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RAT PROTEIN SV-IV (SEMINAL VESICLE PROTEIN NO. 4) ACCELERATES HUMAN BLOOD COAGULATION *IN VITRO* BY SELECTIVE INHIBITION OF ANTITHROMBIN III

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Abstract—The seminal vesicle protein No. 4 (SV-IV) secreted from the rat seminal vesicle epithelium, possesses immunosuppressive and anti-inflammatory properties and it is a potent inhibitor of platelet aggregation both *in vivo* and *in vitro*. This research aimed to investigate the possible effect of SV-IV on the process of human blood coagulation. Preliminary experiments showed that the recalcification time (RT) of platelet-poor plasma (PPP) samples, obtained from both normal subjects and patients affected by some hemorrhagic disorders, was found to be markedly reduced in the presence of micromolar amounts of SV-IV. It was demonstrated that the concentration of free antithrombin III (AT III) occurring in blood sera obtained from PPP samples recalcified in the presence of SV-IV was significantly decreased in comparison with sera obtained from PPP recalcified in the absence of SV-IV. It was also shown that PPP treatment with SV-IV significantly reduced the concentration of free AT III without affecting the levels of other plasma serine protease inhibitors, such as α_2-macroglobulin, α_1-antitrypsin and C_1-inhibitor. In addition, the RT of PPP treated with a specific rabbit anti-AT III polyclonal antiserum (anti-AT III treated PPP) was not modified by SV-IV. These findings were confirmed by the observation that the addition of SV-IV into an *in vitro* coagulation system, containing pure fibrinogen, α-thrombin and AT-III, resulted in complete suppression of thrombin inhibition by AT III. No other steps of the blood clotting process (prothrombinase complex, factor XIII, fibrinogen concentration) were affected by SV-IV.

Key words: protein SV-IV; blood coagulation; antithrombin III

SV-IV§ (according to its electrophoretic mobility in SDS–PAGE) is a secretory product synthesized in milligram amounts by the epithelium of rat seminal vesicles [1] under strict androgen transcriptional control [2]. This protein has a low M_r (9758) [3], is basic (pH = 8.9) [4], polymorphic [5], possesses only about 14% α-helix and has remarkable concentration-dependent properties in aqueous solution (self-association) at neutral pH [6]. The sequence of its 90 amino acids has been established [3], and much is known about the structure and expression of the gene coding for it [2, 4, 7–12]. Proteins immunologically cross-reactive with SV-IV have been found in several rat tissues of both sexes [8] and in human seminal fluid, both free in seminal plasma and bound to the surface of ejaculated spermatozoa [13, 14]. Numerous studies on the biochemical properties of the protein have demonstrated that SV-IV is a good substrate for TGase (EC 2.3.2.13) [15, 16], possesses a marked ability to inhibit, both *in vivo* and *in vitro*, PLA2 activity [17, 18] and the PAF biosynthetic pathway [18]. SV-IV has also been found to be endowed with powerful immunosuppressive [17], anti-inflammatory [17], antiphagocytic and antichemotactic [19, 20] properties. More recently these authors demonstrated that SV-IV possessed a potent inhibitory effect on the process of platelet aggregation induced by different agents both *in vivo* and *in vitro* [21]. On the basis of these results, and taking into account that biological properties of SV-IV seem to be nonspecies-specific [17], it was of interest to investigate whether the protein influenced the process of human blood coagulation by acting at some critical level of its complex biochemical mechanism. The present paper reports that micromolar concentrations of SV-IV were able to markedly accelerate the process of human blood clotting *in vitro* with a molecular mechanism involving a selective inhibition of AT III, a plasma protein physiologically devoted to keep thrombin activity under control.

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§ Abbreviations: SV-IV, seminal vesicle protein No.4; TGase, transglutaminase; PLA2, phospholipase A2; PAF, platelet-activating factor; AT III, antithrombin; PAGE, polyacrylamide gel electrophoresis; PPP, platelet-poor plasma; PT, prothrombin time test; PTT, partial thromboplastin time; RT, recalcification time; SRPT, serum residual prothrombin test; TGT, thrombin generation test.

