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Novel Recombinant Glycosylphosphatidylinositol (GPI)-Anchored ADAMTS13 and Variants for Assessment of Anti-ADAMTS13 Autoantibodies in Patients with Thrombotic Thrombocytopenic Purpura

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Summary

Immunoglobulin Gs (IgGs) against ADAMTS13 are major causes of acquired (idiopathic) thrombotic thrombocytopenic purpura (TTP). We report here a novel cell-based assay using glycosylphosphatidylinositol (GPI)-anchored ADAMTS13 or variants expressed on cell membrane for assessment of autoantibodies in patients with TTP. We showed that IgGs from all 26 patients with acquired TTP bound to cells expressing a GPI anchored full-length ADAMTS13 (gFL) and a variant truncated after the spacer domain (gS). Also, IgGs from 25/26 (96.7%) of these TTP patients bound to cells expressing a GPI-anchored C-terminal fragment, TSP1 2-8 plus CUB (gT2C). In contrast, none of the 20 healthy blood donors showed detectable binding of their IgGs to the cells expressing gFL, gS, and gT2C. A moderate, but statistically significant correlation was observed between plasma concentrations of anti-ADAMTS13 IgG and positive cells expressing gFL ($r=0.65$), gS ($r=0.67$), and gT2C ($r=0.42$). These results suggest that the microtiter-plate assay and the cell-based assay may detect differential antigenic epitopes. Moreover, antigens clustered on cell membrane may enhance antibody binding affinity, thereby increasing analytical sensitivity. Finally, our assay was able to determine kinetic changes of plasma levels of anti-ADAMTS13 IgGs in TTP patients during plasma therapy. Together, our findings suggest that the novel cell-based assay may be applicable for rapid identification and mapping of anti-ADAMTS13 autoantibodies in patients with acquired TTP.

Keywords

von Willebrand factor cleaving protease; thrombotic microangiopathies; diagnostic test; autoantibody

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a potentially fatal syndrome, characterized by profound thrombocytopenia and microangiopathic hemolytic anemia ¹. Some patients may have signs of major organ dysfunction including change of mental status and/or renal failure. TTP may be classified into at least three distinct categories: 1) hereditary TTP, which is caused by mutations in *ADAMTS13* gene ²; 2) acquired idiopathic TTP, which is

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mainly caused by polyclonal immunoglobulin Gs (IgGs) that inhibit plasma ADAMTS13 activity (or anti-ADAMTS13 autoantibodies) ^{3,4}; and 3) acquired non-idiopathic TTP, which is associated with pregnancy ⁵, hematopoietic progenitor cell transplantation ⁶, infections ⁷, disseminated malignancy⁸, and certain drugs such as ticlopidine and clopidogrel ⁹. The mechanisms underlying acquired non-idiopathic TTP remain to be determined.

Severe deficiency of plasma ADAMTS13 activity (5–10% of normal) and presence of anti-ADAMTS13 autoantibodies may be highly specific for diagnosis of acquired idiopathic (or autoimmune) TTP ^{10–12}. Moreover, the positive anti-ADAMTS13 autoantibodies are correlated with the persistence of low plasma ADAMTS13 activity in remission, increased relapses, and reduced survival ^{13–16}. Clinical interventions to eliminate anti-ADAMTS13 autoantibodies such as the use of immunosuppressive drugs including cyclosporine ¹⁷, cyclophosphamide ^{18;19}, and rituximab ^{20;21} have been shown to be highly efficacious for treatment of acquired TTP. Therefore, the determination of anti-ADAMTS13 autoantibodies in patients with acquired idiopathic TTP may be crucial for confirming diagnosis, predicting outcome, and guiding the selection of adjunctive therapy.

To date, anti-ADAMTS13 autoantibodies can be determined by either functional assays or immunological assays. The former detect only the inhibitory anti-ADAMTS13 autoantibodies ^{4;22–24}, whereas the latter identify both inhibitory and non-inhibitory autoantibodies ^{23–27}. The sensitivity of functional assays for identification of anti-ADAMTS13 autoantibodies ranges from 44% to 90% ^{4;15;28} even in patients with less than 5% of plasma ADAMTS13 activity. The results obtained from different functional assays (i.e. FRETs-vWF73 vs. Western blotting) do not always agree with each other ^{14;24;29}. The immunological assays such as enzyme-linked immunosorbent assay (ELISA) may be more sensitive than functional assays for identification of anti-ADAMTS13 IgGs ^{25;26;30}, however, the test specificity may also be low. For example, ~5% of healthy individuals and 13% of patients with systemic lupus erythematosus showed positive ELISA results despite normal ADAMTS13 activity in plasma ^{30;31}.

