overlapping between the two studies were well consistent. In addition, we observed a decrease of plasminogen activity in the elderly, between 70–79 years and 80–89 years (Fig. 1). The low plasminogen activity in the elderly is probably affected by liver's ability to generate protein, because a decrease of albumin level with age also was observed ($r = -0.35$, $P < 0.0001$).

In the present study, we found the prevalence of plasminogen deficiency to be 4.30% in the Japanese general population ($n = 4517$), the prevalences of type I and II heterozygous plasminogen deficiency being 0.42% and 3.87%, respectively. The prevalence of type II plasminogen deficiency in the present study agreed with previously reported results in Japanese [6–9]. We also obtained the prevalence of plasminogen deficiency in patients with DVT and in patients with cardioembolic stroke and found that those prevalences were not different from those obtained from age-matched and sex-matched control groups selected from the general population. Most of these patients were residents in the northern Osaka area where the cohort study took place. Therefore, our study indicated that heterozygous plasminogen deficiency is not a risk factor for thrombotic complications.

We identified 173 heterozygotes and two homozygotes in 4517 individuals. If we assume that all those with type II deficiency carried the plasminogen Tochigi mutation, the Ala→Thr substitution at position 601, we can calculate the allele frequency of plasminogen Tochigi to be 1.96% in the Japanese general population. This allele frequency is similar to those of factor V Leiden mutation (2–7%) [26] and prothrombin 20210 A mutation (0.35–2.0%) [27] found in the Caucasian general population. We also identified 19 heterozygotes of type I plasminogen deficiency. The prevalence of type I plasminogen deficiency thus obtained (0.42%) showed quite good agreement with the previously observed prevalence in the Scotland population (28/9,611, 0.29%) [18].

There are several reports addressing a phenotype of mice with homozygous type I plasminogen deficiency [28,29]. The plasminogen gene in these mice was abnormal so that no plasminogen activity was present in plasma, resulting in spontaneous fibrin deposition due to impaired thrombolysis. One of the useful features of engineered mice is that although a transgenic or knockout gene may have no phenotype, a phenotype may become apparent with a physiological or pathological challenge. For example, mice deficient in plasminogen exacerbated renal injury in experimental crescentic glomerulonephritis [30]. Those mice also abolished wound healing after myocardial infarction [31]. These studies suggest that even though individuals with plasminogen deficiency did not show venous thrombosis, they may express a certain phenotype after a challenge or insult. Therefore, careful continuous observation in individuals with plasminogen deficiency is required for assessment of relation of plasminogen deficiency with its phenotype.

In conclusion, the prevalence of heterozygous plasminogen deficiency is about 4% in Japanese, and plasminogen deficiency is not a primary cause of thrombosis. This conclusion was also supported by the phenotypes of 19 patients with homozygous type II plasminogen deficiency.

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Addendum of the roles of authors

Drs Okamoto, Sakata, Mannami, and Miyata were responsible for the study design, interpretation of the data and preparation of the article. Drs Baba, Katayama, Matsuo, Yasaka, Minematsu, and Tomoike were responsible for sample collection, steering and discussion.

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The ubiquitin-like domain of Herp is involved in Herp degradation, but not necessary for its enhancement of amyloid β-protein generation

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Abstract Herp is an endoplasmic reticulum (ER)-stress-inducible membrane protein, which has a ubiquitin-like domain (ULD). However, its biological function is as yet unknown. Previously, we reported that a high expression level of Herp in cells increases the generation of amyloid β-protein (Aβ) and that Herp interacts with presenilin (PS). Here, we addressed the role of the ULD of Herp in Aβ generation and intracellular Herp stability. We found that the ULD is not essential for the enhancement of Aβ generation by Herp expression and the interaction of Herp with PS, but is involved in the rapid degradation of Herp, most likely via the ubiquitin/proteasome pathway. Thus, the ULD of Herp most likely plays a role in the regulation of the intracellular level of Herp under ER stress.
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Key words: Presenilin; Herp; Amyloid β-protein; Endoplasmic reticulum stress; Ubiquitin-like domain; Proteasome

1. Introduction

Amyloid β-protein (Aβ), which is the major component of senile plaques in the brains of patients with Alzheimer's disease (AD), is generated from the amyloid precursor protein (APP) through its sequential proteolytic cleavage catalyzed by β- and γ-secretases (reviewed in [1]). β-Secretase was identified as a membrane-tethered aspartyl protease [2]; γ-secretase, however, remains to be clarified, although presenilin (PS) was found to be essential for inducing γ-secretase activity [3–6]. Mutations in the PS genes, *PS1* and *PS2*, cause early-onset familial AD (reviewed in [1]). PS is a multiple transmembrane protein and forms a high-molecular-weight complex with several other proteins (reviewed in [6]). Recent studies have shown that the PS complex, including nicastrin, PEN-2 and APH-1, is responsible for inducing γ-secretase activity [7–10]. However, it still remains unknown how the PS-mediated γ-cleavage is regulated. In order to elucidate how the γ-cleavage is regulated, we have recently developed a new functional screening method for identifying cDNAs that enhance γ-cleavage using the combination of puromycin resistance assay and Aβ quantitation [11]. Previously, we have identified *Herp* using this screening method [12]. *Herp* was originally identified as a homocysteine-inducible gene and was found to be also inducible by endoplasmic reticulum (ER) stress [13,14]. Homocysteine or the inducers of ER stress, including thapsigargin, induce the expression of Herp mRNA approximately 50-fold over levels observed in untreated cells [13]. We have demonstrated that a high expression level of Herp, which is comparable to the level induced by homocysteine or ER stress, increases PS-mediated Aβ generation, possibly through Herp binding to PS [12]. Interestingly, elevated levels of homocysteine in plasma are likely to be a risk factor for AD [15]. Therefore, Herp induced by a high level of homocysteine may play an important role in Aβ accumulation, including the formation of senile plaques or vascular Aβ deposits in AD. Herp is a membrane protein localized in the ER and it has an N-terminal ubiquitin-like domain (ULD) [13]; however, the precise function of this protein and the role of the ULD of Herp have not been established.

Ubiquitin is a highly conserved small protein widely present in all eukaryotic cells. The modification of cellular proteins with ubiquitin targets them for degradation by proteasome. Accumulating evidence has shown that ubiquitin-like proteins (ULPs), which have structural similarity to ubiquitin, are also present in cells. ULPs are divided into two subclasses (reviewed in [16]): small, type-1 ULPs, such as SUMO-1 and NEDD8, which are ligated to target proteins in a manner analogous to the ubiquitination pathway (reviewed in [17]), and type-2 ULPs that contain a ubiquitin-like structure in large proteins, such as elongin B [18], Rad23 [19], parkin [20] and Herp [13]. Although type-1 ULPs constitute a new type of post-translational protein-modifying system, the biological significance of type-2 ULPs remains to be clarified. The ULD of elongin B appears to have a chaperone-like function [18], whereas a recent report showed that the intracellular level of parkin is regulated by the ULD [21]. In this

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Abbreviations: Aβ, amyloid β-protein; PS, presenilin; AD, Alzheimer's disease; APP, β-amyloid precursor protein; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; ULD, ubiquitin-like domain; ULP, ubiquitin-like protein; ERAD, ER-associated degradation

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study, we determined the role of the ULD of Herp. For this purpose, we first investigated whether the ULD of Herp is involved in the pathway leading to the enhancement of Aβ generation caused by a high expression level of Herp, since the precise mechanism underlying this action is not known. Second, we determined whether the cellular level of Herp is regulated by this domain via the ubiquitin/proteasome pathway similar to the function of ubiquitin.

2. Materials and methods

2.1. Antibodies, reagents and cell lines

An affinity-purified rabbit anti-Herp antibody was prepared as previously described [13]. A rat anti-PS1 antibody (for the N-terminal fragment (NTF) of PS1) and a rabbit anti-PS2 antibody (for the NTF) were purchased from Chemicon International (Temecula, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. A monoclonal antibody 6E10 specific to human Aβ1–17 was purchased from Senetek (St. Louis, MO, USA). BA27 specific to the Aβ40 terminal site, BC05 specific to the Aβ42 terminal site and BNT77 raised against human Aβ11–28 have all been characterized previously [22]. The 369 antibody was raised against the C-terminal residues of APP695 [23]. A monoclonal anti-FLAG antibody and a rabbit polyclonal anti-FLAG antibody were purchased from Sigma. Anti-multi-ubiquitin antibody was purchased from MBL. Cycloheximide was purchased from Wako. Lactacystin was purchased from Kyowa. Monoclonal antibody against β-tubulin was purchased from Babco (Richmond, CA, USA). HEK293 cells stably transfected with APP695 and PS1, which were used for the study of the interaction between PS1 and Herp, were generated as previously reported [12].

2.2. Plasmids and retrovirus-mediated infection

pMX-Herp was generated as previously described. The ΔUb-Herp cDNA encoding Herp lacking Val[10] to Cys[86] of Herp was inserted at the *Eco*RI and *Sal*I sites of pMX (designated pMX-ΔUb-Herp). We generated C-terminally FLAG-tagged ΔUb-Herp using the polymerase chain reaction method (sense primer: GGAATTCGCCACCATGGAGTCCGAGACCGA; antisense primer: GCGTCGACTCACTTATCGTCATCGTCCTTGTAGTCGTTTGCGATGGCTGGGGGC) from Herp cDNA. This cDNA was inserted into pCI (Promega) at *Eco*RI and *Sal*I sites (designated pCI-ΔUb-Herp-F). The *Eco*RI-*Sal*I fragment of pCI-ΔUb-Herp-F was also inserted into pcDNA 3.1 (Invitrogen) at *Eco*RI and *Sal*I sites (designated pcDNA-ΔUb-Herp-

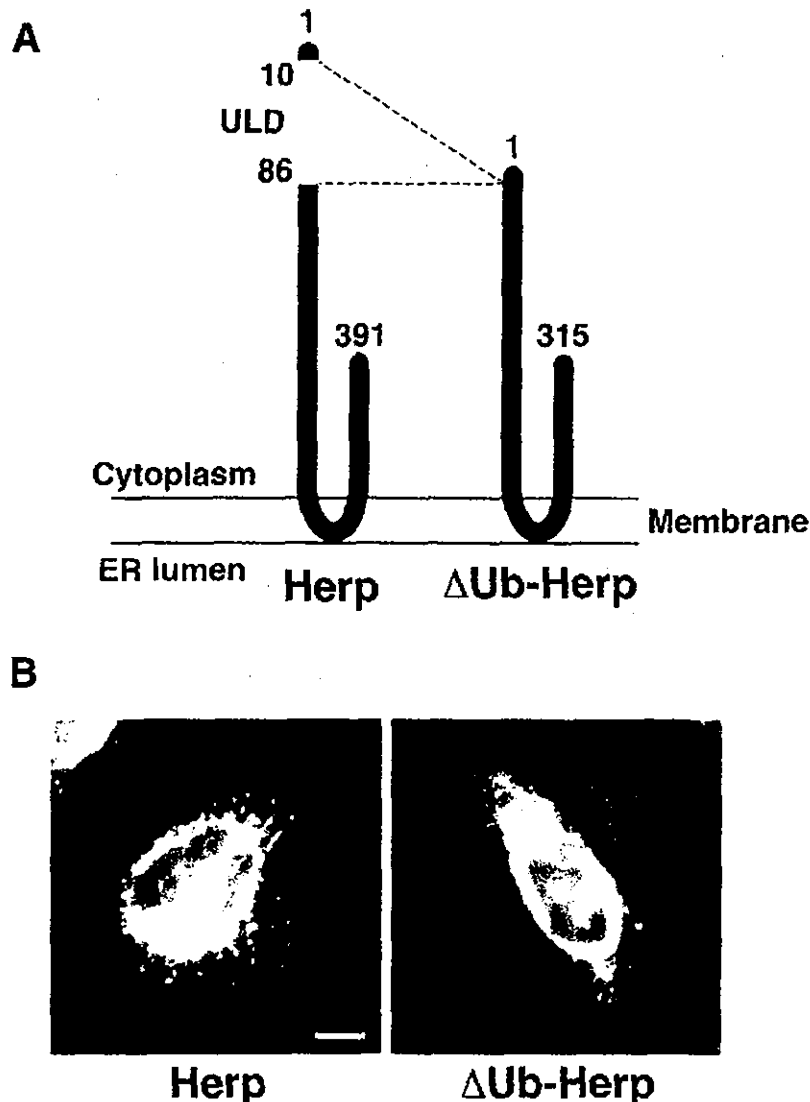

Fig. 1. Schematic representation of Herp lacking the ULD (ΔUb-Herp) (A) and the cellular localization of ΔUb-Herp (B). A: ΔUb-Herp encodes Herp that lacks the residues Val[10]–Cys[86]. The gray box illustrates the ULD. B: HeLa cells were transiently transfected with pCI-Herp-F or pCI-ΔUb-Herp-F. The cells were immunostained with anti-FLAG antibody. Scale bar, 10 μm.

