Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) using forward or reverse PCR primers, according to the manufacturer's protocol. The sequencing products were then precipitated with 0.15 M NaOAc (pH 8.0) and cold ethanol, washed once with 70% ethanol, dried, resuspended in 25 μL of Template Suspension Reagent (Applied Biosystems), and analysed by an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Analysis of FV mRNA

To investigate the presence of FV transcripts from the mutant allele in platelets, we analysed platelet RNAs from the proband by mRNA-based PCR-restriction fragment length polymorphisms (RFLPs). In brief, total RNA extracted from the platelets was reverse-transcribed using the respective gene-specific primers: 12GSP (5′-TCTGTTCTGGTAATCA TAGT-3′) for 1943insC or 15GSP (5′-GTGCTG TTTATTGCCATTTT-3′) for A5279G, and Super Script II RT reverse transcriptase (Invitrogen Japan, Tokyo, Japan). To detect the 1943insC mutation, a nested PCR was performed using the following primers: 12rPCR-UP (5′-CCCTATAGCATTTAC CCTCA-3′) and 12GPS for the first PCR, and 12mut-UP (5′-ACTTCTGTAGTGTGGGGggCC-3′; bold lower case characters are mismatched nucleotides) and 12 M-LW (5′-TTCATCATCATCTGGG-ATAC-3′) for the second PCR, introducing a new *Apa*I restriction site in the mutant PCR products, as a single PCR using the first or second PCR primer set failed to amplify authentic PCR products. The 1943insC mutant RT-PCR products would yield 19- and 221-bp fragments, whereas the wild-type products would not be digested (239 bp). To detect the A5279G mutation, PCR was performed with the following primers: 15 M-UP (5′-AAAAATCATCA GAGGGAAAG-3′) and 15mut-LW (5′-CTGGGT TCACAGCTGAcTAG-3′) introducing a *Spe*I restriction site in the wild-type PCR products. Thus, the wild-type RT-PCR products would yield 18- and 159-bp fragments, while the A5279G mutant products would not be digested (177 bp). These fragments were run on a 4% agarose gel and stained with ethidium bromide. We evaluated the allele-specific mRNA levels by the quantitative densitometric analyses using the NIH image software (version 1.62) (National Institutes of Health, Bethesda, MD, USA).

Preparation of mutant FV expression vectors

We prepared individual FV expression vectors bearing the identified mutations, 1943insC (FS592X; the initial Met residue is denoted amino acid +1) and A5279G (Y1702C), based on pMT2 containing a full-length cDNA of human FV (pMT2-FV). Both mutations were introduced individually into the pMT2-FV expression vector using the recombinant PCR method described elsewhere [14]. After recombinant PCRs, each DNA fragment encoding the 1943insC or A5279G mutation was isolated as *Bsp*36I-*Bsp*EI or *Bsp*MI-*Sna*BI fragments, and separately replaced into the appropriate position for the pMT2/FV expression vector. DNA sequencing confirmed that no unexpected mutation was found in any of the whole mutant inserts in either construct.

Transient expression of recombinant FVs in COS-1 cells

African green monkey kidney COS-1 cells were cultured in a 5% CO_2 humidified atmosphere at 37°C in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with fetal calf serum (10%), glutamine (1%), and antibiotics (penicillin and streptomycin, 100 IU mL^{-1} and 100 μg mL^{-1} respectively). Cells in 30-mm dishes were transfected with either wild type or individual mutant plasmids using the Fu-GENE6^{TM} transfection reagent (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions. After 48-h culture of the transfected cells in serum-free DMEM, conditioned media containing the secreted recombinant proteins were collected, then concentrated using Centrisart I (cut off MW 20000; Sartorius, Goettingen, Germany), and subjected to one-stage clotting assay as well as ELISA (Cedarlane Lab. Ltd) for recombinant FV antigen measurements as described above.

Results and discussion

Case report

The patient (individual II-1, Fig. 1) is a 39-year-old Japanese woman who had recurrent episodes of bleeding such as epistaxis, joint region haematoma and hypermenorrhea, which were treated with FV replacement therapy by transfusion of fresh frozen plasma. When the patient was 4 years old, she had been diagnosed as having coagulation FV deficiency, since laboratory tests revealed that the prothrombin time and the activated partial thromboplastin time were prolonged, and FV activity was below the measurable limit. There was no history of bleeding tendencies in her other family members tested, since FV activities in plasma of both her mother and a sibling were 65%, suggesting that they might be

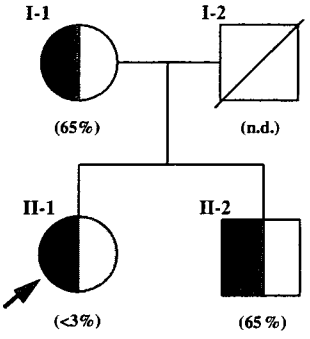

Fig. 1. Pedigree of the factor V-deficient family. The propositus is subject II-1 (arrow). Circle and square indicates male and female respectively. Values in parentheses represent plasma factor V activities (n.d., not done). Subjects with 1943insC and A5279G mutations are demonstrated with solid and shaded areas respectively.

heterozygous for FV-deficiency causing mutation. Consanguinity in the family was excluded.

DNA sequencing

In order to identify causative FV gene mutation(s) in such an FV-deficient patient, we analysed nucleotide sequences of all 25 exons and exon–intron boundaries of the FV gene. Results from direct sequencing of the FV gene revealed that the patient had a C insertion in three consecutive cytosine nucleotides [^{589}Thr(ACC)–^{590}Gln(CAG)] in exon 12 at nucleotide positions 1940–1942 (1943insC), and an A–G transition in exon 15 at nucleotide position 5279 (A5279G) (Fig. 2). DNA samples from her mother and brother also showed heterozygosity for the 1943insC mutation, but no A5279G mutation (data not shown), which are consistent with the data of plasma FV activity, i.e. about half that of normal subjects; 1943insC is a novel mutation, which can cause a frame-shift resulting in a substitution of the amino acids after ^{590}Gln with two abnormal residues (^{590}Pro–^{591}Glu) followed by a stop codon (FS592X). The A5279G will cause the amino acid substitution Y1702C, which was previously designated FV Seoul 2 [15]. The A5279G FV gene mutation has also been found in Italian and Slovenian subjects [16,17], and is thought to be a very ancient and/or recurrent mutation. In this study, we demonstrated that this mutation also occurred in a Japanese subject, suggesting that the A5279G might be a hot-spot mutation rather than a founder mutation.

mRNA analysis (RT-PCR RFLPs)

We analysed the expression of mutant FV gene transcripts from the patient's platelets by mRNA-mediated PCR-RFLPs (RT-PCR RFLPs). For 1943insC (FS592X-FV mRNA), the nested RT-PCR followed by *Apa*I digestion yielded 239- and 221-bp bands, representing transcripts from the normal and mutant alleles, respectively, although the mutant signal was markedly reduced (Fig. 3a). For A5279G (Y1702C-FV mRNA), the RT-PCR products digested with *Spe*I yielded 159- and 177-bp bands, representing transcripts from the normal and mutant alleles respectively (Fig. 3b). Thus, both mutant transcripts were present in the patient's platelets. However, the FS592X-FV mRNA signal was markedly reduced to 12% of the wild type in the quantitative densitometric analysis, whereas the Y1702C-FV mRNA signal was more intense (250% of the wild type). These data suggest that the patient could be compound heterozygous for these mutations, and that her RNA surveillance system would eliminate most of the FV mRNA derived from the mutant allele encoding a premature termination by the frame-shift mutation, FS592X [18]. On the other

