División de Ciencias de la Salud
Facultad de Medicina

EL COMPLEJO FACTOR VIIa - FACTOR TISULAR Y SU PAPEL COMPENSATORIO EN LAS DISFUNCIONES HEMOSTÁTICAS

Tesis presentada por Raúl Tonda Hernández, licenciado en Biología por la Universidad de Barcelona para optar al grado de Doctor.

Tesis dirigida por el Dr. **GINÉS ESCOLAR ALBALADEJO** y la Dra. **ANA MARÍA GALÁN SILVO.**

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4. ARTÍCULOS PUBLICADOS POR EL DOCTORANDO

4.1 ARTICULOS INCLUIDOS EN ESTA TESIS

Galan AM, **Tonda R**, Altisent C, Maragall S, Ordinas A, Escolar G. Recombinant factor VIIa (Novoseven) restores deficient coagulation: experience from an ex vivo model. Semin Hematol 2001; 38(4 Suppl 12):10-14.

Galan AM, **Tonda R**, Pino M, Reverter JC, Ordinas A, Escolar G. Increased local procoagulant action: a mechanism contributing to the favorable hemostatic effect of recombinant FVIIa in PLT disorders. Transfusion 2003; 43(7):885-892.

Tonda R, Galan AM, Pino M, Cirera I, Bosch J, Hernandez MR et al. Hemostatic effect of activated recombinant factor VII (rFVIIa) in liver disease: studies in an in vitro model. J Hepatol 2003; 39(6):954-959.

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Tonda R, Lopez-Vilchez I, Galan AM, Navalon F, Pino M, Hernandez MR et al. Tissue Factor immobilized on surfaces promotes platelet adhesion and fibrin formation under flow conditions: importance of shear rate and FVIIa. 2007.

Tonda R, Lopez-Vilchez I, Pino M, Altisent C, Escolar G, Galan AM. Recombinant FVIIa (rFVIIa) improves platelet dysfunction in patients with hemophilia: studies under flow conditions with collagen-tissue factor surfaces. 2007.

Recombinant Factor VIIa (NovoSeven®) Restores Deficient Coagulation: Experience From an Ex Vivo Model

Ana Maria Galán, Raul Tonda, Carmen Altisent, Santiago Maragall, Antonio Ordinas, and Gines Escolar

The action of recombinant factor VIIa (rFVIIa) in coagulation deficiencies with increased risk of bleeding was investigated using in vitro perfusion. Blood samples were drawn from healthy donors, a patient with hemophilia A and inhibitors, and six patients undergoing oral anticoagulant treatment. Fragmin 10 U/mL was used as anticoagulant. rFVIIa (10 μg/mL in plasma) was added to blood samples, incubated for 1 minute at 37°C, and perfusion studies performed for 10 minutes at 600 s^{-1} through annular chambers containing damaged vascular segments. Subendothelial fibrin and platelets were expressed as a percentage of subendothelial surface screened. Under different conditions, rFVIIa consistently restored or improved fibrin formation on the damaged vascular subendothelium exposed to circulating blood. It restored fibrin deposition in blood from the hemophilia A patient; in patients undergoing acenocoumarol treatment, it reduced the international normalized ratio (INR) from 2.47 to 1.25 with a significant increase in fibrin deposition. Platelet deposition varied slightly between clinical conditions but was less evident in the hemophilia A patient. These data support the concept that rFVIIa facilitates fibrin formation in these clinical situations, promoting procoagulant activity at sites of vascular damage where tissue factor is exposed. This could improve hemostasis in patients with hemophilia A and inhibitors, and in patients treated with oral anticoagulants.
Semin Hematol 38(suppl 12):10-14. Copyright © 2001 by W.B. Saunders Company.

RECOMBINANT FACTOR VIIa (rFVIIa, NovoSeven®, Novo Nordisk, Copenhagen, Denmark) has been reported to be clinically effective in patients with FVII deficiency[1] and is being successfully used in the control of bleeding episodes in hemophilic patients who have developed inhibitors.[2-4] The rationale for the mechanism of action for FVIIa in hemophiliacs has been investigated in detail in cellular models,[5,6] but the evidence has not been confirmed clinically.

Patients taking oral anticoagulants have an increased risk of bleeding.[7,8] The prognosis of patients with bleeding episodes is adverse when the cerebrovascular territory is affected.[9] Treatment with plasma or concentrates containing activated factors can improve the outlook in such circumstances.[7,8,10,11] There is experimental[12] and clinical evidence to show that preparations containing FVIIa can be used to reverse the hypocoagulable state that develops in individuals taking oral anticoagulants.[13,14]

Perfusion techniques have facilitated the investigation of mechanisms involved in hemostasis. A technical variation of the original technique has proved useful to assess the procoagulant action of different platelet preparations[15] and to evaluate the potential thrombogenic profile of activated coagulation factors.[16] Here we describe in vitro perfusion techniques that have been used to gain more knowledge about the mechanisms of action of rFVIIa in clinical conditions where coagulation is impaired.

Materials and Methods

Patients, Blood Collection, and International Normalized Ratio Determinations

Blood samples were drawn from healthy donors, one patient with severe hemophilia A who had developed inhibitory antibodies to FVIII, and a group of patients taking oral anticoagulants. The patient with hemophilia A was an 18-year-old man with a severe deficiency in FVIII who had developed antibodies 13 years previously. He was also human immunodeficiency virus (HIV)-negative and hepatitis C virus–positive with normal hepatic function. Immunotolerance protocols had been unsuccessful and at the time of the extraction inhibitor titers were at 6 Bethesda units.

Patients taking oral anticoagulants were randomly chosen from those attending our clinic for control of oral anticoagulation (aceno-

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coumarol). None of the participants had taken drugs affecting platelet functions over the previous 10 days. All individuals gave their informed consent to participate in the study.

For determinations of the international normalized ratio (INR), a sample of blood was obtained in citrate 0.129 mol/L. Citrated blood specimens were centrifuged and INRs were calculated using a Sysmex, CA-6000 (Dade Behring, Marburg, Germany) testing plasma samples with reference preparations of thromboplastin (Tromborel S, Dade Behring).

Preparation of Perfusates

Blood samples were anticoagulated with low-molecular-weight heparin (LMWH; Fragmin, Pharmacia & Upjohn, Stockholm, Sweden) at a concentration of 10 U/mL. This concentration of LMWH maintains anticoagulation but allows fibrin formation once blood is exposed to a thrombogenic surface.[17] To test the effects of rFVIIa, blood samples were incubated for 1 minute with a neutral diluent (saline) or with volumes of the recombinant factor calculated to reach a concentration of 10 μg/mL in plasma. Concentrations chosen correspond approximately to 180 μg/kg bodyweight.

Perfusion Studies and Morphometric Evaluation

After incubation, blood samples were immediately perfused through annular chambers containing denuded arterial segments, as described by Baumgartner.[18] Blood was recirculated for 10 minutes at 37°C through the perfusion system by means of a peristaltic pump with the flow previously adjusted to rise to a shear rate equivalent to 600 s^{-1}. At the end of the perfusions, segments were rinsed with 20 mL of phosphate-buffered saline (pH 7.2), and fixed with the same buffer containing 2.5% glutaraldehyde. The fixed segments were histologically processed as described by Escolar et al.[15]

Fibrin deposition and platelet interactions with the subendothelium were evaluated using a light microscope equipped with a split prism. A specially devised computer program automatically classified and quantified platelet and fibrin coverage,[19] following the method described by Turitto and Baumgartner.[20] For simplicity, platelet interactions were globally expressed as the total percentage of the surface of the vessel covered by platelets (% covered surface = %CS). The presence of fibrin in the same microscopic fields was also expressed as a percentage of fibrin (%F) deposited on the surface length of the vessel screened.

Statistics

Results were expressed as mean ± SEM. Student's *t* test for paired data was used for comparisons before/after perfusion. The level of statistical significance was established at $P < .05$.

Results

Control Studies

The percentages of the subendothelial surface covered by platelets in control experiments with normal blood anticoagulated with LMWH at 10 U/mL reached values of 24% ± 3.7%. The percentage of fibrin deposition in the same microscopic fields reached values of 50% ± 10%.

Effects of rFVIIa in Blood From a Hemophilia Patient With Inhibitor

Perfusion of blood from a patient with hemophilia A and demonstrated inhibitor through annular chambers containing denuded vessel segments resulted in %CS values of 34% ± 8%, which was slightly above values observed in control experiments. Fibrin deposited on the exposed subendothelium was dramatically reduced to less than 10% (Fig 1).

In vitro addition of rFVIIa at 10 μg/mL of plasma to blood from this patient dramatically enhanced levels of fibrin generated on the subendothelium during perfusions. Percentages of the subendothelial surface covered by platelets (%CS, 21% ± 9%) were slightly reduced with respect to baseline levels. Despite the presence of a FVIII inhibitor at 6 Bethesda units, rFVIIa was able to restore levels of fibrin deposition to levels slightly superior to those observed in control studies (%F, 68% ± 8%). The quality of the fibrin masses formed was structurally indistinguishable from those formed in perfusion studies with normal blood.

Effects of rFVIIa Blood From Patients Taking Oral Anticoagulants

Baseline INR values in blood samples from the population of patients taking oral anticoagulants used in our studies ranged from 1.8 to 4.35 with an average of 2.47. Addition of 10 μg/mL rFVIIa to these blood samples corrected the INR values to an average of 1.25.

Rates of platelet and fibrin interaction were slightly decreased with respect to those found in

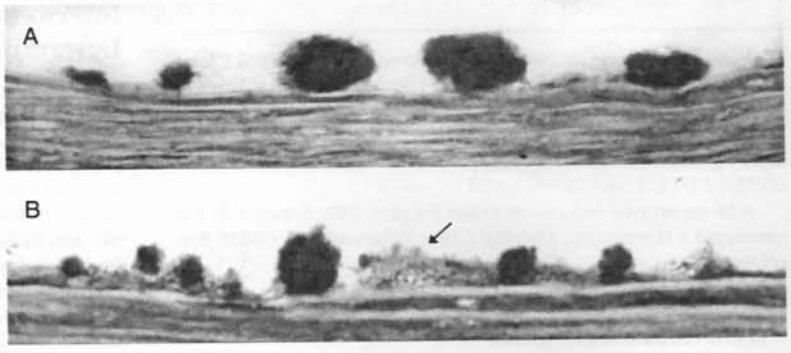

Figure 1. Light micrographs show fields observed in perfusion studies of blood from a patient with hemophilia A with inhibitor (6 Bethesda units) before (A) or after (B) the addition of 10 μg/mL rFVIIa. Fibrin formation was almost absent in baseline studies and improved dramatically after addition or rFVIIa (arrow). However, the presence of platelet aggregates and their size seemed slightly reduced with respect to baseline. (Original magnification: A = 450×, B = 560×.)

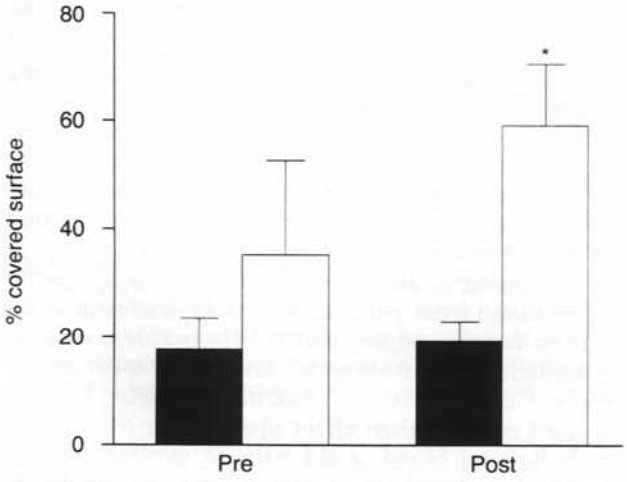

Figure 2. Bar diagrams of morphometric results calculated in perfusion studies of blood from patients under oral anticoagulant therapy (n = 6). Average INR in baseline samples was 2.47 and returned to 1.25 after addition of rFVIIa (10 μg/mL). Deposition of platelets (□) remained basically unchanged or slightly increased. A statistical significant increase ($P < .05$) in fibrin formation (■) was observed in perfusion studies performed in the presence of rFVIIa.

perfusion studies with blood from normal donors. Deposition of platelets on the subendothelium (%CS) perfused with blood from this group of patients averaged 18% ± 5.2% (n = 6) with fibrin covering 35% ± 17.9% of the screened surface (Fig 2). In vitro addition of rFVIIa at 10 μg/mL of plasma to aliquots of the same blood did not modify the overall interaction of platelets with the subendothelium, but dramatically enhanced levels of fibrin generated during perfusions. Percentages of the subendothelial surface covered by platelets (%CS, 19% ± 3.6%) were slightly increased with respect to baseline levels. The presence of rFVIIa in the perfusates was able to restore levels of fibrin deposition to levels similar to those observed in control studies (%F, 58% ± 11%). Figure 3 illustrates modifications in the morphometric parameters already mentioned.

Discussion

The present study has investigated possible mechanisms through which rFVIIa could reverse deficiencies of blood coagulation known to result in bleeding complications. Two situations were explored, each with a different risk of occurrence and severity of bleeding complications. With this in mind, we chose a patient with severe hemophilia A complicated by an inhibitor, a condition known to result in frequent and severe bleeding. The results from this patient were compared with those from studies performed on blood from patients taking oral anticoagulants, a treatment known to reduce levels of vitamin K–dependent factors. Risk of bleeding is usually reduced in such patients, although the prognosis may be poor if the cerebrovascular area is affected. Data from our studies provide morphological evidence supporting the concept that rFVIIa facilitates fibrin formation in both clinical conditions. This mechanism of action could explain the beneficial action of this recombinant factor in the correction of abnormal hemostasis in different clinical situations where coagulation deficiencies result in bleeding complications.