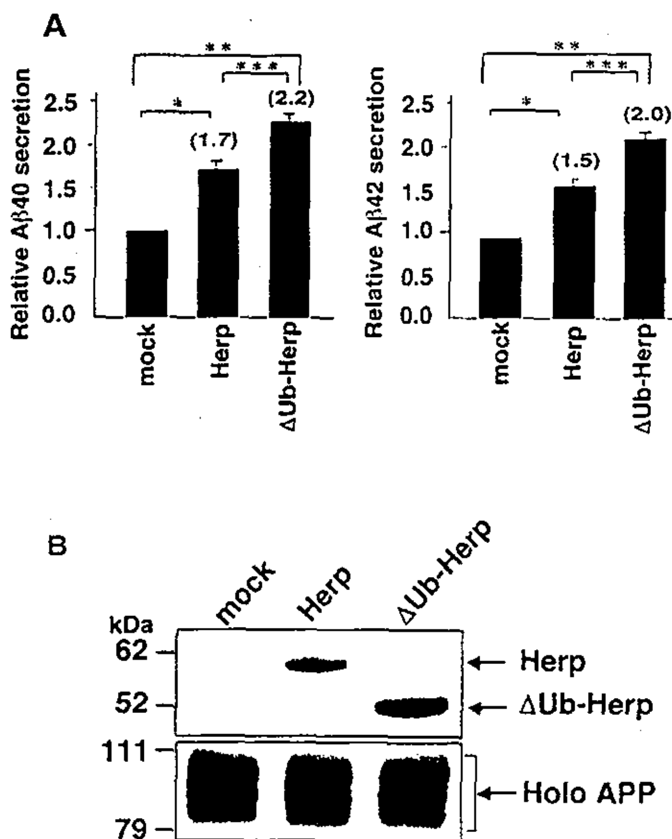

Fig. 2. Effect of the deletion of the ULD of Herp on Aβ generation. A: Murine fibroblasts (2×10^5) expressing APP695 were retrovirally transfected with pMX (mock), pMX-Herp or pMX-ΔUb-Herp. Aβ secreted during a 72-h culture was detected by ELISA. Values are means ± S.D. of four independent dishes ($n=4$). Similar results were obtained from four independent experiments. Relative amounts of Aβ (figures in parentheses) were determined by calculating the ratio of the amounts of Aβ secreted from the cells expressing Herp or ΔUb-Herp to the mean amount of Aβ secreted by a mock transfectant (normalized to 1). For Aβ40, $*P<0.01$; $**P<0.01$; $***P<0.01$ (Mann–Whitney *U*-test). For Aβ42, $*P<0.05$; $**P<0.01$; $***P<0.05$. B: Lysates (10 μg) were immunoblotted with the anti-Herp antibody (upper panel). Intracellular APP level (holo APP) was detected with the anti-C-terminal APP antibody, 369 (lower panel). WB, Western blots.

F). The *Bsu*36I-*Sal*I fragment of ΔUb-Herp-F cDNA was replaced with that of wild-type Herp and inserted at the *Eco*RI and *Sal*I sites of pMX, producing pMX-ΔUb-Herp. The retrovirus-mediated infection was carried out as previously reported [11].

2.3. *Fluorescent immunocytochemistry*

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum on 35-mm dishes. The cells were transfected with pCI-Herp-F or pCI-ΔUb-Herp-F using FuGENE6 (Roche). At 24 h post transfection, the cells were immunostained with anti-FLAG rabbit polyclonal antibody (Sigma) followed by Alexa Fluor 488-labeled secondary antibody (Molecular Probes) as previously described [13]. Fluorescence was visualized with the Axiovert 200 microscope equipped with AxioCam (Carl Zeiss).

2.4. *Immunoprecipitation, immunoblotting and sandwich ELISA*

Cultured cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.5, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 0.2% sodium deoxycholate) containing a protease inhibitor cocktail. The solubilized proteins were subjected to immunoprecipitation as previously described [24]. The precipitated proteins were resolved by SDS–polyacrylamide gel electrophoresis on 4–20% gel for the detection of PS and Herp, and on 7.5% gel for the detection of intracellular APP. Immunoblotting was performed as previously reported [24]. Enzyme-linked immunosorbent assay (ELISA) for Aβ was performed as previously described [22]. The capture antibody used was BNT77. Detector antibodies were horseradish peroxidase (HRP)-conjugated BA27 (for Aβ40) and HRP-conjugated BC05 (for Aβ42). ELISA data were statistically analyzed by ANOVA using StatView-J.4.11.

2.5. *Cycloheximide and lactacystin treatment*

Cells (5×10^5) plated on 60-mm tissue culture dishes were grown for 24 h, then cycloheximide and/or lactacystin were added to final concentrations of 30 μg/ml and 100 μM, respectively. At various times after the addition of cycloheximide and/or lactacystin, the cells were harvested and then lysed in RIPA buffer.

3. Results and discussion

As previously reported, Herp has the N-terminal region, Val^{14}–Val^{85}, which includes the stretch sharing 32% identity with the Val^{5}–Val^{70} stretch of ubiquitin [13]. In order to elucidate the role for this ULD of Herp, we generated Herp lacking the residues Val^{10}–Cys^{86} (designated ΔUb-Herp) (Fig. 1A). We confirmed with the immunostaining study that there is no significant difference in intracellular localization between Herp and ΔUb-Herp (Fig. 1B). Previously we demonstrated that a high expression level of Herp enhances PS-mediated Aβ generation. Therefore, in this study, we first investigated whether the expression of ΔUb-Herp also increases Aβ generation to determine whether the ULD is necessary for the increase in Aβ generation caused by a high expression level of Herp. As shown in Fig. 2A, ΔUb-Herp expression also increased the level of Aβ. Thus, we concluded that the ULD of Herp is not necessary for the enhancement of Aβ generation. It was noted in our previous study that Herp

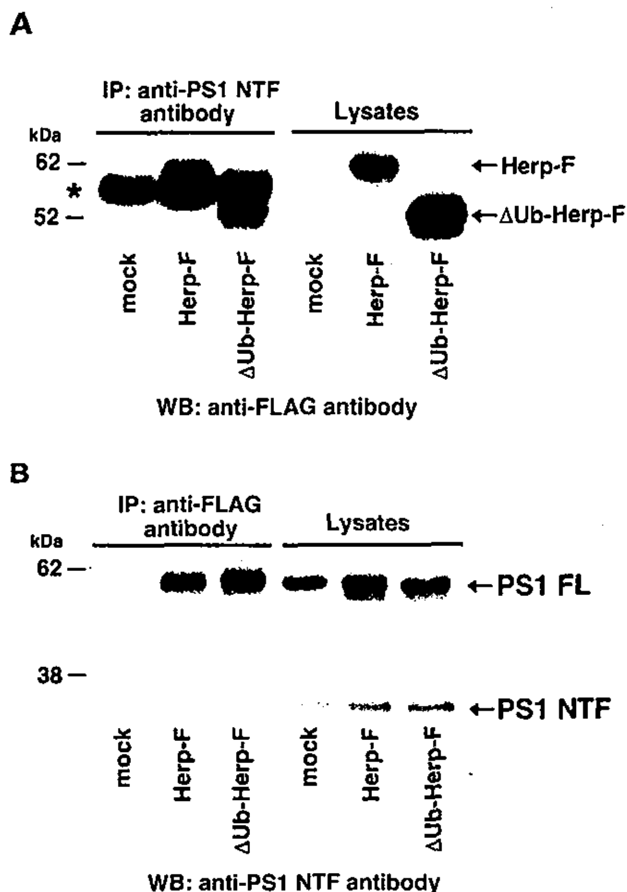

Fig. 3. Interaction between PS and Herp lacking the ubiquitin-like domain. A: HEK293 cells stably expressing APP and PS1 transiently transfected with pcDNA (mock), pcDNA-Herp-F or pcDNA-ΔUb-Herp-F. RIPA-solubilized lysates (100 μg) were immunoprecipitated with the anti-PS1 NTF antibody and immunoblotted with the anti-FLAG antibody (left, three lanes). The lysates (10 μg) were also immunoblotted with the anti-FLAG antibody (right, three lanes). IP, Immunoprecipitation; WB, Western blots. Herp-F, C-terminally FLAG-tagged Herp; ΔUb-Herp-F, C-terminally FLAG-tagged ΔUb-Herp. The asterisk indicates a non-specific band. B: The same RIPA-solubilized lysates (100 μg) were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-PS1 NTF antibody (left, three lanes). The lysates (10 μg) were also immunoblotted with the anti-NTF antibody (right, three lanes). Note: The anti-FLAG antibody was used for the immunoprecipitation of Herp-F and ΔUb-Herp-F since the anti-Herp antibody used in this study cannot immunoprecipitate ΔUb-Herp.

induced a greater increase in Aβ40 level than Aβ42 level in HEK293 cells [12], but in fibroblasts the extents of increase in both Aβ40 and Aβ42 levels were almost the same (Fig. 2A). The effect of Herp on Aβ42 generation appears to be different between the two cell types. It was also noted that, as shown in Fig. 2A, the extent of increase induced by the expression of ΔUb-Herp was slightly higher than that induced by the expression of wild-type Herp, probably because the protein level of ΔUb-Herp was higher than that of Herp (Fig. 2B; see below: the ULD is involved in Herp degradation). We next determined whether ΔUb-Herp can also bind to PS, since Herp interacts with the full-length PS as previously reported [12]. As shown in Fig. 3, ΔUb-Herp bound to the full-length PS1, but failed to bind to PS1 NTF. We also noted that ΔUb-Herp bound to the full-length PS2 (data not shown). Taken together, these data show that the ULD of Herp is not involved in the pathway leading to the enhancement of Aβ generation and in the interaction with PS.

