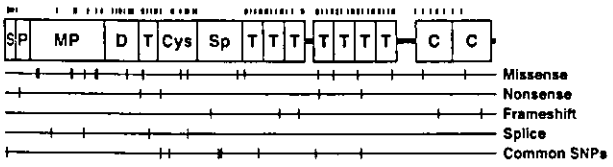

Figure 1. ADAMTS-13 mutations. The mutated sites responsible for hereditary TTP and common SNPs are shown below the domain structures of human ADAMTS-13. All cysteine residues are shown above. S, signal peptide; P, propeptide; MP, metalloprotease domain; D, disintegrin-like domain; T, TSP1 motif; Cys, cysteine-rich domain; Sp, spacer domain; C, CUB domain.

VWF cleavage.[25,26] Enzymatic analysis of these mutants showed that removal of the cysteine-rich/spacer domains caused loss of proteolytic activity towards VWF. The more C-terminal TSP1 and CUB domains were dispensable for VWF-cleaving activity, at least in vitro.

Thirty-three Mutations Responsible for TTP

To date, 33 mutations responsible for hereditary TTP have been identified in the *ADAMTS13* gene[19,27-30] (Table 1 and Fig 1). Five result in frameshift deletions or insertions and four are splice mutations. The remaining 24 mutations lead to codon changes, 19 of which are nonconservative missense and five of which are nonsense mutations. The mutated sites in *ADAMTS13* are distributed across many exons and introns throughout the gene, which spans approximately 37 kb on chromosome 9q34. The absence of clusters of mutations within the metalloprotease domain implies structural and functional importance of other regions.

Missense Mutations

Nineteen missense mutations have been identified: H96D, R102C, R193W, T196I, L232Q, S263C, R268P, P353L, R398H, C508Y, R528G, I673F, R692C, C908Y, C951G, C1024G, R1123C, C1213Y, and R1336W. Nine mutations are substitutions to/from cysteine residues, suggesting that disulfide-bond formation is important for proper conformation and function.

Among these, only six mutations were characterized by expression analysis.[27,31] We made mammalian expression plasmids for the R193W, R268P, C508Y, I673W, C908Y, and R1123C mutants, as well as wild-type ADAMTS-13, and then transfected them into cultured HeLa cells. A FLAG-tag sequence was added to the C terminus to aid in immunochemical detection. Transient expression of wild-type ADAMTS-13 produced a single immunoreactive band in culture medium, whereas none of the mutants were secreted, except for the R193W mutant, which was secreted at very low levels. Therefore, these six mutations must affect some aspect of the secretory pathway, possibly causing changes in protein folding and stability. The majority of the 19 identified missense mutations may cause secretory problems, similar to those in these five mutations.

Nonsense Mutations

Five nonsense mutations have been found: Q44X, W390X, Q449X, R910X, and R1034X. Among these, only one mutation, Q449X, was characterized by expression analysis.[26,27] The Q449X mutant was secreted efficiently like wild-type ADAMTS-13, but showed little cleavage activity towards VWF multimers.

As described above, the ADAMTS-13 spacer region is necessary for normal enzymatic activity. Since the W390X and Q449X mutants do not contain the spacer region, they would not possess VWF-cleaving activity, even if they are secreted. Alternatively, mRNAs derived from these nonsense mutant *ADAMTS13* genes may be destroyed by the nonsense-mediated decay (NMD) pathway (mRNA surveillance), which

Table 1. *ADAMTS13* Gene Mutations Responsible for Hereditary TTP

Exon/ Intron	Nucleotide	AA	Domain	Reference
Ex. 2	130C→T	Q44X	Propeptide	29
Ex. 3	286C→G	H96D	Metalloprotease	19
Ex. 3	304C→T	R102C	Metalloprotease	19
Int. 4	414+1G→A	Splice	Metalloprotease	31
Ex. 6	577C→T	R193W	Metalloprotease	31
Ex. 6	587C→T	T196I	Metalloprotease	19
Int. 6	686+1G→A	Splice	Metalloprotease	31
Ex. 7	695T→A	L232Q	Metalloprotease	28
Ex. 7	788C→G	S263C	Metalloprotease	28
Ex. 7	803G→C	R268P	Metalloprotease	27
Ex. 9	1058C→T	P353L	Disintegrin-like	28
Ex. 10	1169G→A	W390X	Tsp1-1	28
Ex. 10	1193G→A	R398H	Tsp1-1	19
Int. 10	1244+2T→G	Splice	Tsp1-1	31
Ex. 12	1345C→T	Q449X	Cysteine-rich	27
Ex. 13	1523G→A	C508Y	Cysteine-rich	27
Ex. 13	1582A→G	R528G	Cysteine-rich	19
Int. 13	1584+5G→A	Splice	Cysteine-rich	19
Ex. 15	1783delTT	Frameshift	Spacer	30
Ex. 17	2017A→T	I673F	Spacer	31
Ex. 17	2074C→T	R692C	Tsp1-2	19
Ex. 19	2376del26	Frameshift	Tsp1-3	19
Ex. 20	2549delAT	Frameshift	Tsp1-4	28
Ex. 21	2723G→A	C908Y	Tsp1-5	31
Ex. 21	2728C→T	R910X	Tsp1-5	28
Ex. 22	2851T→G	C951G	Tsp1-5	19
Ex. 24	3070T→G	C1024G	Tsp1-7	19
Ex. 24	3100A→T	R1034X	Tsp1-7	28
Ex. 25	3367C→T	R1123C	Tsp1-8	31
Ex. 26	3638G→A	C1213Y	CUB1	19
Ex. 27	3769insT	Frameshift	CUB1	19
Ex. 28	4006C→T	R1336W	CUB2	29
Ex. 29	4143insA	Frameshift	CUB2	28

Table 2. Mutated Alleles of Each Patient With Hereditary TTP

Patient	Allele 1	Allele 2	Reference
A1	R692C	R692C	9
A2	H96D	C951G	19
A3	R528G	Frameshift (3769insT)	19
A4	R398H	C1024G	19
A5	R102C	T196I	19
A6	Frameshift (2376del26)	C1213Y	19
A7	Splice (1584+5G→A)	ND	19
B1	R268P	C508Y	27
B2	Q449X	Q449X	27
C1	S263C	Frameshift (4143insA)	28
C2	P353L	R910X	28
C3	R1034X	Frameshift (4143insA)	28
C4	L232Q	L232Q	28
C5	W390X	Frameshift (2549delAT)	28
C6	P353L	Frameshift (4143insA)	28
C7	R910X	Frameshift (4143insA)	28
D1	Q44X	R1336W	29
E1	Frameshift (1783delTT)	Frameshift (1783delTT)	30
F1	Splice (414+1G→A)	Splice (414+1G→A)	31
F2	Splice (414+1G→A)	1673F	31
F3	1673F	C908Y	31
F4	R193W	Splice (1244+2T→G)	31
F5	Splice (686+1G→A)	R1123C	31

is one of the quality-control mechanisms that ensure high fidelity of gene expression. The NMD pathway destroys aberrant mRNAs that contain premature termination codons.[32,33] Therefore, ADAMTS-13 mutants with nonsense mutations may not be translated in vivo.

Frameshift Mutations

Five frameshift mutations have been identified: 1783delTT, 2376del26 (2376-2401del), 2549delAT, 3769insT, and 4143insA. If these mutated forms of ADAMTS-13 were to be translated, truncated mutants with aberrant C-terminal ends would be expressed. Each mutation replaces L595-T1427 with GGEDRRALCRGWEDEHLP, A793-T1427 with PALPCQVGGVRAQLMHISWWSRPGLGERDLCARGRWPGGSSD, D850-T1427 with GEAACP, L1258-T1427 with VGHDFQLQDQHAGGEAALRAARRWGAAAVWEPACS, or E1382-T1427 with REQPG, respectively. Some of these forms may not be secreted, and others may be secreted but dysfunctional. However, like the nonsense mutations described above, mRNAs with these frameshift mutations might be eliminated by gene expression quality-control systems.

Splice Mutations

Four splice mutations have been found: 414+1G→A, 686+1G→A, 1244+2T→G, and 1584+5G→A. The 414+1G→A (intron 4), 686+1G→A (intron 6), and 1244+2T→G (intron 10) mutations cause inconsistencies with the GT-AG rules of mRNA splicing. The 1584+5G→A (intron 13) mutation also alters the donor splice site from the consensus sequence. In fact, the effect of each of these mutations on mRNA splicing was examined experimentally. Total RNAs were isolated from patients' lymphoblasts or whole blood cells and used as templates for reverse-transcriptase polymerase chain reaction (RT-PCR). The data indicated that little or no normally spliced products were generated from the mutant alleles.[19,31]

Frequency of TTP-Causing Mutations

All 33 mutations were excluded as common sequence polymorphisms by screening a large panel of unaffected chromosomes. However, nine of these mutations occur on several occasions in 23 TTP patients (Table 2). Notably, this multiplicity occurs within the subjects of each investigator group. This suggests that *ADAMTS13* mutations may be specific to limited areas rather than be shared across the world. Specifically, Schneppenheim et al identified four alleles with the frameshift mutation 4143insA in seven unrelated German patients,[28] suggesting that the mutation is a relatively common polymorphism in areas as compared with other rare mutations.

Although most patients with hereditary TTP are compound heterozygotes at the *ADAMTS13* gene, some patients are homozygotes. Levy et al identified three patients with homozygous R692C mutations,

Table 3. Common Missense SNPs Identified in the *ADAMTS13* Gene

Exon	Nucleotide	AA	Domain	References
1	19C→T	R7W	Signal peptide	19,29
12	1342C→G	Q448E	Cysteine-rich	19,27,29
12	1423C→T	P475S	Cysteine-rich	27
16	1852C→G	P618A	Spacer	19,29
16	1874G→A	R625H	Spacer	19
18	2195C→T	A732V	Tsp1-2	19,29
21	2699C→T	A900V	Tsp1-5	19
24	3097G→A	A1033T	Tsp1-7	19

who all came from the same small village where the families lived for several generations.[19] We identified a homozygous patient with a Q449X mutation, whose two great-grandparents came from the same village in the northeastern region of the Japanese mainland at the end of the 19th century.[27] The patient with the homozygous 414+1G→A splice mutation had a family history of consanguineous marriage.[31] The patient with the homozygous L232Q mutation also likely came from a consanguineous pedigree, based on analysis of an intragenic haplotype.[28] The patient with the homozygous 1783delTT frameshift mutation was born to parents who were first cousins of Yemenite background.[30] Thus, all five homozygous mutations appear to be the progeny of some blood relationship.

Common Single-Nucleotide Polymorphisms

In all, eight missense mutations were reported as common single-nucleotide polymorphisms (SNPs)[19,27,29] (Table 3). In addition, Levy et al reported eight silent SNPs in eight exons and 10 SNPs in eight introns.[19] Thus far, no relationship between these SNPs and the development of TTP has been suspected.