MATERIALS AND METHODS

Purification of SV-IV. SV-IV was purified to

homogeneity from adult rat (Fisher–Wistar) seminal vesicle secretion according to a published technique [1]. The purity of the protein was assessed by 15% PAGE in denaturing and nondenaturing conditions [22], by the fingerprint technique [9], by amino acid composition analysis [22] and by fast atom bombardment mass spectrometry [16]. Protein concentration was determined by the method of Lowry *et al.* [23]. The concentration of the purified SV-IV was evaluated by its molar absorption at 276 nm (4100 per M/cm), calculated on the basis of the tyrosine and phenylalanine residues present in the SV-IV polypeptide chain [24].

Preparation of PPP. Most of the experiments were performed with PPP of normal, healthy individuals. In some experiments, however, PPP was prepared from patients affected by hemorrhagic disorders. Venous blood, taken from these subjects and mixed with 0.1 M sodium citrate (9:1, v/v), was centrifuged at 3000 *g* for 30 min at room temperature. The supernatant, containing between 2 and 20 platelets/μL, was carefully separated from the pellet and immediately used as PPP in all the experiments [25, 26].

RT. The evaluation of the RT was carried out with a technique similar to the Howel's original method [26, 27], except that PPP was used instead of platelet-rich plasma. The test was performed at 37° by mixing 0.1 mL of 30 mM $CaCl_2$ with 0.1 mL of PPP in the presence of 0.1 mL of either SV-IV at various micromolar concentrations or 150 mM NaCl (control) and recording the coagulation time. Where indicated, 0.1 mL of 15 μM protamine or histone H1 were used in place of SV-IV. In some experiments 0.1 mL of factor XIII-deficient plasma (Behring, Scoppito, Italy) was substituted for PPP. The RT was also evaluated by using AT III treated with a specific rabbit anti-AT III polyclonal antiserum (anti-AT III treated PPP; Sigma, St Louis, MO, U.S.A.). In more detail, 200 μL of normal PPP were mixed with 50 μL of anti-AT III antiserum and the reaction mixture was incubated at 37° for 10 min to allow the specific antigen–antibody reaction to occur. At the end of the incubation, 0.1 mL aliquots of the anti-AT III-treated PPP were used in the RT assay to measure the coagulation time in the absence or presence of different concentrations of SV-IV. In control experiments, where PPP was treated with a pool of normal rabbit sera, the SV-IV accelerating effect on RT was similar to that obtained with untreated PPP.

PTT. The assay was carried out by mixing 0.1 mL of celite-containing PTT reagent [28] with 0.1 mL of PPP in the presence of either 15 μM SV-IV or 150 mM NaCl (control). The mixture was incubated at 37° for 3 min. At the end of the incubation time, 0.1 mL of 30 mM $CaCl_2$ was added and the coagulation time recorded. The PTT test was carried out with a PTT kit from Boehringer, Milano, Italy.

TGT. This test evaluated the amount of thrombin generated during the coagulation process of PPP treated with suitable amounts of PTT reagent and calcium ions. 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 [26]. The TGT reaction mixture (0.4 mL) contained 0.1 mL PPP, 0.1 mL PTT reagent, 0.1 mL 40 mM $CaCl_2$ and 0.1 mL of either 20 μM SV-IV or 150 mM NaCl (control). The reaction mixture was incubated at 37° for 3 min and, following fibrin removal, 50 μL of fibrin-free aliquots taken from the total incubation mixture were combined with 150 μL of 1.9 mM Chromozym contained in a thermostated (37°) cell of a double beam Cary 219 spectrophotometer. The thrombin activity was expressed as the optical density at 405 nm of the *p*-nitroanilide generated at different incubation times by the chromogenic substrate in the presence of the enzyme occurring in 50 μL of fibrin-free mixture.

PT. The reaction mixture contained 0.1 mL of PPP, 0.1 mL of brain extract and 0.1 mL of 40 mM $CaCl_2$ [29, 30]. Where required, 0.1 mL of either 20 μM SV-IV or 150 mM NaCl (control) were added to the reaction mixture. The clotting time was recorded upon incubation of the mixture at 37°. This test was performed with a PT kit from Boehringer.

SRPT. A PT kit from Boehringer was used to perform this test. The assay mixture was prepared by adding 0.1 mL of 30 mM $CaCl_2$ and 0.1 mL of either 15 μM SV-IV or 150 mM NaCl (control) to 0.1 mL of PPP. After allowing 30 min of incubation at 37° for clot formation, 0.1 mL of serum produced from each plasma clot was added into a reaction mixture containing 0.1 mL aliquots of prothrombin-deficient plasma, 0.1 mL of brain extract and 0.1 mL of 30 mM $CaCl_2$. The clotting time was recorded, its value being inversely proportional to the amount of residual prothrombin present in the serum analysed. The concentration of prothrombin in these sera was calculated from a standard titration curve obtained with sera having a known titre of prothrombin.

