SV-IV Peptide1–16 reduces coagulant power in normal Factor V and Factor V Leiden

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Abstract

Native Factor V is an anticoagulant, but when activated by thrombin, Factor X or platelet proteases, it becomes a procoagulant. Due to these double properties, Factor V plays a crucial role in the regulation of coagulation/anticoagulation balance.

Factor V Leiden (FVL) disorder may lead to thrombophilia. Whether a reduction in the activation of Factor V or Factor V Leiden may correct the disposition to thrombophilia is unknown. Therefore we tested SV-IV Peptide 1–16 (i.e. a peptide derived by seminal protein vesicle number IV, SV-IV) to assess its capacity to inhibit the procoagulant activity of normal clotting factor V or Factor V Leiden (FVL). We found that SV-IV protein has potent anti-inflammatory and immunomodulatory properties and also exerts procoagulant activity. In the present work we show that the SV-IV Peptide 1–16, incubated with plasma containing normal Factor V or FVL plasma for 5 minutes reduces the procoagulant capacity of both substances. This is an anticoagulant effect whereas SV-IV protein is a procoagulant. This activity is effective both in terms of the coagulation tests, where coagulation times are increased, and in terms of biochemical tests conducted with purified molecules, where Factor X activation is reduced.

Peptide 1–16 was, in the pure molecule system, first incubated for 5 minutes with purified Factor V then it was added to the mix of phosphatidylserine, Ca2+, Factor X and its chromogenic molecule Chromozym X. We observed a more than 50% reduction in lysis of chromogenic molecule Chromozym X by Factor Xa, compared to the sample without Peptide 1–16. Such reduction in Chromozym X lysis, is explained with the reduced activation of Factor X by partial inactivation of Factor V by Peptide 1–16. Thus our study demonstrates that Peptide 1–16 reduces the coagulation capacity of Factor V and Factor V Leiden in vitro, and, in turn, causes factor X reduced activation.

Introduction

We have recently studied the Protein SV-IV (Seminal vesicle number IV) [1-3]. According to its mobility in SDS/PAGE, SV-IV protein is a basic, thermostable, secre-
to activated Protein C, which is altered in carrier of FVL as
compared [19-21]. The absent lysis in FVL causes throm-
bohilia by activating Factor X, therefore leading to an
increase of the procoagulant potency, because normal
Factor V is joined with FVL, that has not been broken
down by activated Protein C. Therefore, we tried to reduce
the procoagulant activity of normal Factor Va and also of
FVL activated by Peptide1–16. Then, we also evaluated the
activities of Peptide1–16 incubated with plasma contain-
ing normal Factor V or FVL in diversified coagulation sys-
tems in vitro and/or incubated with Factor V in a system
of pure molecules.

Methods
INBIOS Company, via Olivetti 1, Pozzuoli, Naples, Italy
provided the organic synthesis on solid phase of SV-IV
Peptide 1–16, since now is well known amino acid
sequence of Protein SV-IV. The amino acids sequence is
RKTKEKYSQSEEVVSE. The Thrombosis Center of the Insti-
tuto Clinico Humanitas, via Manzoni 56, Rozzano, Milan,
Italy and the Department of Internal Medicine of the
Buon Consiglio Fatebenefratelli Hospital of Naples pro-
vided plasma samples taken from twelve patients carriers
of FVL.

Selected patients carriers of FVL were affected by previous
thrombotic disease except 2 subjects that had not experi-
enced any thrombotic manifestation in their anamnesis
and that have been monitored for a couple of years.

Blood samples were obtained by venipuncture from
antecubital vein and collected in sodium citrate 0.11 M
tubes in 9:1 v/v in three aliquots of 5 mL and also in EDTA
in one aliquot of 5 mL from all subjects.

All samples were tested to confirm the presence of FVL
in order to confirm the thrombophilic state. The presence
of FVL was confirmed with DNA extraction from the sample
in EDTA and using the "NUCLEON BACC" kit (Amers-
shan, Germany); Factor V G1691A gene polymorphism
(i.e. FVL) was researched using PCR amplification with
specific primers and the Light Cycler apparatus (Roche,
Milan, Italy). Behring Institute (Protein C reagent, Dade
Bhering, Milan, Italy) provided the kit to assess resistance
to activated Protein C, which is altered in carrier of FVL as
it contains Agkistrodom Contortix venom as the Protein C
activator.