The allele frequencies of R7W, Q448E, P475S, and P618A SNPs have been reported. We sequenced 364 Japanese subjects without TTP, and identified 125 heterozygotes and eight homozygotes of Q448E, and 35 heterozygotes and one homozygote of P475S. The allele frequencies were calculated to be 19% and 5%, respectively.[27] Since a 43% frequency of Q448E was also determined among 120 European alleles,[29] this SNP seems to be spread worldwide. In contrast, the P475S SNP was only reported by us. We sequenced 95 Caucasian subjects and found no alleles positive for the SNP (unpublished data, January 2003). Therefore, the P475S SNP may be localized to restricted areas around Japan. Antoine et al also reported the allele frequencies of R7W and P618A, as 10% and 9%, respectively.[29]

We prepared recombinant ADAMTS-13 with Q448E or P475S mutations and measured their cleavage activities toward VWF multimers.[27] The Q448E mutant showed normal activity, whereas the P475S mutant had a significantly reduced activity (~5% to 10% of wild type). The allele frequency of P475S is about 5%, as described above, suggesting that approximately 10% of the Japanese population are heterozygotes and may possess significantly reduced ADAMTS-13 activity. Theoretically, one in 400 Japanese may be P475S homozygotes, with quite low activity.

Sequencing Methods

We have performed *ADAMTS13* sequence analysis as described previously[27] with a slight modification. This analysis was carried out with the permission of ethics committees. Human genomic DNA was isolated from whole blood using the FlexiGene DNA kit (Qiagen, Hilden, Germany) or an automated nucleic acid isolation system, NA-3000 (Kurabo, Osaka, Japan). All exons of the *ADAMTS13* gene, including the intron-exon boundaries, were PCR-amplified with corresponding intronic primers (Table 4). Twenty-six primer sets were used. PCR was performed using HotStarTaq DNA polymerase (Qiagen) or the GC-RICH PCR system (Roche, Tokyo, Japan). For all exons, except 7 and 8, 10 μL of HotStarTaq Master Mix was added to 10 μL of water containing 50 ng of genomic DNA, 1 μmol/L forward primer, and 1 μmol/L reverse primer. For the amplification of exon 7, 0.4 μL of GC-RICH Enzyme Mix was added to 19.6 μL of water containing 50 ng of genomic DNA, 0.25 μmol/L forward primer, 0.25 μmol/L reverse primer, 0.2 mmol/L dNTP, and 4 μL of 5× GC-RICH PCR reaction buffer. For exon 8, 10 μL of HotStarTaq Master Mix was added to 10 μL of water containing 250 ng of genomic DNA, 4 μmol/L forward primer, 4 μmol/L reverse primer, and 5% dimethyl sulfoxide. The thermal cycle conditions for each reaction are summarized in Table 4. PCR products were purified using the QIAquick PCR Purification kit (Qiagen), and sequenced in both directions using a BigDye Terminator Kit (Applied Biosystems, Foster City, CA) and a 3700 DNA Analyzer (Applied Biosystems).

Future Issues

The coming sequence analysis of hereditary TTP patients will likely reveal still more *ADAMTS13* mutations. At present, it is difficult to define a correlation between each genotype and phenotype for TTP patients. Patients with hereditary TTP differ greatly in the age of onset and severity of symptoms. About half of hereditary TTP patients have their first acute episode during childhood, while the other half are

Table 4. Amplification of *ADAMTS13* Exons

Exon (bp)	Forward Primer	Reverse Primer	Product (bp)	PCR Step 1	PCR Step 2
1 (549)	GATTGCCAGGCCGTTTGTGAT	GCAAACCCCAAAGCTGATGTA	768	95°C/15 min	(94°C/20 s, 63°C/20 s, 72°C/45 s) × 40
2 (67)	CCTCGGTCTCCCCAAGTGTTA	GAACCCTGGCCTGGCTGGAAC	348	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
3 (158)	GGTGGGGGTGACACGCAATGT	CCAGGGGAGGGAGGGAGAAGA	437	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
4 (84)	TGTTTTCCTTGCGTTAGTTGG	GAGGATGGAGATGCGATGACT	382	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
5-6 (125, 147)	AACAAACCGACCGCAGTCAGC	GGTTCCCCTGTCCTCACACCT	638	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
7 (138)	GCTGGCGCTGCGGCACTAGGG	GTTGGACGGAGGGGTGGGTTG	399	95°C/3 min	(95°C/30 s, 64°C/30 s, 72°C/45 s) × 38
8 (163)	ACTCCTCCGTCCCGCCTCCTC	GCCCTCCCAGGACTAGCTACA	477	95°C/15 min	(94°C/20 s, 64°C/20 s, 72°C/30 s) × 40
9 (105)	GTGCAGAGTGTTGGCTGTGTC	CTCTGCCCCATACTGGTCCTG	334	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
10-11 (152, 64)	TGAGGATGTTGGGGGACTCTC	CAAATGTGTCCTGGTGTGAAC	512	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
12 (127)	TGAGGCCACACCCACATCTTG	ATGCCAGAGCCTGAACCACTT	366	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
13 (149)	ATAGAAACCCTTGCCCCAGAT	ATCCTTTTCCCCAGCACCACT	390	95°C/15 min	(94°C/20 s, 63°C/20 s, 72°C/45 s) × 40
14 (121)	CAGGGCTGCAGAGTCATTGAG	GAAGGGTGGCGAAGTGGAAGA	358	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
15 (81)	CTCCCTTTGTCTGTGGTGTGG	ACTATCAAGCCTGAGGGTGGT	279	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
16 (182)	GGGACCCCGGGAAGGAGAGTC	GTAAGTGACCGCTGAATGAAT	393	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
17-18 (136, 130)	GGCCAGGCTGGAGTGCTATGT	CAGAATGGGGGCACTCACAGA	770	95°C/15 min	(94°C/20 s, 63°C/20 s, 72°C/45 s) × 40
19 (186)	ACCAGCCTGTGATTCGGTTGT	AGGAACTCTGACAGCAGCACT	548	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
20 (190)	CTCTTTGGGCTCCTGGATGTT	CAATGGGTGCTCCTCGTTCTC	386	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
21 (121)	AAGGATACCCGCTGCGAGACC	AGCCAATCAACACCCACATTT	489	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
22 (130)	CCATGCGGGCCTTATGTGCTA	TCTGGGTTGCAGTCCTCAAAG	439	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
23 (183)	GGGGGCCTCCAGAAAGAGAAC	GTGTTGCCCAGGTTGGACTTG	476	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
24 (205)	GGCTCAGTGGCTGCACTTTCC	TCCAGCGTCCCCAAACCTAAG	576	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
25 (319)	GACAGGGACCCAGACTTGAAT	AAGTTACTTCCCCTTGATAGT	729	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
26 (147)	CTGCATGTGCCCCCTCTTGCT	TGGGCACATCACTTAATCTCT	574	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
27 (177)	GTGCATTCCCACCTGTAGTTT	TCCCTGGCACGTGCAGACTGA	583	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
28 (185)	CCAGAGCCCAGAACATTTAGC	GCCACTATTTCACTCTTGTAG	581	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35
29 (412)	GTGTCCTTGGGGAAGTGATGT	GATTGGATTTTCTTCCTGGAT	757	95°C/15 min	(94°C/20 s, 60°C/20 s, 72°C/45 s) × 35

NOTE. All exons except exon 7 are amplified using HotStarTaq DNA polymerase. Exon 7 is amplified using GC-RICH PCR system. The reaction mixture for exon 8 contains 5% dimethyl sulfoxide.

affected in adulthood. Symptoms in adults often develop in association with stress of infection or pregnancy.[34] Genetic backgrounds other than *ADAMTS13* or environmental factors may be associated with the etiology of TTP.

From the viewpoint of clinical genetics, some problems still remain to be resolved. First, is the genetic variation in *ADAMTS13*, including common SNPs, associated with acquired TTP? Although the majority of TTP is the acquired type, with autoantibodies to ADAMTS-13, almost all subjects analyzed so far have had hereditary TTP. Sequence analysis of many patients with acquired TTP may help answer this question.

Second, are common SNPs that influence VWF-cleaving activity associated with other diseases? Although the almost complete loss of ADAMTS-13 activity results in TTP, the decreased activity may also be a risk factor for some thrombotic diseases, due to large circulating VWF multimers. In fact, a recent report suggested that levels of ADAMTS-13 activity are decreased in patients with coronary heart disease.[35] Common SNPs causing less than normal activity may also be linked to other disorders. The epidemiologic assessment of the P475S SNP will be important, at least in Japan.

Third, can VWF mutation be responsible for TTP? All hereditary TTP patients analyzed so far had mutations in *ADAMTS13*, and no *VWF* mutations were identified. Many mutations of *VWF* that enhance susceptibility to ADAMTS-13 and lead to the loss of large VWF multimers have been identified as the cause of type 2A von Willebrand disease. In contrast, no mutations conferring resistance against ADAMTS-13 proteolysis have been found. Such mutations might be lethal in the uterus. Analysis of genetically engineered animals may be useful to investigate this issue.

Fourth, do mutations causing deficiency of *THBS1*, which encodes thrombospondin-1, cause TTP? Two molecules are known that control the size of VWF multimers, ADAMTS-13 and thrombospondin-1. The latter is a trimeric glycoprotein that uncouples VWF multimers by reducing the disulfide-bonds that link individual subunits.[36,37] A combination of common genetic variations of *ADAMTS13*, *VWF*, and *THBS1* may be associated with TTP and other thrombotic diseases.

Additional Information

At the same time as the deadline for this manuscript, the 19th Congress of the International Society on Thrombosis and Haemostasis was held in Birmingham, UK. Eighteen additional mutations causing TTP were reported there by three research groups.[38-40] Reduced activity of some mutants was confirmed by expression analysis. The activities of recombinant ADAMTS-13 with common SNPs, R7W, P618A, A900V, and A1033T, were also measured.[41] The P475S SNP was also found in the Chinese population, but its allele frequency was remarkably lower than that in the Japanese population.[42]

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Identification of Strain-specific Variants of Mouse *Adamts13* Gene Encoding von Willebrand Factor-cleaving Protease*

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Human *ADAMTS13* was recently identified as a gene encoding von Willebrand factor-cleaving protease, hADAMTS13. Both congenital and acquired defects in this enzyme can cause thrombotic thrombocytopenic purpura. hADAMTS13 consists of 1,427 amino acid residues and is composed of multiple structural domains including thrombospondin type 1 motifs and CUB domains. To analyze the functional roles of these domains *in vivo*, we determined the cDNA sequence of the mouse ortholog, mADAMTS13. Unexpectedly, two forms of the mouse *Adamts13* gene were isolated that differed in the insertion of an intracisternal A particle (IAP) retrotransposon including a premature stop codon. The IAP insertion was found in BALB/c, C3H/He, C57BL/6, and DBA/2 strains but not in the 129/Sv strain. The outbred ICR strain had either the IAP-free or IAP-inserted allele or both. IAP-free *Adamts13* encoded mADAMTS13L, a protein of 1,426 amino acid residues with the same domain organization as hADAMTS13. In contrast, IAP-inserted *Adamts13* encoded a C-terminally truncated enzyme, mADAMTS13S, that is comprised of only 1,037 amino acid residues and lacking the C-terminal two thrombospondin type 1 motifs and two CUB domains. Strain specificity was also confirmed by reverse transcription-PCR and Northern blot analyses. Both recombinant mADAMTS13L and mADAMTS13S exhibited von Willebrand factor cleaving activities *in vitro*. The natural variation in mouse ADAMTS13 should allow for the determination of hitherto unknown functions of its C-terminal domains *in vivo*.

von Willebrand factor (VWF)[1] is a large glycoprotein that mediates adhesion between the platelet surface and damaged subendothelium (1, 2). VWF is mainly synthesized in endothelial cells and secreted into the circulating blood as unusually large VWF (UL-VWF) multimers (1, 2). In healthy individuals, UL-VWF multimers are cleaved to smaller sizes in plasma (3). If cleavage is impaired, however, UL-VWF multimers accumulate in the plasma. Because UL-VWF multimers possess an extremely high thrombotic activity (4, 5), UL-VWF multimers in the circulation lead to platelet clumping at the sites of vascular injury. The importance of VWF proteolysis is best illustrated by the severe consequences of thrombotic thrombocytopenic purpura, a condition associated with increased levels of UL-VWF multimers (6). This disease is characterized by microangiopathic hemolytic anemia, thrombocytopenia, neurological dysfunction, renal failure, and fever (7). The mortality of affected patients may exceed 90% without treatment such as plasma exchange.

