

Recovery and Half-Life of von Willebrand Factor-Cleaving Protease after Plasma Therapy in Patients with Thrombotic Thrombocytopenic Purpura

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Summary

Plasma exchange using fresh-frozen plasma (FFP) for replacement was given to two brothers during a relapse of thrombotic thrombocytopenic purpura (TTP). A constitutional deficiency of von Willebrand factor(vWF)-cleaving protease had been previously established in both patients. No inhibitor of vWF-cleaving protease was present in patients' plasmas. They received plasma exchange for four and three consecutive days, respectively. In both patients, the activity of vWF-cleaving protease after the first plasmapheresis session was evaluated and was found to be virtually identical to anticipated activity calculated from predicted patient plasma volume and volume of exchanged plasma. Pathologic platelet counts and lactate dehydrogenase levels were normalized in both patients within 4-6 days. The biologic half-life of vWF-cleaving protease was determined in these patients following the last plasma exchange. The respective half-lives of 3.3 and 2.1 days represent the lowest known clearance rates of proteases in circulating human plasma.

Another patient with relapsing TTP was treated with plasma exchange and/or plasma infusion for 10 consecutive days during the first relapse, 221-231 days after the initial TTP event. Pharmacokinetic studies of vWF-cleaving protease were performed after plasma exchange on day 221 and after plasma infusion on day 231. High level of an IgG in patient plasma, capable of completely inhibiting protease activity in an equal volume of normal plasma, had been established prior to first plasmapheresis. There was no measurable protease activity at any time during plasma therapy. Following plasma exchange, the level of the inhibitor was transiently slightly depressed. After 10 days of plasma therapy, the concentration of the inhibitor in patient plasma was increased about 5-fold. We suggest that, in contrast to protease deficient patients without circulating inhibitor, complementary therapy including immunosuppressive treatment, vincristine and/or splenectomy is indicated in patients with acquired inhibitors of vWF-cleaving protease. Testing for vWF-cleaving protease inhibitor may be useful in predicting the response to plasma exchange in patients with TTP.

Introduction

Von Willebrand factor (vWF) multimers larger than those in normal plasma, but similar to those produced by normal endothelial cells in culture, have been observed in plasma of patients with thrombotic thrombocytopenic purpura (TTP) (1). These unusually large vWF multimers have been shown to attach to circulating platelets and lead to platelet clumping (2), especially at elevated levels of shear stress (3). It has been suggested that a substance in normal human plasma is capable of reducing the size of unusually large vWF multimers. This "reductase" activity may be attributed to a plasma protease that has been shown to degrade large vWF multimers in the high shear environment (4), but also at low ionic strength (5), in the presence of urea (5) or guanidinium chloride (6), suggesting that a conformational change in the subunit of vWF is required to expose the cleavable peptide bond thus enhancing its susceptibility to proteolysis. Circulating vWF has been proposed to become proteolytically degraded by a calcium-dependent protease cleaving the peptide bond Tyr842-Met843 (7). This protease was partially purified from normal human plasma (5,6) and was shown in vitro to generate fragments of vWF subunit that are indistinguishable from those found in normal plasma.

Deficient activity of the vWF-cleaving protease has been recently established in four patients, that included two brothers, with relapsing TTP (8). In none of these patients, an inhibitor of vWF-cleaving protease was found. The activity of vWF-cleaving protease was also found to be deficient in another patient with recurring episodes of TTP showing an acquired autoantibody against the protease. Splenectomy performed one year after the initial TTP event resulted in disappearance of the antibody, normalization of the protease activity and of the platelet count (9). These observations suggest that the thrombocytopenia in patients with TTP is associated with a deficiency of vWF-cleaving protease that may be either inherited (8) or acquired due to an autoimmune mechanism (9).

The infusion of fresh-frozen plasma (FFP) or plasma exchange using FFP for replacement are effective in controlling or preventing relapses in patients with relapsing TTP (10, 11). Although the survival of patients with TTP has been dramatically improved following introduction of plasma therapy, there are still many patients who are refractory to plasma exchange. Early recognition of such patients would be important, since they might benefit from alternative treatments complementary to plasma therapy. The amount of transfused plasma is critical in the treatment of TTP. No attempt has thus far been undertaken to determine the recovery and the biological half-life of vWF-cleaving protease.