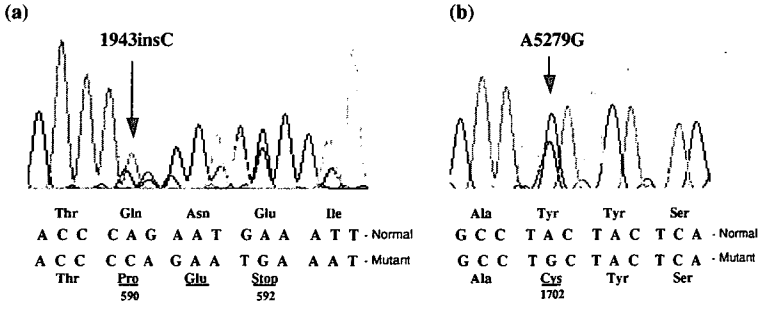

Fig. 2. Patient's nucleotide and amino acid sequences surrounding the mutations. (a) Nucleotide and amino acid sequences surrounding 1943insC. Arrow indicates mutation point. The mutation predicts an abnormal sequence of two amino acid residues and a stop codon. (b) Nucleotide and amino acid sequences surrounding A5279G. Arrow denotes mutation point. Patient's heterozygous sequencing pattern is shown.

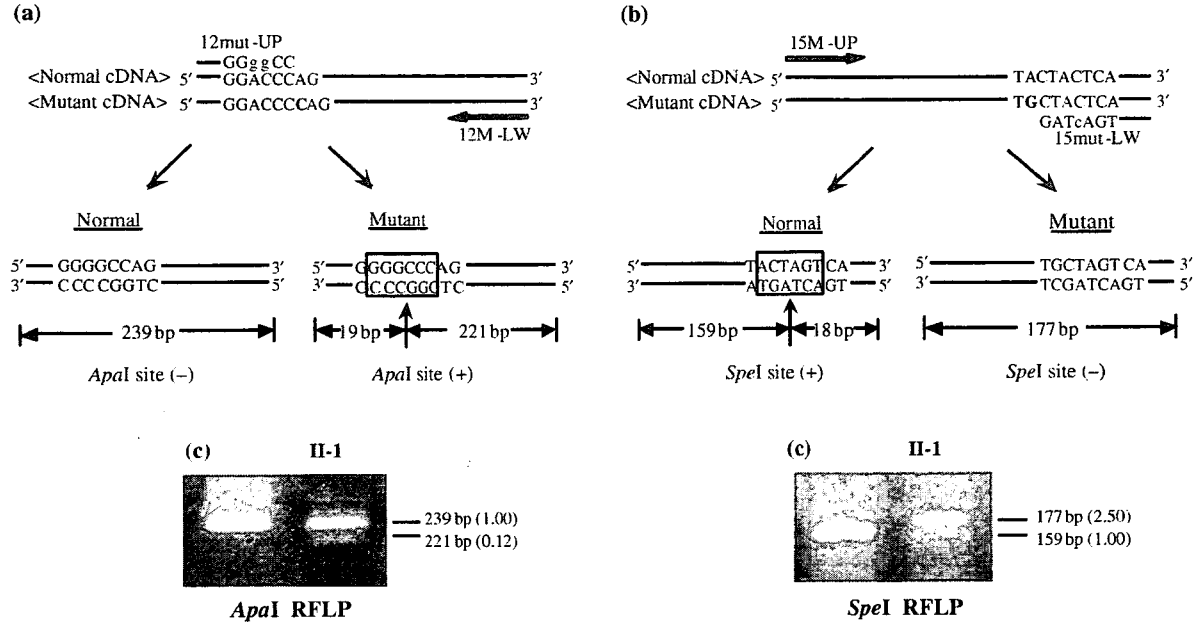

Fig. 3. Detection of mutant factor V mRNAs in patient's platelets. (a) Reverse transcriptase polymerase chain reaction (RT-PCR) products (239 bp) using primers 12mut-UP and 12M-LW were digested with *Apa*I, then electrophoresed on 4% NuSieve 3:1 agarose gel. Wild-type RT-PCR product migrated as an uncleaved 239-bp band, while FS592X (1943insC') RT-PCR product is represented by an *Apa*I cleaved 221-bp band. II-1, proband; C, control donor. Numbers in parentheses are relative amounts of signals measured by the quantitative densitometric analysis (wild type = 1.00). (b) RT-PCR products (177 bp) using primers 15M-UP and 15mut-LW were digested with *Spe*I, then electrophoresed on 4% NuSieve 3:1 agarose gel. Wild-type RT-PCR product migrated as *Spe*I cleaved 159-bp band, whereas Y1702C (A5279G) RT-PCR product is represented by an uncleaved 177-bp band. II-1, proband; C, control donor. Numbers in parentheses are relative amounts of signals measured by the quantitative densitometric analysis (wild type = 1.00).

hand, both the FV antigen and activity in her plasma were below the detectable limit, suggesting that the mutant Y1702C-FV might be impaired during the post-transcriptional process of protein synthesis and/or in secretion. Indeed, it has also been previously reported that the FV allele bearing the Y1702C mutation was expressed normally at the mRNA level, but not at the protein level in plasma [15].

Expression of wild type and mutant recombinant FVs in COS-1 cells

It is important to determine the patient's phenotype on Met1736Val polymorphism, as it will exert a great influence on the expression level of the recombinant FV [19]. Sequence analysis revealed that the patient was homozygous for 1736Met, which is the same phenotype encoded in the pMT2-FV, and thus its influence may not be revealed in expression experiments for her Y1702C-FV.

We investigated the effects of the identified mutants on FV secretion by conducting transient transfection experiments in COS-1 cells, which do not express endogenous FV. We observed that the wild type recombinant FV proteins were secreted efficiently into culture media with an adequate specific activity (0.94), but that the mutant Y1702C-FV showed an impaired secretion (1.8% of the wild type) and inadequate FV procoagulant activity (0.56) (Fig. 4). These data tend to support the conclusion that the Y1702C mutation could be causative for the FV deficiency as reported previously [15]. Indeed, plasma levels of FV activity in her mother and brother, who had only the Y1702C mutation in heterozygous, were reduced to 65% of normal. The 1702Y is a highly conserved amino acid not only in FV molecules among various species, but also in human FVIII and ceruloplasmin [15]. Moreover, an X-ray crystal structure analysis of wild type FV has demonstrated that the FV Y1702C mutation leads to the disappearance of two hydrogen bonds with P1618, and that its structure was significantly altered by a new hydrogen bond bridge formed between this cysteine and one of the other free cysteines [15]. These data suggest that 1702Y may play an important role in maintaining the structure and function of the FV molecule.

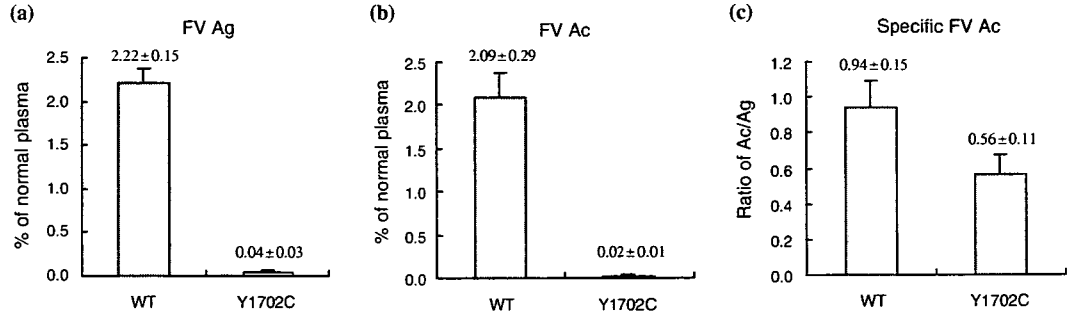

Fig. 4. Transient expression of wild-type factor V (FV) and 1702C mutant FV in COS-1 cells. Plasmids containing wild type (pMT2/FV) or mutant (pMT2/FV-Y1702C) FV cDNA were transiently transfected in COS-1 cells using FuGene reagent. Antigen and activity levels of recombinant FVs were measured in conditioned media 48 h after transfection (a, FV antigen; b, FV activity; c, FV-specific activity). Bars represent mean values ± SD of three independent experiments, each performed in duplicate. FV levels were expressed as percentage of normal pooled plasma from 25 healthy individuals.

