In vitro characterization of missense mutations associated with quantitative protein S deficiency

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Summary. *Objective:* To elucidate the molecular consequences of hereditary protein S (PS) deficiency, we investigated the *in vitro* synthesis of the PS missense mutants in COS-1 cells and their activated protein C (APC) cofactor activities. *Patients:* Four patients with quantitative PS deficiency suffering from venous thrombosis were examined. *Results:* We identified 3 distinct novel missense mutations, R275C, P375Q and D455Y, and 2 previously reported missense mutations, C80Y and R314H. The P375Q and D455Y mutations were found in one patient and observed to be in linkage on the same allele. The R314H mutant showed the lowest level of expression (32.7%), and the C80Y, P375Q+D455Y, and R275C mutants exhibited a moderate impairment of expression, i.e., 43.8%, 49.5%, and 72.3% of the wild type, respectively. Furthermore, pulse-chase experiments demonstrated that all mutants showed impaired secretion and longer half-lives in the cells than the wild type PS. In the APC cofactor assays, the C80Y mutant showed no cofactor activity, and the R275C mutant showed reduced activity, 62.3% of the wild type PS, whereas the R314H and P375Q+D455Y mutants exhibited normal cofactor activity. *Conclusion:* These data indicate that the C80Y and R275C mutations affect the secretion and function of the PS molecule, and that the R314H and P375Q+D455Y mutations are responsible for only secretion defects, causing the phenotype of quantitative PS deficiency observed in the patients.

Keywords: venous thrombosis, protein S, missense mutation, quantitative deficiency, activated protein C cofactor activity.

Introduction

Protein S (PS) is a vitamin K-dependent plasma glycoprotein that acts as a cofactor for activated protein C (APC) in the inactivation of the procoagulant factor Va and factor VIIIa [1,2]. Since, PS also directly inhibits factor Va and factor Xa independently of protein C [3-5], it plays an important role in the regulation of blood coagulation, and a deficiency in PS is a risk factor for venous thrombosis [6,7]. PS is a modular protein comprised of a γ-carboxyglutamic acid (Gla) domain, a thrombin-sensitive region (TSR), 4 epidermal growth factor (EGF)-like domains, and a large domain homologous to the sex hormone binding globulins (SHBG-like domain) [1,2]. The Gla domain is pivotal for Ca^{2+}-dependent binding of PS to phospholipid membranes where it exerts its biological function [8], and also associated with the expression of APC cofactor activity [9]. The TSR and the EGF-like domain are shown to be important for expression of the APC cofactor function using the epitope mapping analysis of monoclonal antibodies and site-directed mutagenesis [10-12]. The SHBG-like domain mediates the binding of PS to a complement-regulator component, C4b-binding protein (C4BP), which results in the inhibition of APC cofactor activity [13]. In human plasma, approximately 60% of PS circulates in conjunction with C4BP, whereas the remaining 40% circulates as a cofactor for APC [14].

PS deficiency has been traditionally classified into type I (quantitative deficiency with low total PS antigen, low free PS antigen, and low APC cofactor activity), type II (qualitative deficiency with normal total PS antigen and normal free PS antigen levels, but low APC cofactor activity), and type III (normal total PS antigen, but low free PS antigen, and low APC cofactor activity) [15]. However, several reports have suggested that both type I and type III are two phenotypic expressions of the same genetic defect [16-18].

There are the few reports investigating the molecular bases of missense mutations responsible for protein S deficiency [19-21]. In the present study, we identified 3 novel

missense mutations associated with the phenotype of quantitative PS deficiency, and investigated the molecular consequences of the missense mutations, together with 2 previously detected naturally occurring missense mutations by *in vitro* expression studies and APC cofactor activity assay.