In the present study, we found an adequate recovery of vWF-cleaving protease following plasma therapy and a half-life of 2–4 days in two patients with relapsing TTP showing constitutional protease deficiency. On the other hand, in a patient with high levels of autoantibody against vWF-cleaving protease, the plasma exchange led to only insignificant and transient neutralization of the inhibitor. We conclude that testing for protease inhibitor may be useful in predicting the response to plasma exchange in patients with TTP.

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Patients and Methods

Patients

Pharmacokinetic data were obtained in two brothers (patients 1 and 2) with relapsing TTP that have been previously described (8): on two occasions, plasma samples had been collected for the assay of vWF-cleaving protease. In both patients, constitutional deficiency of vWF-cleaving protease was demonstrated. None of the plasma samples contained an inhibitor of the protease (8).

Patient 1 (born 1975) was admitted to the hospital in February 1998, four years after the last severe acute episode of TTP. Upon admission, he suffered from abdominal pain, nausea, headache, and diarrhea. He had fever (38.9° C), slight elevation of serum creatinine, but no neurologic deficit. His initial platelet count was 59×10^9/l and serum lactate dehydrogenase (LDH) was 1606 U/l (normal, < 430 U/l). Treatment consisted only of plasma exchange therapy, using 11, 10, 10, and 10 U of fresh-frozen plasma (FFP) for replacement on days 1, 2, 3, and 4, respectively. The patient readily recovered and was discharged from the hospital on day 7. His body weight and height were 69 kg and 181 cm, respectively. Laboratory data are shown in Table 1.

Patient 2 (born 1964), brother of patient 1, has had frequent relapses (more than 20) of TTP since 1985 and received both therapeutic and prophylactic plasma exchange at irregular time intervals. The acute event, leading to hospitalization in March 1998, might have been associated with pharyngitis acquired a few days earlier. Upon admission, his platelet count was 13×10^9/l and LDH was 297 U/l but increased to 976 U/l on day 2 (Table 1). The patient received the following therapy: plasma exchange with replacement of 6 U FFP each on days 1, 2 and 3, in addition to 100 mg prednisolone and 2 mg clemastine i.v. His platelet count and LDH values normalized within 4 days. His body weight and height were 88.7 kg and 181 cm, respectively.

The recovery of vWF-cleaving protease was examined in another patient with relapsing TTP (patient 3, born 1962), in whom an inhibitor of vWF-cleaving protease had been established (9). Activity of vWF-cleaving protease in this patient was measured in plasma samples collected after plasma exchange with 10 U FFP, at the beginning of the first relapse, corresponding to day 221 after his initial TTP episode. The platelet count was 10×10^9/l while LDH was 1518 U/l. Another pharmacokinetic study was undertaken 10 days later (day 231), after the platelet count (231×10^9/l) and LDH (440 U/l) had normalized and the patient received only an infusion of 4 U FFP. As reported earlier (9), this patient had no measurable activity of vWF-cleaving protease between day 126 and day 378, due to an acquired autoantibody that disappeared only after splenectomy on day 365 and corticosteroid treatment. The body weight and height of patient 3 were 76.8 kg and 160 cm, respectively.

Table 1 Laboratory parameters in patients 1 and 2 during the course of the TTP relapse. Hb, hemoglobin; LDH, lactate dehydrogenase. Blood samples were taken from the patients prior to plasma exchange (PEx)

Days	Platelets (x 10^{-9}/l)	Hb (g/dl)	LDH (U/l)	Creatinine (µmol/l)	Bilirubin (µmol/l)	PEx (l)
Normal range	125-400	14-18	< 430	< 110	< 20	
			Patient 1			
1	59	15.3	1606	140	128	2.75
2	15	14.9	1139	120	119	2.50
3	18	11.7	343	93	35	2.50
4	47	12.1	283	91	21	2.50
5	101	12.6	349	89	19	—
6	126	12.1	425	87	14	—
7	204	12.9	439	91	12	—
12	508	12.4	345	95	14	—
19	368	12.5	333	104	14	—
			Patient 2			
1	13	9.9	297	106	31	1.50*
2	33	9.8	976	107	12	1.50*
3	123	7.7	472	90	4	1.50*
4	179	7.7	416	87	4	—
5	399	8.8	—	—	—	—
10	525	10.2	—	97	6	—

* + 100 mg prednisolone, 2 mg clemastine i.v.

