Aspirin resistance is not a common biochemical phenotype explained by unblocked cyclooxygenase-1 activity.

Hillarp, Andreas; Lethagen, Stefan; Mattiasson, Ingrid

Published in:
Journal of Thrombosis and Haemostasis

DOI:
10.1046/j.1538-7836.2003.00012.x

2003

Journal of Thrombosis and Haemostasis, **1**: 186–202

LETTERS TO THE EDITORS

Outpatient treatment of pulmonary embolism is feasible and safe in a substantial proportion of patients[1]

J.H. BEER,* M. BURGER,* S. GRETENER,* S. BERNARD-BAGATTINI† and H. BOUNAMEAUX†

**Department of Internal Medicine, Kantonsspital Baden; and †Division of Angiology and Hemostasis, University Hospital of Geneva, Switzerland*

Dear Sir,

Asymptomatic pulmonary embolism (PE) is frequent in proximal deep vein thrombosis (DVT) and DVT patients are now largely treated as outpatients [1–4]. It was therefore tempting to treat at least some patients with symptomatic PE in an outpatient setting. The aim of the present study was to evaluate the proportion of symptomatic PE patients with a low predicted risk (according to Wicki *et al*. [3] who could effectively be treated in an outpatient setting). Patients ($n = 255$; median age 69 years, range 18–96) were diagnosed during the period from May 1999 to December 2001 with symptomatic, objectively confirmed PE in the two participating centers. 150 patients had predefined exclusion criteria, including contraindication for anticoagulants, history or presence of drug addiction, high probability of noncompliance, body weight >110 kg, psychiatric conditions, clinically relevant impairment of renal function (creatinine clearance $<30\,\mathrm{mL\,min^{-1}}$), thrombocytopenia ($<120\,\mathrm{G\,L^{-1}}$), any contraindications for low molecular weight heparins (LMWHs) or standard heparin, concomitant fibrinolytic therapy, treatment with oral anticoagulants during the 24 h prior to entering the study. Recruitment was not possible on weekends, which excluded 57 additional patients. Thus, 105 patients could be included in the study, and 43 (41%) could in fact be treated entirely as outpatients (Fig. 1). Treatment consisted of a single daily subcutaneous (s.c.) injection of a weight-adjusted dose of nadroparin calcium [Fraxiforte®, 171 UI anti-factor Xa (FXa) $\mathrm{kg^{-1}}$, Sanofi-Synthelabo, France], and phenprocoumon until the international normalized ration was in the therapeutic range of 2–3 for two consecutive days (LMWH for a minimum of 5 up to 10 days). Oral anticoagulation was continued for 6–12 months. Among the 62 patients treated in hospital, only 11 had a high risk score [3], while the main reasons for the inpatient treatment was either the patient's choice or the presence of comorbidity (Fig. 1). All patients were followed up for 3 months for clinical manifestation of thromboembolism or bleedings. During the 3-month follow-up, clinically suspected recurrent DVT or PE was objectively confirmed in three patients (two in the inpatient and one in the outpatient group). No patients died, and there were no major bleeding incidents, although two minor bleedings occurred in the inpatient group. In conclusion, a substantial proportion of patients with symptomatic PE at low predicted risk of adverse outcome could be treated in an outpatient setting by means of a once daily s.c. injection of LMWH nadroparin. These data, along with an earlier report from Canada [5], call for a large-scale randomized study, with particular emphasis on safety and cost-effectiveness in PE patients with a predicted low risk of adverse outcome.

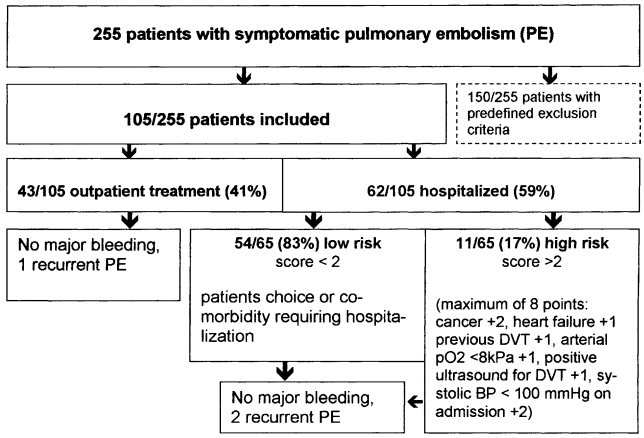

Fig. 1. Study flow chart.

Acknowledgements

The project was supported by an unrestricted grant from Sanofi Synthelabo, Geneva, and in part by the Swiss National Foundation of Science (grant no. 32–059 449) and the Swiss Heart Foundation.

References

1 Kearon C, Gent M. Heparin therapy for deep vein thrombosis. from hospital to home. *Am J Med* 2001; **110**: 501–2.

2 Buller HR. Outpatient therapy with low-molecular weight heparins: new perspectives for treatment of deep vein thrombosis. *Haemostasis* 1998; **28** (Suppl. 3): 91–4.

3 Wicki J, Perrier A, Perneger TV, Bounameaux H, Junod AF. Predicting adverse outcome in patients with acute pulmonary embolism: a risk score. *Thromb Haemost* 2000; **84**: 548–52.

Correspondence: Professor Dr Jürg H. Beer, Head of the Department of Internal Medicine, Kantonsspital, CH-5404 Baden, Switzerland.
Tel.: +41 56 486 2501; fax: +41 56 486 2509; e-mail: hansjuerg.beer@ksb.ch

[1]This work was presented in part at the XVIIIth Meeting of the International Society on Thrombosis and Haemostasis, in Paris, 6–12 July 2001 and published in abstract form in *Thrombosis and Haemostasis* 2001; **86**: abstract no. 971.

Received 19 March 2002, revised 15 April 2002, accepted 5 May 2002

4 Schraibman IG, Milne AA, Royle EM. Home versus in-patient treatment for deep vein thrombosis. *Cochrane Database Syst Rev* 2001; **2**: CD003076.

5 Kovacs MJ, Anderson D, Morrow B, Gray L, Touchie D, Wells PS. Outpatient treatment of pulmonary embolism with dalteparin. *Thromb Haemost* 2000; **83**: 209–11.

Immunoadsorption for the treatment of a patient with severe thrombotic thrombocytopenic purpura resistant to plasma exchange: kinetics of an inhibitor of ADAMTS13

P. KNÖBL,* M. HAAS,† K. LACZIKA,* K. VARADI‡ and P. L. TURECEK‡

**Department of Medicine 1; and †Department of Medicine 3, University of Vienna, Austria; and ‡Baxter BioScience, Vienna, Austria*

Dear Sir,

Thrombotic thrombocytopenic purpura (TTP) [1] is characterized by a decreased activity of ADAMTS13 [von Willebrand factor (vWF)-cleaving metalloprotease], caused either by a congenital deficiency (in cases of familial TTP) or by inhibition by an autoantibody (in acquired TTP) [2–4]. ADAMTS13 has been characterized and cloned [5–7]. Plasma exchange is considered to be the treatment of choice to remove unusually large vWF multimeres (UL vWF-MM) and inhibitors of ADAMTS13 and to substitute normal vWF and ADAMTS13 [8]. However, sometimes the response is slow despite intensive plasma exchange. We present the case of a patient with severe TTP, resistant to plasma exchange, and show the kinetics of a high titer inhibitor of ADAMTS13 during plasma exchange, immunoadsorption and follow-up.

The 38-year-old female patient with a history of systemic lupus erythematosus was admitted after a grand mal seizure which occurred after a short period of cephalea, fatigue and 'restless legs'. On admission, dysarthria and a right-sided hemiparesis were obvious. A cranial computerized tomography showed no signs of bleeding, hypoperfusion or vasculitis. Red blood cell count was 2.7 T L^{-1}, hemoglobin 8.0 g dL^{-1}, platelet count 13 G L^{-1}, lactate dehydrogenase (LDH) 745 U L^{-1}, creatinine 1.3 mg dL^{-1}. Fragmented red cells were visible in the blood smear. Tests of plasmatic coagulation were normal. Antibodies to platelet antigens could not be detected. Coombs-negative hemolytic anemia with red cell fragmentation, together with severe thrombocytopenia, neurological disorder and elevated serum creatinine were highly suggestive for TTP.

Serial determinations of vWF antigen (vWF:Ag), vWF-collagen-binding activity (vWF:CB), factor VIII (FVIII) activity and the multimeric composition of vWF were performed as described elsewhere [9]. The activity of ADAMTS13 was measured according to Gerritsen *et al.* [10], modified by using a purified recombinant vWF (Baxter BioScience, Vienna, Austria) [11] as substrate, and a different ELISA plate (Exiqon, Vedbaek, Denmark) to measure the residual vWF:CB activity [9]. The titer of the inhibitor of ADAMTS13 was measured as follows: the inhibitor-containing plasma samples undiluted and diluted with physiological saline up to 1 : 32 were mixed 1 : 1 with normal plasma and subsequently incubated for 30 min at 37 °C. After incubation, the samples were diluted 1 : 10 and 1 : 20 with 1.5 mol L^{-1} urea, 5 m mol L^{-1} Tris, pH 8.0 and the residual ADAMTS13 activity was measured. The amount of inhibitor was calculated according to the assay principle of the Bethesda method initially described for FVIII inhibitors [12] and expressed as inhibitor units per milliliter (InhU mL^{-1}). One inhibitor unit per milliliter was defined as the amount of the inhibitor which decreases the ADAMTS13 activity to 50% in the assay mixture in 30 min compared with a control negative sample.

At admission, analysis of the vWF-multimer pattern showed the presence of UL vWF-MM (Fig. 1), vWF:Ag was slightly elevated (2.1 U mL^{-1}), vWF:CB and F VIII:C were in the normal range (1.18 and 0.82 U mL^{-1}, respectively). The activity of ADAMTS13 was below the detection limit of our assay, and an inhibitor against the metalloprotease could be detected at an initial titer of 3.7 InhU mL^{-1}. These findings were consistent with the current hypothesis of the pathophysiology of TTP [3,4].

Plasma therapy was initiated by daily exchange of 50 mL kg^{-1} body weight of plasma with fresh frozen plasma according to a standard procedure. Additionally, prednisone (100 mg daily) was given. Red blood cells were transfused at hemoglobin values below 8.0 g dL^{-1}. No platelet concentrates were given.

During the first days of plasma exchange the patient's platelet count increased and her neurological state improved (Fig. 1). On the sixth day, however, the platelet count decreased from 34 to 6 G L^{-1}, and LDH increased from 235 to 763 U mL^{-1} despite continuing plasma exchange. The patient's neurological state deteriorated, and she was intubated and mechanically ventilated to avoid aspiration. Because the patient had an underlying autoimmune disease that had probably triggered the thrombotic microangiopathy, extracorporeal immunoadsorption on anti-human immunogobulin-Sepharose columns (Ig-Therasorb®) was initiated. It was carried out using a continuous extracor-

Correspondence: Professor Dr Paul Knöbl, Department of Medicine 1, University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria.
Tel.: + 43 1 40 400 4409; fax: + 43 1 402 6930;
e-mail: paul.knoebl@akh-wien.ac.at

