Linear Relationship between ADAMTS13 Activity and Platelet Dynamics Even Before Severe Thrombocytopenia

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Abstract. Von Willebrand factor (VWF) cleaving metalloprotease, ADAMTS13, known for its causative relation to thrombotic thrombocytopenic purpura (TTP), also decreases to variable degree in other clinical conditions associated with thrombocytopenia, indicating a possible contribution of moderate deficiency of ADAMTS13 to platelet dynamics. We measured ADAMTS13 activity along with VWF activity, collagen binding activity (VWF:CB), and thrombin/antithrombin complex (TAT) in plasma drawn from patients with consumptive coagulopathy, in whom the platelet count was closely followed. ADAMTS13 activity was significantly but variably decreased in the patients, and VWF activity and VWF:CB were markedly increased as expected. The platelet count itself was not correlated with ADAMTS13 activity, VWF activity, or VWF:CB. However, the rate of decline of log-scaled platelet count (ΔLnPLT/day) correlated well with ADAMTS13 activity and VWF:CB. ADADMTS13 activity showed inverse correlation with VWF:CB. Moreover, the correlation between ADAMTS13 and ΔLnPLT/day was preserved even after VWF:CB was controlled. Multiple regression analysis showed that ADAMTS13 activity was the sole factor explaining ΔLnPLT/day among ADAMTS13, VWF:CB, TAT, prothrombin time, d-dimer, and fibrinogen. TAT level and d-dimer as indicators of systemic fibrinolytic activity did not correlate with ADAMTS13 activity. In conclusion, we found that the decrease of ADADMTS13 activity in consumptive coagulopathy has stronger relationship to platelet dynamics than has generally been recognized.

Keywords: platelet dynamics, thrombocytopenia, ADAMTS13, thrombin-antithrombin complex

Introduction

ADAMTS13, a metalloprotease in plasma that cleaves von Willebrand factor (VWF) multimer, was first known for its association with Upshaw-Schulman syndrome, a congenital form of thrombotic microangiopathy [1-7]. It was also implicated in thrombotic thrombocytopenic purpura (TTP), in which proteolytic activity is inhibited by autoantibodies [8-10]. Depressed ADAMTS13 activity allows the ultra-large form of VWF (UL-VWF) to remain uncut and results in unopposed adhesion of platelets to VWF circulating in blood or anchored on endothelial surface, with subsequent platelet plug formation. ADAMTS13 activity also decreases to variable degree in clinical conditions associated with thrombocytopenia other than the two conditions just mentioned [11,12].

Based on these findings, it may be presumed that decreased ADAMTS13 activity plays a role in the development of thrombocytopenia. The fact that von Willebrand disease type 2B originating from abnormally high affinity of VWF for platelet glycoprotein Ib often accompanies mild thrombocytopenia is an attractive example. Although ADAMTS13 activity does not seem to be affected, this mutant VWF simulates hyperactive UL-VWF.
escaping ADAMTS13 cleavage. Most previous studies evaluating both ADAMTS13 and platelet count emphasize the importance of ADAMTS13 activity for the accurate diagnosis of TTP. The cause and effect relationship between ADAMTS13 activity and thrombocytopenia itself was not seriously considered, given the presentation of inconclusive data. To clarify this we measured ADAMTS13 activity in plasma obtained from patients with suspected disseminated intravascular coagulation (DIC) or consumptive coagulopathy, which were known to be associated to variable degrees both with decreased ADAMTS13 activity and thrombocytopenia. We followed the platelet count throughout the day plasma was obtained. Because of difficulty in putting platelet counts directly into analysis due to the wide range of baseline values, we introduced a derived value calculated from the declining rate of log-scaled platelet count and found good correlation with ADAMTS13 activity.

To probe the cause of depressed ADAMTS13 activity in consumptive coagulopathy, we measured thrombin/antithrombin (TAT) complex as an indicator of systemic thrombin activity and examined its relationship with ADAMTS13 activity to see if over-activated thrombin contributes to diminution of ADAMTS13 activity. This concept was based upon a recent report that thrombin cleavage of ADAMTS13 at the site C-terminal to the spacer domain causes inactivation of ADAMTS13 in vitro [13].