Materials and methods

Blood samples and DNA sequencing

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine. Following informed consent, venous blood samples from patients with PS deficiency as well as normal individuals were collected in a 1:10 volume of 3.13% (wt/vol) trisodium citrate. Genomic DNA was isolated from the peripheral blood leukocytes according to standard procedures [23]. Fifteen exons and their boundaries of the PS gene were amplified from genomic DNA of the patients by a polymerase chain reaction (PCR) as described previously [24]. The PCR products were directly sequenced using a Big-Dye Terminator Cycle Sequencing kit and a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Mutagenesis and construction of expression vectors

A full-length human PS cDNA (a generous gift from Dr. B. Dahlbäck) subcloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA) was designated wild type PS cDNA in this study. Mutants were generated by recombinant PCR [25], and the final mutated PCR fragments were treated with the restriction enzymes, and then inserted into the vector with those same enzymes.

Expression of recombinant protein S

Monkey kidney COS-1 cells were cultured in Dulbecoo's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 3.5mM glutamin, and administration of antibiotics-antimycotics. Approximately 80% confluent COS-1 cells in 3.5-cm diameter 6-well plates were transiently transfected with 1.3 μg/well of pcDNA3 containing wild type or mutant PS cDNA, using the Lipofectin mediated method [26]. The

cells were incubated in serum-free medium, Optimem I (GIBCO-BRL) supplemented with antibiotics-antimycotics and 10 μg/ml vitamin K1 (Isei, Yamagata, Japan) for 24 hours, and PS containing media were harvested for further expression analysis. In order to measure APC cofactor activities, we also established stable transformants expressing recombinant PS molecules in human embryo kidney (HEK) 293 cells as described previously [27].

Western blot analysis

The samples were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nylon membranes. PS was detected colorimetrically by immnuoblotting with a rabbit polyclonal antibody and a swine anti-rabbit IgG antibody conjugated with alkaline phosphatase (DAKO, Glostrup, Denmark).

Quantification of protein S expression

PS antigen concentration in the conditioned media of transfected cells was measured by an enzyme-linked immnunosorbent assay (ELISA), essentially following a described method [20]. The plates were developed with 100 μL of TMB substrate (DAKO) for 2 minutes, and the absorbance was then measured at 490 nm. A calibration curve was constructed using the recombinant wild type PS of a known concentration diluted in fresh media.

Pulse-chase experiments

Pulse-chase experiments of the recombinant PS by radioactivity labeling were performed essentially as previously described [20]. The gels were analyzed with an ImageQuant® (Molecular Dynamics, sunnyvale, CA) to quantify the radioactivity of the bands on the gels.

Determination of APC cofactor activity of recombinant protein S

Stable transformants were grown in DMEM supplemented with 10% fetal calf serum, 3.5mM glutamin, antibiotics-antimycotics, and 10 μg/ml vitamin K1. The serum-free conditioned media were harvested, and the concentrations of recombinant PS in the conditioned media were measured by ELISA. The conditioned media were diluted in 10 mM Tris-HCl, 150 mM NaCl; pH 7.4 to provide a range of final recombinant PS concentrations 0 to 100 ng/ml. APC cofactor activity of recombinant PS was determined on the basis of a clotting assay (Statclot, Diagnostica-Stago) using ST art4 (Roche).

Results

Clinical characteristics and gene abnormalities in patients with PS deficiency

The clinical characteristics and gene abnormalities in 4 patients with PS deficiency are shown in Table 1. All patients had episodes of recurrent deep vein thrombosis or cerebral infarction. All parameters in coagulation and fibrinolysis other than PS were within normal limits (data not shown). The C80Y and R314H mutations have been identified previously [22], while 3 others, R275C, P375Q and D455Y, were novel, with the latter 2 being found in the same patient. The P375Q and D455Y mutations were detected on the same allele of the PS gene of one patient by sequencing of a PCR-amplified DNA fragment spanning the region of exons 11 to 12 that might contain both mutations. All these patients were found to be heterozygous for the respective mutations. We also performed PCR-RFLP analyses for 3 novel missense mutations to search for the same nucleotide substitutions in healthy Japanese volunteers, and did not find the R275C, P375Q and D455Y mutations in any of 104 healthy control subjects (data not shown).