On the other hand, recombinant FS592X-FV molecule was not detected in cultured media of the transfected COS-1 cells (data not shown). The transcripts of FS592X-FV were detected in the patient's platelets, but were found to be markedly reduced compared with normal allele transcripts (Fig. 3a). Moreover, as the FS592X-FV is a truncated molecule in the A2 domain, it would not be processed normally as reported for other mutant coagulation factors [20,21].

Conclusion

In this study, we investigated the molecular basis of a severe coagulation FV deficiency in a Japanese woman and identified two distinct mutations (1943insC/FS592X and A5279G/Y1702C) in her FV gene. The data indicated that both mutant FV molecules would be impaired, at least in part, during the post-transcriptional process of protein synthesis and/or in secretion. Taken together with the above observations, it seems to suggest that each mutation could be separately responsible for severe FV deficiency, while this phenotype is due to the in-trans combination of the two defects.

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Letter to the Editor

Recurrent Mutations of Factor XI Gene in Japanese

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Factor XI (FXI) deficiency is most commonly found in individuals of Ashkenazi Jewish origin and consists of a splice-junction abnormality (type I), a nonsense mutation in exon 5 (type II), or a missense mutation in exon 9 (type III) [1,2]. However, it has also been reported in other ethnic populations, including Japanese, as a rare bleeding disorder inherited as an autosomal recessive condition, and it is often diagnosed following abnormal bleeding after trauma or surgical operation [3].

In the present study, we investigated the molecular basis of FXI deficiency in Japanese patients under ethical approval from the Ethics Committee of Nagoya University School of Medicine. Direct sequencing of polymerase chain reaction (PCR) products specific for the FXI gene revealed 4 distinct and potentially causative mutations in 5 unrelated Japanese patients with FXI deficiency (Table 1). Although 81 mutations associated with FXI deficiency have been reported to date [1], we found a novel mutation, C773T (Gln226Stop) in exon 7, and 3 previously reported mutations in unrelated Japanese subjects: 2 missense mutations (G1296T [Gly400Val] and T759C [Phe221Ser]), and a G to A transition at position +1 at the exon 10 splicing donor site (exon 10 +1 G→A).

C773T was identified in case 1 as a homozygous mutation and in case 2 as a heterozygous mutation. In case 2, The other FXI gene mutation, G1296T, was found to be heterozygous in case 2 and homozygous in case 3. T759C in case 4 was a homozygous mutation, and exon 10 +1 G→A was detected in case 5 as a heterozygous mutation. These FXI gene abnormalities were confirmed by PCR–restriction fragment length polymorphism (RFLP) analyses (data not shown), such as a *Sty*I-RFLP for C773T, a *Hin*fI-RFLP for G1296T, a mismatch PCR mediated *Xba*I-RFLP for T759C, and a mismatch PCR mediated *Bcl*I-RFLP for exon 10 +1 G→A.

C to T transition at nucleotide 773 of the FXI gene results in a nonsense mutation, Gln226Stop, and disrupts the third apple domain structure of the FXI molecule, which is essential for ligand binding to platelets, heparin, and factor IX. It also loses the fourth apple domain, which is required for dimerization and secretion, as well as a serine protease domain of FXI. Therefore, mutant Gln226Stop FXI would not have normal FXI activity, even if it could be secreted. Moreover, we suppose that mutant mRNA as well as dysfunctional truncated protein may be rapidly broken down intracellularly, resulting in reduced production of abnormal protein, as suggested previously in other plasma proteins [4,5]. In case 1, Southern blot analysis using full length FXI cDNA as a probe and dose evaluation of FXI gene by real-time PCR revealed that the patient had a homozygous C773T mutation rather than a large deletion in one allele of the FXI gene (data not shown). These data suggested that the severe FXI deficiency in case 1 could be explained by homozygosity for the nonsense mutation, Gln226Stop.

In case 2, the patient showed compound heterozygosity for C773T (Gln226Stop) and G1296T (Gly400Val) mutations. The latter was previously identified in a Japanese patient and was also observed in a man with Italian/Czechoslovakian heritage and his family, as well as a Chinese woman and her son [6], suggesting that it may be a hot spot mutation in humans. The residue 400Gly is located near the highly conserved histidine loop in the catalytic domain, suggesting that it may have an important role in maintaining FXI conformation, and that the replacement of 400Gly by Val is expected to change the structure and function of the FXI molecule. Indeed, it has been reported that Gly400Val reduced secretion to approximately 50% of the wild-type, consistent with a dominant-negative effect, in cotransfection experiments with a wild-type FXI construct [6]. We also observed severely

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Table 1.
Gene Abnormalities and Clinical Features of Patients with FXI Deficiency*

Case	Age, y	Sex	Mutation	Predicted Amino Acid Change	Location	FXI:C, %	FXI:Ag, %
1	39	F	773C→T†	Gln226Stop†	Exon 7	< 3	ND
2	62	M	773C→T	Gln226Stop	Exon 7	< 3	ND
			1296G→T	Gly400Val	Exon 11		
3	58	M	1296G→T	Gly400Val	Exon 11	18‡	21‡
4	43	M	759T→C	Phe221Ser	Exon 7	< 3	7
5	29	F	Exon 10 + 1G→A	—	Intron J	50	ND

*F indicates female; ND, not done, M, male.

†cDNA and amino acid numbers are according to Fujikawa et al [12], except for those located within intronic sequences where the location is given to the intron-exon boundary.

‡Data at posttransfusion of fresh frozen plasma.

impaired production and secretion of Gly400Val FXI compared with those of wild-type FXI in a transient expression experiment (data not shown). Thus, compound heterozygosity for these 2 mutations, C773T and G1296T, might have caused the severe FXI deficiency in case 2. We also expected that the Gly400Val mutation would be homozygous in case 3, because Southern blot analysis and dose evaluation of the FXI gene by real-time PCR suggested that the patient would not have a large deletion of the FXI gene in 1 allele (data not shown).

T to C transition at nucleotide 759 in exon 7 of the FXI gene, which causes a missense mutation, Phe221Ser, located at the third apple domain, has been reported previously [7]. In our transient expression experiments, it was revealed that secretion of Phe221Ser FXI was significantly reduced compared with that of wild-type FXI (data not shown), consistent with plasma data on the FXI antigen in case 4 (7% of normal). Recently, it was suggested that Phe221Ser FXI synthesis in the cell was normal but there was a fault in secretion [8].

In case 5, we identified a previously reported splicing donor site mutation, exon 10 +1 G→A [9], in a Japanese patient with mild FXI deficiency. This mutation changed an invariant nucleotide of the splicing site recognition sequence, and likely resulted in aberrant splicing of FXI mRNA causing FXI deficiency [10].

Additionally, we previously reported compound heterozygous mutations for severe factor XI deficiency in a Japanese patient [11], but 1 of 2 mutations, 501/502insG, was independently found in unrelated Japanese patients with FXI deficiency [7], suggesting that this mutation also might be a founder mutation in Japanese population.