Factor V Leiden (FVL) is a gene variant of clotting Factor V
and it is responsible for the major forms of activated Pro-
tein C resistance. The structural difference of normal Fac-
tor V compared to Factor V Leiden consists in a substi-
tution of an Arg with Gln in position 506, that is
responsible for FVL absent lysis by the activated Protein C,
but as far as factor X activation is concerned they are com-
parable [19-21]. The absent lysis in FVL causes throm-
bohilia by activating Factor X, therefore leading to an
increase of the procoagulant potency, because normal
Factor V is joined with FVL, that has not been broken
down by activated Protein C. Therefore, we tried to reduce
the procoagulant activity of normal Factor Va and also of
FVL activated by Peptide1–16. Then, we also evaluated the
activities of Peptide1–16 incubated with plasma contain-
ing normal Factor V or FVL in diversified coagulation sys-
tems in vitro and/or incubated with Factor V in a system
of pure molecules.

Factors and immunomodulatory properties and also exerts
procoagulant activity [4,10-15]. We sought to find a corre-
lation between a function of the molecule and its structure
and to assess if portions of the protein, fragments or pep-
tides, individually tested, would reveal one or more of the
molecule's reported functions [4,10-15]. We already
showed that the fragment 1–70 of SV-IV is responsible for
the procoagulant function of the molecule [14-18]. We
decided to study a small segments of such fragment 1–70
in order to assess which sequence was specifically respon-
sible of its activity [4,17]. We synthesised small peptides
such as SV-IV Peptide 1–8, Peptide 8–16 and Peptide 1–
16 of the fragment 1–70 [4].

Factor V is synthesized by megacaryocytes, endothelial
cells and hepatocytes. Native Factor V is an anticoagulant,
but when activated by thrombin, Factor X or platelet pro-
teases, it becomes a procoagulant. Physiologically, it can
be activated by Factor X activated (Xa) and platelet pro-
teases but usually it is largely activated by thrombin, so
Factor V becomes Factor V activated (Va) [19]. It is a large
protein with a molecular weight of 300.000 d. Factor V
binds the phospholipids of cellular membranes, such as
platelets, endothelial cells, and monocytes, in the pres-
ence of calcium, and forms a receptor for Factor X and Pro-
thrombin [20,21]. This last complex, named
Prothrombinase, is responsible for cleaving prothrombin
to form thrombin. This complex is much more efficient
than Factor Xa alone in the prothrombin conversion. The native
(i.e. inactivated) Factor V, however, is an anticoag-
ulant. Native Factor V and Protein S are synergistic co-fac-
tors in the anticoagulant-inhibitory action of the activated
Protein C [19,21], which inhibits coagulation by breaking
down Factor Va (i.e. after its activation) and Factor VIIIa
thus leading to a slow thrombin generation.

When activated, Factor V loses this anticoagulant property
[19]. Consequently, due to its double properties in its
native and activated forms, Factor V plays a relevant role
in coagulation/anticoagulation balance [4,19]. Should
Factor V anticoagulant capacity be utilised, it would be
sufficient to inhibit or reduce the activation of Factor V.
The activated partial thromboplastin time (aPTT) reagent (Ellagic acid and phospholipids) necessary for aPTT technique was also provided by Bhering Institute (Thromboplastin time, Milan, Italy).

Sigma, Milan, Italy, provided Factor V deficient plasmas, purified Factor V, purified Factor X, Phosphatidylserine and Chromozym X (Human Factor V deficient plasma, Sigma, Milan, Italy; Human Plasma Factor V, Sigma, Milan, Italy; Human Plasma Factor X, Sigma, Milan, Italy; Phosphatidylserine, Sigma, Milan, Italy; Chromozyn X, Sigma, Milan, Italy, respectively).

Once obtained all materials we developed study techniques to assess possible interference of Peptide 1–16 with Factor V and Factor V Leiden. We had to prove before the factor V activation to factor Va: in pure molecules factor V, phosphatidylserine, Ca2+ and factor X system, we attempted to obtain factor X activation to factor Xa by factor V activation to Va; we have varied the physiological concentrations, increasing some component concentration of the system: phosphatidylserine, not phospholipid complex, and factor X increased concentration, because we have further coactivators lacking in vitro. After testing the 50 or 5 or 1 µM peptide1–16 concentrations both with plasmas and pure molecules we noted that 1 mM not produced variation whereas 50 and 5 µM produced same variations and both the concentrations required the incubation time of 5 minutes. Here we report the reagents used to perform the in vitro’s experiences.

1) NaCl solution: 0.15 M
2) CaCl2 solution: 25 mM
3) aPTT reagent (phospholipids plus ellagic acid from the kit)
4) Peptide 1–16 solution: 50 µM
5) Factor V solution: 16.6 µM; concentration in tests 0.166 µM
6) Factor X solution: 61 µM;
7) Phosphatidylserine solution: 12.5 µg/µL
8) solution of Chromozym X substrate: 1.9 mM.