Received 19 March 2002, revised 8 July 2002, accepted 24 July 2002

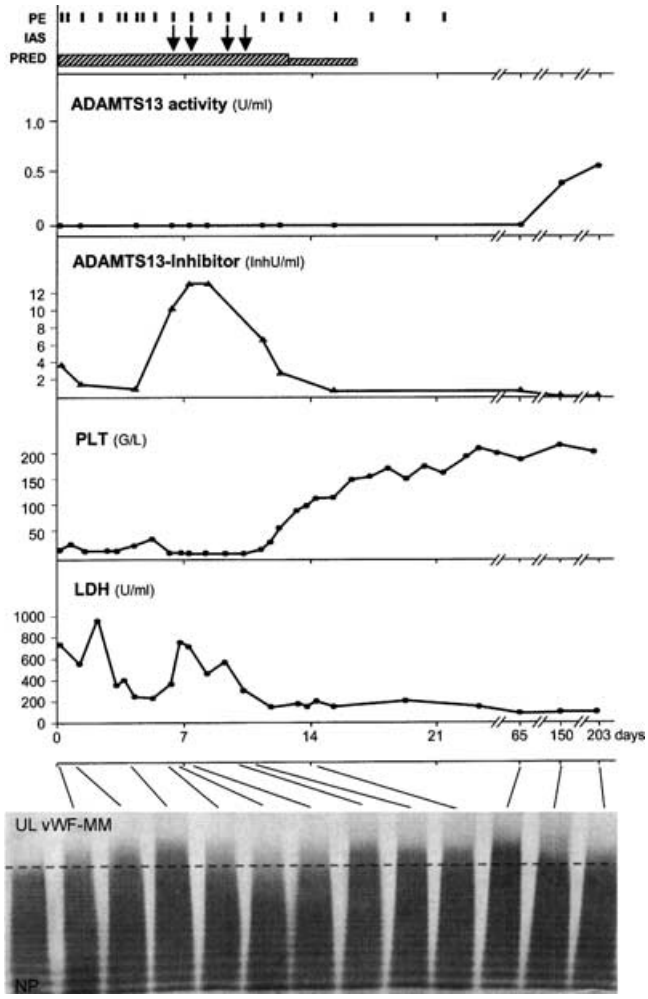

Fig. 1. Treatment modalities, ADAMTS13 activity and inhibitor, platelet count, LDH, and vWF multimer pattern of a patient with TTP poorly responding to plasma exchange are shown. The lanes of the multimer analysis are assigned to the respective days of blood sampling. PE, plasma exchange; IAS, immunoadsorption; PRED, prednisone; InhU ml^{-1}, inhibitor units per milliliter; PLT, platelet count; LDH, lactate dehydrogenase; NP, normal plasma; vWF-MM, multimer pattern of von Willebrand factor; bands above the dotted line indicate unusally large (UL) vWF-MM.

poreal system at a flow rate of 40 mL min^{-1} as described previously [13]. The blood was anticoagulated with citrate dextrose and the plasma separated with a Fenwal Autopheresis-C system (Baxter, Unterschleißheim, Germany). The plasma was loaded alternately onto one of two columns filled with Sepharose-coupled with goat anti-human immunoglobulins (Ig-Therasorb®, Plasmaselect, Teterow, Germany), while the other column was regenerated. After immunoadsorption, the plasma was reinfused together with the separated blood cells. A total of 7000 mL of plasma was processed in one session lasting about 4 h.

Additionally, 500 mg of cyclophosphamide were given for immunosuppression. Daily plasma exchange and prednisone were continued. Four immunoadsorption sessions were carried out within 5 days. After the third immunoadsorption, the platelet count began to increase and LDH concentrations returned to normal. The patient's neurological state improved rapidly. She could be extubated on day 13 and discharged without residues after seven further plasma exchanges on the day 24 after admission with a platelet count of 200 G L^{-1}.

Prospective serial determinations of vWF-MM, ADAMTS13 activity and ADAMTS13 inhibitor were performed (Fig. 1). During the first days of intensive plasma exchange the inhibitor titer decreased from the initial 3.7 InhU mL^{-1} to approximately 1 InhU mL^{-1}. On day 6, ADAMTS13 inhibitor titer increased suddenly to 13 InhU mL^{-1}, concomitant with an increase of UL vWF-MM and signs of accelerated formation of platelet microthrombi (LDH increase, drop of platelet count, neurological deterioration) (Fig. 1). The reason for this increase is unclear, but would explain the poor or prolonged response to plasma exchange of some patients with TTP. In their retrospective analysis of a patient with chronic relapsing TTP, Furlan *et al.* [14] described a similar phenomenon during a relapse treated with plasma exchange and plasma infusion.

The inhibitor titer decreased rapidly after initiation of immunoadsorption and immunosuppression, but ADAMTS13 activity was still below the detection limit. The UL vWF-MM disappeared during immunoadsorption (probably due to consumption during the exacerbation of the disease), but returned thereafter (although the inhibitor titer was very low) and were detectable until day 65. At this time the patient's platelet count, LDH, renal function and neurological state were normal, but ADAMTS13 activity was still below the detection limit due to a low titer inhibitor (below 0.5 InhU mL^{-1}). This suggests that [1] the inhibitor completely suppresses ADAMTS13 activity, even at low titers; and [2] an additional triggering situation is necessary to induce platelet aggregation when UL vWF-MM are present. Therefore, a patient with TTP in remission may be at risk of relapse when ADAMTS13 activity is low and UL vWF-MM are present. In contrast, in cases of severe hereditary ADAMTS13 deficiency even very low ADAMTS13 levels (about 0.05 U mL^{-1}) seem to protect from relapse [3]. On day 150, the inhibitor disappeared and ADAMTS13 activity increased to 0.3 U mL^{-1}. By day 203, the ADAMTS13 activity was 0.62 U mL^{-1} and no UL vWF-MM could be detected. vWF:Ag, vWF:CB and FVIII were within the upper normal range (1.5, 1.4 and 1.4 U mL^{-1}, respectively).

Immunoadsorption is a useful method to effectively deplete the plasma of immunoglobulins, and it has been used successfully for short- and long-term treatment of several immunological disorders [13]. Our patient's good response suggests that different treatment strategies, similar to the therapy of autoimmune diseases (immunosuppression, immunoadsorption, high-dose immunoglobulin infusions) may be more effective than plasma exchange in patients with TTP and inhibitors to ADAMTS13. Before these strategies can be implemented, fast and reliable assays are required to measure ADAMTS13 activity and inhibitor titer as well as studies that prove their effectiveness.

In conclusion, the time course of clinical symptoms, and the changes in ADAMTS13 activity and its inhibitor observed in this patient during and after treatment show some remarkable features of TTP. The determination of ADAMTS13 activity and inhibitor, and the vWF multimer pattern is useful to diagnose

and monitor patients with TTP. These measurements can also be used to justify the inclusion of immunoadsorption or immunosuppression in a treatment protocol, and to identify patients at risk of relapse. Prospective studies are still necessary to verify the effectiveness of these new approaches.

Acknowledgements

We gratefully acknowledge the work of the staffs of the intensive care unit (13I2) and the apheresis unit (13H2) of the University Hospital of Vienna and the skillful technical assistance of Jutta Schreiner and Ingrid Neunteufel (Baxter BioScience, Vienna, Austria).

References

1 Moschcowitz E. Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. *Proc N Y Pathol Soc* 1924; **4**: 21–4.
2 Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 1996; **87**: 4235–44.
3 Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, Krause M, Scharrer I, Aumann V, Mittler U, Solenthaler M, Lammle B. Von Willebrand factor–cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic uremic syndrome. *N Engl J Med* 1998; **339**: 1578–84.
4 Tsai HM, Lian EC. Antibodies to von Willebrand factor–cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 1998; **339**: 1585–94.
5 Gerritsen HE, Robles R, Lämmle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood* 2001; **98**: 1654–61.
6 Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, Sarode R, Shurin SB, Chandrasekaran V, Stabler SP, Sabio H, Bouhassira EE, Upshaw JD, Ginsburg D, Tsai HM. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001; **413**: 488–94.
7 Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 2001; **276**: 41059–63.
8 Rock GA. Management of thrombotic thrombocytopenic purpura. *Br J Haematol* 2000; **109**: 496–507.
9 Siekmann J, Turecek PL, Schwarz HP. The determination of von Willebrand factor activity by collagen binding assay. *Haemophilia* 1998; **4**: 15–24.
10 Gerritsen A, Turecek PL, Schwarz HP, Lämmle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF. *Thromb Haemost* 1999; **82**: 1386–9.
11 Turecek PL, Gritsch H, Pichler L, Auer W, Fischer B, Mitterer A, Mundt W, Schlokat U, Dorner F, Brinkman HJ, van Mourik JA, Schwarz HP. *In vivo* characterization of recombinant von Willebrand factor in dogs with von Willebrand disease. *Blood* 1997; **90**: 3555–67.
12 Kasper CK, Aledort LM, Counts RB, Edson JR, Fratantoni J, Green D, Hampton JW, Hilgartner MW, Lazerson J, Levine PH, McMillan CW, Pool JG, Shapiro SS, Shulman NR, Van Eys J. A more uniform measurement of factor VIII inhibitors. *Thrombos Diathes Haemorrh* 1975; **34**: 869–72.
13 Knöbl P, Derfler K, Korninger L, Kapiotis S, Jäger U, Maier-Dobersberger T, Hörl W, Lechner K. Elimination of acquired factor VIII antibodies by extracorporeal antibody-based immunoadsorption (Ig-Therasorb). *Thromb Haemost* 1995; **74**: 1035–8.
14 Furlan M, Robles R, Solenthaler M, Lämmle B. Acquired deficiency of von Willebrand factor-cleaving protease in a patient with thrombotic thrombocytopenic purpura. *Blood* 1998; **91**: 2839–46.

Autoimmune disease antigen U1 snRNP neutralizes heparin

M. F. SCULLY and M. A. GEBSKA
Thrombosis Research Institute, Emmanuel Kaye Building, Manresa Road, London, UK

Dear Sir

The RNA-binding protein, U1 (small nuclear ribonucleoprotein-associated 68/70 kDa protein; U1-snRNP) is the major antigen of autoantibodies found in the serum of patients with mixed connective tissue disease and to a lesser extent in serum of patients with systemic lupus erythematosus [1]. A well-established symptom in individuals with autoimmune diseases of this type is an increased risk of thrombosis usually associated with the presence of antiphospholipid antibodies [2].

Because of the arginine-rich regions within snRNP 68/70 [3], we investigated whether the protein has an antiheparin activity according to the hypothesis that abnormal exposure could also contribute to the increased risk of thrombosis by abrogating the normal functioning of the antithrombin pathway of anticoagulation (blocking the interaction of the serpin with heparan sulfate on the vessel wall). Indeed we have observed that U1-SnRNP has a potent antiheparin activity comparable to that of protamine when observed under two separate experimental conditions. Firstly, the clotting time of heparinized normal human plasma (1.25 IU mL^{-1} of plasma, equivalent to around 500 nmol L^{-1} heparin) was completely reversed by raising the concentration of snRNP 68/70 (obtained from Diarect AG, Freiburg, Germany) to 750 nmol L^{-1} (Fig. 1a; a similar concentration of protamine was required). Secondly, the accelerated rate of inhibition of thrombin by antithrombin in the presence of heparin was reduced by U1-snRNP, as compared to that observed in the absence of heparin by U1-snRNP (Fig. 1b, IC_{50} 13 nmol L^{-1} compared to 22 nmol L^{-1} for protamine).

Correspondence: Thrombosis Research Institute, Emmanuel Kaye Building, Manresa Road, London SW3 6LR, UK,
Tel.: + 44 207 351 8305; fax: + 44 207 351 8324;
e-mail: mscully@tri-london.ac.uk

Received 2 April 2002, accepted 5 May 2002

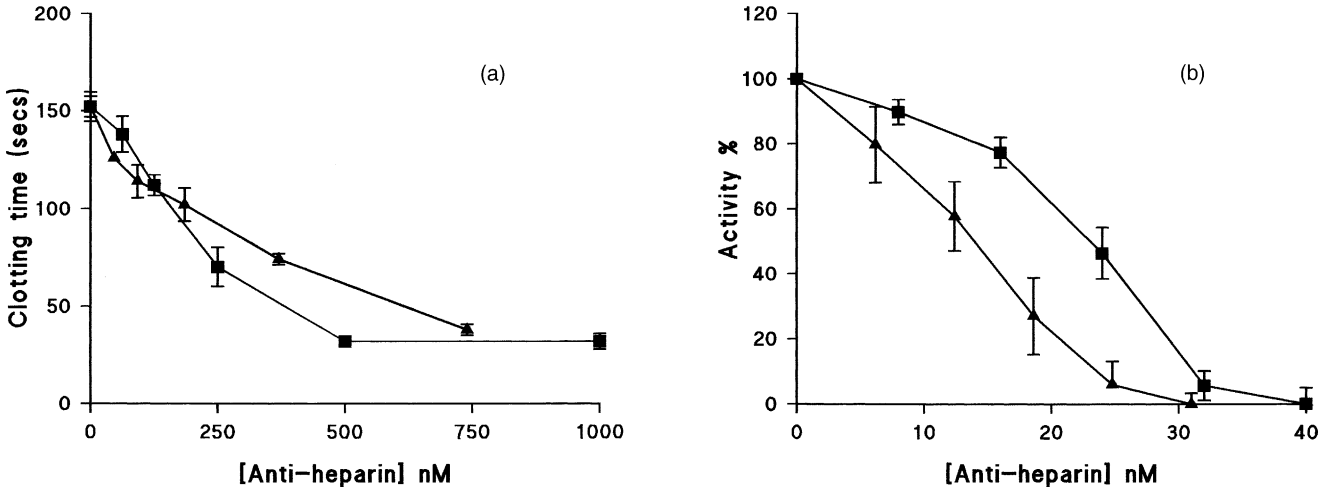

Fig. 1. (a) The APTT of heparinized human pooled plasma (1.25 IU of unfractionated heparin mL^{-1}) was determined in the presence of an increasing concentration of U1-snRNP (▲–▲) or protamine (■–■). Results are shown as the mean ±SEM, $n = 3$. (b) The rate of inhibition of thrombin by antithrombin in the presence of heparin (10 nmol L^{-1}, unfractionated heparin) was determined according to a modification of a previously described method [7] in the presence of an increasing concentration of U1-snRNP (▲–▲) or protamine (■–■). Results are calculated as the percentage of heparin-dependent activity and shown as the mean ± SEM, $n = 3$.