**Materials and Methods**

**Patients.** Blood was drawn in sodium citrate tubes from 46 patients suspected to have consumptive coagulopathy. The patients were 26 males and 20 females and their ages ranged from 9 mo to 87 yr with a median age of 55 yr. The underlying conditions of the patients included cancer in 13 patients, sepsis or septic shock in 16, stroke in 6, and miscellaneous clinical conditions in 11. All had prolonged prothrombin time and the diagnosis of DIC was established according to the scoring system proposed by SSC/ISTH, taking in account platelet count, D-dimer level, and fibrinogen level. The values of the clinical laboratory tests are summarized in table 1.

**Platelet count and ΔLnPLT/day.** Platelet counts of all patients were followed throughout the day the blood was drawn. Transfusion history was carefully examined, since blood transfusion would disturb the changing course of platelet count and could be the source of exogenous ADAMTS13 and other plasma proteins. We intended to calculate the rate at which platelet count changes to prevent the extreme variability of baseline platelet counts among individuals from influencing the analysis of the relationship between platelets and other parameters. In patients who develop thrombocytopenia, the platelet count usually does not decrease linearly but rather exponentially. Thus, if calculated directly from platelet count, the rate of change will diminish as platelet count approaches the nadir. Blunted rate of decrease in low platelet count does not indicate that the cause of thrombocytopenia has subsided. We converted the platelet counts into a log scale so that the change in low platelet count is not overlooked and we calculated the rate of change from the converted values, which we called ΔLnPLT/day. By doing this, the rate at which thrombocytopenia develops remains relatively stable from the baseline platelet count to the nadir platelet count. Patients who received transfusion of platelet or other blood components during the interval were excluded from consideration (4 of 46). Patients with a follow-up interval of blood cell count longer than 2 days were also excluded (2 of 46), along with those whose platelet count rose steadily during the follow-up interval (3 of 46).

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Table 1. Patient characteristics and summary of laboratory data related to DIC scoring (mean ± SD; RI = reference interval).

<table>
<thead>
<tr>
<th>Disease (n)</th>
<th>age (yr)</th>
<th>Sex (M/F)</th>
<th>DIC score</th>
<th>Platelet count (RI = 15-40/μl)</th>
<th>Prothrombin time (RI = 11-15 sec)</th>
<th>Fibrinogen (RI = 154-358 mg/dl)</th>
<th>D-dimer (RI = 0.0-0.4 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis (16)</td>
<td>49±28</td>
<td>9/7</td>
<td>4 ± 2</td>
<td>162 ± 122</td>
<td>21.3 ± 9.2</td>
<td>452 ± 201</td>
<td>26.64 ± 52.09</td>
</tr>
<tr>
<td>Cancer (13)</td>
<td>62±10</td>
<td>6/7</td>
<td>4 ± 1</td>
<td>103 ± 73</td>
<td>21.0 ± 7.7</td>
<td>361 ± 198</td>
<td>11.73 ± 10.91</td>
</tr>
<tr>
<td>Stroke (6)</td>
<td>53±25</td>
<td>3/3</td>
<td>3 ± 1</td>
<td>226 ± 202</td>
<td>17.5 ± 4.4</td>
<td>518 ± 104</td>
<td>4.70 ± 3.88</td>
</tr>
<tr>
<td>Miscellaneous†(11)</td>
<td>41±17</td>
<td>8/3</td>
<td>4 ± 2</td>
<td>155 ± 134</td>
<td>21.8 ± 8.2</td>
<td>281 ± 170*</td>
<td>14.77 ± 28.65</td>
</tr>
<tr>
<td>Total (46)</td>
<td>51±22</td>
<td>26/20</td>
<td>4 ± 2</td>
<td>152 ± 129</td>
<td>20.8 ± 8.2</td>
<td>394 ± 198</td>
<td>16.73 ± 34.39</td>
</tr>
</tbody>
</table>

† Miscellaneous category includes trauma, postpartum hemorrhage, and peptic ulcer bleeding.

* p = 0.037 by Kruskal-Wallis test.
**ADAMTS13 activity.** Plasma ADAMTS13 activity was measured by a fluorescence resonance energy transfer method using a fluorogenic substrate, FRETs-VWF73 (Peptide International, KY, USA). It contains the 73-amino acid residues from D1596 to R1668 of VWF, which is the minimal essential portion for proteolytic activity of ADAMTS13 on VWF. Residues Q1599 (P7) and N1610 (P5') of the 73 amino acid peptide are converted to a 2,3-diaminopropionic residue (A2pr) modified with a 2-(N-methylamino)-benzoyl group (Nma) and with a 2,4-dinitrophenyl group (Dnp), respectively. Dnp acts as a quencher and when the Nma group is excited at 340 nm, with the bond between Y1605 and M1606 cleaved, the energy transfer that quenches the fluorescence does not occur, allowing the emission of fluorescence at 440 nm from Nma [14]. Briefly, FRETs-VWF73 dissolved in 25% dimethyl sulfoxide /water to 100 μmol/L was used as stock solution. Zero to 8 μl of pooled human plasma as standards, and 4 μl of each test plasma were diluted in 100 μl of assay buffer (5 mmol/l Bis–Tris, 25 mmol/l CaCl2, 0.005% Tween-20, pH 6.0) in a 96-well white plate (Nunc, Denmark). One hundred micro liters of 4 μmol/L FRETs-VWF73 assay buffer was added to each well and fluorescence was measured at 30°C every 5 min in a FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany) with excitation at 340 nm and emission at 450 nm. The reaction rate calculated by linear regression was compared with standards.

**VWF activity and collagen binding activity.** Plasma VWF activity and its collagen binding ability (VWF:CB) were measured by enzyme-linked immunosorbent assay (ELISA) using a VWF activity kit (Axis-Shield Diagnostic, UK) that detects a functional epitope of VWF, and a collagen binding assay kit (Gradipore, Australia), according to the procedure provided by the vendor. In the collagen binding assay, plasma VWF binds to the collagen-coated surface of a micro-well plate during incubation. Subsequently, the VWF bound to collagen after washing is detected photometrically by peroxidase conjugated anti-human VWF antibody and TMB substrate.

**Thrombin/antithrombin complex.** Thrombin/antithrombin complex (TAT) was measured with Enzygnost TAT micro ELISA kit (Dade-Behring, Germany) according to the procedure provided by the vendor. TAT is known to increase in patients predisposed to DIC. Briefly, during the first incubation, the TAT in plasma binds antibodies against thrombin that are attached to the surface of a microtiter plate. In a second incubation, peroxidase-conjugated antibodies to human antithrombin are bound to the free antithrombin determinants whose enzyme activity is measured photometrically after washing.

**Statistical analysis.** Plasma VWF activity, VWF:CB, TAT, and ADAMTS13 values were expressed as percentages of the values observed in normal plasma. The results of ADAMTS13 activity were normally distributed and Student's t test was used to compare the ADAMTS13 activity between patients and control. Differences in values of the measured parameters between each disease group were examined by Kruskal-Wallis test. For correlations between parameters, Pearson's correlation coefficients were calculated. Results are presented as mean ± SD. P values <0.05 were considered statistically significant.

**Results**

**VWF activity, VWF:CB, and platelet counts in consumptive coagulopathy.** Because uncleaved hyperactive large VWF is known to lead to platelet consumption, we examined the presence of a relationship between VWF activity, VWF:CB, and platelet count or ΔLnPLT/day that was calculated as indicated above. VWF activity and VWF:CB could be measured in 39 of the 46 patients. Both were significantly increased (186 ± 42% and 237 ± 55%, respectively, expressed as mean ± SD; Fig. 1a)
compared with the laboratory established normal reference intervals (49 to 164 % and 46 to 164 %, respectively). As plasma VWF level is elevated in inflammatory conditions, we examined the correlation of VWF and DIC scores, which we regarded as a rough indicator of disease severity. No correlation was found between the DIC score and VWF activity or VWF:CB (p = 0.296, p = 0.610, respectively). The difference of VWF activity and VWF:CB between patients who were scored as overt DIC and the remaining patients was not significant. There were no significant differences of VWF activity or VWF:CB between disease groups, either. The mean platelet count of the patients was slightly decreased (123 x 10³/μl) compared with normal reference interval (150 - 450 x 10³/μl). The platelet count itself did not show any correlation with VWF activity or VWF:CB. However, while increased VWF activity was not necessarily associated with increased ΔLnPLT/day, a significant correlation was observed between VWF:CB and ΔLnPLT/day (r = 0.409, p=0.025; Fig. 1b). Since VWF:CB effectively represents large form VWF multimer in plasma, our finding may reflect the fact that large VWF multimers interact more efficiently with platelets and are more apt to contribute to platelet consumption.

**ADAMTS13 activities in consumptive coagulopathy and their relationship to platelets.** ADAMTS13 activities measured in the 46 patients were significantly decreased compared with healthy controls (36.5 ± 24.5% vs 95.0 ± 17.0%, p <0.01; Fig. 2a). There were no significant differences in ADAMTS13 activities among disease categories of the patients. ADAMTS13 activity was not correlated with the DIC score, which served as an indicator of severity of coagulopathy or primary clinical condition (p = 0.167). However, VWF:CB activity representing large VWF multimers showed a significant inverse correlation with ADAMTS13 activity (r = -0.380, p = 0.019; Fig. 2b), probably reflecting the enzyme substrate relationship of ADAMTS13 and VWF. We tested for the presence of correlation between platelet counts and ADAMTS13 activity in the first place, which could be anticipated from extreme clinical conditions where severe ADAMTS13 deficiency results in

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Fig. 2. ADAMTS13 activity in patients and its relationship to platelet count. (a, upper panel) ADAMTS13 activity in 46 patients was greatly decreased compared to controls (36.5 ± 24.5% vs 95.0 ± 17.0%, p<0.01). Patient results are shown on the left and those of controls on the right. Horizontal lines indicate medians (35.0 and 93.0, respectively). (b, middle panel) VWF:CB increased as ADAMTS13 activity decreased, showing negative correlation (r = -0.380, p = 0.019), probably because UL-VWF was incompletely degraded as ADAMTS13 activity decreased. (c, lower panel) Negative correlation was found between ADADMTS13 activity and ΔLnPLT/day (r = -0.427, p = 0.008).
thrombocytopenia, as in TTP. There was no correlation between platelet counts and ADAMTS13 activities, which were evenly distributed from 0.0 to 96.1% irrespective of the platelet count. Then we examined the relationship between ADAMTS13 activity and ΔLnPLT/day and observed a significant inverse correlation between ΔLnPLT/day and ADAMTS13 activity (r = -0.427, p = 0.008; Fig. 2c), as was found between ΔLnPLT/day and VWF:CB. When VWF:CB was controlled the correlation between ADAMTS13 and ΔLnPLT/day was still preserved (r = -0.418, p = 0.024) while correlation between VWF:CB and ΔLnPLT/day was lost after ADAMTS13 was controlled. ADAMTS13 also inversely correlates with VWF:CB, which precludes the possibility of VWF:CB possessing the determining role in this triangular relationship. The data were reanalyzed after removing cases with <5.0% of ADAMTS13 activity to prevent extremely low ADAMTS13 values from biasing the analysis. Again, the correlation between ADAMTS13 and ΔLnPLT/day existed with or without VWF:CB controlled (r = -0.578, p = 0.0003; r = -0.418, p = 0.024). Finally, we performed multiple regression analysis with age (as an indirect indicator of bone marrow thrombopoiesis compensating thrombocytopenia), ADAMTS13, VWF:CB, TAT, and other DIC related parameters included in the SSC/ISTH scoring system (PT, d-dimer, fibrinogen) as independent variables that could supposedly determine ΔLnPLT/day. Only ADAMTS13 was proved to be meaningful as a determining factor of ΔLnPLT/day (p = 0.023).

**TAT level irrelevant to ΔLnPLT/day and ADAMTS13.** TAT, as an indicator of systemic thrombin activity, was measured in 40 of the 46 patients in whom ADAMTS13 activities were measured. TAT levels were found to be significantly increased (16.9 ± 20.0 μg/L; laboratory established normal reference interval <4.2 μg/L). The TAT level correlated well with DIC scores (r = 0.429, p = 0.003, Fig. 3a) although it was not included in the scoring system, reaffirming TAT levels as an indicator of severity of consumptive coagulopathy. Because thrombin can activate platelets, we examined for correlation between thrombin and platelet count or ΔLnPLT/day. No correlation between them could be found (TAT and platelet count p = 0.251; TAT and ΔLnPLT/day p = 0.929). Platelet counts and ΔLnPLT/day were not different between patients with normal and above normal level of TAT (p = 0.139, p = 0.137, respectively). As for the possibility of lowered ADAMTS13 activity having a relation to systemic thrombin or fibrinolytic activity, there was no significant correlation between ADAMTS13 activity and TAT level (p = 0.644), nor between ADAMTS13 activity and d-dimer level as an indirect indicator of fibrinolysis (p = 0.086). ADAMTS13 activity varied among patients regardless of the TAT level. Patient groups categorized by ADAMTS13 activity below or above 20%, as adopted from a study of Ono et al [15],
showed no significant difference in TAT levels ($p = 0.713$, Fig. 3b). The VWF:CB value was also unrelated to the TAT level ($p = 0.693$). Thus, the TAT level seems to be independent from the triangular relationship among ADAMTS13, VWF:CB, and $\Delta \text{Ln PLT/day}$.

**Discussion**

In this study we intended to determine if decreased ADAMTS13 activity in consumptive coagulopathy can influence platelet homeostasis as in case of TTP. We envisioned that severe thrombocytopenia in TTP is not a threshold phenomenon that abruptly develops as ADAMTS13 activity falls below a certain point. We expected that there would be some degree of subclinical disturbance of platelet homeostasis related to decreased ADAMTS13 activity before profound thrombocytopenia manifests itself, even with nearly normal platelet counts. Thus we collected plasma of patients who were suspected to have consumptive coagulopathy and were shown to have elevated d-dimer level and we measured ADAMTS13 activity. We also determined VWF:CB and VWF activity, because thrombocytopenia in TTP is mediated by hyperactive UL-VWF. VWF:CB depends on the presence of both intact collagen binding domain on VWF and high molecular weight VWF multimer. It fully demonstrates the capability to detect the absence of large and intermediate vWF multimers and is used as a substitute for conventional immunoblotting in detecting residual large multimers after digestion of VWF [16,17]. Both VWF activity and VWF:CB were significantly increased in our patients compared to the normal ranges. These increases are attributed to endothelial secretion of VWF, accelerated by inflammation and reduced clearance from the circulation.

Significantly decreased ADAMTS13 activity in patients with consumptive coagulopathy compared with healthy controls was reconfirmed. This finding is comparable to previous reports showing significant decrease of ADAMTS13 in clinical conditions similar to those included in our study [11,12,15]. However, the physiological meaning of decreased ADAMTS13 activity as to platelet function in clinical conditions other than TTP has not been thoroughly considered. Platelet counts did not correlate with ADAMTS13 activity in these studies, although Moore et al [11] observed a small number of non-TTP cases where thrombocytopenia and ADAMTS13 deficiency coincided. This is the same as our findings. There was no significant correlation between platelet counts and ADAMTS13 activity. However, this apparent anomaly does not preclude the assumption that ADAMTS13 has some pathophysiologic implications for platelets. An important notion to consider is that the platelet count included in the analysis merely reflects a point in a dynamic process. It can change by several fold within a brief period and varies widely between individuals at the nadir as well as at the baseline. Moreover, clinical factors associated with thrombocytopenia affect the dynamics of platelet generation and clearance rather than determining their levels. Thus we were interested in the rate at which platelet counts decrease. In calculating the rate we converted platelet counts into a logarithmic value as mentioned above ($\Delta \text{Ln PLT /day}$). In contrast to the platelet count per se, there was a significant inverse correlation between ADAMTS13 activity and the $\Delta \text{Ln PLT /day}$. VWF:CB also showed a correlation with $\Delta \text{Ln PLT /day}$ and it can be assumed that progressively more high molecular weight VWF escapes enzymatic degradation to interact with platelets as plasma ADAMTS13 activity decreases [18,19]. However, unexpectedly the relationship observed between ADAMTS13 and $\Delta \text{Ln PLT /day}$ was preserved even after VWF:CB was controlled. Multiple regression analysis including VWF:CB revealed ADAMTS13 activity was the only meaningful predictor of $\Delta \text{Ln PLT /day}$.