Human ADAMTS13 (hADAMTS13), an enzyme responsible for the proteolytic processing of UL-VWF multimers, was recently purified, and its partial amino acid sequence was determined (8–10). hADAMTS13 cleaves a peptidyl bond between Tyr^{1605} and Met^{1606} in the VWF A2 domain (11–13). The gene encoding hADAMTS13 was identified as a member of the "a disintegrin-like and metalloprotease with thrombospondin type 1 motif (ADAMTS)" family and designated as *ADAMTS13* (8, 14, 15). *ADAMTS13* contains 29 exons and spans ~37 kb on chromosome 9q34 (8, 14, 15). The mRNA is detected primarily in liver (8, 14, 15). Analysis of genomic DNA in patients with congenital thrombotic thrombocytopenic purpura revealed that mutations of *ADAMTS13* could lead to an inactive enzyme (15–20). Notably, a common single nucleotide polymorphism, P475S, with ~10% heterozygosity in the Japanese population, resulted in a decrease of enzymatic activity (16).

hADAMTS13 consists of several different domains: a signal peptide, a propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type 1 (TSP1) motif, a cysteine-rich domain, a spacer domain, seven additional TSP1 repeats, and two CUB domains. *In vitro* studies using C-terminally truncated hADAMTS13 constructs revealed that the C-terminal TSP1 motifs and CUB domains were dispensable to maintain the VWF cleaving activity (21, 22). However, the biochemical and physiological roles of these domains *in vivo* remain to be resolved. As a first step to develop suitable animal models for following potential roles of this enzyme *in vivo*, we have cloned the mouse ortholog of hADAMTS13, mADAMTS13, and determined its complete genomic structure. In the present study, we report two types of the *Adamts13* gene in mice caused by the strain-specific insertion of an intracisternal A-particle (IAP) retrotransposon. We further examine the VWF cleaving activity of mADAMTS13.

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB071302, AB095445, and AB112362.

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[1] The abbreviations used are: VWF, von Willebrand factor; TSP1, thrombospondin type 1; UL-VWF, unusually large VWF; IAP, intracisternal A-particle; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends; RT, reverse transcription; ORF, open reading frame; h, human; m, mouse; HRP, horseradish peroxidase.

EXPERIMENTAL PROCEDURES

Animals—Male 129/Sv mice were purchased from Clea Japan, Inc. Male BALB/c, C3H/He, C57BL/6, DBA/2, and ICR mice were purchased from Japan SLC, Inc. Blood (~100 μl) was collected by cardiac puncture into a syringe containing 10 μl of 3.8% sodium citrate and centrifuged to obtain plasma. Spleen and liver were excised, rinsed in phosphate-buffered saline, and immediately used for DNA and RNA preparation.

DNA Sequencing—All of the sequence analyses were performed by 373A or 3700 automated DNA sequencer (Applied Biosystems) with a BigDye Terminator Kit (Applied Biosystems).

Determination of the mADAMTS13 cDNA Sequence—Total RNA was prepared from the livers of C57BL/6 and 129/Sv mice with Isogen (Nippon Gene), and poly(A)$^+$ RNA was purified with an mRNA purification kit (Amersham Biosciences) according to the manufacturer's instruction. The cDNA was synthesized from the poly(A)$^+$ RNA with a first strand cDNA synthesis kit (Amersham Biosciences). PCR was carried out with primers designed from the genomic DNA sequence (forward sequence in 5′-untranslated region, 5′-AGGAAGCTCCCAAGAGTAAACACTGCCT-3′; reverse sequence within the metalloprotease domain, 5′-TCAGAGAGGGTGATTAGCTTCACCAGGT-3′). PCR products were cloned into pCR2.1 vector using a TA Cloning™ kit (Invitrogen) and sequenced.

In addition, 3′-RACE was performed using a 3′-Full RACE Core Set (Takara), according to the manufacturer's instructions. After reverse transcription from liver poly(A)$^+$ RNA, PCR was performed using the Adaptor Primer provided with the kit and a gene-specific forward primer within the metalloprotease domain, 5′-TGGAGTTGCCTGATGGCAACCAGCA-3′. The second PCR was performed using the first PCR products as a template with Adaptor Primer and a gene-specific internal forward primer, 5′-CATCACCTTTTCCTACTTTCAACTGAAGCAG-3′. The cycling parameters were as follows: 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, followed by 72 °C for 7 min. PCR products were cloned into pCR2.1 vector and sequenced.

PCR Analysis of IAP Insertion in the Adamts13 Gene—Genomic DNA was extracted from ear punches of 129/Sv, BALB/c, C3H/He, C57BL/6, DBA/2, and ICR mice by DNeasy Tissue Kit (Qiagen). Presence or absence of an IAP insertion in the *Adamts13* gene was determined by PCR with HotStarTaq DNA polymerase (Qiagen). The amplification was carried out using mixture of three primers; the intron 23-specific forward primer, 5′-ACCTCTCAAGTGTTTGGGATGCTA-3′, the IAP-specific reverse primer, 5′-TCAGCGCCATCTTGTGACGGCGAA-3′, and the primer downstream of the IAP target site, 5′-TGCCAGATGGCCATGATTAACTCT-3′. PCR products were directly sequenced.

Southern Blot Analysis—Genomic DNA prepared from spleens was digested with EcoRV, separated on 0.7% agarose gel, and transferred to a nylon membrane by standard capillary blotting techniques. A genomic fragment (842 bp) upstream of the IAP target site was produced by PCR with the primers 5′-TAGGCAGCCATGGATCTGTATTAG-3′ and 5′-TGTCTGCTCTTCCAGAAATCCTTA-3′ and labeled with fluorescein-11-dUTP using Gene Images random prime labeling module (Amersham Biosciences). The blot was hybridized with the probe, and the hybridized probe was detected using the Antifluorescein-AP conjugate and the CDP-*Star* detection reagent (Amersham Biosciences) according to the manufacturer's instructions. Chemiluminescence was measured by an LAS-1000plus image analyzer (Fujifilm).

Determination of the Adamts13 Genomic Sequence—A λ phage library constructed from Sau3AI-digested genomic DNA of the 129/Sv strain was screened by a PCR-based method as described previously (23). Three independent positive phages were obtained. Each phage insert DNA was subcloned into pBluescript II SK(+) vector (Stratagene) and sequenced using the GPS-1 Genome Priming System (New England Biolabs) according to the manufacturer's instructions. The sequence data were assembled and analyzed using the Sequencher software (Gene Codes). Sequence gaps of each target DNA were filled by primer walking sequencing.

Reverse Transcription-PCR—Total RNA was extracted from the livers of six mouse strains with Isogen (Nippon Gene), and poly(A)$^+$ RNA was purified using PolyATtract mRNA Isolation Systems (Promega) and subjected to one-step RT-PCR (Qiagen). The exon 21/22-specific sense primer (5′-TTGTGGGAGAGGTCTGAAGGAACT-3′), the pseudoexon 24-specific antisense primer (5′-TCAGCGCCATCTTGTGACGGCGAA-3′), and the exon 24/25-specific antisense primer (5′-ACAGGAGACAGAGCACTCTGTCCA-3′) were simultaneously used for the amplification. The PCR products were excised from the agarose gel and sequenced.

Northern Blot Analysis—The specific fluorescein-labeled probe (1.3 kb) was synthesized by PCR from mouse *Adamts13* cDNA as described previously (23). The primers used were a sense primer located in exon 3 (5′-ATTCTGCACCTGGAACTCCTGGTA-3′) and an antisense primer located in exon 13 (5′-CGGCTGACAATGAAGCTTTCTCCA-3′). Poly(A)$^+$ RNA (10 μg) from mouse livers were separated on 1% agarose gel containing 2% formaldehyde and transferred to a nylon membrane. Hybridization and detection using the Antifluorescein-AP conjugate and the CDP-*Star* detection reagent (Amersham Biosciences) were performed according to the manufacturer's instructions. Commercially available premade Northern blot membranes containing poly(A)$^+$ RNA from the BALB/c strain (Multiple Tissue Northern blot; Clontech) and the Swiss Webster strain (FirstChoice Northern blot; Ambion) were also analyzed using the Antifluorescein-HRP conjugate and the DNA Thunder Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Chemiluminescence was measured by an LAS-1000plus image analyzer (Fujifilm).

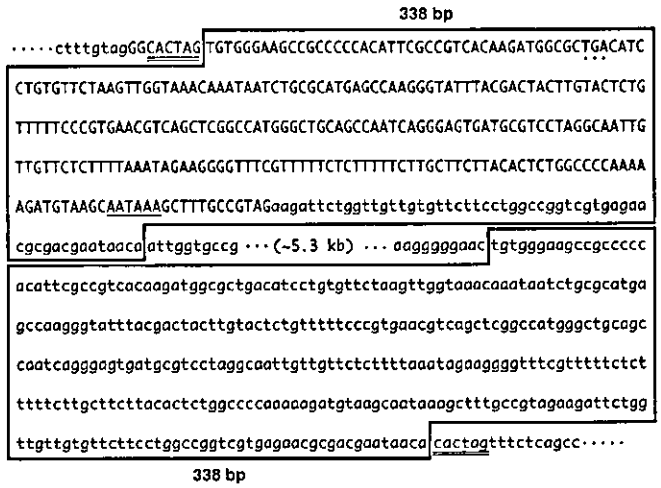

FIG. 1. **Nucleotide sequence of the retrovirus-like element observed in *Adamts13* intron 23 of the C57BL/6 strain.** The 338-bp repetitive sequences of a retrovirus-like element are *boxed*. The 6-bp duplication of target DNA at the insertion site is *double underlined*. Exon 24 of the C57BL/6 strain, shown in *uppercase letters*, contains a stop codon TGA (*dotted*) and a putative polyadenylation signal AATAAA (*underlined*).