To develop a better assay, we engineered and expressed a recombinant chimeric glycosylphosphatidylinositol (GPI) anchored ADAMTS13 or variants on the plasma membrane of Chinese hamster ovary (CHO) cells. Such a modification helps maintain antigens to be detected in their native conformations, which greatly facilitates the binding of specific IgGs to both linear and non-linear epitopes. Our results demonstrate that this novel cell-based assay may be applicable for rapid identification and mapping of anti-ADAMTS13 IgGs in patients with acquired idiopathic TTP. Our findings also suggest differential antigenic epitopes may be detected under different assay conditions. Further investigation of the clinical significance of these anti-ADAMTS13 autoantibodies with various assay methods may shed more light on pathogenesis of TTP.

Methods

Construction of GPI-anchored ADAMTS13 and variants

A cDNA fragment encoding 41 amino acid residues (His³⁰⁷-Thr³⁴⁷) of decay accelerating factor (DAF), the sequence required for GPI anchoring signal ³², was amplified by PCR using a pDF4 encoding human full-length DAF in pBluescript KS+ vector as a template (kindly provided by Dr. Douglas Lublin at Department of Pathology and Immunology, Washington University in St. Louis). Primers used for amplification of GPI-anchoring signal were 5'-act gcg gcc gcc atg aaa caa ccc caa ata aag ga-3' (forward) and 5'-tca gcg gcc gct caa gtc agc aag ccc atg gtt act ag-3' (reverse). The Not I restriction enzyme digestion site (underlined) was introduced at either end. PCR-amplified GPI-anchoring sequence was then

digested with NotI and ligated into pcDNA3.1-ADAMTS13-V5-His containing a full length human ADAMTS13 (FL) or a variant truncated after the spacer domain (S) tagged at the C-terminus with V5-His epitope^{33,34}. Because a stop codon was also introduced after the GPI-anchoring signal, the resulting plasmids, pcDNA3.1-gFL and pcDNA3.1-gS, contained no V5-His epitope. There was a short peptide sequence (-KGNSADIQHSG-) derived from the vector linking FL or S to the GPI-anchoring signal at the very end of the C-terminus (Fig. 1A).

To generate a construct containing a GPI-anchor signal and the C-terminal fragment of ADAMTS13 from TSP1 2-8 repeats and CUB domains, we used pcDNA3.1-ADAMTS13-V5-His containing a full-length ADAMTS13 as a template and primers (forward: 5'-gcc tgg tgg tgg gcc gct gtg cgt-3' and reverse: 5'-gtt aag tca gca agc cca tgg tta-3') for amplification of the cDNA fragment. The resulting cDNA fragment was purified using a gel purification kit and ligated into pSecTag/FRT TOPO vector (Invitrogen) according to the manufacturer's instruction. The resulting plasmid designated pSecTag/FRT-gT2C contained an Igk-chain signal peptide, a short linker, the C-terminal fragment (TSP1 2-8 repeats and CUB domains), and a GPI-anchoring signal, but no V5-His epitope (Fig. 1A). All plasmids were sequenced at the Core Facility of the Children's Hospital of Philadelphia Research Institute to confirm the accuracy of the inserts. Each plasmid was purified using a Qiagen kit for transfection.

Transfection and establishment of stable cell lines

Chinese hamster ovary (CHO)-Flip In cells (Invitrogen,) were cultured in 5% CO₂ and 95% air at 37 °C with Ham's F-12 medium supplemented with 1% penicillin/streptomycin, 10% fetal calf serum and 100 µg/ml Zeocin. The CHO Flip-In cells in 6-well plate were co-transfected with plasmid pOG44 (0.4 µg) plus pSecTag/FTR-gT2C (4 µg) using lipofectamine2000 reagents (Invitrogen). Also, CHO Flip-In cells were transfected with 4 µg of pcDNA3.1-gFL or pcDNA3.1-gS using lipofectamine2000 (Invitrogen). Twenty-four hours after transfection, cells transfected with pOG44 plus pSecTag/FTR-gT2C were treated with 0.5 mg/ml of hygromycin B (Cellgro) in a complete medium for 8 days. However, cells transfected with pcDNA3.1-gFL and pcDNA3.1-gS were treated with 1 mg/ml of geneticin for 5–7 days. The resistant clones expressing human full-length ADAMTS13 and variants were identified by immunofluorescent microscopy and flow cytometry using rabbit anti-ADAMTS13 IgG (1:200) or mouse anti-ADAMTS13-Dis IgG (1:200) as described in the immunofluorescent microscopy and flow cytometry sections.

Patient blood samples

Pooled and individual normal human plasmas were obtained from George King Biomedical (Overland Park, KS) for controls. Patient blood was collected during the acute episode or during treatment or after remission in some cases into a tube containing 0.38% sodium citrate as an anticoagulant. After centrifugation at 1500×g for 15 min, plasma was aspirated and stored in aliquots at -80 °C until assay. The Institutional Review Board (IRB) at the Children's Hospital of Philadelphia approved the study protocol. Informed consent was obtained from the participants.

Assays for plasma ADAMTS13 activity and inhibitors

Plasma ADAMTS13 activity and inhibitors were determined with a fluorogenic peptide, FRETs-VWF73, according to the method described previously³⁵. A positive inhibitor was defined if proteolytic activity in the pooled normal human plasma was reduced by 30% after being incubated for 60 minutes with heat-inactivated patient plasma (50:50 mix) as previously described²³.

ELISA

Plasma concentrations of anti-ADAMTS13 IgGs were determined by a modified immunoassay (Technoclone, Vienna, Austria). Briefly, a high-binding microtiter plate was coated with either recombinant full-length ADAMTS13 or C-terminal fragment comprising TSP1 2-8 and CUB domains (T2C) with V5-His epitope (0.2 µg/well). After being blocked with blocking reagent (Technoclone), 100 µl of diluted plasma (1:20) were added to the wells and incubated at 25 °C for 2 hours. The bound IgGs were detected by peroxidase-conjugated anti-human IgG, followed by 3,3',5,5'-tetramethylbenzidine (TMB) according to the manufacturer's instruction. TTP patient plasma with known concentration of anti-ADAMTS13 IgGs provided by Technoclone was used for calibration. One tenth of the standard anti-ADAMTS13 IgGs was used for determination of anti-ADAMTS13 IgG specifically targeted at the C-terminal ADAMTS13. Values greater than 15 U/ml was defined as positive.

Immunofluorescent microscopy

Stably transfected or non-transfected cells were cultured on a chamber slide at 70–100% confluence. After being washed with PBS, cells were fixed with 1% paraformaldehyde in PBS (non-permeabilized) for 10 minutes. The cells were blocked with 2.5% BSA in PBS for 30 minutes and incubated with a primary antibody (monoclonal anti-disintegrin IgG, mAb360 or rabbit anti-ADAMTS13 IgG, pAb480) (1:200) for 2 hours at 22 °C, followed by a fluorescein-conjugated secondary antibody diluted in 0.5% BSA in PBS for 1 hour. The cells were then incubated with Alexa Fluor® 647 conjugated transferrin (Invitrogen) (1:500) for 10 minutes, and mounted with medium containing 4',6 diamidino-2-phenylindole (DAPI) (1:5) (Vector Lab, Burlingame, CA) after three washes with PBS. The fluorescent signals were determined under a multi-color fluorescent microscope. The images were obtained using SPORTadvanced software (Sterling Heights, MI) at magnifications of 100X and 600X.

Flow Cytometry

Cells (untransfected or transfected) on 10-cm dishes were dissociated with 5 ml of 0.05% trypsin-EDTA for 2 minutes. The cells were washed with ice-cold PBS and incubated with a specific antibody against ADAMTS13 (monoclonal anti-ADAMTS13-Dis IgG, 1:200) (custom made in Green mountain antibodies, Burlington VT) or rabbit anti-ADAMTS13 IgG (1:200) (custom made in Open Bioscience, Huntsville, AL) for 60 minutes at 4 °C. After being washed with ice-cold 0.5% BSA in PBS, cells were incubated with a fluorescent conjugated secondary antibody at 4 °C for 30 minutes. In addition, 7-Amino-actinomycin D (7-AAD) (Biolegend, San Diego, CA) at concentration of 1 µg/ml was added to exclude the dead cells. For detection of autoantibodies against ADAMTS13 in patients with TTP, diluted plasma (1:40) was first incubated for 60 minutes with 3–4×10⁶ of untransfected CHO cells to pre-absorb non-specific IgGs. After centrifugation at 13,000 rpm for 30 minutes at 4 °C, the resulting clear plasma (1:40, 1:80, and 1:160) was incubated with 1×10⁶ of untransfected cells or cells expressing gFL, gS, and gT2C for 60 minutes. After being washed with ice-cold PBS, the cells were incubated with a fluorescent conjugated secondary antibody on ice for 30 minutes. Again, 7-AAD was added at the end for 10 minutes to exclude the dead cells. The stained cells were analyzed by flow cytometry using a BD FACS sorter (BD Biosciences, San Jose, CA). Only the viable cells were gated for analysis of the antibody binding (Suppl. Fig. 1).

Definition

For the functional assay, a patient was defined as positive for anti-ADAMTS13 inhibitors if greater than 30% of ADAMTS13 activity in pooled normal human plasma (NHP) was

inhibited after 50:50 mixing with patient plasma²³. For the flow cytometry assay, a patient was defined as negative for anti-ADAMTS13 IgGs if the percentage of IgG-binding cells was less than 5%. In contrast, a patient was defined as 1+ positive if the percentage of IgG binding cells detected was greater than 5% at 1:40, 2+ at 1:80, 3+ at 1:160, and 4+ if greater than 25% at 1:160. This cut-off value (5%) was based on the assessment of normal IgG binding to the transfected cells and binding of patients' IgGs to untransfected cells, in which none had greater than 5% of positive rate.

Results

Patient characteristics

Patients' demographic, clinical information and anti-ADAMTS13 IgGs or inhibitors are summarized in Table 1. Twenty-six patients with idiopathic TTP and 20 healthy donors were recruited for the study. The mean age of TTP patients was 44.2 years old with predominantly female patients (F/M ratio=22/4). Most patients (17/26) had their initial episode, whereas the remaining (9/26) were relapsed or recurrent. Twenty-three patients had severe deficiency of plasma ADAMTS13 (less than 5% of normal), but 3 patients had moderate deficiency (5–50%) of plasma ADAMTS13 because of plasma exchange treatment. Twenty-five of the 26 (96%) patients were positive for anti-ADAMTS13 autoantibody by FRETs-VWF73 assay and had detectable anti-ADAMTS13 IgGs toward full-length ADAMTS13 (FL) IgGs and C-terminal fragment TSP1 2-8 plus CUB (T2C) (>15 U/ml). The plasma levels of anti-FL IgGs and anti-T2C IgGs were 90.0 ± 56.0 (means $\pm$ SD) U/ml and 37.4 ± 22.7 (means $\pm$ SD) U/ml, respectively (Table 1).

Characterization of GPI-anchored ADAMTS13 and variants expressed on CHO cells

ADAMTS13 is a soluble protein that does not normally interact with cell membrane or extracellular matrix³⁴. To convert a soluble protein to a membrane-anchored form, we attached a 41-amino acid residue peptide derived from GPI-anchor of DAF onto the C-terminal end of human full-length ADAMTS13 or variants. The engineered proteins were stably expressed on CHO cells and used as a source of antigens. Cell lysate containing either gFL or gS, but not gT2C (no protease domain) was able to cleave rF-VWF73 peptide. The proteolytic activity in cells expressing gS was 1.2 unit/ 1.3×10^7 cells, which was almost twice as much as that in cells expressing gFL (0.7 unit/ 1.3×10^7 cells), suggesting that the expression levels of the truncated form are higher.

By flow cytometry, nearly 100% of cells were found to be positive for gFL (Fig. 1B), gS (Fig. 1D), and gT2C (Fig. 1F). Immunofluorescent staining with monoclonal anti-Dis (for gFL and gS) or polyclonal anti-ADAMTS13 IgG (for gT2C) under nonpermeabilized conditions confirmed that almost 100% of these cells express gFL (Fig. 1C), gS (Fig. 1E), and gT2C (Fig. 1G). The expression levels for truncated variants (i.e. gS and gT2C) appeared to be higher than those of gFL based on the fluorescent intensity (Fig. 1B, 1D, and 1F), consistent with the results from activity assay. Under higher magnification, the expressed gFL, gS, and gT2C clustered on the membrane could be easily visualized (Fig. 2, A, E, and I), which was co-localized with the binding of fluorescein-labeled transferrin (Fig. 2, B, F, and J and Fig. 2, D, H, and L). The punctuated, but not smooth staining of the expressed gFL, gS, and gT2C on plasma membrane suggests clustering of these chimeric proteins into the glycosphingolipid-cholesterol rich microdomains. No membrane staining was detected on cells expressing a wild-type ADAMTS13 without permeabilization (data not shown). When permeabilized, the staining was predominantly intracellular or perinuclear (not shown), suggesting the normal distribution of soluble ADAMTS13 along the secretory pathway. The results demonstrated that the expressed GPI-anchored ADAMTS13 and variants were functional and localized to the cell plasma membrane.

Pre-adsorption of plasma with untransfected cells reduced non-specific IgG binding

Human plasma contains a variety of IgGs that may bind cell surface proteins non-specifically, which masks the detection of specific anti-ADAMTS13 IgG binding to the transfected cells. To reduce the background, we pre-incubated patient plasma (1:40) with various numbers ($2\sim 4\times 10^6$) of untransfected CHO cells. We then tested the binding of plasma IgGs to both untransfected and transfected CHO cells expressing gS. As shown, pre-adsorption of TTP patient plasma with 2×10^6 , 3×10^6 , and 4×10^6 of untransfected CHO cells dramatically reduced the binding of plasma IgGs to the untransfected CHO cells (Fig. 3A). However, this process did not significantly reduce specific anti-ADAMTS13 IgG binding to the CHO cells expressing gS (Fig. 3B-D). These results suggest that the pre-adsorption is a critical step to ensure the removal of non-specific IgGs from patient samples.

Detection of plasma anti-ADAMTS13 IgG binding by flow cytometry

When pre-adsorbed patients' plasmas (1:40, 1:80, and 1:160) were incubated with stably transfected cells expressing gFL, gS, and gT2C, the percentage of positive cells reduced as a function of increasing plasma dilutions in each patient (Fig. 4A-C). Most patients had high percentage binding rates greater than 20% even at dilution of 1:160. The percentage of patients who had positive IgG binding (defined by having greater than 5% of positive cells at any given dilution) to the cells expressing gFL, gS, and gT2C was 100%, 100%, and 96%, respectively (Table 1, Fig. 4A-C). The positive rates for detecting patients' IgGs using cells expressing gFL and gS were rather consistent with those by ELISA or immunoprecipitation plus Western blotting²⁷ (data not shown). However, the positive rate (96% of patients had greater than 5% of positive cells) for anti-T2C IgGs was much higher than those (0–46%) previously reported using other techniques^{3,27,36}. Intriguingly, patient 6 has mild deficiency of proteolytic activity, but strong IgG binding to the transfected cells expressing gFL, gS, and gT2C (Table 1), suggesting the presence of non-inhibitory binding epitopes in this patient. These results suggest that our cell-based assay is more sensitive than other assays for identification of anti-ADAMTS13 IgGs, particularly those recognizing the more distal C-terminal domains of ADAMTS13.

To determine the specificity of this assay, we performed similar binding experiments in 20 plasma samples from healthy blood donors. An incubation of pre-absorbed normal human plasma (1:40) with untransfected or transfected cells expressing gFL, gS, and gT2C resulted in only less than 5% of positive rate (not shown). None of 20 healthy blood donors had their IgG binding to the cells at levels of greater than 5% after plasma was diluted 1:40. The ranges of binding rates to the cells expressing gFL, gS, and gT2C were 0.40–0.85%, 0.88–1.22%, and 0.61–1.11%, respectively. Therefore, 5% positive rate was used as a cut-off for the presence of anti-ADAMTS13 IgGs in patients.

Correlation between flow cytometry assay and ELISA for detection of plasma anti-ADAMTS13 IgGs

To determine whether the percentage of positive cells was correlated with the concentrations of plasma anti-ADAMTS13 IgGs, we performed Pearson correlation coefficient analysis. The results showed that the numbers of positive IgG binding cells expressing gFL, gS, and gT2C by flow cytometry were significantly correlated with the concentrations of plasma anti-ADAMTS13 IgGs by ELISA with the correlation coefficients of 0.65 ($p<0.001$) (Fig. 5A), 0.67 ($p<0.001$) (Fig. 5B), and 0.42 ($p<0.001$) (Fig. 5C), respectively. The correlation coefficient between the percentage of IgG positive cells expressing gT2C and plasma concentration of anti-ADAMTS13 IgGs recognizing the C-terminal TSP1 2-8 plus CUB (T2C) was weak ($r=0.33$), but significant (Fig. 5D). These results suggest that the antigenic epitopes identified by cell-based assay may be different from those determined by microtiter

plate assay. Alternatively, clustering antigen on plasma membrane may increase the binding affinity of anti-ADAMTS13 IgGs, thereby increasing the analytic sensitivity.

Monitoring changes of plasma anti-ADAMTS13 IgGs during treatment

To determine whether the cell-based assay was useful or not for monitoring the changes in plasma levels of anti-ADAMTS13 IgG during therapy, we assayed the binding rates of plasma anti-ADAMTS13 IgGs to the cells expressing either gFL or gS in two patients with acquired idiopathic TTP. For comparison, plasma concentrations of anti-ADAMTS13 IgGs were also determined by the ELISA method. As shown in Figure 6, the numbers of positive anti-ADAMTS13 IgG binding cells expressing either gFL (Fig. 6A and 6B) or gS (Fig. 6C and 6D) were high prior to therapy and dramatically reduced once plasma exchange therapy was initiated and remained low during remission (Fig. 6A-D). These results were highly consistent with the improvement of platelet counts (Fig. 6E and 6F), rise in plasma ADAMTS13 activity and reduction in plasma anti-ADAMTS13 IgG concentrations (Fig. 6G and 6H). These results suggest that our cell-based assay may be useful for monitoring therapy of acquired TTP patients.

Discussion

We report here a novel cell-based assay using GPI-anchored ADAMTS13 and variants for assessment of anti-ADAMTS13 IgGs and determination of the binding epitopes at the same time. The expressed chimeric ADAMTS13 and variants were localized to the plasma membrane of the cells (Fig. 1 and Fig. 2). These punctuated staining patterns on the cell surface suggests that clustering of these GPI-anchored proteins on spingolipid-cholesterol rich microdomains (or rafts)³⁷. The cell surface anchored antigens are presumably in their native conformations, so that both linear and non-linear epitopes may be recognized by polyclonal anti-ADAMTS13 IgGs in patients with acquired TTP.

This novel assay appears to be highly sensitive and specific for identification of circulating anti-ADAMTS13 IgGs in TTP patients. Of 26 TTP patients and 20 healthy blood donors tested, 100% of TTP patients' IgGs bound to the cells expressing gFL and gS in addition to 96% of patients IgGs that bound to the cells expressing gT2C (Table 1 and Fig. 4). In contrast, none of healthy blood donors had their IgGs binding to any of these stable cell lines detectably (data not shown).