In conclusion, we identified 4 distinct and possible causative FXI gene mutations, including a novel nonsense mutation, in 5 unrelated Japanese patients with FXI deficiency. As in previous reports, these FXI gene abnormalities were identified repeatedly in unrelated Japanese individuals, indicating that they may be founder or hot spot mutations in Japanese.

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ORIGINAL ARTICLE

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 three distinct novel missense mutations, R275C, P375Q and D455Y, and two 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, that is, 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.

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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 (F) Va and FVIIIa [1,2]. Since PS also directly inhibits FVa and FXa 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), four 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 PS deficiency [19–21]. In the present study, we identified three novel missense mutations associated with the phenotype of quantitative PS deficiency, and investigated the molecular consequences of the missense mutations, together with two 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 Nagoya University School of Medicine, Japan. 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, USA).

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, USA) 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 PS

Monkey kidney COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 3.5 mM 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 per 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^{-1} vitamin K1 (Isei, Yamagata, Japan) for 24 h, 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 293 cells as described previously [27].

Western blot analysis

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

Quantification of PS 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 min, 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, USA) to quantify the radioactivity of the bands on the gels.

Determination of APC cofactor activity of recombinant PS

Stable transformants were grown in DMEM supplemented with 10% fetal calf serum, 3.5 mM glutamin, antibiotics-antimycotics, and 10 μg mL^{-1} 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^{-1}. APC cofactor activity of recombinant PS was determined on the basis of a clotting assay (Statclot; Diagnostica-Stago, Asinères, France) using ST art4 (Roche, Basel, Switzerland).

Results

Clinical characteristics and gene abnormalities in patients with PS deficiency

The clinical characteristics and gene abnormalities in four 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

Table 1 Clinical characteristics and gene abnormalities in patients with protein S (PS) deficiency

Case	Age (years)	Sex	PS Ag (%)		PS Ac (%)	OAC	Thrombosis	Mutation	Predicted AA change	Location
			Total	Free						
1	62	M	49	39	21	(+)	DVT	TGT → TAT	C80Y	Exon 5
2	31	F	77	49	36	(−)	DVT	CGT → CAT	R314H	Exon 10
3	34	F	63	ND	38	(+)	Cerebral infarction	CGT → TGT	R275C	Exon 9
4	40	M	66	26	26	(−)	DVT	CCG → CAG	P375Q	Exon 11
								GAT → TAT	D455Y	Exon 12

The novel mutations are underlined.
M, male; F, female; ND, not done; DVT, deep vein thrombosis; OAC, oral anticoagulant therapy at PS assays.

[22], while three others, R275C, P375Q and D455Y, were novel, with the latter two 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-restriction fragment length polymorphism (RFLP) analyses for three 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%.

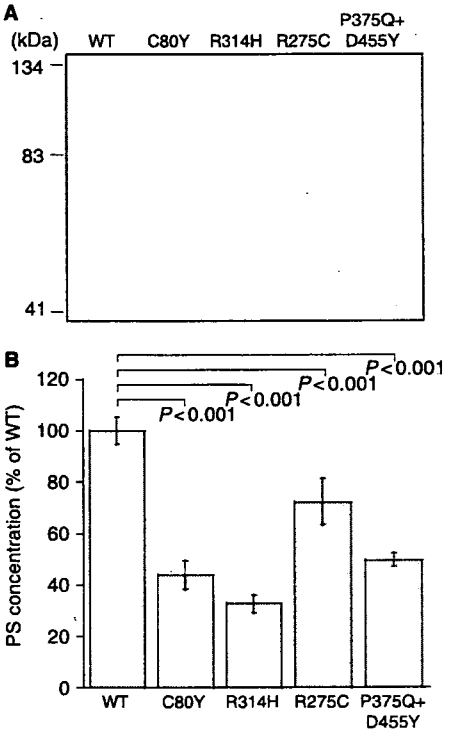

Fig. 1. Transient expression of wild type and mutant recombinant protein S (PS) in COS-1 cells. (A) Western blot analysis of 15 μL of conditioned media was performed under reducing conditions. Note that some mutants appeared as doublet bands, with the lower band representing cleaved-PS by proteolysis 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 six transfection experiments per mutant. Comparison between mutant and wild type expression levels was performed using unpaired *t*-test.

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 h 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 h in the C80Y and P375Q + D455Y mutants, and 8 h 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 h, was higher in the wild type PS (70% of the initial value), whereas it was significantly reduced in the four 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 two 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

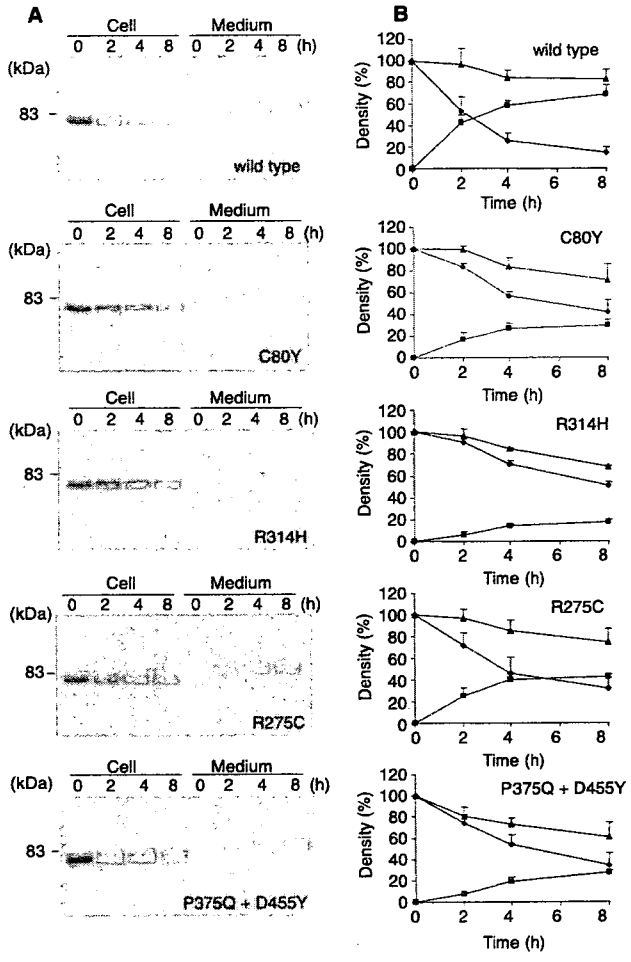

Fig. 2. Pulse-chase analysis using transient expression in COS-1 cells. Radiolabeled media and cell lysates were immunoprecipitated and electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis. (A) A representative experiment of wild type PS and each mutant pulse-chase. (B) Radioactivity 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 three or four independent experiments.

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

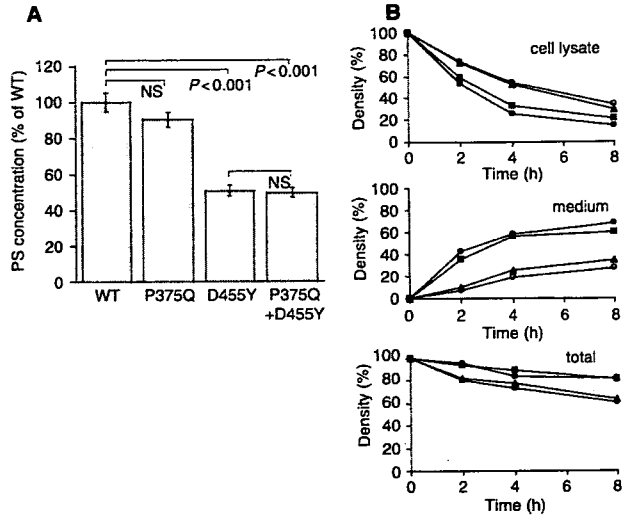

Fig. 3. Effects of each amino acid substitutions (P375Q and D455Y) on protein-expression pathway. (A) ELISA determination of concentrations of different mutants. Histograms and bars represent mean ± SD ($n = 6$). Mean value of wild type PS is assigned as 100%. Comparison between mutants and wild type 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 h is assigned as 100% for each construct.