Preparation of Recombinant Substrate (mVWF73) for Enzymatic Assay—To examine the enzymatic activities of mADAMTS13, we prepared the recombinant substrate as described previously (24). In brief, a D1596-T1668 region of mouse VWF was amplified by RT-PCR using total RNA from a C57BL/6 mouse liver. The primers, 5′-cgggatccGACCGGGTAGAGGCACCTAACC-3′ and 5′-cggaattcTCAGTGATGGTGATGGTGATGTGTCTGCAGGACCAGGTCAGGA-3′ were used for the amplification. Lowercase letters indicate added restriction enzyme sites, and the underlined sequence is the inserted C-terminal His_6 tag (H). The PCR product was digested with BamHI and EcoRI and cloned into the corresponding sites of pGEX-6P-1 (Amersham Biosciences), a glutathione S-transferase (GST) fusion expression vector. The resulting plasmid encoding GST-D1596T1668-H was introduced into *Escherichia coli*, BL21 (Stratagene), and expression was induced by the addition of isopropyl-β-D-thiogalactoside. The bacterial cells were collected and lysed with CelLytic B (Sigma), followed by centrifugation. The soluble fraction was subjected to a nickel-nitrilotriacetic acid Spin Kit (Qiagen) and further to a MicroSpin GST purification module (Amersham Biosciences). The purified protein, designated GST-mVWF73-H, was used as substrates for enzymatic assays. The molecular mass of GST-mVWF73-H was 35.7 kDa. If mADAMTS13 cleaves the expected site, the size of the N-terminal portion including the GST tag will be 28.0 kDa.

Transient Expression of mADAMTS13—The entire open reading frame (ORF) constructs with C-terminal FLAG sequence (DYKDDDDK) were prepared for two types of mouse ADAMTS13, mADAMTS13L (GenBank™ accession number AB112362) and mADAMTS13S (GenBank™ accession number AB071302), by PCR. Each PCR product was inserted into pCAGG-neo mammalian expression vector (25). The resulting plasmids were transfected into HeLa cells using FuGENE 6 (Roche Applied Science) as described previously (16). Forty-eight hours after transfection, the media were collected and concentrated using Centricon YM-30 (Millipore). The cells together with extracellular matrix were lysed in SDS sample buffer (10 mM

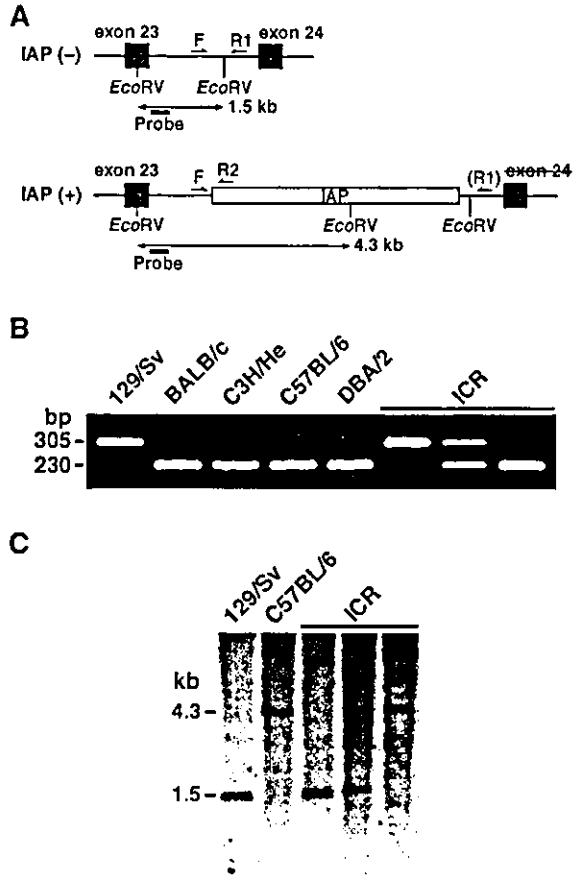

FIG. 2. **Genotyping of mouse *Adamts13*.** *A*, diagram of a segment of the *Adamts13* gene around the IAP insertion site. The sites of primers used for the genotyping PCR are indicated by *arrows*. The EcoRV fragments detected in Southern blot analysis are indicated by *double-headed arrows*. *B*, PCR analysis. In mixture of three primers, F and R1 primers generate a 305-bp product specific for the IAP-free *Adamts13* gene, whereas F and R2 primers generate a 230-bp product specific for the IAP-inserted *Adamts13* gene. *C*, Southern blot analysis. Genomic DNA from each mouse strain was digested with EcoRV and hybridized with the probe that detects a 1.5-kb fragment in the IAP-free allele and a 4.3-kb fragment in the IAP-inserted allele.

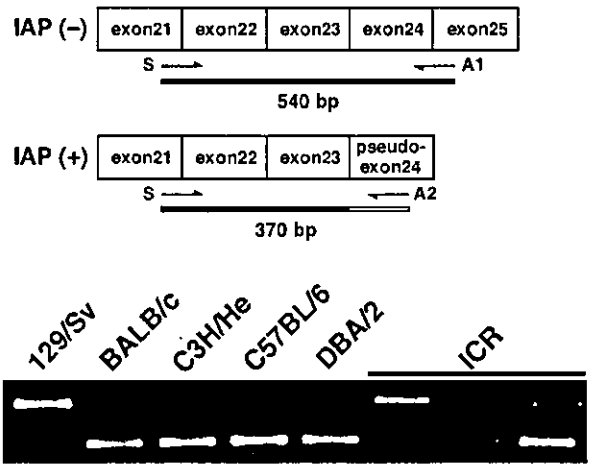

FIG. 3. **RT-PCR of *Adamts13* mRNA in liver.** PCR primers are shown as *arrows* indicating direction at their approximate locations. In combination of three primers, S and A1 primers generate a 540-bp product specific to the IAP-free transcript, whereas primers S and A2 generate a 370-bp product specific to the IAP-inserted transcript.

Tris-HCl, 2% SDS, 50 mM dithiothreitol, 2 mM EDTA, 0.02% bromphenol blue, 6% glycerol, pH 6.8).

Recombinant proteins were detected by SDS-PAGE and Western blot as described previously (16). For culture media, a rabbit anti-

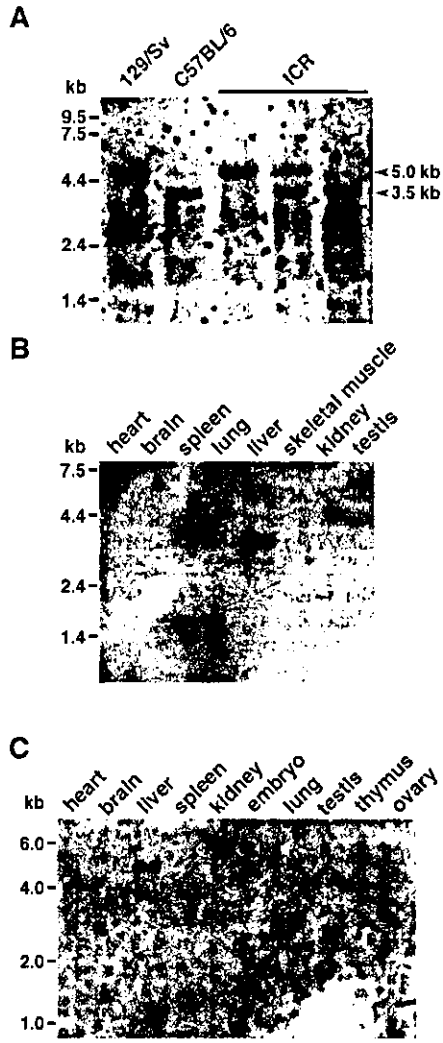

FIG. 4. **Northern blot analysis of mouse *Adamts13* mRNA.** *A*, expression of *Adamts13* mRNA in liver. Poly(A)$^+$ RNA isolated from liver of indicated strains was probed with a 1.3-kb *Adamts13* cDNA corresponding to exons 3–13. The approximate sizes of the IAP-free (5.0 kb) and the IAP chimeric (3.5 kb) transcripts are indicated by *arrowheads*. *B*, expression of *Adamts13* mRNA in tissues from BALB/c mice. *C*, expression of *Adamts13* mRNA in tissues from SwissWebster mice. The sizes of RNA markers are shown at the *left*.

mADAMTS13 polyclonal antibody (described below) and an HRP-labeled goat anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories) were used for detection after SDS-PAGE under nonreducing condition. For cell lysates, an anti-FLAG M2 monoclonal antibody (Sigma) and an HRP-labeled goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories) were used for detection after SDS-PAGE under reducing condition. Chemiluminescence was developed using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and detected by an LAS-1000plus image analyzer (Fujifilm).

Preparation of Polyclonal Antibody against Mouse ADAMTS13—A polyclonal antiserum against mADAMTS13 was raised by DNA-based immunization protocols. Rabbits were immunized by intradermal injection with ~1 mg of mADAMTS13S expression plasmid at 25 sites on the back. Booster immunizations were carried out by the same protocol 3 weeks after the primary immunization. Serum was collected 3 weeks after the second immunization. The IgG fraction was then prepared by an affinity chromatography using a protein G column (Amersham Biosciences).

Enzymatic Assay—Purified GST-mVWF73-H (500 ng) was incubated with recombinant mADAMTS13L or mADAMTS13S in 40 μl of reaction buffer (5 mM Tris-HCl, 10 mM $BaCl_2$, 0.01% Tween 20, and 1 mM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, pH 8.0) at 37 °C for 1 h. The reaction was stopped by adding 10 μl of SDS sample buffer (50 mM Tris-HCl, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA, 0.1% bromphenol blue, 30% glycerol, pH 6.8). The samples were sub-

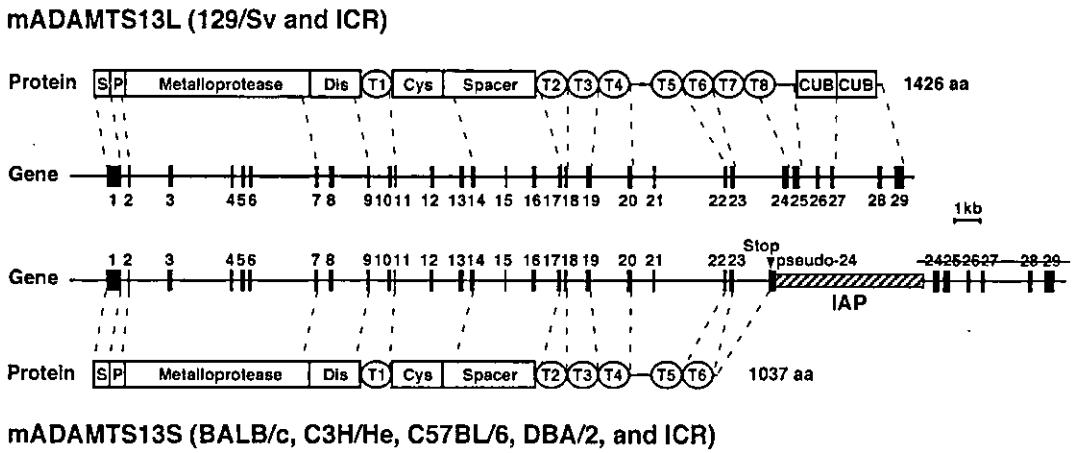

FIG. 5. **Schematic structure of two forms of the *Adamts13* genes and proteins.** Genomic and protein structures of mADAMTS13L in the 129/Sv strain and of mADAMTS13S in the BALB/c, C3H/He, C57BL/6, and DBA/2 strains are shown. The outbred ICR strain has both type alleles. The exons and introns are drawn to scale. A *hatched box* represents the IAP insertion in intron 23. *S*, signal peptide; *P*, propeptide; *Dis*, disintegrin-like domain; *T1–T8*, thrombospondin type1 motifs; *Cys*, cysteine-rich domain.

jected to Western blot using a rabbit anti-GST antibody (Molecular Probes) and an HRP-labeled goat anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories) as described (24). We also analyzed the proteolytic activity of plasma from five mouse strains in the same way.