We next examined the effect of the deletion of the ULD of Herp on intracellular Herp stability, because it is well established that ubiquitin is involved in the protein degradation via the ubiquitin/proteasome pathway. Western blot analysis of intracellular degradation of Herp and ΔUb-Herp in fibroblasts following cycloheximide treatment revealed that Herp is rapidly degraded within 6 h, while ΔUb-Herp remains stable (Fig. 4A). The quantification of band intensity showed that the half-life of Herp is approximately 2.5 h, while that of ΔUb-Herp is more than 24 h (Fig. 4B). Furthermore, (i) Herp degradation was completely inhibited after treating the cells with the proteasome inhibitor lactacystin (Fig. 5A); (ii) the immunoprecipitated Herp and ΔUb-Herp contained smear bands on the gel, which were immunoreactive with the anti-polyubiquitin antibody (Fig. 5B), strongly suggesting that Herp and

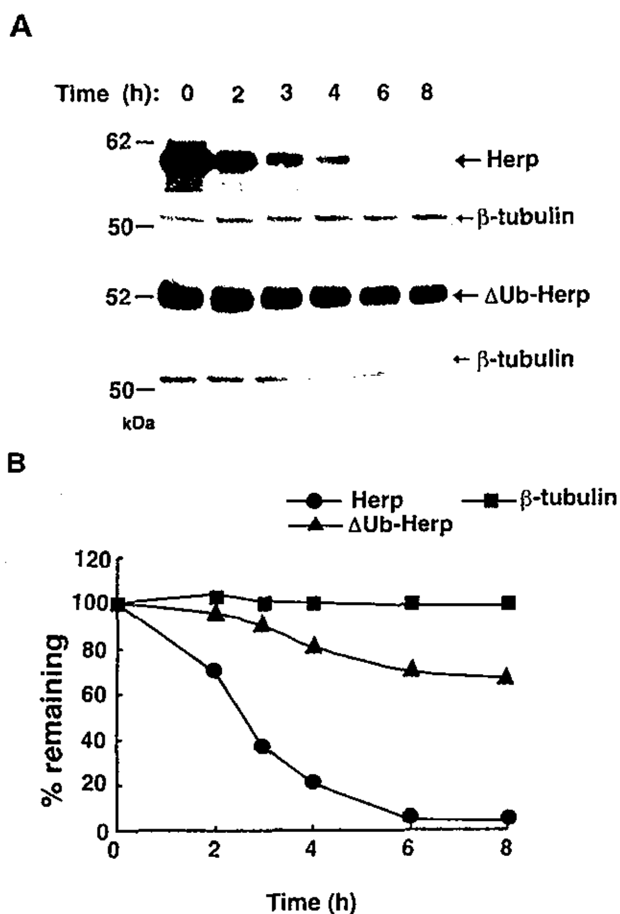

Fig. 4. Stabilization of Herp by deletion of the ULD. A: Wild-type fibroblasts retrovirally expressing Herp and ΔUb-Herp were treated with 30 μg/ml cycloheximide and then harvested at the times indicated. Herp or ΔUb-Herp in the RIPA-solubilized lysates (10 μg) was detected by immunoblotting with the anti-Herp antibody. As a control for the stable protein in the lysates, β-tubulin was immunodetected with anti-β-tubulin antibody. Upper panel: lysates from cells expressing Herp; lower panel: lysates from cells expressing ΔUb-Herp. The blots are representative of four independent experiments. B: The intensities of the bands corresponding to Herp, ΔUb-Herp and β-tubulin in A were quantified densitometrically using NIH Image software (PDI). Relative intensity was calculated as percentage of the intensity at time 0. Time, the time period of cycloheximide treatment.

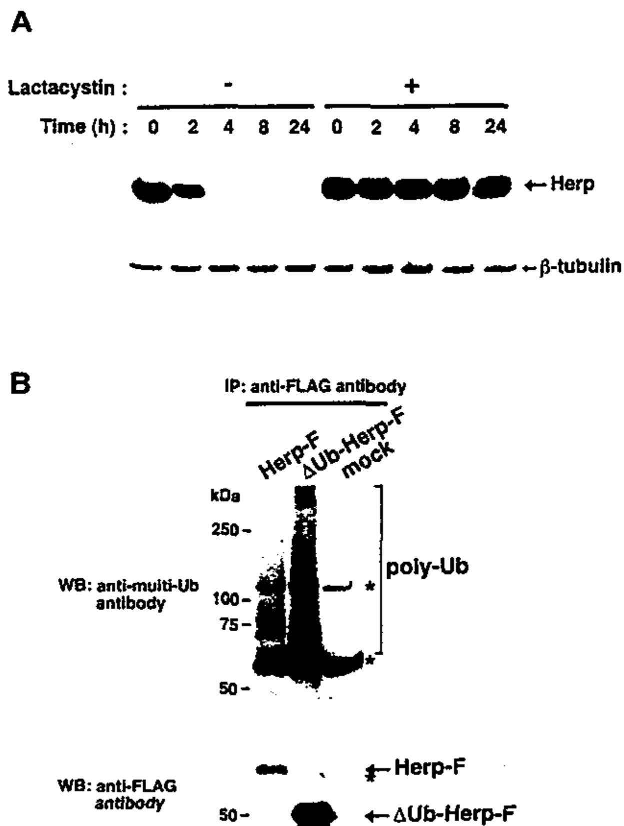

Fig. 5. Effect of lactacystin treatment on Herp degradation (A) and detection of Herp polyubiquitination (B). A: Wild-type fibroblasts retrovirally expressing Herp were treated with 30 μg/ml cycloheximide in the absence (−) or presence (+) of lactacystin and then harvested at the times indicated. Herp in the RIPA-solubilized lysates (10 μg) was detected by immunoblotting with the anti-Herp antibody. As a control for the stable protein in the lysates, β-tubulin was immunodetected with anti-β-tubulin antibody. B: Fibroblasts transiently transfected with pcDNA-Herp-F or pcDNA-ΔUb-Herp-F or pcDNA (mock) were treated with cycloheximide and lactacystin for 8 h, and then harvested. The cell lysates were immunoprecipitated with anti-FLAG mouse antibody and then immunodetected with anti-multi-ubiquitin antibody (upper panel) or anti-FLAG rabbit antibody (lower panel). Ub, ubiquitin, Herp-F, C-terminally FLAG-tagged Herp; ΔUb-Herp-F, C-terminally FLAG-tagged ΔUb-Herp. The asterisk indicates a non-specific band. IP, immunoprecipitation; WB, Western blots.

ΔUb-Herp undergo polyubiquitination. Taken together, Herp is degraded most likely via the ubiquitin/proteasome pathway, and the ULD is not involved in Herp polyubiquitination.

In this study, we noted that the ULD of Herp is not essential for the increase in Aβ generation mediated by Herp expression. We also demonstrated that the ULD of Herp is indeed involved in the degradation of Herp, most likely via the ubiquitin/proteasome pathway. Recently, it has been shown that the ULD of parkin regulates the intracellular parkin level and parkin polyubiquitination does not require the ULD [21]. In this regard, the ULD of Herp has a role very similar to that of parkin. However, it is not known how the ULD of Herp is involved in Herp degradation. Further study of the role of the ULD in Herp degradation is necessary. At present, the function of this protein is not known; Herp may, however, mediate the degradation of misfolded proteins induced by ER stress through the ULD, since Herp is the ER-stress-inducible ER-resident protein. The ER-associated degradation (ERAD) pathway is known to direct the ubiquitin-mediated degradation of various ER-associated misfolded proteins (reviewed in [25]). In response to ER stress, a series of ERAD-related genes are induced [26,27]. Parkin is also suggested to be involved in ERAD (reviewed in [28]). Therefore, *Herp* may also be an ERAD-related gene.

Since a strong ubiquitin immunoreactivity is associated with the pathogenesis of AD [29], the ubiquitin/proteasome protein degradation system appeared to be impaired in the brains of AD patients. We demonstrated that a high expression level of Herp enhanced Aβ generation, and Herp is degraded via the ubiquitin/proteasome pathway. Therefore, an impairment of the ubiquitin/proteasome system in AD may reduce Herp degradation, resulting in the enhancement of Aβ generation induced by a higher level of Herp.

Taking these data together, the ULD of Herp most likely plays a role in the regulation of the intracellular level of Herp under ER stress, and also possibly in AD pathogenesis. Further study of the mechanism underlying Herp degradation and the Herp-mediated increase in Aβ generation is required.

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VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13

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ADAMTS-13 was recently identified as a new hemostatic factor, von Willebrand factor (VWF)–cleaving protease. Either congenital or acquired defects of the enzymatic activity lead to thrombotic thrombocytopenic purpura (TTP). ADAMTS-13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF. Here, we determined the minimal region recognized as a specific substrate by ADAMTS-13. A series of partial deletions in the A2 domain flanked with N- and C-terminal tags were expressed in *Escherichia coli* and affinity-purified. These purified proteins were incubated with human plasma, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blot. Judging from mobility shifts, all constructs except one were cleaved at the expected site. Data suggested that a minimal region as a functional substrate consisted of 73 amino acid residues from D1596 to R1668 of VWF, designated VWF73, and that further deletion of the E1660-R1668 region led to the loss of cleavage by ADAMTS-13. VWF73 was not cleaved by plasma from patients with congenital or acquired TTP, but cleaved by plasma from patients with hemolytic uremic syndrome, suggesting that VWF73 is a specific substrate for ADAMTS-13. Thus, VWF73 will be a useful seed to develop a new rapid assay to determine ADAMTS-13 activity. (Blood. 2004;103:607-612)

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Introduction

Thrombotic thrombocytopenic purpura (TTP), a serious disease with high mortality, is typically characterized by 5 features: thrombocytopenia, microangiopathic hemolytic anemia, renal failure, fever, and neurologic dysfunction.[1] In patients with TTP, formation of platelet thrombi within the microvasculature is observed. TTP can be categorized into congenital and acquired types. In congenital cases with neonatal onset, the patients quickly respond to infusion of fresh frozen plasma, but the episodes of thrombocytopenia and hemolytic anemia are repeated. TTP with neonatal onset and frequent relapses is often diagnosed as Upshaw-Schulman syndrome (USS).[2] The majority of clinically observed TTP is acquired, often affecting adolescents and adults.

The 2 key molecules involved in the pathogenesis of TTP are the plasma proteins, von Willebrand factor (VWF)[3-6] and ADAMTS-13.[7-11] The platelet-adhesive blood-coagulation protein, VWF, is synthesized primarily in vascular endothelial cells and released into plasma as large multimeric forms, which are highly active in interactions with platelets and collagen.[12,13] In patients with both congenital and acquired TTP, unusually large VWF multimers circulate in plasma, resulting in the promotion of microvascular thrombosis, platelet consumption, and hemolysis. In normal plasma, VWF multimers are rapidly cleaved into smaller forms ranging in size from 500 to 20 000 kDa. This physiologically important cleavage is achieved by a newly identified plasma protease, ADAMTS-13. Functional deficiency of ADAMTS-13 caused by genetic mutation,[14-18] inhibitory autoantibodies,[19,20] or other etiologies leads to the accumulation of unusually large VWF multimers in plasma.