There is unquestionable evidence that rFVIIa is an effective treatment in the control of bleeding episodes in patients with hemophilia who had developed inhibitors.[2,4,21-23] Experimental studies have demonstrated that the interaction of FVIIa with tissue factor has a bypassing effect on coagulation mechanisms which is the key for the hemostatic action of this activated factor in patients with hemophilia and inhibitors.[24]

It is difficult to test experimental hypotheses, for the models cannot easily be replicated in a live situation. Patients suffering bleeding episodes are frequently treated on an emergency basis, with control of the bleeding taking priority over further investigations. Perfusion devices offer the possibility of studying mechanisms of hemostasis in vitro. A damaged vessel is exposed to circulating blood and the use of LMWH as anticoagulant facilitates the study of platelet- and coagulation-mediated mechanisms.[15]

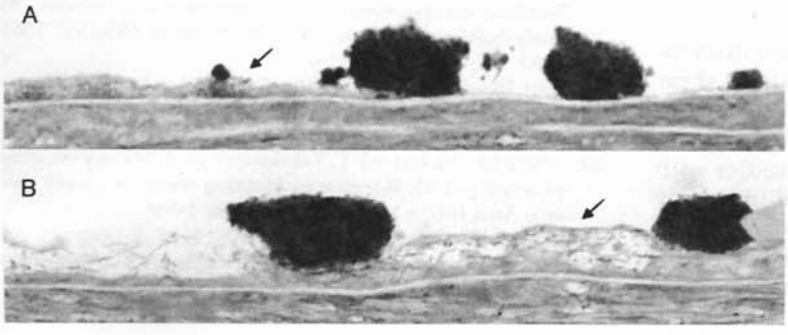

Figure 3. Light micrographs of fields observed in perfusion studies of blood from one patient under treatment with an oral anticoagulant before (A) or after (B) addition 10 μg/mL rFVIIa. Fibrin formation (arrows) was improved in the presence of rFVIIa. (Original magnification: A = 450×, B = 450×.)

Therapeutic agents can be tested in vitro avoiding unnecessary exposure of patients to drugs. In the studies reported here we found that the use of 10 U/mL of LMWH as anticoagulant provided maximal sensitivity to detect the effects of rFVIIa in studies with blood from patients with pre-established coagulation deficiencies.

According to more recent information, FVIIa would always require exposure of tissue factor on the subendothelium or on activated monocytes to initiate coagulation mechanisms.[5,6,25] The participation of tissue factor in mechanisms of hemostasis has been previously investigated by Weiss and Lages.[26] Exposure of tissue factor at sites of vascular damage would initiate a coagulation mechanism that would lead to thrombin generation. The thrombin generated would be important not only for fibrin generation, but also for platelet activation leading to primary arrest of bleeding.[27] Under the different clinical and experimental conditions used in our studies, rFVIIa was consistently able to restore or enhance fibrin formation on the damaged vascular subendothelium exposed to the circulating blood samples. However, the effects on platelet deposition varied between the hemophilia and the decreased vitamin K–dependent factors.

Early studies in perfusion models using native blood had reported a reduction of fibrin formation in studies with blood from patients with hemophilia A.[28] Our present data confirm this observation in a patient with severe hemophilia with a FVIII inhibitor. It is worth mentioning that despite the known elevated risk of spontaneous bleeding in such patients, the function of platelets is usually normal. In fact, interaction of platelets with the subendothelium in our hemophilia case was absolutely normal or even slightly elevated with respect to that observed in normal individuals. Interestingly, while a dramatic increase in fibrin formation was observed in studies with blood from the hemophilic patient in the presence of rFVIIa, the deposition of platelets and the size of the aggregates was apparently reduced. That phenomenon was not observed in studies performed with blood from patients receiving oral anticoagulant therapy in whom fibrin deposition was enhanced, but rates of platelet interaction seemed to remain constant or slightly elevated.

A possible explanation for the apparently discrepant results could be that at the intermediate shear rates used in our studies (600 s^{-1}) fibrin formation prevails over platelet-mediated events.[29,30] The decreased deposition of fibrin in baseline studies with blood from the hemophilic patient would indirectly facilitate the interaction of platelets with the naked subendothelial surface. Once fibrin formation is restored by addition of rFVIIa, the augmented deposition of fibrin could actually compete with platelets for the available subendothelial surface. With the limited information provided by our morphological studies we cannot rule out that rFVIIa could induce a sudden burst in thrombin generation with a very early fibrin deposition. In this situation, the fibrinogen-derived peptides generated could themselves interfere with platelet adhesive and aggregating capacities. These aspects are currently under investigation in our laboratories.

In summary, our experimental results in studies using blood from patients with deficiencies in coagulation factors suggest that rFVIIa is able to restore coagulation mechanisms by favoring a local procoagulant effect at sites of vascular damage. This increased procoagulant effect observed in our studies with flowing blood could help to explain the improvement of hemostasis in patients with coagulation deficiencies.

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Increased local procoagulant action: a mechanism contributing to the favorable hemostatic effect of recombinant FVIIa in PLT disorders

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BACKGROUND: Recombinant FVIIa (rFVIIa) has been shown to improve hemostasis in patients with thrombocytopenia and to prevent or control bleeding episodes in patients with inherited deficiencies of major PLT glycoproteins, but the mechanism of action is not well understood.

STUDY DESIGN AND METHODS: Effects of rFVIIa on hemostasis were explored with an in vitro perfusion technique. Blood samples, from healthy donors or from patients with congenital defects of PLT glycoprotein IIb–IIIa (GPIIb–IIIa), were anticoagulated with low-molecular-weight heparin. Experimental thrombocytopenia (<6000 PLTs/μL) was induced by a filtration procedure. rFVIIa was added to blood samples at therapeutic concentrations. A severe GPIIb–IIIa impairment was also induced by exposure of normal blood samples to a specific antibody. Perfusion studies were performed through annular chambers containing damaged vascular segments. The presence of fibrin and PLTs on the perfused subendothelium was morphometrically quantified.

RESULTS: Under conditions of experimental thrombocytopenia, addition of rFVIIa enhanced fibrin formation in a dose-dependent manner ($p < 0.05$). Improvements in local fibrin generation and partial restoration of PLT interactions were also observed after incubation of blood from patients with Glanzmann's thrombasthenia with rFVIIa at 5 μg per mL (180 μg/kg). Similar improvements were observed in blood samples incubated with antibodies to GPIIb–IIIa. rFVIIa in whole normal blood also enhanced fibrin formation but PLT deposition was unaffected. Evaluation of prothrombin fragments 1 and 2 in the perfusates confirmed that rFVIIa increased thrombin generation in all cases.

CONCLUSION: Our data indicate that rFVIIa promotes a procoagulant activity at sites of vascular damage. This mechanism could explain the beneficial hemostatic effect of rFVIIa in patients with thrombocytopenia or with Glanzmann's thrombasthenia.

FVIIa present in prothrombin complex concentrates provides their FVIII-bypassing activity.[1,2] Recombinant FVIIa (rFVIIa, NovoSeven, Novo Nordisk, Denmark) has been shown to be clinically effective in patients with FVII deficiency[3] as well as in patients with hemophilia A and B who have developed inhibitors to FVIII and F IX.[4] Treatment with rFVIIa has also shown to improve hemostasis in patients with thrombocytopenia.[5,6] Several clinical studies have confirmed the efficacy of rFVIIa in preventing or controlling bleeding episodes in patients with inherited deficiencies of PLT glycoprotein IIb–IIIa (GPIIb–IIIa).[7–9]

Exposure of tissue factor (TF) at sites of endothelial damage plays a critical role in the initiation of hemostasis in vivo.[10,11] Interaction of TF with FVIIa activates FX and causes the local generation of thrombin which, in turn, facilitates PLT activation.[12] The exposure of anionic phospholipids on PLTs already activated by proteins present at sites of vascular damage provides a further burst of thrombin generation, which maintains hemostasis.[13–15] A recent study suggests that the hemostatic action of rFVIIa in patients with hemophilia could be explained through an enhanced procoagulant action and a reduced fibrinolytic response.[16] While these mechanisms could explain the favorable hemostatic action of activated coagulation factors in cases of severe hemophilia A or B, their effects

ABBREVIATIONS: F1 + 2 = fragments 1 and 2; GPIIb–IIIa = glycoprotein IIb–IIIa; rFVIIa = recombinant FVIIa; TF = tissue factor.

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in patients with severe PLT function impairment are still unknown.

Perfusion models have facilitated the investigation of mechanisms involved in hemostasis.[17–19] We have developed a variation of the original technique, which has allowed us to evaluate the transfusional effectiveness of different therapeutic strategies under conditions of experimental thrombocytopenia,[20] the hemostatic action of different PLT preparations,[21,22] and the potential thrombogenic profile of activated coagulation factors.[23] In the present study, the hemostatic effect of therapeutic concentrations of rFVIIa in human blood under conditions of experimental thrombocytopenia was studied in this modified perfusion model. Furthermore, the potential mechanisms of action of rFVIIa was explored in blood from patients with severe deficiencies of GPIIb–IIIa, because of the low frequency of severe congenital deficiencies of GPIIb–IIIa, our studies included samples of normal blood incubated with specific antibodies known to produce a thrombasthenic like status in exposed patients.[24] Finally, the potential prothrombotic activity of rFVIIa in blood samples from normal donors, incubated with increasing concentrations of this activated factor, was evaluated.

MATERIALS AND METHODS

Blood collection and preparation of perfusates

Blood samples were drawn from healthy donors who had not been exposed to drugs known to affect either PLTs or the coagulation system and from two patients with Glanzmann's thrombasthenia. All participants in the study provided informed consent. Blood samples were always anticoagulated with 20 U per mL low-molecular-weight heparin (Fragmin, Pharmacia & Upjohn, Stockholm, Sweden).[25] For experimental purposes, samples of normal blood (n = 4) were incubated with 10.5 µg per mL abciximab (ReoPro, Centocor B.V, Leiden, Holland), a humanized MoAb specific for human GPIIb–IIIa.[26] To reproduce conditions found in patients with severe thrombocytopenia (<10,000 µL) caused by marrow failure, PLTs and WBCs were filtered from normal blood samples with WBC reduction filters (RC100 PALL Corp., Glen Cove, NY) (n ≥ 6). Details of this procedure have been described elsewhere.[20,27] Before the initiation of perfusion, blood samples were incubated for 1 minute with either diluent in the absence of rFVIIa (baseline) or aliquots of rFVIIa calculated to reach concentrations of 2.5, 5, and 10 µg per mL at the plasma interface (n ≥ 6). These concentrations approximately correspond to 75, 150, and 300 µg per kg body weight if extrapolated to patient therapy.

Experimental design

All experimental procedures were conducted according to a single-blind design. The technician performing the evaluations was not aware of the origin of samples. After incubation, blood samples were immediately perfused through annular chambers containing denuded arterial segments, as described by Baumgartner.[17] Inhibition of TF present on the subendothelium was performed by incubation of vascular vessels with saturating concentrations of a polyclonal antibody against TF (American Diagnostica Inc., Greenwich, CT) for 15 minutes at 37°C (n = 6).

Blood was recirculated for 10 minutes at 37°C with a peristaltic pump with the flow previously adjusted to give rise to a shear rate equivalent to 600 per second. The hemostatic effectiveness was assessed with morphometric procedures to evaluate PLT and fibrin deposition onto the subendothelium of the damaged arterial segments. Aliquots of plasma were obtained before and after perfusion for prothrombin fragments 1 and 2 (F1 + 2) determinations.

Processing of vessel segments and morphometric evaluation

At the end of each perfusion, the arterial segments were rinsed with 20 mL of PBS (pH 7.2), removed from the rod, and fixed with the same buffer containing 2.5 percent glutaraldehyde. The fixed segments were processed histologically, as described elsewhere.[28]

Fibrin deposition and PLT interactions with the subendothelium were evaluated with light microscopy and a specially devised software that automatically classifies and quantifies PLT and fibrin coverage[20,28] following the criteria described by Turitto and Baumgartner.[29] For simplicity, PLT interactions were globally expressed as the total percentage of the vessel surface covered. The presence of fibrin in the same microscopic fields was also morphometrically quantified and expressed as percentage of fibrin deposited on the surface length of the vessel screened.[28]

Evaluation of thrombin generation

The level of thrombin generation during perfusion was monitored through assessment of the F1 + 2 in plasma samples. Aliquots of blood were systematically collected before and after the perfusion. Blood aliquots were immediately mixed with sodium citrate (129 m*M*) to prevent any further activation of the coagulation system. Plasma was separated by centrifugation of the anticoagulated blood samples (1800 × *g* for 20 min) and frozen at –70°C. Levels of F1 + 2 were determined in plasma samples with commercially available EIAs (Enzygnost, Behring, Germany).[30]

Data analysis

Results are expressed as means ± SEM. In experiments performed with whole blood, with thrombocytopenic

blood, and with antibodies against TF for each concentration and controls were at least n = 6. Experiments with blood from Glanzmann's thrombastenia patients were performed with blood samples taken from two different patients (n = 2) and experimental studies with blood samples exposed to a specific MoAb against GPIIb–IIIa was n = 4. A *t* test for paired data was used to compare measurements collected before and after perfusion. The level of statistical significance was established at $p < 0.05$.

RESULTS

Effects of rFVIIa in normal whole-blood samples

Perfusions with normal whole blood in the absence of rFVIIa (baseline) resulted in a PLT coverage surface of 21.7 ± 5.2 percent. Addition of 2.5, 5, or 10 µg per mL rFVIIa to whole samples of normal blood had no effect on PLT interaction (19.48 ± 2.98, 21.83 ± 2.47, and 17.17 ± 1.10%, respectively), but caused a progressive increase in fibrin deposition (46.97 ± 12.39, 52.16 ± 15.98, and 54.87 ± 18.23%, respectively). These differences did not reach the level of significance. These results are summarized in Fig. 1.

F1 + 2 levels measured in the samples obtained before perfusion involving blood from normal donors were 0.54 ± 0.05 n*M*. Incorporation of rFVIIa at the different concentrations tested did not result in any significant difference following the 1-minute incubation period (see Table 1). After perfusion, F1 + 2 levels significantly increased compared with preperfusion values. Statistical differences were observed when levels of F1 + 2 in perfusates containing rFVIIa were compared with the corresponding control studies with diluent (Table 1).

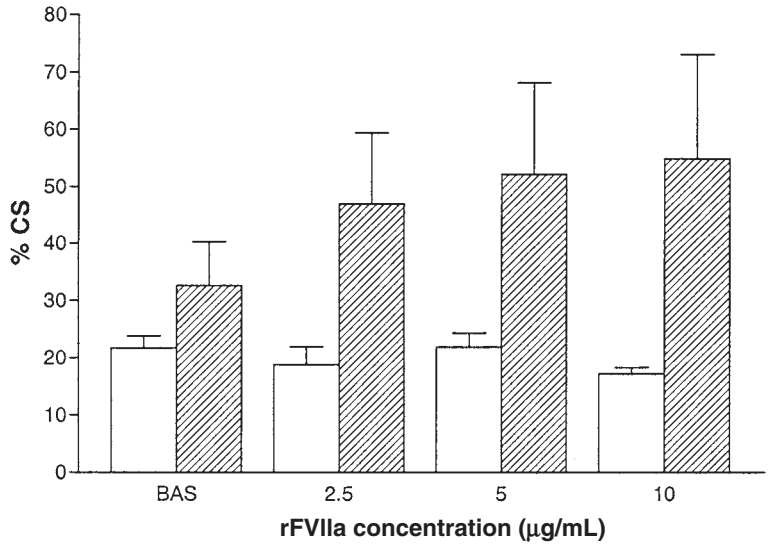

Fig. 1. Effect of rFVIIa in experiments performed with whole blood. Percentage of subendothelium (% CS) covered by PLTs (□) or fibrin (▨) in perfusion studies performed with blood samples drawn from healthy volunteers before (baseline) and after addition of rFVIIa at concentrations equivalent to 2.5, 5, and 10 µg per mL in plasma. Results are expressed as means ± SEM; n = 6.