RESULTS

Identification of Adamts13 in the C57BL/6 Strain—To identify the orthologous mouse gene of human *ADAMTS13*, we performed a BLAST search in the public data base, based on the human *ADAMTS13* cDNA sequence (GenBank™ accession number AB069698, 4,284-nucleotide ORF) reported by Soejima *et al.* (8). This search led us to identify a compatible genomic sequence (GenBank™ accession number AC090008) derived from the C57BL/6 strain. This sequence was located on chromosome 2, band A3 and contained 29 conserved exons similar to human *ADAMTS13*. To obtain the cDNA to corresponding mRNA, we performed RT-PCR and 3′-RACE using poly(A)$^+$ RNA from the liver of a C57BL/6 mouse. Unexpectedly, the cDNA sequence (GenBank™ accession number AB071302) included only a 3,114-nucleotide ORF derived from 24 exons.

From a comparison between the cDNA and the genomic sequences of the C57BL/6 strain, we found a 6-kb retrovirus-like sequence in intron 23 of the *Adamts13* gene (Fig. 1). This sequence was flanked by the identical 338-bp sequence with a 6-bp (CACTAG) duplication of target site, as is often observed for retrotransposition. A BLAST search identified the insertional element as an intracisternal A-particle (IAP), which is one of the retrotransposons present at about 2,000 sites in the mouse genome (26). This insertion of IAP seemed to be responsible for loss of the original mRNA 3′-end by splicing exon 23 to pseudo-exon 24 that contains a premature stop codon.

Identification of Adamts13 in the 129/Sv Strain—To determine whether the IAP insertion into the *Adamts13* gene is common to a variety of mouse strains, we carried out PCR genotyping of five inbred and one outbred strains (Fig. 2*A*). A mixture of three primers was used for the reaction; Primers F and R1 were designed to produce a 305-bp band in the absence of IAP, and primers F and R2 were designed to produce a 230-bp band in the presence of IAP. This experiment revealed that the IAP insertion was present in the BALB/c, C3H/He, and DBA/2 strains but not in the129/Sv strain (Fig. 2*B*). The outbred ICR strain was genetically heterogeneous with respect to the IAP insertion into the *Adamts13* gene. Southern blot analysis using probes upstream of the IAP target site revealed that *Adamts13* was a single copy gene and confirmed the strain-specific insertion of IAP (Fig. 2*C*). These results implied that the *Adamts13* gene transcript of the 129/Sv mice and some ICR mice might contain residual exons lost in the other mouse strains.

To confirm this hypothesis, RT-PCR was performed using liver poly(A)$^+$ RNA from a 129/Sv mouse. The obtained sequences indicated that the *Adamts13* cDNA (GenBank™ accession number AB112362) contained a 4,281-nucleotide ORF similar to human *ADAMTS13* (4,284-nucleotide ORF). To determine the complete genomic sequence of *Adamts13* in the 129/Sv strain, we screened a 129/Sv mouse λ genomic library. Sequence analysis of positive phage clones confirmed the absence of IAP in the *Adamts13* gene. *Adamts13* in the 129/Sv strain (GenBank™ accession number AB095445) contained 29 exons like human *ADAMTS13* and spanned ~30 kb.

To examine the effect of IAP on *Adamts13* mRNA splicing, RT-PCR was performed using liver poly(A)$^+$ RNA from six mouse strains (Fig. 3). The exon 21/22-specific sense primer, the exon 24/25-specific antisense primer, and the pseudo-exon 24-specific antisense primer were mixed and used for the amplification. We detected the IAP chimeric transcript in four inbred strains with the IAP insertion. In contrast, the IAP-free transcript was observed in the 129/Sv strain. The heterogeneous expression of two types of transcripts was observed in samples from the ICR strain. To characterize the transcripts in more detail, Northern blot analysis of liver RNA was carried out using a 1.3-kb probe spanning exons 3–13 of *Adamts13* cDNA (Fig. 4*A*). The RNA was prepared from the same animals as used for the Southern blot analysis. An ~3.5-kb mRNA corresponding to the size of IAP chimeric transcript was detected in the C57BL/6 and the ICR strains. The IAP-free transcript of ~5.0 kb was observed in the 129/Sv and the ICR strains.

Thus, these results clearly indicate the presence of two types of mouse *Adamts13* in a strain-specific manner (Fig. 5). *Adamts13* of the 129/Sv strain encodes an ADAMTS13 protein containing 1,426 amino acid residues with the same domain structure as hADAMTS13, designated mADAMTS13L. *Adamts13* of the BALB/c, C3H/He, C57BL/6, and DBA/2 strains encodes the shorter ADAMTS13 protein including only 1,037 amino acid residues, designated mADAMTS13S. In this protein, the C-terminal two TSP1 and two CUB domains are replaced with the 16-amino acid sequence, ALVWEAAPTFAVTRWR, derived from the IAP. The outbred ICR strain carries either the IAP-free or IAP-inserted allele or both.

Expression of the Adamts13 mRNA in Mouse Tissues—To study the expression pattern of the mouse *Adamts13* gene, we analyzed Northern blots containing poly(A)$^+$ RNA from various tissues of the BALB/c and the Swiss Webster strains. As shown

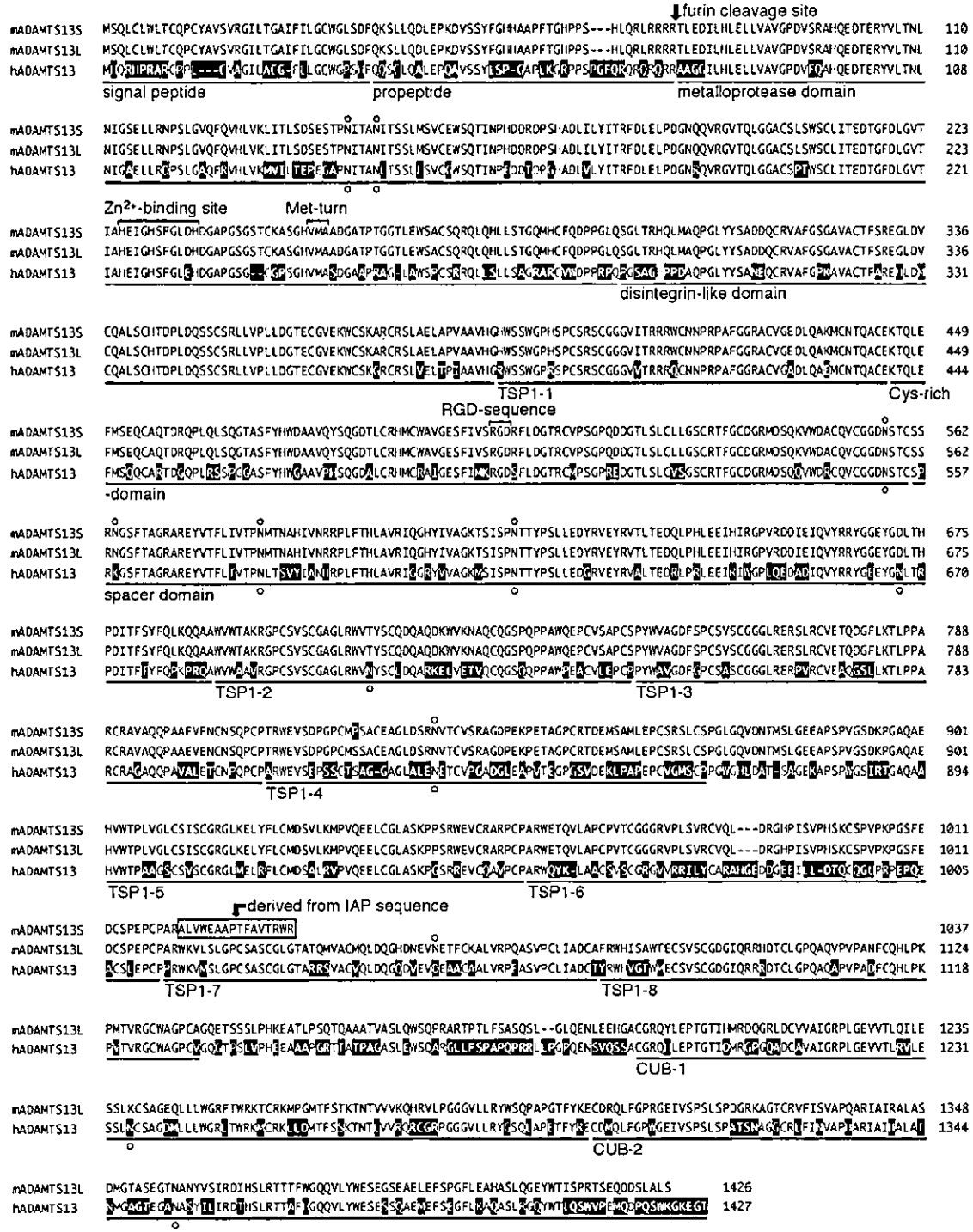

FIG. 6. **Alignment of deduced amino acid sequences of mADAMTS13 and hADAMTS13.** Sequences are represented with the *single-letter code*, and residues that differ from mADAMTS13L are *shaded*. Each structural domain is *underlined*. The predicted furin cleavage site (R*X*(K/R)R) is marked with an *arrow*. The IAP-derived 16 amino acid residues in mADAMTS13S are *boxed*. *Open circles* indicate the potential *N*-glycosylation sites.

in Fig. 4 (*B* and *C*), *Adamts13* mRNA in both strains was exclusively observed in the liver, suggesting that mADAMTS13 is primarily synthesized in the liver, similar to hADAMTS13. A single transcript of 3.5 kb was expressed in liver of the BALB/c strain with the IAP insertion. In contrast, two transcripts of 5.0 and 3.5 kb were detected in liver of the outbred Swiss Webster strain, suggesting that this strain may carry two types of alleles, like the ICR strain. Both SwissWebster and ICR strains are derived from a colony of Swiss mice.