Recently it has been shown that proteins of the nuclear matrix of which U1-snRNP is one example segregate into discrete structures following transcriptional arrest [4]. These structures are observed to move from the nucleus onto the surface of cells during apoptosis and necrosis. We have also reported similar heparin binding structures forming within the nucleus of apoptotic cells, which are subsequently transferred onto the surface or released, into the medium [5,6]. The exposure of U1 snRNP through this mechanism may initiate the generation of antibodies and may also, we propose, enhance the risk of thrombosis by inhibition of the antithrombin pathway of anticoagulation.

References

1 Bosman FT. The nuclear matrix in pathology. *Virchows Arch* 1999; **435**: 391–9.
2 Arnout J. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost* 2001; **86**: 83–91.
3 Theissen H, Etzerodt M, Reuter R, Schneider C, Lottspeich F, Argos P, Luhrmann R, Philipson L. Cloning of the human cDNA for the U1 RNA-associated 70K protein. *Embo J* 1986; **5**: 3209–17.
4 Biggiogera M, Pellicciari C. Heterogeneous ectopic RNP-derived structures (HERDS) are markers of transcriptional arrest. *Faseb J* 2000; **14**: 828–34.
5 Gebska MA, Titley I, Paterson HF, Morilla RM, Davies DC, Gruszka-Westwood AM, Kakkar VV, Eccles S, Scully MF. High-affinity binding sites for heparin generated on leukocytes during apoptosis arise from nuclear structures segregated during cell death. *Blood* 2002; **99**: 2221–7.
6 Morita S, Gebska MA, Kakkar AK, Scully MF. High affinity binding of heparin by necrotic tumour cells neutralises anticoagulant activity – implications for cancer related thromboembolism and heparin therapy. *Thromb Haemost* 2001; **86**: 616–22.
7 Scully MF, Ellis V, Kakkar VV. Heparan sulphate with no affinity for antithrombin III and the control of haemostasis. *FEBS Lett* 1988; **241**: 11–4.

Multi-therapeutic approach to manage delivery in an alloimmunized patient with type 3 von Willebrand disease

C. BOYER-NEUMANN,*† M. DREYFUS,†‡ M. WOLF,*† A. VEYRADIER*† and D. MEYER*†
**Service D'Hèmatologie biologique, Höpital Antoine-Bèclëre, Clamart, France; †Inserm U143, Le Kremlin-Bicétre, France; and ‡Service D'Hèmatologie biologique, Höpital de Bicétre, Le Kremlin-Bicétre, France*

Dear Sir,

Type 3 von Willebrand disease (VWD) is characterized by a virtually complete deficiency of von Willebrand factor (VWF) associated with markedly reduced factor VIII (FVIII) procoagulant activity (FVIII:C) [1]. This combined deficiency of both VWF and FVIII results in severe mucosal bleeding as well as hemophilia-like symptoms, requiring multiple transfusions and leading to development of alloantibodies to VWF in about 10% of patients [2]. The presence of these alloantibodies not only renders replacement therapy with VWF concentrates ineffective, but may even be dangerous, because of severe allergic reactions [3]. Recombinant activated factor VII (rFVIIa) has

Correspondence: Dominique Meyer, MD, Service d'Hèmatologie biologique, Höpital Antoine-Bèclëre, 157, rue de la Porte de Trivaux, 92140 Clamart Cedex, France Tel.: + 33 1 4537 4305; fax: + 33 1 4632 4055; e-mail: dmeyer@infobiogen.fr

Received 15 April 2002, revised 2 July 2002, accepted 4 July 2002

been classically proposed for the treatment of patients with severe hemorrhagic diseases with alloantibodies, mainly hemophilia and Glanzmann's thrombasthenia [4]. Up to now, few reports on the use of rFVIIa in alloimmunized type 3 VWD patients have been published [5,6]. Alternatively, recombinant factor VIII (rFVIII) has been successfully used in a type 3 VWD woman with precipitating alloantibodies to VWF during abdominal surgery [3,7]. However, none of the latter treatments has ever been experienced in obstetrical situations. We here report, for the first time, the successful management of cesarean section and postpartum period using the sequential combination of recombinant FVIII and FVIIa in an alloimmunized type 3 VWD patient.

This patient was a 29-year-old Algerian woman with a lifelong bleeding history from puberty, requiring multiple transfusions. Familial inquiry revealed parental consanguinity (first cousins) and the death, related to severe bleeding, of two sisters and two brothers among seven siblings. At the time of her first visit to France, FVIII coagulant activity (FVIII:C), VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo) levels were undetectable in plasma and molecular analysis showed a total deletion of VWF gene. Because of prolonged menorrhagia leading to severe anemia, she received VWF/FVIII concentrates (Innobrand®; LFB, Les Ulis, France). This transfusion was ineffective and complicated by lumbar pains and chills. Laboratory analysis using classical mixing studies demonstrated the presence of an inhibitor to VWF:RCo (titer of 16 Bethesda Units [BU] per mL) and also to FVIII:C (titer of $4\,BU\,mL^{-1}$). The inhibitor was identified as an antibody of the IgG class and induced, as previously described, a nonspecific but steric hindrance-linked inhibition of FVIII [8].

A few months later, the patient became pregnant and was referred to our center for obstetrical follow-up and hematological management (Fig. 1). At day 0, just prior to the cesarean section (performed with a tubal ligation after informed and signed consent), a central venous line was inserted after a starting bolus of $100\,IU\,kg^{-1}$ of recombinant FVIII (Kogenate®; Bayer, Puteaux, France) immediately followed by a continuous infusion of $35\,IU\,kg^{-1}\,h^{-1}$. This treatment resulted in a rise of FVIII:C levels up to $60\,IU\,dL^{-1}$, allowing the cesarean section without any bleeding, and the birth of a healthy full-term boy whose VWF phenotype in venous blood at day 1 of life was as follows: VWF:Ag: $94\,IU\,dL^{-1}$, VWF:RCo: $93\,IU\,dL^{-1}$, FVIII:C: $107\,IU\,dL^{-1}$. The same dose regimen was effective in obtaining FVIII:C levels close to $50\,IU\,dL^{-1}$, and elicited no side-effects until day 3. At this time, the patient developed cutaneous urticaria, which was treated with antihistamine drugs and corticosteroids. Despite this treatment, the allergy persisted and at day 7, Kogenate® was switched to Recombinate® (Baxter, France). In addition, as the recovery of FVIII:C levels decreased with levels close to $20\,IU\,dL^{-1}$, we decided to start discontinuous infusions of $115\,IU\,kg^{-1}$ per 4 h, leading to a $40\,IU\,dL^{-1}$ peak of VIII:C (10 min after infusion) and a $10\,IU\,dL^{-1}$ residual FVIII:C (just before the following infusion). This treatment improved both the allergic reaction and FVIII recovery. Moreover, the absence of gynecological bleeding allowed the progressive decrease of Recombinate® (from $100\,IU\,4\,h^{-1}$ at day 9 to $57\,IU\,4\,h^{-1}$ at day 14). At day 15, treatment by Recombinate® was tentatively interrupted, but the onset of gynecological bleeding 12 h later led us to reintroduce Recombinate® infusions together with oral methylergometrin, without any clinical improvement. As the uterine bleeding was thought to be related to an early resumption of menstruation, it was decided to perform a subcutaneous injection of gonadotrophin-releasing hormone agonist (Enantone®) at day 16 [9]. Unfortunately, 36 h later, despite the addition of aprotinin, gynecological hemorrhage dramatically worsened, with the emission of large clots. This persistent uterine bleeding could be partially explained by a 'flare-up' effect of Enantone® [9] and forced us to stop both Recombinate® and aprotinin and to begin a treatment by recombinant FVIIa (Novoseven®; Novo Nordisk, Denmark), as previously used in alloimmunized type 3 VWD patients by Ciavarella *et al*. [5]. At day 18, an infusion of $4\,kIU\,Novoseven^{*}\,kg^{-1}\,4\,h^{-1}$ was started through a femoral central line. Clinical improvement was obvious already after the second infusion of Novoseven® with a sharp decrease of uterine bleeding. However, at day 19, after the fifth infusion of Novoseven®, biological follow-up revealed a five-fold increase of D-dimer levels leading to the discovery of an ilio-femoral venous thrombosis close to the femoral central line. Thus, NovoSeven® had to be stopped and a pulmonary embolism was excluded by tomodensitometry. A few hours later, uterine bleeding dramatically reappeared, requiring multiple red cell transfusions. This dramatic therapeutic deadlock led the medical staff to place a cava filter as a first step and to embolize the uterine arteries as a second step, using in both cases a single NovoSeven infusion ($4\,kIU\,kg^{-1}$). The embolization of the uterine artery was remarkably successful on bleeding, with no additional requirement for erythrocyte transfusions nor any hemostatic drugs. Interestingly, ultrasonography performed 24 h after uterine artery embolization demonstrated the unexpected complete dissolution of the iliofemoral thrombus. In less than 48 h, uterine bleeding definitively stopped allowing the patient's discharge 1 week later.

The retrospective analysis of this complex case led us to discuss several critical points. In addition to the severity of the congenital disease, the presence of a high titer of alloantibodies to VWF constituted a major risk of bleeding, which had to be considered for both the baby and his mother. Regarding the baby, a cesarean section was decided on, because the late passing of IgG alloantibodies through the placenta could have accounted for a transitory acquired VWD. Regarding the mother, management of delivery was extensively discussed with a multidisciplinary staff. The removal of alloantibodies by immunoadsorption was first considered [10], but this therapeutic option was finally discarded for two main reasons: insufficient experience in the obstetrical context and the allergic history of our patient, requiring a total removal of alloantibodies, which could not be guaranted by the immuno-adsorption. In cases of treatment with VWF concentrates, even minimal residual alloantibody levels have been demonstrated to be capable of inducing the formation of immune complexes that

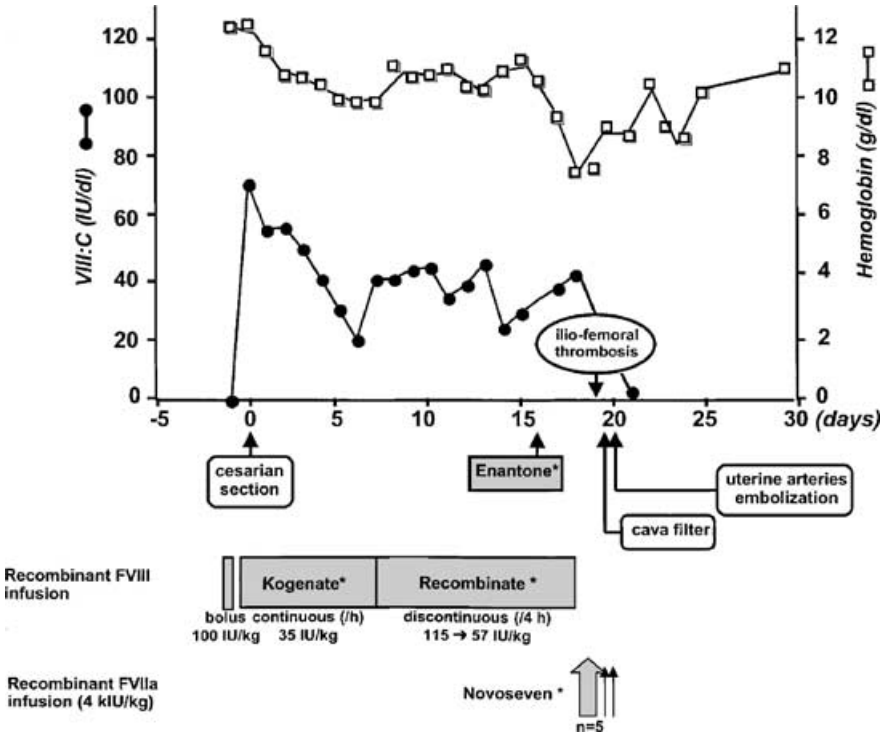

Fig. 1. Clinical, biological and therapeutic follow-up from cesarean section to about one-month postpartum of the alloimmunized type 3 von Willebrand disease patient. The evolution of both hemoglobin level (Hb) and factor VIII clotting activity (FVIII:C) are shown on the graph. Cesarean section was performed at day 0. The drugs used for the therapeutic management are indicated in gray and mainly include recombinant factor VIII, recombinant activated factor VII and one Enantone® injection. Ilio-femoral thrombosis occurred at day 19 leading to placement of a cava filter and to embolization of the uterine arteries.

