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European Journal of Pharmacology 391 (2000) 1–9

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Inhibition of antithrombin by protein SV-IV normalizes the coagulation of hemophilic blood

Biagio Di Micco [a], Jacques Caen [b], Giovanni Colonna [a,c], Maria A. Macalello [a], Magda Marchese [d], Paola Stiuso [a], Pierpaolo Di Micco [a], Francesco Morelli [d,e], Salvatore Metafora [d,e,*]

[a] *Department of Biochemistry and Biophysics, II University of Naples, 80138 Naples, Italy*
[b] *I.V.S. Hopital Lariboisiere, Paris, France*
[c] *Research Center of Computational and Biotechnological Sciences, II University of Naples, 80138 Naples, Italy*
[d] *CNR International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy*
[e] *Servizio Tecnologie Biomolecolari, Area di Ricerca del CNR, 80128 Naples, Italy*

Received 6 December 1999; accepted 4 January 2000

Abstract

The aim of the study was to evaluate the effect of the protein Seminal Vesicle Protein No. 4 (SV-IV), a potent inhibitor of antithrombin III (antithrombin), on the coagulation of blood obtained from patients affected by hemophilia A. In the coagulating blood of these patients, the antithrombin/thrombin ratio was found to be markedly higher (about 44) than in normal individuals (about 4.4). This high ratio was related to the low efficiency of thrombin-generating reactions induced by the factor VIII deficiency and to the high levels of free (not bound to serine proteases) antithrombin present in the hemophilic serum (antithrombin concentration was the same in normal and hemophilic plasma). The elevated concentration of free antithrombin in hemophiliacs was primarily a consequence of a reduced consumption caused by the scarce availability in the hemophilic serum of factors Xa and IIa, which are serine proteases possessing strong binding affinity for antithrombin. Addition of SV-IV to coagulating hemophilic blood reduced markedly the serum antithrombin and thrombin–antithrombin complexes, normalizing, as a consequence, the clotting time and other coagulation parameters. Similar results were obtained by using appropriate concentration of factor VIII.

Keywords: Hemophilia A; Blood coagulation control; Antithrombin consumption; Thrombin–antithrombin complex; Factor Xa/antithrombin complex; Protein SV-IV

1. Introduction

Hemophilia A (factor VIII deficiency) is a hereditary, sex-linked hemorrhagic disorder that may result from many different mutations of the gene coding for factor VIII (Tuddenham et al., 1994; Antonarakis et al., 1995; Giannelli and Green, 1996). The blood coagulation cascade has a multitude of protease zymogens that require proteolytic activation and two non-enzymatic protein cofactors: factors V and VIII (Furie and Furie, 1988; Kalafatis et al., 1997). Factor VIII is activated by thrombin to yield activated factor VIIIa (Butenas et al., 1997). The latter serves as a cofactor in the factor IXa/VIIIa/phospholipid enzyme complex (intrinsic tenase) that activates factor X to factor Xa (Kalafatis et al., 1997). The mechanism, by which factor VIII deficiency produces hemorrhages in hemophilia A, is based on the role played by this factor in the formation and activity of prothrombinase, an enzyme complex on the surface of activated platelets or tissue cells, which contains factor Xa associated with factor Va and a negatively charged phospholipid (Furie and Furie, 1988; Walker and Krishnaswamy, 1994; Kalafatis et al., 1997; Krishnaswamy and Walker, 1997). The prothrombinase in the presence of Ca^{2+} cleaves the prothrombin in two fragments: thrombin and fragment F_{1+2}, whose function has not yet been completely elucidated (Owen et al., 1974; Lau et al., 1979; Teitel et al., 1982; Furie and Furie, 1988). The scarcity of factor Xa induced by the deficiency of factor VIII in hemophilia A renders a prothrombinase

* Corresponding author. CNR International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy. Tel.: +0039-81-5607312; fax: +0039-81-5607593.
E-mail address: metafora@iigbna.iigb.na.cnr.it (S. Metafora).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00020-0

that is inefficient and hence brings about a very low thrombin production. As a result, the small amount of thrombin produced, being unable to generate a sufficiently extended and stable fibrin plug, leads to a spontaneous or traumatic hemorrhage (Kane and Davie, 1988; Samama et al., 1990; Hathaway and Goodnight, 1993; Kalafatis et al., 1997).

