Molecular cloning, tissue distribution and androgen regulation of rat protein C inhibitor

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Abstract Protein C inhibitor (PCI) is the plasma serine protease inhibitor of activated protein C, the active enzyme of the anticoagulant protein C pathway. Recently, PCI was also detected in human seminal plasma and reproductive organs (testis, seminal vesicle and prostate) suggesting that PCI may also play an important role in the reproductive system. In this study, we cloned the full length of rat PCI cDNA, and determined its amino acid sequence and tissue distribution. We also evaluated the effect of androgen on PCI mRNA expression in seminal vesicles and testes. The isolated 2074-bp rat PCI cDNA was composed of a 47-bp 5′-non-coding region, a 1218-bp coding region of a 406-amino acid precursor protein, a stop codon and a 806-bp 3′-non-coding region. The deduced amino acid sequence of rat PCI showed 85.7%, 64.1% and 62.2% homology with that of mouse, rhesus monkey and human PCIs, respectively. Northern blot analysis showed that the rat PCI mRNA is expressed strongly in the seminal vesicle, moderately in the testis, but not in the liver. PCI mRNA expression in seminal vesicles and testes was found to increase during the process of development, suggesting that it is under androgen control. Subsequently, we examined the effect of castration and/or treatment with 17β-estradiol or testosterone on PCI mRNA expression in the mature rat seminal vesicles. The PCI mRNA expression in seminal vesicles was significantly decreased after castration or 17β-estradiol treatment. Testosterone itself did not affect PCI mRNA expression, but treatment in castrated rats significantly enhanced its mRNA expression. These findings suggest that the PCI gene expression in rat seminal vesicles is regulated by androgen.

Key words: Protein C inhibitor; Serine protease inhibitor; mRNA expression; Testosterone; Seminal vesicle

1. Introduction

Protein C inhibitor (PCI) is a member of the plasma serine protease inhibitor (serpin) family proteins. It was originally described as an inhibitor of the anticoagulant protease, activated protein C [1], and subsequently found to inhibit a variety of other proteases involved in blood coagulation and fibrinolysis, such as thrombin [2], thrombin-thrombomodulin complex [3], factor Xa [2], factor XIa [4], plasma kallikrein [4] and urokinase-type plasminogen activator (uPA) [5]. Recently, human PCI was also detected in various extracellular fluids, its concentration being particularly high in seminal plasma [6], and it was found to inhibit several serine proteases involved in the process of reproduction (e.g. prostate-specific antigen (PSA) [7] and sperm acrosin [8]).

Human PCI is synthesized in the liver, kidney and in male reproductive organs (e.g. testis, seminal vesicle and prostate). Interestingly, the level of PCI in seminal plasma was found to be low in some infertile men [6]. Moreover, PCI was reported to inhibit the binding of human sperm to the oocyte; in this binding, a site other than the reactive site of PCI is involved [9,10]. Recently, we reported that seminal plasma PCI effectively inhibits PSA by forming a complex with it, and that PCI binds to the seminal coagula proteins, semenogelins I and II, protecting them from degradation by proteases [11]. These observations suggest that PCI participates in the regulation of reproduction.

PCI cDNAs of human [12] and rhesus monkey [13] were previously isolated and characterized. Human PCI gene consists of five exons and four introns [14,15]. This genetic organization is similar to that of other members of the serpin family such as α1-antitrypsin [16], α1-antichymotrypsin [17] and heparin cofactor II [18]. The mouse PCI gene was also recently reported to have the same gene organization, and its amino acid sequence was described to be highly homologous to that of human PCI [19]. Interestingly, mouse PCI mRNA is only expressed in organs of the reproductive system such as testis, seminal vesicle and ovary, but not in the liver [20]. These reports also suggest that PCI plays a role in the regulation of the reproductive system.

In the present study, we cloned the rat PCI cDNA and characterized its structure and distribution in different rat tissues. We also assessed the hormonal regulation of PCI mRNA in seminal vesicles and testes. The results suggest that PCI mRNA expression in the seminal vesicles is regulated by androgen.