activate the complement system responsible for anaphylactic reactions [3,11]. The use of recombinant FVIIa was also discussed but we decided to reserve this treatment as a last resort in reason of the well-known thrombotic risk associated with pregnancy and postpartum. Thus, in the current case, the lack of *in vitro* inhibitory effect of the alloantibody against FVIII:C prompted us to choose rFVIII (completely devoid of VWF). Although rFVIII infusion has never been used in type 3 VWD pregnant patients,it has been successfully experienced in a type 3 vWD patient with alloantibodies during surgery [7]. We therefore decided to use for the first time rFVIII for the management of delivery and postpartum in this type 3 alloimmunized VWD patient. In this patient, the efficacy of recombinant FVIII alone was clearly demonstrated during the cesarean section, delivery and the following 2 weeks,as previously reported in an alloimmunized type 3 VWD patient during abdominal surgery [7]. Nevertheless, the predictable resumption of menstruation, which was quite early in our patient, was dramatically hemorrhagic and was only overcome by recombinant FVIIa. However, the onset of thrombosis must lead to cautious use of recombinant FVIIa in the peripartum period.

The current observation emphasizes the complex therapeutic management of alloimmunized type 3 VWD patients in any surgical and especially obstetrical situation. It also clearly demonstrates that pregnancy in such patients remains a risky challenge.

References

1 Sadler JE. A revised classification of von Willebrand disease. *Thromb Haemost* 1994; **71**: 520–5.

2 Mannucci PM, Bloom AL, Larrieu MJ, Nilsson IM, West RR. Atherosclerosis and von Willebrand factor. I. Prevalence of severe von Willebrand's disease in western Europe and Israel. *Br J Haematol* 1984; **57**: 163–9.

3 Mannucci PM, Tamaro G, Narchi G, Candotti G, Federici A, Altieri D, Tedesco F. Life-threatening reaction to factor VIII concentrate in a patient with severe von Willebrand disease and alloantibodies to von Willebrand factor. *Eur J Haematol* 1987; **39**: 467–70.

4 Aledort LM. Recombinant factor VIIa is a pan-hemostatic agent ? *Thromb Haemost* 2000; **83**: 637–8.

5 Ciavarella N, Schiavoni M, Valenzano E, Mangini F, Inchingolo F. Use of recombinant factor VIIa (NovoSeven) in the treatment of two patients with type III von Willebrand's disease and an inhibitor against von Willebrand factor. *Haemostasis* 1996; **26** (Suppl. 1): 150–4.

6 Grossmann RE, Geisen U, Schwender S, Keller F. Continuous infusion of recombinant factor VIIa (NovoSeven) in the treatment of a patient with type III von Willebrand's disease and alloantibodies against von Willebrand factor. *Thromb Haemost* 2000; **83**: 633–4.

7 Bergamaschini L, Mannucci PM, Federici AB, Coppola R, Guzzoni S, Agostoni A. Posttransfusion anaphylactic reactions in a patient with severe von Willebrand disease: role of complement and alloantibodies to von Willebrand factor. *J Lab Clin Med* 1995; **125**: 348–55.

8 Batlle J, Loures E, Vila P, Hernandez MC, Mendez JA, Torea J, Rendal E, Couselo MJ, Filgueira A, Lopez Fernandez MF. Alloantibody from a patient with severe von Willebrand disease inhibits von Willebrand factor–factor VIII interaction. *Ann Hematol* 1997; **75** (3): 111–5.

9 Yen SSC. Clinical application of gonadotrophins releasing hormone analogs. *Fertil Steril* 1983; **39**: 257–61.

10 Gjörstrup P, Watt RM. Therapeutic protein A immunoadsorption: a review. *Tranfusion Sci* 1990; **11**: 281–302.

11 Mannucci PM, Ruggeri ZM, Ciavarella N, Kazatchkine MD, Mowbray JF. Precipitating antibodies to factor VIII/von Willebrand factor in von Willebrand's disease: effects on replacement therapy. *Blood* 1981; **57**: 25–31.

Serum concentration of α1-proteinase inhibitor and α2-macroglobulin correlates with late lumen loss following coronary stent implantation

Y. IKARI,* H. HASHIMOTO,† H. NAKAJIMA* and K. HARA*

**Division of Cardiology, Mitsui Memorial Hospital; and †Department of Hygiene and Public Health, Teikyo University School of Medicine, Tokyo, Japan*

Dear Sir,

Restenosis is a well-appreciated limitation of coronary intervention. Fibrin deposition becomes histologically detectable around stent struts soon after implantation [1]. Smooth muscle cell attachment and migration into fibrin is considered an important initiating event in stent restenosis [2,3]. Integrins participate in this process [4,5]. Furthermore, plasma proteinase inhibitors such as α1-proteinase inhibitor (α1PI), α1-antichymotrypsin (α1ACT), and α2-macroglobulin (α2M) are essential for smooth muscle cells to attach, migrate and survive in fibrin [6,7]. These *in vitro* data suggested that plasma proteinase inhibitors may participate in the mechanism of stent restenosis.

In 88 elective patients, 107 *de novo* lesions were treated with stent implantation. Serum concentrations of proteinase inhibitors were measured by nephelometry. Reagent kits for α1PI and α2M were obtained from Dade Behring Marburg (Germany). The kit for α1ACT was obtained from Hoechst Japan (Tokyo, Japan). Follow-up angiography was performed at 170 ± 38 days. Quantitative coronary angiography was performed using QCA-CMS (MEDIS, Leiden, the Netherlands) by an expert cardiologist who was kept unaware of clinical or blood test data.

The average age of the patients was 64 ± 9 years; 85% were male; 13% of patients had a history of bypass surgery; and 6% were hemodialysis patients. Diabetes was found in 34%. Cilostazol was prescribed for 44% of patients while probucol was prescribed only for 5%. Serum concentrations of the proteinase inhibitors at 24 h after stent implantation were within the normal range. Of all the lesions, 46% were located in left anterior descending arteries; type B2 and C accounted for more than 60% of lesions. The lesion length was 11.7 ± 5.0 mm, but stent length was 19.5 ± 8.1 mm. Atherectomy was performed prior to stenting in 16 lesions, rotational atherectomy in 15 and directional atherectomy in one. Stents were selected at the discretion of individual operators. The minimum luminal diameter was 1.11 ± 0.49 mm before the procedure, 2.60 ± 0.52 mm after stent implantation, and 1.97 ± 0.60 mm at 6 months follow-up. The reference diameter was 2.70 ± 0.70 mm before the procedure, 3.00 ± 0.47 mm after stent implantation, and 2.80 ± 0.51 mm at 6 months follow-up. Average acute gain was 1.48 ± 0.57 mm, average late loss was 0.63 ± 0.44 mm, and average loss index was 0.44 ± 0.30. Binary restenosis determined to be more than 50% by QCA was observed in 14 lesions (13%). Target lesion revascularization (TLR) was performed in six lesions (6%) based on clinical recurrence of angina due to restenosis. The serum concentrations of α1PI, α1ACT and α2M were compared according to TLR. Serum concentrations of α1PI and α2M were significantly higher with TLR than that without TLR, but no difference was found in α1ACT [α1PI, 2.14 (2.03–2.51) vs. 1.94 (1.80–2.12) $\times 10^{-5}$ mol L^{-1}, $P = 0.0312$; α2M, 0.36 (0.23–0.44) vs. 0.18 (0.16–0.23) $\times 10^{-5}$ mol L^{-1}, $P = 0.0016$, respectively]. Scatter plots are shown in Fig. 1. Loss index showed linear correlation with α1PI ($R = 0.2501$, $P = 0.0094$) and with α2M ($R = 0.2154$, $P = 0.0259$), but no correlation was observed with α 1ACT. As shown in Table 1, we prepared a set of candidate variables from previous known factors that predict restenosis: diabetes, left anterior descending lesion, type C lesion, lesion length, reference diameter, prior bypass surgery, calcification and hemodialysis. Stepwise regression analysis selected reference diameter, diabetes and hemodialysis as significant predictors. Using these three variables as a basal model, α1PI, α1ACT, and α2M were additionally included in the model. This second model found α1PI and α2M as significant predictors of loss index, independent of the three variables in the basal model.

Clinical factors to predict restenosis have been reported, however, many serum factors failed to predict restenosis such as C-reactive protein, tumor necrosis factor-α, interleukin-6 or lipoprotein(a) [8]. Although plasma fibrinogen may predict restenosis [9], no association was found in another report [8]. The weak but significant correlation between plasma proteinase inhibitors and late loss may imply that α1PI and α2M are involved in the complex mechanism of restenosis. Proteinase inhibitors are the third most abundant proteins in plasma, next to albumin and globulin. Since α2M is a highly conserved protein found even in invertebrates, it may be essential to animal survival. An obvious question concerns the target enzymes of α1PI and α2M. α1PI specifically inhibits all serine proteinases, while α2M is a universal inhibitor of proteinases. Previous *in vitro* data suggest that the major target enzyme(s) is(are) one or more serine proteinases. Unfortunately, the key serine proteinases produced by vascular smooth muscle cells

Correspondence: Dr Y. Ikari, Division of Cardiology, Mitsui Memorial Hospital, 1, Kanda-Izumi-cho, Chiyoda-ku, Tokyo 101–8643, Japan.
Tel.: + 81 3 3862 9111, fax: + 81 3 5687 9765;
e-mail: ikari-tky@umin.ac.jp

Received 17 April 2002, accepted 11 June 2002

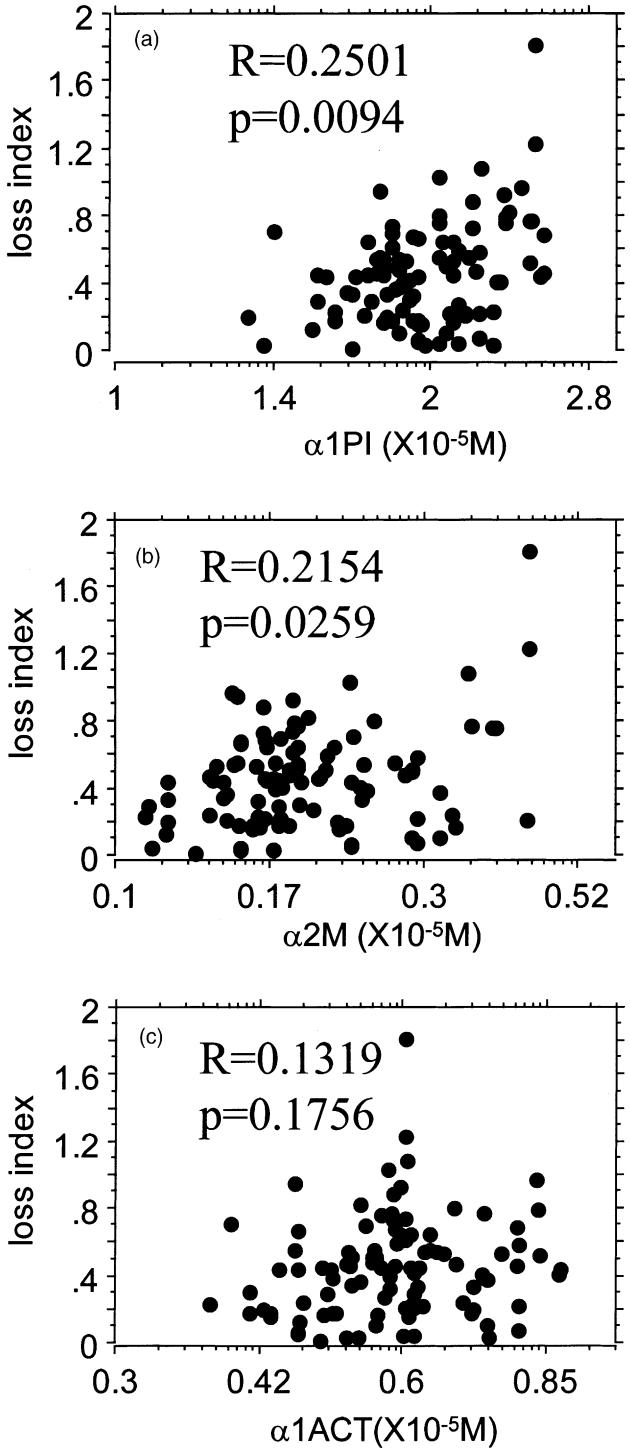

Fig. 1. Scatter plots of lumen loss index and concentrations of three proteinase inhibitors. (a) α1-proteinase inhibitor (α1PI); (b) α2-macroglobulin (α2M); and (c) α1-antichymotrypsin (α1ACT).

have not been identified. Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) have received much attention with respect to the pathophysiology of atherosclerosis. However, MMPs are not central to investigations because only α2M would correlate with the loss index if MMPs were the target.

