95:864–971.

7. Carrell, R. W., and D. R. Boswell. 1986. Serpins: the superfamily of plasma proteinase inhibitors. *In* Proteinase Inhibitors. R. W. Carrell and D. R. Boswell, editors. Elsevier Science Publishers B.V., Amsterdam. 403–420.

8. Suzuki, K., J. Nishioka, H., Kusumoto, and S. Hashimoto. 1984. Mechanism of inhibition of activated protein C by protein C inhibitor. *J. Biochem. (Tokyo)*. 95:187–195.

9. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitor. *Annu. Rev. Biochem.* 52:655–709.

10. Kazama, Y., M. Niwa, R. Yamagishi, K. Takahashi, N. Sakuragawa, and K. Takeheiko. 1987. Specificity of sulfated polysaccharides to accelerate the inhibition of activated protein C inhibitor. *Thromb. Res.* 48:179–185.

11. Radtke, K. P., T. W. Stief, and N. Heimburger. 1988. A simple isolation procedure for human protein C inhibitor. *Biol. Chem. Hoppe-Seyler.* 369:965–974.

12. Meijers, J. C. M., D. H. A. J. Kanters, R. A. A. Vloswijk, H. E. vanErp, M. Hessing, and B. B. Bouma. 1988. Inactivation of human plasma kallikrein and Factor XIa by protein C inhibitor. *Biochemistry.* 27:4231–4237.

13. Pratt, C. W., B. G. Macik, and F. C. Church. 1989. Protein C inhibitor: purification and proteinase reactivity. *Thromb. Res.* 53:595–602.

14. Espana, F., M. Berettini, and J. H. Griffin. 1989. Purification and characterization of plasma protein C inhibitor. *Thromb. Res.* 55:369–384.

15. Comp, P. C., R. M. Jacocks, G. L. Ferrell, and C. T. Esmon. 1982. Activation of protein C in vivo. *J. Clin. Invest.* 70:127–134.

16. Heeb, M. J., and J. H. Griffin. 1988. Physiologic inhibition of human activated protein C by alpha 1-antitrypsin. *J. Biol. Chem.* 263:11613–11616.

17. Stump, D. C., M. Thienpoint, and D. Collen. 1986. Urokinase related proteins in human urine. *J. Biol. Chem.* 261:1267–1273.

18. Stump, D. C., M. Thienpont, and D. Collen. 1986. Purification and characterization of a novel inhibitor of urokinase from human urine. Quantization and preliminary characterization in plasma. *J. Biol. Chem.* 261:12759–12766.

19. Stief, T. W., K. P. Radtke, and N. Heimburger. 1987. Evidence for identity of PCI and plasminogen activator inhibitor 3. *Biol. Chem. Hoppe-Seyler.* 368:1427–1433.

20. Heeb, M. J., F. Espana, M. Geiger, D. Collen, D. C. Stump, and J. H. Griffin. 1987. Immunologic identity of heparin-dependent plasma and urinary Protein C inhibitor and plasminogen activator inhibitor-3. *J. Biol. Chem.* 262:15813–15816.

21. Laurell, M., and J. Stenflo. 1989. Protein C inhibitor from human plasma: characterization of native and cleaved inhibitor and demonstration of inhibitor complexes with plasma kallikrein. *Thromb. Haemostasis.* 62:885–891.

22. Meijers, J. C. M., and D. W. Chung. 1990. Evidence for a glycine residue at position 316 in human protein C inhibitor. *Thromb. Res.* 59:389–393.

23. Lilja, H., J. Oldbring, G. Rannevik, and C. B. Laurell. 1987. Seminal vesicle-secreted proteins and their reaction during gelation and liquefaction of human semen. *J. Clin. Invest.* 80:281–285.

24. Lilja, H., and P. A. Abrahamsson. 1988. Three predominant proteins secreted by the human prostate gland. *Prostate.* 12:29–38.

25. Laurell, M., T. H. Carlson, and J. Stenflo. 1988. Monoclonal antibodies against the heparin dependant protein C inhibitor; suitable for inhibitor purification and assay of inhibitor complexes. *Thromb. Haemostasis.* 60:334–339.

26. Harlow, E., and D. Lane. 1988. Storing and purifying antibodies. *In* Antibodies: A Laboratory Manual. E. Harlow and D. Lane, editors. Cold Spring Laboratory, Cold Spring Harbor, NY. 288–318.

27. Angeles-Cano, E., and Y. Sultan. 1984. A solid phase fibrin immunoassay for the specific detection of monoclonal antibodies against different epitopic determinants of tissue plasminogen activators. *J. Immunol. Methods.* 69:115–127.

28. Hsu, S. M., L. Raine, and H. Fanger. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29:577–580.

29. Sternberger, L. A. 1979. *In* Immunocytochemistry. Second edition. John Wiley & Sons, Inc., New York.

30. Gultekin, M., and K. M. Heermann. 1988. The use of polyvinylidenedifluoride membranes as a general blotting matrix. *Anal. Biochem.* 172:320–329.

31. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. *Anal. Biochem.* 112:195–203.

32. Johnsson, D. A., J. W. Gautch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3–8.

33. Blake, M. S., K. H. Johnston, G. J. Russel-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175–179.

34. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

35. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 545.

36. Laurell, C. B. 1966. Quantitive estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* 15:45–52.

37. Harlow, E., and D. Lane. 1988. Immunoassays. *In* Antibodies: A Laboratory Manual. E. Harlow and D. Lane, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 578–583.

38. Lilja, H., P. A. Abrahamsson, and Å. Lundwall. 1989. Semenogelin, the predominant protein in human semen. *J. Biol. Chem.* 264:1894–1900.

39. Rosenberg, R. D., and P. S. Damus. 1973. The purification and mechanism of action of antithrombin-heparin cofactor. *J. Biol. Chem.* 248:6490–6505.

40. Chimielewska, J., M. Rånby, and B. Wiman. 1988. Kinetics of the inhibition of plasminogen activators by the plasminogen-activator inhibitor. Evidence for "second-site interactions." *Biochem. J.* 251:327–332.

41. Lilja, H. 1985. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J. Clin. Invest.* 76:1899–1903.

42. Chapdelaine, P., G. Paradis, R. R. Tremblay, and J. Y. Dubé. 1988. High level of expression in the prostate of a human glandular kallikrein mRNA related to prostate-specific antigen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 236:205–208.

43. Polakoski, K. L., and R. A. McRorie. 1973. Boar Acrosin. I. Purification and preliminary characterization of a proteinase from boar sperm acrosomes. *J. Biol. Chem.* 248:8178–8182.

44. Polakoski, K. L., and R. A. McRorie. 1973. Boar Acrosin. II. Classification, inhibition, and specificity studies of a proteinase from sperm acrosomes. *J. Biol. Chem.* 248:8183–8188.

45. Schleuning, W. D., and H. Fritz. 1974. Some characteristics of highly purified boar sperm acrosin. *Biol. Chem. Hoppe-Seyler.* 355:125–130.