While the generation of a normal fibrin plug is required to stop excessive bleeding, unregulated clotting results in occlusion of blood vessels and thrombosis. In addition, to the fine regulation of the plasmin-based fibrinolytic system (Samama et al., 1990; Lijnen and Collen, 1995; Neshheim et al., 1997), specific inhibitors of clotting factors are also required for a precise control of the coagulation process (Lammle and Griffin, 1985; Furie and Furie, 1988; Samama et al., 1990; Hathaway and Goodnight, 1993; Kalafatis et al., 1997). The most important of these is the antithrombin III (antithrombin), a member of the serine proteinase inhibitor (serpin) family that has the ability to primarily inactivate factors Xa and IIa (thrombin) by forming irreversible complexes with each (Lammle and Griffin, 1985; Samama et al., 1990; Hathaway and Goodnight, 1993; Ling et al., 1995). Under physiological conditions, antithrombin circulates in the blood in a conformationally inactive form. When this serpin binds to the heparan sulfate side chains expressed on normal endothelial cell surfaces, or to the therapeutically administered heparin, it undergoes a conformational change that results in a dramatic acceleration of its inhibitory activity (Nawroth et al., 1985; Fan et al., 1994; Huntington et al., 1996; Jurd et al., 1996; Meagher et al., 1996; Wu and Thiagarajan, 1996; Ersdal-Badju et al., 1997; Jin et al., 1997; Desai et al., 1998).

The amount of thrombin and antithrombin in normal coagulating blood are balanced to achieve rapid clotting at the site of injury (Furie and Furie, 1988; Samama et al., 1990; Hathaway and Goodnight, 1993; Kalafatis et al., 1997). It is possible that this fine balance is disturbed in the blood clotting process of the subjects affected by hemophilia A. We hypothesize that the antithrombin concentration in the serum of these patients is higher than in normal subjects as a result of its reduced consumption caused by the low availability of factor Xa and thrombin induced by the cofactor VIIIa deficiency (see above). Should this be the case, then adding appropriate concentrations of specific antithrombin inhibitors to hemophilic blood is expected to markedly increase the activity of the small amount of thrombin produced in these patients with a consequent significant decrease of the coagulation time.

On the basis of these considerations, we first investigated, by in vitro studies, the involvement of antithrombin in the blood clotting of patients with hemophilia A. Our findings in this paper show that, as expected, the amount of thrombin produced in vitro during hemophilic blood coagulation was markedly reduced compared to normal controls, and was strongly inhibited by high concentrations of free antithrombin that is conformationally activated by the binding of heparin-like molecules during the process of blood coagulation (Lammle and Griffin, 1985; Samama et al., 1990; Hathaway and Goodnight, 1993).

Next, we studied the effect of the protein SV-IV, a potent in vitro inhibitor of AT (Di Micco et al., 1994; Di Micco et al., 1997), on the coagulation process of hemophilic blood. It is a long time that we have been studying the biological activities and the relevant molecular mechanism(s) of action of the protein SV-IV [seminal vesicle protein no. 4, according to its electrophoretic mobility in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE); SwissProt ID, SVS4-RAT]. This protein is a major, small ($M_r = 9758$), basic ($pI = 8.9$), secretory protein synthesized by the rat seminal vesicle epithelium under strict androgen transcriptional control (Mansson et al., 1979; Ostrowski et al., 1979; Abrescia et al., 1986). Its amino acid sequence is known (Pan and Li, 1982), and the structure and expression of the SV-IV gene have been extensively studied (Mansson et al., 1979; Ostrowski et al., 1979; Harris et al., 1983; Abrescia et al., 1986; D'Ambrosio et al., 1993). The biological function of SV-IV is multifaceted. The binding of the protein after ejaculation to the surface of epididymal spermatozoa markedly decreases their strong immunogenicity, thus facilitating their perilous journey to the place where they encounter the egg (Paonessa et al., 1984; Peluso et al., 1994). In addition to a potent procoagulant activity (Di Micco et al., 1994, 1997), SV-IV has also an immunomodulatory (Metafora et al., 1989; Vuotto et al., 1993; Peluso et al., 1994; Romano-Carratelli et al., 1995; Tufano et al., 1996) and an anti-inflammatory (Metafora et al., 1989; Camussi et al., 1990) nonspecies specific properties. The modulatory effects on the immune response are due to its ability to interfere with the macrophage T-cell cooperation (Metafora et al., 1989; Vuotto et al., 1993; Peluso et al., 1994; Romano-Carratelli et al., 1995; Tufano et al., 1996). The inhibition of phospholipase A_2 activity by SV-IV is at the basis of its anti-inflammatory properties (Metafora et al., 1989; Camussi et al., 1990). The procoagulant activity of SV-IV has been explained by its ability to inhibit the heparin-dependent activation process of antithrombin (Di Micco et al., 1994, 1997). In this paper, we report data showing that the addition to hemophilic blood clotting systems of micromolar amounts of protein SV-IV markedly accelerated the coagulation time and normalized the concentration of serum antithrombin and thrombin–antithrombin complexes.