Comparison of the Deduced Amino Acid Sequences of mADAMTS13 and hADAMTS13—The deduced amino acid sequences of mouse and human ADAMTS13 were aligned (Fig. 6). The overall sequence identity between mADAMTS13L and hADAMTS13 was ~70%. The highest identity (>80%) was observed in the disintegrin-like (81%), TSP1–1 (89%), cysteine-rich (80%), and TSP1–8 (83%) domains, whereas relatively low conservation (<60%) was observed in the signal peptide (43%), propeptide (52%), TSP1–4 (46%), and TSP1–6 (40%) domains. mADAMTS13L contained eight potential *N*-glycosylation sites, six of which were conserved in hADAMTS13. mADAMTS13L also included several motifs characteristic for each domain of hADAMTS13, such as a furin cleavage sequence at the end of

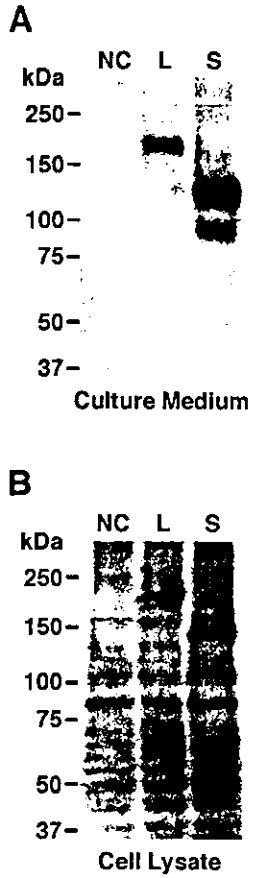

FIG. 7. **Transient expression of recombinant mADAMTS13.** *A*, mADAMTS13 in the culture medium. HeLa cells were transfected with plasmids encoding mADAMTS13L (*L*) and mADAMTS13S (*S*). The concentrated culture media were analyzed by Western blot with an anti-mADAMTS13 antibody under nonreducing conditions. *B*, mADAMTS13 in the cell lysate. The cell lysates including extracellular matrixes were analyzed by Western blot with an anti-FLAG antibody under reducing condition. *NC* is the culture medium or the lysate of untransfected cells. The size of protein markers is indicated at the *left*. The small size difference of recombinant enzymes in medium and cell lysates was due to the difference of electrophoretic condition. The faster migrating bands seen in medium and cell lysates expressing mADAMTS13S may result from proteolysis during cell culture. The typical result of three experiments is shown.

the propeptide, a zinc-binding site in the metalloprotease domain, and an RGD sequence in the cysteine-rich domain. The sequences of mADAMTS13L and mADAMTS13S were almost identical, except for the deletion of C-terminal regions.

Expression and Enzymatic Activity of Recombinant mADAMTS13L and mADAMTS13S—We transiently expressed the mADAMTS13L and mADAMTS13S proteins in HeLa cells. Western blot analysis using a polyclonal antibody against mADAMTS13 revealed that both were secreted into the culture media (Fig. 7*A*). Transient expression of mADAMTS13L produced an immunoreactive band of ~200 kDa, and mADAMTS13S exhibited a 130-kDa band. The level of mADAMTS13S in the medium was almost 10-fold higher than that of mADAMTS13L. This was not due to a preferential accumulation of mADAMTS13L on the cell surface or the extracellular matrix, because a relatively high amount of mADAMTS13S was also observed in the cell lysates (Fig. 7*B*). It was conceivable that mADAMTS13S was effectively synthesized in HeLa cells compared with mADAMTS13L in our experimental conditions. Whether mADAMTS13S is also preferentially expressed *in vivo* remains unknown. Further analysis is required to determine the plasma levels of mADAMTS13L and mADAMTS13S in mice.

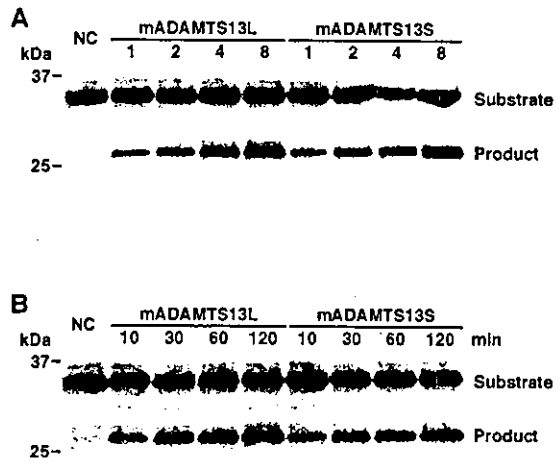

FIG. 8. **Enzymatic activity of recombinant mADAMTS13.** *A*, cleavage of GST-mVWF73-H by serial dilutions of mADAMTS13. GST-mVWF73-H was incubated with recombinant mADAMTS13L or mADAMTS13S at 37 °C for 1 h. A negative control reaction using the culture medium of untransfected cells (NC) was also performed simultaneously. The products were analyzed by Western blot using an anti-GST antibody. The *numbers 1*, *2*, *4*, and *8* indicate relative amounts of mADAMTS13L and mADAMTS13S in the reaction mixtures. The typical result of three experiments is shown. *B*, time course of GST-mVWF73-H cleavage by mADAMTS13. GST-mVWF73-H was incubated with recombinant mADAMTS13L or mADAMTS13S for the indicated time at 37 °C. The reaction mixtures contained the equivalent amounts of the recombinant enzyme. Products were analyzed by Western blot using an anti-GST antibody. The typical result of three experiments is shown.

The VWF cleaving activities of recombinant proteins were measured by the degradation of the specific recombinant substrate, GST-mVWF73-H. The relative concentration of recombinant mADAMTS13 in the culture medium was determined by chemiluminescent intensities on Western blot, and equal amounts were used for the enzymatic assay. The substrate, GST-mVWF73-H, was incubated with serial dilutions of the culture medium, and the cleavage product including the N-terminal GST tag was visualized by Western blot using anti-GST (Fig. 8). When the substrate was incubated with the medium of mADAMTS13L-transfected cells, a band appeared with the expected size of the N-terminal portion (28 kDa) in a concentration-dependent manner, indicating the cleaving activity of recombinant mADAMTS13L (Fig. 8*A*). No degradation was observed after the incubation of GST-mVWF73-H with the medium from untransfected cells. The cleaved band was also detected after incubation with the mADAMTS13S culture medium, and the chemiluminescent intensities of the product bands were almost equal to those obtained by mADAMTS13L (Fig. 8*A*). We confirmed that the degradation of GST-mVWF73-H by mADAMTS13 was also time-dependent, and the rate of the product formation by mADAMTS13S was similar to that by mADAMTS13L (Fig. 8*B*).

The VWF Cleaving Activity of Mouse Plasma—To examine the ADAMTS13 activity in plasma from various mouse strains, we collected plasma samples from five strains and carried out the enzymatic assay using GST-mVWF73-H. As shown in Fig. 9, plasma from all strains cleaved GST-mVWF73-H. Comparison of the product levels did not reveal a significant difference among strains. This suggested that the IAP insertion into the *Adamts13* gene does not affect the *in vitro* cleavage of GST-mVWF73-H by plasma.

DISCUSSION

In this study, we identified two isoforms of the mouse *Adamts13* gene that result from the strain-specific insertion of

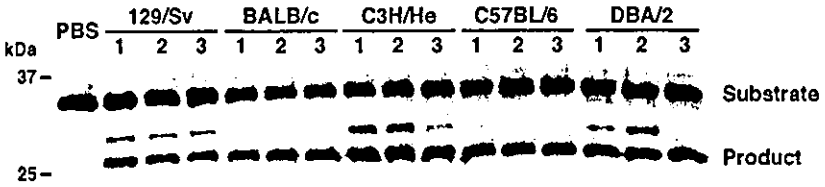

FIG. 9. **Cleavage of GST-mVWF73-H by mouse plasma.** GST-mVWF73-H was incubated with plasma samples from mice with (BALB/c, C3H/He, C57BL/6, and DBA/2) or without (129/Sv) the IAP insertion in the *Adamts13* gene. The products were analyzed by Western blot using an anti-GST antibody. The results from three animals/strain are shown.

an IAP-retrotransposon. The IAP-free *Adamts13* gene contained 29 exons, and the deduced protein sequence included 1,426 amino acid residues with the same domain organization as hADAMTS13. In contrast, the IAP-inserted *Adamts13* gene contained only 24 exons encoding 1,037 amino acids having a truncated C terminus.

The inserted IAP is one of the endogenous transposable elements, which is closely related to retroviruses and transposed via the reverse transcription of an RNA intermediate (26, 27). The IAP element contains two long terminal repeats with the signals for the initiation/regulation of transcription and for the polyadenylation of transcripts (28). IAP insertions into introns have been shown to cause formation of chimeric transcripts (29–32), similar to our findings in the *Adamts13* gene. We noted that the presence of IAP in the *Adamts13* gene induces the appearance of a cryptic splicing site followed by a premature in-frame stop codon and a polyadenylation signal derived from the IAP long terminal repeat. As a result, the insertion leads to replacement of the last 405 amino acid residues corresponding to two TSP1 motifs and two CUB domains with the IAP-encoded 16 amino acid residues.

Northern blot and RT-PCR analyses confirmed that the IAP chimeric short transcript (3.5 kb) and the IAP-free long transcript (5 kb) were expressed in a strain-specific manner. Both types of transcripts were specifically expressed in the liver, consistent with expression of the human *ADAMTS13* gene. It should be noted that the IAP insertion could not completely abolish the formation of mADAMTS13L mRNA. The RT-PCR products (540-bp; Fig. 3) characteristic of mADAMTS13L mRNA were also detectable in the strains with the IAP insertion when using a large amount of template (data not shown). A small amount of mADAMTS13L protein may be expressed in mice with the IAP-inserted *Adamts13* gene such as the BALB/c, C3H/He, C57BL/6, and DBA/2 strains. Incidentally, the RT-PCR and 3′-RACE data did not show any splicing variants that encoded mADAMTS13S-like protein in the IAP-free strains.

Recently, we developed a novel recombinant substrate, GST-VWF73-H, to measure hADAMTS13 activity (24). GST-VWF73-H is a partial region of human VWF flanked by GST and His_6 tags. Because of difficulty in isolating VWF from mouse plasma, we have also prepared the recombinant substrate, GST-mVWF73-H, based on the mouse VWF cDNA sequence. Both mouse and human plasma efficiently cleaved GST-mVWF73-H and produced a fragment of the expected size. Mouse plasma also cleaved the substrate for hADAMTS13, GST-VWF73-H (data not shown).