Human ADAMTS-13 was purified from plasma[21-23] and its cDNA was cloned.[23-25] At the same time, the *ADAMTS13* gene was also identified as a gene responsible for congenital TTP by linkage analysis.[14] ADAMTS-13 mRNA is predominantly expressed in liver.[14,23,24,26] The protease consists of 1427 amino acid residues, containing an N-terminal signal peptide, a propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type-1 motif (TSP1), a cysteine-rich domain, a spacer domain, 7 more TSP1 repeats, and 2 CUB domains. The only known physiologic substrate for ADAMTS-13 is VWF multimers. The spacer domain of ADAMTS-13 is necessary for normal VWF-cleaving activity, and the more C-terminal domains are dispensable for the catalytic activity in vitro.[27,28]

ADAMTS-13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF.[29-32] Although several methods have been developed to measure plasma ADAMTS-13 activity, they are not widely used at the clinical level due to various difficulties. The symptoms of TTP are similar to hemolytic uremic syndrome (HUS), a syndrome that is also characterized by thrombocytopenia, microangiopathic hemolytic anemia, and renal failure. HUS occurs mostly in young children after *Escherichia coli* O157 infection, but in some cases, it is difficult to discriminate between TTP and HUS. Therefore, the diagnosis of ambiguous TTP/HUS is made occasionally. For adequate therapy, the establishment of a consistent

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diagnosis system for TTP is eagerly anticipated by physicians and patients. The clinical assay of ADAMTS-13 activity is the most effective instrument for the diagnosis of TTP.[33,34]

To develop a more rapid and convenient method than previously described, an artificial specific substrate that can be easily processed by ADAMTS-13 will be useful. Here, we report that the minimal substrate for ADAMTS-13 is composed of 73 amino acid residues, and we designate this substrate as VWF73.

Materials and methods

Materials

Human plasma was obtained by centrifugation from whole blood that had been anticoagulated with 1:10 volume of 3.8% sodium citrate. Plasma from 3 patients with USS (congenital TTP), 6 patients with acquired TTP, 3 patients with HUS, and healthy individuals were used to measure the ADAMTS-13 activity.

Construction of bacterial expression vectors

Plasmid DNA to express partial regions of human VWF tagged with N-terminal glutathione *S*-transferase (GST) and C-terminal 6xHis (H) were constructed as follows. First, the D1459-R1668 region of VWF was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA prepared from cultured human umbilical vein endothelial cells. We used 2 primers for amplification: 5′-cgggatccGACCTTGCCCCT-GAAGCCCCTC-3′ and 5′-cggaattcTCAGTGATGGTGATGGTGAT-GCCTCTGCAGCACCAGGTCAGGA-3′. Lowercase letters indicate added restriction enzyme sites, and the underlined sequence is the inserted C-terminal H-tag. The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the corresponding site of pGEX-6P-1 (Amersham Biosciences, Buckinghamshire, England), a *Schistosoma japonicum* GST fusion expression vector. The other plasmids for E1554-R1668, D1587-R1668, D1596-R1668, and D1596-R1659 regions of VWF were also prepared in the same way by combinational use of primers as follows: 5′-cgggatccGAGGCACAGTCCAAAGGGGACA-3′, 5′-cgggatccGACCA-CAGCTTCTTGGTCAGCC-3′, 5′-cgggatccGACCGGGAGCAGGCGC-CCAACC-3′, and 5′-cggaattcTCAGTGATGGTGATGGTGATGTCGGG-GGAGCGTCTCAAAGTCC-3′.

Expression and purification of recombinant proteins

To obtain the different recombinant proteins, expression vectors encoding GST-D1459R1668-H, GST-E1554R1668-H, GST-D1587R1668-H, GST-D1596R1668-H, and GST-D1596R1659-H were introduced into *E coli*, BL21 (Stratagene, La Jolla, CA). After isopropyl-β-D-thiogalactoside (IPTG) induction in liquid culture, bacterial cells were collected and lysed with CelLytic B (Sigma, St Louis, MO), followed by centrifugation to separate soluble and insoluble fractions. GST-D1587R1668-H, GST-D1596R1668-H, and GST-D1596R1659-H were collected in soluble fractions, whereas GST-D1459R1668-H and GST-E1554R1668-H were in insoluble fractions. First, all these proteins were purified by Ni-NTA Spin Kit (Qiagen, Hilden, Germany) in a denaturing condition containing 8 M urea and 20 mM 2-mercaptoethanol according to the instruction. The eluates (pH 4.3) were diluted to a 40-times volume of phosphate-buffered saline and left overnight at 4°C for refolding. Then, the proteins were purified by MicroSpin GST Purification Module (Amersham Biosciences) according to the instruction. Eluted proteins (10 mM glutathione) were dialyzed against 20 mM Tris (tris(hydroxymethyl)aminomethane)-HCl (pH 8.0) and quantified by DC Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Cleavage of recombinant proteins by plasma

Purified recombinant proteins (300 ng) were incubated with 1 μL plasma in 40 μL reaction buffer (5 mM Tris-HCl, 10 mM $BaCl_2$, and 1 mM amidinophenylmethanesulfonyl fluoride hydrochloride, pH 8.0) at 37°C for the indicated time. The reaction was stopped by adding 10 μL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA (ethylenediaminetetraacetic acid), 0.1% bromophenol blue, and 30% glycerol; pH 6.8). Alternatively, to detect inhibitors of ADAMTS-13 in plasma from patients, normal plasma was preincubated with an equal volume of heat-inactivated patient plasma for one hour at room temperature, and then incubated with recombinant substrate proteins at 37°C for 1 hour.

Western blot analysis

The samples were subjected to SDS-PAGE (10%-20% gradient gel) and transferred to a polyvinylidene fluoride membrane (Bio-Rad). Following blocking with 3% skim milk, the membrane was incubated with 1 μg/mL anti-GST (Molecular Probes, Eugene, OR) and then with 0.1 μg/mL peroxidase-labeled anti-rabbit immunoglobulin G (IgG; Kirkegaard & Perry Laboratories, Gaithersburg, MD). Chemiluminescence was developed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Warrington, United Kingdom) and detected on an image analyzer LAS-1000plus (Fujifilm, Tokyo, Japan).

Results

Preparation of substrate proteins

To identify the minimal region of VWF recognized as a substrate by ADAMTS-13, we prepared 5 recombinant proteins containing a partial region of human VWF. First, 2 criteria were set: (1) The region should contain the cleavage site by ADAMTS-13, Y1605 and M1606, in the A2 domain of VWF. (2) It should not contain any cysteine residues that often interfere with the proper folding of artificially engineered proteins. The longest region that satisfied the criteria ranged from D1459 to R1668 of VWF. These 210 amino acid residues were flanked with N-terminal GST and C-terminal H tags for convenient purification and detection, and designated GST-D1459R1668-H or substrate I (Figure 1). The other 4 substrates, GST-E1554R1668-H (substrate II), GST-D1587R1668-H (substrate III), GST-D1596R1668-H (substrate IV), and GST-D1596R1659-H (substrate V), were shorter derivatives of this VWF region.

When expressed in *E coli*, a band corresponding to the expected size of each substrate was visualized (substrate I, 50.8 kDa; II, 40.4 kDa; III, 36.7 kDa; IV, 35.7 kDa; V, 34.7 kDa) after IPTG induction

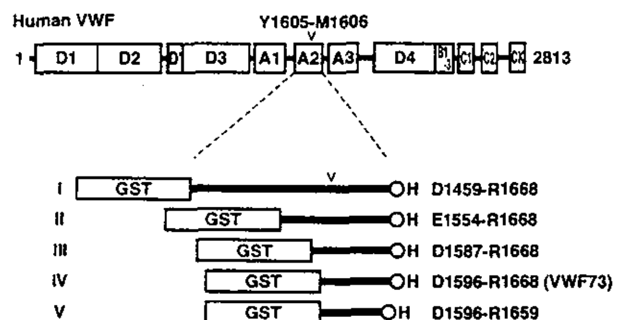

Figure 1. Structures of VWF and fusion proteins for ADAMTS-13 substrate. The domain structure of human preproVWF is shown above the structures of recombinant fusion proteins designed in the present study. Amino acid residues of preproVWF are numbered from the initiating Met codon. The locations of 5 kinds of structural domains (A, B, C, D, and CK) are indicated. The mature VWF secreted from cells consists of 2050 residues (S764-K2813) from the D′ domain to the C-terminal CK domain. ADAMTS-13 cleaves the Y1605-M1606 peptidyl bond in the A2 domain (D1498-L1664). We made 5 different recombinant proteins flanked with GST- and H-tags: GST-D1459R1668-H (I), GST-E1554R1668-H (II), GST-D1587R1668-H (III), GST-D1596R1668-H (IV), and GST-D1596R1659-H (V).

(Figure 2A). Substrates I and II were collected from the insoluble fractions (inclusion bodies), whereas substrates III, IV, and V were mainly recovered in soluble fractions (Figure 2B). All of the recombinant proteins were purified by 2 steps, nickel-ion chelating column chromatography and glutathione-affinity column chromatography, using C-terminal H and N-terminal GST tags, respectively (Figure 2C).

Cleavage of substrate proteins by normal plasma

If ADAMTS-13 cleaves the expected site of substrates I, II, III, IV, and V, the sizes of N-terminal portion including the GST-tag will be 43.1, 32.7, 29.0, 28.0, and 28.0 kDa, respectively. To explore the proteolytic effects of human plasma, these substrate proteins were incubated with normal plasma and analyzed by Western blot using an anti-GST antibody (Figure 3). When substrate I was incubated with normal human plasma for one hour, a very faint band (arrowhead in lane 2) appeared with the apparent size of approximately 43 kDa; this band was not detected before incubation (lane 1). This implied that substrate I was cleaved by some protease in plasma. In the presence of 50 mM EDTA, the substrate was not cleaved (data not shown), suggesting that this cleavage was catalyzed by a metalloprotease, possibly ADAMTS-13. For substrates II, III, and IV, the N-terminal fragments with expected sizes were also detected only after incubation with plasma (arrowheads in lanes 4, 6, and 8). Substrates III and IV were cleaved more effectively than I and II. This might be caused by different refolding efficiency during purification, because substrates I and II were recovered from inclusion bodies. Adding urea to the reaction, which is expected to expose the proper cleavage site of these substrates, did not enhance the cleavage (data not shown). Interestingly, substrate V was not cleaved by plasma, suggesting that it was not recognized as a substrate by ADAMTS-13. Thus, the shortest cleavable substrate in these, IV (GST-D1596R1668-H), was characterized further. Hereinafter, the peptide with 73 amino acid residues corresponding to the region from D1596 to R1668 of VWF is referred to as VWF73.