2. Materials and methods

2.1. Amplification of partial cDNA encoding mouse PCI using polymerase chain reaction (PCR)

A sense primer located in exon III (5′-GTATTCCTACTACCTGGACC-3′, nucleotide residues 2124–2143) and an antisense primer in exon V (5′-CAGCTGCTGTGGTTCCTGAC-3′, complementary to nucleotide residues 4121–4102) of the genomic DNA of mouse PCI [19] were prepared and reverse transcriptase (RT)-PCR was performed. Reverse transcription was done using the T-Primed First-Strand kit (Pharmacia, Uppsala, Sweden) with 5 μg of total RNA isolated from the testes of BALB/c mice by standard protocol [21]. Using 1 μg of this first-strand cDNA as a template, PCR amplifica-

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Abbreviations: PCI, protein C inhibitor; Serpin, serine protease inhibitor; PSA, prostate-specific antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EIA, enzyme immunoassay; uPA, urokinase-type plasminogen activator

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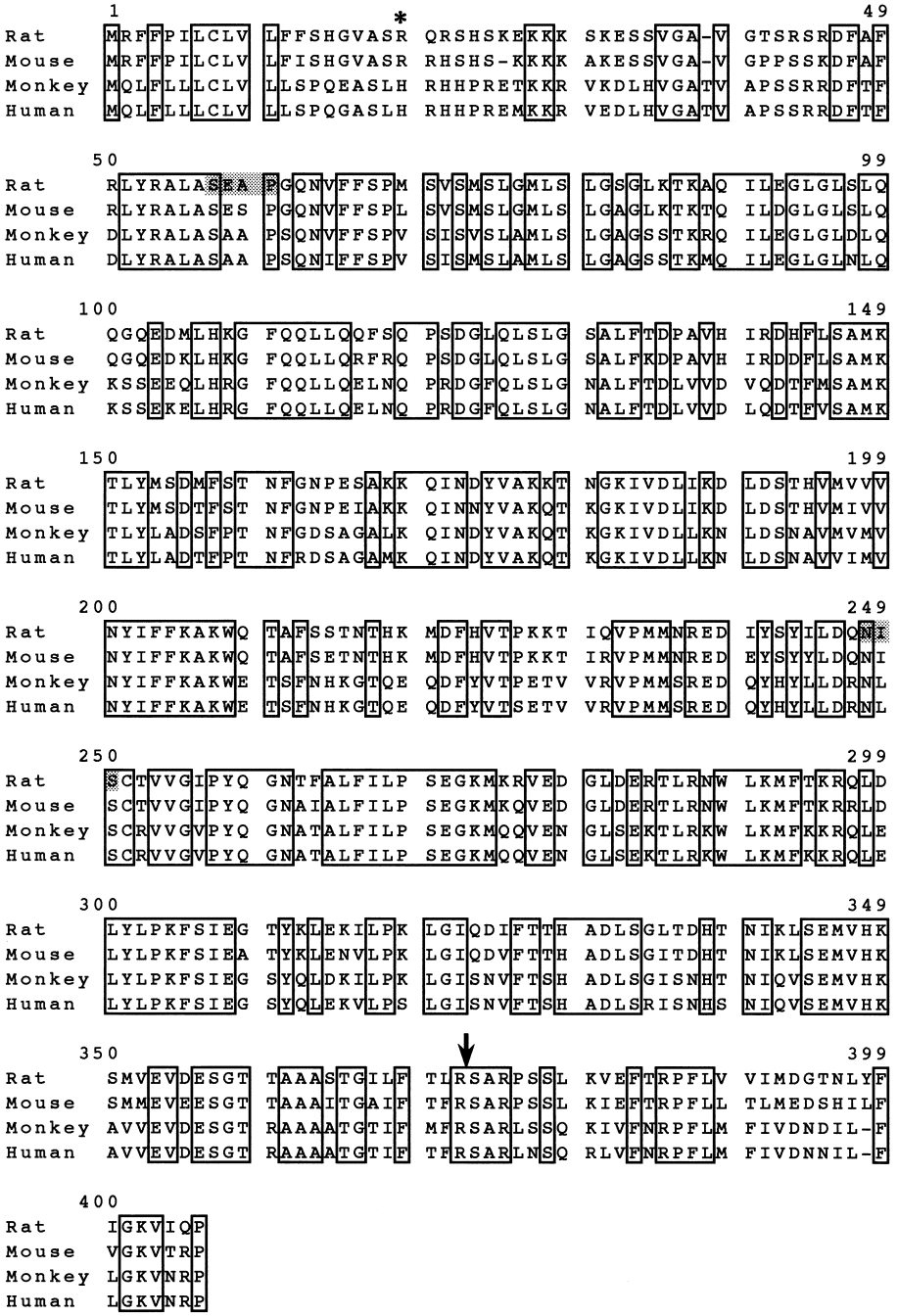