In conclusion, α1PI and α2M have correlation with late loss following stent implantation. α1PI, α2M and their unidentified serine proteinase targets are likely to be involved in the pathogenesis of restenosis.

Table 1 Results of stepwise regression analysis

Parameter	Partial R [2]	Model R [2]	*P*-value
Model 1*			
Reference diameter	0.1041	0.1041	0.0007
Diabetes mellitus	0.0247	0.1288	0.0887
Hemodialysis	0.0181	0.1469	0.1421
Model 2†			
Basal model	0.1469	0.1469	0.0009
α1PI	0.0930	0.2399	0.0006
α2M	0.0413	0.2812	0.0178

*Model 1 includes the following variables as candidate predictors for loss index: diabetes, left anterior descending lesion, AHA/ACC type C lesion, lesion length, reference diameter, prior bypass surgery, calcification and hemodialysis. †Model 2 tests the additional explanatory power of α1PI, α2M and α1ACT over model 1.

Acknowledgements

We acknowledge the Ueda Memorial Trust Fund for Research of the Heart Disease.

References

1 Farb A, Sangiorgi G, Carter AJ, Walley VM, Edwards WD, Schwartz RS, Virmani R. Pathology of acute and chronic coronary stenting in humans. *Circulation* 1999; **99**: 44–52.
2 Yee KO, Schwartz SM. Why atherosclerotic vessels narrow: the fibrin hypothesis. *Thromb Haemost* 1999; **82**: 762–71.
3 Yee KO, Ikari Y, Schwartz SM. An update of the Grutzbalg hypothesis: the role of thrombosis and coagulation in atherosclerotic progression. *Thromb Haemost* 2001; **85**: 207–17.
4 Ikari Y, Yee KO, Schwartz SM. Role of alpha5beta1 and alphavbeta3 integrins on smooth muscle cell spreading and migration in fibrin gels. *Thromb Haemost* 2000; **84**: 701–5.
5 Ikari Y, Yee KO, Hatsukami TS, Schwartz SM. Human carotid artery smooth muscle cells rarely express alpha(v) beta3 integrin at sites of recent plaque rupture. *Thromb Haemost* 2000; **84**: 338–44.
6 Ikari Y, Fujikawa K, Yee KO, Schwartz SM. Alpha(1)-proteinase inhibitor, alpha(1)-antichymotrypsin, or alpha(2)-macroglobulin is required for vascular smooth muscle cell spreading in three-dimensional fibrin gel. *J Biol Chem* 2000; **275**: 12799–805.
7 Ikari Y, Mulvihill E, Schwartz SM. alpha 1-Proteinase inhibitor, alpha 1-antichymotrypsin, and alpha 2-macroglobulin are the antiapoptotic factors of vascular smooth muscle cells. *J Biol Chem* 2001; **276**: 11798–803.
8 Pietersma A, Kofflard M, de Wit LE, Stijnen T, Koster JF, Serruys PW, Sluiter W. Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment. *Circulation* 1995; **91**: 1320–5.
9 Montalescot G, Ankri A, Vicaut E, Drobinski G, Grosgogeat Y, Thomas D. Fibrinogen after coronary angioplasty as a risk factor for restenosis. *Circulation* 1995; **92**: 31–8.

Protein Z, a vitamin K-dependent protein in patients with renal failure

J. MALYSZKO,* E. SKRZYDLEWSKA,†J. S. MALYSZKO* and M. MYŚLIWIEC
**Department of Nephrology and Internal Medicine, and †Department of Analytical Chemistry, Medical School, Zurawia, Poland*

Dear Sir,

Disturbances in hemostasis are common complications of kidney diseases. Both bleeding diathesis and thromboembolism may complicate the course of chronic uremia [1]. The pathogenesis of uremic bleeding and prothrombotic state in dialyzed patients is multifactorial [1,2]. Results of both *in vitro* and *in vivo* studies suggest that protein Z, a vitamin K-dependent plasma glycoprotein, plays an important role in dampening coagulation [3]. In the presence of phospholipid vesicles and calcium ions, protein Z serves as a cofactor for the inhibition of activated factor X (FX) by protein Z-dependent protease inhibitor. Deficiency of protein Z might therefore result in a prothrombotic state [3,4]. As far as we know, there is limited data about protein Z in kidney diseases. Usalan *et al*. [5] have reported high plasma levels of protein Z in hemodialyzed patients. The aim of our work was to examine soluble plasma protein Z and vitamin K concentrations in patients with kidney diseases.

The study was performed on 22 patients with chronic renal insufficiency (10 patients with nephrotic syndrome, 12 patients with glomerulonephritis without nephrotic syndrome), 22 peritoneally dialyzed (continuous ambulatory peritoneal dialysis; CAPD), and 27 age–sex-matched healthy controls. Inclusion criteria were no diabetes mellitus, no inflammation (C-reactive protein within normal range) and no liver dysfunction (prothrombin time, alanine aminotransferase within normal range). We evaluated protein Z with commercially available kit (Protein Z, Diagnostica, Stago, France). Vitamin K concentration was analyzed by HPLC as described previously [6]. ANOVA and linear regression was used in statistical analysis employing computer software STATISTICA 5.0. Data are presented as means ± SD in Fig. 1.

Protein Z was highest in CAPD patients when compared with any other group. In nephrotic syndrome, protein Z was significantly lower when compared with the healthy volunteers, but it did not differ significantly between two groups of patients with chronic renal failure (both with and without nephrotic syndrome). Protein Z correlated negatively with fibrinogen in CAPD ($r = -0.68$, $P < 0.05$) and nephrotic patients ($r = -0.79$, $P < 0.05$). Vitamin K was significantly lower in nephrotic syndrome when compared with non-nephrotic patients

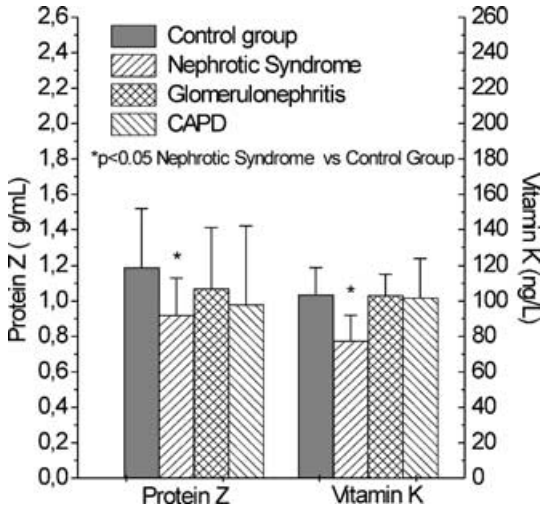

Fig. 1. Protein Z and vitamin K concentrations in patients with nephrotic syndrome (NS), glomerulonephritis (GN), on continuous ambulatory peritoneal dialyzes (CAPD) and in the healthy volunteers (CG).

($P = 0.03$). In nephrotic patients, vitamin K concentration was low and almost reached statistical significance when compared with the patients maintained on CAPD ($P = 0.05$). Concentrations of vitamin K were not significantly related to either age or gender in patients on renal replacement therapy (CAPD), but in CRF vitamin K correlated positively with age ($r = 0.73$, $P = 0.017$).

Protein Z is highly homologous to FVII, FIX, FX, proteins C and S, and prothrombin [3], which are lost in urine and their plasma concentrations are lowered in nephrotic syndrome. In our study, we found that in patients with nephrotic syndrome, protein Z concentration was significantly lower than in the healthy volunteers and in CAPD patients and it was significantly higher than in the control group. To our knowledge, this is the first report concerning protein Z levels and vitamin K status in patients with nephrotic syndrome. CAPD appears to mimic the metabolic abnormalities that account for the hypercoagulability in the nephrotic syndrome [7]. In our previous study, we observed a hypercoagulable state in CAPD patients when compared with either hemodialyzed patients or healthy volunteers [8]. It has been suggested that due to a peritoneal ultrafiltration, CAPD patients usually exhibit a reduced plasma volume and hemoconcentration. Significant protein losses through the peritoneum may be counterbalanced by the possible increase in protein synthesis in CAPD as a result of a non-

Correspondence: Dr J. Malyszko, Department of Analytical Chemistry, Medical School, Bialystok 15–540 Bialystok, Zurawia 14, Poland. Tel.:/fax: + 48 85 741 2391; e-mail: jolmal@poczta.onet.pl

Received 13 May 2002, revised 15 July 2002, accepted 24 July 2002

specific stimulation of the liver to protein production due to a lowered oncotic pressure. Thus, protein Z synthesis in CAPD patients may be increased via this mechanism, however, there was only negative correlation between protein Z and fibrinogen in nephrotic syndrome and CAPD. It is known that vitamin K is needed for the carboxylation of osteocalcin, matrix Gla protein in bone, prothrombin, and proteins S and Z [9]. However, the specific function of these proteins is still unknown. In our study, none of the patients investigated was on oral anticoagulants and all of them had international normalized ratio within normal ranges. As protein Z is one of the vitamin K-dependent proteins, we attempted to find any correlations between vitamin K status and protein Z.

In conclusion, the most interesting finding of our study is the observation that Z concentration is low in nephrotic syndrome protein. It may be due to the urinary losses and/or vitamin K deficiency. Protein Z deficiency might contribute to the enhanced risk of thromboembolic complications in nephrotic syndrome.

References

1 Sagripanti A, Barsotti G. Bleeding and thrombosis in chronic uremia. *Nephron* 1997; **75**: 125–39.
2 Malyszko J, Malyszko JS, Pawlak D, Pawlak K, Buczko W, Mysliwiec M. Platelet function, hemostasis and serotonin in acute and chronic renal failure. *Thromb Res* 1996; **83**: 351–61.
3 Broze GJ. Protein Z-dependent regulation of coagulation. *Thromb Haemost* 2001; **86**: 8–13.
4 Broze GJ. Protein-Z and thrombosis. *Lancet* 2001; **357**: 900–2.
5 Usalan C, Erdem Y, Altun B, Arici M, Haznedaroglu YC, Yasavul U, Turgan C, Caglar S, Protein Z levels in hemodialysis patients. *Int Urol Nephrol* 1999; **31**: 541–5.
6 Van-Haard PMM, Engel R, Postma T. Routine clinical determination of carotene, vitamin E, vitamin A, 25-hydroxy vitamin D3 and vitamin K1 (phylloquinone) in human serum by straight phase HPLC. *Biomed Chromatogr* 1987; **2**: 79–88.
7 Llach F. Hypercoagulability, renal vein thrombosis in the nephrotic syndrome. *Kidney Int* 1985; **28**: 429–39.
8 Malyszko J, Malyszko JS, Mysliwiec M. Comparison of hemostasis between CAPD and HD patients. *Perit Dial Int* 2001; **21**: 158–65.
9 Vermeer C. Gamma-carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. *Biochem J* 1990; **266**: 625–36.