Fibrinogen quantitation. An immunological precipitation technique [31] was used to evaluate the fibrinogen concentration in PPP. An appropriate concentration of antibody specific to fibrinogen was included in the agar. A hole was punched in the agar and filled with the antigen [5 μL from a mixture of 0.1 mL PPP and 0.1 mL of either 10 μM SV-IV or 150 mM NaCl (control), preincubated at 37° for 10 min]. After 48 hr of incubation at room temperature the antigen, diffused into the agar, produced a ring of precipitate around the hole, indicating where the antigen and antibody had reacted at equivalence. The diameter of the ring was proportional to the concentration of antigen in the well and to the concentration of antibody in the agar. By setting up suitable fibrinogen standards of known titre, fairly accurate figures were obtained for the amount of fibrinogen present in the tested PPP, pre-treated or not with SV-IV.

AT III quantitation. The AT III concentration was assessed by two different techniques. The clotting technique to quantitate AT III in the serum was an interaction test between fibrinogen and thrombin. The fibrinogen coagulation time could vary only as a consequence of the inhibitory action of AT III occurring in the serum sample, the fibrinogen and thrombin being present in the system at determined, pre-fixed, suitable concentrations. In this technique

0.5 mL of PPP were mixed with 0.3 mL of 30 mM $CaCl_2$ and 0.2 mL of either 25 μM SV-IV or 150 mM NaCl (control), and incubated for 30 min at 37°. The AT III, occurring in the serum obtained from the coagulum by a proper test tube jolting, was titrated with a suitable kit (Coagulometric Kit for AT III determination) from Behring according to the manufacturer's instructions. The assay mixture, containing 0.2 mL of serum and 0.2 mL of α-thrombin kit solution, was incubated at 37° for 4 min. At the end of incubation, 0.1 mL of this mixture was added to an equal volume of bovine fibrinogen solution and the coagulation time (whose value was proportional to the amount of AT III present in the serum) recorded. The AT III concentration in the serum was calculated by a standard titration curve obtained with serum samples of known AT III concentrations. The quantitation of AT III by the immunological technique was performed with agar plates (Boehringwerke, Scoppito, Italy) containing antibodies specific to AT III. The plate wells were loaded with appropriate volumes of the sera obtained from PPP clotted with $CaCl_2$ either in the presence or absence of SV-IV. Following incubation at room temperature for 48 hr, the diameters of the precipitation rings were measured and the concentration of AT III in the tested sera evaluated by using a standard AT III titration curve prepared with serum samples of known AT III concentrations. In detail, the control PPP/NaCl clots and the PPP/SV-IV clots were prepared by adding 0.3 mL of 30 mM $CaCl_2$ to 0.5 mL of PPP in the presence of 0.2 mL of either 150 mM NaCl or 25 μM SV-IV. After incubation at 37° for 30 min the serum expressed from the clots following test tube jolting was taken and 5 μL aliquots loaded into the wells of the agar plates used for the immunological quantitation of AT III. Purified AT III, used for the preparation of the standard titration curve, was obtained from Sigma Chemical Co. The AT III purity was tested by FPLC as well as SDS–PAGE; a single symmetric protein peak or band, respectively, was always demonstrated by these techniques. In addition, the functional integrity of the AT III provided by Sigma was demonstrated by mixing, at 37° 0.1 mL of bovine fibrinogen (300 μg; from Behring) in saline with 0.1 mL of bovine thrombin/heparin (2 U/0.2 U; from Behring) in saline containing or not 0.4 nmol of AT III and measuring the coagulation time. In the absence of AT III the coagulation time was 50 ± 5 sec, whereas in the presence of AT III the coagulation time was 180 ± 9 sec.

Plasma serpin quantitation. To investigate a possible inhibitory effect of SV-IV on other plasma serpins (α_2-macroglobulin, α_1-antitrypsin and C_1-inhibitor), the latter were quantitated by an immunological technique on special agar plates (from Boehringwerke) containing antibodies specific for the single serpins. Briefly, 0.1 mL of PPP were incubated with 0.1 mL of either 10 μM SV-IV or 150 mM NaCl (control) at 37° for 15 min. After incubation, 5 μL aliquots of the reaction mixtures were separately loaded into the well of agar plates specific for these three different serpins. Following incubation at room temperature for 48 hr, the diameters of the precipitation rings were measured and the concentration of the specific serpins in the tested plasma was assessed by using standard serpin titration curves prepared with plasma samples of known serpin concentration.