TABLE 1. Modifications in F1 + 2 levels in perfused blood samples during perfusion experiments in the absence or in the presence of rFVIIa*

	Whole blood		Thrombocytopenic blood	
	Before perfusion	After perfusion	Before perfusion	After perfusion
Baseline	0.54 ± 0.05	1.32 ± 0.35†	0.49 ± 0.06	1.16 ± 0.21†
2.5 µg/mL	0.53 ± 0.07	2.77 ± 0.29†‡	0.51 ± 0.05	3.23 ± 0.58†‡
5.0 µg/mL	0.65 ± 0.06	3.90 ± 0.56†‡	0.58 ± 0.06	3.84 ± 0.83†‡
10.0 µg/mL	0.77 ± 0.14	4.53 ± 0.43†‡	1.1 ± 0.5	5.80 ± 1.19†‡

* Results are expressed in n*M*. Values are given as means ± SEM. n = 6.
† $p < 0.05$ versus levels before perfusion.
‡ $p < 0.05$ versus experiments performed in the absence of rFVIIa (diluent).

Effects of rFVIIa under conditions of severe thrombocytopenia

After filtration of blood samples PLT counts ranged from 2000 to 6000 PLTs per µL, while WBC counts were less than 100 WBCs per µL. Although the addition of rFVIIa to PLT-depleted blood samples did not improve PLT interactions (percentage of vessel surface covered, <10%) there was a dose-dependent increase in the percentage of fibrin deposited on the perfused vessel segments. Differences reached levels of significance at all rFVIIa concentrations tested (percentage of fibrin, 24.9 ± 5.9, 27.3 ± 7.5, and 29.8 ± 8.0% with concentrations of 2.5, 5 and 10 µg/mL, respectively; $p < 0.05$ vs. 9.7 ± 2.2% in thrombocytopenic blood without rFVIIa [baseline]; n = 6). These results are summarized in Fig. 2.

F1 + 2 levels in the perfusion samples of blood taken from patients with severe thrombocytopenia were 0.49 ± 0.06 n*M*. After perfusion, plasma levels of F1 + 2 increased significantly to 1.16 ± 0.21 n*M*. Incorporation of rFVIIa into the perfusates at concentration of 2.5, 5, and 10 µg per mL induced a very mild increase in the before-perfusion F1 + 2 levels compared with controls, but differences did not reach significance. However, postperfusion F1 + 2 levels in rFVIIa-treated blood increased significantly in a dose-dependent manner with

respect to both preperfusion values and values found in thrombocytopenic blood samples in the absence of rFVIIa (Table 1).

Effect of rFVIIa in blood from patients with severe GPIIb-IIIa deficiency

Increases in fibrin deposition were observed when 5 µg per mL rFVIIa was added to blood samples taken from two patients with severe congenital deficiency of GPIIb–IIIa (Glanzmann's thrombasthenia).

A marked increase in PLT deposition after incubation with 5 µg per mL rFVIIa with percentage of vessel surface covered being twice the value observed in the absence of rFVIIa (see individual results in Table 2). PLT interactions in these blood samples occurred with upper layers of the fibrin masses formed (Fig. 3).

F1 + 2 levels before perfusion were not modified by the addition of rFVIIa compared to baseline. In contrast, F1 + 2 levels were found increased in postperfusion samples (Table 3).

Effects of rFVIIa in samples of blood exposed to a specific antibody to PLT GPIIb-IIIa

Incubation of blood samples with 10.5 µg per mL of a humanized MoAb against GPIIb-IIIa caused a dramatic inhibition of PLT deposition and a marked reduction of fibrin formation. This pattern of interaction was similar to that initially observed in patients with Glanzmann's thrombasthenia. Addition of rFVIIa (5 µg/mL plasma) caused by an increase in fibrin deposition. Similarly to the findings observed in patients with the congenital defect, an increased presence of single PLTs adhered to fibrin masses was observed. Micrographs illustrating the more remarkable features of these data are shown in Fig. 4.

Role of subendothelial TF in perfusion experiments

Incubation of vascular segments with a polyclonal antibody against TF resulted in a significant decrease of fibrin formation (19.95 ± 3.70 vs. 46.41 ± 7.12%, $p < 0.05$; $n = 8$). However, no differences were observed in PLT deposition (31.31 ± 5.28 vs. 28.35 ± 6.93%, $p < 0.05$). Addition of rFVIIa (5 µg/mL plasma) to perfusates did not modify PLT interaction. However, the presence of rFVIIa partially restored fibrin formation on the TF-blocked subendothelium (32.41 ± 9.14%). Micrographs illustrating the more remarkable features of these perfusions are shown in Fig. 5.

DISCUSSION

Our study has explored potential mechanisms by which rFVIIa could exert its hemostatic effect in quantitative and qualitative disorders of PLT function. Using an experimental model we have tested concentrations of rFVIIa similar or slightly superior to those applied for the clinical practice. Data raised in our experimental model indicate

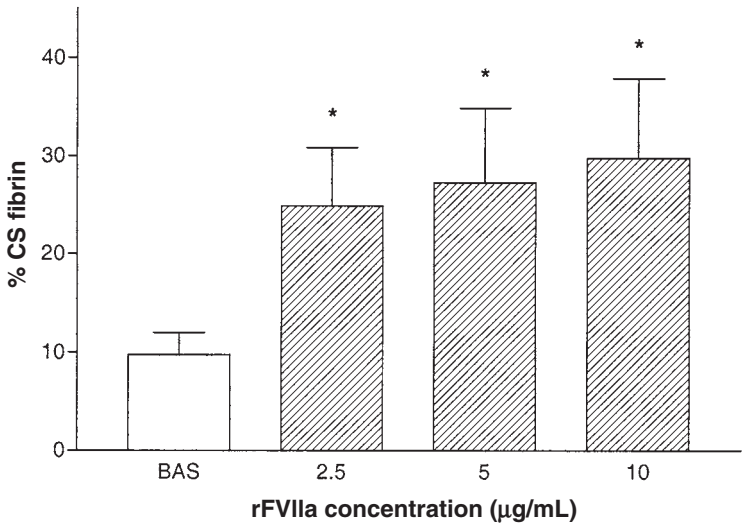

Fig. 2. Effect of rFVIIa under thrombocytopenic conditions. Deposition of fibrin on the subendothelium in studies with blood experimentally depleted of PLTs (thrombocytopenia <6000 PLTs/µL) before (baseline [BAS]) and after addition of rFVIIa equivalent to 2.5, 5, and 10 µg per mL in plasma. Results are expressed as percentage of covered surface by fibrin (% CS fibrin) (mean ± SEM; *$p < 0.05$ vs. BAS; $n \geq 6$).

TABLE 2. Effect of rFVIIa on PLT interaction and fibrin deposition in experiments performed with blood samples from Glanzmann's thrombasthenia patients and blood samples incubated with an antibody against GPIIb-IIIa*

	Glanzmann's thrombasthenia (n = 2)		Blood samples incubated with abciximab (n = 4)	
	Percentage of CS PLTs	Percentage of CS fibrin	Percentage of CS PLTs	Percentage of CS fibrin
Baseline	6.97/16.34	9.74/46.12	13.82 ± 3.51	30.88 ± 10.33
5.0 µg/mL rFVIIa	11.33/31.67	22.47/91.77	18.15 ± 2.60	54.27 ± 10.71

* Results are expressed as percentage of the vessel surface (CS) covered by PLTs or fibrin. Individual values are given for each one of the Glanzmann's patients. Values for experiments with abciximab are expressed as means ± SEM.

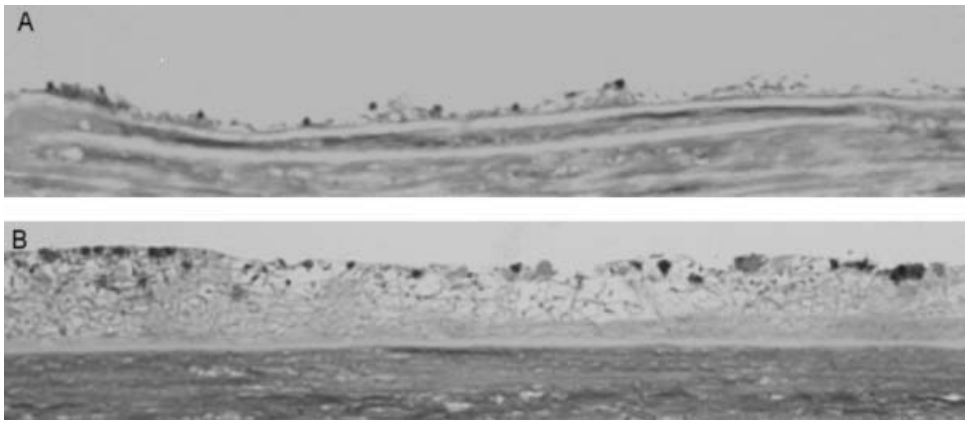

Fig. 3. Light micrographs illustrating microscopic fields observed in experiments performed with blood from one of the patients with Glanzmann's thrombasthenia (A) before and (B) after addition of 5 µg per mL rFVIIa in plasma. Deposition of PLTs and formation of aggregates were reduced in studies in the absence of rFVIIa. Formation of fibrin was dramatically increased in perfusions performed in the presence of rFVIIa. PLT interactions were often observed with the more superficial layers of fibrin deposited on the subendothelium. Magnification, ×400.

TABLE 3. Modifications in F1 + 2 levels in perfused blood samples from Glanzmann's thrombasthenia patients and blood samples incubated with an antibody against GPIIb-IIIa in the absence or in the presence of rFVIIa*

	Glanzmann's thrombasthenia (n = 2)		Blood samples incubated with abciximab (n = 4)	
	Before perfusion	After perfusion	Before perfusion	After perfusion
Baseline	0.73/0.77	1.19/3.49	1.26 ± 0.19	2.25 ± 0.54
5.0 µg/mL rFVIIa	0.71/0.83	2.27/3.44	1.28 ± 0.13	4.42 ± 0.62†‡

* Results are expressed in n*M*. Individual values are given for Glanzmann's patients. Values for abciximab are expressed as means ± SEM.
† $p < 0.05$ versus levels before perfusion.
‡ $p < 0.05$ versus control experiments.

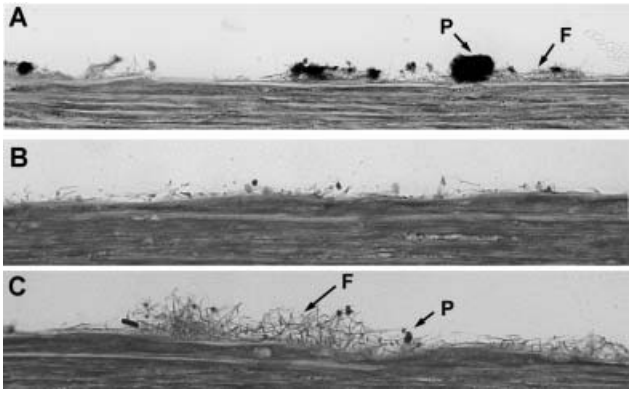

Fig. 4. Light micrographs illustrating microscopic fields observed in experiments performed with blood incubated with an antibody against GPIIb–IIIa (abciximab): (A) Perfusion studies performed with whole-blood samples; (B) experiments performed with blood samples incubated with abciximab; and (C) incubation of blood samples with abciximab and rFVIIa (5 µg/mL plasma). The presence of rFVIIa increases fibrin formation and interaction of PLT (P) with fibrin deposits (F). Magnification, ×400. n = 4.

that rFVIIa promotes a procoagulant activity at sites of vascular damage. This mechanism of action could explain the beneficial action of rFVIIa reported in a wide variety of disorders of primary hemostasis.

rFVIIa has been successfully used in the control of bleeding episodes in patients with hemophilia with inhibitors.[31,32] There is clinical evidence suggesting that rFVIIa can be used in the treatment and control of bleeding in patients with quantitative and qualitative PLT disorders.[5,7–9] While the mechanism of action of rFVIIa in patients with hemophilia has been investigated in detail,[1,2,4] these activities contributing to its effects in other disorders of primary hemostasis are not yet fully understood.

The presence of collagen and TF at the level of a damaged vessel trigger PLT interaction and coagulation mechanisms.[21,33,34] Recent data indicate that rFVIIa requires the presence of TF to initiate coagulation.[13,14] Exposure of TF at sites of vascular damage initiates coagulation and leads to thrombin generation.[10] Thrombin induces fibrin generation and promotes local PLT activation. Both mechanisms contribute to the maintenance of correct hemostasis.[11,22,28] According to the more recent lit-

erature there are at least three sources of TF: present in the subendothelium, expressed on WBCs, and carried out in microparticles.[35,36] In the perfusion model that we have used, collagen and TF are exposed at the level of the damaged vessel exposed to circulating blood.[28] The fact that incubation of vessel with an antibody to TF markedly reduced fibrin formation suggests that TF present in the subendothelium would be the main contributor to the procoagulants effects we observed. Interestingly, inhibition of subendothelial TF did not totally block fibrin formation. This finding would be in agreement with the concept of other sources of circulating TF that are not inhibited by the antibody bound to the damaged vessel.

In our study, rFVIIa consistently enhanced fibrin formation on the damaged vascular subendothelium exposed to circulating blood of severe thrombocytopenia (<6000 PLTs/µL).[22] It is interesting to note that because of the filtration procedure used to remove PLTs, the number of WBCs was also drastically reduced. Thus the contribution of TF on whole WBCs should be minimal or almost absent in our system. Recent studies seem to indicate that circulating microparticles containing TF could be present in the circulating blood under certain conditions.[37,38] Our present data suggest that the procoagulant action of rFVIIa would not necessarily require a high number of circulating PLTs and could be supported under very low PLT counts or even in the presence of circulating microparticles. Although PLT microparticles can improve fibrin formation under experimental conditions,[39] further studies are required to confirm the importance of circulating microvesicles in the possible hemostatic action of rFVIIa under conditions of thrombocytopenia.