2. Materials and methods

2.1. Purification of SV-IV

The protein SV-IV was purified to homogeneity from the seminal vesicle secretion of adult rats (Fisher–Wistar) (Ostrowski et al., 1979). Protein purity was evaluated by

15% PAGE in denaturing and nondenaturing conditions (Metafora et al., 1987), amino acid composition analysis, fingerprint technique (Abrescia et al., 1986), and fast atom bombardment mass spectrometry (Porta et al., 1991). The SV-IV preparations were free of lipopolysaccharide and tumor necrosis factor (Thye et al., 1972; Rubin et al., 1985). The concentration of purified SV-IV was assessed by its molar absorption at 276 nm (4100 M^{-1} cm^{-1}) (Di Micco et al., 1994).

2.2. Preparation of serum and plasma

Most of the experiments were carried out with sera obtained from coagulated blood of either normal, healthy, nonsmokers, or Human Immunodeficiency Virus-negative patients affected by hemophilia A with factor VIII levels in 1.5–3% of normal range and without anti-factor VIII antibodies (courtesy of Dr. Marisa Papa, Hemophilia Center, Ospedale Nuovo Pellegrini, Naples, Italy). The absence of anti-factor VIII antibodies in the sera was demonstrated by the Nijmegen assay (Verbruggen et al., 1995). Venous blood was incubated at 37°C in glass tubes and the serum was separated from coagulated blood at different times (25, 35, and 50 min) after clot formation. To stop the Ca^{2+}-dependent clotting reactions in the sera taken at different times of the coagulating process, the serum samples were quickly mixed with 0.2 M sodium citrate (9:1, v/v) and then used in the experiments aiming to quantitate antithrombin, thrombin–antithrombin, and Xa/antithrombin complexes by immunological or biochemical techniques. In other experiments, the sera were used as such without added citrate. The plasma was prepared by centrifuging blood samples, made unclottable by 0.1 M sodium citrate (9:1, v/v), at room temperature for 30 min and at $3000 \times g$. The supernatant, containing between 2 and 20 platelets/μl, was separated from the pellet and immediately used as platelet-poor plasma (PPP) in the experiments.

2.3. Blood coagulation time

The blood samples (0.9 ml) were mixed with 0.1 ml of 150 mM NaCl [with or without rFactor VIII (Kogenate, from Bayer Italia, Milan, Italy) or purified protein SV-IV] and allowed to coagulate in glass test tubes at 37°C. The coagulation time was recorded and its value was expressed in minutes.

2.4. Coagulation time of recalcified plasma

The evaluation of the recalcification time was carried out with a technique similar to the Howel's original method (Howel, 1935), using PPP instead of platelet-rich plasma. The assay was performed at 37°C by mixing $CaCl_2$ with PPP in the presence or absence of SV-IV at various micromolar concentrations and recording the coagulation time (Di Micco et al., 1994).

2.5. Serum residual protrombin

A Prothombin Time (PT) kit from Boehringer (Milan, Italy) was used to perform this test. Normal or hemophilic blood was coagulated with $CaCl_2$ in the presence or absence of rFactor VIII (Kogenate). At different times after clot formation, a suitable aliquot of the serum was added to a reaction mixture containing prothrombin-deficient plasma, rabbit brain acetone extract, and $CaCl_2$. The clotting time was recorded and the concentration of prothrombin (expressed in μM) in the sera was calculated from a calibration curve obtained with sera having a known titer of residual prothrombin (Di Micco et al., 1994).

2.6. Thrombin generation test

This test evaluated the amount of thrombin generated during the coagulation process of PPP treated with suitable amounts of Partial Prothrombin Time reagent (Boehringer) and $CaCl_2$. Upon coagulation, the fibrin was removed by a glass rod and the levels of thrombin were measured in the incubation mixture at different times after clot formation by a spectrophotometric method using the tosyl-gly-pro-arg-*p*-nitroanilide (Chromozym TH from Boehringer) as chromogenic substrate (Di Micco et al., 1994). The concentration of thrombin (expressed in μM) in the samples was calculated from a calibration curve obtained by incubating the chromogenic substrate with known concentrations of pure thrombin.

