研究成果の刊行に関する一覧表

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Regular Article

Identification of epitopes on ADAMTS13 recognized by a panel of monoclonal antibodies with functional or non-functional effects on catalytic activity ☆

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Epitope mapping

ABSTRACT

Introduction: von Willebrand factor (VWF) cleavage by ADAMTS13 is mediated by multi-step interactions between their multi-domain structures. To clarify the relationship between inhibitory effects of monoclonal antibodies and epitopes on each ADAMTS13 domain, we analyzed how each ADAMTS13 domain contributes to catalyze VWF using a mouse anti-ADAMTS13 monoclonal antibody panel.

Materials and Methods: FRETS-VWF73 assay was used to examine the effects of 14 anti-ADAMTS13 monoclonal antibodies on the catalytic activity of plasma ADAMTS13. Epitope mapping was performed using phage surface display. Libraries expressing peptide fragments of ADAMTS13 were screened with the monoclonal antibodies.

Results: Eleven epitopes of 14 monoclonal antibodies were successfully defined. Three monoclonal antibodies recognizing metalloprotease or disintegrin-like domains strongly inhibited the catalytic activity and their epitopes were on Gln159-Asp166, Tyr 305-Glu327, and Asn308-Glu376. Five monoclonal antibodies recognizing TSP1-3 to -7 repeats showed weak inhibitory effects, and their epitopes were on Pro744-Ala806, Pro856-Cys864, Gln892-Gly940, Cys1007-Cys1072, and Gln1163-Asn1185. Four monoclonal antibodies recognizing the TSP1-1, TSP1-2, CUB1 or CUB2 domains had no inhibitory effects, and their epitopes, except that for TSP1-1, were Pro682-Cys742, Thr1200-Cys1213, and Gln1409-Glu1414. Two monoclonal antibodies recognizing cysteine-rich and spacer domains showed moderate inhibitory effects, but their epitopes were not determined.

Conclusions: We revealed the epitopes of 11 monoclonal anti-ADAMTS13 antibodies on each of the domains and clarified their association with inhibitory effects on VWF catalysis under static conditions. Catalytic activity correlated strongly with the epitopes on metalloprotease and disintegrin-like domains, weakly with those on TSP1-3 to -7 repeats, and negatively with those on TSP1-1, -2, and CUB domains.

Introduction

ADAMTS13 catalyzes the multimeric von Willebrand factor (VWF) to reduce its size in the bloodstream, thereby controlling its affinity to platelets under shear stress. ADAMTS13 deficiency contributes to the accumulation of ultra-large VWF multimers, resulting in the formation of platelet-rich thrombi in the microcirculation, which may lead to the onset of a lethal disease, thrombotic thrombocytopenic purpura (TTP) [1]. This protease is a member of the family of disintegrin and metalloprotease with thrombospondin type 1 repeats, and comprises multi-domain structures: a signal peptide, a propeptide, a metalloprotease (M) domain, a disintegrin-like (D) domain, a thrombospondin type 1 repeat (T), a cysteine-rich (C) domain, a spacer (S) domain, seven TSP1-repeats (T2~T8), and CUB1~2 (C1, C2) domains [2]. ADAMTS13 cleaves VWF at a single peptide bond between Tyr1605 and Met1606 in the unfolded A2 domain under shear stress in blood flow [3]. Under static conditions, domains from metalloprotease to spacer are sufficient to cleave the unfolded VWF-A2 domain and the spacer domain plays a pivotal role in the specific substrate binding [4–7]. Crystal structures from disintegrin-like to spacer domains recently revealed that three linearly-aligned discontinuous binding exosites reside in disintegrin-like, cysteine-rich, and spacer domains [8]. Under flow conditions, however, carboxyl-terminal domains from TSP1 repeats to CUB are considered to have significant roles in the recognition and subsequent VWF cleavage processes [9,10]. Furthermore, carboxyl-terminal domains of ADAMTS13 are reported to interact with the VWF-D4 domain exposed

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on the surface of globular VWF under static conditions [11]. These findings suggest that the VWF cleavage process by ADAMTS13 in the blood flow is controlled by reciprocal multi-step reactions of multi-domains.

To address the conformational association between ADAMTS13 and VWF, we raised 14 monoclonal anti-ADAMTS13 antibodies and assessed their inhibitory effects on VWF proteolysis using a FRETS-VWF73 assay. Based on the results of their binding domains confirmed with truncated ADAMTS13 mutants [12], precise epitope sequences were analyzed using a lambda phage surface display system. By expressing various peptides of ADAMTS13 on its surface, this phage library allows us to define the epitope sequences of monoclonal antibodies [13]. We previously analyzed the VWF-binding sequence in the spacer domain [14] and the epitopes of autoantibodies to ADAMTS13 in patients with TTP [15] using the same strategy.

In the present study, we defined 11 epitope sequences of 14 monoclonal anti-ADAMTS13 antibodies with or without inhibitory effects on the catalytic function to elucidate the structure-function relationship of ADAMTS13.

Materials and Methods

Monoclonal Anti-ADAMTS13 Antibodies

Ten monoclonal antibodies, W688X3-91A, WH2-22-1A, W688X3-27, W688X6-1, W688X3-69, WHS40-3, WH10, WH2-11-1, WH2-1-1, and WH63-1 were generated by immunization of mice with a purified full-length wild-type recombinant ADAMTS13 or a mutant recombinant ADAMTS13 protein carboxyl-terminally truncated at residue 688. Pep4-5B-1 was generated with a peptide fragment from residue1169 to 1190. Cterm20 was also generated with a carboxyl-terminal peptide fragment from residue1406 to 1427. CUB1-3 was generated with a recombinant carboxyl-terminal fragment from CUB1 to 2 domains [12]. A10 was generated with a full-length wild-type recombinant ADAMTS13 purified by anti-FLAG M2 agarose affinity chromatography [16,17]. Their binding domains (Fig. 1) were defined with a series of carboxyl-terminally truncated recombinant ADAMTS13 mutants [12,16,17].

FRETS-VWF73 Assay

To assess the effects of the monoclonal antibodies on ADAMTS13 activity, a FRETS-VWF73 assay [18] was performed according to the manufacturer's protocol (Peptide Institute, Osaka, Japan) with slight modification. Pooled standard human plasma from healthy donors was used as a source of ADAMTS13. Briefly, each of the anti-ADAMTS13 antibodies was serially diluted, mixed with 4 μl standard plasma, and incubated for 30 min at room temperature in 50 μl reaction buffer (5 mM bis-Tris, 25 mM $CaCl_2$, and 0.05% Tween-20 at pH 6.0). Then, 50 μl of the 4 μM FRETS-VWF73 substrate solution was added and the emitted fluorescence intensity was measured every 2 min at 30 °C in a fluorescence spectrophotometer (Wallac ARVO SX) with λex = 340 nm and λem = 450 nm.

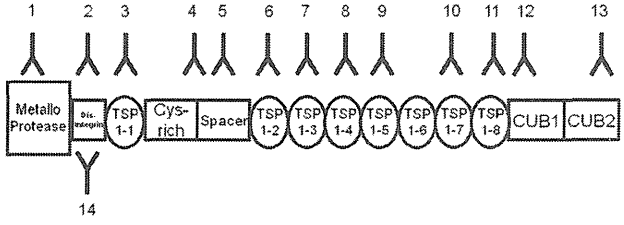

Fig. 1. A panel of mouse monoclonal anti-ADAMTS13 antibodies. Molecular structure of ADAMTS13 is depicted as a chain of domains. Binding regions of 14 monoclonal antibodies to ADAMTS13 are shown on the schema. 1: W688X3-91A, 2: WH2-22-1A, 3: W688X3-27, 4: W688X6-1, 5: W688X3-69, 6: WHS40-3, 7: WH10, 8: WH2-11-1, 9: WH2-1-1, 10: WH63-1, 11: Pep4-5B-1, 12: CUB1-3, 13: Cterm20, 14: A10.