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 four patients with quantitative PS deficiency associated with recurrent thrombotic complications. In the DNA sequence analyses, we identified three novel (R275C, P375Q and D455Y) and two 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, five of them, identified in four 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 four

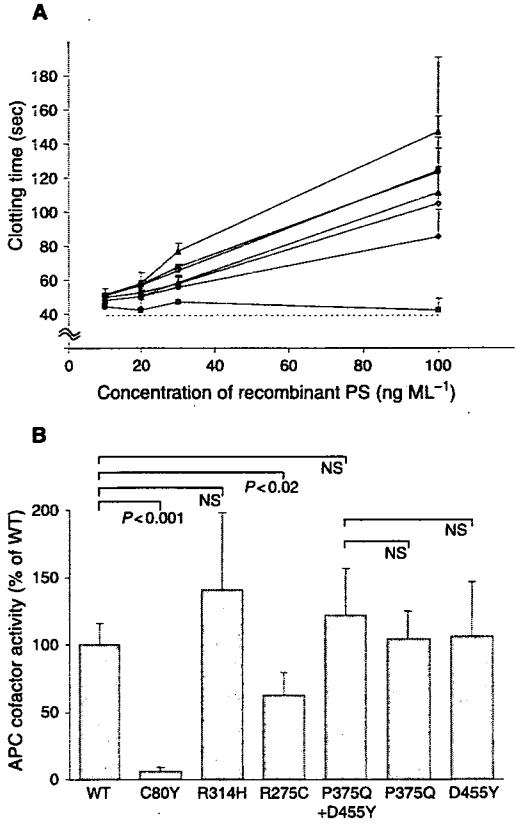

Fig. 4. Activated protein C (APC) cofactor activity of recombinant PS. (A) A range of recombinant PS concentrations (0–100 ng mL^{-1}) in serum-free conditioned media of stable transformants was incubated with PS-depleted plasma, factor Va, and APC for 2 min. Clotting was initiated by addition of $CaCl_2$, and clotting time was measured using ST art4. Values represent mean ± SD of four or five 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^{-1}) of different mutants. Mean value of wild type PS is assigned as 100%. Values represent mean ± SD of four or five independent experiments. Comparison between clotting times of 100 ng mL^{-1} of each mutant and that of wild type PS was determined using unpaired *t*-test.

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 h) was longer than that of the wild type (2 h, 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 Case 2 than in 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, as 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 two missense mutations, P375Q and D455Y, which were linked on the same allele. To determine the individual effects of these two mutations, we performed expression studies using two 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 two 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 three 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 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 two 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 FV during the APC-mediated inactivation of FVIIIa [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 by 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

Patients: K. Yamamoto, J. Takamatsu, T. Matsushita; molecular genetics: H. Okada, A. Takagi, T. Murate; expression and characterization: H. Okada, S. Kunishima, T. Yamazaki; specific and general supervisors: T. Naoe, M. Hamaguchi, H. Saito, T. Kojima.

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Disclosure of Conflicts of Interest

The authors state that they have no conflict of interest.

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PROTEINASE 3 EXPRESSION ON NEUTROPHIL MEMBRANES FROM PATIENTS WITH INFECTIOUS DISEASE

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ABSTRACT—Proteinase-3 (PR3) is an abundant serine proteinase stored in the azurophilic granules of neutrophils and released to the cell surface upon activation where it contributes to local tissue destruction and inflammation. The subpopulation of membrane PR3 (mPR3) high expression (PR3-high) varies among individuals. There are many reports about PR3 in Wegener's granulomatosis, but few about PR3 expression in patients with common inflammatory disorders, such as sepsis. The mPR3 expression on neutrophils from 56 patients with inflammatory disorders and from 64 healthy volunteers was examined by flow cytometry. High variability in the percentage of PR3-high (%PR3-high) neutrophils was observed in healthy volunteers and patients with inflammatory disease, and the %PR3-high was significantly greater in the patients (72 ± 19% vs 55 ± 20%, $P < 0.0001$). Overall neutrophil PR3 expression in patients with infectious diseases, especially systemic inflammatory response syndrome *(SIRS)* was significantly high ($P < 0.01$) and showed a positive correlation with C-reactive protein (CRP). Even under inflammatory conditions not involving autoimmune vasculitis, there are significant increases in both the absolute surface expression of PR3 and the numbers of neutrophils expressing high levels of PR3 and these correlate with CRP levels. The data are consistent with a model in which neutrophil membrane expression of PR3 is greatly influenced by an in vivo inflammatory environment.

KEYWORDS—PR3, inflammatory diseases, neutrophil, SIRS

INTRODUCTION

Proteinase-3 (PR3) is an abundant serine proteinase stored in the azurophilic granules of neutrophils along with neutrophil elastase and cathepsin G, and is released to the cell surface upon activation (1–3). This 29-kD glycoprotein also is stored in secretory vesicles and specific granules and is generally thought of as a soluble enzyme, in the sense of being released into the local inflammatory milieu upon neutrophil activation and disgorgement of granular contents. However, several recent studies have observed that most of the neutrophil PR3 remains associated with the cell membrane upon activation, with very little released into the medium (4).

PR3 is possibly best known as the primary target antigen of the PR3 anti-neutrophil cytoplasmic antibodies (PR3-ANCA) in Wegener's granulomatosis (WG), a debilitating autoimmune disease characterized by necrotizing vasculitis (5–7). PR3-ANCA are also found in patients with microscopic polyangiitis, a systemic vasculitic disease (8). PR3 has activities that include degradation of extracellular matrix proteins (9), regulation of myeloid differentiation (10, 11), potentiation of platelet activation (12), and antibacterial action that is independent of its enzymatic activity (13). Structurally, PR3 is very similar to neutrophil elastase (14), but it does have unique substrates, including the membrane-bound precursors of the pro-inflammatory tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) cytokines (15–17). Thus, PR3 expression near a vascular surface would very likely contribute to local tissue destruction and inflammation.

New diseases entities have recently emerged, such as systemic inflammatory response syndrome (SIRS) (18) sepsis in the emergency department and intensive care unit, which frequently. become fatal outcome. SIRS is frequently associated with elevated levels of plasma cytokine, organ failure and disseminated intravascular coagulation (19, 20). Neutrophils play a major role in mediating innate inflammatory reactions and PR3 activity contributes to production of pro-inflammatory cytokines. One study shows membrane PR3 expression in normal volunteers that is stable with time, but variable in magnitude, and a relatively high expression of PR3 on neutrophils of patients with WG and rheumatoid arthritis (21). In SIRS, expression of PR3 may play an important role in the progress of inflammation.

In this study, we measured the expression of PR3 on neutrophil membrane from patients with infectious diseases to examine the relationship between inflammation and PR3 expression.