Table 1. RT of PPP in the presence of different concentrations of SV-IV*

Addition	RT (sec)	
	Untreated PPP	Anti-AT III treated PPP
None (control)	196 ± 10	74 ± 6
SV-IV (1 μM)	150 ± 8	76 ± 7
SV-IV (2 μM)	74 ± 7	72 ± 4
SV-IV (5 μM)	68 ± 4	77 ± 5
SV-IV (10 μM)	106 ± 9	72 ± 5
SV-IV (15 μM)	118 ± 10	78 ± 7

* Experimental details are described in Materials and Methods. The values reported in the table are the means ± SEM of determinations performed in triplicate on 30 blood samples.

$P < 0.01$, Student's t-test.

In vitro *analysis of the SV-IV and/or AT III effect on a purified coagulation system.* In these experiments only purified proteins were used. The coagulation reaction mixture contained, in 0.3 mL of 150 mM NaCl, 300 μg of bovine fibrinogen (Behring), 2 U bovine α-thrombin (Behring) and 0.2 U heparin. Where indicated, SV-IV and/or human AT III (Sigma) were added to the mixture at a final concentration of 5 and 1.45 μM, respectively. In some assays normal PPP, diluted 1:3 with saline, was used as the AT III source. The reaction mixtures were incubated at 37° and the coagulation time recorded.

RESULTS

SV-IV produced a marked decrease of RT

The data reported in Table 1 clearly demonstrate that the plasma RT was markedly reduced when micromolar amounts of SV-IV were present in the clotting system, thus indicating that the protein SV-IV possesses a strong procoagulant activity *in vitro*. In other experiments in which anti-AT III treated PPP was used, the plasma RT, even though markedly reduced in comparison with the RT of the normal PPP, was not modified by the presence of various micromolar concentrations of SV-IV (Table 1). The ability of SV-IV to decrease the plasma RT seemed to be rather specific, because other basic proteins of similar M_r, such as protamine and histone H1, exhibited, in contrast, a potent anticoagulant activity (data not shown). It is noteworthy that in the assay in which normal PPP was used RT decreased progressively with an increase in SV-IV concentration, reaching a minimum value at about 5 μM (Table 1). Further increases in the SV-IV concentration significantly reduced the inhibitory effect. This biphasic pattern in the concentration-related response curve was not surprising because it

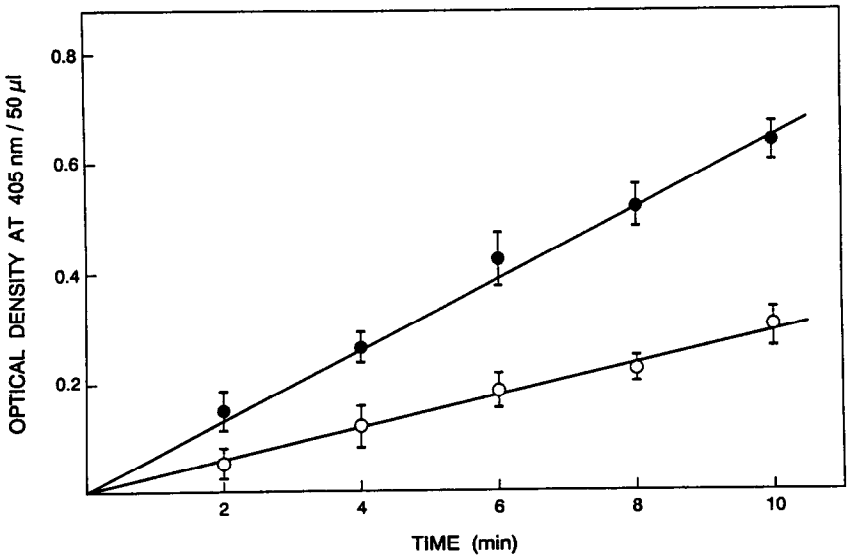

Fig. 1. Effect of 5 µM SV-IV on the thrombin activity (expressed as optical density at 405 nm produced by the enzyme from the chromogenic substrate) occurring in 50 mL of fibrin-free PPP clotting reaction mixture obtained at different intervals of time after clot formation. Each value was corrected by subtracting the optical density measured at 405 nm immediately after the chromogenic substrate addition. Experimental details are described in Materials and Methods. (○——○) – SV-IV; (●——●) + SV-IV (5 µM).

