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Detection of von Willebrand factor-cleaving protease (ADAMTS-13) in human platelets☆

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Abstract

The hemostatic activity of von Willebrand factor (vWF) is strongly dependent on its multimeric structure, with the highest activity in 'unusually large' multimers secreted from endothelial cells. The multimeric structure is regulated by a plasma protease, vWF-cleaving protease (vWF-CP, or ADAMTS-13). ADAMTS-13 mRNA is variably expressed in liver and other tissues. Because 15–25% of circulating vWF is stored in platelets, the presence and function of ADAMTS-13 in platelets are important issues. Here we report ADAMTS-13 expression in human platelets. Western blot analysis and flow cytometric analysis on permeabilized platelets revealed the presence of ADAMTS-13 protein. Real-time PCR demonstrated that ADAMTS-13 mRNA is present in platelets of six healthy volunteers, with little quantitative difference. The presence of ADAMTS-13 in platelets may imply the functional role of this enzyme in the local regulation of platelet function at the site of vascular injury or thrombus formation, and provide a useful tool for the analysis of structure and function of this enzyme.

Keywords: Platelets; vWF-CP; ADAMTS-13; vWF; TTP; Thrombosis; Expression; Real-time PCR

von Willebrand factor (vWF) plays a key role in normal hemostasis, by mediating platelet adhesion to the site of vascular damage and by acting as a carrier protein for coagulation factor VIII. vWF is synthesized in vascular endothelial cells and megakaryocytes and is present not only in the plasma but also in the subendothelial matrices. Fifteen to 25% of circulating vWF is stored in the α-granules of platelets. Stimulation of endothelial cells or platelets by thrombin or ADP induces vWF release into plasma as unusually large multimeric forms (UL-vWF), which are more active than the smaller multimeric forms of vWF. UL-vWF are rapidly degraded to smaller multimeric forms ranging in size from 500 to 20,000 kDa in normal human plasma. The degradation depends on the presence of the von Willebrand factor-cleaving protease (vWF-CP or ADAMTS-13), which cleaves vWF at the peptide bond between residues Tyr 842 and Met 843 [1,2].

Thrombotic thrombocytopenic purpura (TTP) is a generalized disorder characterized by the classic pentad of thrombocytopenia, microangiopathic hemolytic anemia, neurologic signs, renal impairment, and fever. UL-vWF are present in TTP patients' plasma. Recent studies revealed that the presence of UL-vWF is due to the loss of vWF-CP activity caused by an acquired autoantibody to vWF-CP [3,4] or by inherited deficiency of vWF-CP in patients with congenital forms of TTP [5]. UL-vWF present in the plasma are believed to cause platelet aggregation in normal circulation, resulting in microthrombus formation, which underlies the pathogenesis of TTP.

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Partial purification and characterization of vWF-CP [1,2] was recently followed by purification of human vWF-CP, allowing for determination of the N-terminal amino acid sequence and the cDNA sequence encoding vWF-CP. vWF-CP is designated ADMTS-13, a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin type1 motif) family [6,7]. Based on the data that a 4.7-kb ADAMTS-13 transcript is detected specifically in liver [6,8,9] while an approximately 2.3-kb transcript is faintly visible in placenta [8], the plasma vWF-CP is considered to be derived from the liver. Partial cDNAs for ADAMTS-13 were isolated from brain and prostate. A strong reverse transcription-polymerase chain reaction (RT-PCR) signal in ovary and variable expression in other tissues points to other potential functions for ADAMTS-13 [8]. There is weak ADAMTS-13 mRNA expression in kidney, pancreas, spleen, thymus, prostate, testis, small intestine, and peripheral blood leukocytes [8]. Semiquantitative PCR analysis revealed ADAMTS-13 mRNA expression in a panel of human tissue. Although a low level of expression is observed in bone marrow, there is no expression in peripheral blood leukocytes [10]. None of the reports mentioned expression in platelets. Because ADAMTS-13 cleaves vWF and there are no other known substrates, the physiologic relevance of ADAMTS-13 expressed in the above-mentioned tissues is unclear.

In view of platelet thrombus formation in which vWF binding to platelets plays an essential role, the presence and function of ADAMTS-13 in platelets are important issues. Therefore, we set out to determine whether ADAMTS-13 protein is expressed in platelets.

Materials and methods

Blood donors. Blood was obtained from six healthy volunteers who had neither previous diseases nor past disease history. Informed consent was obtained prior to performing all experiments using blood samples.