Levels of F1 + 2 were consistently elevated in post-perfusion values, when rFVIIa was added to blood perfusates. This observation supports the concept that rFVIIa promotes thrombin generation. Unfortunately, levels of F1 + 2 seem not sensitive enough to detect quantitative differences among the different experimental and clinical situations. It is important to emphasize that elevations of F1 + 2 levels observed in our experiments with normal blood incubated with the highest concentrations of rFVIIa were still below those found in clinical or experimental conditions after the administration of prothrombin complex concentrates.[23,40]

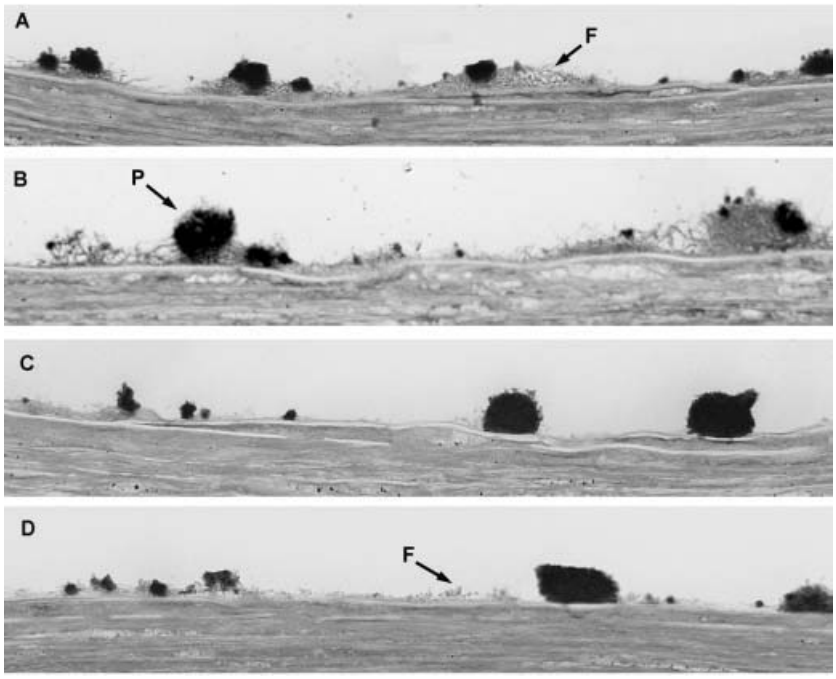

Fig. 5. Effect of rFVIIa and TF in studies with normal blood: (A) PLT interaction (P) and fibrin formation in control experiments in the absence of rFVIIa; (B) addition of 10 µg per mL rFVIIa in plasma of resulted in clearly enhanced the formation of fibrin (F) on the perfused subendothelium, but did not appear to improve PLT adhesive or aggregation functions; (C) incubation of vascular segment with anti-TF decreased significantly fibrin deposits on the subendothelium versus control experiments; and (D) addition of rFVIIa partially restored fibrin deposition but did not modify PLT interaction. Magnification, ×400. n ≥ 6.

Interestingly, our studies show that while fibrin generation always increased after addition of rFVIIa regardless of clinical or experimental conditions, the level of PLT interaction remained, for the most part, unmodified. Only those studies performed with blood from patients with Glanzmann's thrombasthenia showed a discrete increase of PLT deposition. Differential roles of fibrinogen and vWF on PLT adhesion might explain why PLTs from patients with Glanzmann's thrombasthenia interacted with the more superficial layer of fibrin deposits.[41,42] At the intermediate shear rates used in our studies (600 per second), fibrin formation prevails over PLT-mediated events.[43] Under such rheologic conditions, the augmented deposition of fibrin could actually compete with PLTs for the available subendothelial surface. Data obtained in perfusion studies where normal blood was exposed to the highest concentrations of rFVIIa (Fig. 5) strongly suggest that, at intermediate shear rates, elevated fibrin deposition could exclude PLTs from interacting with the damaged vessel.

Although rFVIIa is seen as a universal hemostatic agent for patients with severe impairments of hemostasis,[44] the extension of its use to patients with better pre-

served hemostatic mechanisms is a subject of debate.[45] There have been reports on rFVIIa being infused into volunteers at doses up to 320 µg per kg,[46] and there are reasonable concerns on possible side effects if high concentrations of rFVIIa are repeatedly injected to patients. Although overall clinical experience has reported low incidence of thromboembolic phenomena,[47,48] isolated reports of delayed thrombotic complications have been communicated in some patients with inherited Glanzmann's thrombasthenia.[49] It should be emphasized that in the latter case, rFVIIa was administered in continuous infusion, a route of administration that is off label, and that thrombotic complications observed were delayed and it was difficult to find a cause-and-effect relationship. Because rFVIIa in sufficient doses can significantly enhance thrombin generation, its use in patients with underlying conditions predisposing them to thrombosis should always be carefully considered.[48]

In summary, our results suggest that rFVIIa triggers a local procoagulant effect at sites of vascular damage. Exposure of subendothelial TF seems to be required for this procoagulant action to occur. The increased procoagulant effect could help to restore defective hemostasis in patients with various quantitative or qualitative PLT disorders. Our experimental data indicate that effects of rFVIIa are mainly located at sites of vascular damage and that its systemic impact should therefore be limited. Data from the present in vitro study provide experimental support favoring the concept that rFVIIa may have a beneficial effect on impaired hemostasis caused by quantitative or qualitative PLT disorders.

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Hemostatic effect of activated recombinant factor VII (rFVIIa) in liver disease: studies in an in vitro model☆

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***Background/Aims*: There is clinical evidence for the efficacy of activated recombinant factor VII (rFVIIa) in patients with cirrhosis. The exact mechanism of action of rFVIIa in this clinical condition is unknown. We have explored effects of rFVIIa on hemostasis in cirrhotic patients using an in vitro perfusion technique.**

***Methods*: Blood samples were drawn from control donors or from 11 patients previously diagnosed with cirrhosis (seven Child-Pugh B and four Child-Pugh C) and anticoagulated with low molecular weight heparin. rFVIIa was added to blood samples at therapeutic concentrations (0.5 or 1 μg/ml of plasma) and blood was recirculated through annular chambers containing damaged vascular segments. Presence of platelets and fibrin on the subendothelium were morphometrically quantified.**

***Results*: Cirrhotic patients showed a diminished platelet interaction with the subendothelium compared to healthy donors (17.3% (9.28–28.88%) vs. 26.16% (19.96–54.5%), $P < 0.05$). After addition of rFVIIa to cirrhotic samples, no differences in platelet covered surface were observed. However, fibrin formation was significantly improved after the addition of rFVIIa (from 51.81% (3.02–86.68%) to 86.94% (30.03–93.18%) and 89.05% (45.65–93.84%), respectively, $P < 0.05$).**

***Conclusions*: Our data confirm a defective interaction of platelets with the subendothelium in cirrhotic patients. rFVIIa improved local fibrin formation at damaged sites and this mechanism could explain the beneficial action of rFVIIa in cirrhotic patients.**

***Keywords*: Activated recombinant factor VII (rFVIIa); Cirrhosis; Hemostasis; Procoagulant action**

1. Introduction

Hemostasis is impaired in patients with liver disease [1,2]. The bleeding problem in cirrhotic patients has a multifactorial origin [3]. Some clinical features of those patients are: thrombocytopenia, platelet function defects, deficiencies of clotting factors and fibrinolytic proteins, hyperfibrinolysis and dysfibrinogenemia [4]. These combined deficiencies can result in an imbalance of the hemostatic system. Several authors have described a platelet dysfunction in these patients, which may contribute to the bleeding episodes observed in these patients [1–4]. Previous studies performed in our laboratory demonstrated that platelet deposition is diminished in patients with cirrhosis [4].

Therapeutic strategies to control hemorrhagic events in cirrhotic patients include: the administration of fresh frozen plasma, desmopressin, vitamin K or vitamin K-dependent coagulation factors. All these therapies have associated risks, such as an increase in the portal pressure, disseminated intravascular coagulation or other biological risks [5,6]. Activated recombinant factor VII (rFVIIa) has proven to be effective in the control of bleeding episodes in patients with hemophilia A or B and in patients with congenital platelet disorders [7]. It has been reported that rFVIIa improves hemostasis in cirrhotic patients, allowing invasive procedures without bleeding complications [8].

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The exact mechanism of action of rFVIIa in cirrhosis is unknown. Potential contributors include: increased thrombin generation; improved platelet activity; and reduced fibrinolysis. The bleeding tendency of cirrhotic patients has been partially related to accelerated fibrinolysis [9]. Interestingly, Lisman and coworkers have found that inhibition of fibrinolysis through thrombin-activatable fibrinolysis inhibitor, a mechanism contributing to the favorable action of rFVIIa in hemophiliacs with inhibitors [10], does not seem to play a significant role in cirrhotic patients [11]. Furthermore, no evidence of an antifibrinolytic effect of rFVIIa was found in patients undergoing orthotopic liver transplantation [12]. Given the increased thrombin generation seen in in vitro models and the clinical efficacy of rFVIIa in disorders of primary hemostasis, we hypothesized that rFVIIa could improve platelet and coagulation deficiencies in cirrhotic patients.

Studies in perfusion models have facilitated the investigation of the mechanisms involved in hemostasis [4,13–15]. In a previous study using a perfusion model, our group demonstrated that rFVIIa promotes procoagulant activity at sites of vascular damage in patients with hemophilia A and in patients treated with oral anticoagulants [16]. In the present study, we have explored the effect of rFVIIa on platelet deposition and fibrin generation using whole blood from patients with cirrhosis circulating through an in vitro perfusion model. The concentrations of rFVIIa that were tested included those reported to reduce the prothrombin time into the normal range in patients with cirrhosis [17].

2. Patients and methods

2.1. Patients

The study was performed in accordance with ethical guidelines. Eleven cirrhotic patients (six women and five men) provided informed consent and were included in the study. The diagnosis of hepatic cirrhosis was established by history, physical examination, laboratory findings, and liver biopsy when not contraindicated. Renal failure, hepatocellular carcinoma or other malignancies, recent gastrointestinal bleeding, and evidence of active disseminated intravascular coagulation were conditions for exclusion. The etiology of cirrhosis was alcoholic in nine patients and hepatitis C virus-related in two patients. Seven patients were classified as Child-Pugh group B and the remaining four were classified as Child-Pugh C. Detailed characteristics of the patients included are shown in Table 1.

2.2. Blood collection

Blood samples were drawn from cirrhotic patients or from healthy donors who in the previous 10 days, had not taken any drug known to affect either platelets or the coagulation system. Blood samples were anticoagulated with 7.5 U/ml low molecular weight heparin (Fragmin, Pharmacia & Upjohn, Stockholm, Sweden).

2.3. Perfusion experiments

Prior to the initiation of perfusion, blood samples were incubated for 1 min with either diluent or aliquots of rFVIIa (rFVIIa, NovoSeven®, Bagsvaerd, Denmark) calculated to reach concentrations of 0.5 and 1 μg/ml at the plasma interface. These concentrations approximately correspond to 15 and 30 μg/kg body weight if extrapolated into patients. After incubation, blood samples were immediately perfused through annular chambers containing denuded arterial segments, as described by Baumgartner [13]. Blood was recirculated for 10 min at 37°C using a peristaltic pump with the flow previously adjusted to give rise to a shear rate equivalent to 600 s^{-1}. The hemostatic effectiveness of the blood samples was assessed using morphometric evaluation of platelet and fibrin deposition onto the subendothelium of the arterial segments [18].

Table 1
Clinical characteristics of the patients

	Mean ± SD	Range	Normal values
Age	60 ± 7	(47–71)	–
Child Pugh score	9.5 ± 1.2	(8–12)	–
Bilirubin (mg/dL)	4.5 ± 6.5	(0.7–24)	(0.1–1.2)
Prothrombin time (%)	57 ± 9	(40–71)	(80–100)
Albumin (g/l)	26.6 ± 2.9	(20–30)	(37–53)
Platelets (platelets/μl)	88,270 ± 27,300	(43,000–131,000)	(150,000–400,000)
Ascites (n)	10	–	–
Encephatopathy (n)	3	–	–

2.4. Processing of vessel segments and morphometric evaluation

At the end of each perfusion, the arterial segments were rinsed with 20 ml of phosphate buffered saline, pH 7.2, sliced off from the chamber and fixed with the same buffer containing 2.5% glutaraldehyde. The fixed segments were processed histologically, as described in previous works [19].

Fibrin deposition on the subendothelium as well as platelet interactions, were morphometrically evaluated in the light microscope. Studies were conducted according to a single blind design. The technician performing the morphometric evaluation was unaware of the experimental design. Platelet interaction and fibrin deposition were analyzed using a specially devised program [20], which automatically classifies and quantifies the total percentage of the vessel surface covered by platelets (% C.S.) or fibrin (% F) in the same microscopic fields were morphometrically quantified and expressed as percentage on the total surface of the vessel screened.

2.5. Evaluation of thrombin generation

Thrombin generation during perfusion was indirectly assessed through measurement of prothrombin fragments F1 + 2 in plasma samples. Aliquots of blood were systematically collected before and after perfusion was stopped. Blood aliquots were immediately mixed with sodium citrate (129 mM) to prevent any further activation of the coagulation system. Plasma was separated by centrifugation of the anticoagulated blood samples (1800 × *g* for 20 min) and frozen at − 70°C. Levels of F1 + 2 were determined in plasma samples using commercially available immunoassay (Enzygnost F1 + 2, Behring, Germany) [21].

2.6. Data analysis

Results were expressed as median and observed range. The number of experiments performed at each concentration of rFVIIa was 11 for the group of cirrhotic patients and 6 for the group of healthy donors. Kruskal–Wallis test was used to compare donors, treatments and measurements collected before and after perfusion. The level of statistical significance was established at $P < 0.05$.

3. Results

3.1. Platelet counts

Platelet counts were performed before and after perfusion. Platelet numbers were 205,000 plts/μl (range: 120,000–311,000 plts/μl) in healthy donors and 91,000 plts/μl (range: 43,000–131,000 plts/μl) in the patients. Post-perfusion counts were always lower with values of 146,000 plts/μ (range: 111,000–294,000 plts/μl) and 50,000 plts/μl (28,000–67,000 plts/μl) for healthy controls and cirrhotic patients, respectively. A slight additional decrease in platelet counts was observed in experiments performed with rFVIIa. However, differences never reached the levels of statistical significance.

3.2. Effect of rFVIIa in perfusion studies

As shown in Fig. 1, studies with blood from cirrhotic patients showed a defective interaction of platelets with the damaged vascular surfaces. Addition of rFVIIa did not improve the deposition of platelets in the group of cirrhotic patients, but significantly enhanced the deposition of fibrin on the perfused vascular surface.

