Platelet-mediated proteolytic down regulation of the anticoagulant activity of protein S in individuals with haematological malignancies

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Introduction

Protein S, a vitamin K-dependent plasma protein, is one of the molecules involved in down-regulation of the coagulation cascade. The importance of protein S as natural coagulation inhibitor is underscored by the occurrence of fatal consumptive coagulopathy in the embryonic stage of homozygous protein S-deficient mice, while heterozygous deficient mice exhibit a thrombotic phenotype (1, 2). In humans, heterozygous protein S deficiency is associated with the development of both venous and arterial thrombosis (3, 4). The anticoagulant activities ascribed to protein S are numerous. Well established is the activated protein C (APC)-cofactor activity of protein S that refers to the cofactor function of protein S in the proteolytic inactivation of both factor (F) Va and FVIIIa by APC (5). More recently, protein S has been recognised as cofactor for tissue factor pathway inhibitor (TFPI) in the inhibition of the tissue factor (TF)/FVIIa/FXa complex (6). Protein S may also display a direct anticoagulant activity, that refers to the ability of protein S to inhibit the assembly and activity of FVIIIa/FIXa and FVa/FXa complexes (7, 8).

Protein S contains a so-called thrombin-sensitive region (TSR) comprising residues Cys47-Ala75, that is susceptible to proteolytic cleavage. In vitro studies employing purified proteins in plasma-free systems have revealed that FXa, thrombin and elastase are able to cleave protein S at the TSR. FXa cleaves at Arg60 only (9–12), while thrombin cleaves at Arg60 and Arg90 (10–12). Elastase is reported to cleave protein S adjacent the protease sensitive loop at Val73 (13). It has been firmly established that upon TSR cleavage at any of these sites the APC-cofactor activity of protein S is abolished (9–11, 14). The full impact of in vivo TSR cleavage on the role of protein S as an anticoagulant in the generation of thrombin in plasma, therefore, remains to be elucidated.

Summary

The natural anticoagulant protein S contains a so-called thrombin-sensitive region (TSR), which is susceptible to proteolytic cleavage. We have previously shown that a platelet-associated protease is able to cleave protein S under physiological plasma conditions in vitro. The aim of the present study was to investigate the role of platelet-associate protein S cleaving activity and in vivo protein S cleavage, and to evaluate the impact of in vivo protein S cleavage on its anticoagulant activity. Protein S cleavage in healthy subjects and in thrombocytopenic and thrombocythaemic patients was evaluated by immunological techniques. Concentration of cleaved and intact protein S was correlated to levels of activated protein C (APC)-dependent and APC-independent protein S anticoagulant activity. In plasma from healthy volunteers 25% of protein S is cleaved in the TSR. While in plasma there was a clear positive correlation between levels of intact protein S and both APC-dependent and APC-independent protein S anticoagulant activities, these correlations were absent for cleaved protein S. Protein S cleavage was significantly increased in patients with essential thrombocythaemia (ET) and significantly reduced in patients with chemotherapy-induced thrombocytopenia. In ET patients on cytoreductive therapy, both platelet count and protein S cleavage returned to normal values. Accordingly, platelet transfusion restored cleavage of protein S to normal values in patients with chemotherapy-induced thrombocytopenia. In conclusion, proteases from platelets seem to contribute to the presence of cleaved protein S in the circulation and may enhance the coagulation response in vivo by down regulating the anticoagulant activity of protein S.

Keywords

Coagulation, thrombin generation, protein S, cellular proteases, platelets

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In plasma, a single-chain molecule as well as a two chain form of protein S can be detected (13, 15). This two-chain variant is the result of in vivo cleavage at position Arg60 (13). To date, the enzyme responsible for the presence of TSR-cleaved protein S in the circulation is not known. Indeed, the so far described proteases targeting the TSR of protein S in vitro, thrombin, FXa and elastase do not cleave protein S in plasma under normal physiological conditions (12, 13, 16). Moreover, the documented in vivo cleavage at Arg60 excludes any contribution of thrombin and elastase to the presence of cleaved protein S in the circulation. Mitchell and Salem (17) have reported protein S cleaving protease activity in platelets. Our group confirmed and extended this observation by showing that a protease from platelets is able to cleave protein S in plasma (12). Concurrent with the presence of Arg60-cleaved protein S in plasma, cleavage of protein S by the platelet protease occurred at Arg60 (12). We hypothesised that platelets might contribute to the presence of cleaved protein S in the circulation. Recently we described the occurrence of acquired APC resistance in patients with essential thrombocythaemia (ET) (18), a phenomenon to which platelet-mediated proteolysis of protein S might be accountable for.

The aim of this study was to extend prior observations on the modulation of the anticoagulant activity of protein S by limited proteolysis in vivo. Particularly, we investigated whether a correlation exists between platelet count and levels of cleaved protein S in the circulation of individuals with pathological conditions characterised by low platelet counts, as occurs in patients under chemotherapy, and high platelet count, i.e. in patients with ET. The present study provides new insights into the contribution of platelets in the regulation of thrombin generation under normal and pathological conditions.

Materials and methods

Study subjects

The study group consisted of 106 randomly selected healthy Dutch blood bank donors (51 males / 55 females), 34 patients with chemotherapy-induced thrombocytopenia (17 males / 17 females), nine patients (4 males / 5 females) with idiopathic thrombocytopenia and 24 patients (11 males / 13 females) with ET. The chemotherapy-induced thrombocytopenia group consisted of patients with haematological malignancies treated with myelo-suppressive chemotherapy, i.e. multiple myeloma (11 individuals), acute myeloid leukaemia (11 individuals), myelodysplastic syndrome (6 individuals), acute lymphoblastic lymphoma (2 individuals) and non-Hodgkin lymphoma (4 individuals). Patients were treated in the Amsterdam Academical Medical Center with high-dose chemotherapy according to standard protocols. Blood samples were taken prior to and during thrombocytopenia. We also included samples from patients with chemotherapy-induced thrombocytopenia collected within 48 hours (h) after platelet support (leukocyte-depleted platelet concentrate containing 350 x 10^9 platelets per unit, Sanquin Blood Supply Foundation, Amsterdam, the Netherlands). Blood samples from ET patients and the corresponding group of 24 healthy controls (16 males / 8 females), were collected at the Bergamo Thrombosis and Hemostasis Center. At the time of blood collection, five ET patients were on aspirin treatment and 17 on cytotherapeutic treatment with hydroxyurea (HU). The present study was conducted with the approval of the ethical committees of the involved medical institutions and all blood samples were obtained after informed consent. The procedures followed were in accordance with the Helsinki declaration of 1975 as revised in 2000.

Blood collection and plasma preparation

Venous blood samples were collected into siliconised tubes containing trisodium citrate (0.129 M, 1/9 v/v). Differential blood cell counts were determined by a Sysmex-XE 2100 hematology analyzer (Sysmex, Kobe, Japan) or a NE800 Analyzer (Dasit, Milan, Italy). Plasma was separated by centrifugation of whole blood for 15 minutes at 4,000 g at room temperature, aliquoted, snap-frozen and stored at –80 ºC until testing.

Determination of cleaved protein S in plasma by Western blotting

Protein S was immune-precipitated from plasma samples using a home-made rabbit anti-human protein S I g G (12) coupled to CNBr-sepharose. Intact and cleaved protein S was then separated by electrophoresis on 7.5% polyacrylamide gels under reducing conditions. Protein bands were visualised by immunblotting with HRP-labelled sheep anti-human protein S IgG. The bands on immunoblot were stained by ECL (Roche Diagnostics GmbH, Mannheim, Germany) and quantified using the Image-J analysing software (National Institute of Health, Bethesda, MD, USA). Percentage of cleavage was calculated from the digitally quantified intact and cleaved protein S bands. Some experiments were performed with plasma in which protein S was cleaved with 100 nM thrombin (12). Coloured protein molecular weight markers (Rainbow™ RPN756) were from Amersham Life Science (Buckinghamshire, UK).

Determination of cleaved protein S in plasma by ELISA

Total and intact protein S antigen levels were measured in citrated plasma samples by in-house ELISAs, as described (12). For both determinations, a home-made polyclonal antibody against human protein S was used as catching antibody. A commercially available HRP-labelled sheep-anti human protein S IgG (Affinity Biologicals Inc, Ancaster, ON, Canada) or HRP-labelled monoclonal anti-
body (MoAb) CLB-PS18 (12, 19) was used to detect total protein S. HRP-labelled MoAb CLB-PS52 was employed as detection antibody for intact protein S. This MoAb is directed against the protein S peptide sequence Phe\textsuperscript{40}-Ala\textsuperscript{59} and recognises TSR-intact protein S only (12, 19). Our reference plasma (pooled citrated plasma from 40 large quantity donations, Blood bank division of our Institute) contained 346 nM total protein S (estimated concentration) (20) and 225 nM intact protein S (calculated from the relative value of 35% cleaved protein S in our plasma pool as determined by Western blotting) and was used for the calibration curve in both ELISAs. The levels of cleaved protein S in plasma samples were calculated from the determined levels of total and intact protein S.

**Determination of protein S anticoagulant activity**

Protein S activity was measured both as APC-dependent and APC-independent activity by the calibrated automated thrombogram (CAT) assay (21) as described by Sere et al. (22) with some modifications. Thrombin generation was performed in polystyrene round-bottom 96-well microtiter plates (Greiner Bio-one, ref. no. 650161, Kremsmünster, Austria) in a final volume of 120 μl. Typically, prewarmed reaction mixtures contained 60 μl plasma, 20 μl additives (APC, MoAb CLB-PS13) diluted in Tris buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) supplemented with 1% w/v albumin and 20 μl of a TF/phospholipid mixture (ThrombinoScope, Maastricht, The Netherlands). Final concentration of TF and phospholipid vesicles (phosphatidylserine / phosphatidylcholine / phosphatidylethanolamine, 2/6/2 molar ratio) was 1 pM and 4 μM, respectively. Reaction was started with 20 μl of a mixture of the fluorogenic substrate (Z-Gly-Gly-Arg-AMC; Bachem, Bubendorf, Switzerland) and CaCl\textsubscript{2} (final concentration 0.5 mM and 15 mM, respectively). Substrate hydrolysis, monitored by recording the fluorescence signal at 460 nm with excitation at 390 nm in a plate fluorimeter (Fluoroscan Ascent, Thermo Labystems, Helsinki, Finland), was correlated with thrombin concentrations using the thrombin calibrator from ThrombinoScope as internal standard. The APC cofactor activity of protein S was measured as the % decrease in peak height of the thrombin generation curve in the presence of 0.7 nM APC (Enzyme Research Laboratories, Uplands Swansee, UK), a concentration of APC that reduced peak thrombin by 50% in normal pooled plasma. The APC-independent anticoagulant activity of protein S was measured as the % increase in peak height of the thrombin generation curve in the presence of our inhibiting anti-protein S MoAb CLB-PS13 (22–24). Titration experiments in normal pooled plasma revealed maximal enhanced peak thrombin values at IgG concentrations > 15 μg/ml. Data reported in the present study were obtained with 100 μg/ml MoAb CLB-PS13. Specificity of the assays was confirmed in plasmas deficient in protein S (Hyphen Biomed, Neuville-sur-Oise, France) or protein C (Siemens Healthcare Diagnostics, Deerfield, IL, USA) and in normal plasma supplemented with 50 μg/ml of an inhibitory anti-protein C polyclonal antibody. Anti-protein C IgG was obtained by immunsing rabbits with human protein C according to standard laboratory protocols. Titration experiments revealed that 50 μg/ml anti protein C IgG was able to inhibit up to 50 nM APC as measured by CAT.

Some experiments were performed with intact and cleaved protein S supplemented in protein S-deficient plasma. Intact protein S was purified from human plasma as described (12). TSR-cleaved protein S was obtained by incubating purified intact protein S with α-thrombin (prepared as described) (12) in a 100/1 molar ratio in Tris buffered saline for 2.5 h at 37°C. Subsequently, thrombin was

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**Figure 1:** Presence of cleaved protein S in plasma from healthy individuals. A) Immunoblot of protein S immune-precipitated from plasma from 12 healthy volunteers. Si, intact protein S. Sc, cleaved protein S. B) Immunoblot of protein S immune-precipitated from normal pooled plasma incubated for 1 hour in the absence and presence of thrombin (100 nM). C) Protein S cleavage examined by ELISA in plasma from 106 healthy individuals.
irreversibly inhibited with D-Phe-Pro-Arg-chloromethyl ketone (PPACK, Bachem) and excess of inhibitor was removed by dialysis against Tris buffered saline. The preparation contained cleaved protein S only, as judged by SDS-PAGE.

Protein S activity in plasma samples was also evaluated by a commercial available functional assay of protein S (Instrumentation Laboratory, Milan, Italy) by measuring the degree of prolongation of a prothrombin time in the presence of TF, phospholipids, calcium ions and APC according to the manufacturer’s instruction.

Statistical analysis

Results are expressed as mean ± SD. Student’s t-test was employed to compare the difference between the mean values of different groups. Differences were considered significant at a p-value < 0.05. Correlation and linear regression statistical analysis was performed using the Graphpad Prism 5 package (La Jolla, CA, USA).

Results

Quantification of circulating cleaved protein S in healthy individuals

To investigate the occurrence of cleaved protein S in plasma, immunoprecipitates from plasma of 12 healthy subjects were subjected to SDS-PAGE under reducing conditions. The immunoblot (Fig. 1A) revealed the presence of two forms of protein S in plasma with an estimated apparent molecular weight of 86 and 77 kDa, respectively. Figure 1B shows that upon incubation of plasma with thrombin the upper band disappeared while the intensity of the lower band increased, indicating the upper band as intact protein S and the lower band as heavy chain of TSR cleaved protein S (15). To exclude protein S cleavage post-venipuncture, blood was collected both in the absence and presence of 55 mM di-isopropylfluorophosphate (DFP) (12). The analysis of protein S in these two types of plasma samples gave a similar portion of cleaved protein S (i.e. 31% and 32%, respectively; data not shown), indicating that the portion of cleaved protein S measured in plasma truly reflects the in vivo cleavage of protein S. We then measured the percentage of cleaved protein S by ELISA in a population of 106 healthy subjects. The results as depicted in Figure 1C show that the percentage of cleaved protein S ranged between 5 and 51%, with an average of 25 ± 8% (mean ± SD).

Effect of in vivo protein S cleavage on its anticoagulant activities

Figure 2 shows the result of measurements on the impact of TSR cleavage on the anticoagulant activities of protein S by the CAT assay performed with purified intact and thrombin-cleaved protein S added to protein S-deficient plasma. Plasma containing intact protein S showed sensitivity in the CAT assay for both APC and the anti-protein S MoAb CLB-PS13 (Fig. 2A, left panel and Fig. 2B). On the contrary, protein S-deficient plasma supplemented with TSR-cleaved protein S was insensitive to the addition of MoAb CLB-PS13 (100 μg/ml). Data are expressed as mean ± SD of three different experiments.

Figure 2: Influence of intact and TSR-cleaved protein S on thrombography. A) Representative thrombin generation profiles (CAT assay) obtained with protein S-deficient plasma supplemented with 140 nM intact protein S or thrombin-cleaved protein S in the absence (solid black curve) and presence of MoAb CLB-PS13 (100 μg/ml) (striped gray curve) or APC (0.7 nM) (solid gray curve). B) Peak thrombin value obtained with the CAT assay in protein S-deficient plasma, in absence or in presence of intact or TSR-cleaved protein S. The experiments were performed in the absence (black bar) and presence of (solid gray bar) APC (0.7 nM) or (striped gray bar) MoAb CLB-PS13 (100 μg/ml). Data are expressed as mean ± SD of three different experiments.
of APC or MoAb CLB-PS13 (Fig. 2A, right panel and Fig. 2B), similar as occurred in protein S-deficient plasma alone (Fig. 2B).

We subsequently analysed plasma from a subset of 47 healthy individuals (Fig. 3) Correlating the change in peak thrombin in the CAT assay upon CLB-PS13 or APC addition with levels of intact protein S in these plasma samples yielded a positive correlation for both CLB-PS13 (Fig. 3, left upper panel) and APC (Fig. 3, middle upper panel). For cleaved protein S this relationship was absent (Fig. 3, lower left and middle panels). A similar correlation was observed in a different subset of normal plasma samples when tested in a prothrombin time based assay for functional protein S (Fig. 3, right upper and lower panels). These results indicate that the anticoagulant activities displayed by protein S in plasma are attributable to intact protein S only.

Contribution of circulating platelets to protein S cleavage in vivo

Experiments were performed to correlate in vivo protein S cleavage with circulating platelets. In 45 healthy individuals showing a wide range of protein S cleavage (6–48%), no correlation was observed between platelet count (133–361 x 10⁹/l) and protein S cleavage (Fig. 4A). Such a correlation was also absent for white blood cells (WBC count 4–9 x 10⁹/l; R² 0.03) (data not shown). On the other hand, a clear correlation between platelet count and protein S cleavage was observed in blood samples from 44 patients with haematological malignancies randomly taken during HU treatment or high dose chemotherapy (Fig. 4B). The effect of platelet number on protein S cleavage was most pronounced at platelet counts below 50 x 10⁹/l and protein S cleavage tend to a maximum value at platelet counts above normal.

![Figure 3: Correlation of protein S anticoagulant activities with levels of intact protein S (upper panels) and cleaved protein S (lower panels) in plasma samples from healthy individuals.](https://www.thrombosis-online.com/)

![CAT: CLB-PS13 sensitivity](https://www.thrombosis-online.com/)

![CAT: APC sensitivity](https://www.thrombosis-online.com/)

![Clotting assay](https://www.thrombosis-online.com/)
Table 1 shows protein S cleavage at conditions characterised by extreme high and low platelet counts. In seven ET patients not taking any platelet reducing agent (platelet count > 800 x 10^9/l) protein S cleavage was significantly increased compared to healthy controls (40%, p<0.05). Differently, in nine ET patients at normalising platelet count upon cytoreductive treatment with HU (platelet count 290–443 x 10^9/l), protein S cleavage was significantly reduced compared to no-HU treated patients (28%, p<0.05) and similar to that in respective healthy control subjects (Table 1). Accordingly, high-dose chemotherapy-induced thrombocytopenia (platelet count < 50 x 10^9/l) (n=15) was associated with a significantly reduced protein S cleavage (16%, p<0.05) as compared to the control subjects (Table 1). In our HU-treated patient population, WBC count (5–10 x 10^9/l) remained in the normal range and a correlation of WBC number with protein S cleavage was absent (data not shown). Thrombocytopenia induced by high dose chemotherapy, however, was inevitably associated with a drop in WBC count (0–2 x 10^9/l). In order to exclude a possible contribution of WBC to protein S cleavage, we therefore performed the measurement of protein S in a subgroup of 11 patients with chemotherapy-induced thrombocytopenia during platelet transfusion. In these subjects the cleavage of protein S rose within the normal range (Table 1). Level of total protein S was not affected upon platelet transfusion (Table 1). In order to discriminate between reduced thrombopoiesis and peripheral platelet destruction, we included nine patients with idiopathic thrombocytopenia. In these patients with autoantibody-mediated peripheral platelet destruction, protein S cleavage was in the normal range (Table 1). Collectively, our data suggests a correlation between thrombopoiesis and in vivo cleavage of protein S.

**Discussion**

This study corroborates and extends prior observations on the origin and anticoagulant activity of cleaved protein S in the circulation. Cleaved protein S accounted for 25% of total protein S in plasma (Fig. 1). The inability of appropriate protease inhibitors in the blood collection tube to prevent protein S cleavage (this study and [13, 15]) suggests that protein S cleavage indeed occurs prior to blood sampling. Since platelets contain a protease able to cleave protein S (12, 17), platelets may contribute to the presence of cleaved protein S in the circulation. This issue was addressed using...
Table 1: Cleavage of protein S in thrombocytopenia and thrombocytopaenia

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<th>Subjects</th>
<th>Protein S cleavage (%)</th>
<th>Total protein S (nM)</th>
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<td><strong>Study 1: Essential thrombocythaemia</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>(n = 24)</td>
<td>29 ± 11</td>
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<tr>
<td>Thrombocytosis</td>
<td>(n = 7)</td>
<td>40 ± 15*</td>
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<tr>
<td>Platelet count normalisation (HU)</td>
<td>(n = 9)</td>
<td>28 ± 6†</td>
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<td><strong>Study 2: Chemotherapy-induced thrombocytopaenia</strong></td>
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<td>Control</td>
<td>(n = 16)</td>
<td>32 ± 9</td>
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<tr>
<td>Thrombocytopenia</td>
<td>(n = 15)</td>
<td>16 ± 7*</td>
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<tr>
<td>Thrombocytopenia + platelet support</td>
<td>(n = 11)</td>
<td>35 ± 14†</td>
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<td><strong>Study 3: Idiopathic thrombocytopaenia</strong></td>
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<td>26 ± 8</td>
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<tr>
<td>Thrombocytopenia</td>
<td>(n = 9)</td>
<td>29 ± 4</td>
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Dienava-Verdoold et al. Platelets and in vivo cleavage of protein S

Thrombosis and Haemostasis 107.3/2012

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plasma from patients with chemotherapy-induced thrombocytopaenia. A significantly reduced cleavage of protein S at a platelet count < 50 x 10^9/l suggests that indeed platelets contribute to protein S cleavage in vivo (Table 1). Strong evidence for platelets as in vivo source of protein S cleaving thrombopoietic protease was provided by the observation that in patients with suppressed platelet production, protein S cleavage was restored to normal values upon platelet transfusion (Table 1). Additional evidence was provided by the observation of increased protein S cleavage in patients with ET at platelet counts > 800 x 10^9/l and normalisation of both parameters upon cytotherapeutic therapy (Table 1). Cleaved protein S present in plasma is completely inactive as an anticoagulant (Figs. 2, 3). Therefore, cleavage of protein S provoked by platelet infusion may contribute to the beneficial effect of platelet transfusion in the treatment of bleeding episodes. On the other hand, cleavage of protein S provoked by high platelet count, e.g. in ET, may promote thrombosis.

Our recently published in vitro study (12) clearly points towards platelets as source of the protein S cleaving proteolytic activity in plasma. Merit of the present study resides in the in vivo approach in correlating platelet count with protein S cleavage. A positive correlation between protein S cleavage and platelet count, although undisclosed in healthy individuals, was evident in patients with haematologic malignancies (Fig. 4). Our results show that the effect of platelet number on protein S cleavage is most prominent at platelet count extremes. In healthy individuals, the platelet count range is too narrow to argue either in favour or against a correlation of protein S cleavage with platelet count. In this group, variability in extent of protein S cleavage is apparently due to individual variability in protein S cleaving activity per se. However, it can not be fully ruled out that a correlation between platelet count and protein S cleavage is restricted to pathological conditions associated with haematological malignancies. Also, contribution of proteases from blood cells other than platelets had to be taken into account. Neutrophil elastase e.g. is known to degrade protein S, a process however that is strongly suppressed in plasma (13, 16). High-dose chemotherapy-induced thrombocytopaenia is not only associated with a reduction in protein S cleavage but also with inevitable low white blood cell counts. Nevertheless, normalisation of protein S cleavage upon transfusion of leukocyte-depleted platelet concentrate to chemotherapy-treated patients (Table 1) excludes contribution of an in vivo cellular source of protein S cleaving protease activity other than platelets. Contribution of donor protein S, nonetheless, should be taken into account. It can be calculated that cleaved donor protein S from plasma present in the transfusion bag as preservative may account, on average, for a 2% increase in the portion of cleaved protein S measured in the patient samples upon platelet transfusion. This amount still gives a 17% increase in the portion of cleaved protein S on the account of transfused platelets. It has been reported that platelets contain 1.6 ng protein S per 10^9 platelets (25). Assuming that all protein S in transfused platelets is released in the circulation, one bolus of 350 x 10^9 transfused platelets may increase the total concentration of plasma protein S by 2.54 nM, an amount too low to significantly contribute to levels of total protein S in transfused patients. Whether platelet-derived protein S is intact or cleaved remains to be elucidated. However, even if donor platelet-derived protein S is fully cleaved and completely secreted from the transfused platelets, the portion of circulating cleaved protein S is increased by less than 1%. The increase in the portion of circulating TSR-cleaved protein S upon platelet transfusion, therefore, is predominantly attributable to proteolytic activity associated with transfused platelets.

The average portion of cleaved protein S that we observed (Fig. 1) in our Dutch population of 106 healthy control subjects (25 ± 8%), is more than twice as high as the 10% reported by Borgel et al. (26). The cause of this apparent discrepancy is unclear, but it might be a methodological issue. In the Borgel study, a MoAb specific for Arg66-cleaved protein S was used in ELISA (13). In our study, cleavage is based on the protein profile on Western blot and loss of response in ELISA with our MoAb CLB-PS 52 that specifically recognises TSR-intact protein S (12, 19). Most intriguing is the observation that the effect of platelet number on protein S cleavage was most pronounced at platelet counts below 50 x 10^9/l and that protein S cleavage tend to a maximum value at platelet counts above normal (Fig. 4B). This observation might be ex-
plained by a reduction in biomass (including the putative protein S cleaving protease) per platelet at increased thrombopoiesis (27). Alternatively, since two third of the total amount of protein S is bound to C4b-binding protein (C4BP) (20), C4BP-bound protein S might be protected from cleavage by the platelet-associated protein S cleaving activity.

The rationale for the presence of cleaved protein S in the circulation is presently unknown. Possibly, controlled protein S cleavage is required to keep the activity of circulating protein S within acceptable limits. Although several studies show loss of protein S anticoagulant activity upon TSR cleavage in vitro, the impact of TSR cleavage on the APC-independent anticoagulant activity of protein S has never been investigated in plasma. Also, in vivo cleavage of protein S has never been correlated to thrombin generation in the CAT assay. This assay has been successfully used by others for the separate measurement of APC-dependent and APC-independent protein S anticoagulant activity (22, 28). The mechanistic background behind the measurement of the APC cofactor activity of protein S lies in the observation that in plasma APC is completely dependent on protein S (29, 30). For the measurement of the APC-independent cofactor activity of protein S by CAT we employed our inhibiting MoAb CLB-PS13. This MoAb is directed against the Gla-domain of protein S (23) and recognises both intact and TSR cleaved protein S (19). In a study by Sere et al. (22), CLB-PS13 was found to increase thrombin generation in the CAT assay in the absence of APC; a phenomenon that later turned out to correspond with the TFPI cofactor activity of protein S (6). The results of our experiments show that TSR-cleaved protein S has no detectable APC-dependent and APC-independent anticoagulant activity in plasma (Fig. 3). Indeed, uncleaved protein S is able to block TF-initiated thrombin generation while plasma containing only TSR-cleaved protein S is devoid of protein S anticoagulant activity with a concomitant hypercoagulable state (Fig. 2) as observed in plasma from type I and type III protein S-deficient individuals (28). Because of the impact of protein S cleavage on its anticoagulant activity, an association of protein S cleavage with haematological disorders seems conceivable. However, cleaved protein S did not emerged as risk factor for thrombosis in a case-control study including 87 patients with VTE (26). On the other hand, a pathologically increased protein S cleavage has been observed in disseminated intravascular coagulation (13). In addition, increased cleavage of protein S in ET may contribute to the occurrence of an acquired APC-resistance phenotype in these patients (18).

Research from the past decades have notified that although protein S deficiency is associated with the development of thrombotic events, the relevance of protein S as marker in coagulation or as a therapeutic target may be limited. On the other hand, in vivo protein S cleavage has largely been ignored and studies on the mechanism of in vivo cleavage of protein S may reveal new strategies in the prevention and treatment of coagulation disorders. Employing plasma samples from healthy individuals and patients with thrombocytopenia or thrombocytosis, we are the first to describe a relationship between in vivo protein S cleavage, protein S anticoagulant activity and platelet-associated protein S cleaving activity. In addition to protein S, the concept of cellular proteases as modulators of the activity of clotting factors have also been suggested for FV, FVIII, FX and TF (31–35). To date, the role of the protein S cleaving protease in haemostasis is only speculative. Vessel trauma needs a rapid response to injury reaction with locally triggered thrombin generation. Down regulation of the anticoagulant activity of protein S by platelet-associated protein S cleaving activity may guarantee full activity of TF-FVIIa, FIXa-FVIIa and FXa-FVa complexes required for the thrombin generation burst.

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Conflict of interest
None declared.

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