Experimental tests were performed by several study techniques:

- biochemical technique (conducted with purified molecules)

Coagulation techniques included the aPTT test technique, performed using factor V deficient plasma and purified Factor V; moreover a further coagulation technique was performed with Leiden plasmas compared to normal plasmas when incubated with Peptide 1–16 or NaCl as control.

A volume of 50 µl of aPTT reagent's was added to 100 µl of Factor V deficient plasmas and was incubated at 37°C for 1 minute and then 10 µl of purified Factor V incubated with 7 µl of Peptide 1–16 or 7 µl of NaCl for 5 minutes; then 100 µl CaCl2 was added and the coagulation time was recorded.

Coagulation techniques were also performed with Leiden plasma incubated for 5 minutes with Peptide 1–16 or NaCl; to 100 µl Leiden plasma plus 7 µl Peptide 1–16 or NaCl incubated for 5 minutes was added 50 mcl aPTT reagent and, after incubation for 1 minute, was furtherly added 100 µl CaCl2; then the coagulation time was registered. Thereafter the same procedure were performed with normal plasma: we incubated normal plasma for 5 minutes with Peptide 1–16 or NaCl; to 100 µl normal plasma plus 7 µl Peptide 1–16 or NaCl incubated for 5 minutes was added 50 µl aPTT reagent and, after 1 minute, we added 100 µl of CaCl2, then the coagulation time was registered again. We chose to incubate the peptide 1–16 with purified factor V or normal or Leiden plasma for 5 minutes because the incubation for 5 or 7 or 9 minutes gave the same results in previous experiences (data not shown). Five tests were conducted on each plasma sample and the result was given as media with SD for all performed tests.

Biochemical techniques with pure molecules aimed to carry out Factor X activation by Factor Va. For Factor X activation, Factor V and FVL operate in the same way. Once activated, Factor X breaks down its chromogenic substrate Chromozym X. The scission of the Chromozym X produces a coloured molecule and its concentration and colour depended directly on the concentration of activated Factor X. The activation of Factor V to Va, that actives Factor X has been obtained with incubation of phosphatidylserine and Ca2+, and then added to Factor X.

The final concentrations of used molecules are shown below:

10 µl Phosphatidylserine (100 ng) and 10 µl of purified Factor V (0.02 µM) and CaCl2, then incubated at 37°C for 3 minutes. Then the process was: 10 µl Phosphatidylserine added to 10 µl of purified Factor V added to CaCl2
added to 20 µl of purified Factor X (12,2 µM) and incubated at 37°C for 3 minutes. Thereafter, a volume of 1,120 µl NaCl (0,15 mM) was added to 20 µl of Chromozym X. OD readings were taken at 405 nm after 15 minutes in order to know the Chromozym X lysis. Obtained values were recorded as control value of Chromozym X lysis.

We substitute Peptide 1–16 to NaCl and we tested the incubation of the mixture of purified Factor V with Peptide 1–16 for 5 minutes; this mix was then added to the phosphatidylserine, CaCl2 and factor X mixture and incubated for 3 minutes. Then, Chromozym X was added and OD readings were taken at 405 nm after 15 minutes incubation. Obtained values were recorded as results of the inhibiting clotting activities of peptide 1–16 against purified factor V.

Five tests were conducted on each samples. Result represent the media with SD of all performed tests.

Statistical analysis
Statistical analysis was based on the one way analysis of variance (ANOVA); differences were considered to be significant if p was < 0.05. Statistical analysis were carried out using SPSS statistical software.

Results
The coagulation time of aPTT test of normal plasmas and plasmas containing FVL and aPTT reagent plus NaCl and CaCl2 was normal and no differences were found between normal plasmas and Leiden plasmas: 36 +/- 3 seconds and 37 +/- 2 seconds respectively (data not shown). No differences were found between aPTT of normal plasmas and Leiden plasmas at time T0, when we performed the incubation with peptide 1–16 or NaCl (respectively 40 +/- 5 seconds and 38 +/- 3 seconds); when we incubated the Peptide 1–16 with normal or Leiden plasmas for 5 minutes, the coagulation time turned respectively to 96 +/- 6 seconds and 100 +/- 5 seconds reaching statistical significance (96 +/- 6 seconds versus 38 +/- 3 seconds, p: < 0.001, s; and 100 +/- 5 seconds versus 38 +/- 5 seconds, p: 0.001, s) (Table 1).