Fig. 2, represents modifications in morphometric values in the different study groups. Platelet interaction in experiments performed with baseline blood samples from healthy donors in the absence of rFVIIa resulted in percentages of platelet coverage (% C.S.) equivalent to 26.16% (19.9–54.5%). Addition of rFVIIa at 0.5 or 1 μg/ml

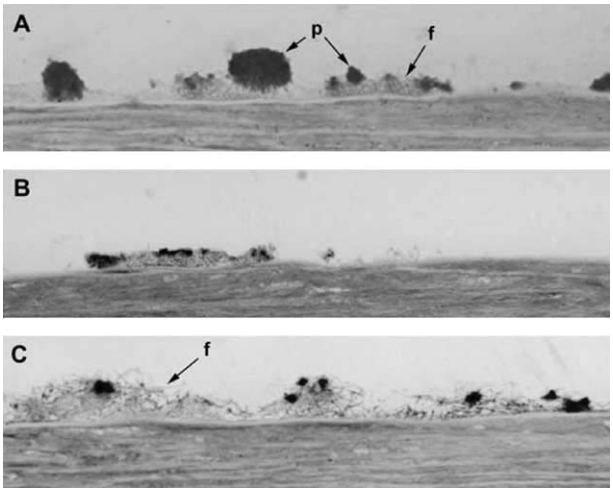

Fig. 1. Light micrographs illustrate the most remarkable features observed in cross-sections of the perfused vascular segments. (A) Pattern of platelet and fibrin interactions observed in studies with blood samples from healthy donors. (B) Platelet interaction was diminished when perfusion experiments were performed with blood samples from cirrhotic patients. (C) Addition of rFVIIa to a cirrhotic blood sample improved fibrin formation on the subendothelium, but not platelet deposition. p: platelet interaction; and f: fibrin deposits (× 400).

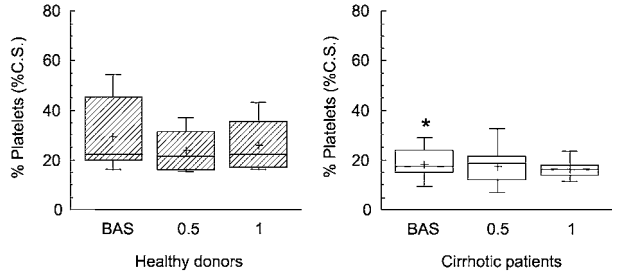

Fig. 2. Effect of rFVIIa on platelet deposition in experiments performed with whole blood from control and cirrhotic patients. Box-and-Whisker Plot representing results of percentage of subendothelium covered by platelets (% C.S.) in baseline studies (BAS) or in the presence of rFVIIa at concentrations equivalent to 0.5 or 1 μg/ml of plasma. The central box covers the middle 50% of the data; the sides of the box are the lower and upper quartiles, and the horizontal line drawn through the box is the median. The whiskers extend from the lower to the upper values of the data (range). Markers (+) show the means. $n = 11$ in cirrhotic samples; $n = 6$ in healthy donors samples; *$P < 0.05$ vs. experiments performed with blood from healthy donors.

did not significantly modify the deposition of platelets. Although the deposition of platelets was significantly reduced in experiments with baseline blood from cirrhotic patients (17.3% (9.2–28.8%); $P < 0.05$ vs. healthy donors) the presence of rFVIIa did not cause significant modifications in the overall interaction of platelets with the subendothelial surface.

As shown in Fig. 3, the deposition of fibrin on the subendothelium observed in experiments performed with baseline blood from healthy donors was 58.1% (27.2–73.5%). A slight reduction was observed for fibrin coverage in experiments performed with baseline blood from cirrhotic patients 51.8% (3.0–86.6). Addition or rFVIIa at 0.5 or 1 μg/ml caused a statistically significant enhancement in the deposition of fibrin in the group of healthy donors

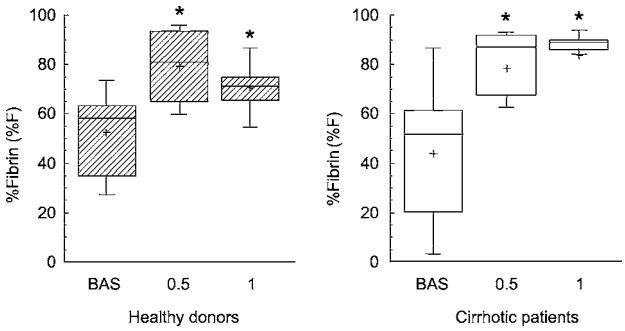

Fig. 3. Effect of rFVIIa on fibrin formation in experiments performed with whole blood from control and cirrhotic patients. Box-and-Whisker Plot representing results of percentage of subendothelium (% F) covered by fibrin in baseline studies (BAS) or in the presence of rFVIIa at concentrations equivalent to 0.5 or 1 μg/ml of plasma. The central box covers the middle 50% of the data; the sides of the box are the lower and upper quartiles, and the horizontal line drawn through the box is the median. The whiskers extend from the lower to the upper values of the data (range). Markers (+) show the means. $n = 11$ in cirrhotic samples; $n = 6$ in healthy donors samples; *$P < 0.05$ vs. experiments performed with blood from healthy donors.

[80.7% (59.9–96.0) and 71.3% (56.4–90.6), respectively], but also in that of cirrhotic patients [86.94% (30.03–93.18) and 89.05% (45.65–93.84), respectively]. In both cases, healthy and cirrhotic individuals, differences reached levels of statistical significance ($P < 0.05$ vs. baseline values).

3.3. Detection of prothrombin fragments F1 + 2

Prothrombin fragments (F1 + 2) levels were measured in plasma samples collected in sodium citrate immediately before and after perfusion experiments were finished. Pre-perfusion values of F1 + 2 were 0.76 nM (0.4–3.74 nM) in experiments performed with blood from healthy donors and 2.06 nM (1.04–3.37 nM) in those of cirrhotic patients. As shown in Table 2, the single addition of rFVIIa to blood samples did not result in statistical modifications of F1 + 2 values with respect to baseline levels.

F1 + 2 levels were markedly increased in post-perfusion samples in all experimental groups (Table 2). F1 + 2 levels rose from 0.76 to 2.6 nM (0.4–3.74 nM) in perfusions studies with blood from healthy donors and from 2.06 to 2.16 nM (1.38–4.47 nM) in perfusions with blood from cirrhotic patients. The presence of 1 μg/ml rFVIIa caused a statistically significant elevation at in post perfusion F1 + 2 values in blood from healthy donors. Concentrations of rFVIIa equivalent to 0.5 and 1 μg/ml resulted also in significant elevations of F1 + 2 levels in post perfusion samples from cirrhotic patients, with values of 3.44 nM (0.18–5.64 nM) and 4.06 nM (2.21–6.66 nM), respectively ($P < 0.05$).

4. Discussion

Data from the present in vitro study provide experimental evidence on the mechanisms of action of rFVIIa involved in the improvement of hemostasis in cirrhotic patients. We have confirmed an impairment in platelet adhesion in cirrhotic patients. Our present results indicate that the addition of rFVIIa into cirrhotic blood samples increases thrombin generation and enhances fibrin deposition onto damaged vessels exposed to flowing blood. We hypothesize that the local procoagulant action of rFVIIa at sites of vascular damage could be responsible for the hemostatic action of rFVIIa in patients with liver cirrhosis.

Recombinant FVIIa is indicated for treatment of bleeding episodes in patients with hemophilia who have developed inhibitors [22]. A recent study from our group has found that rFVIIa facilitates fibrin formation on damaged vascular areas using blood from patients with hemophilia and inhibitors [16]. Clinical evidence suggests that rFVIIa could be used in the treatment and control of bleeding in patients with quantitative and qualitative platelet disorders [23–26]. Recent clinical studies suggest that rFVIIa would improve hemostasis in patients with advanced liver cirrhosis [8,27]. However, the mechanisms involved in the clinical effectiveness of rFVIIa in the latter conditions have not been elucidated.

It is well established that rFVIIa requires the presence of tissue factor (TF) to initiate coagulation [28,29]. Earlier studies by Weiss and coworkers [30] indicated that TF exposed at sites of vascular damage would play an important role in the initiation of hemostasis in vivo [31]. Interaction of the exposed TF with FVIIa would activate coagulation mechanisms leading to a local generation of thrombin, fibrin generation and further activation of platelets, that would contribute to further thrombin formation [29,32,33]. Interestingly, while fibrin generation was found always increased after addition of rFVIIa under our experimental conditions, the level of platelet interaction remained, for the most part, unmodified. Although our results did not show an increase in platelets deposition, we cannot rule out the possibility that rFVIIa could have an effect on platelets by increasing the generation of thrombin. We are convinced that under the flow conditions produced in our experiments, the augmented deposition of fibrin observed in studies with rFVIIa, could actually exclude platelets from interacting with the damaged vessel [34]. A recent work published by Butenas and coworkers has suggested that FVIIa could appear to function effectively and locally by the combined effect of TF expression and platelet accumulation at site of a vascular lesion [32].

A mechanism for rFVIIa independent of TF has been suggested by two groups [35–37] using different experimental approaches. However, recent evidence suggests that platelets and microparticles may contain residual levels of TF [38] and it seems reasonable that these sources could still provide minimal amounts of TF in experimental approaches. Whether or not these mechanisms independent of TF play a role in the hemostatic mechanisms of rFVIIa in

Table 2
Thrombin generation measured as prothrombin fragments 1 + 2 (F1 + 2)

	Healthy donor ($n = 6$)		Cirrhotic patients ($n = 11$)	
	Pre-perfusion	Post-perfusion	Pre-perfusion	Post-perfusion
Baseline (BAS)	0.76 (0.4–3.74)	2.6 (0.4–3.93)	2.06 (1.04–3.37)	2.16 (1.38–4.47)
0.5 μg/ml rFVIIa	0.66 (0.45–3.1)	3 (1.38–5.24)	2.05 (1.08–2.99)	3.44 (0.18–5.64)*
1 μg/ml rFVIIa	0.59 (0.47–1.14)	3.32 (1.67–4.6)*	2.9 (1.4–3.68)***	4.06 (2.21–6.66)***

Results are expressed in nM as median and observed range. *$P < 0.05$ vs. pre-perfusion values; **$P < 0.05$ vs. BAS; and ***$P < 0.05$ vs. healthy donors.

patients is an issue that must be addressed in future clinical studies.

Previous studies showed that rFVIIa was able to correct the prolonged prothrombin time in cirrhotic patients, showing a dose dependent effect in the duration of this action [27]. There is evidence that administration of rFVIIa reduces bleeding episodes in those patients [8,39]. Clinical studies performed in liver transplantation showed a decrease of blood loss in patients after infusion of rFVIIa [40]. It has been reported that the beneficial effects of rFVIIa in hemophilia patients with inhibitor would be partially contributed by an antifibrinolytic action mediated by TAFI [10]. Interestingly, the same group have been unable to detect this antifibrinolytic mechanisms to play a role in cirrhotic patients [11,12]. Our results do not exclude the possibility that fibrin formed in our experimental model, could be more resistant to fibrinolysis. It has been suggested that the fibrin structure of the hemostatic plug is important for hemostasis [7]. Fibrin plugs that could be easily dissolved by normal fibrinolytic activity would be less effective to maintain hemostasis. Recently, it has been demonstrated that addition of rFVIIa to FVIII- or FIX-deficient systems normalizes fibrin clot permeability altering network structure [41].

Previous studies from our group have shown that the presence of rFVIIa results in an increment of the procoagulant action on the subendothelium under thrombocytopenic conditions or in severe alterations of platelet glycoproteins [34]. Results from the present study suggest that the beneficial effects of rFVIIa in the deterred hemostasis in cirrhotic patients would be mainly related to an improvement of coagulation at sites of vascular damage which would result in an increase of fibrin generation. However, further studies are required to evaluate whether an increase in fibrin formation would be enough to restore hemostasis in other acquired disorders of the platelet function.

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Hemostatic effect of activated recombinant factor VIIa in Bernard-Soulier syndrome: studies in an in vitro model

To the Editor:

Bernard-Soulier syndrome (BSS) is a rare disorder associated with the lack or dysfunction of the platelet (PLT) glycoprotein complex Ib-V-IX. These PLTs have impaired interaction with the subendothelium and affected persons experience repeated mucosal bleeding. Transfusion of PLT concentrates may be required to control severe bleeding. Unfortunately, some patients develop antibodies and refractoriness to further PLT transfusions. Recombinant factor VIIa (rFVIIa) is indicated for treating bleeding episodes in patients with hemophilia A or B with inhibitors. A recent study from our group found that rFVIIa facilitates fibrin formation on damaged vasculature using blood from patients with hemophilia and inhibitors.[1] Clinical experience suggests that rFVIIa may have a potential role in the treatment of bleeding in patients with quantitative and qualitative PLT disorders.[2] Moreover, there are reports demonstrating effectiveness of rFVIIa in the management of bleeding in patients with BSS.[3] We report our findings using an in vitro perfusion system to assess the effect and mechanism of action of rFVIIa in blood samples from a patient with BSS.

Blood samples were anticoagulated with 20 U per mL low-molecular-weight heparin (LMWH; Fragmin, Pharmacia & Upjohn, Stockholm, Sweden).[4] This concentration of LMWH keeps blood anticoagulated, but allows thrombin generation when blood is exposed to a damaged vascular segment. Previous studies from our group have already demonstrated that rFVIIa enhances fibrin formation[1,4] in the presence of the LMWH concentrations used in our present investigations. For the purpose of the studies described here, blood samples were incubated for 1 minute with either diluent (baseline) or an aliquot of rFVIIa calculated to achieve a concentration of 5 μg per mL in plasma (approximately 150 μg/kg body weight). Immediately after, samples were perfused through annular chambers containing denuded arterial segments for 10 minutes at 37°C at a shear rate of 600 per second. The hemostatic effectiveness of rFVIIa added to the samples was assessed using morphometric evaluation of PLT and fibrin deposition onto the subendothelium of the arterial segments as we described previously.[4] Thrombin generation was monitored through assessment of F1+2 in plasma samples.

Our experimental data confirm impairment in PLT adhesion related to the glycoprotein complex Ib defect in this patient (Fig. 1). The percentage of the damaged vascular segment covered by PLTs increased modestly following incubation with rFVIIa (15.8% vs. 13.2% under baseline conditions). The average cross-sectional area covered by PLT aggregates in the presence of rFVIIa was twice that found under baseline conditions (110 μm^2 vs. 51 μm^2). The percentage of fibrin deposited also showed a marked increase from less than 5 percent in the baseline study to 30.8 percent in the presence of rFVIIa. F1+2 levels were not modified by the addition of rFVIIa (1.02 nmol/L vs. 1.09 nmol/L). A marked increase, however, was observed in postperfusion samples (2.57 nmol/L vs. 1.18 nmol/L in baseline) indicating an activation of prothrombin to thrombin.