2.7. Antithrombin titration

Antithrombin was titrated by a clotting technique (Di Micco et al., 1994), in which a definite amount of pure fibrinogen interacted in vitro with a suitable concentration of pure thrombin in the presence of heparin and aliquots of antithrombin-containing serum or plasma from normal or hemophilic individuals. In this clotting system, the fibrinogen coagulation time varies only as a result of the inhibitory action of the antithrombin in the serum or plasma sample, the fibrinogen and thrombin being present in the system at determined, prefixed, and appropriate concentrations. The assay mixture, containing 0.2 ml of serum or plasma (diluted 1:100 with saline) and 0.2 ml of α-thrombin/heparin solution (from the Coagulometric Kit for AT III determination; Behring, Marburg, Germany), was incubated at 37°C for 4 min. At the end of incubation, 0.1 ml of the mixture was added to an equal volume of bovine fibrinogen solution (1.5 mg/ml, Behring) and the coagulation time (depending on the amount of antithrombin in the tested sample) was recorded. The antithrombin concentration in the serum or plasma was calculated by a standard titration curve using serum or plasma samples of known antithrombin concentration (Behring). Standard titration curves were also prepared using pure antithrombin (Sigma,

St. Louis, MO, USA). Antithrombin purity was tested by Fast Protein Liquid Chromatography and SDS-PAGE (Di Micco et al., 1994), whereas its functional activity was demonstrated by a coagulation technique where only highly purified components were used (Di Micco et al., 1994, 1997).

Where required, the antithrombin concentration was immunologically measured by the NOR-Partigen-Antithrombin III Kit (Behring). The determination was based on an immunodiffusion technique using either rabbit or sheep polyclonal antibodies (components of the Behring kit mentioned above) as immunological reagents; the experimental procedure was performed according to the manufacturer's instructions (see also Di Micco et al., 1994).

The antithrombin levels were also evaluated spectrophotometrically by a technique described in detail elsewhere (Di Micco et al., 1997). In this technique, the assay was kinetic, the absorbance measurements were performed at 405 nm, and the chromogenic substrate used was the tosyl-gly-pro-arg-5-amino-2-nitrobenzoic acid isopropylamide (Behring).

2.8. Evaluation of thrombin–antithrombin complexes

The amount of thrombin–antithrombin complexes in the normal or hemophilic sera separated from coagulated blood at different times (25, 35, and 50 min) after clot formation, was evaluated in microtiter plates by the Enzyme Immunoassay for the Determination of human Thrombin/Antithrombin III complex (Enzygnost TAT, Behring) assay kit according to the manufacturer's instructions (see also Pelzer, 1986; Di Micco et al., 1994).

2.9. Titration of serum factor Xa / antithrombin complexes

The level of the factor Xa/antithrombin complexes, occurring in normal or hemophilic sera separated from coagulating blood at different times (25, 35, and 50 min) after clot formation, was measured in microtiter plates by an immunoenzymatic technique using: (1) specific immunoplates with microwell walls coated with either anti-factor Xa (American Diagnostica, Greenwich, CT, USA); (2) reagents included in the Enzygnost TAT assay kit (Behring) and required for the titration of the antithrombin in the factor Xa/antithrombin complex. Briefly, 50 μl aliquots of normal or hemophilic serum were mixed with 50 μl of incubation buffer (from the Baxter's kit used for the evaluation of Dade ELISA fragment F1.2 of prothrombin: Baxter Diagnostics, Deerfield, IL, USA) in the immunoplate microwells, whose walls were coated with anti-factor Xa antibodies, and incubated at 37°C for 20 min. At the end of the incubation, the mixture was discarded, 100 μl of peroxidase-bound anti-antithrombin antibody solution (from the Behring's Enzygnost TAT assay kit) were added to the microwells previously washed three times with 200 μl of washing buffer (from the Behring's kit), and the plates were incubated for 30 min at 37°C. After incubation, the amount of factor Xa/antithrombin–(anti-antithrombin-peroxidase) complexes attached to the walls of microwells was evaluated by a peroxidase-based colorimetric assay according to the Behring kit's instructions (see also Di Micco et al., 1997). The concentration of factor Xa/antithrombin complex present in the analyzed sera was calculated by comparing the serum factor Xa/antithrombin titration curve with the standard titration curve obtained using different plasma samples of known thrombin–antithrombin complex concentration (from Behring).

With this technique, we found that the factor Xa/antithrombin complexes were, as expected, undetectable in normal and hemophilic plasma (data not shown). This finding indicates that the immunoenzymatic technique described in this paragraph is reliable for the evaluation of these complexes in serum samples.