Preparation of monoclonal antibody against the peptides of ADAMTS-13. Antipeptide monoclonal antibody was generated using a region between the eighth Tsp1 motif and the first CUB domain as an antigen according to the standard protocols. Briefly, Balb/c mice were immunized with a keyhole limpet hemocyanin (KLH)-conjugated peptide (KLH-CFSPAPQPRRLLPGPQENSVQSS) using Freund's adjuvant. Conditioned media from hybridoma cultures were screened by ELISA and Western blot analysis using recombinant ADAMTS-13 protein.

Western blot analysis. Blood obtained from peripheral vein was immediately mixed with sodium citrate at a final concentration (f.c.) of 3.8%. Platelet-rich plasma (PRP) was prepared by centrifugation at 100*g* for 15 min at room temperature. Two-third volumes of ACD-A solution (sodium citrate 2.2 w/v%, citric acid 0.8 w/v%, and glucose 2.2 w/v%, pH 4.5–5.5, Terumo, Tokyo, Japan), prostagrandin E1 (PGE1, 1.2 μM, f.c.), and EDTA (1.2 μM, f.c.) was added to PRP to prevent aggregation. The PRP was filtrated twice through Sepacell PLX (Asahi Medical, Tokyo, Japan) for the purpose of leukocyte depletion, followed by centrifugation at 780*g* for 7 min at room temperature. Platelet pellet was washed with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes)–Tyrode's buffer in the presence of ACD-A and PGE1, and resuspended in the Hepes–Tyrode's buffer. Washed platelets were then mixed with Laemmli sample buffer (1:1) containing 5% of 2-mercaptoethanol and subjected to SDS/PAGE (5–15% gradient gel). Approximately 2×10^7 platelets were applied for one lane. Recombinant ADAMTS-13 was used as a positive control. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, CA, USA), followed by blocking of the membrane with 1% skim milk. The membrane was then incubated with the mouse anti-ADAMTS-13 monoclonal antibody (1 μg/ml). After washing three times, the membrane was incubated with horseradish-peroxidase-conjugated sheep IgG against mouse Ig (Amersham–Pharmacia Biotech, NJ, USA). Chemiluminescence was developed by using the Western Lightening Chemiluminescence Reagent Plus (Perkin–Elmer, MA, USA). Recombinant ADAMTS-13 was prepared according to the method previously described [10]. The culture medium of HEK293 cells in which recombinant ADAMTS-13 was expressed was collected and 3-fold concentrated.

Flow cytometric analysis. PRP (4.8×10^8 platelets/ml) was incubated with a mouse anti-ADAMTS-13 monoclonal antibody (1 μg/ml, f.c.), anti-human CD41 (5B12, 2.25 μg/ml, f.c.; Dako Cytomation, Kyoto, Japan), anti-β-actin antibody (AC15, 25 μg/ml, f.c.; Sigma, MO, USA), or anti-human CD45 (T29/33, 3 μg/ml, f.c.; Dako Cytomation) for 1 h at room temperature. For a control study, mouse IgG1 (1.125 μg/ml, f.c.; Dako Cytomation) was used as the primary antibody. Platelets were pretreated with 0.1% NP40 for permeabilization. Permeabilized samples were incubated with primary antibodies under the same conditions as non-permeabilized samples. After incubation with primary antibodies, all the samples were fixed with 4% paraformaldehyde (PFA), followed by incubation with FITC-conjugated rabbit F (ab′)2 fragments of anti-mouse IgG antibody (10 μg/ml, f.c.; Dako Cytomation) for 30 min at room temperature. They were then washed with PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, Bio-Rad) containing PGE1 and analyzed with a flow cytometer (COULTER EPICS XL-MCL Flow-Cytometer; Beckman, FL, USA). Data were analyzed using System II software.

Analysis of platelet mRNA. Platelet RNA was isolated as previously described [11] with some modifications. PRP was prepared as described above. Leukocyte depletion was performed using Sepacell. Absence of leukocyte contamination was confirmed by negative amplification of CD20 on quantitative RT-PCR. Platelet pellets were treated with TRIZOL (Invitrogen, CA, USA). Total RNA was extracted from the solubilized platelet pellet with chloroform and precipitated with isopropanol. Dried platelet RNA was dissolved in RNase-free water. To prevent RNA degradation, platelet RNA was amplified by RT-PCR as soon as possible. Platelet RNA (3 μg) was heated for 10 min at 70 °C and reverse-transcribed for 65 min at 42 °C with the TrueScript II Reverse Transcriptase kit (Sawady Technology, Tokyo, Japan) in a final volume of 20 μl with reagent concentrations according to the manufacturer's instructions. Specific oligonucleotide primers were used for reverse transcription. The primers for amplification of the cDNA, corresponding to exon 1–7 of VWF-CP were: a sense primer, RT-1(5′-ATGCACCAGCGTCACCCCCG-3′) and an anti-sense primer RT-2 (5′-AATGGTGACTCCCAGGTCGA-3′). PCR was performed in a total volume of 50 μl containing 0.5 U TaKaRa LA *Taq* with GC buffer (Takara Shuzo, Shiga, Japan), 4 μM dNTP, and 0.5 μM each of the forward and reverse primers. Samples were incubated for 5 min at 96 °C and amplified for 35 cycles with the following cycle profile: 1 min at 96 °C, 1 min at 52 °C, and 1 min at 72 °C. Samples were fractionated on a 2% agarose gel stained with ethidium bromide. The amplified DNA was purified and recovered from agarose gels using a GENECLEAN II kit (Bio 101, CA, USA) according to the manufacturer's protocol. The nucleotide sequences were determined by the direct sequencing method with an ABIPRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer, Chiba, Japan) using a Model 310 DNA autosequencer (Applied Biosystems, CA, USA).

Real-time PCR. After RNA isolation and cDNA preparation of each sample, the specific cDNA of interest and β-actin reference cDNA

were separately PCR-amplified using a fluorescent oligonucleotide probe with a 5′ reporter dye (FAM) and a downstream 3′ quencher dye (TAMRA). During PCR, the 5′ to 3′ nuclease activity of *Taq* DNA polymerase releases the reporter, whose fluorescence can be detected by the laser detector of the ABI Model 7700 Sequence Detection System (Applied Biosystems). After crossing the fluorescence threshold, the PCR-amplification results in linear curves of the corresponding PCR products. After establishing the threshold fluorescence intensity, the cycle at which this occurred was defined as the threshold cycle. Internal positive and negative controls (samples without polymerase) were performed in parallel during different determinations to assure equivalent assay conditions. The following oligonucleotides were used: forward ADAMTS-13 primer, 5′-CCCAACCTGACCAGTGTCTA CA-3′ (exon 15); reverse ADAMTS-13 primer, 5′-CTTCCCAGCCA CGACATAGC-3′ (exon16); and TaqMan probe, 5′-FAM-ACAGG CCTCTCTTCACACACTTGGCG-TAMRA-3′ (exon 15–16). The PCR mixture (50 μl) consisted of 0.5 μM of each primer, 0.25 μM probe, and TaqMan Universal Master Mix (2×). The following cycle conditions were used: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min, using the ABI Prism 7700 sequence detection system. Real-time PCR data were expressed as relative quantity based on the ratio of fluorescent change. CD20 was also amplified to monitor the contamination of leukocytes.

Results

Western blot analysis

The monoclonal antibody directed against a region between the eighth Tsp1 motif and the first CUB domain of ADAMTS-13 detected an immunoreactive band at approximately 220 kDa, which was identical to the size of the band detectable in the culture medium containing recombinant ADAMTS-13 (Fig. 1). These findings indicate monospecificity of the antibody and the expression of ADAMTS-13 in human platelets.

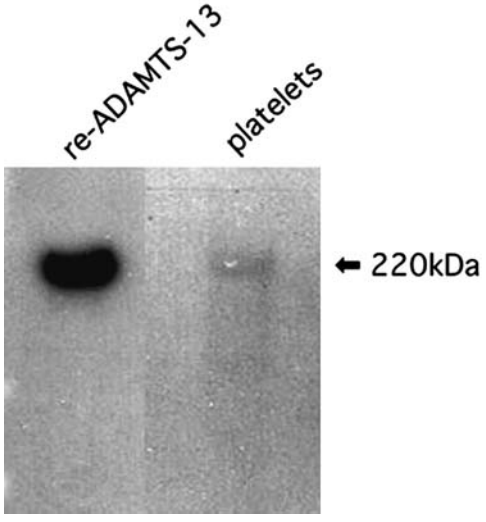

Fig. 1. Western blot analysis of ADAMTS-13 expression in platelets. Approximately 2×10^7 washed platelets and culture medium containing recombinant ADAMTS-13 were subjected to Western blot analysis as described in Materials and methods. Left lane, culture medium containing recombinant ADAMTS-13; right lane, platelets.

Flow cytometric analysis

As shown in Fig. 2A, permeabilized platelets showed a significant increase in the reactivity to the anti-ADAMTS-13 antibody, although the reactivity was weaker than that of β-actin used as a positive control. Fig. 2B indicated the different reactivity toward the anti-ADAMTS-13 antibody between permeabilized and non-permeabilized platelets, suggesting that ADAMTS-13 antigen is localized inside the platelets. On the other hand, no difference was observed between non-permeabilized and permeabilized platelets when a control antibody was used (data not shown). To prove that the particles analyzed in flow cytometry were platelets, we

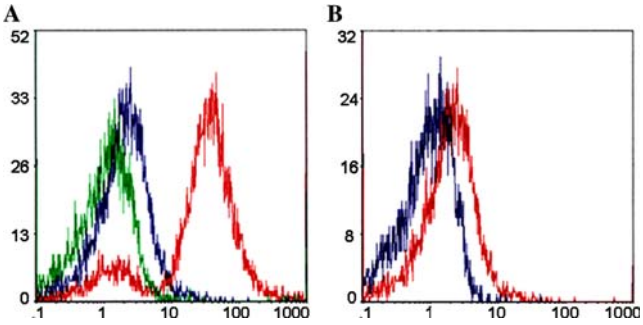

Fig. 2. Flow cytometric analysis of ADAMTS-13 expression. (A) Platelets were treated with 0.1% NP40 for permeabilization and incubated with mouse anti-ADAMTS-13 monoclonal antibody (navy line), mouse monoclonal anti-β-actin antibody (red line) as a positive control, or mouse IgG1 as a negative control (green line). (B) Permeabilized vs non-permeabilized platelets. The same experiment was carried out using non-permeabilized platelets (navy line) comparing with permeabilized platelets (red line). All the samples fixed with 4% PFA were incubated with FITC-conjugated rabbit F (ab′)2 fragments of anti-mouse IgG antibody as a second antibody.

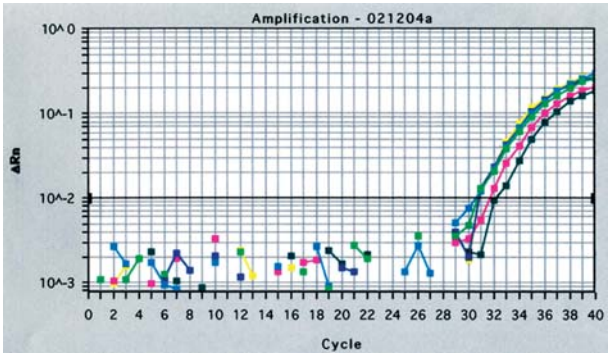

Fig. 3. Representative presentation of quantitative real-time PCR for expression of platelet ADAMTS-13 mRNA. PCR amplification was performed separately for mRNAs from six volunteers with specific primers and specific fluorescent probes for ADAMTS-13. The relative normalized fluorescence change (ΔR_n) versus time (PCR cycle number) is plotted for each sample. After defining the threshold fluorescence intensity (0.01, solid horizontal line), the threshold cycle was determined for each sample. Relative gene expression was determined based on the threshold cycles of the gene of interest and of the internal standard.

examined whether leukocyte common antigen, CD45, was expressed in non-permeabilized and permeabilized samples and found no CD45 expression, excluding the possibility of significant leukocyte contamination.

RT-PCR analysis and sequencing of ADAMTS-13

Platelet RNA isolated from citrated PRP was amplified by RT-PCR. The amplified DNA was purified and recovered from agarose gels. A cDNA fragment of the expected size (660 bp) was obtained and subjected to direct sequencing analysis. The determined nucleotide sequences were identical to ADAMTS-13 cDNA (exon 1–7), when compared using the BLAST (Basic local alignment search tool) program.

Real-time PCR

Platelet ADAMTS-13 mRNA expression was quantitatively analyzed by real-time PCR. As shown in Fig. 3, ADAMTS-13 mRNA was expressed in platelets of all six samples analyzed and there was little difference in the threshold cycles (triplicate) between them: PRP 1: 28.38, 29.02, and 28.18; PRP 2: 28.46, 27.83, and 28.19; PRP 3: 28.79, 29.05, and 29.39; PRP 4: 29.78, 29.60, and 29.47; PRP 5: 28.92, 29.01, and 28.56; and PRP 6: 29.98, 30.85, and 30.59. Data were also expressed as the ratio of the threshold cycle of the internal standard β-actin and that of ADAMTS-13 [12]. The results were as follows: PRP 1, 0.563 ± 0.007; PRP 2, 0.570 ± 0.005; PRP 3, 0.567 ± 0.005; PRP 4, 0.572 ± 0.003; PRP 5, 0.583 ± 0.004; and PRP 6, 0.563 ± 0.007 (means ± SD from three independent determinations; Table 1). Efficacy of leukocyte depletion using a filter system was confirmed by the absence of CD20 amplification by real-time PCR (Table 2).

Table 1
Ratio of the threshold cycle of β-actin versus that of ADMTS-13

	Ct of β-actin	Ct of ADAMTS-13	β-Actin/ADAMTS-13
PRP 1	15.99 ± 0.24	28.52 ± 0.36	0.563 ± 0.007
PRP 2	16.06 ± 0.11	28.16 ± 0.26	0.570 ± 0.005
PRP 3	16.47 ± 0.03	29.08 ± 0.25	0.567 ± 0.005
PRP 4	16.95 ± 0.06	29.62 ± 0.13	0.572 ± 0.003
PRP 5	16.79 ± 0.34	28.83 ± 0.19	0.583 ± 0.004
PRP 6	17.13 ± 0.04	30.47 ± 0.36	0.563 ± 0.007

Data are expressed as the ratio of the threshold cycle (Ct) of the internal standard β-actin vs. ADAMTS-13. PRP 1–PRP 6 are from six individuals. Values are means ± SD from three independent determinations.

Table 2
Quantitation of platelet ADAMTS-13 mRNA and CD20 mRNA using real-time fluorescence PCR

	Ct of ADAMTS-13	Ct of CD20
Platelet-1	38.75/38.71	40.00/39.49
Platelet-2	36.62/36.59	40.00/40.00
Lym-1	32.47/34.10	26.43/27.29
Lym-2	35.91/35.25	27.30/27.53
Buffy-1	29.34/27.52	26.46/26.27
Buffy-2	34.43/34.57	26.36/26.22

PCR was performed separately on mRNAs from two volunteers (1 and 2) with specific primers and specific fluorescent probes for ADAMTS-13 or CD20. Platelet-1 and 2 are cDNA of platelets after leukocyte depletion. Lym-1 and 2 are cDNA of lymphocytes collected using Lympho-Prep (AXIS-SHIELD ProC AS, Oslo, Norway). Buffy-1 and 2 are cDNA of buffy coat.

Discussion

In the present study, we demonstrated for the first time the expression of ADAMTS-13 in platelets. Our results indicate that ADAMTS-13 protein exists inside the platelets, but not on the surface of platelets. We established real-time PCR as a useful method for quantitative evaluation of ADAMTS-13 mRNA expression. ADAMTS-13 mRNA was expressed in platelet samples from all six healthy subjects with little quantitative difference. The slight difference between samples seemed to reflect differences in the RNA turnover time in each sample. Our results suggest that platelets have a fairly constant amount of ADAMTS-13 mRNA and protein, and that ADAMTS-13 in platelets is not derived from plasma by endocytosis.

In this study, the primers corresponding to exon 1–7 of vWF-CP were used for RT-PCR and the amplified fragment was sequenced. Moreover, the fluorescent oligonucleotide probe located in exon 15–16 of ADAMTS-13 was used for real-time PCR. The results excluded the amplification of possible contaminating genomic DNA in the test samples and suggested that ADAMTS-13 mRNA corresponding to at least exon 1–16 was expressed in platelets. Alternative splicing is a common feature of ADAMTS gene families and there are some variant transcripts for ADAMTS-13 in liver, prostate, and brain [6]. Further investigation into alternative splicing in ADAMTS-13 is needed for blood cells. In addition, morphologic studies to localize ADAMTS-13 in the platelet structure might provide clues to the role of ADAMTS-13 in platelets. Platelet ADAMTS-13 is a good source for functional analysis of ADAMTS-13.

Fifteen to 25% of circulating vWF is stored in the α-granules of platelets and is released into the plasma by stimulation of thrombin or ADP. ADAMTS-13 in platelets might regulate the multimeric size of vWF. Because the character of vWF derived from platelets might be one factor that regulates platelet thrombus formation in local tissues, the establishment of a method for evaluating ADAMTS-13 activity in platelets might be of clinical relevance for predicting a predisposition to thrombosis. An autoantibody to ADAMTS-13 could bind to platelets in TTP patients, a possible mechanism of autoimmune thrombocytopenia, which complicates

TTP. In such cases, detection of anti-ADAMTS-13 antibody might be a tool for the diagnosis of TTP. Thus, the presence of ADAMTS-13 in platelets is important in the investigation of the pathogenesis of platelet thrombus formation.

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