Brief report
Cleavage of ultralarge multimers of von Willebrand factor by C-terminal–truncated mutants of ADAMTS-13 under flow

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A disintegrin-like and metalloprotease with thrombospondin type 1-motif 13 (ADAMTS-13) cleaves the A2 domain of von Willebrand factor (VWF), converting the ultralarge (UL) and hyperactive VWF multimers freshly released from endothelial cells to smaller and less active forms found in plasma. Recombinant ADAMTS-13 lacking the C-terminal region is active under static conditions, but its functions under flow conditions have not been determined. Here, we show that VWF-cleaving activity measured under flow was preserved in an ADAMTS-13 mutant lacking the second to eighth thrombospondin-1 motifs and the complement components C1r/C1s, Uegf sea urchin fibropellins, and bone morphogenetic protein 1 (CUB) domains, but was severely deficient in a mutant that was further truncated to remove the spacer domain. We also show that the mutant lacking the TSP-1 and CUB domains was hyperactive under flow, suggesting that the C-terminal region may negatively regulate ADAMTS-13 activity. The wild type and the mutant without the spacer were more active in the presence of plasma, raising the possibility of ADAMTS-13 cofactors in plasma. (Blood. 2005;106:141-143)

Introduction

Thrombotic thrombocytopenic purpura (TTP) is caused by widespread microvascular thrombi rich in platelets and von Willebrand factor (VWF).1–2 The disorder is associated with the deficiency of the VWF-cleaving metalloprotease, a disintegrin-like and metalloprotease with thrombospondin type 1-motif 13 (ADAMTS-13), which cleaves the Y842/M843 peptide bond in the VWF A2 domain,4,6 converting the ultralarge (UL) and hyperactive VWF multimers newly released from endothelial cells to smaller and less active forms.7 ADAMTS-13 deficiency, caused either by congenital defects of the ADAMTS-13 gene or the presence of autoantibodies against the metalloprotease,8 results in the accumulation of UL-VWF multimers in plasma and systemic thrombosis in the microvasculature.1 ADAMTS-13 activity is mostly measured under static conditions that require denaturing the VWF substrate. We recently showed that freshly released ULVWF multimers are anchored to the endothelial surface and form long platelet-decorated strings under venous and arterial shear stresses.9,10 These ULVWF strings are cleaved within seconds to minutes by normal plasma, but not plasma from patients with TTP9 suggesting that ULVWF proteolysis in vivo may occur on the surfaces of endothelial cells and is regulated by fluid shear stress.

ADAMTS-13 contains multiple domains characteristic of the ADAMTS family of metalloproteases. Experiments performed under static conditions suggest that many of these domains are dispensable for protease activity, as recombinant ADAMTS-13 mutants lacking much of the C-terminal region, including 7 thrombospondin-1 (TSP-1) motifs and 2 complement components C1r/C1s, Uegf sea urchin fibropellins, and bone morphogenetic protein 1 (CUB) domains, can still cleave plasma VWF.11,12 However, more than 50% of mutations identified in patients with congenital TTP were located in the deleted regions. One possibility for such discrepancy is that the C-terminal regions are required for ADAMTS-13 to function in vivo under flow conditions. To examine this possibility and further investigate the relationship between ADAMTS-13 structure and function, we examined the activity of 2 ADAMTS-13 truncation mutants under flow.

Study design

ADAMTS-13 activity was measured under flow conditions by a previously described method.5 Briefly, human umbilical vein endothelial cells (HUVECs) were stimulated with 25 μM histamine and then perfused with washed platelets suspended in either buffer or citrated plasma at 2.5 dynes/cm² of shear stress. Platelets tethered to newly released ULVWF multimers to form long and platelet-decorated strings on endothelial cells in the absence of ADAMTS-13, whereas perfusion of platelets in normal plasma produced no strings because of their cleavage by plasma ADAMTS-13. Using this system, we examined the ability of 2 truncation mutants to cleave ULVWF strings. The mutants are described previously (Figure 1A)13 and named in this study as MDTCS, which represents the domains remained in the mutant (metalloprotease, disintegrin, the first TSP-1 motif, Cys-rich, and spacer), lacking TSP-1 motifs second through eighth and the C-terminal CUB domains, and MDTC, which also lacks the spacer. Wild-type (WT) ADAMTS-13 and the 2 mutants were transiently

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with a high-titer ADAMTS-13 autoantibody. Briefly, plasma was first prepared from the plasma of a patient with acquired TTP. Human blood was used for the studies presented in the manuscript and a myc-tag antibody (Sigma, St Louis, MO). Protein concentrations were determined using a commercial kit (Pierce Chemicals). WT and ADAMTS-13-depleted plasma were perfused over stimulated HUVECs. ULVWF protelysis was determined after 2-minute perfusion.

Human blood was used for the studies presented in the manuscript for platelets and ADAMTS-13. The use of human blood has been approved by the institutional review board of the Baylor College of Medicine. All donors signed informed consent in accordance with the Declaration of Helsinki before blood was drawn. ADAMTS-13-depleted plasma was prepared from the plasma of a patient with acquired TTP and a myc-tag antibody (Sigma, St Louis, MO). Protein concentrations were determined using a commercial kit (Pierce Chemicals). WT and ADAMTS-13 mutants suspended in either Tyrode buffer or ADAMTS-13-depleted plasma were perfused over stimulated HUVECs. ULVWF protelysis was determined after 2-minute perfusion.

Figure 1. Cleavage of ULVWF strings under flow in the absence of plasma. Recombinant ADAMTS-13 and 2 truncation mutants (A) were resuspended in the complete Tyrode buffer to a final concentration of 670 nM and perfused over histamine-stimulated HUVECs at a shear stress of 2.5 dyne/cm². (B) Perfusion of WT or MDTC mutant resulted in 100% cleavage of ULVWF strings after 2-minute perfusion, whereas the cleavage by the MDTC mutant was significantly less. (C) The dose-dependent cleavage of ULVWF strings by WT ADAMTS-13 and the MDTC mutant suspended in Tyrode buffer. At the concentrations of 42, 84, and 168 nM, the cleavage activity of the MDTC mutant was significantly greater than that of WT. The figures are mean ± SEM; n = 96 for panel B and n = 9 for panel C; *P < .005 compared with WT.

Results and discussion

ADAMTS-13 activity was defined as the number of ULVWF strings present in 20 continuous view fields (X 400). There were 78 ± 24 strings counted when the Tyrode buffer containing platelets was perfused and defined as 0% activity, whereas 0 to 7 strings were detected when recombinant ADAMTS-13 (670 nM) was perfused and defined as 100% activity. At the equal molar concentration, the MDTC mutant demonstrated 100% cleavage under these conditions, whereas the MDTC mutant cleaved only 26.5 ± 2.5% of strings, significantly less than the percentage cleaved by MDTCs (n = 96, P < .001, Figure 1B). ADAMTS-13 activities at the maximal concentration of 670 nM were comparable between the WT and the MDTC mutant, but the latter exhibited significantly greater activities at lower concentrations (Figure 1C). Similar results were obtained using recombinant ADAMTS-13 expressed in Hela and sf9 cells (data not shown), suggesting that difference in activity is an intrinsic property of the mutant. This result is consistent with that obtained under static condition, suggesting that the C-terminal domains are not critical for, but may regulate, the enzymatic activity under flow conditions. This is different from recent studies that the C-terminus CUB domains either directly bind to VWF or regulate the affinity of VWF-ADAMTS-13 interaction. These results suggest that the C-terminal regions (especially CUB domains) may serve as or be a part of the docking site for ULVWF, a critical step for cleaving endothelial cell–anchored ULVWF under flow. The reason for such a discrepancy is unclear, but may suggest that there are either multiple docking sites on ADAMTS-13 or new sites created by the truncations. Additional studies to solve the discrepancy are critical for understanding how ADAMTS-13 cleaves ULVWF under flow conditions.

One potential problem with the studies described in the previous section is that plasma, in which ADAMTS-13 functions in vivo, was not present. We therefore tested the activity of WT and 2 mutants in ADAMTS-13-depleted plasma, which presumably contains any potential ADAMTS-13 modifiers. As shown in Figure 2A, both WT and the MDTC mutant fully restored the ability of ADAMTS-13-depleted plasma to cleave ULVWF strings at concentrations comparable with those used in buffer. To our surprise, the presence of plasma increased the cleavage of ULVWF strings by the MDTC mutant (800 nM) from 26.5 ± 3.5% in buffer to 54.8 ± 6.8% in plasma (Figure 2A, Student t test, n = 9, P < .05), even though activity was not detected at lower concentrations (400 and 200 nM). To investigate whether plasma also regulates the activity of WT ADAMTS-13, we then measured ULVWF cleavage by various concentrations of WT ADAMTS-13 in the presence and absence of plasma. We found that WT ADAMTS-13 at 84 nM cleaved 19.5 ± 4.3% more strings in the presence of plasma than in its absence (Figure 2B, Student t test, n = 4, P < .05). In summary, we show that the truncation mutant that lacks the second to eighth TSP-1 motifs and the CUB domains (MDTCs) is active under flow conditions, consistent with observations under

Figure 2. Cleavage of ULVWF strings in plasma under flow. (A) WT ADAMTS-13, MDTCs, and MDTC were mixed with ADAMTS-13-depleted plasma to final concentrations of 670, 670, and 800 nM, respectively, and were perfused over HUVECs under a shear stress of 2.5 dyne/cm². The percent cleavage of ULVWF strings for MDTC mutant was similar to that of WT at 100%, whereas it was 54.8 ± 6.8% for the MDTC mutant, significantly lower than those of WT and MDTC mutant, but higher than its activity in the absence of plasma. The figures are mean ± SEM; n = 22; *P < .01 compared with WT. (B) WT ADAMTS-13 was perfused over histamine-stimulated HUVECs in the presence or absence of the enzyme-depleted plasma. The percent cleavage for the 2 enzyme preparations was similar at 670, 335, and 168 nM, but significantly higher for ADAMTS-13 in the depleted plasma at 84 nM. The figures are mean ± SEM; n = 4; *P < .05.
static conditions.\textsuperscript{11,12} Furthermore, the mutant appears to be more active than WT ADAMTS-13 (Figure 1C), suggesting that the C-terminal domain may regulate the enzyme activity. This hypothesis is supported by a study showing that the activity and substrate specificity of ADAMTS4 is regulated by the C-terminal domains, including the TSP-1 motif.\textsuperscript{16,17} In contrast to the hyperactivity of the MDCTS mutant, truncation to further remove the spacer domain (MDTC) rendered the mutant severely deficient in this system.\textsuperscript{18} More importantly, the metalloprotease is significantly more active in the presence of plasma (Figure 2), suggesting that ADAMTS-13 activity may be regulated by plasma factors. The nature and identity of these cofactors remains to be investigated, but a recent study shows that glycoprotein Ibα (GP Ibα), the platelet VWF receptor, enhances ADAMTS-13 activity.\textsuperscript{19} Glycocalcin, the soluble extracellular portion of GP Ibα that normally circulates in plasma in various amounts, may therefore be one of such cofactors. Studying how ADAMTS-13 is regulated by plasma factors is important not only for understanding the ADAMTS-13 function in vivo, but also for identifying new factors that affect ULVWF proteolysis and contribute to bleeding or thrombotic phenotypes.

References

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