Table 2. Effect of SV-IV on different parameters of the blood clotting cascade *in vitro**

Addition	PTT (sec)	PT (sec)	SRPT (mg/L)	Fibrinogen (mg/L)
None (control)	50 ± 4	12.3 ± 0.75	174 ± 28	30.2 ± 4.7
SV-IV (5 µM)	49 ± 3	11.7 ± 1.02	173 ± 29	29.4 ± 2.8

* Experimental details are described in Materials and Methods. The values reported in the table are the means ± SEM of determinations performed in triplicate on 50 blood samples.

was also observed when other biological properties of SV-IV were analysed [17]. In addition, Fig. 1 shows that the treatment *in vitro* of plasma with SV-IV was able to increase, by about 100%, the thrombin activity present in the reaction mixture. This result suggested that the SV-IV-induced RT decrease was related to a marked activation of the coagulation process caused by an increase of thrombin activity in the PPP system. This hypothesis was strongly supported by the results obtained with the AT III treated PPP which, in turn, suggested an involvement of AT III in the SV-IV induced decrease of RT (Table 1).

SV-IV neither interfered with prothrombinase activity nor with fibrinogen concentration.

To determine whether SV-IV was able to affect the activity of the prothrombinase (a complex enzymatic system produced on assembly of factors II, Va and Xa on a lipid membrane in the presence of calcium ions [32]) the effect of this protein on PTT, PT and SRPT was measured. The results obtained (Table 2) did not reveal any significant difference between the tests performed either in the presence or absence of micromolar amounts of SV-IV. These data strongly suggested that SV-IV did not interfere with the prothrombinase activity. Furthermore, by using a suitable and specific immunoassay, it was also found the PPP fibrinogen level did not change following addition of SV-IV to the clotting system (Table 2).

Factor XIII was not involved in the procoagulating effect of SV-IV

Factor XIII (fibrin-stabilizing factor) plays an important role in the final step of the common pathway of blood coagulation. This factor, following its activation by thrombin and calcium, is transformed in an active TGase that, by catalysing the formation of numerous isopeptide bonds between fibrin molecules, cross-links them in a solid clot very resistant to dissolution. Since it has previously been demonstrated [15, 16] that SV-IV is a substrate of the enzyme, the presence of SV-IV in a clotting system containing normal levels of factor XIII could accelerate clot formation as a consequence of a

Table 3. Evaluation of AT III in sera obtained from PPP clotted by $CaCl_2$ addition in the presence or absence of 5 μM SV-IV*

Addition	AT III concentration (mg/mL)	
	Coagulation technique	Immunoassay
None (control)	306 ± 20	300 ± 24
SV-IV	132 ± 12 (−57)	144 ± 16 (−52)

* Experimental details are described in Materials and Methods and in the legend to Table 4. The negative values reported in parenthesis express the percentage decrease in the levels of AT III occurring in the sera obtained from PPP clotted by $CaCl_2$ additions in the presence of SV-IV. The values reported in the table are the means ± SEM of determinations performed in triplicate on 50 different blood samples.

$P < 0.01$, Student's *t*-test.

Table 4. Immunological evaluation of AT III following its *in vitro* interaction with different concentrations of SV-IV*

Addition	AT III concentration (mg/L)	Control (%)
None (control)	316 ± 17	100
SV-IV (1 μM)	216 ± 12	68
SV-IV (2 μM)	168 ± 10	53
SV-IV (5 μM)	120 ± 8	38
SV-IV (10 μM)	192 ± 9	61
SV-IV (15 μM)	204 ± 12	65

* The *in vitro* interaction between AT III and SV-IV was studied by incubating at 37° for 15 min, pure AT III (1.45 μM) with various concentrations of SV-IV (0–15 μM) in 60 μL of 50 mM Tris–HCl buffer, pH 7.5. At the end of the incubation period the amount of immunoreactive AT III occurring in 5 μL aliquots of the different incubation mixtures was titrated by a suitable specific immunoassay (see Materials and Methods for experimental details). Each value represents the mean ± SEM of six determinations.