Data from this study provide experimental evidence on a favorable action of rFVIIa by improving hemostasis in blood obtained from a BSS patient. Our results indicate that the addition of rFVIIa in BSS blood samples increases thrombin and fibrin generation, but also facilitates the recruitment of PLT aggregates onto damaged vessels. This procoagulant mechanism, previously observed by our group in other disorders of hemostasis,[4,5] could explain the beneficial action of rFVIIa reported in patients with BSS. A similar mechanism has been described in other primary hemostasis disorders.[2]

Although enhanced fibrin generation is the more prominent and consistent effect after adding rFVIIa,[1,4,5] in this study we also observed a discrete improvement in PLT deposition. In our opinion, the presence of collagen and tissue factor in the damaged subendothelium is important for the localization of procoagulant activity observed in our experimental setting. Interaction of tissue factor with FVIIa would activate coagulation mechanisms leading to a local generation of thrombin. Recent reports suggest that PLTs simultaneously activated with thrombin and collagen reveal a subfraction of PLTs (COAT-PLTs) with high procoagulant activity on their surface.[6] All these events taking place in an environment of a damaged vessel would result in fibrin generation and further activation of PLTs, thus contributing to the formation of a more stable hemostatic plug.[7,8]

Overall, results of our in vitro study provide experimental support favoring the concept that rFVIIa may have a beneficial effect on correcting impaired hemostasis in patients with BSS.

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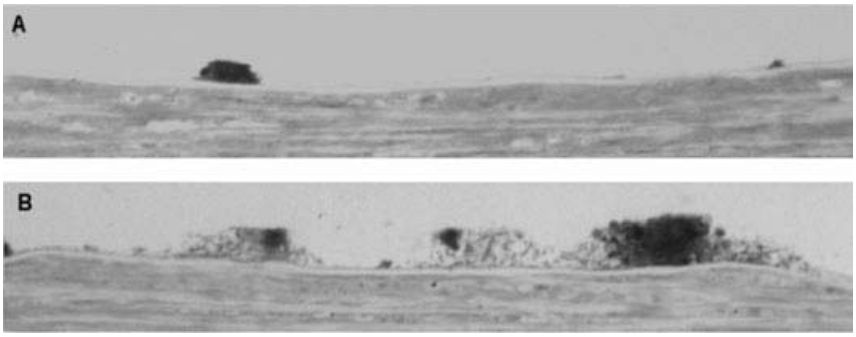

Fig. 1. Light micrographs of microscopic fields observed in experiments performed with blood from a BSS patient: (A) before and (B) after addition of 5 µg per mL rFVIIa. Deposition of PLTs and fibrin was minimal at baseline. Fibrin deposition was markedly improved in the presence of rFVIIa with clumps of PLTs appearing to be recruited into the fibrin nets.

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Red blood cell fragmentation before hematopoietic progenitor cell transplantation

To the Editor:

In their review of thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP-HUS) following allogeneic hematopoietic progenitor cell (HPC) transplantation, George and colleagues state that "RBC fragmentation occurs in almost all patients after allogeneic HPCT and therefore may also be an unreliable diagnostic criterion for TTP-HUS."[1] They did not evaluate the quantity or the degree of red blood cell (RBC) fragmentation during the clinical course, however, probably because this information was lacking in the journal reports they reviewed.

Recently, we developed a system to quantitate fragmented RBCs (FRCs) using an automated hematology analyzer (XE-2100, Sysmex Co., Kobe, Japan) (Fig. 1A). The correlation with conventional manual counts was excellent.[2,3] With this system, we are beginning to evaluate sequential quantitative data of FRCs in patients who have undergone HPC transplantation. We report the findings of three patients who had prominent FRCs before HPC transplantation.

The first case is a 36-year-old woman with chronic myelogenous leukemia. After 5 years of interferon therapy, she progressed to an accelerated phase and then to a second chronic phase (CP) in response to imatinib mesilate. She underwent an allogeneic cord blood stem cell transplantation. FRCs, not obvious during her first CP, became prominent as her disease progressed, even in her second CP. Before transplantation, the peak percentage of FRCs was 11.1 percent (10% by manual counting; Figs. 1B and 2). There were no signs of microangiopathic hemolysis. Serum lactate dehydrogenase (LDH), bilirubin, thrombomodulin (2.2 FU/mL), and antithrombin III (28.1 mg/dL) were normal. Fibrinogen degradation product (FDP) and fibrinogen were also within normal range. The percentage of FRCs was 6 percent on Day 0 and then gradually decreased after transplantation to 1 to 2 percent after Day 50 (Figs. 1E and 2).

The second case is a 2-year-old male patient with histiocytosis X. After a chemotherapy-resistant relapse, he underwent HPC transplantation from an HLA-identical sibling cord blood donor. FRCs appeared 1 month before transplantation without an increase in serum LDH, bilirubin, or FDP, but with elevated fibrinogen (454 mg/dL) and platelet (PLT) count (500×10^9/L). On Day 0, the percentage of FRCs was 4.6 percent (4.3% by manual count;

Platelet Membrane Fragments Enhance the Procoagulant Effect of Recombinant Factor VIIa in Studies With Circulating Human Blood Under Conditions of Experimental Thrombocytopenia

Raúl Tonda, Ana M. Galán, Roberto Mazzara, James G. White, Antonio Ordinas, and Ginés Escolar

The mechanism of action of recombinant factor VIIa (rFVIIa), which is being considered as an alternative treatment for the control of bleeding episodes in patients with thrombocytopenia, has not been fully characterized. This study was undertaken to explore the effects of rFVIIa and platelet microvesicles on hemostasis in an experimental model of thrombocytopenia. Damaged arterial segments were exposed to thrombocytopenic blood (shear rate 600 s^{-1}) either with or without the addition of rFVIIa and/or platelet microvesicles. The presence of fibrin and platelets on the subendothelium were morphometrically quantified and immunolocalization techniques and electron microscopy were used for a more detailed analysis. Both rFVIIa and platelet microvesicles consistently improved fibrin formation on the damaged vascular subendothelium, and microvesicles were shown to be localized at different levels of the fibrin lattice. Further, under conditions of moderate thrombocytopenia, addition of platelet microvesicles potentiated the procoagulant action of rFVIIa. This effect may be due to the phospholipid surface provided by the platelet microvesicles. These studies support the concept that, under conditions of thrombocytopenia, both rFVIIa and platelet microvesicles enhance fibrin formation at sites of vascular damage.

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CLINICAL BLEEDING related to quantitative platelet defects can be controlled and prevented by the transfusion of platelet concentrates. Consequently, in recent years, the demand for platelet concentrates has increased progressively. New and more aggressive oncohematological treatments, poorer platelet recoveries after transfusion, and the development of refractoriness after platelet transfusion from several donors have contributed to this increased demand.[22] In recent years, these concerns have prompted the development of alternative therapeutic strategies.[15]

Recombinant factor VIIa (rFVIIa) is clinically effective in the management and prevention of bleeding episodes in patients with FVII deficiency,[2] as well as in patients with hemophilia A and B who have developed inhibitors.[11,23] Further studies have provided evidence that rFVIIa clinically improves hemostasis in patients with inherited deficiencies of platelet glycoprotein (GP)IIb-IIIa[6,20,26] and thrombocytopenia.[4,14]

While the mechanism of action of rFVIIa in patients with hemophilia has been investigated in detail,[11,12,24,25] those features contributing to its beneficial effects in quantitative and qualitative disorders of primary hemostasis are still under investigation. We have previously demonstrated that platelet microvesicles can improve fibrin generation and hence overall hemostasis under conditions of severe thrombocytopenia.[9] More recently, we confirmed that rFVIIa can exert similar procoagulant effects in blood from patients with severe thrombocytopenia and major glycoprotein deficiencies.[10] Such effects could explain the beneficial actions of rFVIIa observed in a wide variety of primary hemostasic disorders, such as thrombopenic conditions or inherited platelet disorders.

In the present study, we explored whether rFVIIa could act in conjunction with platelet microvesicles to promote hemostasis in human blood under conditions of experimental thrombocytopenia. Our experiments were performed using intermediate shear rates and denuded arteries to mimic circulating blood flow and a damaged vessel, respectively.

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Material and Methods

Blood Collection and Preparation of Thrombopenic Blood

Blood was obtained from healthy volunteers, who had not taken drugs capable of affecting platelet density in the previous 10 days. Blood was anticoagulated with low-molecular-weight heparin (LMWH; Fragmin, Pharmacia & Upjohn, Stockholm, Sweden) at a concentration of 20 U/mL. This concentration was sufficient to maintain anticoagulation, but still allowed fibrin formation following the exposure of blood to a thrombogenic surface.[17] Platelet and leukocyte depletion was performed using a filtration procedure and a RC100 filter (PALL Corp, Cortland, NY).[9] Different platelet counts were obtained by mixing varying proportions of filtered and unfiltered blood. For these studies, we used two predetermined levels of thrombocytopenia: (1) severe with platelet counts ranging from 2,000 to 6,000 platelets/μL; and (2) moderate with platelet counts from 25,000 to 30,000 platelets/μL.

Preparation of Perfusates

Blood samples were incubated for 1 minute with either a neutral diluent (saline) or with sufficient rFVIIa (NovoSeven®, Novo Nordisk, Bagsvaerd, Denmark) to reach a plasma concentration of 10 μg/mL, which approximates to 300 μg/kg body weight.

Infusible platelet membranes (IPM; Cyplex, Cypress Bioscience Inc, San Diego, CA) were used as the standardized source of platelet microvesicles.[5] IPM are produced by freeze/thawing outdated platelets, followed by viral inactivation using wet heat and lyophilization.

Sufficient platelet microvesicle concentrations were added to the thrombocytopenic blood to achieve concentrations equivalent to 1 mg/kg body weight in a standard 70-kg patient. This concentration has been proved effective in previous studies.[9] Studies investigating the combined effects of rFVIIa and microvesicles used each agent at the previously described concentrations.

Perfusion Studies

After incubation, blood samples were immediately perfused through annular chambers containing denuded arterial segments, as described by Baumgartner.[3] Whole or thrombocytopenic blood was circulated through the perfusion system for 10 minutes at 37°C via a peristaltic pump. The flow was adjusted to achieve a shear rate equivalent to 600 s^{-1}. At the end of perfusion, the segments were rinsed with phosphate-buffered saline (PBS; pH 7.2) and prefixed in 4% paraformaldehyde.

Detection of Microvesicles on the Subendothelium

The prefixed vascular segments were washed with PBS and frozen at −40°C to facilitate cryosectioning; the detection of microvesicles was undertaken using immunofluorescence of the perfused vascular segment cryosections. Sections were incubated at room temperature for 45 minutes with Annexin V (ANV; Sigma-Aldrich, St Louis, MO) labeled with fluorescein (FITC; dilution 1:100 in Hank's buffer supplemented with 2 mmol/L of $CaCl_2$). After removing excess ANV by washing three times with PBS, the cryosections were mounted for fluorescence microscopy.

Morphometric Evaluation of Platelets and Fibrin Formation

The vascular segments for morphometric or ultrastructural evaluations were post-fixed with 2.5% glutaraldehyde prepared in PBS (pH 7.2) and were histologically processed as described elsewhere.[8]

Fibrin deposition and platelet interactions with the subendothelium were evaluated using a light microscope equipped with a split prism. A specially devised computer program, which automatically classifies and quantifies platelet and fibrin coverage, was used. For simplicity, platelet interactions were globally expressed as the total percentage of the surface of the vessel covered by platelets (% covered surface = %CS). The presence of fibrin in the same microscopic fields was expressed as percentage of fibrin (%F) deposited on the surface length of the screened vessel.[9,10]

Electron Microscopy

The samples of vessel segments for electron microscopy were postfixed for 60 minutes in 2.5% glutaraldehyde in White's saline (pH 7.3).[7] Second fixation was achieved by exposure for 90 minutes in 1% OsO_4 containing 1% potassium ferrocyanide. Thereafter, samples were dehydrated in a graded series of alcohol and then embedded in Epon 812. Cross-sections of the vessel segments were obtained using an ultramicrotome. Contrast of ultrathin sections was enhanced with uranyl acetate and lead citrate.

Statistics

Results are expressed as mean ± SEM. Student's *t* test for paired data was used for comparisons before and after perfusion. The level of statistical significance was established at $P < .05$.

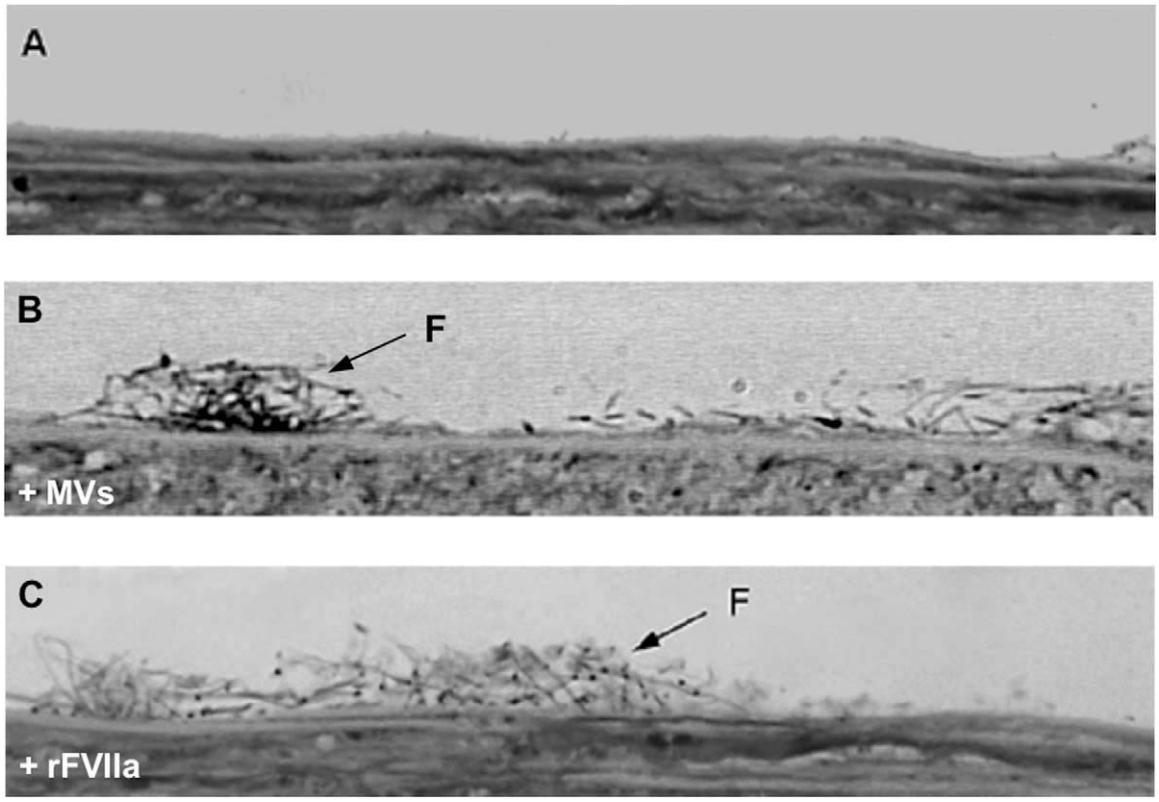

Figure 1. Light micrographs illustrating the most remarkable features observed in cross-sections of vascular segments after being exposed to circulating thrombocytopenic blood for 10 minutes at 600 s^{-1}. Under severe conditions of thrombocytopenia (A), presence of platelets and fibrin are extremely reduced. Addition of 1 mg/mL of purified platelet membrane fragments (B) or 10 μg/mL rFVIIa (C) resulted in an increase of fibrin deposition (F) on the subendothelium.