Aspirin resistance is not a common biochemical phenotype explained by unblocked cyclooxygenase-1 activity

A. HILLARP,* S. LETHAGEN† and I. MATTIASSON‡

*Departments of *Clinical Chemistry, †Coagulation Disorders and ‡Vascular Diseases and Angiology, University Hospital, Malmö, Sweden*

Dear Sir,

The term aspirin resistance has been used to describe patients who, despite being on therapeutic doses of aspirin, experience thrombotic vascular events. Aspirin resistance can also be a biochemical phenotype based on an inadequate platelet inhibition response. Previous studies involving platelet reactivity or aggregation tests have estimated that between 8 and 45% of patients with ischemic stroke or cardiovascular disease are aspirin resistant [1–6]. Recently, it was shown that urinary concentrations of 11-dehydrothromboxane B_2, a stable marker of thromboxane A_2 (TXA_2) production, can be used to predict the risk of future cardiovascular events [7]. Although the causative factor of aspirin resistance has not been elucidated, the observed aspirin-resistant biosynthesis of thromboxane could imply that the platelets are incompletely blocked.

The aspirin-sensitive cyclooxygenase pathway in platelets is initiated when arachidonic acid is released from membrane phospholipids. TXA_2, an important activator of platelets, is produced from arachidonic acid by sequential action of the enzymes cyclooxygenase-1 (Cox-1) and thromboxane synthetase. Aspirin blocks the platelet production of TXA_2 by irreversible acetylation of serine 529 of the Cox-1 molecule. The serine residue is not directly involved in the cyclooxygenase activity but its acetylation leads to blocked access of arachidonic acid in the active site [8]. We have now investigated 122 patients and healthy individuals in order to see if aspirin resistance, defined as arachidonic-induced whole blood aggregation and/or ATP release, is a common finding after treatment with low-dose aspirin.

Blood was collected in tubes containing 3.8% citrate from 41 healthy individuals (21 female, 19 men; mean age 40 years; range 23–57 years), 34 patients with ischemic stroke (12 female, 22 male; mean age 72 years; range 50–89 years) and 47 patients with myocardial infarction (9 female, 38 male; mean age 70 years; range 40–86 years). The healthy individuals were analyzed before and after taking aspirin (one tablet of 75 mg aspirin 24 and 1 h before blood sampling). The two groups of patients were all taking 75–160 mg day^{-1} of aspirin for various lengths of time (range 1–310 months). Whole blood aggregation using impedance technique [9,10] was performed using a Chronolog 550 Lumi-Aggregometer (Chronolog Corp., Haverstown, USA). Citrated blood was analyzed after dilution 1 : 1 with 0.9% NaCl with 0.5 mmol L^{-1} arachidonic acid as agonist. Aggregation recorded as impedance response (in ohms) was measured 6 min after addition of agonist. Release of ATP (in nanomoles) from dense granules

Correspondence: Dr A. Hillarp, Department of Clinical Chemistry, Lund University, University Hospital, SE-205 02 Malmö, Sweden.
Tel.: + 46 40 333155; fax: + 46 40 337044;
e-mail: andreas.hillarp@klkemi.mas.lu.se

Received 13 May 2002, revised 4 June 2002, accepted 10 June 2002

was detected with luminescence from an added luciferin–luciferase reagent.

The group of healthy individuals showed a mean aggregation of $26.0 \pm 9.0\,\Omega$ and ATP release of 0.49 ± 0.33 nmol. The responses to arachidonic acid are similar to what have been reported by others [10]. The same group of healthy individuals was analyzed after intake of low-dose aspirin and none of the subjects showed any sign of platelet aggregation or ATP release with arachidonic acid as agonist. A completely inhibited response was also observed for the patients with myocardial infarction and, with the exception of a single patient, in the stroke group. The deviating patient in the stroke group showed a weak aggregation response together with an ATP release within the normal range for individuals not taking aspirin. This patient was tested at a later occasion and was then found to be completely blocked by aspirin. Thus, non-compliance cannot be ruled out in this case.

Taken together, aspirin resistance, due to an unblocked Cox-1, appears to be rare in this group of patients and in healthy control subjects. We found only a single patient out of 122 investigated (0.8%) with reactive platelets after intake of low-dose aspirin. This is by far less than the previous reports of 8–45% of aspirin resistance in patients with ischemic stroke or cardiovascular disease [1–6]. In these studies, the definition of aspirin resistance is based on various biochemical definitions based on the platelet reactivity test, optical aggregation with various agonists or using an *in vitro* bleeding time test. However, none of these studies have revealed the mechanism(s) behind aspirin resistance. With our approach, using arachidonic acid-induced whole blood platelet aggregation, we aimed to investigate if the phenomenon of aspirin resistance can be a biochemical phenotype derived from an aspirin insensitive Cox-1 molecule. It has been hypothesized that variants of the Cox-1 gene could result in an aspirin resistant Cox-1 molecule leading to thromboxane generation [7]. However, such a phenotype cannot be identified in this setting. With only a single exception, all samples showed a completely blocked Cox-1 activity indicating that low-dose aspirin is efficient of blocking the Cox-1 activity.

References

1 Grotemeyer K-H. Effects of acetylsalicylic acid in stroke patients. Evidence of nonresponders in a subpopulation of treated patients. *Thromb Res* 1991; **63**: 587–93.

2 Grotemeyer K-H, Scharafinski HW, Husstedt IW. Two-year follow-up of aspirin responder and aspirin non responder. A pilot study including 180 post-stroke patients. *Thromb Res* 1993; **71**: 397–403.

3 Helgason CM, Hoff JA, Kondos GT, Brace LD. Platelet aggregation in patients with atrial fibrillation taking aspirin or warfarin. *Stroke* 1993; **24**: 1458–61.

4 Helgason CM, Bolin KM, Hoff JA, Winkler SR, Mangat A, Tortorice KL, Brace LD. Development of aspirin resistance in persons with previous ischemic stroke. *Stroke* 1994; **25**: 2331–6.

5 Pappas JM, Westengard JC, Bull BS. Population variability in the effect of aspirin on platelet function. Implications for clinical trials and therapy. *Arch Pathol Laboratory Med* 1994; **118**: 801–4.

6 Gum PA, Kottke-Marchant K, Poggio ED, Gurm H, Welsh PA, Brooks L, Sapp SK, Topol EJ. Profile and prevalence of aspirin resistance in patients with cardiovascular disease. *Am J Cardiol* 2001; **88**: 230–5.

7 Eikelboom JW, Hirsch J, Weitz JI, Johnston M, Yi Q, Yusuf S. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation* 2002; **105**: 1650–5.

8 Shimokawa T, Smith WL. Prostaglandin endoperoxide synthase: the aspirin acetylation region. *J Biol Chem* 1992; **267**: 12387–92.

9 Feinman RD, Lubowsky J, Charo I, Zabinshy J. The lumi-aggregometer: a new instrument for simultaneous measurement of aggregation and secretion. *J Laboratory Clin Med* 1977; **90**: 125–9.

10 Podczasy JJ, Lee J, Vucenik I. Evaluation of whole-blood lumiaggregation. *Clin Appl Thromb-Hem* 1997; **3**: 190–5.

Only excessive III concentrations release prostacyclin in human dermal microvascular cells

M. MITTERMAYR,* A. LERCHER,† A. KOUNTCHEV,† W. SCHOBERSBERGER* and N. SEPP‡

**Department of Anesthesia and Intensive Care Medicine, and †Institute of Medical Chemistry and Biochemistry, The Leopold-Franzens University of Innsbruck; and ‡Department of Dermatology, The Leopold-Franzens University of Innsbruck, Innsbruck, Austria*

Dear Sir,

Antithrombin (AT) is an important inhibitor of serine proteases that are part of the coagulation cascade. Recent interest has focused on the anti-inflammatory properties of AT. One mechanism suggested for AT's anti-inflammatory effect is the release of prostacyclin (PGI_2). AT has been shown to induce the release of PGI_2 in several *in vitro* and *in vivo* studies. It stimulated PGI_2 production by cultured bovine aortic endothelial cells which was inhibited by anti-AT antiserum and heparin [1]. AT increased the release of PGI_2 in human umbilical vein endothelial cells. This effect was abolished by addition of heparin and heparinase [2]. The intravenous administration of AT in rats significantly increased the plasma levels of

Correspondence: Dr M. Mittermayr, Department of Anesthesia and Intensive Care Medicine, The Leopold-Franzens University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria.
Tel.: + 43 512 504 2400; fax: + 43 512 504 2450;
e-mail: markus.mittermayr@uibk.ac.at

Received 3 June 2002, accepted 10 June 2002

PGI_2. Treatment with heparin and AT had no effect on the release of PGI_2 [3]. These observations suggested that AT promotes the release of PGI_2 by interacting with endothelial heparin-like glycosaminoglycans. However, recent results from Uchiba and Okajima [4] did not support these previous findings; incubation of cultured human umbilical vein endothelial cells (HUVECs) and cultured bovine aortic endothelial cells (BAECs) did not induce PGI_2 release. Thus it seems likely that the release of PGI_2 by AT is not common to all endothelial cells and the exact mechanism, therefore, is still unclear. At present, AT-induced PGI_2 formation and release has not been studied in endothelial microvascular cells. Thus, we incubated primary cultures of human dermal microvascular cells (HDMEC), which are derived from human foreskins (for details see [5]) with AT (1, 5, 10 and 25 U mL^{-1} Kybernin P®) for 60, 120, 180, 240 and 480 min, both without as well as with indomethacin (10 µmol L^{-1}). PGI_2 release was estimated by measuring its stable analog 6-keto-prostaglandin-$F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) with an enzyme-immunoassay (EIA) system (Biotrack, Amersham, Bucks, UK)

Incubations of HDMEC with AT at 1, 5 and 10 U mL^{-1} had no effect on the release of 6-keto-$PGF_{1\alpha}$ in the culture supernatant. Compared with untreated controls, incubation of HDMEC with 25 U mL^{-1} AT resulted in an increase of 6-keto $PGF_{1\alpha}$ after 180 min (25.5 ± 8.3 pg mL^{-1} vs. 17.1 ± 6.0 pg mL^{-1}; $P = 0.006$; $n = 6$), after 240 min (35.6 ± 12.6 pg mL^{-1} vs. 23.5 ± 10.1 pg mL^{-1}; $P = 0.003$; $n = 6$) and after 480 min (104.8 ± 68.7 pg mL^{-1} vs. 37.7 ± 11.4 pg mL^{-1}; $P = 0.008$; $n = 6$) (Fig. 1). Coincubation of 25 U mL^{-1} AT with indomethacin completely abolished the release of 6-keto-$PGF_{1\alpha}$.

AT has been suggested to act as an anti-inflammatory agent. In several studies, administration of high concentration of AT improved organ failure and mortality in animals exposed to lipopolysaccharides [6,7]. Clinical studies showed a beneficial effect of high concentrations of AT on the incidence of multiple organ failure in patients with severe sepsis [8,9]. However, a recent study with septic patients maintaining high plasma concentration of AT (150%) by substitution of AT did not result in a reduction of 28-day mortality [10]. One mechanism suggested for the anti-inflammatory effect of AT is the release of prostacyclin (PGI_2). AT (250 U kg^{-1}) reduced coagulation abnormalities and pulmonary vascular injury in endotoxin-exposed rats, and this was accompanied by elevated plasma levels of PGI_2. These effects were completely inhibited by indomethacin [11]. In a recent study, AT had beneficial effects on ischemia/reperfusion injury of the liver by releasing PGI_2 [12]. In contrast with others [1,2], we cannot demonstrate an increase in PGI_2 by AT treatment of cultured cells in concentrations which are of clinical relevance. Only by applying excessive doses of AT (25 U mL^{-1}) could a significant increase of PGI_2 in the culture supernatant be measured. The reasons for these differences are not clear at present. It seems that there are cell-type specific mechanisms involved in AT-induced PGI_2 formation. HDMEC are microvascular endothelial cells derived from human foreskins and are different with regard to cell morphology, proliferation and stimulation by proinflammatory agents as compared with other endothelial cells, e.g. HUVEC, BAEC [13]. We used early passages (3–6) in order to avoid later dedifferentiation. Whether the release of PGI_2 is in fact the crucial mechanism for some of the anti-inflammatory properties of AT seems to be questionable. Dschietzig *et al*. [14] demonstrated that AT treatment failed to increase PGI_2 levels in isolated endotoxin-exposed rat lungs, but augmented release of endothelin 1. Thus, it is possible that mechanisms other than the release of PGI_2 are responsible for the anti-inflammatory effects of AT.