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MATERIALS AND METHODS

Patients and controls

The patient group consisted of 56 patients with infectious diseases (34 men; 22 women; mean age, 59.0 ± 17.3 yr; range, 18 to 86 years) seen at Mie University Hospital between December 24, 2003 and February 17, 2004. Sixty-four healthy volunteers (40 men; 24 women; mean age, 26 ± 8 year; range 19 to 65 yr) were included in control group. Patients' diagnoses were as follows: 24 pneumonia, 19 peritonitis, 9 tonsillitis, 2 phlegmon and 2 pyelonephritis. None of the patients had vasculitis or ANCA titres in those patients whom we could examine (data not shown). Disseminated intravascular coagulation (DIC) was diagnosed according to the criteria established by the Japanese Ministry of Health and Welfare (22). SIRS was diagnosed by the criteria delineated by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) Consensus Conference Committee (19). Briefly, patients demonstrated more than 3 of 4 criteria: (1) temperature > 38° or < 36°; 2) heart rate > 90 beats/minute; 3) respiratory rate 20 breaths/minute or $PaCO_2$ < 32 mmHg; and 4) white blood cell count >12,000/mm^3, or < 4,000/mm^3, or > 10 % immature (band) forms). All patients and healthy volunteers gave informed consent before participation in the study and the study was approved by the Mie University's Review Board for human studies.

Materials

Dextran was purchased from Wako (Osaka, Japan). Hanks Balanced Salt Solution (HBSS) was from Gibco BRL (NY, USA). Trypan blue was from Acros Organics (New Jersey, USA). Recombinant TNF-α was from Peprotech EC LTD (London, UK). N-formyl-L-methionyl-L-phenylalanine (FMLP) was from Sigma-Aldrich Co. (St. Louis, USA).

Monoclonal antibody 1549 recognizing human PR3 was obtained from hybridoma supernatants using mice immunized with PR3 purified from human neutrophils (Athens Research, GA). Supernatants were screened for binding to neutrophil-derived PR3 immobilized in wells of 96 well plates and bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG and substrate. The 1549mAb does not recognize neutrophil elastase (data not shown). For production, hybridoma cells were cultured in bags (I-MAB Monoclonal Antibody Production Kit, Diagnostic Chemicals Ltd) in RPMI-1640, 10% bovine calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 0.35 mg/mL L-glutamine. Antibodies were purified from conditioned media using Mep Hypercel resin (Life Technologies, Inc) equilibrated in 50 mM Tris HCl, ph 7.5, 0.1 M NaCl, 0.02% sodium azide and eluted with 50 mM sodium acetate, pH 4.0. Antibody was labeled for flow cytometry studies with the Alexa488–succinimidyl ester conjugate (Molecular Probes, Eugene, OR) using standard methods and free probe was removed with extensive dialysis.

Neutrophil isolation

For experiments using purified neutrophils, 3 mL of EDTA-anticoagulated whole blood was mixed with a half volume of 2% dextran in 0.9% NaCl. RBCs were allowed to sediment for 45–60 min at room temperature. The supernatant was removed and centrifuged at 400 ×g (5 min, 4°C). The pellet was re-suspended in 5 ml of ice-cold 0.2% NaCl with mixing for 25 s, followed by 5 ml of ice-cold 1.6% NaCl. The cells were centrifuged and the pellet gently re-suspended in 5 ml of HBSS with 3mM calcium chloride containing 1% bovine serum albumin (HBSS+ buffer). This was transferred to a 15 ml conical centrifuge tube and underlaid with 5 mL of Lymphocyte Separation Media (density 1.077 ± 0.001 g/ml at 22°C; Cellgro, Herndon, VA). The sample was centrifuged at 400 ×g for 30 min at 4°C. The pellet of purified neutrophils was re-suspended in 5–10 ml HBSS+ buffer. Cell viability was determined for every cell preparation by trypan blue exclusion and was consistently greater than 99%.

In vitro stimulation and measurement of membrane PR3 expression by flow cytometry

Isolated neutrophils were re-suspended in HBSS+ buffer at a final concentration of 2 × 10^6/mL. The cell suspension was incubated with TNF-α (10 ng/ml) and FMLP (1.0 μM) for 20 min at 37° and washed with 1 mL ice-cold HBSS+ buffer. Cells were pelleted and re-suspended in HBSS+ buffer before incubation with labeled antibodies. Data acquisition by flow cytometry and subsequent analysis was done with a FACScan using CellQuest software (Becton Dickinson, Heidelberg, FRG). Neutrophils were gated according to relative size (forward scatter) and relative granularity (side scatter) properties. Data is reported either as the percentage of mPR3 high expression (%PR3-high) neutrophil population, or the level of mPR3 expression is reported as mean fluorescence intensity (MFI) after correction for non-specific binding (NSB) as assessed using an irrelevant isotype-matched antibody and appropriate conjugate. Data are expressed as: (MFI-NSB) × %PR3-high to take into account differences in surface PR3 expression between individuals (23).

Measurement of plasma concentrations of granulocyte elastase derived fibrin degradation products (GE-XDP), D-dimer, soluble fibrin (SF) and thrombomodulin (TM).

GE-XDP was measured by latex-agglutination assay using monoclonal antibody IF-123. IF-123 specifically recognizes elastase-digests of human fibrinogen and fibrin, but not their plasmin-digests. The epitope for this antibody is located at Aα Leu-196 to Ile-204. D-dimer, SF and sTM were determined with D-dimer test Kokusai-F (Kokusai-Shiyaku), Enzymum test (Boehringer Mannheim, Mannheim, Germany) and sTM-test Kokusai (Kokusai-Shiyaku), respectively.

Statistical analysis

Differences in continuous variables between two groups were analyzed by means of Mann Whitney U test. Differences in continuous variables between more than three groups were analyzed by means of Steel-Dwass test. Correlations were analyzed by the nonparametric Spearman correlation coefficients. A two-sided $P < 0.05$ was considered to be statistically significant.

RESULTS

mPR3 expression on activated neutrophils from healthy volunteers and septic patients

Similar to other studies, we observed PR3 antigen on the surface of purified neutrophils and a sub-population of neutrophils expressing higher membrane PR3 antigen appears after activation (Fig. 1). However, the distribution of PR3 antigen after neutrophil activation with TNF-α and FMLP varied considerably between individuals. For example, in cells from four healthy volunteers, the percentage of cells expressing high levels of membrane PR3 (%PR3-high) varied from 0.19% to 84.36% (Fig. 1A–D), and was not related to the phenotype before activation. This contrasts with results of a previous study in which the distribution of PR3 antigen between sub-populations remained constant regardless of stimulation (21). This latter study activated neutrophils with cytochalasin B and

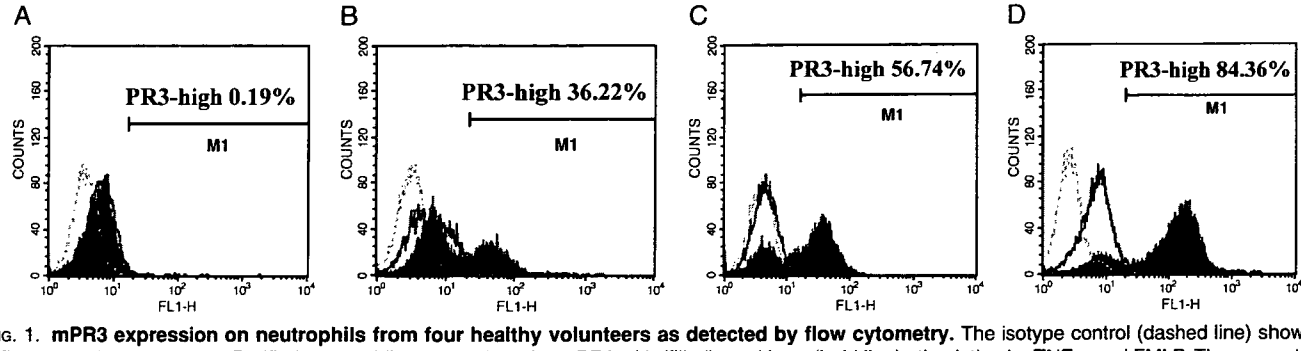

FIG. 1. **mPR3 expression on neutrophils from four healthy volunteers as detected by flow cytometry.** The isotype control (dashed line) showed no significant membrane staining. Purified neutrophils were stained for mPR3 with (filled) or without (bold line) stimulation by TNF-α and FMLP. The percentage of cells expressing high levels of mPR3 are shown as %PR3-high.