Results

Effect of rFVIIa and Microvesicles Under Conditions of Severe Thrombocytopenia

Under conditions of severe thrombocytopenia (<6,000 platelets/μL) and using a shear rate of 600 s^{-1}, only very low percentages of the vessel surface were covered by platelets. The addition of platelet microvesicles (1 mg/kg) or rFVIIa (10 μg/mL plasma) did not significantly affect the deposition of platelets. However, the same concentrations of agents, used independently, caused a significant increase in the amount of fibrin generated on the damaged vascular surface ($P < .05$; Fig 1).

Presence of Microvesicles in Thrombocytopenic Blood

Whole platelets were rarely identified in the perfusion studies with severely thrombocytopenic blood. However, immunocytochemical studies performed with ANV-FITC on cryosections of vascular segments perfused in the presence of microvesicles revealed the presence of fluorescent spherical or oval structures involved in the formed fibrin masses (Fig 2).

Further studies using the electron microscope confirmed the involvement of microvesicles in fibrin lattices generated on the damaged subendothelial structures. These microvesicles were observed at different levels of the formed fibrin layers (Fig 3).

Effect of rFVIIa and Microvesicles Under Moderate Thrombocytopenic Conditions

As shown in Fig 4, under conditions of moderate thrombocytopenia, exogenously added platelet microvesicles enhanced fibrin formation on the subendothelium at a shear rate of 600 s^{-1}. A similar tendency was observed in the presence of rFVIIa.

Under these experimental conditions, there were slight, but not statistically significant, increases in the platelet coverage on the formed fibrin masses (Fig 4). Selected micrographs illustrating some of the most remarkable features of these perfusions are shown in Fig 5.

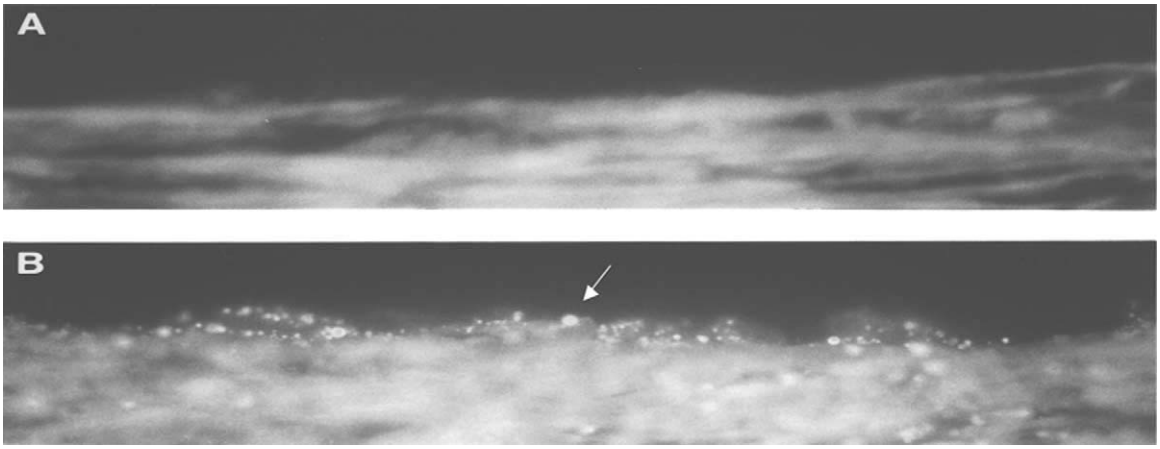

Figure 2. Immunolocalization experiments using ANV-FITC on cryosections from vascular segments perfused with severely thrombocytopenic blood. Presence of positive labeling was infrequent (A). Perfused subendothelium with thrombocytopenic blood in the presence of microvesicles. Fluorescent microvesicles were readily observed in close relation with fibrin masses in those experiments in which blood was enriched with exogenously added platelet microvesicles (B).

Discussion

The control of bleeding episodes in patients with thrombocytopenia, who are refractory to platelet transfusions, poses critical problems to transfusion services. Although there are several reports of rFVIIa improving hemostasis in patients with quantitative[4,14] and qualitative deficiencies of platelet GPIIb-IIIa,[6,20] the possible mechanism of action remains only partially understood.[10,13,16] The present study provides further insight into the mechanisms involved in the hemostatic action of rFVIIa under conditions of thrombocytopenia.

Our results demonstrate that the presence of platelet microvesicles or rFVIIa can enhance procoagulant action localized at sites of vascular damage. These initial impressions confirm our previous findings,[9,10] that is, exposure to tissue factor at sites of vascular damage supports local coagulation mechanisms and thus avoids more generalized thrombin action. Immunocytochemical analysis of our perfused vascular samples confirmed

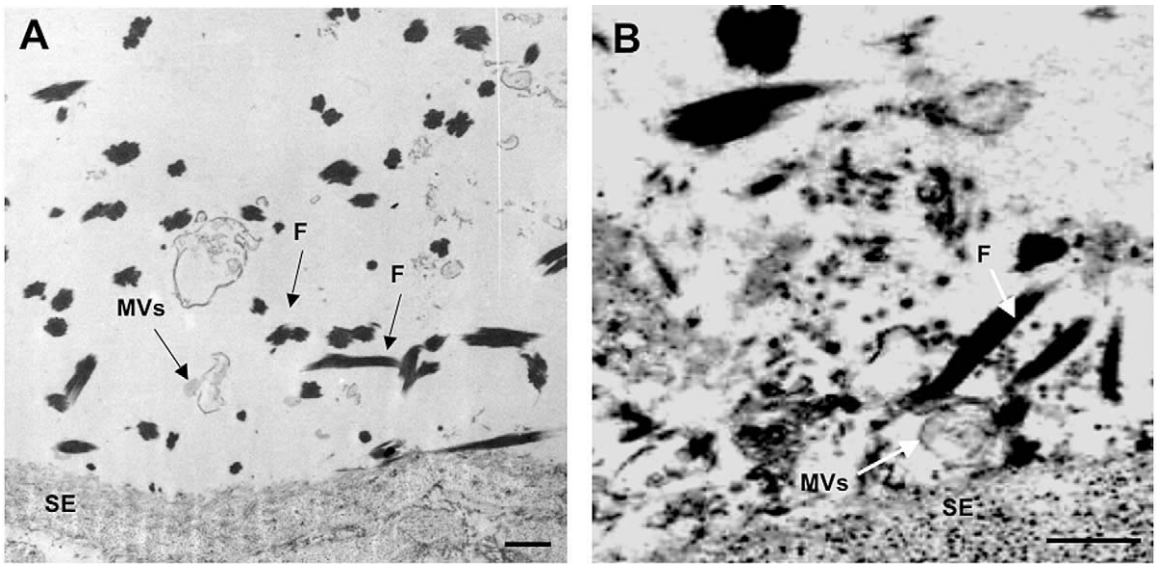

Figure 3. Electron micrographs of ultrathin sections of vascular segments perfused with severely thrombocytopenic blood enriched with platelet microvesicles. Microvesicles (MVs) were observed at different layers of fibrin strands (F) formed on the subendothelium (SE) (A). Occasionally, microvesicles are observed attached to the subendothelium at specific sites where fibrin strands are originating. Bar equals 500 nm (B).

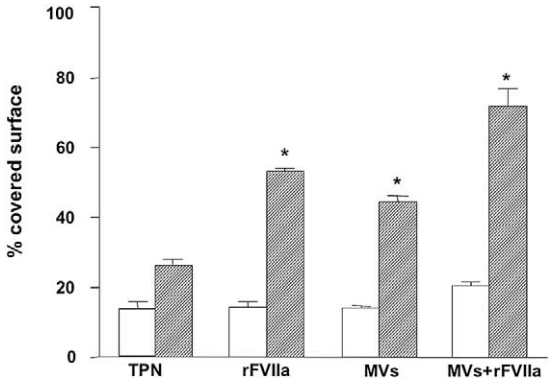

Figure 4. Bar diagram showing percentage of subendothelium covered by platelets (□) or fibrin (▨) in perfusion studies performed with blood experimentally manipulated to produce a moderate thrombocytopenia (TPN) (25,000 platelets/μL), after the addition of rFVIIa 10 μg/mL, platelet microvesicles (MVs) 1 mg/mL, or a combination of both agents. Results are expressed as mean ± SEM.

that fibrin generated under conditions of severe thrombocytopenia was often localized on areas where microvesicular structures showed positive labeling for ANV. Furthermore, ultrastructural studies confirmed the presence of microvesicles in different layers of the fibrin lattices formed on the subendothelium.

Studies from our group have suggested that nonviable platelets,[1] and even platelet microvesicles, retain major glycoprotein receptors[5] that could support residual adhesive functions. In this respect, Owens reported platelet microvesicles attached to the subendothelium in similar perfusion studies.[19] It is suggested that due to their reduced size, platelet microvesicles circulate in the boundary layer and the presence of some functional receptors or even anionic phospholipids could be sufficient to permit anchorage to the subendothelial structures.

Flow conditions are known to have a great impact on the balanced participation of platelet or coagulation mechanisms in different vascular territories. At elevated shear rates, von Willebrand factor plays a crucial role in initial platelet attachment through its interaction with platelet GPIb.[21] However, local thrombin generation and coagulation mechanisms seem to be more important at low shear rates.[27] It is important to emphasize that our studies were performed at intermediate shear rates (600 s^{-1}) where both platelet and coagulation events coexist. Whether our results could be extrapolated to bleeding situations taking place at vascular areas exposed to other shear conditions is a matter that deserves further investigation.

It has been suggested that platelets are critical for the hemostatic action of rFVIIa.[12,18] However, our present observations indicate that whole platelets may not be essential provided that sufficient phospholipids are available from platelet membrane fragments. Since, platelet microvesicles are not detected by current automated blood-cell counters, it is important that their potential presence should be considered when rFVIIa is scheduled for use in patients with apparent thrombocytopenia. Indeed, the presence or absence of circulating platelet microvesicles

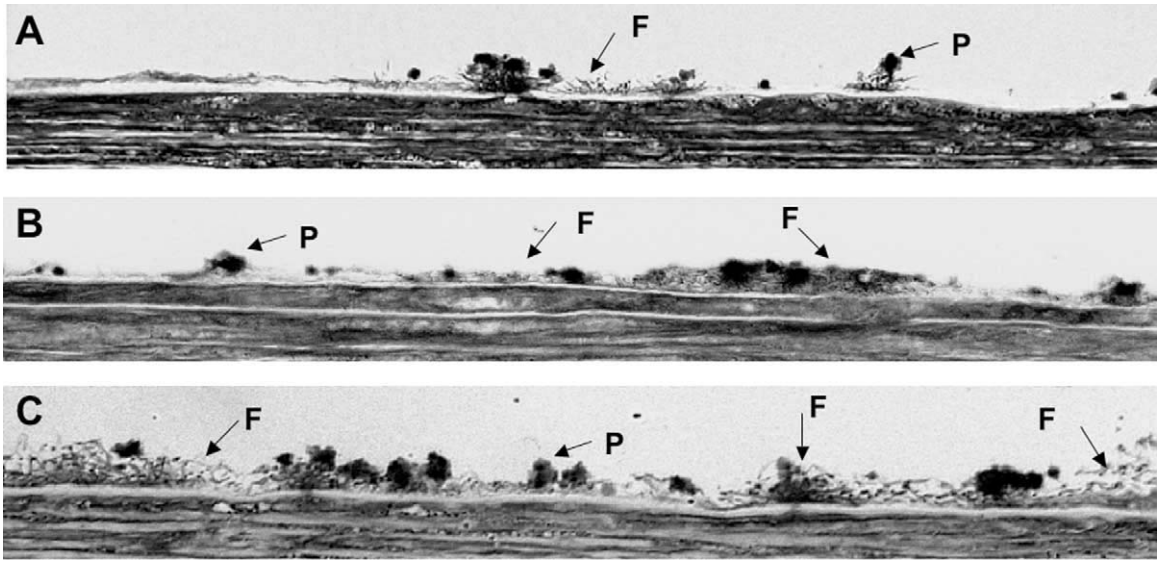

Figure 5. Light micrographs illustrating the most remarkable features observed in cross-sections of vascular segments after being exposed to blood manipulated to produce a moderate thrombocytopenia (25,000 platelets/μL) for 10 minutes at a shear rate of 600 s^{-1} (A). Recombinant FVIIa improved fibrin (F) formation (B). Combined presence of rFVIIa and microvesicles caused a further increase in the generation of fibrin on the damaged vascular surface (C). P, platelets.

could help explain current inconsistencies in the effects of rFVIIa[14] and, under special circumstances, might even contribute to an additional risk of unwanted thrombotic complications.

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The authors wish to thank Montserrat Viñas and Marc Pino for their excellent technical assistance.

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TISSUE FACTOR IMMOBILIZED ON SURFACES PROMOTES PLATELET ADHESION AND FIBRIN FORMATION UNDER FLOW CONDITIONS: IMPORTANCE OF SHEAR RATE AND FVIIa

Running head: Platelet adhesion on TF

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SUMMARY

Background: Reactivity of platelets to human tissue factor (hTF) has not been studied in detail.

Methods: We explored the interaction of platelets with hTF firmly adsorbed on a synthetic surface using different shear rates. For studies at 250 and 600s^{-1}, TF adsorbed on a synthetic surface was exposed to flowing anticoagulated blood in flat perfusion devices. Deposition of platelets and fibrin were evaluated by morphometric, immunocytochemical and ultrastructural methods. Experiments at 5000s^{-1}, were performed on the PFA-100™ with experimental cartridges with collagen or collagen-hTF. Effect of rFVIIa was assessed in the previous experimental settings. F1+2 levels were also measured.