2.10. Effect of antithrombin, SV-IV, or both on the coagulation time of a purified clotting system

The reaction mixture of the purified clotting system contained 300 μg of bovine fibrinogen (Behring), 2.5 U of bovine α-thrombin (Behring), and 0.2 U of heparin (Liquemin; Roche, Milan, Italy) in 0.3 ml of 150 mM NaCl. The heparin was included in the mixture to activate the antithrombin when this protein was added to the system. Where indicated, human purified antithrombin (Sigma) or purified protein SV-IV (a potent in vitro inhibitor of the heparin-dependent activation process of antithrombin: Di Micco et al., 1994, 1997), or both, were added to the reaction mixture at a final concentration of 1.2 and 5 μM, respectively, to analyze their effect on the coagulation time of the system. In other assays, normal or hemophilic plasma or serum (diluted 1:3 with saline, and supplemented or not with SV-IV) were used as antithrombin sources. The reaction mixtures, in which coagulation was started by thrombin addition, were incubated at 37°C, and the coagulation time, expressed in seconds, was recorded.

2.11. Statistical analysis

The data are reported as means ± standard error of the mean (S.E.M.) of determinations performed in triplicate on a suitable number (see footnotes to Tables 1–4) of different blood samples. The means were compared using analysis of variance (ANOVA) plus Bonferroni's *t*-test, and a *P*-value of less than 0.05 was considered significant.

3. Results

3.1. In hemophilic sera, the antithrombin / thrombin ratio is higher than in normal ones

The coagulation time and different coagulation parameters [concentration of thrombin–antithrombin complexes,

Table 1
Measurement of normal or hemophilic coagulation time and evaluation of different coagulation parameters in sera separated from coagulated blood of normal or hemophilic subjects at different times (25, 35, and 50 min) after clot formation[a]

Coagulation parameters	Time (min) of serum separation	Normal	Hemophiliacs	
		No addition of rFactor VIII	No addition of rFactor VIII	Addition of rFactor VIII[b]
Coagulation time (min)	–	11 ± 2	20 ± 3**	11 ± 1
Serum residual prothrombin (μM)	25	0.50 ± 0.072	1.32 ± 0.060**	0.53 ± 0.082
	35	0.32 ± 0.063	1.22 ± 0.070**	0.35 ± 0.050
	50	0.10 ± 0.010	1.15 ± 0.050**	0.12 ± 0.018
Antithrombin (μM)[c]	25	2.64 ± 0.192	4.76 ± 0.184*	2.70 ± 0.103
	35	2.40 ± 0.143	4.58 ± 0.172*	2.40 ± 0.124
	50	2.20 ± 0.125	4.48 ± 0.156*	2.28 ± 0.072
Thrombin–antithrombin complexes (μM)	25	0.043 ± 0.015	0.021 ± 0.012*	0.042 ± 0.010
	35	0.049 ± 0.013	0.036 ± 0.024*	0.048 ± 0.010
	50	0.055 ± 0.012	0.042 ± 0.003*	0.052 ± 0.008
Total thrombin (μM)[d]	25	0.45 ± 0.063	0.055 ± 0.007**	0.45 ± 0.032
	35	0.54 ± 0.102	0.105 ± 0.010**	0.54 ± 0.046
	50	0.65 ± 0.065	0.140 ± 0.011**	0.65 ± 0.052
Percent of total thrombin in thrombin–antithrombin complexes[e]	25	9.60 ± 1.44	38.20 ± 4.64**	9.40 ± 1.32
	35	9.06 ± 1.54	34.20 ± 4.90**	8.80 ± 1.40
	50	8.46 ± 1.08	30.00 ± 4.52**	8.00 ± 1.20

[a] Experimental details are described in Section 2.

[b] Final concentration of 2 U/ml; the hemophilic coagulation time was determined with the standard technique (see Section 2) in which 0.1 ml of saline was substituted or not (no addition of rFactor VIII) with 0.1 ml of a suitable concentration of rFactor VIII.

[c] The data reported in the table were acquired by using the immunological assay; similar results were obtained with the spectrophotometric assay (the relevant values were about 30% lower) and with the clotting technique (the relevant values were about 15% higher).

[d] Total thrombin was calculated as follows: (plasma prothrombin − serum residual prothrombin)/2; similar results were obtained when the thrombin concentration was directly measured with the thrombin generation test (see Di Micco et al., 1994).

[e] Percent of total thrombin in thrombin–antithrombin complexes = [(thrombin–antithrombin complexes)/total thrombin] × 100. Plasma prothrombin in normal subjects = 1.4 ± 0.12 μM. Plasma prothrombin in hemophiliacs = 1.43 ± 0.14 μM. Plasma antithrombin in normal subjects = 4.65 ± 0.60 μM, as determined by the immunological assay (see also Hathaway and Goodnight, 1993). Plasma antithrombin in hemophiliacs = 4.82 ± 0.70 μM, as determined by the immunological assay. The values reported in the table are the means ± S.E.M. of determinations performed in triplicate on single blood samples obtained from 20 different normal and 33 different hemophilic individuals. As a whole, the results reported in the table indicate that the low efficiency of thrombin-generating reactions during the hemophilic blood coagulation process in the absence of factor VIII produces a marked increase of the antithrombin /thrombin ratio.