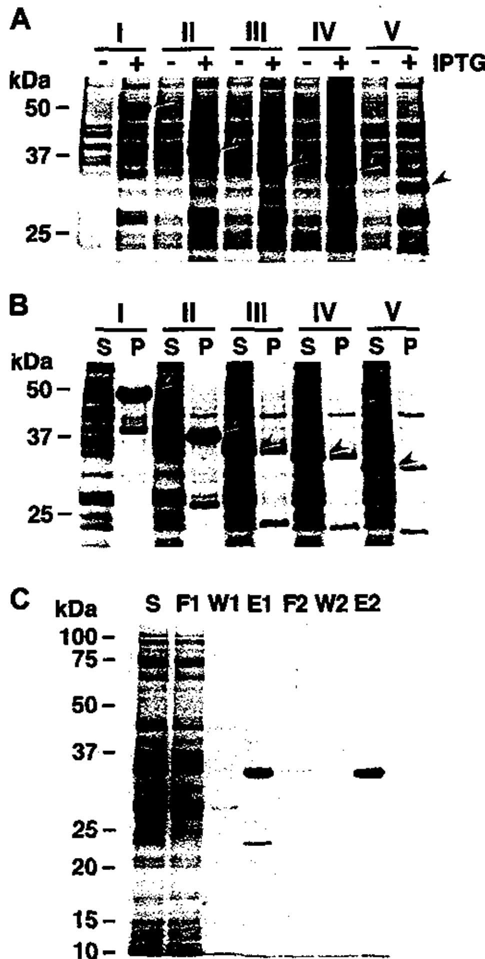

Figure 2. Preparation of bacterial recombinant proteins. *(A) Expression.* Arrowheads indicate 5 recombinant proteins expressed in *E coli* after IPTG induction: GST-D1459R1668-H (I), GST-E1554R1668-H (II), GST-D1587R1668-H (III), GST-D1596R1668-H (IV), and GST-D1596R1659-H (V). Gels after SDS-PAGE were stained with GelCode Blue (Pierce, Rockford, IL). The sizes of the protein standards are indicated at the left. (B) Fractionation. Recombinant proteins I and II were collected in pellet fractions (P) after centrifugation, whereas III, IV, and V were in soluble fractions (S). (C) Purification. All the recombinant proteins were purified by 2 sequential column-chromatography procedures, nickel-ion chelating chromatography and glutathione-affinity chromatography. The representative pattern of GST-D1596R1668-H is shown. S indicates soluble fraction of bacterial lysate; F1, flow-through of nickel-ion column; W1, wash; E1, eluate; F2, flow-through of glutathione column; W2, wash; and E2, eluate.

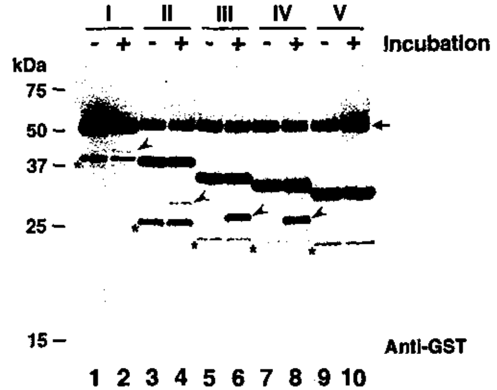

Figure 3. Cleavage of recombinant proteins by normal plasma. The recombinant substrates (I-V) were incubated with normal plasma at 37°C for 0 hours (lanes 1, 3, 5, 7, and 9) or 1 hour (lanes 2, 4, 6, 8, and 10). Both substrates and products were detected by Western blot using anti-GST. The product bands including N-terminal GST-tag are indicated by arrowheads. Substrates III and IV were cleaved more efficiently than I and II, and substrate V was not cleaved. The arrowed bands observed in all lanes are nonspecific signals derived from plasma albumin. The bands with asterisks, probably contaminating degradation products, are reproducible background signals.

Characterization of cleavage

It was previously reported that ADAMTS-13 cleaves VWF in vitro preferentially in the presence of urea and in low ionic strength.[4] We examined the effect of urea and NaCl on the cleavage efficiency of GST-VWF73-H. In hypotonic buffer including 5 mM Tris-HCl and 10 mM $BaCl_2$, GST-VWF73-H was efficiently cleaved by normal plasma in a time-dependent manner (Figure 4, lanes 1-3). In the presence of either 1.5 M urea or 150 mM NaCl, however, the production of the N-terminal fragment was quite low (lanes 4-9). The inhibitory effect of physiologic ionic strength was consistent with a previous report.[4] No requirement of urea for efficient cleavage suggests that the structure surrounding the Y1605-M1606 peptidyl bond is different between GST-VWF73-H and intact VWF multimers.

Cleavage of GST-VWF73-H by patient plasma

To confirm that the cleavage of GST-VWF73-H is catalyzed by ADAMTS-13, the substrate was incubated with plasma prepared from patients with congenital TTP, USS (Figure 5). Previously, we confirmed that these 3 patients have no VWF-cleaving activity,[2]

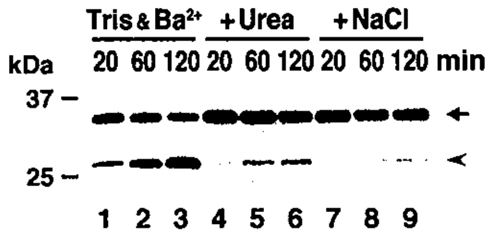

Figure 4. Effect of urea and ion strength on cleavage. GST-VWF73-H was incubated with normal plasma for the indicated time in reaction buffer (5 mM Tris-HCl, 10 mM $BaCl_2$, pH 8.0) (lanes 1-3) or in the same buffer supplemented with either 1.5 M urea (lanes 4-6) or 150 mM NaCl (lanes 7-9). The substrate and product bands are shown by an arrow and an arrowhead, respectively.

and that USS patients 2 and 3 are a homozygote of the ADAMTS-13 Q449X mutation and a compound heterozygote of R268P/C508Y, respectively.[15] In the present assay, none of their plasma cleaved GST-VWF73-H, strongly suggesting that the cleavage of GST-VWF73-H by normal plasma is catalyzed by ADAMTS-13. The plasma derived from patients with ticlopidine-associated TTP also showed no cleavage of the substrate (Figure 5), consistent with our previous reports.[35,36] Among 9 patients with idiopathic TTP examined, 5 patients had no ADAMTS-13 activity, but the remaining 4 patients had some apparent activity. This result was also consistent with previous data.[37] Conversely, the plasma from 4 patients with HUS produced a fragment of the substrate. Thus, the recombinant substrate, GST-VWF73-H, was confirmed to be a specific substrate for ADAMTS-13.

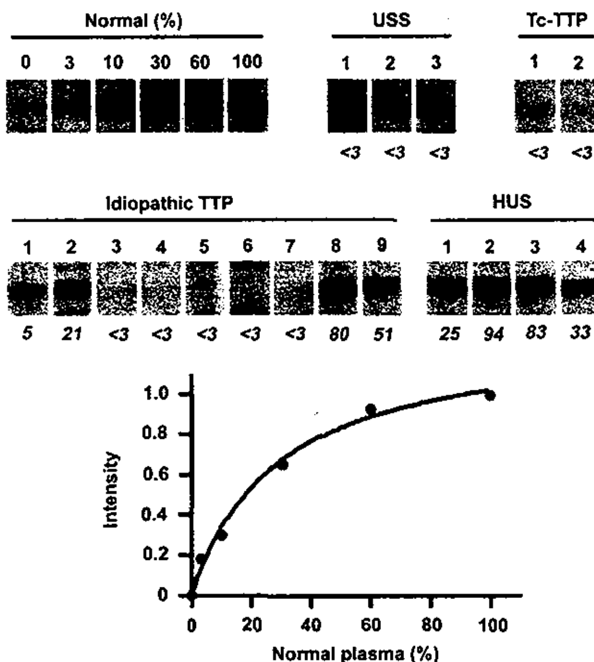

Figure 5. Cleavage of GST-VWF73-H by patients' plasma. GST-VWF73-H was incubated with serially diluted normal plasma (0%-100%) or with plasma from patients with USS, ticlopidine-associated TTP (Tc-TTP), idiopathic TTP, and HUS. The band intensities of reaction products were measured by chemiluminescence on Western blot, and the relative activities of patients' plasma (%, shown by italic numbers) to normal plasma were calculated from nonlinear regression by serially diluted normal plasma. USS patients 1 to 3 correspond to ST-III-4, SY-III-1, and KI-III-2 by previous report.[2] Tc-TTP patients 1 and 2 were reported by Sugio et al[35] and Orimo et al,[36] respectively. The idiopathic TTP patients 1 to 9 correspond to the case numbers 1-3, 5, 6, 11-13, and 17, and HUS patients 1 to 4 correspond to the case numbers 2, 4, 6, and 9 in the previous report.[37]

Inhibitors of ADAMTS-13 in plasma from patients

Most patients with acquired TTP have autoantibodies that inhibit ADAMTS-13 activity in their plasma.[19,20] No inhibitors are detected in plasma from patients with USS.[19] After incubation of normal plasma with plasma from the patients with USS or acquired TTP, the cleavage of GST-VWF73-H was examined (Figure 6). Preincubation with plasma from 3 patients with acquired TTP inhibited the cleavage of GST-VWF73-H, whereas preincubation with plasma from 3 USS patients had no effect. This indicates that the assay system using recombinant substrate VWF73 can be also useful to measure inhibitors of ADAMTS-13.

Discussion

Several assay methods have been reported to measure plasma ADAMTS-13 activity. The original method was developed by Furlan et al[31] and Tsai,[32] independently. They purified human VWF and incubated it with plasma in the presence of urea or guanidine-HCl as well as divalent cations such as Ba^{2+} and Ca^{2+}. Subsequently, Furlan et al separated the degraded material by SDS-agarose gel electrophoresis followed by Western blot using anti-VWF antibodies to detect a decrease in VWF-multimer ladders. Alternatively, Tsai separated the reaction materials by SDS-PAGE and detected the degraded products by Western blot. The former is visually attractive and sensitive, but time and skill are required. From an enzymologic viewpoint, the latter approach is superior in that it visualizes the product of the reaction, and not the disappearance of the substrate.

Gerritsen et al developed a different method based on the preferential binding of high–molecular-weight forms of VWF to collagen.[38] The proteolytic degradation of VWF leads to low-molecular-weight forms of VWF, which show impaired binding to microtiter plates coated with collagen. The collagen-bound VWF is quantified using antibodies against VWF. Obert et al reported an immunoradiometric assay using 2 site-directed monoclonal antibodies to VWF.[39] In this assay, the residual full-length VWF after proteolytic incubation was estimated by a sandwich enzyme-linked immunosorbent assay. Böhm et al recently reported a method based on the positive correlation between VWF multimeric size and Ristocetin cofactor activity.[40] After digestion of VWF with plasma, the residual cofactor activity of the samples was assessed to calculate the ADAMTS-13 activity of the samples. Although these assay methods may be more suitable for clinical applications because they require less time to complete, they provide only an indirect detection of the cleavage reaction compared with the original methods developed by Furlan et al[31] and Tsai.[32]

In the present study, we provide a new substrate for ADAMTS-13, VWF73, by which convenient clinical assays can be developed.

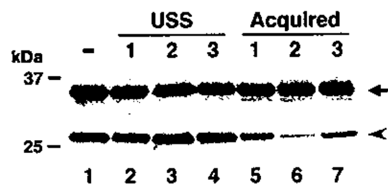

Figure 6. Inhibitory activity of plasma from patients. Normal plasma was preincubated with equal volumes of heat-inactivated plasma from patients with USS (lanes 2-4), Tc-associated TTP (lanes 5-6), and idiopathic TTP (lane 7). Then, cleavage of GST-VWF73-H was compared with normal plasma without preincubation (lane 1). Plasma from patients with acquired TTP but not with USS inhibited substrate cleavage.

Compared with the previous methods, VWF73 has several advantages. First, it is the only ADAMTS-13–specific substrate obtained by bacterial expression system. For an enzymatic assay to measure ADAMTS-13 activity, protease-free VWF should be purified from human plasma. To overcome this obstacle, the bacterial recombinant expression system is one of the most convenient alternative methods. Whole VWF, however, is not suitable because of its large size and many disulfide bonds. Therefore, short and soluble VWF73 will be a good molecule for this purpose. Second, VWF73 can be used with N- and C-terminal tag sequences, which are often used for convenient purification and detection. Here, we used both an N-terminal GST-tag and C-terminal H-tag for purification and the GST-tag for immunodetection. These tags could be used to develop a new assay system suitable for clinical usage. Third, no denaturing reagents such as urea or guanidine-HCl as used in the previous methods are needed to cleave VWF73 efficiently. To use whole VWF as a substrate, pretreatment with high concentrations of urea or guanidine-HCl and/or carrying out the proteolytic reaction in the presence of the denaturing reagents is required. VWF73 is efficiently cleaved by ADAMTS-13 in the absence of these reagents, therefore undesired damage on the enzyme can be avoided.