Fig. 1. Comparison of the deduced amino acid sequences of the PCI precursor protein of four species. Amino acid numbering is based on the amino-terminal residue of rat PCI precursor protein (position +1). Conserved amino acids in four species are enclosed within open boxes. The estimated amino-terminal amino acid residue (Arg^{20}) of the rat mature protein is marked by an asterisk, the estimated glycosylation sites of rat PCI are indicated by shaded boxes and the reactive site of PCI is indicated by an arrow.

tion was conducted during 35 cycles (94°C×1 min, 58°C×1 min, 72°C×2 min) using Taq polymerase (TaKaRa Shuzo, Kyoto, Japan) and a thermal cycler (model 480; Perkin-Elmer, Norwalk, CT, USA) [22]. PCR products were then subcloned into pBluescript $SKII^+$ (Stratagene, La Jolla, CA, USA) and sequenced using Dye Terminator Cycle Sequencing FS Ready Reaction kit and ABI 373A DNA sequencer (PE Applied Biosystems, Cheshire, UK) [23]. The mouse PCI cDNA fragment prepared in this way was then used as a probe for screening the rat testes cDNA library.

2.2. Cloning of rat PCI cDNA

The λZAP II rat testes cDNA library (Stratagene) was screened using a probe prepared from a portion of the mouse PCI cDNA (368 bp), as described above. Screening was performed as described previously [24]. Bacteriophage plaques giving positive hybridization signals were re-screened and purified. Isolated λZAP clones were converted into pBluescript SK^- phagemid clones by in vivo excision, according to the manufacturer's instruction.

2.3. DNA sequence analysis

Phagemid DNAs were digested with *Eco*RI, and Southern blot hybridization was performed as described previously [25]. The cDNA clones were cleaved by several restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I), and both strands of the cDNA clones were sequenced by primer walking using ABI 373A DNA sequencer.

2.4. Animals

Wistar rats used in this study were housed under a constant 12-h light and 12-h dark cycle, and were allowed free access to standard food and water. Adult male and female rats (9 weeks old; body weight 180–200 g) were used to study the tissue distribution of PCI mRNA. Male rats from 2 to 9 weeks old (each age group $n=3$) were used to study the PCI mRNA expression in seminal vesicles and testes during development. Adult male rats (9 weeks old) were classified into six groups (each group $n=3$): (a) castrated rats under pentobarbital anesthesia, (b) sham-operated rats but without removal of the testes, (c) rats receiving daily subcutaneous injections of 1 mg of testosterone propionate (Sigma, St. Louis, MO, USA) on days 4–12 after castration, rats treated with daily subcutaneous injections of (d) 100 μg of 17β-estradiol (Sigma), (e) 1 mg of testosterone propionate, or (f) vehicle alone (i.e. 200 μl of olive oil). Three rats were killed by overdose injection of pentobarbital on days 2, 4, 6 and 8 after the treatments. In case of groups a and c, the rats were killed using the same method used on days 10 and 12 after castration. The dose of hormone was the same as reported in a previous study [26]. Seminal vesicles and testes of the rats were removed and immediately frozen in liquid nitrogen and then stored at −80°C. These samples were used for the extraction of total RNA and for Northern blot analysis. At the same time, citrated plasma of each rat was also prepared and stored at −30°C; plasma samples were then used to measure the levels of testosterone.