Moreover, the coagulation time was also obtained by the aPTT test with deficient Factor V plasma and purified Factor V incubated with NaCl or Peptide 1–16 for 5 minutes plus aPTT reagent (Ellagic acid and phospholipids) and CaCl2. When we incubated NaCl, the coagulation time was normal: 35 +/- 4 seconds and 39 +/- 4 seconds respectively; when the Peptide 1–16 substituted NaCl, the coagulation time was significantly longer: 96 +/- 7 seconds, p: < 0.001 (Table 2).

As control of previous experiments, in the biochemical tests, purified Factor V activation was obtained by the incubation for 3 minutes with phosphatidylserine, CaCl2, in presence of factor X with Chromozym X, and NaCl (as control of next tests) in order to activate Factor X with pure molecules: the Factor X incubated for 3 minutes and Chromozym X, incubated for 15 minutes. We obtained a coloured product with an optical density (O.D.) of 0.420 nm. On the other hand, when we incubated Factor V with Peptide1–16 (instead of NaCl) for 5 minutes and then added to the mixture of phosphatidylserine and CaCl2 with subsequent addition of factor X and Chromozym X, we obtained a significantly different optical density (O.D.) of 0.190 nm, p: < 0.0001, s (Table 3).

Discussion
Whether factor V has sufficient endogenic activity to allow small amount factor Xa generation is still a matter of debate [22]. Data from literature report that factor V circulates as a single chain protein with about 0.27% of proteolitic activity comparable to Va [23]. Foster et al.[24] postulate that factor V appears to be a procofactor with 1/

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**Table 1**: aPTT coagulation time of normal plasma and Leiden plasma (100 µL) with and without incubation for 5 minutes with peptide 1–16 or NaCl as control.

| Plasmas incubated with peptide 1–16 | aPTT coagulation time (sec.) | Plasmas incubated with NaCl | aPTT coagulation time (sec.) | p |
|-------------------------------------|------------------------------|-----------------------------|------------------------------|---|
| Normal plasma incubated at T0 with peptide 1–16 | 40 ± 5 | Normal plasma incubated at T0 with NaCl | 38 ± 3 | 0,16 |
| Leiden plasma incubated at T0 with peptide 1–16 | 41 ± 4 | Leiden plasma incubated at T0 with NaCl | 40 ± 4 | 0,59 |
| Normal plasma incubated with peptide 1–16 at T1 | 90 ± 6 | Normal plasma incubated at T1 with NaCl | 37 ± 4 | 0,000 |
| Leiden plasma incubated at T1 with peptide 1–16 | 120 ± 9 | Leiden plasma incubated at T1 with NaCl | 38 ± 3 | 0,000 |

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aPTT: activated partial thromboplastin time
sec.: seconds
T0: time of incubation
T1: 5 minutes after the incubation
NaCl: sodium clorure
400 the activity of full activated factor V (i.e. factor Va). The proteolytic conversion of factor V to factor Va is catalysed by thrombin and Xa. However, because factor Va is required for thrombin generation, the initial participation of factor V in the expression of prothrombinase activity is not well understood [24]. Orfeo et al. demonstrated [25] that prothrombinase complex consists of the protease factor Xa, Ca2 and factor V assembled on phospholipid membrane. Factor Va functions both as a receptor for factor Xa and as a positive effector for factor Xa catalytic efficiency, thus being a key factor for an efficient conversion of prothrombin to thrombin. The activation of the procofactor factor V to factor Va is an essential reaction that occurs early in the process of tissue factor pathway, however the catalytic sequence leading to formation of factor Va is still a controversial issue [25]. We attempted, by a system consisting of pure molecules (i.e. factor V, phosphatidylserine, Ca2 and factor X) to obtain factor X activation to Xa through factor V activation to factor Va. We varied the physiological concentrations, increasing the same components of the system and chose to work only with phosphatidylserine and not phospholipid complex and to increase factor X concentration. This system produced factor X activation to Xa. We tested factor Xa peptidolytic activity with Chromozym X, that is a specific chromogenic molecule of factor Xa; when we added Chromozym X to this system, it generated colour, indicating that factor X in our system became Xa. Then, we incubated purified factor V with Peptide 1–16 for 5 minutes, and thereafter we added it to the same mixture, in order to prove if this Peptide 1–16 hampers factor V activation. In this system, in fact, factor X activation is reduced, as demonstrated by reduced lysis of Chromozym X.