Both recombinant mADAMTS13L and mADAMTS13S were secreted into the culture medium of HeLa cells. This result indicates that the IAP insertion does not abolish secretion of mADAMTS13 from cells. The recombinant mADAMTS13L and mADAMTS13S cleaved GST-mVWF73-H with nearly the same efficiency. Similarly, a deletion mutant of hADAMTS13 in mimicry of mADAMTS13S was also secreted efficiently from HeLa cells and cleaved GST-VWF73-H with normal activity (data not shown). In previous reports, we and others found that deletion mutants of hADAMTS13 devoid of the C-terminal TSP1 motifs and CUB domains retained VWF cleaving activity (21, 22). Therefore, our current observation on mouse and human recombinant proteins was consistent with these previous studies. Moreover, the plasma VWF cleaving activities in mice were also comparable among the strains with or without the IAP insertion in the *Adamts13* gene. The C-terminal two TSP1 motifs and two CUB domains of mADAMTS13 may contribute to activity but are not essential for the VWF cleavage, at least *in vitro*.

The fact that several common strains of mice have a naturally truncated form of ADAMTS13 allows us to hypothesize that the truncated domains are not necessary *in vivo*. However, several mutations in TSP1–7, TSP1–8, CUB-1, and CUB-2 domains of hADAMTS13 were reported to associate with congenital thrombotic thrombocytopenic purpura (15, 17–20). It is still unclear whether these mutants are secreted from cells, as is the case with mADAMTS13S. To date, two mutations, R1123C and 4143insA, were characterized by expression analysis, and both impaired secretion of the enzyme from cells (19, 20). The C-terminal mutations found in thrombotic thrombocytopenic purpura patients may influence their synthesis or secretion.

Bernardo *et al.* (33) reported that several short peptides within the regions from TSP1–6 to the C terminus of hADAMTS13 block VWF cleavage on the endothelial cell surface under flow conditions. This finding suggests an important role for the C-terminal domains *in vivo*. Although our results clearly show that the mouse has managed without full-length ADAMTS13, the relative importance of ADAMTS13 for regulation of VWF activity may be different between human and mouse. A gene targeting technique of mouse *Adamts13* will help to clarify the physiological contribution of mADAMTS13.

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High Prevalence of Anti-Prothrombin Antibody in Patients With Deep Vein Thrombosis

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The present study was designed to determine the prevalence of lupus anticoagulant (LA) antibody and several antibodies for antiphospholipid syndrome (APS) in patients with deep vein thrombosis (DVT)/pulmonary embolism (PE) (n = 48), cerebral thrombosis (CT, n = 30), systemic lupus erythematosus (SLE, n = 22), and idiopathic thrombocytopenic purpura (ITP, n = 30). The presence of antibodies was examined by using the respective ELISA kits. LA was positive in 38.6% of patients with DVT/PE, suggesting that LA is one of the most important risk factors in DVT/PE. The highest prevalence of anti-β_2 glycoprotein I (β_2GPI) IgG was in CT and SLE, followed by DVT, and none in ITP and healthy volunteers (control, n = 40), suggesting that it is related to thrombosis, particularly arterial thrombosis. The highest prevalence of anti-prothrombin (aPT) IgG antibody was in DVT, followed by CT and SLE, and none in ITP and the control, suggesting that it is related to thrombosis, especially venous thrombosis. The highest prevalence of antiphospholipid (aPL) IgG was in DVT, CT, and SLE, but 0% in ITP and control. On the other hand, aPL IgM, anti-annexin V IgG, and anti-annexin V IgM were positive in patients both with and without thrombosis, suggesting that they are not related to thrombosis. Our results indicated that among the anti-phospholipid antibodies, LA is the most sensitive marker for APS while anti-β_2GPI IgG, aPT IgG, and aPL IgG are risk factors for thrombosis. In particular, aPT IgG is a significant marker for DVT/PE. Am. J. Hematol. 76:338–342, 2004. © 2004 Wiley-Liss, Inc.

Key words: PE; DVT; DRVVT; anti-prothrombin antibody; anti-β2GPI antibody

INTRODUCTION

Patients with antiphospholipid syndrome (APS) develop episodes of thrombosis of the arteries and/or veins, miscarriage, and thrombocytopenia, associated with positivity for antiphospholipid antibody [1,2]. In patients with venous thromboembolism, the prevalences of anti-cardiolipin antibody (aCL) and lupus anticoagulant (LA) antibodies vary from 3% to 17% and from 3% to 14%, respectively [3–5]. A high prevalence of antiphospholipid antibodies is present in patients with systemic lupus erythematosus (SLE), with estimates varying between 30% and 60% [6,7]. In stroke patients, 18% of young patients (mean age, 38 years) were positive for antiphospholipid antibodies (LA and aCL) and 9.7% of patients who developed stroke for the first time were positive for aCL according to the Antiphospholipid Antibodies in Stroke Study Group (APASS) [8,9]. In patients with myocardial infarction, the prevalence of aCL is reported to be between 5% and 15% [10]. In 543

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blood donors under 65 years of age, 2% were positive for aCL [11]. Among 179 patients who were confirmed to be free of deep vein thrombosis (DVT), 18% were positive for aCL and 2% were positive for LA [12].

Approximately 50% of patients with APS do not have underlying systemic disease and are labeled as having primary APS [13]. The age of first thrombosis in primary APS ranges between 32 and 45 years [14], suggesting that APS tends to occur at a young age. However, many APS patients are more than 45 years of age. There are many tests for APS, such as aCL [15], LA [16], anti-β_2 glycoprotein I (β_2GPI) [17], and anti-prothrombin (aPT) antibody [18]. aCL antibody was first established as an anti-phospholipid antibody [15]. The phospholipid-dependent coagulation inhibitor, which did not appear to be associated with a bleeding tendency, was named LA. At present, however, LA is also known as anti-β_2GPI antibody or aPT antibody. The present study was designed to identify the most sensitive antibody marker(s) for thrombosis in APS and other conditions. For this purpose, we determined the prevalence of several antiphospholipid antibodies in 48 patients with DVT and examined the cause of antiphospholipid antibodies for LA.

MATERIALS AND METHODS

From 1 January 2000 to 31 June 2002, we examined several anti-phospholipid antibodies in 48 patients with DVT (ratio of females/males = 14:34, mean age, 55.5 ± 17.8 years), 30 with idiopathic thrombocytopenic purpura (ITP) (21:9, 44.4 ± 14.8 years), 22 with SLE (15:7, 43.9 ± 13.4 years), 30 cerebral thrombosis (CT) (10:20, 45.8 ± 11.3 years), and 40 healthy volunteers (control) (13:27, 38.0 ± 10.7 years). In SLE, six patients were associated with DVT and 5 patients were associated with CT. A total of 38 patients were diagnosed as having APS (22:16, 50.5 ± 15.6 years), including 21 patients with DVT and 17 with CT, and 18 patients were positive for LA without thrombosis (2:16, 49.8 ± 10.9 years). APS was diagnosed on the basis of Sapporo criteria [6]. None of the patients had congenital abnormalities of protein C, protein S, antithrombin, or Factor V Leiden.

The Diluted Russell Viper Venom test (DRVVT) was measured using the DVV-test (American Diagnostica Inc. [ADI], Greenwich, CT) and DVV-confirm (ADI). Patients with a DVV-test/DVV-confirm ratio of more than 1.2 were considered LA-positive.

Anti-phospholipid (aPL), anti-annexin V, aPT, and anti-β_2GPI antibodies were measured by enzyme-linked immunosorbent assay (ELISA) using the Imuclone aPL IgG ELISA kit (ADI), Imuclone aPL IgM ELISA kit (ADI), Imuclone Anti-annexin V IgG ELISA kit (ADI), Imuclone anti-Annexin V IgM ELISA kit (ADI), Imuclone anti-Prothrombin IgG ELISA kit (ADI), anti-prothrombin IgM ELISA kit ADI), Imuclone anti-β_2GPI IgG, ELISA kit (ADI), and anti-β_2GPI IgM ELISA kit (ADI), respectively. The aPT antibody ELISA uses human prothrombin for coating the solid ELISA plate. There are no anionic phospholipids on the aPT ELISA plate saturated with goat serum. The detecting antibody used is a goat anti-human Ig (G or M) antibody coupled with peroxidase. The buffer and diluent used in the kits are both calcium-free. In the Imuclone aPL IgG and IgM ELISA kits, the antigen is a mixture of negatively charged phospholipids instead of cardiolipin, and it has β_2GPI. In the Imuclone anti-β_2GPI IgG and IgM ELISA kits, anti-β_2GPI was prepared in human sera. The cutoff value is defined by testing a large group (>60) of normal individuals and some patients with APS. The cutoff value corresponds to the mean value of normal subjects ± 2SD in the LA assay and to the mean value of normal subjects ± 5SD in ELISA assays. All data were expressed as mean ± SD. Statistical analysis was performed by Fishers' test. A P value less than 0.05 denoted statistical significance. The likelihood ratio of a positive result (LR^+) was calculated by the formula LR^+ = sensitivity/(1 − specificity).

RESULTS

The prevalence of LA in patients with thrombosis (DVT and CT) was higher than in those free of thrombosis ($P < 0.01$) (Table I) and was 23.3% in patients with ITP and 54.5% in patients with SLE. The prevalence of anti-β_2GPI IgG in patients with thrombosis was higher than in those without thrombosis ($P < 0.01$) and was 45.5% in patients with SLE and 0% in patients with ITP. There was no significant difference in the prevalence of anti-β_2GPI IgM between patients with thrombosis and those without thrombosis, and the prevalence of the same antibody was 4.5% in SLE and 0% in ITP (Table I). The prevalence of aPT IgG antibody in patients with thrombosis was significantly higher than in those without thrombosis ($P < 0.01$) and was 18.2% in SLE and 0% in ITP. On the other hand, there was no significant difference in the prevalence of aPT IgM antibody between patients with thrombosis and those without thrombosis, and was 0% in SLE and 0% in ITP (Table I). The prevalence of aPL IgG was higher in patients with thrombosis than in those without thrombosis ($P < 0.01$), and was 40.9% in SLE and 6.7% in ITP. On the other hand, there was no

TABLE I. Prevalence of Lupus Anticoagulant (LA), Anti-β_2 Glycoprotein I (Anti-β_2GPI), Anti-prothrombin, Anti-phospholipid, and Anti-annexin V Antibodies in Patients With Deep Vein Thrombosis (DVT)/Pulmonary Embolism (PE), Cerebral Thrombosis (CT), Thrombosis, and Without Thrombosis and in Healthy Volunteers (Control)