Methods

All samples for vWF analysis and vWF-cleaving protease assay were obtained from blood samples anticoagulated with ⅛ volume 0.106 mol/l Na_3-citrate. Platelet-poor plasma was recentrifuged for 15 min at 3000 rpm at 25° C and stored at –20° C for subsequent testing. Platelet count, LDH, hemoglobin (Hb), creatinine, and bilirubin determinations were performed by conventional methods.

Before the assay of vWF-cleaving protease activity, Pefabloc® SC (Boehringer, Mannheim, Germany) was added at 10 mmol/l final concentration to citrated normal plasma or patient plasmas, and further dilutions of these plasmas were made with 0.15 mol/l NaCl-10 mmol/l Tris, pH 7.4 (TBS) containing 1 mmol/l Pefabloc. The protease was activated by 5-min incubation of diluted plasma at 37° C with 10 mmol/l $BaCl_2$ as previously described (9). One hundred microliters of the incubation mixture were added immediately to 50 µl of protease-free vWF (adjusted to about 5 U vWF:Ag/ml) purified by gel filtration on Sepharose CL-2B (Pharmacia-LKB, Uppsala, Sweden) of a normal human plasma cryoprecipitate. Thus, the ratio of patient plasma vWF to purified normal vWF was about 1:50. The reaction mixture was dialyzed on the surface of a hydrophilic filter (VSWP, 25 mm diameter, Millipore, Bedford, MA) for 24 h at 37° C against 1.5 mol/l urea/5 mmol/l Tris-HCl, pH 8.0. The reaction was stopped by addition of 10 µl 0.2 mol/l EDTA, pH 7.4 and the extent of vWF degradation was assayed by multimer analysis using SDS-electrophoresis in 1.4% agarose gels. Following electrophoresis, the proteins were electrotransferred to nitrocellulose, and vWF was visualized with peroxidase-conjugated rabbit antibodies against human vWF (P0226, Dako, Glostrup, Denmark). A citrated normal human plasma pool (NHP), prepared from 42 healthy male subjects and stored at –70° C, was used for calibration of the protease assay in patient plasmas. The calibration curve for vWF-cleaving protease activity was made with NHP dilutions between 1:20 and 1:960, and the corresponding activities were defined as 100% and 2.1%, respectively. The immunoblotted multimeric patterns of degraded vWF substrate were each scanned and the resulting images were processed by the program Image Tool V.1.27 (UTHSCSA, San Antonio, Texas). A histogram of the staining intensity (pixel value versus pixel number) was imported into the program SigmaPlot V.2.0 (Jandel, Erkrath, Germany) and the total area under the histogram, between pixel numbers 1 and 285, was integrated. Subsequently, the pixel number of the vertical line, cutting the histogram area into two equal halves, was calculated. Pixel numbers of these area-halving lines were plotted against protease concentrations and fitted to logistic dose response curve. The resulting calibration curve had the steepest slope between 2 and 8% protease activity. Therefore, appropriate dilutions of patient plasmas were made that resulted in area-halving pixel numbers of about 200.

Inhibitor of the vWF-cleaving protease was assayed by measuring the protease activity in mixtures of patient plasma and normal plasma at two different volume/volume (vol/vol) ratios, ie, 1:1 and 1:5, following 10-min preincubation at 37° C. An arbitrary inhibitor unit was defined as the amount of inhibitor that completely inhibits the vWF-cleaving protease in 1 ml of NHP.