As far as we examined, no significant discrepancy in the plasma ADAMTS-13 activity could be found between assays using intact VWF multimers (multimer analysis) and recombinant VWF73. The discrepancies, however, could be found in the future, because the ADAMTS-13 mutants with different activity against intact VWF and VWF73 may be identified. Alternatively, the autoantibody inhibitors in acquired TTP patients might bind the protease and interfere with recognition of large VWF but not VWF73.

In general, a specific chromogenic assay for each protease is useful for routine clinical measurement. Therefore, trials to find a chromogenic oligopeptide substrate for ADAMTS-13 were carried out but were not successful,[41] suggesting that the cleavage at Y1605-M1606 of VWF depends on not only specific residues in the close vicinity of the scissile bond but also some more remote sequences in the VWF subunit. The present study was quite consistent with this assumption. VWF73 (D1596-R1668, substrate IV in Figure 1) was a good substrate for ADAMTS-13, whereas D1596-R1659 (substrate V) was not degraded, indicating that 9 residues between E1660 and R1668 contain essential residues for cleavage. This region may contribute to the structural preservation of the cleavage site for ADAMTS-13 or interact directly with the protease. This will be interesting from the viewpoint of the enzymology of metalloproteases. In order to further define the role of residues E1660 to R1668, we tested whether substrate V could be cleaved by normal plasma in the presence of 1 to 100 μM nonapeptide, EAPDLVLQRR (corresponding to E1660-R1668 of VWF), but substrate V was not still cleaved (data not shown). The nonapeptide also had no effect on the cleavage of VWF73, suggesting that the region may not bind directly to ADAMTS-13 but contribute to proper presentation of the cleavage site to ADAMTS-13.

Causative mutations of the *ADAMTS13* gene have been identified in patients with congenital TTP.[14-18] In addition, we identified a common missense single-nucleotide polymorphism, P475S, with approximately 5% allele frequency in the Japanese population.[15] When this mutant was transiently expressed in cultured cells, it was efficiently secreted from cells like the wild-type molecule but exhibited low VWF-cleaving activity. This suggested that approximately 10% of the Japanese population (heterozygotes of P475S) may possess significantly reduced activity of ADAMTS-13 with the normal antigen level. Other unknown common genetic variations or environmental factors might be involved in abnormal activity of ADAMTS-13. In these cases, enzymatic assays to measure the ADAMTS-13 activity will be more important than the measurement of the antigen levels. Although the almost complete loss of the ADAMTS-13 activity results in TTP, the weakened ADAMTS-13 activity may also be a risk factor for some thrombotic complications due to circulating large VWF multimers. In fact, a recent report suggested decreased levels of the ADAMTS-13 activity in coronary heart disease.[42] Well-designed and large-scale studies to assess the relation between ADAMTS-13 and disease will be one of the most important issues in this field.

In conclusion, we here identified the minimal specific substrate for ADAMTS-13, VWF73, which could be a powerful tool to establish clinical enzymatic assays. We strongly hope that it will be widely used and contribute to improving the prognosis and prevention of TTP.

Acknowledgment

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Molecular characterization of *ADAMTS13* gene mutations in Japanese patients with Upshaw-Schulman syndrome

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We report here 7 new mutations in the *ADAMTS13* gene responsible for Upshaw-Schulman syndrome (USS), a catastrophic phenotype of congenital thrombotic thrombocytopenic purpura, by analyzing 5 Japanese families. There were 3 mutations that occurred at exon-intron boundaries: 414+1G>A at intron 4, 686+1G>A at intron 6, and 1244+2T>G at intron 10 (numbered from the A of the initiation Met codon), and we confirmed that 2 of these mutations produced aberrantly spliced messenger RNAs (mRNAs). The remaining 4 mutations were missense mutations: R193W, I673F, C908Y, and R1123C. In expression experiments using HeLa cells, all mutants showed no or a marginal secretion of ADAMTS13. Taken together with the findings in our recent report we determined the responsible mutations in a total of 7 Japanese patients with USS with a uniform clinical picture of severe neonatal hyperbilirubinemia, and in their family members, based on *ADAMTS13* gene analysis. Of these patients, 2 were homozygotes and 5 were compound heterozygotes. The parents of one homozygote were related (cousins), while those of the other were not. Molecular models of the metalloprotease, fifth domain of thrombospondin 1 (Tsp1-5), and Tsp1-8 domains of ADAMTS13 suggest that the missense mutations could cause structural defects in the mutants. (Blood. 2004;103:1305-1310)

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disorder, and its diagnosis is made according to the criteria of Moschcowitz's pentad[1]: thrombocytopenia, microangiopathic hemolytic anemia (MAHA), fluctuating neurologic signs, renal failure, and fever. These criteria, however, are almost undistinguishable from those of hemolytic-uremic syndrome (HUS) with Gasser's triad[2]; MAHA, thrombocytopenia, and renal insufficiency. Thus, the comprehensive term "TTP/HUS" or "thrombotic microangiopathy"[3] has frequently been used in clinical practice.

Recent advances in elucidating the proteolytic processing of plasma von Willebrand factor (VWF) multimers have established assays for the activity of VWF-cleaving protease and its inhibitor (autoantibody).[4-7] These assays have largely made it possible to distinguish TTP from HUS, because the former has defective VWF-cleaving activity, whereas the latter has VWF-cleaving activity.[6,7] Studies by several groups of investigators have led to the identification of this enzyme as a new metalloprotease belonging to the ADAMTS (a disintegrinlike and metalloprotease with thrombospondin type 1 motif) family, which has been designated ADAMTS13.[8-12] This enzyme is produced in the liver.[10-12] The deduced amino acid residue number is 1427, and the gene contains 29 exons and is located on chromosome 9q34.[10-12]

Upshaw-Schulman syndrome (USS) was originally reported as a disease complex with repeated episodes of thrombocytopenia and hemolytic anemia that quickly respond to infusions of fresh frozen plasma (FFP).[13-16] Clinical signs often develop in the patients during the newborn period or early infancy. In fact, the earliest and most frequently encountered clinical manifestation is severe hyperbilirubinemia with negative Coombs test soon after birth, which requires exchange blood transfusions. Pediatric hematologists have long been more familiar with this disease than general physicians, but this diagnostic name appears to be lacking or ignored in most medical textbooks because of its uncertain clinical entity. Thus, a variety of alternative nomenclatures has been given to this disease, such as chronic relapsing TTP, congenital MAHA, and familial TTP/HUS.

Under these circumstances, the report of Furlan et al[17] in 1997 showing that 4 cases of chronic relapsing TTP lacked ADAMTS13 activity was notable. Of these cases, 2 were siblings with a

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consistent deficiency of the enzyme activity, suggesting that they had an inheritable form, whereas the other 2 were unrelated and apparently had an acquired form. Since then, there have been several reports on TTP/HUS associated with congenital deficiency of ADAMTS13 activity, and, accordingly, the diagnostic name of USS has been re-evaluated and has gained a position as an independent disease entity. Usually, parents of USS patients have reduced plasma ADAMTS13 activity, roughly half of the normal control level, indicating that they are asymptomatic carriers.[15] Among them, one father detected in our laboratory was particularly interesting because he had an extremely low level of plasma ADAMTS13 activity, 4.5% to 7% of the normal control level on 3 different occasions, but so far, at the age of 36, he has no apparent clinical signs.[15] The unusually low level of ADAMTS13 activity in this man was further confirmed by both genetic and biochemical analyses,[18] that is, he was compound heterozygote, R268P/P475S. In expression analysis, the R268P mutant was not secreted from the cells, but the P475S mutant was secreted normally and showed low but significant activity. The P475S mutation is prevalent in the Japanese population (heterozygosity ~ 10%). Thus, the discovery of new *ADAMTS13* mutations in different countries or races appears to be very important for the analysis of a potential linkage with other thrombotic generic risks.

Here, we identified 7 new mutations in the *ADAMTS13* gene responsible for USS by analyzing 5 patients and 16 relatives belonging to 5 different families from widely separated regions in Japan. All the patients had an episode of severe hyperbilirubinemia during the newborn period, and received exchange blood transfusions, except for one. Furthermore, structural changes of the mutant ADAMTS13 molecules associated with impaired enzyme activity were predicted using a homology modeling method.

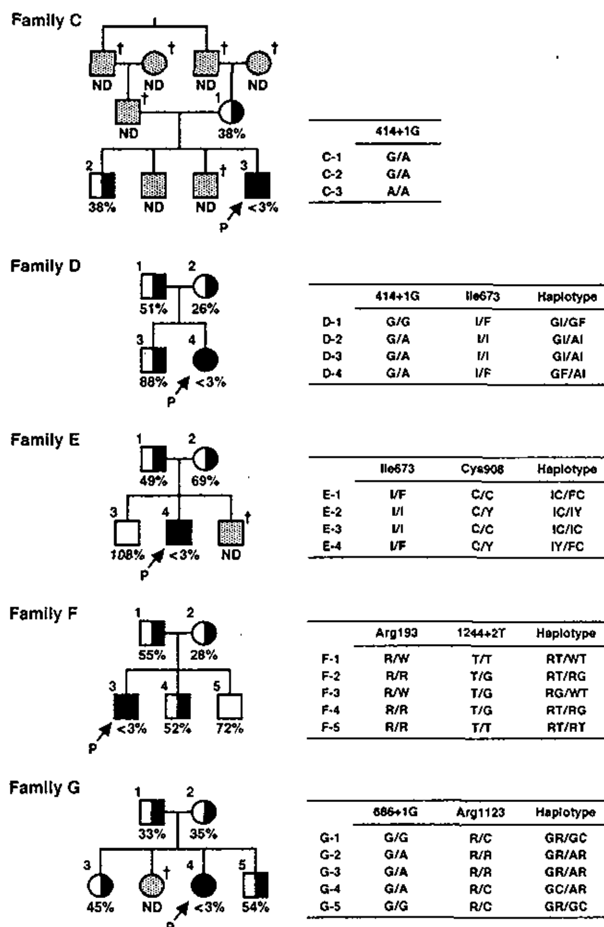

Figure 1. Pedigree and haplotypes of patient families. Squares and circles indicate males and females, respectively. Closed circles and squares with arrows with a P indicate probands. The half-closed circles and squares represent asymptomatic carriers. The cross indicates deceased. The ADAMTS13 activity is shown as a percentage of the normal control. ND indicates not determined. Mutations found in the *ADAMTS13* gene are shown as one-letter amino acid abbreviations numbered from the initial Met or as nucleotides numbered from the A of the translation initiation Met codon.

Patients, materials, and methods

Families A to G with USS

Families C to G are shown in Figure 1.