$P < 0.01$, Student's *t*-test.

pronounced increase in cross-linking among SV-IV and other plasma proteins (fibrin, fibronectin and α_2-antiplasmin) able to act as substrates of TGase. If this was the case, the deficiency of factor XIII in the PPP used for the RT assay should abrogate the procoagulant effect of SV-IV. In contrast, it was found that the RT was similarly decreased (about 70%) in the presence of micromolar amounts (5 μM) of SV-IV also when factor XIII-deficient PPP was used instead of normal PPP. This result clearly indicated that SV-IV-induced clotting acceleration was not a consequence of SV-IV polymerization catalysed by the activated factor XIII.

SV-IV accelerated the clotting process by inhibiting the biological activity of AT III

In order to investigate the possible effect of SV-IV on AT III biological activity the concentration of AT III in sera obtained from PPP clotted by $CaCl_2$ in the presence or absence of 5 μM SV-IV was measured using a coagulation technique. It was found that in sera obtained from PPP clotted in the presence of SV-IV the detectable concentration of AT III was 57% lower than in controls (Table 3). Similar results were obtained when AT III was titrated by an immunoassay using specific antibodies against AT III (Tables 3 and 5). To confirm the hypothesis of a direct interaction of SV-IV with AT III pure AT III (Sigma) was incubated at 37° for 15 min with various micromolar concentrations of SV-IV. At the end of the incubation period the amount of immunoreactive AT III was titrated by an appropriate immunoassay. The data reported in Table 4 indicated that the AT III titre progressively decreased with an increase in SV-IV concentration, reaching a minimum at about 5 μM SV-IV. The pattern of the titration curve was, however, biphasic, a further increase in SV-IV concentration produced a parallel increase in the AT III titre. These data strongly suggested that the decrease of immunoreactive AT III in the SV-IV-treated PPP could be related to the formation of a molecular complex between AT III and SV-IV. SV-IV, by masking some specific AT III structural sites, would render the clotting regulator nonreactive with both its specific antibodies and thrombin. Finally, Table 5 shows that the interaction of SV-IV with AT III is rather specific, the titre of other plasma serpins (α_1-antitrypsin, α_2-macroglobulin and C_1-inhibitor) being unmodified by the presence of micromolar concentrations of SV-IV.

Table 5. Immunological evaluation of plasma serpins in PPP incubated in the presence or absence of 5 μM SV-IV*

Plasma serpins	Serpin concentration in PPP (mg/mL)	
	−SV-IV	+SV-IV
AT III	312 ± 25	150 ± 15
α_1-antitrypsin	3340 ± 270	3360 ± 180
α_2-macroglobulin	3240 ± 202	3220 ± 150
C_1-inhibitor	274 ± 18	268 ± 14

* For experimental details see Materials and Methods. The values reported in the table are the means ± SEM of determinations performed in triplicate on 10 blood samples.

$P < 0.01$, Student's *t*-test.

The protein SV-IV completely suppressed the inhibition effect of AT III on thrombin in a coagulation system containing only pure protein components

The data reported in Table 6 clearly show that in a coagulation system constituted only by purified fibrinogen, thrombin, heparin and AT III, SV-IV (5 μM) was able to completely inhibit the potent anti-thrombin effect of pure AT III (1.45 μM). Similar results were obtained when 0.1 mL of diluted PPP was added to the clotting system as source of AT III.

Table 6. Effect of SV-IV (5 μM) on the fibrinogen/thrombin reaction in the presence or absence of AT III (1.45 μM) by using purified proteins*

Addition	Coagulation time (sec) (fibrinogen/thrombin/heparin coagulation system)
None	50 ± 7
SV-IV	52 ± 5
AT III	188 ± 9
AT III + SV-IV	49 ± 8
PPP†	191 ± 6
PPP + SV-IV†	51 ± 4

* Experimental details are described in Materials and Methods. The values reported in the table are the means ± SEM of six determinations performed in triplicate.

† In these assays normal PPP, diluted 1:3 with saline, was used as AT III source, 0.1 mL of diluted PPP being substituted for 0.1 mL of pure AT III in the final volume (0.3 mL) of the coagulation system (see Materials and Methods, and other text).