Calculations

Body surface area (SA) was calculated according to the equation:
$SA\ (m^2) = (\text{body weight, kg})^{0.425} \times (\text{body height,cm})^{0.725} \times 71.84$ (12).
Blood volume (BV) was predicted according to Feldschuh and Enson (13):
$BV\ (mL) = 391 + 2566 \times SA$.

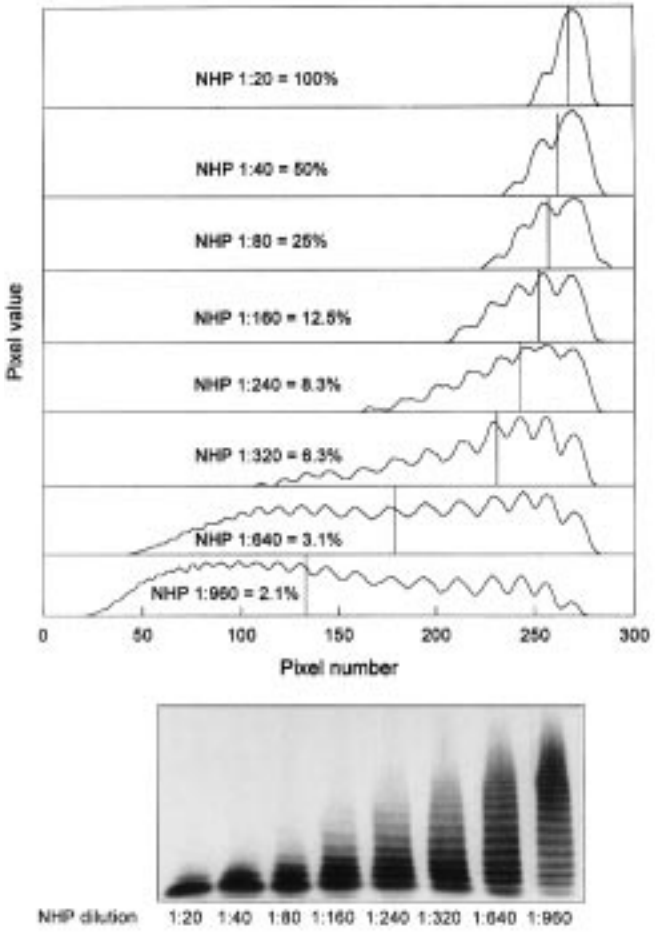

Fig. 1 Assay of vWF degradation by vWF-cleaving protease in plasma. Dilutions of NHP were incubated for 24 h at 37° C with purified vWF, and then the polymeric size of the digested vWF was analyzed by electrophoresis in SDS-1.4% agarose gel (bottom panel). Incubation mixtures were applied on top of the gel. Following electrophoresis, vWF was detected by immunoblotting. Dilutions of NHP are shown beneath the immunoblot of the electrophoretic gel. The stained immunoblots were scanned and the areas of the histograms were cut by vertical lines into identical halves (top panel). Pixel numbers 1 and 285 correspond to the top and the bottom of the electrophoretic gel, respectively

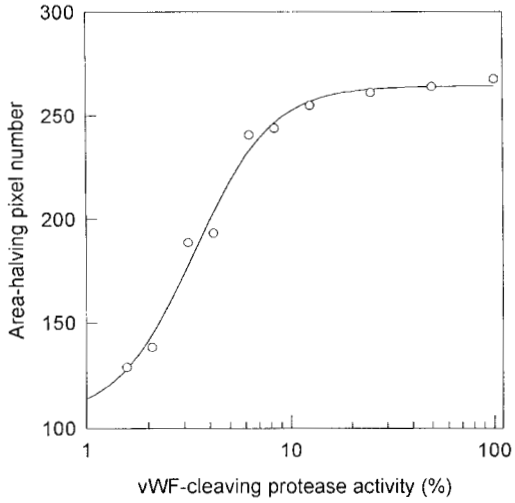

Fig. 2 Calibration of the assay of vWF-cleaving protease. Dilutions of NHP (1:20 to 1:960) were incubated with purified vWF and the extent of substrate degradation was assayed by multimer analysis of vWF by electrophoresis in SDS-agarose and by scanning of the immunoblots. The 1:20 dilution of NHP was denoted as 100% activity. Pixel numbers of the vertical lines cutting the areas of the histograms into identical halves (area-halving pixel numbers) are plotted against protease activity. The calibration curve was obtained by fitting data points to the logistic dose-response curve

The venous hematocrit value was used to calculate plasma volume (PV) from BV.