Results: Platelet deposition on hTF reached 19.8±1.3% and 26.1±3.4% of the total surface respectively. Our results suggest that von Willebrand factor could mediate these interactions. Fibrin formation was significantly higher at 250 s^{-1} ($p<0.05$). FVIIa did not influence platelet deposition but significantly raised fibrin formation and thrombin generation ($p<0.05$). At 5000 s-1, closure times (CT) in the PFA-100 were significantly shortened en the presence of hTF (154.09 ±14.69s vs 191.45± 16.09s with collagen alone; $p<0.05$). Addition of rFVIIa did not cause a further reduction of CT.

Conclusions: Our studies demonstrate that hTF is an adhesive substrate for platelets. At low and intermediate shear rates, rFVIIa enhanced the procoagulant action of hTF, but this effect was not observed at very elevated shear rates.

Keywords: flow conditions, PFA-100, platelets, rFVIIa, tissue factor.

INTRODUCTION

Physiological mechanisms of hemostasis are initiated after an injury disrupts the integrity of the vessel wall. The main proteins involved in the initiation of prohemostatic mechanisms are collagen, von Willebrand Factor (vWF) and Tissue Factor (TF). There is abundant information on the effect of collagens and vWF on platelet reactivity [1;2] and it is also accepted that TF is the main trigger of the coagulation mechanisms [3].

Rupture of a unstable atherosclerotic plaque and subsequent exposure of TF and subendothelial components is believed to lead to the formation of occlusive thrombi and sudden ischemia downstream vascular territories [4]. It has been postulated that exposure of TF to circulating blood and successive thrombin generation are the major contributors to acute thrombotic events on arterial vasculature. Platelets are known to play a central role in the pathogenesis of occlusive events [4]. Interestingly, there is no convincing evidence in the literature that platelets could interact directly with TF.

Early vascular biology studies evidenced the presence of TF on the subendothelium [5;6], at the inner layers of the vessel wall, the more superficial layers constituting a barrier to prevent activation of the coagulation cascade in case that only the endothelium would get damaged. The coagulation mechanisms initiated by the assembly of complex tissue factor-FVIIa in damaged areas is the most important mechanism for thrombin generation [7]. This thrombin leads to platelet activation, thus facilitating thrombus formation [8].

Evidence raised in experimental models with circulating blood predict that at elevated shear rates taking place at the arterial circulation, platelet deposition is enhanced, but fibrin formation is markedly reduced [9;10]. Moreover, elevated shear rates also result

in increased detachment of platelets, having a negative impact on the size of platelet accumulation [11].

Using diffusion and kinetic models, Hathcock et al [12] postulated important limitations for the progression of an occlusive thrombi initiated by TF on the vessel wall due to platelets themselves covering the initially exposed TF. It was hypothesized that there should be a source of circulating TF (blood borne TF) that would facilitate thrombus progression by transfer of new TF to the thrombotic interface, which may result in further activation of the coagulation system at sites of vessel injury [13]. The precise source of circulating tissue factor is the object of investigations. Monocytes and neutrophils are likely to be the main source of this TF, but other recent reports propose that platelets contain small amounts of TF that may be expressed upon platelet activation [14;15] facilitating thrombus propagation. In contrast with these hypothesis on circulating sources of TF, Day et al [16] have recently suggested that vascular TF could still be the major contributor to microvascular thrombosis.

To gain more insight into the contribution of TF to hemostasis and thrombosis mechanisms we employed a parallel perfusion device based on models previously described [17] using polyvinilidene based surfaces as a support for sprayed human TF. We performed immunocytochemical studies to determine the contribution of vWF and fibrinogen to TF related hemostatic mechanisms. For investigation on the role of the thrombogenic potential of TF at very elevated shear rates we used experimental cartridges coated with collagen alone (COL) or a mixture of collagen and TF (COL-TF) in the PFA-100™.

MATERIALS AND METHODS

Experimental design

We explored the role of TF in hemostasis under different shear rates. For this purpose, we applied two different approaches: i) TF adsorbed on a synthetic surface and exposed to circulating human blood at low-intermediate shear rates (250 and 600s^{-1}) using perfusion systems [17], and ii) TF adsorbed to experimental collagen cartridges and exposed to extremely high shear rates (5000s^{-1}) using the Platelet Function Analyzer (PFA-100™)[18]. The effect of FVIIa, the natural ligand of TF, was also explored. After perfusion experiments, some of the coverslips were treated for Scanning Electron Microscopy and others for immunocytochemical studies.

Blood samples

The study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. Blood samples were obtained by arm venipuncture from healthy donors (n=12) who had not taken any drug known to affect either platelets or the coagulation system in the previous 10 days. Blood samples were anticoagulated with 20 U/ml low molecular weight heparin (Fragmin, Pharmacia & Upjohn, Stockholm, Sweden), which keeps blood anticoagulated but allows thrombin generation [19].

Chemical reagents and antibodies

Thromboplastin from human placenta (Thromborel S®, Dade-Behring, Marburg, Germany) was used as a source of TF. Polyvinilidene difluoride membranes (PVDF) were purchased from Bio-Rad (Hercules, CA). Experimental collagen cartridges were provided by Dade-Behring. rFVIIa was from NovoSeven® (NovoNordisk, Bagsvaerd,

Denmark). IgG and primary antibodies against von Willebrand Factor (vWF) and fibrinogen were purchased from DAKO (DAKO A/S, Denmark). The antibody against TF was from American Diagnostica (Greenwich, CT). Phosphate buffered saline (PBS) was from Roche (Indianapolis, IN). Fixing solutions were prepared diluting 25% glutaraldehide (Merck, Darmstadt, Germany) in PBS to reach final concentrations of 2.5%.

Studies with flowing blood: Perfusion Studies

TF from Tromborel was reconstituted following manufacturer's instructions and 100µl of the suspension were sprayed with an air brush onto 18 x 18 mm^2 PVDF surfaces. The sprayed coverslips were kept at 4ºC overnight. For each perfusion, two PVDF coverslips were inserted into the separate receptacles of a parallel-plate perfusion chamber [17]. Prior to the initiation of perfusion, blood samples were incubated for 1 minute with either diluent or aliquots of rFVIIa calculated to reach concentrations of 5 µg/ml at the plasma interface. This concentration approximately corresponds to 190 µg/kg body weight if extrapolated into patients. After incubation, blood samples were immediately perfused through parallel-plate perfusion chambers. Blood samples at 37ºC were recirculated through the perfusion chamber at shear rates of 250 or $600s^{-1}$ for 10 minutes. The perfused surfaces were rinsed with PBS and fixed with 2.5% glutaraldehyde at 4ºC for 24 hours. For each experiment, one of the coverslips was processed histologically for cross-section analysis [20]. The other coverslip was used for Scanning Electron Microscopy or for immunolabelling studies.

For cross-section analysis, the surfaces were dehydrated through increasing ethanol concentration gradient, embedded in JB-4 plastic compound (Polyscience Warrington, PA), thin sectioned for bright field microscopy and then stained with 1% Toluidine blue. Fibrin deposition on the subendothelium as well as platelet interactions, were

morphometrically evaluated in a bright field microscope equipped with a split prism that projects a virtual image of the preparation on a digitizing tablet. The profiles of the platelet aggregates were introduced in a computer and analyzed with a specially designed software [20] which automatically classifies and quantifies the total percentage of the screened vessel surface covered by platelets (% CS) or fibrin (%F). Studies were conducted according to a single blind design. The technician performing the morphometric evaluation was unaware of the experimental design.

Platelet Function Analyzer (PFA-100) studies

To explore the hemostatic potential of TF associated to collagen at high shear rates ($5000s^{-1}$) properties we used the Platelet Function Analyzer (PFA-100, Dade-Behring) [18] with experimental cartridges containing a membrane coated with collagen alone or collagen/Tissue Factor (COL/TF). For the latter purpose incubated for 2 minutes the collagen coated apertures of the cartridges with 40 µl of human Thromboplastin (Thromborel S®). Blood samples were aspirated under the controlled flow conditions through the 150 µm aperture cut into the COL or COL/TF membrane and the platelet hemostatic capacity of the blood sample was measured by the time (expressed in seconds) required for the platelet plug to occlude the aperture (closure time or CT).

Scanning Electron Microscopy

Fixed coverslips were rinsed in PBS and postfixed with 1% osmium tetraoxide plus potassium ferricyanide (0.8%) for 1 hour at 4ºC. Samples were then rinsed in water and dehydrated in a progressive series of alcohols. Critical point drying techniques were applied to the samples in a CPD 7501 apparatus, and finally, were mounted on a holder,

where they were coated with a thin layer of gold. Samples were observed by Scanning Electron Microscopy using a Zeiss DSM 940A microscope at 15 KV of acceleration.

Evaluation of prothrombin fragment F1+2 generation

Thrombin generation during perfusion at low and moderate shear rates was indirectly assessed through measurement of prothrombin fragments F1+2 in plasma samples collected before and after perfusions as described elsewhere [21].

Immunocytochemical detection in sections and silver enhancement

For immunocytochemical studies we used whole blood from healthy donors anticoagulated with LMWH or the same blood depleted of platelets and leukocytes using RC100 filters (PALL Corp., Glen Cove, NY) as described elsewhere [22]

Perfused PVDF surfaces were fixed with 4% paraformaldehyde in 0.15M PBS, pH 7.4, dehydrated, embebed in glycol-methacrylate and processed histologically to obtain thin sections. Sections of the perfused samples were incubated for 60 min with specific antibodies. The antibodies against vWF and Fibrinogen were diluted 1:50 in PBS. After removing the excess of antibodies by washing (3X) with PBS, coverslips were incubated with a gold-conjugated secondary antibody. The excess of the secondary antibody was removed by washing the coverslips with PBS (3X) and distilled water (3X). Finally, samples were treated with an IntenSE Silver Enhancement reagent (Amersham Pharmacia Biotech) [23].

Parallel studies were conducted to confirm the homogenous coating of the PVDF membranes. For that purpose, non-perfused surfaces coated with human thromboplastin were incubated with an anti TF diluted 1:50 in PBS and processed as described above.

In all experimental settings non-specific binding was assessed with an irrelevant antibody (IgG).

Statistics

Results were expressed as mean ± standard error of the mean (SEM). The number of experiments for perfusion studies was at least n=6, and n=10 for PFA-100 studies. Student's t-test and paired t-test were used for statistical comparisons in perfusion studies and PFA-100 data. Means Fisher's least significant difference (LSD) procedure was used to compare F1+2 levels. The level of statistical significance was established at $p<0.05$.

RESULTS

Studies with flowing blood at low and intermediate shear rates

At low shear rate, TF induced a basal percentage of platelet coverage (%C.S.) equivalent to 19.8±1.3%. Exogenous addition of 5µg rFVIIa/ml of plasma increased platelet deposition up to 28.6±7.5%. A similar tendency was observed in experiments performed at $600s^{-1}$, with values of 26.1±3.4% platelet coverage in basal conditions vs 30.9±5.2% in the presence of rFVIIa. Despite the tendency to increase platelet interaction in the presence of FVIIa, differences did not reach statistical significance in both cases.

Fibrin formed in the same microscopic fields was evaluated showing a statistically significant reduction ($p<0.05$) in experiments performed at 600 s^{-1} vs 250 s^{-1}. Experiments performed at $250s^{-1}$ showed a 13.1±1.1% fibrin coverage of the screened surface. Addition of FVIIa significantly increased fibrin formation up to 29.0±2.4% ($p<0.05$). Fibrin formation at $600s^{-1}$ was 3.14±1.61 and increased significantly up to 12.2±1.2% in the presence of rFVIIa ($p<0.05$). Figure 1 summarizes morphometric values in the different groups of study and Figure 2 illustrates the phenomena above described.

Scanning electron microscopic analysis of the non-perfused PVDF surface sprayed with human thromboplastin showed a homogenous coating of the area (see figure 3 panel a). Pictures of surfaces perfused at 600 s^{-1} confirmed results obtained using bright field microscopy and revealed platelet aggregates deposited onto the fibrin layer (see figure 3 panel b).

Studies with flowing blood at elevated shear rate ($5000s^{-1}$)

Basal closure times in cartridges coated with collagen alone were 191.45±16.07 seconds. The presence of human TF in the membrane, shortened closure times to 154.09±14.69 s ($p<0.05$). Using COL alone cartridges, addition of FVIIa to blood did not reduce the closure time in a significant manner (179.6±23.49s). Incubation of blood with FVIIa and exposure to COL-TF membranes did not result in a significant further shortening of the closure time (143.2±14.26s) when compared to the same cartridges but in absence of rFVIIa. These results are summarized in figure 4.

Evaluation of thrombin generation

Pre-perfusion values of F1+2 were 0.73±0.09 nM. As shown in table 1, the single addition of FVIIa to blood samples resulted in a slight increase of pre-perfusion F1+2 values compared to baseline levels, but differences did not reach the level of statistical significance.

Prothrombin fragment 1+2 levels in plasma significantly increased throughout perfusion in all experimental groups (Table 1). F1+2 levels rose from 0.73±0.09 nM to 2.42±0.39 nM at 250 s^{-1} and to 3.81±0.49 at 600 s^{-1}. Addition of 5 µg rFVIIa/ml of plasma increased post perfusion F1+2 levels in a significant manner compared to pre perfusion values. F1+2 results are summarized in table 1.

Immunolocalization assays in the perfused surfaces

TF immunolocalization studies revealed an intense labeling on the interface of the thromboplastin coated surfaces. After perfusion with filtered blood at $600s^{-1}$ for 10 minutes, immunostaining revealed high presence of fibrinogen and von Willebrand Factor on the TF rich surfaces (Figures 5a, 5b, 6a and 6b), suggesting that these proteins can

interact with TF somehow.

In experiments conducted with whole blood under the same perfusion conditions, vWF labeling was very positive and homogenous on the TF-rich surface, not only in the areas not covered but also under the platelet aggregates (Figure 5c and 5d). Surprisingly, FGN labeling was positive on the TF interface but was almost absent under platelets deposits (Figure 6c and 6d). These observations suggest that vWF would be involved in the first steps of platelet interaction with TF and FGN would play a more relevant role in the following hemostatic phases.

Discussion

We have explored the response of platelets to a preparation of human TF, exposed to blood circulating at low (250 s^{-1}), moderate (600 s^{-1}) and very high (5000 s^{-1}) shear rates. Our results demonstrate that human TF used in our studies acts as a proadhesive substrate for platelets when presented firmly immobilized on a surface. Our data indirectly suggest that vWF may play a role facilitating platelet adhesion under our experimental conditions. Presence of activated FVII enhances the procoagulant activity of the hTF surface under low and intermediate shear rates, but this action was not observed at very elevated shear rates.

For studies in blood circulating at low and moderate shear rates we applied a variation of the original parallel-plate perfusion technique [17] using PVDF membranes as a support for purified proteins. