Analysis of the pathogenesis of thrombotic thrombocytopenic purpura

Doctoral Theses

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is an episodic, rare, life-threatening disorder. TTP is characterized by severe thrombocytopenia, Coombs negative hemolytic anemia and various involvements of other organs. TTP patients are usually presenting with wide spectrum of neurologic symptoms as headache, dizziness and stroke like symptoms like paresis, aphasia, ataxia etc.
However, in other members of thrombotic microangiopathies (TMA) such as hemolytic uremic syndrome (HUS) kidney failure is common, in TTP the serum creatinine level is very rarely higher than 200 umol/L and patient are not anuric. In most cases the presentation of autoantibodies against von Willebrand Factor (VWF)-cleaving protease (A Disintegrin and Metalloproteinase with ThromboSpondin-1 like motifs-13, ADAMTS13) predispose the disease. In rare cases TTP is associated with ADAMTS13 mutation. Severe deficiency of ADAMTS13 leads to the increased presence of ultra-large VWF (UL-VWF). UL-VWF is an adhesive surface for platelets so it can promote a platelet rich trombi in capillaries and small vessels that can lead to an organ ischaemia or failure.
The diagnosis of TTP is based on one or more episodes of thrombocytopenia with microangiopathic hemolytic anemia (increased LDH level, fragmented erythrocytes in the periferial blood smear) and ADAMTS13 deficiency (activity of ADMATS13 <10%).
Plasma-exchange (PEX) is the most effective treatment of TTP that can eliminate autoantibodies and restore ADAMTS13 activity by new enzymes through fresh frozen plasma (FFP). During PEX sessions, immunosuppressant such as methyprednisolon or rituximab can be also given.
In the past years, several studies pointed that fact ADAMTS13 deficiency alone cannot explain the onset of all TTP episodes. In many cases, there is mild infection (gastroenteritis, upper airway infection), pregnancy or surgery before the manifestation of TTP. Moreover, autoantibodies and ADAMTS13 deficiency can be detected in patient plasma during remission without any symptoms and signs of hemolytic

anemia or thrombocytopenia. In addition, the onset of congenital TTP sometimes can be seen in adulthood or can be associated with pregnancy which also point to the need of other trigger factors.

Objectives

Studies emphasized the importance of other triggering events besides ADAMTS13 deficiency in TTP therefore our aim was to investigate other potentially involved trigger factors. Previously our group reported on the presence of complement activation in patients with acute TTP. Our data indicated the presence of all three complements pathway. Because of complement system and neutrophil granulocytes can be both present in inflammation or infection and in pregnancy we hypothesized that neutrophil granulocytes can be involved in the pathogenesis of TTP. Hence, our aim was to investigate the association of neutrophil activation with acute and remission TTP, to assess whether neutrophil activation changes during PEX therapy and to show if complement-, disease activity marker and neutrophil activation are parallel.
We also wanted to investigate the endothelial cell activity in disease process. Endothelial activation is one of the key events in the pathogenesis of TTP and another TMA-s as well. However, there were many studies confirmed the activation of the endothelial cell in TTP, none of them used a specific endothelial marker or a well standardized, bigger patient group. In this way, our further aim was to investigate the endothelial cell activity with a specific marker, to asses if endothelial cell activity changes during therapy and if there is any association between complement- and endothelial cell activity.

Methods

Our laboratory provides diagnostic services (ADAMTS13 and complement measurements) for patients suspected to have HUS or TTP. So we enrolled those who were fulfil the diagnosis of TTP, briefly one or more episodes of Coombs negative microangiopathic hemolytic anemia (elevation of LDH (>450U/L) level, and appearance of fragmented erythrocytes) with thrombocytopenia (PLT<150 G/L) and ADAMTS13 deficiency (activity <10%) or history for it. The patient enrolment for this study was started in August 2007 and closed in January 2014. Patients with acute oligo-anuric renal failure or concomitant diseases such infection, malignancy, autoimmune diseases were excluded from the study.

Because the procedure of PEX can influence the measurements acute TTP samples were separated to “before PEX” and “during PEX” samples. Active TTP was defined as: thrombocytopenia (<150 G/L), direct Coombs negative hemolysis with fragmentocytes in the periferial blood smear and ADAMTS13 deficiency. The term remission refers to inactive disease, i.e. hematologic remission (HR) was determined when platelet counts were more than 150 G/L on two consecutive days without any signs of hemolysis even if there were any neurological, renal or other residual clinical symptoms. Complete remission (CR) was established when platelet count remained above the lower limit continuously for at least 1 month without signs of hemolysis, symptoms of active organic involvement or residual symptoms.

Thirty-eight patient (21 acute disease (“before PEX”), 17 in remission) and 20 age and sex matched healthy control were enrolled in to the investigation of neutrophil activity. Some patients had more than one sample taken in different times therefore we used 49 samples of the 38 patients.

We collected further TTP samples to the analysis of endothelial cell activity and altogether 54 TTP patients were enrolled (25 acute disease, “before PEX”, 12 acute disease “during PEX”, 17 remission). There were some patients who had more than one sample therefore altogether eighty-two samples from 54 TTP patients were available for this study. Fifty-seven age and sex matched healthy control were also enrolled.

The fluorogenic substrate, FRETS-VWF73, was applied for the determination of ADAMTS13 enzyme activity and the presence of ADAMTS13 inhibitors. Levels of complement parameters (C3, Factor-H, -I, -B) and complement activity products (C1rC1sC1-inh, C3bBbP, C3a, Bb, C4d, SC5b-9) were measured by sandwich ELISA or RID (radial immundiffusion). PMNE (plasma polymorphonuclear cell elastase) was used for the assessment of neutrophil activity. A stable complex of PMNE-alpha1-proteinase-inhibitor was measured by sandwich type ELISA, according to the instructions given by the manufacturer. MPO (myeloperoxidase) were also determined by ELISA. Endothelin-1 (ET-1) was used for the evaluation of endothelial cell activity. ET-1 is hardly measurable in human plasma due to its very short half-life (1-2 minutes in circulation). Therefore, a stable precursor (Carboxi-Termianl (CT)-proET-1, half-life is 42 hours in room temperature), which is produced in equimolar concentration to endothelin-1, was measured. CT-proET-1 was measured in EDTA plasma with immunoluminometric assay by following the manufacturer's instruction. Another endothelial cell marker, von Willebrand antigen (VWF ag), were measured in EDTA plasma by an in-house ELISA assay.

The continuous variables showed skewed distribution and failed Shapiro-Wilk's test in this study. Non-parametric tests were used for group comparisons. Continuous variables between two groups were compared with Mann-Whitney U test, for three or more groups with the Kruskal-Wallis ANOVA by rank test, and for repeated measures with the Friedman test. Dunn's post-test was used for group comparisons after analysis of variance. Spearman's correlation coefficients were calculated by non-parametric method. Wilcoxon test was used for comparisons of sample pairs. Two tailed p values were calculated and significance level was put at a value of $p<0.050$.

Results

The distribution of PMNE, VWF-antigen and CT-proET-1 levels were investigated in acute disease, remission and in healthy groups. Acute disease was associated with significantly increased PMNE ($p<0.001$), VWF-antigen ($p<0.0001$) and CT-proET-1 ($p<0.0001$) levels compared to healthy controls, whereas the group medians were similarly low in TTP patients in remission and in healthy controls. However, PMNE, VWF-antigen and CT-proET-1levels were lower in both remission TTP and healthy groups, all marker levels were significantly increased in remission compared to healthy controls (PMNE: $p<0.017$, VWF-antigen: $p<0.0001$, CT-proET-1: $p<0.01$). Different subgroups were made according to disease activity (platelet number and ADAMTS13 activity). Active TTP was defined by low platelet count (<150 G/L) and ADAMTS13 deficiency (activity <10%) whereas remission TTP was defined by normal platelet count with or without ADAMTS13 deficiency. Increased PMNE levels and deficient ADAMTS activity together characterized hematologically active disease (PLT<150G/L). PMNE levels were significantly higher in active disease group compared to remission (active disease vs. remission with ADAMTS13 deficiency: $p=0.0129$, active disease vs. remission with normal ADAMTS13 activity: $p=0.0011$). The presence or absence of ADAMTS13 deficiency did not influence PMNE levels in remission. In line with this observation there was significant association between amounts of functional ADAMTS13 inhibitors (as assessed by measuring the ADAMTS13 activity of the mixed patient/normal plasma samples) and PMNE levels in acute TTP ($r=-0,547$; $p=0,012$). Patients with active disease (PLT<150 G/L) and ADAMTS13 deficiency had the highest VWF-antigen and CT-proET-1 levels compared healthy controls (VWF-antigen: $p<0.0001$, CT-proET-1: $p<0.0001$). However, the level of VWF-antigen and CT-proET-1 were lower in remission compared to active TTP, the difference between remission and healthy controls was also significant ($p<0.0001$). In samples taken in remission the presence or absence of ADAMTS13 deficiency did not influence VWF-antigen or CT-proET-1 levels.

We investigated changes of PMNE, VWF-antigen and CT-proET-1 levels during therapy and in remission TTP. There were 6 patients with available samples collected on the day of admission, before the initiation of PEX series and in hematologic remission at least 1 week after the last session of the PEX series. Achievement of remission was associated with significant reduction of circulating plasma PMNE levels in patients with TTP ($p=0.031$). If we restricted the analysis to the subjects with available sample pairs before PEX and during PEX, a significant decrease in both the VWF-antigen ($p<0.05$) and CT-proET-1 ($p<0.05$) levels were observed in response to PEX therapy. However, VWF-antigen levels did not decrease significantly in patients achieving remission, the change of CT-proET-1 levels was significant in the same samples compared to the first sample taken before PEX ($p<0.01$).
Next, correlations between TTP activity markers and PMNE levels were analyzed. The amount of PMNE showed inverse correlation to platelet count in acute TTP ($r=-0.521$; $p=0.015$) and with hemoglobin level in all TTP patients (acute TTP and remission together, $r=-0.382$; $p=0.018$).
In a subset of these patients, levels of complement factors and activation products have been measured previously. Concentrations of the activity markers of the alternative pathway convertase's enzymatic component Factor B (Bb) and anaphylatoxin C3a showed significant, positive correlation to PMNE levels (Factor B (Bb): r=0,392; p=0,015, C3a: r=0,367; p=0,024).
CT-proET-1 did not show any association with platelet count or hemoglobin levels, but showed a significant positive correlation with the activity marker of the alternative pathway (C3bBbP: $r=0.528$; $p=0.013$) and terminal pathway complex (SC5b-9: $r=0.306$; $p=0.135$). No association was observed between the endothelial cell markers CT-proET-1 and VWF antigen.
Because complement factors showed strong correlations with PMNE and CT-proET-1 we wanted to investigate if there is any change in the level of Factor H in TTP. Factor H, which is an important regulator of the alternative pathway, showed significant decrease in acute TTP compared to healthy controls ($p<0.01$). The lowest Factor H levels were observed in patients with active TTP and ADAMT13 deficiency. Patients in remission had higher Factor H levels than acute TTP

patients, but the levels of Factor H were also significantly decreased compared to healthy controls ($p<0.05$). The change of Factor H levels was not clear in follow-up patients.

Discussion

Our aim was to understand more deeply the pathogenesis of TTP. Here we report that the elevated levels of PMNE and CT-proET-1 are characteristics of acute TTP. The highest levels of PMNE and CT-proET-1 were observed in acute TTP, and lower, but still increased levels of both markers were observed in remission, when compared with healthy controls. PMNE levels were directly related to disease activity markers hemoglobin and platelet count, and correlated with amounts of functional ADAMTS13 inhibitor. Secondly, according to the presented data, neutrophil- and complement activation are present parallel in acute TTP and correlate each other. Furthermore, we looked at the relationship between endothelial cell activation and complement alternative and terminal pathway activation in TTP and observed a moderate positive correlation between the endothelial cell activation and alternative pathway activation. We also quantified levels of complement Factor H, an important regulator of the alternative pathway, and observed its decrease in acute and remission TTP when compared with healthy subjects.

Elevated neutrophil activation marker, PMNE, was shown to be increased in acute disease flare. PMNE levels were directly related to disease activity markers hemoglobin and platelet count, and correlated with the amounts of functional ADAMTS13 inhibitor. The potential link between thrombotic microangiopathy and neutrophils was studied previously and elevated plasma DNA and MPO levels were detected in acute TTP. Authors concluded that circulating DNA and histons in patients with TMA could have originated from “neutrophil extracellular traps” (NET). Neutrophils can release NET to trap and kill pathogens during their activation. NETs are implicated in immune defense, sepsis and autoimmunity. NET is formed by a DNA-histones scaffold that contains granule proteins such elastase and MPO. PMNE showed to be an important component of NET. Therefore, based on our results we conclude that increased PMNE levels in acute TTP may originate from neutrophils in a process of cell activation and NET release. Activation of neutrophils, as assessed by a specific biomarker, elastase, is characteristic for hematologically active TTP. Patients with normal

platelet counts and deficient ADAMT13 activity had similar mean PMNE level to those in remission without ADAMTS13 deficiency, and also similar to healthy controls. Furthermore, treatment of acute TTP by a series of PEX sessions resulted decrease of PMNE levels in the majority of patients during PEX whereas the reduction was significant after reaching of hematologic remission.

Acute TTP flares often follow infections or precipitate during or after pregnancy and neutrophil activation is known to be increased in these clinical states. During neutrophil activation, potentially harmful molecules such as enzymes, reactive oxygen species (ROS) and NET can be released by neutrophil cells. In genetically (rare or common variants in *ADAMTS13*) or immunologically (ADAMTS13 inhibitors) predisposed individuals, activation of neutrophils and release of NET may precipitate acute TTP by multiple mechanisms. NET can stimulate thrombosis and promote cytotoxicity. In addition, ROS released by activated neutrophils have prothrombotic effect, mediated in part by inhibition of VWF cleavage by ADAMTS13. NETs may activate complement (alternative pathway) leading to C3b deposition to NET components and generation of C5a. Previously we and others documented the activation of the classical/lectin, the alternative and the terminal pathways of complement in TTP. Our current results indicate the association between PMNE and C3 activation (C3a), and the presence of NETs may be a potential link between these factors. The presence of correlation between PMNE and Bb levels point to the initiation of alternative amplification loop extending complement activation. Complement activation attracts and activate neutrophils and in turn may exacerbate NET release, initiating a positive feed-back loop. Taken together, evolutionarily conserved innate mechanism acting in concert may stimulate thrombosis, endothelial damage and contribute to the precipitation of acute TTP. Currently PMNE measurement is possible only by manual immune- and enzymatic assays, therefore this restricted availability and relatively high costs limits potential clinical activation. However, if these observations can be repeated in independent cohort markers of neutrophil activation may potentially help to stratify patients between therapeutic modalities and to guide time and frequency of PEX treatment and follow disease activity.

Next to our neutrophil study we looked endothelial cell activity in TTP patients. We have presented the largest study to date on the association of increased CT-proET-1 and von Willebrand factor levels with acute and remission TTP. Endothelial cell activation was demonstrated by comparing groups of patients with acute and remission TTP, in relation with healthy controls. The highest levels of CT-proET-1 and VWF were observed in acute TTP, and lower, but still in- creased levels of both markers were observed in remission, when compared with healthy controls. Neither VWF, nor CT- proET-1 levels were associated with ADAMTS13 deficiency in remission. We looked at the relationship between endothelial cell activation and complement alternative and terminal pathway activation in TTP and observed a moderate positive correlation between the endothelial cell activation and alternative pathway activation. We also quantified levels of complement Factor H, an important regulator of the alternative pathway, and observed its decrease in acute and remission TTP when compared with control subjects. The finding of endothelial activation in TTP is interesting, particularly its association with disease activity. Although both VWF and endothelin-1 are considered endothelial markers, their regulation of expression, storage and release are different. Whereas VWF is expressed in, and released by platelets and endothelial cells, endothelin-1 is a specific endothelial marker and its expression and release reflect regulation at the level of gene expression. This observation constituted the foundation of our hypothesis that changes in CT-proET-1 levels in TTP are specific for the activation state of endothelial cells, and rapidly follow activation level changes during therapy. Our results are consistent with previous observations on the presence of endothelial activation in TTP nevertheless we based our measurements on a well standardised, reliable and commercially available specific endothelial biomarker, CT-proET-1 and our study represents a higher number of subjects with de novo acute phase disease and remission.
The high VWF antigen and CT-proET-1 levels in active TTP are intriguing. Increased levels of both markers were observed in pre-PEX TTP samples when compared with healthy controls. When comparing pre-PEX and remission samples in matched pairs analysis only CT-proET-1 showed significant decrease, while VWF antigen did not. Levels of the two markers tended to decrease during PEX.

We have recently observed that activation of complement is a feature of ADAMTS13 deficient TTP and we wanted to analyze the relationship between complement- and endothelial activation. In acute TTP samples, CT-proET-1showed a positive, significant correlation with the alternative pathway activation marker, C3bBP and a mild positive correlation with the terminal pathway activation marker, SC5b-9. Furthermore, we observed decreased Factor-H levels in acute and remission samples compared to healthy controls. Our data on the decreased Factor H antigen concentration in TTP is notable and is consistent with the findings published previously. ULVWF present in TTP may increasingly bind and consume circulating Factor H, a mechanism that may further increase the risk of alternative pathway dysregulation. The fact, however, that the alternative pathway activation marker C3bBbP correlated with endothelin-1 levels supports the idea that complement activation in TTP may contribute to the progression of TMA. First, the activation of platelets by ULVWF is an important step in the pathogenesis of TTP, and this process is accompanied by thrombin development. Second, thrombin is able to directly activate C3 and initiate complement activation, and in parallel, thrombin is a known activator of endothelial cells resulting in in- creased production and release of VWF and endothelin-1. In addition, Factor H dysfunction may contribute to complement deposition to platelets and to their activation. These processes may jointly turn into a self-expanding circle of increased platelet adhesion and activation, thrombin production and complement activation, propagating endothelial damage, microangiopathy, increased shear-stress and progression of TMA.
Our results suggest elevated CT-proET-1 levels in TTP may indicate the activated state of endothelium and active disease process. The observation of higher CT-proET-1 and also VWF antigen levels in remission (when compared with control) may be related to damage or activation of endothelium in these patients. It is the task of further studies to identify those factors that may cause constant activation of endothelium during remission in TTP patients, and to investigate if elevated CT-proET-1 is also a prognostic marker of an exacerbation and relapse in TTP. Elucidating the role of endothelial activation in TTP is important, given the necessity and potential importance of prognostic and therapy guiding biomarkers as suggested by recent studies.

ADAMTS13 deficiency is an important determinant of the increased risk for the development of TTP, but this factor alone is insufficient to explain the development of active TTP. Additional factors, such as infections, pregnancy or surgery may all contribute to the precipitation of an acute TTP episode, and these states and diseases are known inducers of profound cytokine response, complement activation and coagulation. Biological effects of these processes, such as generation of thrombin, increase of pro-inflammatory cytokines like interleukin-6 and tumor necrosis factor-alpha and complement activation products, may all activate transcription of endothelin-1 and increase its production. Therefore, we suggest CT-proET-1 as a specific, rapidly regulated and well- measurable endothelial biomarker to be used in TTP and potentially in other forms of thrombotic microangiopathies as well. Our results suggest in the pathogenesis of TTP many self-expanding, positive feedback-loop systems, such as neutrophil-, endothelial- and complementsystem can be activated through delivery, infection or surgery. In a patient with ADAMTS13 deficiency these trigger events may contribute to the manifestation of thrombotic microangiopathy.

The bibliography of the candidate's publications

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