In conclusion, this study demonstrates that only excessive concentrations of AT release PGI_2 in human dermal microvascular cells. Clinically relevant concentrations had no impact. In synopsis with recent data [4], it seems questionable whether the release of PGI_2 by AT is a uniform mechanism in the vascular system.

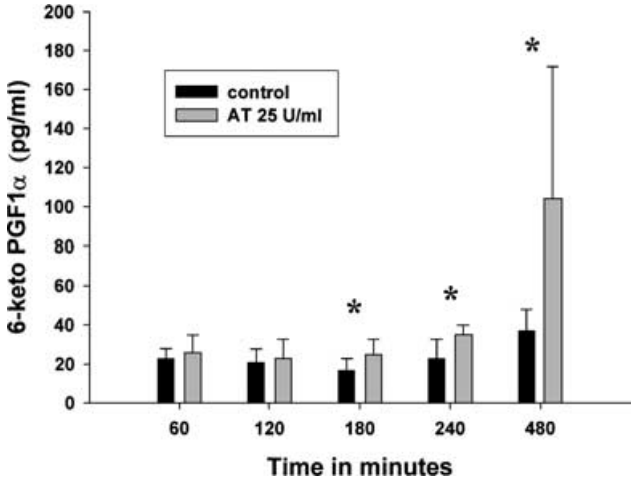

Fig. 1. Time-dependent release of PGI_2 in controls and in AT-treated group (25 U mL^{-1}). Data are expressed as mean ± SD. $^*P < 0.05$ vs. control as compared with corresponding times (Student's *t*-test).

References

1 Yamauchi T, Umeda F, Inoguchi T, Nawata H. Antithrombin III stimulates prostacyclin production by cultured aortic endothelial cells. *Biochem Biophys Res Commun* 1989; **163**: 1404–11.

2 Horie S, Ishii H, Kazama M. Heparin-like glycosaminoglycan is a receptor for antithrombin III-dependent but not for thrombin-dependent prostacyclin production in human endothelial cells. *Thromb Res* 1990; **59**: 895–904.

3 Uchiba M, Okajima K, Murakami K, Okabe H, Takatsuki K. Effects of antithrombin III (AT III) and Trp49-modified AT III on plasma level of 6-keto-PGF1 alpha in rats. *Thromb Res* 1995; **80**: 201–8.

4 Uchiba M, Okajima K. Antithrombin does not directly promote the endothelial production of prostacyclin in cultured endothelial cells. *Thromb Haemost* 2001; **86**: 722–3.

5 Hoffmann G, Schobersberger W, Rieder J, *et al.* Human dermal microvascular endothelial cells express inducible nitric oxide synthase in vitro. *J Invest Dermatol* 1999; **112**: 387–90.

6 Emerson Te Jr, Fournel MA, Redens TB, Taylor Fb Jr. Efficacy of antithrombin III supplementation in animal models of fulminant Escherichia coli endotoxemia or bacteremia. *Am J Med* 1989; **87**: 27S–33S.

7 Taylor Fb Jr, Emerson Te Jr, Jordan R, Chang AK, Blick KE. Antithrombin-III prevents the lethal effects of *Escherichia coli* infusion in baboons. *Circ Shock* 1988; **26**: 227–35.
8 Eisele B, Lamy M, Thijs LG, *et al.* Antithrombin III in patients with severe sepsis. A randomized, placebo- controlled, double-blind multicenter trial plus a meta-analysis on all randomized, placebo-controlled, double-blind trials with antithrombin III in severe sepsis [see comments]. *Intensive Care Med* 1998; **24**: 663–72.
9 Inthorn D, Hoffmann JN, Hartl WH, Muhlbayer D, Jochum M. Antithrombin III supplementation in severe sepsis: beneficial effects on organ dysfunction. *Shock* 1997; **8**: 328–34.
10 Warren BL, Eid A, Singer P, *et al.* Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial. *JAMA* 2001; **286**: 1869–78.
11 Uchiba M, Okajima K, Murakami K, Okabe H, Takatsuki K. Attenuation of endotoxin-induced pulmonary vascular injury by antithrombin III. *Am J Physiol* 1996; **270**: L921–30.
12 Harada N, Okajima K, Kushimoto S, Isobe H, Tanaka K. Antithrombin reduces ischemia/reperfusion injury of rat liver by increasing the hepatic level of prostacyclin. *Blood* 1999; **93**: 157–64.
13 Sepp N, Li LJ, Lee KH, *et al.* Basic fibroblast growth factor increases expression of alpha V and beta 3 integrin complex on human microvascular endothelial cells. *J Invest Dermatol* 1994; **103**: 295–9.
14 Dschietzig T, Alexiou K, Laule M, *et al.* Stimulation of pulmonary big endothelin-1 and endothelin-1 by antithrombin III. a rationale for combined application of antithrombin III and endothelin antagonists in sepsis-related acute respiratory distress syndrome? *Crit Care Med* 2000; **28**: 2445–9.

A procyanidin extract prolongs bleeding time but does not prevent thrombosis in rats

A. DE CURTIS,* C. AMORE,* M. B. DONATI,† G. De GAETANO† and L. IACOVIELLO*

**'Angela Valenti' Laboratory of Genetic and Environmental Risk Factors for Thrombotic Disease, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy; and †Center for High Technology Research and Education in Biomedical Sciences, Catholic University, Campobasso, Italy*

Red wine and derived products have been tested for their capacity to interfere with molecular and cellular mechanisms relevant to the pathogenesis of thrombosis and vascular occlusive disease [1]. Polyphenolic flavonoids present in wine, vegetables, fruits and tea are known for their ability to inhibit platelet aggregation and polymorphonuclear leukocyte function *in vivo*, both in humans and in animal models [2].

In this study the effects of a procyanidin extract from seed of *Vitis vinifera* (Leucoselect™, Indena, Milan, Italy) [3,4] were studied on template bleeding time (BT), platelet adhesion to fibrillar collagen, and venous thrombosis in rats [5] after both intravenous (i.v.) injection and long-term oral administration with the diet.

The compound, dissolved in isotonic saline at pH 7.3, and administered i.v. at a dose range between 5 and 15 mg kg^{-1}, induced a significant prolongation of BT in a dose and time-dependent manner. A statistically significant difference was observed after 5 mg kg^{-1} injection of the procyanidin extract and reached a plateau at 7.5 mg kg^{-1} (Fig. 1a) and 15 min after i.v. injection (Fig. 1b). An increase in BT was still measurable after 60 min but disappeared after 90 min.

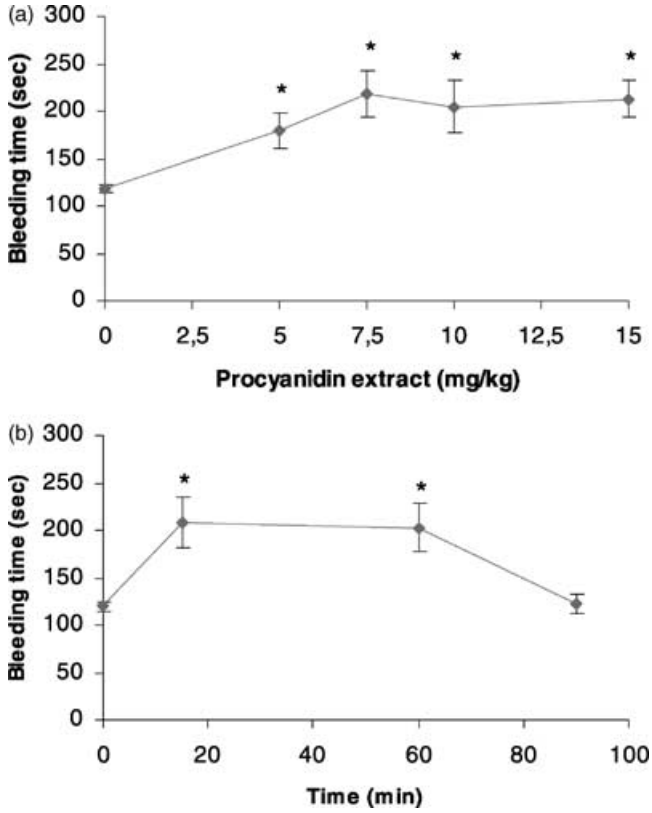

Fig. 1. Effects of a procyanidin extract on bleeding time in rats: (a) dose–response; (b) time–course at 75 mg kg^{-1} (means ± SE, $n = 15$; $^{*}P = 0.05$ vs. controls).

Correspondence: Dr Licia Iacoviello, 'Angela Valenti' Laboratory of Genetic and Environmental Risk Factors for Thrombotic Disease. Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy.
Tel.: + 39 0872 570298; fax: + 39 0872 570299; e-mail: iaco@negrisud.it

Received 29 July 2002, revised 31 July 2002, accepted 31 July 2002

No modification of prothrombin time (PT) and activated partial thromboplastin time (APTT) or platelet adhesion to fibrillar collagen was observed after injection (not shown).

Thirty days of a diet ($3.4\,g\,day^{-1}$ per rat) enriched in procyanidin extract (Leucoselect™ Phytosome, Indena, Milan, Italy; 34% mixed with 66% lipoid S 30 carrier) also determined a significant prolongation of BT (205 ± 25 s) compared with control animals (only lipoid S 30 carrier) (123 ± 11 s, $P < 0.05$). Procyanidin-enriched diet did not affect either thrombus formation (73% vs. 70% in control animals) or thrombus weight (4.5 ± 1.4 mg vs. 4.2 ± 1.8 mg in control animals), in a model of venous thrombosis, induced by inferior vena cava ligature [5]. Procyanidin-enriched diet did not change PT, APTT or platelet adhesion to fibrillar collagen (not shown).

Our results show that procyanidins, similar to de-alcholated red wine [5], prolong BT but, at variance with wine, they do not inhibit platelet adhesion to collagen or thrombosis. It is possible that the prolongation of BT in our test model might be due to a vasodilatory effect of the extract [6] rather than to reduced platelet–vessel wall interactions. We speculate that procyanidins contribute to the protective effect of red wine or its derived total polyphenols [5,6] by a vasomodulatory role [6] without directly affecting factors of the hemostatic system.

Acknowledgements

The authors wish to thank Indena (Milan, Italy) and Ministero Sanità (ICS 030.6/RF00-49) for their support.

References

1 Di Castelnuovo A, Rotondo S, Iacoviello L, Donati MB, De Gaetano G. Meta-analysis of wine and beer consumption in relation to vascular risk. *Circulation* 2002; **105**: 2836–44.
2 Rotondo S, Di Castelnuovo A, de Gaetano G. The relationship between wine consumption and cardiovascular risk: from epidemiological evidence to biological plausibility. *Ital Heart J* 2001; **2**: 1–8.
3 Gabetta B, Fuzzati N, Griffini A, Lolla E, Pace R, Ruffilli T, Peterlongo F. Characterization of proanthocyanidins from grape seeds. *Fitoterapia* 2000; **71**: 162–75.
4 Maffei Facino R, Carini M, Aldini G, Berti F, Rossoni G, Bombardelli E, Morazzoni P. Diet enriched with procyanidins enhances antioxidant activity and reduces myocardial postischaemic damage in rats. *Life Sci* 1999; **64**: 627–42.
5 Wollny T, Aiello L, Di Tommaso D, Bellavia V, Rotilio D, Donati MB, de Gaetano G, Iacoviello L. Modulation of haemostatic function and prevention of experimental thrombosis by red wine in rats: a role for increased nitric oxide production. *Br J Pharmacol* 1999; **127**: 747–55.
6 de Gaetano G, De Curtis A, Di Castelnuovo A, Donati MB, Iacoviello L, Rotondo S. Antithrombotic effect of polyphenols in experimental models: a mechanism of reduced vascular risk by moderate wine consumption. *Ann NY Acad Sci* 2002; **957**: 174–88.