There is moderate, but statistically significant correlation between the plasma concentrations of anti-ADAMTS13 IgGs as determined by ELISA and the percentage of positive IgG-binding cells expressing gFL ($r=0.65$, $p<0.001$), gS ($r=0.67$, $p<0.001$), and gT2C ($r=0.42$, $p<0.001$) (Fig. 5). These results suggest that the binding epitopes of anti-ADAMTS13 IgGs to the immobilized antigen on microtiter plate and to the clustered antigens on plasma membrane may be fundamentally different. It is also likely that clustering antigens into the membrane rafts may enhance the affinity or avidity of anti-ADAMTS13 IgG binding, which is not detectable by the microtiter plate assay. This may be particularly intriguing for the anti-ADAMTS13 IgGs that target at the middle and distal C-terminal domains of ADAMTS13.

In spite of the difference in antibody binding characteristics, our assay appears to be rapid and cost-effective. The assay does not require purified antigens or additional equipment except for a flow cytometer, which is pretty routine diagnostic tool in clinical laboratory. The assay allows us to determine the concentrations and potential binding epitopes of anti-ADAMTS13 IgGs in patients with TTP at the same time. The assay appears to be more sensitive than other assays (i.e. ELISA and immunoprecipitation plus Western blotting) for the detection of anti-ADAMTS13 IgGs, particularly those targeting against the middle and

distal C-terminal domains. In our previous study, we showed that that most of acquired TTP patients had their anti-ADAMTS13 IgGs binding weakly to the C-terminal TSP1 2-8 repeats and CUB domains using the immunoprecipitation assay²⁷. Our current study shows that almost all patients (except for one) have positive IgG binding to the cells expressing gFL and gT2C (Table 1 and Fig. 4).

To determine the utility of the cell-based assay monitoring therapy, anti-ADAMTS13 IgG binding rates were determined in serial samples from two patients with acquired idiopathic TTP. As shown, the IgG binding rates to the cells expressing gFL and gS are high prior to treatment, dramatically decreased over time during plasma exchange, and remained low during remission (Fig. 6). The kinetic changes in anti-ADAMTS13 IgG binding cells are in agreement with clinical resolution of TTP syndrome, alterations of plasma ADAMTS13 activity and anti-ADAMTS13 IgG concentrations (Fig. 6), although dissociation of clinical responses from plasma levels of anti-ADAMTS13 IgG has been observed¹⁵. Nevertheless, the persistence of high concentrations of anti-ADAMTS13 IgGs and/or low ADAMTS13 activity (<10%) during remission has been shown to have increased relapse and/or mortality rates^{14;15;28}. Therapies aiming at elimination of anti-ADAMTS13 IgGs, such as the use of corticoid steroid³⁸, cyclosporine¹⁷, cyclophosphamide^{18;19} and rituximab^{21;39}, may result in long-term remission and potentially cure of acquired autoimmune TTP. These clinical observations warrant a sensitive and specific assay for the assessment of anti-ADAMTS13 autoantibodies.

We conclude that our cell-based assay utilizing recombinant ADAMTS13 and variants anchored on plasma membrane may provide a novel and rapid tool for assessing plasma anti-ADAMTS13 IgGs in patients with acquired TTP, which may help confirm diagnosis, predict outcome, and guide adjunctive therapy. Further investigation of IgG-binding rates toward the cells expressing these chimeric GPI-ADAMTS13 and variants in different patient populations in hospital or clinic will help define true sensitivity, specificity and predictive values.

What is known about this topic?

- Anti-ADAMTS13 IgG is the major cause of acquired idiopathic thrombotic thrombocytopenic purpura (TTP).
- Functional assays (FRETs-vWF73 and Western blotting) and enzyme-linked immunosorbent assay (ELISA) are used to detect plasma anti-ADAMTS13 IgGs. The former lack sensitivity, but the latter may not detect conformationally sensitive (or non-linear) epitopes.
- Detection of anti-ADAMTS13 IgGs in plasma helps confirm diagnosis, predict relapses, and guide potential adjunctive therapies of TTP patients.

What does this paper add?

- Novel recombinant GPI-anchored ADAMTS13 and variants are expressed on cell plasma membrane, providing antigens in their native conformations and allowing anti-ADAMTS13 IgGs to bind under optimal conditions
- This novel cell-based assay appears to be sensitive and specific for detection of anti-ADAMTS13 IgGs in patients with acquired (idiopathic) TTP with severe to moderate deficiency of plasma ADAMTS13 activity.
- Only moderate correlation was observed between plasma concentrations of anti-ADAMTS13 IgGs by ELISA and the percentage of positive cells expressing

gFL, gS, and gT2C by cell-based assay, suggesting differential epitopes being identified under these conditions.

- Our findings suggest that the assay may be useful for rapid identification and mapping of anti-ADAMTS13 IgGs in patients with acquired TTP, which help confirm diagnosis, predict outcome, and guide adjunctive therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of ADAMTS13 protein and expression of GPI-anchored ADAMTS13 and variants in CHO cells

A. the domain composition of GPI-anchored human full-length ADAMTS13 (gFL), a variant truncated after the spacer domain (gS), and a fragment containing TSP1 2-8 repeats and CUB domains (gT2C). **B, D, and F** show the percentage of positive cells by flow cytometry, whereas **C, E, and G** are the immunofluorescent staining images obtained under a fluorescent microscope with 100x magnifications. Monoclonal anti-Dis IgG (**B, C, D, and E**) or polyclonal anti-ADAMTS13 IgG (**F and G**) was used as a primary antibody. Alexa488 anti-murine IgG (green) or Cy3-conjugated anti-rabbit IgG (red) was used as a secondary antibody for detection. The shaded area is the negative control with only a secondary antibody.

Figure 2. Co-localization of GPI-anchored full-length ADAMTS13 and variants with transferrin-binding receptor to plasma membrane

CHO cells stably expressing gFL (**A-D**), gS (**E-H**), and gT2C (**I-L**) or untransfected (**M-P**) were incubated with a monoclonal anti-Dis IgG (1:200) (**A** and **C**) or a polyclonal anti-ADAMTS13 IgG (1:200) (**I** and **M**), followed by Alexa488-conjugated anti-murine IgG (1:200) (green) or a Cy2-conjugated anti-rabbit IgG (1:200) (green). After being washed, the cells were incubated with Alexa688-conjugated transferrin (1:500) (red) (**B**, **F**, **J**, and **N**) for 10 minutes. The nuclear DNA was stained with DAPI (**C**, **G**, **K**, and **O**). The fluorescent signals were detected under a multi-filtered fluorescent microscope (magnification, 600X). The merged images were shown in panels **D**, **H**, **L**, and **P**. Arrowhead indicates the fluorescent signal on plasma membrane.

Figure 3. Pre-adsorption of plasma IgGs with untransfected cells dramatically reduced non-specific IgG binding

Diluted plasma from a TTP patient was pre-adsorbed without (0) or with 2×10^6 , 3×10^6 , and 4×10^6 of untransfected CHO cells as indicated by colored lines. The resulting plasma was incubated with untransfected cells (A) or with the cells expressing gS at dilutions of 1:40 (B), 1:80 (C), and 1:160 (D). The percentage of positive cells was determined by flow cytometry as described in the Materials and Methods. The shaded areas in red (A-D) indicate fluorescent intensity of the untransfected cells after only secondary antibody.

Figure 4. Binding of plasma anti-ADAMTS13 IgGs to the cells expressing GPI-anchored ADAMTS13 and variants in all 26 TTP patients

The percentages of anti-ADAMTS13 IgG binding cells expressing gFL (A), gS (B), and gT2C (C) were detected by flow cytometry after incubation of pre-adsorbed cells with diluted plasma at 1:40 (solid bar), 1:80 (gray bar), and 1:160 (grid bar) from 1–26 TTP patients. None of 20 healthy blood donors with plasma dilution of 1:40 showed more than 5% of positive cells expressing gFL, gS, and gT2C (not shown).

Figure 5. Correlation between plasma concentrations of anti-ADAMTS13 IgGs and positive cells Pearson correlation coefficients (r) were determined between plasma concentrations of anti-ADAMTS13 IgGs measured by ELISA using full-length ADAMTS13 (**A**, **B**, and **C**) or C-terminal fragment T2C (**D**) as antigen and the positive anti-ADAMTS13 IgG-binding cells expressing gFL (**A**), gS (**B**), and gT2C (**C**, **D**) with patient plasma diluted 1:40.

Figure 6. Kinetic changes of anti-ADAMTS13 IgG binding cells in TTP patients during plasma therapy

A and **E** are platelet counts in two patients during and after plasma exchange therapy; **B** and **F** are plasma ADAMTS13 activity by FRET573 assay (open) and anti-ADAMTS13 IgG concentrations by ELISA (closed). **C**, **D**, **G**, and **H** are the percentage rates of plasma anti-ADAMTS13 IgG-binding to the cells expressing gFL (**C** and **G**) and gS (**D** and **H**) by flow cytometry.

Table 1

Demographic information, clinical data, plasma ADAMTS13 activity and anti-ADAMTS13 IgGs in 26 patients with acquired idiopathic TTP

anti-FL														anti-T2C													
NO.	Age	Sex	TTP	%	FRETS		IgG		gFL		Interp.		gS		IgG		gT2C		Interp.								
					Inh	U/ml	1:40	1:80	1:160	P/N	1:40	1:80	1:160	P/N	U/ml	1:40	1:80	1:160		P/N							
1	62	F	Initial	<5	(-)	61.7	64.7	9.0	6.1	3+	41.9	7.0	<u>1.5</u>	2+	26.8	86.7	72.2	45.5	4+								
2	73	F	Rel.	<5	(+)	116.6	19.7	9.9	5.1	3+	92.9	50.6	11.8	3+	17.8	62.8	37.8	22.2	3+								
3	39	F	Initial	14	(+)	129.2	50.9	17.1	12.4	3+	99.2	94.4	67.4	4+	23.1	94.8	65.5	38.9	4+								
4	44	F	Rel.	<5	(+)	67.8	72.0	39.0	<u>1.9</u>	2+	78.7	25.2	<u>4.0</u>	2+	18.0	85.0	43.5	24.7	3+								
5	52	F	Rel.	<5	(+)	56.3	17.1	5.9	6.0	3+	34.0	6.8	<u>1.1</u>	2+	16.0	53.2	24.4	11.7	3+								
6	47	F	Initial	34	(+)	86.5	40.6	26.5	6.7	3+	79.4	63.4	6.6	3+	21.6	53.3	43.5	11.5	3+								
7	42	F	Initial	<5	(+)	59.6	10.3	6.3	<u>4.1</u>	2+	13.8	<u>3.0</u>	<u>0.7</u>	1+	32.0	74.8	48.3	24.5	3+								
8	58	F	Initial	<5	(+)	54.0	29.2	14.0	4.8	2+	46.6	7.2	5.7	3+	24.9	77.3	72.3	52.1	4+								
9	34	M	Initial	<5	(+)	124.4	87.2	34.9	<u>16.7</u>	3+	99.9	99.5	25.5	4+	56.8	97.9	83.9	55.7	4+								
10	45	F	Initial	<5	(+)	73.9	85.6	50.0	23.1	3+	96.7	81.1	13.3	3+	24.3	83.0	60.1	35.3	4+								
11	44	F	Initial	<5	(+)	34.0	9.3	<u>4.1</u>	<u>3.3</u>	1+	19.5	<u>3.1</u>	<u>0.3</u>	1+	8.7	<u>3.1</u>	<u>2.5</u>	<u>2.5</u>	0								
12	34	M	Initial	<5	(+)	159.8	65.8	35.5	16.7	3+	98.6	92.4	61.9	4+	34.7	95.3	86.9	64.4	4+								
13	51	F	Rel.	<5	(+)	90.5	44.1	18.7	8.5	3+	40.9	13.4	8.5	3+	78.1	98.0	93.3	85.8	4+								
14	30	F	Initial	<5	(+)	32.4	19.2	7.5	<u>3.7</u>	2+	6.8	<u>2.0</u>	<u>0.8</u>	1+	22.5	31.7	10.9	6.3	3+								
15	38	F	Initial	<5	(+)	14.1	10.7	7.2	<u>1.5</u>	2+	5.6	<u>3.5</u>	<u>0.6</u>	1+	16.4	15.0	7.1	5.8	3+								
16	50	F	Initial	<5	(+)	237.8	66.4	16.1	<u>4.6</u>	2+	54.5	39.7	14.5	3+	96.5	85.6	78.7	62.8	4+								
17	19	F	Initial	<5	(+)	31.7	15.1	8.2	<u>4.5</u>	2+	14.3	7.3	<u>2.8</u>	2+	35.8	11.4	9.1	<u>4.4</u>	2+								
18	61	F	Rel.	<5	(+)	147.2	83.8	13.4	6.3	3+	87.1	55.5	18.5	3+	24.8	19.1	<u>2.3</u>	<u>0.3</u>	1+								
19	28	F	Initial	<5	(+)	89.5	43.3	8.9	<u>3.5</u>	2+	72.0	14.0	5.6	3+	54.8	60.7	21.4	<u>4.9</u>	2+								
20	47	M	Rel.	<5	(+)	217.8	70.6	22.3	11.6	3+	93.0	93.0	54.7	4+	65.8	60.0	50.7	31.7	4+								
21	46	F	Initial	<5	(+)	82.2	34.8	9.2	6.4	3+	29.4	9.9	<u>1.8</u>	2+	59.7	23.4	6.4	<u>1.1</u>	2+								
22	42	M	Rel.	<5	(+)	40.9	9.5	5.5	<u>4.9</u>	2+	34.6	5.1	<u>1.7</u>	2+	32.3	77.8	33.1	7.3	3+								
23	40	F	Rel.	<5	(+)	28.9	9.1	6.3	5.5	3+	10.0	<u>1.3</u>	<u>0.4</u>	1+	20.6	29.6	12.6	<u>4.7</u>	2+								
24	63	F	Initial	10	(+)	122.4	42.2	20.0	13.8	3+	78.1	52.0	39.3	4+	68.0	59.6	32.7	19.3	3+								
25	38	F	Initial	<5	(+)	121.4	44.0	19.9	13.6	3+	94.2	49.3	14.5	3+	62.7	68.4	28.1	11.9	3+								
26	21	F	Rel.	<5	(+)	58.3	36.6	7.6	<u>3.2</u>	2+	61.4	20.9	<u>3.2</u>	2+	30.5	31.6	21.0	5.6	3+								