Families A and B. There were 2 USS probands, A and B, belonging to different families described and characterized in detail in a recent publication.[18]

Family C. Proband C is a male born in Fukui in 1972 whose history during childhood was reported in 1984.[19] His parents are cousins. His father died of cerebral infarction at 63 years of age, and the third brother died of melena soon after birth. His mother and 2 other brothers have no thrombotic or hemorrhagic signs. Proband C showed hyperbilirubinemia during the newborn period, and he received phototherapy for 3 days without exchange blood transfusion. Thereafter, he had repeated episodes of thrombocytopenia and hemolytic anemia, and he has received prophylactic infusion of 2 units (160 mL) of FFP every 2 to 4 weeks since he was 8 years old. However, he has gradually developed chronic nephritis and was required to receive continuous ambulatory peritoneal dialysis starting in March 1995. Because of repeated peritonitis associated with continuous ambulatory peritoneal dialysis, however, his therapy for renal insufficiency was completely switched to hemodialysis starting in May 1999.

Family D. Proband D is a female born in Yamaguchi in 1978 whose history until the age of 4 years was reported in 1982.[20] Her parents are unrelated. She had an episode of severe hyperbilirubinemia soon after birth that required 2 exchange blood transfusions. Since 4 years of age, she received 1 unit (80 mL) of FFP every 3 weeks until the age of 21 years. Under this treatment regimen, however, her renal function test, including serum creatinine level, was getting worse, and therefore the volume of FFP infused was increased stepwise. She now receives 5 units (400 mL) of FFP infusion every 2 weeks for prophylaxis.

Family E. Proband E is a male born in Kagawa in 1985. He is the second child of unrelated parents. His younger brother with Down syndrome died of an unknown cause soon after birth. His parents and elder brother are apparently healthy. This proband developed severe hyperbilirubinemia and thrombocytopenia in the next day after birth. He received 2 exchange blood transfusions on the second day of life, resulting in excellent clinical improvement. At 5 years of age, he had an episode of thrombocytopenia and hemolytic anemia. Such clinical manifestations quickly improved after 2 plasma exchanges, together with the correction of abnormal laboratory test results that confirmed a clinical diagnosis of USS. After that, he receives 2 to 3 units (160-240 mL) of FFP infusion upon episodes of thrombocytopenia and hemolytic anemia.

Family F. Proband F is a male born in Aomori in 1993. He is the first child of unrelated parents. His parents and brothers are all apparently healthy. Soon after birth, this proband developed severe hyperbilirubinemia and received 2 exchange blood transfusions. At 10 months after birth, he had generalized petechiae with hemolytic anemia and thrombocytopenia. Therefore, he was once diagnosed with idiopathic thrombocytopenic purpura. At 2 ½ years of age, he received 1 unit (80 mL) of FFP infusion for the aforementioned clinical signs, which dramatically improved both his clinical and laboratory findings, resulting in a clinical diagnosis of USS. Now, he receives 1 unit of FFP infusion for each of his occasional hemolytic crises.

Family G. Proband G is a female born in Tokyo in 1987. She is the third child of unrelated parents. One elder sister had an episode of severe

hyperbilirubinemia soon after birth and received exchange blood transfusion. She died of intracranial bleeding after a traffic accident at 8 years old. Her parents, remaining elder sister, and brother are all apparently healthy. Soon after birth the proband developed severe hyperbilirubinemia and received exchange blood transfusion. Thereafter, she had repeated episodes of thrombocytopenia and hemolytic anemia that were quickly improved by FFP infusion. Finally, at 14 years of age, she was clinically diagnosed with USS. Now, she receives 10 mL/kg FFP infusion for each of her occasional hemolytic crises.

Assays of ADAMTS13 activity

Plasma ADAMTS13 activity was assayed by the method of Furlan et al[4,6] based on VWF multimer analysis, with a slight modification as described.[15] The ADAMTS13 activity of pooled normal plasma was defined as 100%. The normal range of ADAMTS13 activity (n = 60; 30 women and 30 men, 20-39 years of age) was 102 ± 23% (mean ± 1 SD).[21]

Sequencing of the *ADAMTS13* gene

All DNA experiments were performed with the permission of the ethics committees of both the sample-collecting hospital and the gene-analyzing institute. Amplification and sequencing of the 29 exons of the *ADAMTS13* gene were performed as recently described.[18]

Transient expression of ADAMTS13

Polymerase chain reaction (PCR)–based mutagenesis was performed for the construction of ADAMTS13 mutants as recently reported.[18] The mutant cDNA was cloned into mammalian expression vector, pCAGG.[22] The DNA sequence of all inserts was confirmed by DNA sequencing.

Each of the expression vectors was transfected into HeLa cells using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's instructions. Briefly, HeLa cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) in humidified air with 5% CO_2 at 37°C. Of each expression plasmid, 5 μg was transfected into subconfluent cells in 90-mm dishes. After 4 to 6 hours of incubation, the medium was changed to 4 mL serum-free OPTI-MEM I (Invitrogen), and the cultures were incubated for 44 hours. The media were collected and the culture media were concentrated to one tenth the original volume. The cells were washed with phosphate-buffered saline, pH 7.4, and lysed with 300 μL sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris [tris(hydroxymethyl)aminomethane]–HCl/2% SDS/50 mM dithiothreitol/10 mM ethylenediaminetetraacetic acid/0.02% bromophenol blue/6% glycerol, pH 6.8).

Western blot analysis

The media and cell lysates were first separated by SDS–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA). After blocking with 3% skim milk, the membrane was incubated with 1 μg/mL anti-FLAG (fludarabine, cytarabine, and granulocyte colony-stimulating factor) M2 monoclonal antibody (Sigma, St Louis, MO) and then 0.1 μg/mL peroxidase-labeled goat antimouse immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Luminographic detection was performed using the Western Lighting Chemiluminescence Reagent (PerkinElmer Life Sciences, Shelton, CT) and detected using an image analyzer LAS 1000 plus (Fujifilm, Tokyo, Japan).

Reverse-transcription PCR

To evaluate the transcription products of the gene with a single nucleotide mutation in introns 4 and 10, we performed reverse transcription (RT)–PCR on mRNA using primers corresponding to the respective exons. RT-PCR for intron 4 using sense primer GGGCAGAACTGCTTCGGGACC (exon 4) and antisense primer AGCATGGCCAGGATCCGTGTC (exon 5) yields a 185–base pair (bp) band from the normally spliced transcript or a 510-bp band from the unspliced transcript. RT-PCR for intron 10 using sense primer GGGTCCCCGAAGTCCTTGCTC (exon 10) and antisense primer AGGTCAGCACCAACACATGCA (exon 11) yields a 115-bp band from the spliced transcript or a 226-bp band from the unspliced transcript. The mRNAs were prepared using a PAXgene Blood RNA system (Qiagen, Valencia, CA), and RT-PCR was performed using Qiagen OneStep RT-PCR (Qiagen).

Construction of 3-dimensional models of metalloprotease, Tsp1-5, and Tsp1-8 domains of ADAMTS13

Model structures of the wild-type (WT) metalloprotease domain, and fifth and eighth domains of thrombospondin 1 (Tsp1-5 and Tsp1-8 domains) were constructed based on homology modeling methods. Searching for the reference proteins and sequence alignments was performed using position-specific iterated (PSI)–BLAST.[23] The reference proteins were adamalysin II (PDB[24] ID: 4AIG[25]) for the metalloprotease domain and thrombospondin-1 type 1 repeats (PDB ID: 1LSL[26]) for Tsp1-5 and Tsp1-8 domains. The sequence alignments produced using PSI-BLAST were manually adjusted taking biologically important regions and secondary structure into consideration using the CHIMERA modeling system.[27,28] The model structures were constructed using the fully automatic modeling system FAMS[29,30] based on each alignment.

Results

ADAMTS13 activity and ADAMTS13 inhibitor in patients with USS and their families

The family pedigrees of 5 patients and their ADAMTS13 activity are shown in Figure 1. The plasma ADAMTS13 activities of all probands were less than 3% of the normal control value. The low activity (<3%) of ADAMTS13 in the proband was confirmed using the plasmas of at least 2 different occasions, with an interval of more than 6 months. The ADAMTS13 activity, which was less than 3% of the normal, of 2 other USS patients A and B in 2 different families was described in our previous paper.[17] Neither the patients' nor their relatives' plasmas contained detectable inhibitor of ADAMTS13 activity (<0.5 Bethesda unit/mL).

The earliest uniform clinical picture of these 7 patients was severe-to-moderate hyperbilirubinemia during the newborn period that required exchange blood transfusion, except in one case (patient C), who was treated with phototherapy. After the clinical diagnosis of USS was made, all patients were treated with a prophylactic infusion of 5 to 10 mL of FFP/kg at 2- to 3-week intervals or with FFP infusion when they had clinical manifestations.

ADAMTS13 gene mutations in patients with USS and their families

We analyzed the *ADAMTS13* gene in 5 patients with USS and 21 of their family members by PCR amplification and sequencing of the 29 exons of the gene. Single-nucleotide mutations at 12 sites were identified (Table 1). There were 4 silent mutations identified: 420T>C, 1716G>A, 2280C>T, and 4221C>A, and all of these mutations have been reported as single nucleotide polymorphisms (SNPs).[12,18] We identified 8 additional mutations in the 5 families. Of 8 mutations, 5 found in the exons were missense mutations: 577C>T (R193W), 1342C>G (Q448E), 2017A>T (I673F), 2723G>A (C908Y), and 3367C>T (R1123C). Of these, 1342C>G (Q448E) has been reported as an SNP.[12,18] The remaining 3 mutations were found in introns at +1 or +2 nucleotides from the exon-intron boundary, and these mutations in introns appeared to render the gene unable to produce a normally spliced transcript.

To investigate the frequencies of the 7 mutations newly identified in this report, we performed sequencing of exons 4, 6, 10, 17, 21, and 25 of the *ADAMTS13* gene in genomic DNAs isolated

Table 1. *ADAMTS13* mutations in 5 USS families

Exon	Family	Nucleotide	Amino acid
Intron 4	C, D	414+1G>A	Splice
5	F	420T>C*	Silent
6	F	577C>T	R193W
Intron 6	G	686+1G>A	Splice
Intron 10	F	1244+2T>G	Splice
12	F, G	1342C>G*	Q448E
15	F, G	1716G>A*	Silent
17	D, E	2017A>T	1673F
19	D, E, F, G	2280C>T*	Silent
21	E	2723G>A	C908Y
25	G	3367C>T	R1123C
29	F, G	4221C>A*	Silent

*Reported by Levy et al[12] and Kokame et al.[18]

from 96 Japanese individuals without TTP. They were all patients of the Division of Hypertension and Nephrology at the National Cardiovascular Center (Suita, Japan). None of these 7 mutations appeared in this panel, indicating that they were rare (<1% heterozygosity) in the Japanese population.

Expression of rADAMTS13 mutants

To determine the effects of the newly identified mutations, WT and mutant forms of recombinant ADAMTS13 (rADAMTS13) were transiently expressed in HeLa cells. The expression of WT rADAMTS13 produced a single immunoreactive band with a molecular mass of about 230 kDa in the culture medium (Figure 2, top). The band was not detected in the medium of untransfected cells. In the culture medium of cells expressing the R193W mutant, a weak band was detected with the same size as WT. The expression level of the R193W mutant was roughly estimated as one fourth of WT by comparing their chemiluminescent intensities on Western blot. On the other hand, no bands were detected for the I673F, C908Y, and R1123C mutants, although these mutants were synthesized within the cells (Figure 2, bottom). These results clearly indicated that these mutants are not secreted normally.