The recovery of vWF-cleaving protease was calculated from the activity in patient plasma 1 h after the initial plasma exchange/infusion. The predicted values for protease concentration (% of NHP) following plasma exchange with a transfused volume (TV, ml) of FFP was calculated as

$$\% \text{ activity} = 100 - ((PV-1)/PV)^{TV} \times 100.$$

Correction for plasma volume increase was done following therapy with plasma infusion. All predicted recovery values were calculated on the assumption that FFP contained 100% vWF-cleaving protease activity and that no equilibration occurred between the intravascular and extravascular space during the course of the procedure. The biological half-life of the vWF-cleaving protease was calculated from the exponential decay curve plotted after the last therapy with FFP.

Results

An example of the calibration procedure is shown in Fig. 1. The shift in the multimeric pattern of vWF substrate is dependent on concentration of vWF-cleaving protease in dilutions of NHP. Mean values of area-halving pixel numbers, each derived from up to 6 measurements, were plotted against the protease concentration (Fig. 2) and the data fitted to the logistic function. Activities of vWF-cleaving protease in patient plasma samples were read from the resulting calibration curve. None of the three patients had any measurable protease activity prior to plasma exchange or infusion.

Assuming a predicted plasma volume (PV) of 2.92 l in patient 1, and a transfused plasma volume (TV) of 2.75 l, an anticipated initial protease activity of 61% was calculated. This value compares well to the measured protease activity of 72% at 1 h after plasma exchange. The predicted PV of patient 2 was 4.09 l and the calculated anticipated protease activity after exchange with replacement of 1.5 l FFP was 31%. The measured protease activity at 1 h after plasma exchange was 29%. These results indicate that virtually all transfused vWF-cleaving protease appeared in the circulation.

Plotting the protease activities against time following the last plasma exchange allowed calculation of the biologic half-life of the vWF-cleaving protease in patients 1 and 2. A protease half-life of 3.3 days (95% confidence interval: 3.09-3.65 days) and an extrapolated initial protease activity of 75% immediately after the fourth plasma exchange were calculated in patient 1 by regression analysis of the decay curve (Fig. 3). In patient 2, the computed half-life and initial activity after the third plasma exchange were 2.1 days (95% confidence interval: 1.91-2.43 days) and 43%, respectively (Fig. 4).

Table 1 shows that in patients 1 and 2 the laboratory parameters normalized 4-6 days after the beginning of the relapse. Both patients also readily clinically recovered and remained symptom-free for several weeks, although the protease activity was reduced to less than 1% about 20 days after the last plasma exchange.

The recovery of vWF-cleaving protease in patient 3 was examined on day 221, on occasion of the first relapse, and 10 days later when his platelet count was normal again. As described previously (9), this patient received daily plasma exchanges using 10 U FFP each for replacement on days 221 to 226, and was subsequently treated with daily infu-