* $P < 0.05$ (Bonferroni's *t*-test) vs. the respective normal value.

** $P < 0.01$ (Bonferroni's *t*-test) vs. the respective normal value.

antithrombin, serum residual prothrombin, and total thrombin; percentage of total thrombin in thrombin–antithrombin complexes] were measured in normal or hemophilic sera separated from coagulated blood at different times (25, 35, and 50 min) after clot formation. The data reported in Table 1 show that in the hemophiliacs, whose coagulation time was expectedly longer than normal, the serum concentration of both total thrombin and thrombin–antithrombin complexes was significantly lower (about 81% and 26%, respectively) than that occurring in

Table 2
Concentration of factor Xa/antithrombin complexes in normal or hemophilic sera separated from coagulated blood at different times (25, 35, and 50 min) after clot formation[a]

Serum molecular complexes	Time (min) of serum separation	Concentration (μM)	
		Normal	Hemophilic
Factor Xa/antithrombin	25	0.0096 ± 0.003	0.00038 ± 0.00005**
	35	0.0117 ± 0.004	0.00057 ± 0.00006**
	50	0.0133 ± 0.004	0.00086 ± 0.00005**

[a] Experimental details are described in Section 2. The values reported in the table are the means ± S.E.M. of determinations performed in triplicate on single blood samples obtained from 18 different normal and 23 different hemophilic individuals.

** $P < 0.01$ (Bonferroni's *t*-test) vs. the respective normal value.

Table 3
Coagulation time of a purified clotting system (fibrinogen/thrombin/heparin) in the presence or in the absence of antithrombin (1.2 μM), SV-IV (5 μM), normal or hemophilic plasma or serum (with or without 5 μM SV-IV) separated from coagulated blood 35 min after clot formation[a]

Addition	Coagulation time (s)
None	26 ± 2
SV-IV	24 ± 4
Purified antithrombin	476 ± 38**
Purified antithrombin + SV-IV	22 ± 3
Plasma (as source of antithrombin)[b]	
Normal	320 ± 26**
Normal + SV-IV	27 ± 3**
Hemophilic	316 ± 23**
Hemophilic + SV-IV	30 ± 2**
Serum (as source of antithrombin)[b]	
Normal	167 ± 15**
Normal + SV-IV	32 ± 4**
Hemophilic	296 ± 26**
Hemophilic + SV-IV	45 ± 5**

[a] Experimental details are described in Section 2. The values reported in the table are the means ± S.E.M. of 14 determinations performed in triplicate. The plasma or sera used as antithrombin sources were obtained from 14 different blood samples of normal or hemophilic individuals.

[b] In these assays (0.3 ml final volume), 0.1 ml of normal or hemophilic plasma or serum, diluted 1:3 with saline and containing or not SV-IV, was used as a source of antithrombin.

** $P < 0.01$ (Bonferroni's *t*-test) vs. the respective value without additions.

normal serum at all the times studied. By contrast, the antithrombin concentration, the percentage of total thrombin in thrombin–antithrombin complexes, and the serum residual prothrombin concentration were substantially higher in the hemophilic serum (about 91%, 277%, and 281%, respectively) than in normal serum at all the times studied.

The data reported in Table 1 also show that the coagulation time and the other coagulation parameters measured in hemophilic sera became normal when appropriate amounts of human recombinant factor VIII were added to the hemophilic blood before the beginning of the clotting process. The experiments with added factor VIII were done not only as controls to show that the low amount of thrombin present in the hemophilic sera was, as expected, a consequence of the factor VIII deficiency, but also to demonstrate that the high concentration of antithrombin in these sera was a consequence of the factor VIII deficiency, as shown by the decrease of antithrombin when the hemophilic blood samples were coagulated in the presence of this factor (Table 1).

3.2. In the hemophilic sera, the concentration of factor Xa-antithrombin complexes is low

The data reported in Table 2 show that the concentration of factor Xa-antithrombin complexes in hemophilic sera is 20–25 times lower than in the normal ones at all the times studied. This result is, most probably, the consequence of the low levels of factor Xa in hemophilic clotting systems (see also Section 1 and Samama et al., 1990; Hathaway and Goodnight, 1993). The low concentration of factor Xa-antithrombin complexes in the hemophilic sera indicates that in this system, there is a small generation of factor Xa and, consequently, a small consumption of antithrombin. This contributes to keep high the concentration of antithrombin in the system.