Transient expression of PS mutants in COS-1 cells

Wild type PS and mutant PS with each of the missense mutations, C80Y, R314H, R275C and P375Q+D455Y, were transiently expressed in COS-1 cells. Using Western blot analysis, PS was detected in all media as doublet bands with the expected molecular size (Fig. 1A). The amount of protein detected, however, varied with different conditioned media. To accurately quantify the PS expression levels of the respective mutants, the concentrations of recombinant PS in the culture media were measured by ELISA (Fig. 1B). The R314H mutant showed the lowest concentration (32.7%), followed by the C80Y mutant (43.8%). The quantity of the P375Q+D455Y mutant in the media was approximately half (49.5%) of that of the wild type PS, and the expression level of the R275C was moderately reduced to 72.3%.

Pulse-chase experiments

A quantitative analysis of the pulse-chase data showed that radiolabeled wild type PS rapidly decreased in the cells with a half-life of 2 hours and immediately appeared in the culture media (Fig. 2). In contrast, the radiolabeled C80Y, R314H and P375Q+D455Y mutants slowly disappeared from the cells, as their half-life was approximately 5 hours in the C80Y and P375Q+D455Y mutants, and 8 hours in the R314H mutant. They were gradually secreted into the culture media. The secretion efficiency, measured as the level of PS in the media at 8 hours, was higher in the wild type PS (70% of the initial value), whereas it was significantly reduced in the 4 mutants (30% for C80Y, 19% for R314H, 28% for P375Q+D455Y and 42% for R275C).

Effects of two mutations, P375Q and D455Y, on one allele

To further determine the effects of the P375Q and D455Y mutations individually, we studied the 2 discrete mutant constructs using expression analysis. In transient expression studies with COS-1 cells, ELISA data showed that expression levels of both the D455Y and P375Q+D455Y mutants were reduced to 40% in the culture media compared with that of the wild type PS, whereas the P375Q mutation did not have impaired expression (Fig. 3A). These findings were again confirmed by pulse-chase experiments (Fig. 3B). Thus, although the D455Y mutant had impaired secretion and a longer half-life in the cells than the wild type PS, the P375Q mutant showed a comparable secretion rate to that of the wild type PS. These data clearly indicate that the impaired secretion of the P375Q+D455Y mutant is caused by the D455Y mutation rather than by the P375Q mutation.

APC cofactor activity assay

We compared the effects of respective mutations on the APC cofactor activity in a clotting assay. The APC cofactor activities of recombinant PSs were examined using the serum-free conditioned media of stable transformants. The concentrations of recombinant PSs in the media were determined by ELISA. The serum-free media of the mock-transfected cells did not affect the clotting time, while those of the wild type, the R314H mutant, and the P375Q+D455Y mutant expressing cells prolonged the clotting time dose-dependently (Fig. 4). In contrast, the C80Y mutant showed no APC cofactor activity, and the R275C mutant was found to be significantly less efficient than the wild type in prolongation of the clotting time (62% of APC cofactor activity of the wild type). In addition, both the P375Q and D455Y mutants did not affect the APC cofactor activities (Fig. 4).

Discussion

We investigated the molecular defect in 4 patients with quantitative PS deficiency associated with recurrent thrombotic complications. In the DNA sequence analyses, we identified 3 novel (R275C, P375Q and D455Y) and 2 previously reported (C80Y and R314H) missense mutations [22]. In the PS gene, the correlation between missense mutations and phenotype of the patients is not always as straightforward as reported in other genes [20, 28]. In this study, to investigate the molecular mechanisms of the quantitative PS deficiency associated with the missense mutations, 5 of them identified in 4 patients with PS deficiency were analyzed by *in vitro* expression studies using COS-1 cells.