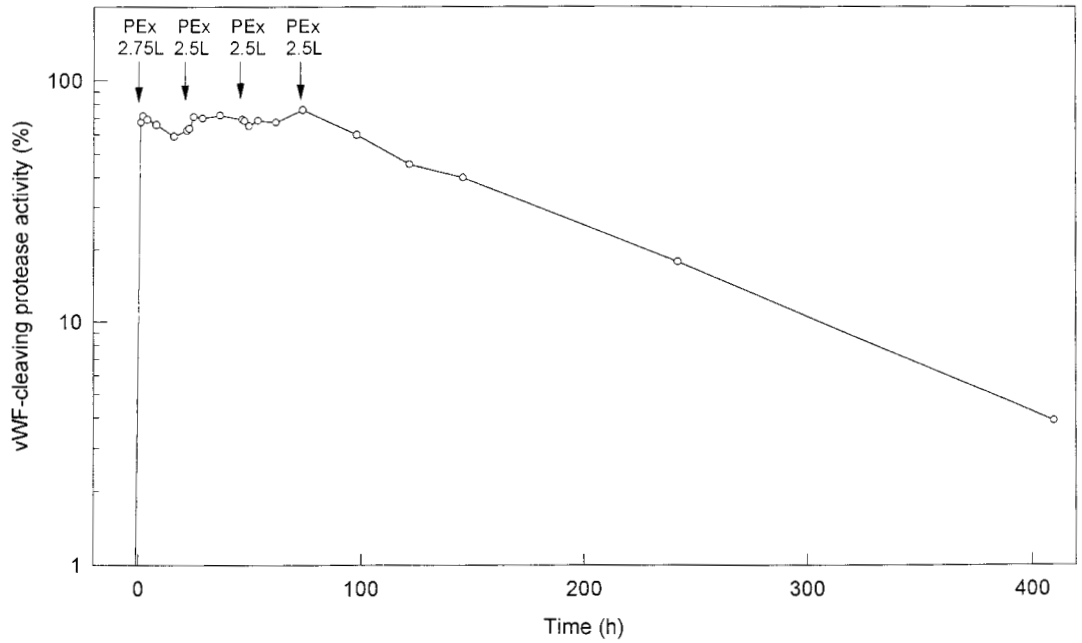

Fig. 3 Activity of vWF-cleaving protease in patient 1 following an acute TTP event. The patient underwent four sessions of plasma exchange (PEx) as the sole treatment. He received 11 U of fresh-frozen plasma on day 1 and 10 U each on days 2, 3, and 4. The baseline level of vWF-cleaving protease was less than 1% of NHP and no inhibitor was present. The recovery of vWF-cleaving protease was estimated after the first plasma exchange session and its half-life was determined by fitting the activities after the fourth session to the exponential decay curve. Each data point is a mean of two assays

sions of 2-4 U FFP each on days 227-231. The predicted plasma volume of the patient was 3.16 l and 3.41 l on day 221 and day 231, respectively. From the exchanged or transfused volumes of FFP, the following anticipated values of protease activity were calculated: 45% following plasma exchange on day 221 and 23% following FFP infusion on day 231.

There was no vWF-cleaving protease detectable in the plasma of the patient after exchange or infusion of FFP. We concluded that the transfused protease had been completely inactivated by the circulating inhibitor. The concentration of protease inhibitor was estimated by measuring the neutralization of vWF-cleaving protease in normal human plasma by the patient plasma. The level of protease inhibitor on day 221