	DVT/PE	CT	With thrombosis	Without thrombosis	Control
LA-positive[a]	21/48 (38.6%)	15/30 (50.0%)	35/77 (45.5%)*	28 (28.0%)	0/40 (0%)
Anti-β_2GPI antibodies					
IgG	8/48 (16.7%)	11/30 (36.7%)	18/77 (23.4%)*	4/100 (4.0%)	0/40 (0%)
IgM	0/48 (0.0%)	3/30 (10.0%)	3/77 (3.9%)	3/100 (3.0%)	0/40 (0%)
Anti-prothrombin antibodies					
IgG	14/48 (29.1%)	5/30 (16.7%)	18/77 (23.4%)*	1/100 (1.0%)	0/40 (0%)
IgM	4/48 (8.3%)	2/30 (6.7%)	6/77 (7.8%)	4/100 (4.0%)	1/40 (2.5%)
Anti-phospholipid antibodies					
IgG	10/48 (20.8%)	3/30 (10.0%)	20/77 (26.0%)*	6/100 (6.0%)	0/40 (0%)
IgM	9/48 (18.8%)	7/30 (23.3%)	15/77 (19.5%)	10/100 (10.0%)	2/40 (5%)
Anti-annexin V antibodies					
IgG	10/48 (20.8%)	8/30 (26.7%)	18/77 (23.4%)**	10/100 (10.0%)	2/40 (5%)
IgM	1/48 (2.1%)	3/30 (10%)	4 (5.2%)	6/100 (6.0%)	3/40 (7.5%)

[a]Based on prolonged DVV test of >40 sec and a DVV-test/DVV-confirm ratio of >1.2.
*$P < 0.01$, compared with patients without thrombosis.
**$P < 0.05$, compared with patients without thrombosis.

significant difference in prevalence of aPL IgM in patients with and without thrombosis, and was 27.3% in SLE and 6.7% in ITP (Table I). The prevalence of anti-annexin V IgG was higher in patients with thrombosis than in those without thrombosis ($P < 0.05$), and was 18.2% in SLE and 7.1% in ITP. There was no significant difference in the prevalence of anti-annexin V IgM among patients with thrombosis and those without thrombosis and was 4.5% in SLE and 6.7% in ITP (Table I). The likelihood ratio of positive results (LR^+) of anti-β_2GP1 IgG was markedly high in patients with CT and with thrombosis. Furthermore, LR^+ of PT IgG was significantly high in patients with DVT and thrombosis (Table II). The prevalences of anti-β_2GP1 IgG, aPT IgG, and aPL IgG were significantly higher in patients with APS than in those with LA without thrombosis ($P < 0.01$ each). However, the frequencies of LA and anti-annexin V IgG were not different in the two groups (Table III).

TABLE II. Likelihood Ratio of Positive Results (LR^+) and Likelihood Ratio of Negative Results (LR^-) for Lupus Anticoagulant (LA), Anti-β_2 Glycoprotein I (Anti-β_2GPI), Anti-prothrombin, Anti-phospholipid, and Anti-annexin V Antibodies in Patients With deep Vein Thrombosis (DVT)/Pulmonary Embolism (PE), Cerebral Thrombosis (CT), Thrombosis, and Without Thrombosis and in Healthy Volunteers (Control)

	DVT/PE		CT		With thrombosis	
	LR^+	LR^-	LR^+	LR^-	LR^+	LR^-
LA-positive[a]	1.344	0.834	1.531	0.742	1.623	0.758
Anti-β_2GPI antibodies						
IgG	1.536	0.935	4.90	0.685	5.884	0.798
IgM	0	1.049	4.90	0.919	1.299	0.991
Anti-prothrombin antibodies						
IgG	13.40	0.738	1.76	0.92	23.4	0.774
IgM	1.792	0.961	1.225	0.987	1.948	0.960
Anti-phospholipid antibodies						
IgG	1.680	0.904	3.593	0.705	4.329	0.788
IgM	1.512	0.928	1.906	0.874	1.948	0.895
Anti-annexin V antibodies						
IgG	1.493	0.920	1.960	0.849	2.338	0.851
IgM	0.299	1.053	2.100	0.945	0.866	1.009

[a]Based on prolonged DVV-test of >40 sec and a DVV-test/DVV-confirm ratio of >1.2.

TABLE III. Prevalence of Anti-phospholipid Antibodies in Patients With Anti-phospholipid Syndrome (APS) and Lupus Anticoagulant (LA)-Positive Patients Without Thrombosis

	LA	Anti-β_2GPI IgG	Anti-PT IgG	Anti-PL IgG	Anti-annexin V IgG
APS	36/38 (94.7%)	19/38* (50.0%)	11/39* (28.2%)	16/39* (29.2%)	10/39 (25.6%)
LA without thrombosis	18/18 (100%)	0/18 (0.0%)	0/18 (0.0%)	0/18 (0.0%)	3/18 (16.7%)

*$P < 0.01$, compared between patients with APS and LA patients without thrombosis.

DISCUSSION

Our results showed that LA was positive in 38.6% of patients with DVT/PE, suggesting that LA is the most important risk factor in DVT/PE. However, previous reports indicated that the prevalence of anti-phospholipid antibodies was less in patients with DVT/PE than in those with arterial thrombosis, although abnormalities of AT [19], protein C [20], and protein S [21] were reported to be more frequent in patients with DVT/PE. With respect to other conditions, our results showed that the prevalence of LA was the highest in collagen diseases. Previous studies reported that APS is frequently detected in patients with collagen diseases [6,7]. Because LA was observed in patients with ITP and those with SLE free of thrombosis as well as in healthy volunteers, this antibody does not seem to be a risk factor for thrombosis directly. In agreement with this conclusion, LA was also reported in patients without thrombosis [22].

Our results also showed a high prevalence of anti-β_2GP1 IgG in CT and SLE, followed by DVT, but was not detected in any of the patients with ITP and the control subjects, suggesting that this antibody is related to thrombosis, especially arterial thrombosis. Cerebral ischemia associated with anti-phospholipid antibody is the most common arterial thrombotic manifestation [23,24]; however, the importance of this antibody as a cardiovascular risk factor is controversial. Since only a few patients with thrombosis were positive for anti-β_2GP1 IgM, this antibody may be low risk factor for thrombosis. In this regard, previous studies [25,26] reported the detection of IgG, and indicated that the presence of IgM anti-β_2GP1 correlated significantly with a history of thrombosis. In particular, Guerin et al. [25] used both anti-β_2GP1 and conventional aCL assays and found that anti-β_2GP1 IgG assays improved the diagnostic specificity relative to the conventional aCL assays.

The present study also showed a moderately high prevalence of aPT IgG antibody in patients with DVT, followed by those with CT and SLE, but in none of the patients with ITP and healthy volunteers, suggesting that it is related to thrombosis, especially venous thrombosis. In 1959, Loeliger [27] was the first to propose that prothrombin, another phospholipid binding protein, is a possible cofactor for LA. A positive correlation between the presence of aPT antibody and DVT was also reported in patients with SLE [28]. In a study of 265 cases with DVT/PE, the risk of thrombotic events was significantly high in carriers of aPT antibody [29]. Because the plasma levels of activated protein C are significantly elevated in patients with DVT/PE [30,31] and LA [32], activation of the coagulation system plays an important role in the onset of DVT/PE, suggesting that aPT is important in these states. On the other hand, activation of platelet plays an important role in the onset of arterial thrombosis. Anti-β_2GP1 antibody may play a more important role in the onset of arterial thrombosis than aPT antibody.

Finally, our results showed moderately high prevalences of aPTIgG, anti-β_2GP1, and aPL IgG antibodies in patients with DVT, CT, and SLE, but these were not present in patients with ITP or in healthy volunteers. Based on the experiences of our hospital, which is a referral center for patients with autoimmune diseases, the prevalence of anti-phospholipid antibodies in our patients with DVT seems to be high. Similarly to the antibodies named above, this antibody may be related to thrombosis. On the other hand, our results demonstrated the presence of aPT IgM, aPL IgM, anti-annexin V IgG, and anti-annexin V IgM antibodies in patients with and without thrombosis, suggesting that they are not strongly related to thrombosis.

In conclusion, we have demonstrated in the present study that LA is the most sensitive antibody for APS among those tested in the study but that it is not specific to APS and LA without thrombosis. Our results also showed that the prevalence of antiphospholipid antibody was high in specific states: aPT IgG in DVT/PE, anti-β_2GP1 IgG in CT and SLE, and aPL in SLE.

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The Japanese Experience With Thrombotic Thrombocytopenic Purpura–Hemolytic Uremic Syndrome

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A total of 290 Japanese patients with thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP-HUS) were analyzed with respect to ADAMTS-13 activity and its inhibitor. Twenty-one patients (17 families) had Upshaw-Schulman syndrome, and 12 patients (six families) had familial HUS of undetermined etiology. The number of patients with acquired HUS and TTP was 44 and 213, respectively. In acquired TTP, patients with severe deficiency of ADAMTS-13 activity secondary to the presence of an inhibitor were high responders to plasma exchange, but others were low responders to plasma exchange. The former patients were associated with "idiopathic" TTP, drugs, and pregnancy, and the latter patients with malignancy and stem cell transplantation. Patients with autoimmune disease-associated TTP fit into both groups.

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THROMBOTIC thrombocytopenic purpura (TTP) is a life-threatening disease, characterized by Moschcowitz's pentad[1]: thrombocytopenia, microangiopathic hemolytic anemia (MAHA), fluctuating neurological signs, renal failure, and fever. The majority of patients with TTP are adults who suddenly develop clinical signs without any underlying disease; this is referred to as "idiopathic" TTP. However, in a significant number of patients TTP is associated with a variety of clinical conditions, including autoimmune disease, stem cell transplantation, malignancy, drugs, pregnancy, and infections. These patients are classified as having "secondary" TTP.[2-4]

By contrast, hemolytic-uremic syndrome (HUS) is characterized by Gasser's triad[5]: MAHA, thrombocytopenia, and renal insufficiency. Most childhood HUS patients have Shiga-like toxin-producing *Escherichia coli* 0157:H7 infection, usually accompanied by bloody diarrhea. Except for these distinctive cases, however, differential diagnosis between TTP and HUS is often difficult in clinical practice, and therefore the comprehensive term TTP-HUS or thrombotic microangiopathy (TMA) has been used.[2-4]

Recent advances in elucidating the proteolytic processing of plasma von Willebrand factor (VWF) multimers have established assays for VWF-cleaving protease (VWF-CP) activity and its inhibitor (autoantibody).[6,7] These assays made it possible to distinguish TTP from HUS, because the former shows defective enzymatic activity with or without presence of its inhibitor, and the latter has exclusively normal activity. VWF-CP is now identified as a metalloproteinase belonging to the ADAMTS (A Disintegrin And Metalloproteinase domain, with ThromboSpondin type I motif) family, termed ADAMTS-13.[8-11] ADAMTS-13 is produced in the liver, exclusively in the perisinusoidal cells.[12]

In early 1998, we began to quantify VWF-CP/ADAMTS-13 activity and its inhibitor, based on previous methods.[13] Over the past 5 years, our laboratory has been the only facility in Japan able to assay these activities. In this regard, we were fortunate to have the opportunity to collect blood specimens and analyze the activities in a variety of diseases, at the request of hospitals across Japan. This system still works, and analysis of the accumulated data is still underway. Here we report on data from 290 patients with TTP-HUS, clinically diagnosed by physicians in referring hospitals, with special reference to the activity of ADAMTS-13 and its inhibitor.

Methods and Patients

Plasma ADAMTS-13 activity was assayed based on VWF multimer analysis,[13] with a slight modification as previously described.[14] The normal range of ADAMTS-13 activity (n = 60) in our laboratory was

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