FVL is an inherited condition leading to thrombophilia through the activated protein C resistance. In presence of FVL, the activated Protein C does not breakdown FVL, that accumulates so increasing the global coagulative power in the coagulative cascade and impairing the coagulation\anticoagulation balance. The results of our experiments demonstrate clearly that Peptide1–16 inhibits the activity both of FVL and of normal clotting factor V, in particular its ability to activate Factor X. We demonstrated that the Leiden Plasmas incubated with the Peptide 1–16 cause an increase in the coagulation time in the test of aPTT, as reported in Table 1.

Data obtained by incubating Peptide 1–16 with purified Factor V for 5 minutes, then added to factor V deficient plasma, confirmed such results. In fact when we added this mixture to deficient Factor V Plasmas in an aPTT test we found a prolonged coagulation time compared to control (i.e. with NaCl without peptide 1–16), as reported in Table 2.

Moreover, considering that in the Factor X activation, Factor V and FVL operate in the same way, to verify Factor X activation, we only tested the ability of purified Factor V activated by pure molecules as phosphatidylserine and CaCl2. Then we added this mixture to Factor X and Chromozym X for 15 minutes. Yet, if we incubate for 5 minutes Factor V and Peptide 1–16 and we add them to the mixture of pure molecules (phosphatidylserine and CaCl2) and thereafter to Factor X and Chromozym X, we observed that Factor X activation caused a

| Test                              | aPTT coagulation time (sec.) | Test                              | aPTT coagulation time (sec.) | p     |
|-----------------------------------|------------------------------|-----------------------------------|------------------------------|-------|
| Deficient factor V plasma added to purified factor V and NaCl | 36 ± 4                      | Deficient factor V plasma added to purified and peptide 1–16 | 119 ± 7                      | 0.000 |

**Table 2: aPTT coagulation time of deficient factor V plasma added to purified factor V (100 µL) and incubated with peptide 1–16 or NaCl as control.**

| Parameter                | Mixture with NaCl | Mixture with peptide 1–16 | p     |
|--------------------------|-------------------|---------------------------|-------|
| O.D. (nm) of Chromozym X | 0.420 ± 0.008     | 0.190 ± 0.006             | 0.000 |

**Table 3: Factor X activation test (O.D.): purified factor V (0.002 µM) incubated with peptide 1–16 or NaCl for 5 minutes and thereafter added to phosphatidylserine and CaCl2 and factor X and Chromozym X for 15 minutes.**

O.D.: optical density

nm: nanometers

CaCl2: calcium chloride

NaCl: sodium chloride
reduced lysis of Chromozym at an optical density of 0.190 nm. Therefore, Factor X activation is significantly slower compared to control (i.e. NaCl and purified Factor V), and this is confirmed by a reduced lysis of Chromozym X (i.e. O.D. = 0.190).

The occurring contact of the two molecules, Factor V and Peptide1–16, is proved by the fact that both incubated at time 0 and added to phosphatidylserine and CaCl2 produced a different effect compared to that obtained if we incubate Factor V, alone for 5 minutes, without Peptide 1–16. This suggests that the observed events were caused by the incubation of the two molecules and that 5 minutes are needed for their effective interaction. Our results indicate that Peptide 1–16 is not only an inhibitor of normal clotting factor V but also of FVL. So, Peptide 1–16 may represent an inhibitor of clotting cascade also in presence of inherited thrombophilic disorder leading to hypercoagulable state as FVL. We demonstrated, in fact, the inhibition of clotting cascade in presence of normal factor V and also in the presence of plasmas containing FVL by both coagulation techniques and/or biochemical approaches.

One may argue that in our studies we used purified factor V to test the normal cascade and FVL from plasma of subjects carrying this kind of alteration. However we point out that in biochemical tests, normal purified factor V alone was incubated with Peptide 1–16 while purified FVL was not incubated with Peptide 1–16 because purified FVL is not available commercially both for experimental studies and/or clinical approaches. Moreover it is really difficult to obtain purified FVL from plasma of subjects carrying this alteration. Yet, the presence of purified FVL is not necessary for our studies because the role of normal Factor V and Factor V Leiden in Factor X activation is comparable.

In conclusion, our study demonstrates that Peptide1–16 may represent a specific antagonist of FVL and one may envision its use in the thrombophilic FVL patients, should further studies confirm our results.

Competing interests
The authors declare that they have no competing interests, nor any financial supports during patients’ selection, experimental tests and article extension.

Authors’ contributions
BDM and ML performed all biochemical tests described in the paper with the exception of genetic tests for factor V Leiden gene variant; IQ and PF performed genetic test to identify factor V Leiden gene variant; LR, GDM and PDM performed patients’ selection; PDM and BDM performed paper extension.

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