2.5. RNA extraction and Northern blot analysis

Frozen tissue samples (kidney, liver, lung, brain, heart, small intestine, colon, ovary, testis, prostate, seminal vesicle, spleen and uterus) were homogenized in RNAzol B (TEL-TEST, Friendswood, TX, USA), without thawing. Total RNA was then extracted by a modification of the method of Chomczynski and Sacchi [21], and quantitated spectrophotometrically. Northern blot analysis was carried out as follows. Total RNA (20 μg for analysis of the tissue distribution of PCI or for studying the change of PCI mRNA expression in testes after treatment, and 10 μg for studying the change of PCI mRNA expression in seminal vesicles after treatment) of each tissue sample was electrophoresed in formaldehyde-agarose gels, transferred onto GeneScreen nylon membranes (NEN Life Science, Boston, MA, USA), UV cross-linked, and hybridized with full-length rat PCI cDNA probe as described previously [24]. The membrane was exposed to imaging plate and analyzed using BAS-2000 Image Analyzer (Fuji Photo Film, Tokyo, Japan). After hybridizing with rat PCI cDNA probe, the membranes were boiled in 0.5% sodium dodecyl sulfate and rehybridized with ^{32}P-labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA, USA) to correct differences in loading or transfer efficiency of RNA in different lanes. In each lane, the ratio between the relative intensities of the PCI mRNA band and the GAPDH band was evaluated using BAS-2000 Image Analyzer; the ratios were used to compare the PCI mRNA levels of different lanes.

2.6. Measurement of testosterone concentration in rat plasma

Testosterone concentration in rat plasma was measured using testosterone enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction.

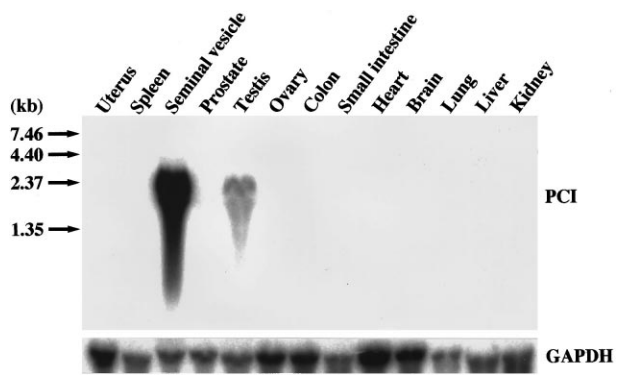

Fig. 2. Tissue distribution of rat PCI mRNA. Total RNA (20 μg) isolated from various tissues was analyzed by Northern blot analysis using ^{32}P-labeled rat PCI cDNA or GAPDH cDNA as a probe.

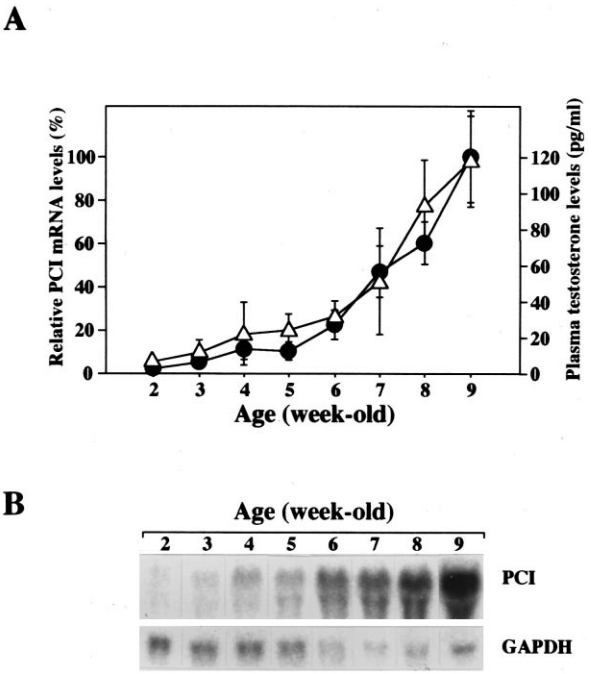

Fig. 3. Change of PCI mRNA expression in seminal vesicles and plasma testosterone levels during development. A: Total RNA (10 μg) isolated from seminal vesicles of each age was analyzed by Northern blotting using ^{32}P-labeled rat PCI cDNA or GAPDH as a probe. Radioactivity of the band corresponding to PCI mRNA and GAPDH mRNA was measured by Image Analyzer as photostimulated luminescence value (PSL), and the ratio of PSL of PCI mRNA to that of GAPDH mRNA was calculated; the ratio obtained in 9-week-old rats was taken as 100%. Plasma testosterone levels of each age group of rats were measured using the testosterone EIA kit. Data are expressed as mean ± S.D. ($n=3$). (●) relative PCI mRNA levels, (△) plasma testosterone levels. B: One typical data point of PCI mRNA and GAPDH mRNA expression of each age group.

3. Results

3.1. Cloning and characterization of rat PCI cDNA

Fourteen independent clones were isolated by screening 6×10^5 plaques of λZAP II rat testes cDNA library. The plaques were then converted into pBluescript SK$^-$ phagemid clones by in vivo excision. Southern blot analysis of the cDNA inserts showed that they consisted of three types of fragments. As the sequence analysis of their 5′- and 3′-ends demonstrated that two smaller fragments were completely included in the largest one, the nucleotide sequence of both strands of the largest fragment was determined, and it was found to contain a 2074-bp nucleotide sequence which encodes a 47-bp 5′-non-coding region, a 406-amino acid precursor protein, a stop codon and a 806-bp 3′-non-coding region. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB013128.

3.2. Comparison of the amino acid sequence of rat PCI with that of mouse, rhesus monkey and human PCIs

Fig. 1 shows the amino acid sequence of the rat PCI precursor protein deduced from its cDNA, and the alignment of the sequence of precursor protein of rat PCI with that of mouse, rhesus monkey and human PCIs. The precursor protein of rat PCI was composed of 406 amino acid residues

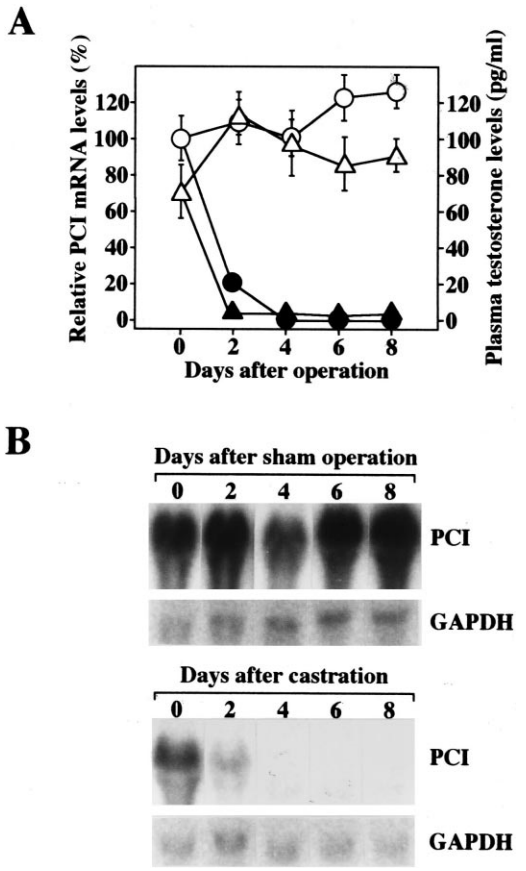

Fig. 4. Effect of castration on PCI mRNA expression in seminal vesicles and on plasma testosterone levels. A: Total RNAs (10 μg) isolated from seminal vesicles of sham-operated (○) or castrated rats (●) were analyzed by Northern blotting using ^{32}P-labeled rat PCI cDNA or GAPDH cDNA as a probe. Relative PCI mRNA levels were obtained as described in the legend to Fig. 3. Plasma testosterone levels of sham-operated rats (△) and castrated rats (▲) were measured using the testosterone EIA kit. Data are expressed as the mean ± S.D. ($n = 3$). B: One typical data point of PCI mRNA and GAPDH mRNA expression of sham-operated or castrated rats.

identical to that of rhesus monkey and human PCIs, but not to that of mouse PCI consisting of 405 amino acid residues. The amino acid sequence of rat PCI showed 85.7%, 64.1% and 62.2% of homology with that of mouse, rhesus monkey and human PCIs, respectively. In the amino acid sequence of the rat PCI precursor protein, the putative reactive site (P1-P1′) at Arg^{372}-Ser^{373}, the potential *O*-glycosylation sites at Ser^{57}, and the potential *N*-glycosylation site at Asn^{248} were estimated. Like mouse, rhesus monkey and human PCIs, the putative reactive site at Arg^{372}-Ser^{373} was located in the same amino acid sequence from P1 to P3′. One cysteine residue at Cys^{251} next to the potential *N*-glycosylation site in rat PCI was conserved in three other species' PCIs. Two potential glycosylation sites located at amino acid residues Ser^{57} and Asn^{248} were also conserved in all species.

3.3. Tissue distribution of rat PCI mRNA

Expression of PCI mRNA in various rat tissues was analyzed by Northern blotting. As shown in Fig. 2, the PCI mRNA (approximately 2.1 kb in size) was expressed strongly in the seminal vesicle and moderately in the testis, but not in the liver and other tissues. The lack of PCI mRNA expression in the liver and other tissues was not the result of RNA degradation, because GAPDH mRNA remained almost intact.

3.4. PCI mRNA expression in rat seminal vesicles and testes during development

Fig. 3A,B shows that PCI mRNA is not expressed in seminal vesicles of 2-week-old rats. However, PCI mRNA expression level gradually increased during development, and this increase was associated with concomitant changes in the plasma level of testosterone. The PCI mRNA expression in testes during development was similar to that in seminal vesicles (data not shown). This finding suggests that the increase of PCI mRNA expression in seminal vesicles and testes may be under androgen control during the process of development.

3.5. Effect of castration or sex hormone treatment on PCI mRNA expression in rat seminal vesicles and testes

PCI mRNA levels in seminal vesicles of rats treated with testosterone showed no difference compared with those of un-

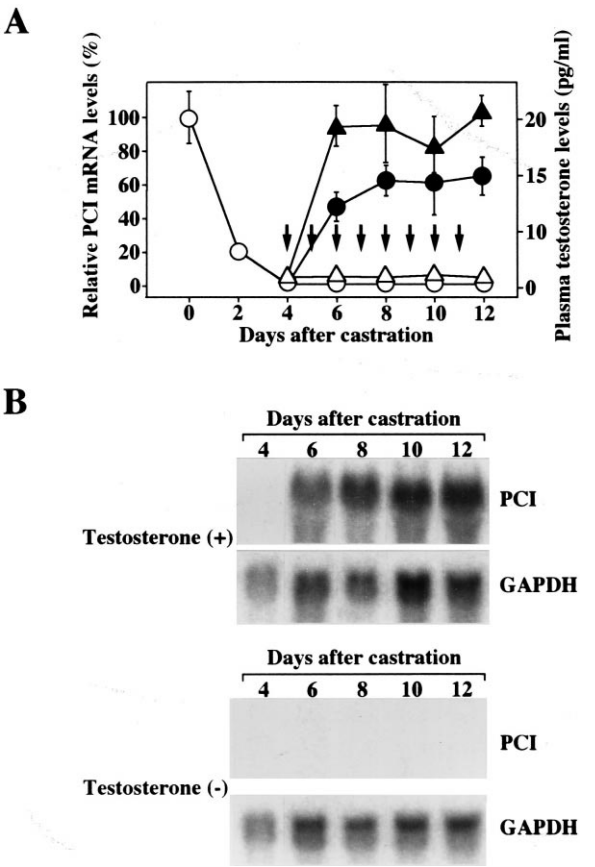

Fig. 5. Effect of testosterone replacement on PCI mRNA expression in seminal vesicles and on plasma testosterone levels. A: Total RNAs (10 μg) isolated from seminal vesicles of castrated rats (○) or of castrated rats treated daily with testosterone (●) from day 4 after castration were analyzed by Northern blotting using ^{32}P-labeled rat PCI cDNA or GAPDH cDNA as a probe. Relative PCI mRNA levels were obtained as described in the legend to Fig. 3. Plasma testosterone levels of castrated rats (△) and castrated rats treated with testosterone (▲) were measured using the testosterone EIA kit. Data are expressed as mean ± S.D. ($n = 3$). The arrow indicates the day when testosterone was injected. B: One typical data point of PCI mRNA and GAPDH mRNA expression of castrated or castrated and testosterone-treated rats.

treated rats (data not shown). In addition, PCI mRNA expression in testes of rats treated with 17β-estradiol did not significantly change as compared with untreated rats (data not shown). However, as shown in Fig. 4A,B, castration resulted in a time-dependent decrease of PCI mRNA expression in rat seminal vesicles. On day 2 after castration, PCI mRNA levels in seminal vesicles were significantly ($P < 0.005$) decreased as compared to those in sham-operated rats, the maximum effect being observed on day 4 after castration. This decrease of PCI mRNA levels after castration was associated with parallel changes in the plasma testosterone levels. The administration of 17β-estradiol also resulted in a time-dependent decrease of PCI mRNA expression in seminal vesicles. On day 2 after 17β-estradiol treatment, PCI mRNA levels in seminal vesicles and plasma testosterone levels were significantly decreased in treated rats as compared with untreated rats (data not shown). These data suggest that PCI mRNA expression in seminal vesicles may be regulated by testosterone.