Table 7. Effect of SV-IV on the RT of PPP from subjects affected by hemorrhagic disorders*

Hemorrhagic disorder	Subject number	RT (sec)	
		−SV-IV	+SV-IV (5 μM)
None (control)	10	200 ± 15	72 ± 8
Thrombocytopenia	10	350 ± 25	240 ± 16
WHS†	10	605 ± 42	256 ± 23
Hepatic cirrhosis	10	387 ± 30	230 ± 18

* For experimental details see Materials and Methods. The values reported in table are the mean ± SEM of determinations performed in triplicate on three blood samples.

† WHS = Warfarin-related hemorrhagic syndrome.

$P < 0.01$, Student's *t*-test.

SV-IV accelerated blood clotting in PPP obtained from patients affected by some hemorrhagic disorders

SV-IV acts as a procoagulant factor *in vitro* for PPP obtained from patients affected by thrombocytopenia, hepatic cirrhosis and Warfarin-dependent hemorrhagic syndrome. In these pathologies, in which a reduced prothrombinase activity is frequently detectable, the RT of PPP was markedly decreased by *in vitro* addition of 5 μM SV-IV to the clotting system (Table 7).

DISCUSSION

The authors have recently demonstrated that SV-IV possesses a potent inhibitory effect on rodent platelet aggregation both *in vitro* and *in vivo* [21]. This, therefore, prompted investigation of the effects of this protein on human blood coagulation. The data reported in this paper demonstrate that SV-IV acted *in vitro* as a procoagulant factor by specifically inhibiting AT III activity. In fact, no evidence of interference of SV-IV with other steps of the coagulation cascade or with other major plasma serpins has been found. It is known that the activated platelets enhance the blood coagulation process by producing a glicoprotein V derived peptide that, by binding to thrombin, is able to prevent the inhibition of this protease by AT III [33]. In contrast, the protein SV-IV seemed to enhance the activity of thrombin generated during blood coagulation by a direct inhibition of AT III. These data demonstrated that by interacting with AT III SV-IV led to the formation of a specific AT III/SV-IV molecular complex. It is worth noting that the maximum effectiveness of SV-IV as inhibitor of AT III was found to be about 5 μM, the inhibitory activity decreasing progressively when higher concentrations of SV-IV were used. This fact was not surprising because similar results were obtained when other biological properties of SV-IV were considered [17, 21, 34]. It was recently demonstrated that in aqueous solution at neutral pH SV-IV acted as a self-associating system [6] in which the degree of association (monomer–trimer equilibrium) was apparently related to its biological activity [35, unpublished results]. In particular, there is evidence that the immunosuppressive activity of SV-IV is detectable only at concentrations lower than 30 μM [17], where the monomeric form is highly represented. Also, the inhibitory activity of SV-IV on IL-1 release and biological activity [34] was found to be detectable only at SV-IV concentrations at which sufficient active monomeric form was present in the system. The authors hypothesize that the inhibitory effect of SV-IV on the biological activity of AT III might be due to a structural interference of the protein SV-IV with the partial insertion of the serpin reactive bond loop as the S4 strand in the six-stranded A β-sheet, a critical event that normally allows the protease inhibitor to tightly bind thrombin in a structurally irreversible inhibitory trap [36, 37]. This hypothesis is supported by recent experiments showing the conversion of AT III from an inhibitor to a substrate of thrombin with enhanced conformational stability by the binding of a synthetic tetradecapeptide corresponding to the P1–P14 region of the putative reactive bond loop of the inhibitor [37]. It is well known that the AT III-induced inhibition of thrombin is generated by the formation of an equimolar complex between the two proteins [38]. Therefore, any reduction in AT III activity would cause blood hypercoagulability and would predispose to thrombosis as a consequence of a decreased thrombin inhibition. The association between AT III deficiency and recurrent venous thrombosis was first reported by Egeberg [39] in a Norwegian family. On the other hand, the presence of a specific mutation in the AT III active site (Ser394 → Leu), by generating the appearance of inactive AT III Denver in the plasma, is known to produce a strong tendency to thrombosis in affected individuals [40]. In theory, any blood hypocoagulability condition caused by a prothrombinase defect could be by-passed by stimulating thrombin activity through AT III inhibition. This type of hemorrhagic disorder seems to be the most

appropriate for a putative utilization of SV-IV or some SV-IV peptide derivative in the prevention and therapy of the pathologies afflicting these patients. This possibility appears to be particularly attractive by taking into account the SV-IV effect on RT observed with PPP obtained from patients affected by hemorrhagic diathesis of various origins (thrombocytopenia, hepatic cyrrosis, Warfarin-dependent hemorrhagic syndrome).

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