3.3. Adding hemophilic serum to a purified clotting system prolongs the coagulation time more than normal serum: this effect is abrogated by the simultaneous addition of SV-IV

The addition of normal, or hemophilic plasma, or serum, as a source of antithrombin to a purified clotting system (fibrinogen, thrombin, and heparin), prolonged the coagulation time of the system on account of the plasma or serum antithrombin concentration (Table 3). When hemophilic serum was added to the system, the coagulation time becomes much longer than by the normal serum addition (Table 3). This indicates that the concentration of antithrombin in hemophilic serum is higher than in normal serum (see Table 1) and is consistent with the hypothesis stating that the small amount of thrombin produced during the clotting of hemophilic blood is strongly inhibited by the large amounts of nonconsumed active antithrombin

Table 4
Normalizing effect of protein SV-IV (5 μM) on blood coagulation time, plasma recalcification time, antithrombin and thrombin–antithrombin complex concentrations in sera separated from coagulated blood of hemophiliacs 35 min after clot formation[a]

Coagulation parameters	Hemophiliacs	
	− SV-IV	+ SV-IV
Coagulation time (min)	19 ± 5**	12 ± 3
Recalcification time (min)	8.5 ± 0.5**	3.5 ± 0.1
Antithrombin (μM)	4.54 ± 0.12**	2.45 ± 0.12
Thrombin-antithrombin complexes(μM)	0.034 ± 0.003**	0.011 ± 0.002

[a] Experimental details are described in Section 2. The hemophilic blood was allowed to coagulate in the absence or in the presence of SV-IV: CT and RT were evaluated by a standard technique (see Section 2) in which 0.1 ml of saline was substituted or not with 0.1 ml of a suitable concentration of SV-IV. Antithrombin and thrombin–antithrombin complexes were measured in the sera separated from hemophilic blood coagulated in the presence or in the absence of SV-IV. Results similar to those reported in the table were obtained with 10 and 15 μM SV-IV. Coagulation time of normal blood = 11 ± 2 min. Recalcification time of normal plasma = 3.2 ± 0.2 min. Concentration of antithrombin in normal serum = 2.40 ± 0.14 μg/ml. The values reported in the table are the means ± S.E.M. of determinations performed in triplicate on 13 different hemophilic blood samples.

** $P < 0.01$ (Bonferroni's *t*-test) vs. the respective values of normal individuals (see above in this note) or hemophiliacs (see in the table) in the presence of SV-IV.

present in hemophilic sera (see data on thrombin, antithrombin, and thrombin–antithrombin complex concentration in Table 1). Plasma from either normal or hemophilic subjects added to the purified clotting system significantly increased its coagulation time. No difference was, however, detectable between the coagulation time of the two different types of plasma, because they contained similar antithrombin concentrations (Tables 1 and 3).

As shown in Table 3, the addition of 5 μM SV-IV to the system did not produce any effect on the coagulation time, because antithrombin was not present in the system. Adding 1 μM of pure antithrombin markedly increased the system coagulation time as a result of the antithrombin inhibitory effect on thrombin. When the same concentration of antithrombin was added in association with 5 μM SV-IV, the antithrombin inhibitory effect was abrogated. Furthermore, when normal or hemophilic plasma or serum was added to the system as a source of antithrombin, their association with SV-IV abrogated their prolonging effect on the coagulation time (Table 3).

3.4. Adding SV-IV to hemophilic clotting systems normalizes their antithrombin-dependent clotting regulatory mechanism

The data reported in Table 4 show that when 5 μM SV-IV was added to hemophilic blood, the concentration of serum thrombin–antithrombin complexes decreased markedly (about 68%), and the coagulation time and other coagulation parameters (recalcification time and serum antithrombin) became normal.

4. Discussion

Our findings (Tables 1–3) show that the thrombin concentration in the hemophilic sera is substantially lower than in the normal controls, whereas the antithrombin concentration is higher. The first finding is clearly related to the low efficiency of thrombin-generating reactions in factor VIII-deficient clotting blood. The second one is not related to an absolute increase of plasma antithrombin [the latter is not increased in the hemophiliacs; what is increased in these individuals in comparison with the normals is the serum antithrombin (see Table 1 and its footnote)] but, rather, to its reduced consumption caused by the scarce generation of thrombin and factor Xa (coagulation factors possessing the ability to form stable complexes with antithrombin) in their clotting systems (Tables 1 and 2). These data indicate that the high antithrombin/thrombin ratio (about 44) observed in hemophilic sera, by reflecting an antithrombin-induced strong inhibition of the low levels of thrombin generated in factor VIII-deficient clotting systems, can play an important role in the molecular mechanism underlying the uncontrolled bleeding of hemophiliacs.

In hemophilic sera, the concentration of thrombin–antithrombin complexes (with thrombin and antithrombin in equimolar amounts) is not only lower than in normal sera, but is also associated with a higher percentage of total thrombin bound in these complexes (Table 1), on account of the high concentration of free antithrombin in these sera. The occurrence of thrombin–antithrombin complexes in normal or hemophilic serum samples indicates that antithrombin, normally inactive in noncoagulating blood or plasma, is present in the serum in a conformationally active form as a result of its binding to heparin-like molecules during the in vitro blood coagulation process (Lammle and Griffin, 1985; Samama et al., 1990; Hathaway and Goodnight, 1993).

The sera used in our experiments were prepared at different timepoints of the coagulation process for a better in vitro evaluation of the progressive changes in prothrombin, antithrombin, and thrombin–antithrombin concentrations, during the normal or pathological clotting cascade. In particular, the data reported in Table 1 demonstrate that the antithrombin concentration in hemophilic sera was higher than in normals at all the times studied.

Should the high antithrombin/thrombin ratio in hemophiliacs be the immediate cause of the coagulation failure, then it is reasonable to design appropriate experiments to verify the effect of antithrombin inhibitors on different clotting parameters of the in vitro coagulating hemophilic blood or plasma. When, in fact, the clotting of a hemophilic system was triggered in vitro in the presence of the antithrombin inhibitory protein SV-IV, it was found that the blood coagulation time, the plasma recalcification time, and the serum antithrombin concentration became nearly normal (Table 4), even though factor VIII concentration in the system was unchanged (data not shown). Furthermore, the thrombin–antithrombin complex concentration in sera, separated from hemophilic blood samples coagulated in the presence of SV-IV, was found to be markedly decreased (about 68%) as a result of the SV-IV-mediated inhibition of the heparin-dependent antithrombin activation process (Table 4). The antithrombin inhibitory effect of SV-IV was also observed when normal or hemophilic plasma or serum was used as a source of antithrombin in purified clotting systems containing only fibrinogen, thrombin, and heparin (Table 3). The presence of SV-IV in the coagulating blood of hemophiliacs, thus, caused a marked decrease of the antithrombin-induced inhibition of the small amount of thrombin generated and led ultimately to a normalization of the hemophilic clotting process.

It is well known that fibrinolysis in hemophiliacs is more active than in normals as a consequence of the low inhibition of thrombin-regulated fibrinolysis (Broze and Higuchi, 1996). In contrast, in the coagulating hemophilic blood treated with SV-IV, the increased levels of free, uninhibited thrombin are expected to be associated with a substantial increase of the thrombin-dependent inhibition

of fibrinolysis as a consequence of the thrombin-induced activation of both Thrombin-Activatable Fibrinolysis Inhibitor (Broze, 1996; Bajzar et al., 1998; Wang et al., 1998) and Factor XIII (Samama et al., 1990). Furthermore, a direct inhibitory effect of the cationic SV-IV protein on the fibrinolytic cascade (competitive inhibition of plasminogen binding to fibrin by the lysine-rich protein SV-IV) can also be hypothesized. In conclusion, the possibility exists that part of the SV-IV procoagulant effect on the hemophilic blood might be related to an expected indirect (SV-IV-induced increase of thrombin-dependent inhibition of fibrinolysis) and/or a possible direct inhibitory action of this protein on the fibrinolytic process. These possibilities certainly warrant further investigation.

The normalization of the blood coagulation parameters obtained by exploiting the antithrombin-inhibitory activity of SV-IV was similar to that obtained by adding appropriate amounts of factor VIII to the hemophilic coagulating blood (Table 1). This result emphasizes the role played by antithrombin in the control of the blood coagulation process both in normal and pathological conditions, and suggests the possibility that any blood hypocoagulability condition caused by a prothrombinase defect could be bypassed by stimulating the thrombin activity by antithrombin inhibition. This possibility appears to be particularly attractive by taking into account the procoagulant effect of the protein SV-IV or its peptide derivatives on plasma samples obtained from subjects affected by hemorrhagic disorders of various origins (thrombocytopenia, hepatic cyrrhosis, Warfarin-dependent hemorrhagic syndrome) (Di Micco et al., 1994, 1997, unpublished data).

Experiments are in progress in our laboratory to unravel the molecular details of the SV-IV inhibitory activity (identification of the protein segments involved in the interactions of SV-IV with antithrombin and heparin) and to prepare human recombinant SV-IV, together with a number of bioactive structural homologues of the protein.

Acknowledgements

The authors wish to express their gratitude to Mr. Salvatore Baiano, Mr. Alessandro Manfredonia, and Mr. Francesco Moscatiello for their excellent technical assistance.

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