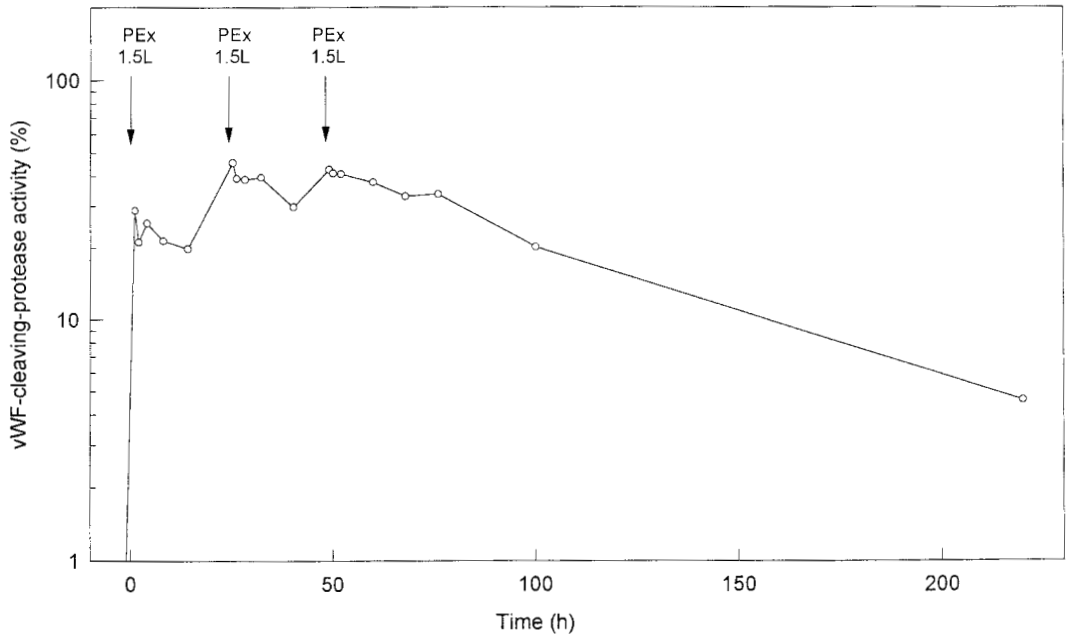

Fig. 4 Activity of vWF-cleaving protease in patient 2 (brother of patient 1) after an acute relapse of TTP. Three sessions of plasma exchange (replacement of 6 U of fresh-frozen plasma each) on days 1, 2 and 3 were complemented by corticosteroid and antihistaminic treatment because of earlier allergic reactions to plasma therapy. The initial protease concentration was less than 1% of normal and there was no protease inhibitor in the patient plasma. The recovery was calculated from the level of protease activity following the first plasma exchange. Biologic half-life was calculated from the slope of the decay curve following the third plasma exchange session. Each data point is the mean value of two assays

was determined in mixtures of equal volumes of NHP and patient plasma, whereas the concentration of inhibitor was markedly increased on day 231 and the assay had to be performed in equal mixtures of NHP and 1:5 dilution of patient plasma.

Fig. 5 shows that the initial level of inhibitor on day 221 was about 1 U/ml. Exchange with replacement of 10 U FFP resulted in only a slight temporary decrease of the inhibitor. The inhibitor level was about 5-fold higher 10 days later in spite of daily therapy with plasma exchange or infusion. On day 231, infusion of 4 U FFP did not significantly affect the level of the inhibitor.

Discussion

The purpose of the present study was to determine the recovery and biological half-life of vWF-cleaving protease supplied to patients with TTP by plasma exchange or infusion. The pharmacokinetic properties might help us identify laboratory tests that could be useful in predicting the response to plasma exchange in patients with TTP.

Whether the benefit of plasma exchange is due to removing a toxic entity from the patient's plasma, to infusing a deficient factor, or both, is still a matter of debate. The Canadian Apheresis Study Group (CASG) showed previously that plasma exchange using replacement with normal FFP was a more effective treatment than infusion of FFP alone (14). Since there is evidence that unusually large vWF multimers are involved in the pathophysiology of TTP (1) it seems plausible that their removal from patient blood should avert formation of platelet aggregates. Furthermore, removal of patient IgG by protein A immunoabsorption led to recovery from TTP in 7 of 10 patients who had failed to respond to therapeutic plasma exchanges (15).

On the other hand, in some other patients, the unusually large vWF multimers decreased or disappeared, at least temporarily, even after a single infusion of FFP or its cryosupernatant fraction (16). The property of normal human plasma to remove the unusually large vWF multimers was tentatively ascribed to a "depolymerase" (1,16-18). A recently described vWF-cleaving protease (5, 6) was found to be deficient in patients with relapsing TTP, suggesting that constitutional (8) as well as acquired (9) deficiency of the vWF-cleaving protease may predispose to TTP.