To confirm this, the effect of testosterone replacement on PCI mRNA expression in seminal vesicles of castrated rats was examined. Fig. 5A,B indicates that PCI mRNA levels in seminal vesicles maximally decreased on day 4 after castration, but they increased significantly by daily administration of testosterone. These data indicate that, in our experimental conditions, testosterone regulates the expression of PCI mRNA in rat seminal vesicles.

4. Discussion

The deduced amino acid sequence of the rat PCI precursor protein showed that the putative reactive site and the site of cysteine residue of human [12], rhesus monkey [13] and mouse [19] PCIs are well-conserved in rat PCI. In addition, a possible heparin-binding site of the mature human PCI located between residues 264 and 283 (these residues are located between residues 283 and 302 in human PCI precursor protein as shown in Fig. 1) [27,28] appears to be conserved between residues 282 and 301 of the rat PCI precursor protein; seven positively charged residues are present in human PCI and five in rat PCI. However, it remains unclear whether rat PCI actually binds to heparin. On the other hand, the potential glycosylation sites in PCI seem to be different. Human and rhesus monkey PCIs have three *N*-glycosylation sites (Asn^{249}, Asn^{262} and Asn^{338}) and two *O*-glycosylation sites (Thr^{39} and Ser^{58}) [12,13], but rat PCI has only one *N*-glycosylation site (Asn^{248}) and two *O*-glycosylation sites (Ser^{57} and Ser^{373}; the latter is not reasonable because this site is involved in the reactive site). The differences of the glycosylation sites between the two types of PCI may influence the specificity of the inhibitor towards the target proteases, their stability in body fluids, and/or the specificity of their tissue expression.

The present analysis showed that the rat PCI mRNA is expressed only in the male reproductive system (seminal vesicle and testis). These observations encouraged us to assess whether PCI expression in seminal vesicles and testes is regulated by sex hormones. The fact that PCI mRNA expression in the seminal vesicles and testes was gradually increased during rat development (from 2 to 9 weeks old) suggests that rat PCI mRNA expression is influenced by androgen. The effect of castration or 17β-estradiol treatment on PCI mRNA expression in rat seminal vesicles suggests that PCI gene expression is under androgen control in seminal vesicles. Testosterone replacement in castrated rats also showed that PCI mRNA expression in seminal vesicles depends on the level of testosterone. Although 17β-estradiol did not significantly affect PCI mRNA expression in rat testes, this was probably due to the small amount of endogenous testosterone released by Leydig cells; this basic level of testosterone probably stimulates constantly PCI mRNA expression in the testes. Long-term administration of 17β-estradiol may down-regulate PCI gene expression in the testes. On the other hand, testosterone itself did not appear to affect the PCI mRNA expression in seminal vesicles and testes, so that the excess amount of testosterone may not affect target organs. The partial structure of the human PCI gene has already been reported [14,15], however there is no androgen-responsive element [29] in the 1.6-kb 5′-non-coding region of the human PCI gene [30].

Several proteases such as acrosin and uPA have been reported to participate in the process of reproduction. Acrosin, which is released from mammalian sperm during the acrosomal reaction, plays a fundamental role in the mechanism of sperm penetration into the zona pellucida [31]. uPA is expressed by epithelial cells from male genital organs (e.g. seminal vesicle, cauda epididymidis and vas deferens) and it is secreted during ejaculation to bind the sperm surface [32,33]. uPA has been suggested to play an important role in sperm capacitation and fertilization processes. The regulatory mechanisms of the proteolytic activity of these enzymes in the reproductive system are not completely understood. It has been suggested that a member of the serpin family, protease nexin I, may inhibit uPA activity in the reproductive system [34]. PCI, another component of the serpin family, is functionally and immunologically the same as plasminogen activator inhibitor-3 [35], which is known to effectively inhibit uPA activity [5]. In addition, PCI has also been reported to modulate the proteolytic activity of acrosin and PSA; this latter is suggested to be an important factor for sperm motility and fertilization. All together, these findings suggest that PCI may play an important function in the regulation of proteolytic processes in the reproductive system.

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