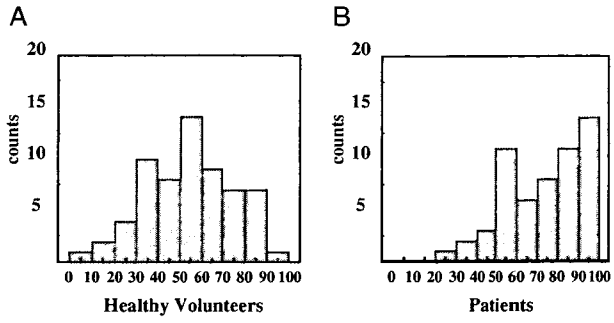

FIG. 2. **Histograms of PR3 high expression neutrophils population.** (A) In a cohort of 64 healthy subjects, the percentage of PR3 high expression neutrophils (%PR3-high) was evenly distributed. (B) In 56 patients with infectious disease, %PR3-high was skewed to a higher phenotype.

FMLP, conditions known to mobilize azurophilic granules, but also to disrupt the cytoskeleton (24), which may result in aberrant re-distribution of surface antigens.

Similar to the data from healthy volunteers (N = 64), this variability in PR3 distribution after in vitro activation with TNF-α and FMLP also was observed in patients with acute infectious disease (N = 56) (Fig. 2). However, the percentage of cells in the PR3-high population was significantly higher in the patients than in healthy volunteers (72 ± 19% vs 55 ± 20%, $P < 0.0001$). In the healthy subjects population, the percentage of PR3-high expressing cells was distributed normally (bell-shaped curve), whereas the distribution of %PR3-high cells in patients with acute infectious disease was skewed to a higher phenotype.

In some cases, there was a significant increase in the percentage of cells in the PR3-high population over time both with and without in vitro activation with TNF/FMLP. Two representative cases are shown in Figure 3. At the onset of sepsis (Day 0), the %PR3-high was higher than at day -14, and this paralleled increases in C-reactive protein (CRP), an acute phase protein and biomarker of inflammation. Though both of the patients seemed to recover from SIRS, they eventually relapsed into SIRS again and died within four weeks.

As shown in Figure 4, overall membrane expression of PR3 on resting neutrophils was higher in the patients than in the healthy volunteers (913 ± 160 vs 393 ± 75; $P = 0.0003$). After in vitro activation by TNF-α and FMLP, expression of PR3 remained significantly higher on neutrophils from patients with inflammatory disease (5242 ± 426 vs 3667 ± 547; $P = 0.00003$).

mPR3 expression and plasma levels of CRP

The mPR3 expression on resting neutrophils and after activation had a slightly correlation with CRP levels (r = 0.465, $P < 0.001$ and r = 0.442, $P < 0.001$, respectively).

It may be important to mention that we did see a few cases that did not follow the general trend described above. This is most likely due to the fact that PR3 expression is governed by multiple complex factors, including genetics, and the inflammatory environment is only one of many contributing factors.

mPR3 expression and SIRS

Each patient was reviewed daily and cardio-respiratory observations and daily blood tests were noted to determine the

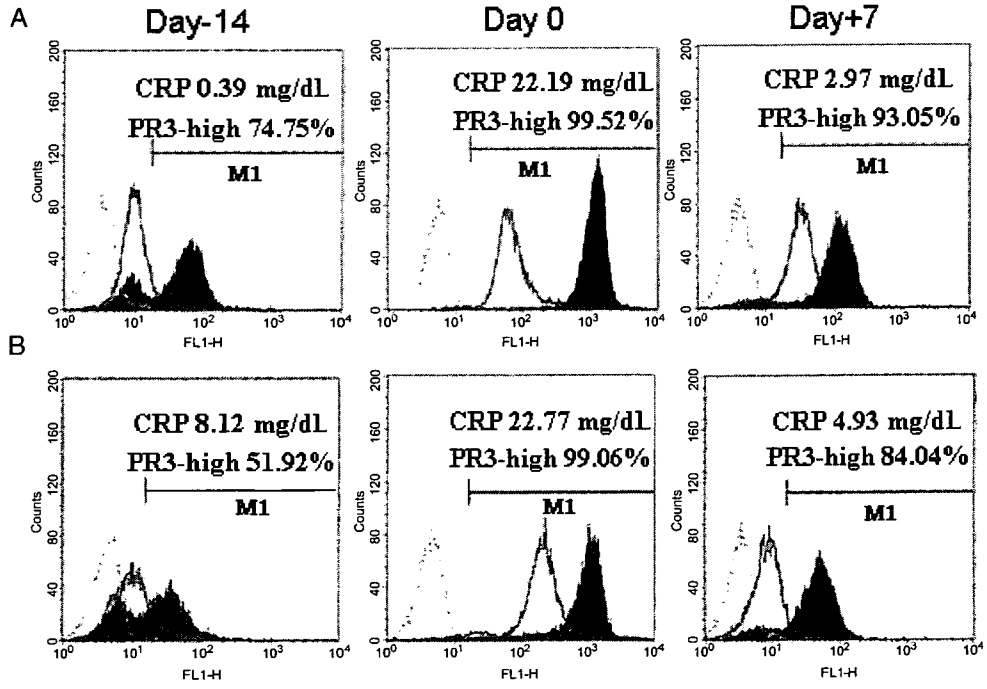

FIG. 3. **Changes in mPR3 expression with time in patients with sepsis.** PR3 expression on neutrophils from sepsis patients was evaluated by flow cytometry with (filled) and without (bold line) stimulation by TNF-α and FMLP. The isotype control (dashed line) showed no significant membrane staining. (A) mPR3 expression in a patient with small cell lung cancer complicated by bacterial pneumonia. (B) mPR3 expression in a patient with cancer of the hypopharynx complicated by bacterial pneumonia. In both cases, mPR3 expression was bimodal before the onset of sepsis (left). At the time of infection, mPR3 expression increased and the %PR3-high also was greater (middle). After treatment for sepsis, mPR3 expression was lower than at the onset of infection whereas the %PR3-high was slower to resolve (right).