If the beneficial impact of plasma exchange/infusion is associated with provision to the patients of the lacking protease, then the therapeutic effect of FFP in patients with TTP must depend upon recovery and biological half-life of vWF-cleaving protease. Our study showed that in two patients with constitutional protease deficiency, the infused vWF-cleaving protease was virtually completely recovered in patient plasmas. The half-life of the protease was 3.3 days in patient 1 and 2.1 days in patient 2. These half-lives are uniquely long for a proteolytic enzyme. Most proteases that become activated or secreted into plasma are rapidly inactivated by circulating inhibitors, such as serine protease inhibitors (serpins) and cysteine protease inhibitors. Another protease inhibitor, α_2-macroglobulin, is known to bind and inhibit serine, cysteine, aspartic, and metallo-proteases. The resulting inhibitor-protease complexes are cleared rapidly from the circulation, reflecting receptor binding and endocytosis (19). Thus, the activated forms of most proteases have plasma half-lives measured in seconds to minutes at most. To our knowledge, the longest known half-life of a protease in human plasma (2.4 h) has been reported (20) for activated coagulation factor VII (FVIIa).

In contrast to patients 1 and 2 who had complete protease deficiency but no inhibitors of vWF-cleaving protease, our patient 3 had acquired protease deficiency due to protease-inhibiting autoantibodies (9). The

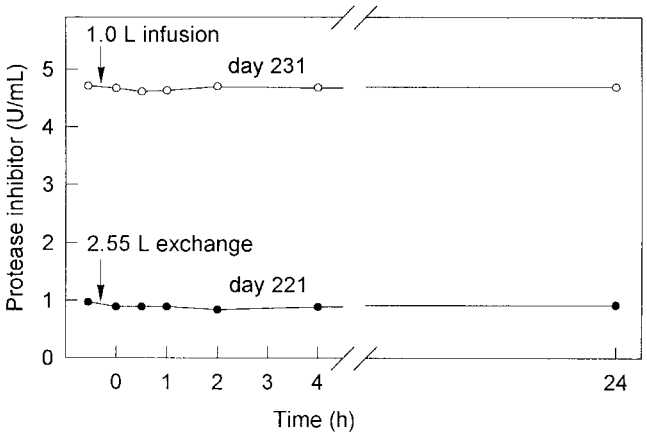

Fig. 5 Levels of protease inhibitor in patient 3 following a TTP relapse that occurred on day 221 after his initial TTP event. The recovery of vWF-cleaving protease was examined on days 221 and 231. The patient received an exchange with replacement of 10 U fresh-frozen plasma on day 221 and an infusion of 4 U fresh-frozen plasma on day 231. The inhibitor concentration was estimated from the exent of protease neutralization in normal human plasma by the patient plasma. Each inhibitor level is the mean value of two assays

amount of the inhibiting IgG in 1 ml patient plasma prior to first plasma exchange (day 221) was sufficient to completely inhibit the vWF-cleaving protease in 1 ml normal plasma. Following 10-day plasma therapy, the level of inhibitor in the patient plasma increased about 5-fold. It was not surprising that no vWF-cleaving protease activity was detected by our assay following plasma exchange and infusion on days 221 and 231, respectively. It must be added, that the assay of vWF-cleaving protease may not adequately reflect the degradation of vWF in circulating blood under shear conditions. Thus, the loss of unusually large multimers in vivo was observed already 0.5-1.5 h after the transfusion of FFP (16), whereas in our assay the vWF substrate was incubated with diluted patient plasma for 24 h at 37° C. It is conceivable that in vivo the infused vWF-cleaving protease is capable of degrading the unusually large vWF multimers before it becomes inactivated by the circulating antibody. This transient appearance of protease activity may explain the increase of the platelet count, observed in patient 3 following plasma therapy (9), although no protease activity was detectable under our assay conditions. It is evident that large volumes of FFP are required for plasma exchange in patients with protease-inhibiting autoantibodies whereas less intensive plasma therapy may be needed in TTP due to constitutional protease deficiency.

In conclusion, our data demonstrate a high recovery and an unusually long half-life of vWF-cleaving protease in inhibitor-free patients with TTP. We propose that in these patients plasma exchange or even plasma infusion may be effective in suppressing or preventing TTP relapses. Much larger amounts of FFP must be transfused to patients with acquired inhibitors of vWF-cleaving protease; in these patients the therapy should be primarily aimed at removal of the autoantibody against vWF-cleaving protease. Therefore, complementary therapeutic strategies, including immunosuppresive therapy, vincristine administration, and/or splenectomy, may be indicated.

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