The ELISA measurement of expressed PS levels in culture media showed that the amounts of all mutants expressed were reduced compared with that of wild type PS (Fig. 1), mimicking the phenotype of quantitative PS deficiency in our 4 patients. To study the steps from primary protein synthesis to secretion in the protein-expression pathway, pulse-chase analysis was performed. We found that the C80Y and P375Q+D455Y mutants had impaired secretion, and that the half-lives of these mutants in the cells were 2.5 times longer than that of the wild type PS (Fig. 2). Among mutants we studied, R314H showed the severest impaired secretion, corresponding to the fact that the expression level of this mutant was also the lowest in the ELISA studies. The expression level of the R275C mutant decreased to 72.3% of that of the wild type PS, while its half-life in the cells (3.5 hours) was longer than that of the wild type (2 hours, Fig. 2) in the pulse-chase. These results support the concept of the missense mutations being responsible for quantitative PS deficiency.

One may find some discrepancies between our *in vitro* expression study data and the *in vivo* plasma data of the patients. For example, total PS antigen levels were higher in the case 2 than in the case 3. However, the ELISA data showed that the expression level of the R314H mutant was significantly lower than that of the R275C mutant. Especially in individuals

heterozygous for a certain missense mutation, an expression level of normal PS derived from a normal allele often hampers the correct diagnosis of PS deficiency, since the normal ranges of PS activity and antigen are relatively wide. In addition, the phenotypic expression of PS *in vivo* varies in individuals, probably depending upon differences in genetic conditions and/or environmental circumstances including age, sex, clinical status, and medical treatment [6,7]. Thus, it is often difficult to directly compare *in vitro* expression study data to the *in vivo* plasma data of the patients. On the other hand, the expression studies and APC cofactor assay in this study indicate the effects by the mutations themselves.

In Case 4, we identified 2 missense mutations, P375Q and D455Y, which were linked on the same allele. To determine the individual effects of these 2 mutations, we performed expression studies using 2 discrete constructs, and compared them to the wild type PS as well as to the P375Q+D455Y mutant (Fig. 3). The ELISA results demonstrated that the D455Y mutant had impaired expression, whereas the P375Q had no effect on the expression level. Pulse-chase analysis also revealed clear differences in the secretion rates between these 2 mutants, indicating that the D455Y mutation played a key role in the impaired expression of the P375Q+D455Y mutant. In this study, the P375Q mutant was found to have normal secretion, and it did not seem that the mutation itself would produce any definite abnormality *in vivo* leading to quantitative PS deficiency. This mutation was not identified in the PCR-RFLP analysis of 104 Japanese healthy volunteers. However, it seemed to be one of the new polymorphisms that only rarely occur in subjects with normal PS levels [7].

Cys80 is present in the first EGF-like domain containing high-affinity calcium ion binding sites for the interaction with APC [6, 29-31], whereas the location of the other 3 mutations, Arg314, Arg275 and Asp455, are in the SHBG-like domain. Amino acid alignments of several mammalian species revealed that these amino acid residues are highly conserved in equivalent positions among those PS molecules, and Arg314 and Asp455 particularly compose α–helix

and β–strand, respectively corresponding to the secondary structure prediction for PS [32]. Thus, it is very likely that the structural roles of these residues are to affect the ternary structure of the PS protein. In addition, the substitution of Tyr for Cys80 destroys the formation of the Cys80-Cys93 disulfide bond [11], and a newly introduced Cys for Arg275 might destabilize the protein by creating an abnormal disulfide bond [19-21].

Although all the missense mutations resulted in a decreased expression of the recombinant PS into media, the efficiency of the secretion was completely different among the mutations (Fig. 1). We hypothesized that not only the secretion defect of these mutations should be responsible for quantitative PS deficiency individually, but also another aspect of the mutations might be associated with the phenotype of PS deficiency in the patients. In order to investigate whether these mutations caused any functional defect in PS, we determined APC cofactor activity of the recombinant PS by measuring clotting time. The 2 PS mutants (R314H and P375Q+D455Y) were found to equally prolong the clotting time with wild type. In contrast, the C80Y did not affect the clotting time, while the R275C showed low APC cofactor activity (Fig. 4). As described above, substitution of Cys80 by Tyr in the first EGF-like domain of PS would interfere with folding of the EGF module. This mutation would, furthermore, increase the susceptibility of PS protein to proteases and cause its reduced affinity for Ca^{2+} [33]. Arg275 is located in the SHBG-liked domain of PS, which is important in the interactions of PS with C4BP and with factor V during the APC-mediated inactivation of factor VIIIa [34]. Taken together with highly conserved Arg275 among species, the change to Cys is likely to disrupt protein folding and its function as a cofactor for APC. On the other hand, the R314H and P375Q+D455Y mutants were found to have no effect on APC cofactor activity by the mutations. However, from the results of the expression studies, it is suggested that these mutations lead to low levels of PS activity in the patients as reducing the secretion of the PS molecule.

In conclusion, our studies reveal that the C80Y and R275C mutations impair both the secretion of the PS molecule and the function as APC cofactor, and that the R314H and P375Q+D455Y mutations are responsible for only secretion defects, causing the phenotype of quantitative PS deficiency. Thus, there is a possibility that the individually quantitative PS deficiency contributing to the risk of thrombosis might be caused by the different mechanisms depend on the missense mutations. This strongly suggests that molecular approaches help to elucidate the relationship between genetic abnormalities and clinical phenotypes.

Addendum

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Molecular genetics: H. Okada, A. Takagi, T. Murate

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Legends to Figures

Fig. 1. Transient expression of wild type and mutant recombinant PS in COS-1 cells

(A) Western blot analysis of 15 μL of conditioned media was performed under reducing conditions. Note that some mutants appeared as doublet bands, with the lower band representing cleaved-protein S by proteolysis as shown in plasma.

(B) ELISA determination of concentration of different mutants. Mean value of wild type PS is assigned as 100%. Values represent mean ± SD of 6 transfection experiments per mutant. Comparison between mutant and wild type expression levels was performed using unpaired *t* test.

Fig. 2. Pulse-chase analysis using transient expression in COS-1 cells

Radiolabeled media and cell lysates were immunoprecipitated and electrophoresed on SDS-PAGE.

(A) A representative experiment of wild type PS and each mutant pulse-chase.

(B) Radioactivity of PS bands on dried gels was measured using an image analyzer. Amount of radioactive PS in cell lysates at beginning of the experiment was assigned a value of 100%. Graphs represent radioactivity recovered from cell lysates (◆), media (■) or total (▲) at each time point. Total radioactivity was calculated as sum of radioactivity recovered from media and lysates. Values represent mean ± SD of 3 or 4 independent experiments.

Fig. 3. Effects of each amino acid substitutions (P375Q and D455Y) on protein-expression pathway

(A) ELISA determination of concentrations of different mutants. Histograms and bars represent mean ± SD (n=6). Mean value of wild type PS is assigned as 100%.

Comparison between mutants and wild type expression levels was performed using unpaired *t* test.

(B) Quantitative results of wild type PS (●), P375Q (■), D455Y (▲) and P375Q+D455Y (○) are shown. Amount of radioactive PS in cell lysates at 0 hour is assigned as 100% for each construct.

Fig. 4. APC cofactor activity of recombinant PS

(A) A range of recombinant PS concentrations (0 to 100 ng/mL) in serum-free conditioned media of stable transformants was incubated with PS-depleted plasma, factor Va, and APC for 2 min. Clotting was initiated by addition of $CaCl_2$, and clotting time was measured using ST art4. Values represent mean ± SD of 4 or 5 independent experiments. (●) indicates wild type PS; (■), C80Y mutant; (▲), R314H mutant; (◆), R275C mutant; (○), P375Q+D455Y mutant; (◇), P375Q mutant; (△), D455Y mutant. Dotted line represents the clotting time of mock transfected media as a control.

(B) APC cofactor activity calculated from clotting time assay compared to same concentration (100 ng/mL) of different mutants. Mean value of wild type PS is assigned as 100%. Values represent mean ± SD of 4 or 5 independent experiments. Comparison between clotting times of 100 ng/mL of each mutant and that of wild type PS was determined using unpaired *t* test.