The enzymatic activity of rADAMTS13 was determined by analyzing the degradation of VWF multimers.[18] In the medium of untransfected cells, the ladders of VWF multimers extended into the high-molecular-weight area (Figure 3, untransfected), indicating the lack of ADAMTS13 activity in the medium. In contrast, the ladder diminished after incubation with the medium of transfected cells expressing WT rADAMTS13, indicating that WT has ADAMTS13 activity. This activity was also observed in the 1:4- and 1:10-diluted WT. As for the R193W mutant, no enzymatic activity was observed (Figure 3), indicating that the R193W mutant had little activity.

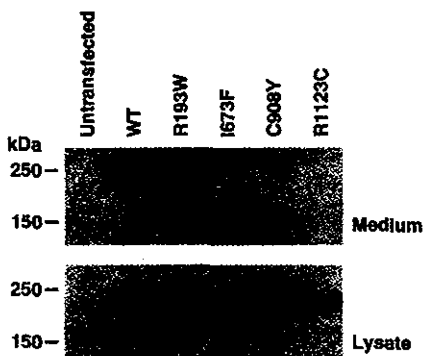

Figure 2. Expression of rADAMTS13. The wild-type (WT) rADAMTS13 and mutants with C-terminal FLAG-tag were transiently expressed in HeLa cells. The culture media (top) and cell lysates (bottom) were analyzed by Western blotting with an anti-FLAG antibody. The sizes of protein standards are indicated at the left.

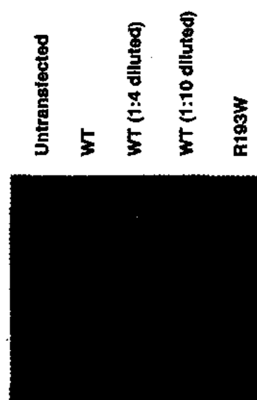

Figure 3. Cleavage of VWF multimer by rADAMTS13. The rADAMTS13 activity was measured by degradation of VWF multimers. The culture media of untransfected, WT, and the R193W mutant were incubated with purified VWF. The multimeric state of VWF was determined by Western blot analysis after SDS–agarose gel electrophoresis.The 1:4 diluted WT medium contained the enzyme amount roughly equivalent to the medium of the R193W mutant.

Aberrant splicing caused by mutations at exon-intron boundary

To test the effects of the mutations in introns 4 and 10, we performed RT-PCR using exon primers. Since no bands corresponding to the RT-PCR products were detected without the reverse-transcription reaction in normal control samples, the possibility that bands were generated due to the contaminating genomic DNA was excluded (Figure 4, lane N(-RT)).

For the 414+1G>A mutation in intron 4, we examined mRNA from 6 individuals: healthy control (N), C-3 (homozygote for the mutation), D-1 (no mutation), and D-2 to D-4 (heterozygotes) (Figure 4, top). In N and D-1, only one band of 185 bp corresponding to the normally spliced product was detected. In D-2 to D-4, a 510-bp band corresponding to unspliced transcript was found together with the 185-bp band derived from the spliced transcript. In contrast, in C-3, only one band of 510 bp was found. Thus, it was clear that the mutation 414+1G>A abolished the splicing at the exon 4–intron 4 boundary.

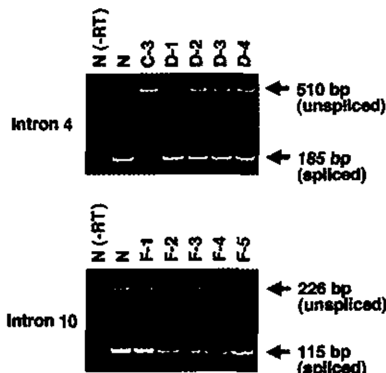

Figure 4. Effects of intronic mutations on mRNA splicing. To evaluate the products of genes with a single splice mutation in intron 4 (top) or intron 10 (bottom), we performed RT-PCR on mRNA using primers corresponding to these exons. RT-PCR for intron 4 yielded a 185-bp band from the normally spliced transcript and a 510-bp band from the unspliced transcript. RT-PCR for intron 10 yielded a 115-bp band from the spliced transcript and a 226-bp band from the unspliced transcript.

For the 1244+2T>G mutation in intron 10, we similarly performed RT-PCR on mRNA from 6 individuals: N, F-1 and F-5 (no mutation), and F-2 to F-4 (heterozygotes) (Figure 4, bottom). In this experiment, we were unable to obtain a sample of a homozygote for the mutation because the proband with this mutation was compound heterozygote. As a result, the product from all 6 individuals had 2 bands of 115 bp derived from the spliced transcript and 226 bp derived from the unspliced transcript. However, it is clear that the intensity of the 115-bp band of F-1 and F-5 was much denser than that of the 226-bp band. In contrast, the intensity of the 115-bp band of F-2 to F-4 was much weaker than that of N, F-1, and F-5. Based on these results, intron 10 appears to be not completely spliced even in healthy individuals, suggesting that in healthy individuals alternative splicing occurred at exon 10–intron 10 boundary or the splicing of this boundary had low efficiency. Furthermore, the transcripts of heterozygous individuals are likely to be less spliced than those of healthy individuals. Thus, the mutation of 1244+2T>G abolishes splicing at the exon-intron boundary.

Molecular modeling of the metalloprotease and Tsp1 domains of ADAMTS13

We constructed 3-dimensional molecular models of the metalloprotease and Tsp1 domains in which the mutations were identified. There are 2 missense mutations located in the metalloprotease domain: R193W identified in the present study and R268P identified in our previous study.[18] The R193W mutation is located close to the active site. Even if the mutant protein is secreted, the mutation is predicted to disturb the activity, as shown in Figures 2-3. The R268P mutation is located in the middle of an α-helix. It is well known that proline is an α-helix breaker. Therefore, it can be predicted that the R268P substitution would cause a secretion defect due to the disruption of one of the α-helices in the molecule.

The C908Y and R1123C mutations are present in the Tsp1-5 and Tsp1-8 domains, respectively (Figure 5B and 5C, respectively). The C908Y mutation may disrupt a potential disulfide bond and the R1123C mutation may create the mixed disulfide bond; both of them could disrupt the proper conformation of the enzyme, leading to the secretion defect.

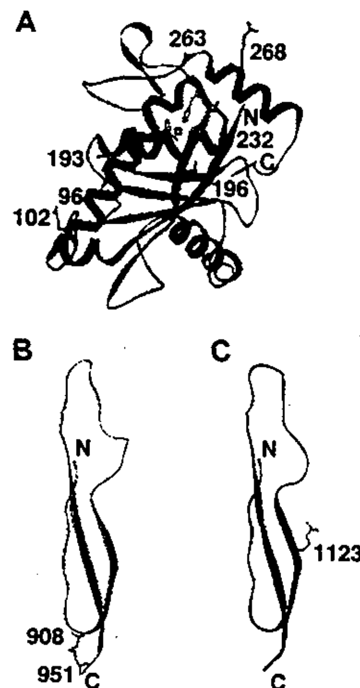

Figure 5. Shown are 3-dimensional models of metalloprotease, Tsp1-5, and Tsp1-8 domains of ADAMTS13. (A) Metalloprotease domain. The side chains of Arg193 and Arg268 are shown in blue with residue numbers. The active site zinc ion coordinated by His224, His228, and His234 is also shown (green). The mutations of H96D, R102C, T196I, L232Q, and S263C reported by Levy et al[12] and Schneppenheim et al[31] are present in the metalloprotease domain. The side chains of His96, Arg102, Thr196, Leu232, and Ser263 are also shown in blue. (B) Tsp1-5 domain. The side chains of Cys908 and Cys951 are shown in red with residue numbers. (C) Tsp1-8 domain. The side chain of Arg1123 is shown in red with residue number.

Discussion

Here, we have identified 7 new mutations in the *ADAMTS13* gene responsible for USS, including 4 missense mutations (R193W, I673F, C908Y, and R1123W) and 3 splice site mutations. We have reported 2 missense mutations (R268P and C508Y) and one nonsense mutation (Q449X).[18] All 6 missense mutant proteins, except for the R193W mutant, were not secreted from the cells in the expression analysis using HeLa cells. Although the R193W mutant was secreted to some extent, it had little activity. This mutation may not cause a dramatic conformational change of the enzyme but may disrupt the active site locally.

To date, the analysis of the *ADAMTS13* gene was reported in a total of 26 patients with congenital TTP, equivalent to USS, including those in the present study.[12,18,31-33] All of the patients with congenital TTP have been shown to have *ADAMTS13* gene mutations. Of these patients, 7 were homozygotes and 19 were compound heterozygotes. It is noteworthy that these mutation sites are distributed over exons 3 to 29 regardless of race. Among our 7 patients with USS, 2 are homozygotes, of whom 1 (patient C) has a family history of consanguineous marriage, and the other (patient B) appears not to have such a history. However, a careful routing analysis performed in this study revealed that 2 great-grandparents of patient B were from the same village in the Northeastern region of the Japanese mainland at the end of the 19th century. More interestingly, one Japanese patient with USS reported by Sasahara et al[34] has the same homozygous mutation (Q449X) as our patient B (personal written communication from Drs David Ginsburg and Shigeru Tsuchiya, May 2003). This patient is a natural habitant of the same Northeastern region of Japan as patient B's ancestors. Our experience suggests that among USS patients consanguinity gives rise to the minor group with homozygous mutation of the *ADAMTS13* gene, whereas the major group consists of individuals with compound heterozygous mutations not arising from consanguinity.

The most striking and earliest clinical sign of USS is a Coombs-test–negative severe hyperbilirubinemia soon after birth. In fact, all of our 7 patients had this history, and 6 of 7 were rescued by exchange blood transfusion and 1 by phototherapy. As noted by others[35] and by us,[16] some patients with USS, despite a severe lack of ADAMTS13 activity, have no acute episode of TTP/HUS during the newborn period, but develop the episode during early or late childhood. In some very unusual female cases, it has been said that the clinical manifestation is first noted after pregnancy.[35] This is perhaps triggered by an increased amount of VWF together with a higher ratio of ultralarge or large VWF multimers than in the nonpregnant state. However, it has not been clarified whether this phenomenon is caused by overproduction of VWF or by a physiologic decrease of ADAMTS13 activity during the second and third trimesters of normal pregnancy, and/or by both. At present we cannot address why phenotypic differences on the clinical onset or signs exist. Besides pregnancy, it has been

speculated that some other precipitating factors may include infections, cytokines, or chemical compounds.

In the present study, we constructed models of the metalloprotease domain, and Tsp1-5 and Tsp1-8 domains of ADAMTS13 using the coordinates of adamalysin II and the thrombospondin-1 type 1 repeats, respectively (Figure 5). The sequence alignments are highly important for obtaining the proper models. In the present study, the sequence alignments produced using PSI-BLAST were manually adjusted taking biologically important regions and secondary structure into consideration. Thus, the sequence identities of 22.1%, 28.8%, and 30.8% were obtained between the metalloprotease domain and adamalysin II, the Tsp1-5 domain and thrombospondin-1 type 1 repeats, and the Tsp1-8 domain and thrombospondin-1 type 1 repeats, respectively. Based on our experiences, the models constructed with the amino acid sequence identities higher than 20% are quite convincing. However, we did not construct the models of the cysteine-rich and spacer domains because their reference proteins were not obtained due to their unique sequences. In addition, the domain-domain interactions presumably present in the ADAMTS13 molecule are not predicted. Thus, the structure determined by the X-ray crystallography remains to be solved.

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