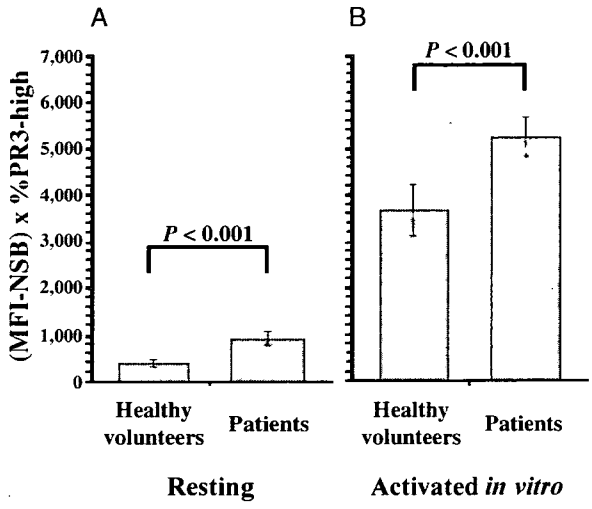

FIG. 4. **Neutrophil PR3 expression in healthy volunteers (N = 64) and patients with infectious diseases (N = 56).** Neutrophil PR3 expression is shown as the product of surface expression levels (MFI-NSB) and relative abundance (%PR3-high) because both parameters change as a consequence of inflammation. Expression levels were significantly higher in patients than in healthy volunteers with (A) and without (B) *in vitro* stimulation, ($P = 0.0003$ and $P = 0.00003$, respectively).

incidence of SIRS. Consequently 14 patients were diagnosed with SIRS on the same day when the blood samples were taken for measurement of membrane PR3 expression by flow cytometry. PR3 expression [(MFI-NSB) × %PR3-high] was significantly greater in the patients with SIRS (N = 14) than non-SIRS (N = 42) on either resting (478 ± 135 vs 1813 ± 361, $P < 0.001$) or activated neutrophils (4170 ± 493 vs 8862 ± 1060, $P < 0.001$), respectively (Fig. 5). We observed a trend that PR3 expression is higher in patients with DIC compared to those without DIC. CRP and D-dimer were significantly higher in larger PR3 than in less PR3, but GE-XDP and TM were not (Table 1).

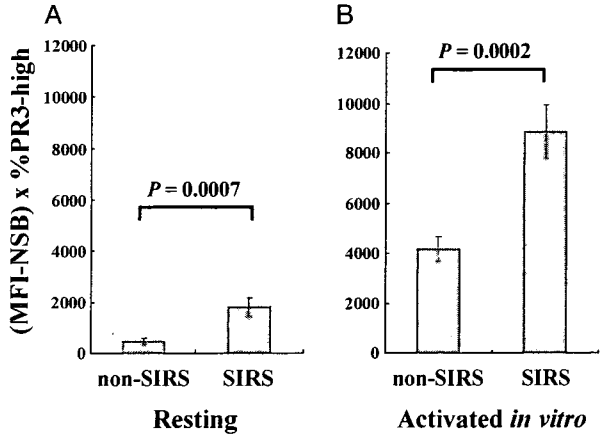

FIG. 5. **Neutrophil PR3 expression in SIRS (N = 14) and non-SIRS (N = 42) patients.** Neutrophil PR3 expression is shown as the product of surface expression levels (MFI-NSB) and relative abundance (%PR3-high) without *in vitro* stimulation (A) or with stimulation (B). Membrane PR3 expression without *in vitro* stimulation was significantly greater in the patients with SIRS (478 ± 135 vs 1813 ± 361, $P = 0.0007$). After stimulation, PR3 was also significantly greater in the patients with SIRS (4170 ± 493 vs 8862 ± 1060, $P = 0.0002$).

TABLE 1. **Clinical and Laboratory Data in Infectious Patients**

		Less PR3 (N = 32)	Larger PR3 (N = 24)	
		n (%)		P
Severe Symptom				
DIC	(N = 11)	2 (63)	9 (37.5)	0.0036**
Organ Failure	(N = 12)	4 (12.5)	8 (33.3)	0.060
Shock	(N = 3)	0 (0)	3 (125)	0.096
Outcome				
Death	(N = 4)	1 (3.1)	3 (125)	0.18
		mean ± SE		
Laboratory Data				
CRP	mg/dL	10.6 ± 1.1	127 ± 1.1	0.009**
GE-XDP	U/mL	9.0 ± 3.4	7.0 ± 1.6	0.27
SF	μg/mL	8.5 ± 1.4	8.4 ± 1.3	0.69
D-dimer	μg/mL	8.8 ± 25	11.6 ± 5.1	0.047*
TM	U/mL	13.0 ± 1.4	15.3 ± 2.2	0.053

DISCUSSION

Previous reports show that both the %PR3-high and level of mPR3 expression are related to disease and relapse in Wegener's granulomatosis patients with PR3-ANCA-associated vasculitis (25, 26). The present study newly shows that, even in patients without vasculitis, but having systemic inflammatory conditions, both the %PR3-high and level of mPR3 expression are increased. Some patients showed a significant increase in %PR3-high with time and clinical severity. The patients with increasing %-PR3-high died from complications due to sepsis, suggesting a correlation with the severity of inflammation (data not shown). This change in neutrophil phenotype also is probably not related to mobilization of new neutrophils from bone marrow, because there was no significant changing in %PR3-high after glucagon injection in healthy volunteers (27). In addition, we find that the %PR3-high does not change even after G-CSF injection (unpublished observations). Thus, the changes in %PR3-high more likely occur in the peripheral circulation as a consequence of an inflammatory environment. We have made many efforts to recreate this phenomenon in vitro, but with limited success, so there are as yet unidentified contributors to changes in %PR3-high observed in patients.

The current observation that PR3 antigen is expressed on the surface of resting, purified neutrophils from patients and healthy volunteers is consistent with previous studies (28). The subpopulations of cells expressing considerable PR3 (PR3-high) or those with less PR3 (PR3-low) varied among individuals. In an earlier study, this expression pattern was found to be stable for each individual over prolonged time periods and may be genetically controlled because the %PR3-high was highly correlated in monozygonic twin pairs but there was no correlation in dizygotic twin pairs (27, 28). After activation in vitro, mPR3 expression increases but the %PR3-high remains stable, again suggesting regulation of expression at

the genetic level (23). The current data in healthy volunteers is consistent with this model because the %PR3-high was constant in an individual regardless of the blood drawing time. However, in a pathophysiologic environment, the expression of mPR3 on neutrophils does differ and will change depending on the severity of the inflammatory challenge.

The present study shows that both the %PR3-high and the absolute level of mPR3 expression are increased in septic patients and related to their CRP concentration. CRP is an acute-phase protein and likely to reflect the presence as well as the severity of sepsis (29). CRP is synthesized predominantly by the liver, mainly in response to interleukin-6 (IL-6) (30, 31). TNF-α and IL-1β also are regulatory mediators of CRP synthesis (30). Actually not only TNF-α, but also IL-1β and IL-6 augment mPR3 expression on neutrophils surface in vitro (data not shown). In addition, activated neutrophils and purified PR3 augment TNF-α and IL-1β release from a stimulated human monocytic cell line (32). It was considered that mPR3 expression was up-regulated by some cytokines in inflammatory environment and contributing to the progress of the inflammation. Thus, neutrophil PR3 expression and activity plays a role in the cytokine networks that modulate an inflammatory response. Some patients did not fall into the general relationship between mPR3 expression and CRP. It is possible that some patients who have genetically dominant PR3-low neutrophils may have difficulty in increasing membrane PR3 expression on neutrophils even with similar inflammatory environment. It is equally possible that others with low CRP and high PR3 expression may be reflecting the fact that an increase in CRP is followed by neutrophil activation.

In the current study we observed that mPR3 expression was increased in patients with SIRS to a level greater than in patients without SIRS, suggesting that the neutrophils in patients with SIRS were either partially activated or shifted to an altered state. In vitro activation studies demonstrated that PR3 is also high in patients with SIRS suggesting that the neutrophils in those patients may be more primed by the inflammatory environment. These findings indicate that patients with SIRS have acquired the ability to express higher levels of PR3. Consequently the amount of PR3 expression was larger in the SIRS patients. It is not clear whether this difference in mPR3 expression is due to greater cellular activation levels in the face of an extreme inflammatory environment or to some other mechanism, but it is clear that elevated expression of mPR3 reflects activation of neutrophils in inflammatory disease.

CONCLUSION

PR3 expression on neutrophils is higher in patients with systemic inflammatory diseases, such as sepsis, in the absence of PR3-ANCA-positive vasculitis, suggesting that membrane expression of PR3 is greatly influenced by an in vivo inflammatory environment.

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