As an explanation for this finding, several experimental and clinical observations can be noted. First, a recent report demonstrated that binding of glycoprotein Ibα to VWF domain A1 dramatically accelerated the cleavage of the VWF domain A2 by ADAMTS13 [20]. Actually, cleavage by ADAMTS13 of VWF with attached platelets on it is very prompt as demonstrated in perfusion studies [18,21]. Second, paradoxical impairment of platelet function in essential thrombocythemia is well explained by acquired VWF deficiency. As platelet counts increase, accompanying the loss of large VWF multimer, 176 kDa and 140 kDa VWF fragments produced by ADAMTS13 also increase...
The fact that cleavage of VWF by ADAMTS13 increases as interaction with platelets occurs more frequently, together with the previously mentioned experimental findings, may suggest, with caution, the possibility that ADAMTS13 directly hampers the entrapment of platelets into microthrombi by cleaving VWF selectively at the site where platelets attach. This immediate response, though somewhat speculative, conforms well with our result indicating a rather direct and independent relationship between ADAMTS13 and platelet dynamics. Thrombocytopenia is an important prognostic factor in sepsis, which is well known cause of consumptive coagulopathy [25]. Whether changes in platelet dynamics associated with decreased ADAMTS13 activity without overt thrombocytopenia has clinical significance has not yet been established and our data need to be bolstered by further investigation.

Regarding decreased ADAMTS13 activity, it is possible that flaring prothrombotic and thrombolytic activity in consumptive coagulopathy contributes to depressed ADAMTS13 activity as Crawley et al [13] speculated, demonstrating that ADAMTS13 was degraded and inactivated by thrombin and plasmin. However, there was no significant correlation between ADAMTS13, TAT, and d-dimer in our study. Ono et al [15] reported similar findings and suggested the importance of granulocyte esterase in clearance of plasma ADAMTS13. However, the physiologic significance of these enzymatic digestions of ADAMTS13 is not yet fully clarified. While several experimental reports have indicated the non-essentiality of the C-terminal portion similar to that removed by thrombin or plasmin for enzymatic activity [26,27], autoantibodies of TTP directed to the C-terminal region and mutations of Upshaw-Schulman syndrome located to C-terminal CUB domains were also reported [2,3,10]. Bernardo et al [28] reported that inflammatory cytokines, especially IL-6, suppressed ADAMTS13 activity directly [28] and this finding can additionally provide an explanation for decreased ADAMTS13 activity. This matter needs further clinical and experimental evaluation, along with the effects of IL-8 and TNF-α on VWF secretion. Moreover, elevated VWF level itself has been shown to induce depression of plasma ADAMTS13 activity supposedly though sequestration of ADAMTS13 to VWF [29-31]. ADAMTS13 levels in these studies ranged from 65 to 79%, a degree far less than the decrease that we observed. Thus elevated VWF level cannot solely explain the profound decrease of ADAMTS13 activity that we observed. Another interesting finding in our study is that ADAMTS13, ΔLnPLT/day and VWF:CB showed no correlations with the TAT level. The three parameters seemed to be separated from the systemic prothrombotic status, which is a dissociation similar to that in TTP.

In conclusion, we observed that even without profound thrombocytopenia as found in TTP, decreased ADAMTS13 activity in consumptive coagulopathy is associated with altered platelet dynamics, the clinical significance of which needs further investigation.

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References