Rebuttal to: Effect of heparin on TAFI-dependent inhibition of fibrinolysis

T. LISMAN and P. G. DE GROOT

Thrombosis and Haemostasis Laboratory, Department of Haematology, University Medical Center, Utrecht, and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands

Dear Sir,

We read with interest the recent paper by Colucci *et al*. [1], who studied the effect of unfractionated heparin on TAFI-dependent inhibition of fibrinolysis. The conclusion of their study was that heparin has no effect on TAFI-dependent clot lysis, as heparin only inhibits thrombin in solution and not clot-bound thrombin. The authors postulate that clot-bound thrombin plays a pivotal role in TAFI-dependent down-regulation of fibrinolysis, and that localized generation of activated TAFI by clot-bound thrombin is sufficient for inhibition of clot lysis.

The conclusion by Colucci *et al*. that heparin, at concentrations which completely inhibit thrombin generation in solution, has no effect on clot lysis, is in sharp contrast with results obtained in our laboratory. Using a plasma-based clot lysis assay, which has been extensively used to study TAFI-dependent down-regulation of fibrinolysis in a variety of pathologies including factor XI deficiency [2], hemophilia [3,4], liver cirrhosis [5], and hemostatic changes associated with the use of oral contraceptives [6], we also studied the effect of heparin on TAFI-mediated down-regulation of fibrinolysis.

In short, in a 96-wells microtitre plate, pooled normal plasma to which different concentrations of unfractionated heparin (Leo pharmaceutical products, Weesp, The Netherlands) was added, was allowed to clot by the addition of tissue factor (Innovin, Dade Behring, 1 : 1000 times diluted), calcium chloride ($17\,mmol\,L^{-1}$), and phospholipid vesicles ($10\,\mu mol\,L^{-1}$), fibrinolysis was induced by addition of tPA ($30\,U\,mL^{-1}$; Chromogenix, Mölndal, Sweden). Coagulation and clot lysis were monitored by continuous turbidity measurements at at a wave-

Correspondence: Ton Lisman, Thrombosis and Haemostasis Laboratory, Department of Haematology, G03. 647, University Medical Center Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.
Tel.: + 31 30 250 6510; fax: + 31 30 251 1893; e-mail: j.a.lisman@azu.nl

Received 26 August 2002, accepted 5 September 2002

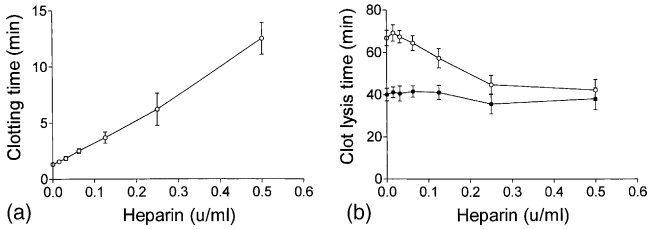

Fig. 1. The effect of heparin on coagulation (panel A) and clot lysis (panel B). Pooled normal plasma containing different concentrations of heparin was allowed to clot by addition of tissue factor, calcium chloride, and phospholipid vesicles. Fibrinolysis was initiated by addition of tPA. Clot formation and subsequent lysis was monitored by tubidity measurements at 405 nm in time. Experiments were performed in absence (○) and presence (●) of CPI. Shown are mean of three independent experiments performed in duplicate. Error bars indicate standard deviation.

length 405 nm, and clotting times and clot lysis times were determined as described [4].

As shown in Fig. 1(A), clotting times dose-dependently increased on addition of heparin. No clot formation was observed at concentrations above 0.5 U mL^{-1}. Clot lysis times dose-dependently decreased on addition of heparin (Fig. 1B, open circles). When carboxypeptidase inhibitor from potato (CPI, Calbiochem, La Jolla, CA), a specific inhibitor of activated TAFI, was added to the plasma, clot lysis time was significantly decreased compared to control values, indicating that TAFI activation contributes to inhibition of fibrinolysis in this assay. Addition of heparin did not affect clot lysis time in the presence of CPI (Fig. 1B, closed circles), indicating that the decrease in clot lysis time was attributable to decreased TAFI activation.

The discrepancy between the experiments of Colucci *et al*. and our own, can probably be explained by substantial differences in experimental set-up. In the experiments performed by Colucci *et al*., first, a fibrin clot is made by adding thrombin and calcium to citrated plasma, which is subsequently incubated for 1 h at 37 °C. Next, the clot is washed with saline, and transferred to defibrinated plasma containing vehicle or heparin. Clot lysis is induced by adding tPA and calcium chloride. In contrast, in our assay, heparin is already present before clot formation.

Thus, in the Colucci assay, the effect of heparin on lysis of a fully matured clot is investigated, whereas in our experiments, the effect of heparin on resistance against fibrinolysis on a developing clot is examined. In the assay performed by Colucci, the TAFI activation in the first part of the assay will already contribute in part to down-regulation of fibrinolysis in the second part of the assay, as some C-terminal lysine and arginine residues will become available during fibrinolysis induced by endogenous tPA. Moreover, and probably of more importance, the thrombin generated in the first part of the assay will bind to the clot, and will contribute to TAFI activation in the second part of the assay. TAFI activation via clot-bound thrombin formed in the first part of the assay, presumably will occur by factor XI-dependent enhancement of thrombin generation [7]. In fact, heparin has been shown to enhance factor XI activation by fibrin-bound thrombin [8]. If this process remains associated to the clot, the generated thrombin will remain insusceptible to inhibition by heparin. Enhancement of thrombin generation via clot-bound thrombin is required for TAFI activation, as absence of calcium- or vitamin K-dependent factors enhances clot lysis (Fig. 3 in the paper by Colucci *et al*.) [1].

In contrast, in our assay, the presence of heparin in plasma before clot formation suppresses thrombin generation throughout the process of clot formation and subsequent lysis, and consequently diminishes the amount of clot-bound thrombin.

In conclusion, we want to modify the conclusion drawn by Colucci *et al*. that heparin does not affect TAFI-mediated down-regulation of fibrinolysis *in vitro*. In our opinion, heparin does not affect resistance against fibrinolysis in a mature clot, but significantly affects clot stability of a developing clot by means of diminishing TAFI activation. If these data can be translated into the *in vivo* situation, this would mean that heparin administered after venous or arterial thromboembolism does not facilitate thrombolysis, as TAFI activation by clot-bound thrombin provides optimal lysis resistance. However, heparin would contribute to the prevention of reocclusion, as TAFI activation in a newly developing clot is efficiently inhibited, making the new thrombus more susceptable to breakdown by endogenous fibrinolysis.

References

1 Colucci M, Pentimone A, Binetti BM, Cramarossa M, Piro D, Semeraro N. Effect of heparin on TAFI-dependent inhibition of fibrinolysis: relative importance of TAFIa generated by clot-bound and fluid phase thrombin. *Thromb Haemost* 2002; **88**: 282–7.

2 von dem Borne PA, Bajzar L, Meijers JCM, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. *J Clin Invest* 1997; **99**: 2323–7.

3 Mosnier LO, Lisman T, van den Berg HM, Nieuwenhuis HK, Meijers JCM, Bouma BN. The defective downregulation of fibrinolysis in haemophilia A can be restored by increasing the TAFI plasma concentration. *Thromb Haemost* 2001; **86**: 1035–9.

4 Lisman T, Mosnier LO, Lambert T, Mauser-Bunschoten EP, Meijers JCM, Nieuwenhuis HK, de Groot PG. Inhibition of fibrinolysis by recombinant factor VIIa in plasma from patients with severe haemophilia A. *Blood* 2002; **99**: 175–9.

5 Lisman T, Leebeek FW, Mosnier LO, Bouma BN, Meijers JC, Janssen HL, Nieuwenhuis HK, de Groot PG. Thrombin-activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis. *Gastroenterology* 2001; **121**: 131–9.

6 Meijers JC, Middeldorp S, Tekelenburg W, van den Ende AE, Tans G, Prins MH, Rosing J, Buller HR, Bouma BN. Increased fibrinolytic activity during use of oral contraceptives is counteracted by an enhanced factor XI-independent down regulation of fibrinolysis: a randomized cross-over study of two low-dose oral contraceptives. *Thromb Haemost* 2000; **84**: 9–14.

7 von dem Borne PA, Meijers JCM, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. *Blood* 1995; **86**: 3035–42.

8 von dem Borne PA, Meijers JC, Bouma BN. Effect of heparin on the activation of factor XI by fibrin-bound thrombin. *Thromb Haemost* 1996; **76**: 347–53.

Rebuttal to: Effect of heparin on TAFI-dependent inhibition of fibrinolysis

M. COLUCCI and N. SEMERARO
Department of Biomedical Sciences, Section of General Pathology, University of Bari, Bari, Italy

Dear Sir,

We agree with Lisman and de Groot [1] that heparin, under certain conditions, may promote fibrinolysis by inhibiting TAFI activation. In fact, it is also our experience that heparin, when added prior to clot formation (in a microplate assay similar to that reported by Lisman and de Groot), shortens the lysis time through a TAFI-dependent mechanism. This finding, however, is quite predictable, considering that in this model heparin exerts a strong thrombin inhibitory effect before fibrin formation, and is not totally new; de Fouw *et al*. [2] reported similar results several years ago, although at that time the thrombin-dependent antifibrinolytic factor was still unknown. As pointed out in our paper [3], the goal of our study was to see whether heparin had a profibrinolytic effect under conditions in which thrombin can be generated on the clot surface. In particular we wished to assess to which extent fibrin-associated thrombin contributed to TAFI-mediated inhibition of fibrinolysis in order to gain new insights on the mechanism of action of heparin in thrombolytic regimens. For this reason the experimental model was set up to mimic the *in vivo* condition during thrombolytic therapy, in which heparin and t-PA are administered after a thrombus has already formed. Quite surprisingly, we observed that, under our experimental conditions, heparin failed to stimulate clot lysis despite complete inhibition of thrombin generation and TAFI activation in the plasma bath in which the blood clot was immersed. We provide evidence suggesting that activation of small amounts of TAFI by the sole fibrin-bound thrombin (formed on the clot surface after recalcification of defibrinated plasma, i.e. after addition of heparin) was sufficient to make the clot resistant to a pharmacologic concentration of t-PA and hypothesize the existence of a localized phenomenon probably involving TAFI cross-linked to fibrin. Obviously, the conclusion that in our model heparin does not stimulate fibrinolysis through a TAFI-dependent mechanism does not imply that heparin is unable to stimulate fibrinolysis under different experimental conditions. Accordingly, any speculation as to the possible clinical implication must be strictly limited to the *in vivo* condition that the *in vitro* model attempts to simulate (in our case, thrombolytic therapy). If TAFI proves to play a role in the *in vivo* regulation of fibrinolysis in humans [4], we, too, believe that heparin might function as a profibrinolytic through the inhibition of TAFI activation under certain conditions such as reocclusion by a newly formed clot or even accretion of an existing thrombus (in our model thrombus accretion did not occur because we used defibrinated plasma). However, if our data are confirmed in more physiological conditions, the suggested use of anticoagulants capable of inhibiting clot-bound thrombin will probably grant a wider profibrinolytic effect that might also influence the resistance of a mature clot.

References

1 Lisman T, de Groot PG. Rebuttal to: effect of heparin on TAFI-dependent inhibition of fibrinolysis. *J Thromb Haemost* 2003; **1**: 201–2.
2 de Fouw NJ, van Tilburg NH, Haverkate F, Bertina RM. Activated protein C accelerates clot lysis by virtue of its anticoagulant activity. *Blood Coagul Fibrinolysis* 1993; **4**: 201–10.
3 Colucci M, Pentimone A, Binetti BM, Cramarossa M, Piro D, Semeraro N. Effect of heparin on TAFI-dependent inhibition of fibrinolysis: relative importance of TAFIa generated by clot-bound and fluid phase thrombin. *Thromb Haemost* 2002; **88**: 282–7.
4 Nagashima M, Yin ZF, Zhao L, White K, Zhu Y, Lasky N, Halks-Miller M, Broze GJ Jr, Fay WP, Morser J. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. *J Clin Invest* 2002; **109**: 101–10.

Correspondence: Prof. Mario Colucci, Dipartimento di Scienze Biomediche, Sezione di Patologia Generale, Policlinico–Piazza G. Cesare, 11 1-70124 Bari, Italy.
Tel./fax: + 39 080 547 84 71; e-mail: mario.colucci@dimo.uniba.it

Received 18 September 2002, accepted 20 September 2002