Epitope Mapping of the Monoclonal Antibodies Using a Phage Surface Display

Lambda phage libraries expressing various ADAMTS13 peptide fragments on the surface were constructed as previously described [14,15]. Two kinds of phage libraries, expressing 30–60 or 60–160 amino acids of the ADAMTS13 peptide sequence, were prepared.

Epitope mapping was performed using the affinity selection of the phage libraries by each of the monoclonal antibodies immobilized on microtiter wells as previously described [13]. Briefly, the libraries were grown with *Escherichia coli* TG1 in CY medium, precipitated with polyethylene glycol, and suspended in blocking buffer (0.25% bovine serum albumin, 5% skimmed milk, 0.1% Tween-20 in 1 × PBS containing 0.1% sodium azide). The amplified phage libraries were applied to the microtiter wells on which each of the monoclonal antibodies was immobilized, and incubated at 4 °C overnight under static conditions. After the wells were washed six times, bound phages were eluted. This panning procedure was repeated three to four times and the concentrated phage clones were collected.

To confirm that the obtained phage clones expressed peptides binding to the monoclonal antibody, the phage plaques were transferred to nitrocellulose membranes and immunostained with the corresponding monoclonal antibody, and positive clones were picked up and subjected to DNA sequence analysis.

Results

Inhibitory Effects of the Monoclonal Anti-ADAMTS13 Antibodies on VWF Cleavage

The inhibitory effects of the monoclonal antibodies on VWF cleavage by ADAMTS13 were assessed using FRETS-VWF73 as a substrate (Fig. 2). Three monoclonal antibodies, W688X3-91A recognizing metalloprotease, and WH2-22-1A and A10 recognizing the disintegrin-like domain, showed strong dose-dependent inhibitory effects on the catalytic activity (IC_{50}; W668X3-91A: 2.5 μg/ml, WH2-22-1A: 4.7 μg/ml, A10: 4.0 μg/ml). Two monoclonal antibodies, W688X6-1 and W688X3-69, recognizing cysteine-rich and spacer domains, showed moderate inhibitory effects (IC_{50}; W688X6-1: 11.0 μg/ml, W688X3-69: 22.7 μg/ml). Five monoclonal antibodies, WH10, WH2-11-1, WH2-1-1, WH63-1, Pep4-5B-1, recognizing TSP1-3 to -8 repeats, showed weak inhibitory effects, resulting in the residual activity of more than 50%, even at the dose of 25 μg/ml. Four monoclonal antibodies, W688X3-27, WHS40-3, CUB1-3, and Cterm20, recognizing TSP1-1, TSP1-2, CUB1 or CUB2 domains, exhibited no inhibitory effects on VWF73 cleavage.

Epitope Analysis of the Monoclonal Anti-ADAMTS13 Antibodies

Each of the 14 monoclonal antibodies was individually immobilized on the microtiter wells and the epitopes were analyzed using the phage display. First, the phage library expressing short peptides of ADAMTS13 (30–60 amino acids) was screened. After three rounds of panning, phage clones that were positively immunostained with the corresponding monoclonal antibody were obtained from eight monoclonal antibodies, W688X3-91A, WH2-22-1A, WHS40-3, WH2-11-1, WH2-1-1, Pep4-5B-1, CUB1-3, and Cterm20. DNA sequence analysis of the positive phage clones revealed the peptide sequences on which the monoclonal antibody binds, thus the shared sequence was thought to be the epitope. Representative positive phage clones immunostained with WH2-1-1 are shown in Fig. 3A and the peptide sequences expressed on the phage clones are depicted in Fig. 3B, revealing the epitope as the shared sequence.

Second, the other six monoclonal antibodies were screened using another phage library expressing longer peptides (60–160 amino acids). As a result, epitopes of three monoclonal antibodies, WH10, WH63-1, and A10, were successfully determined. We failed to find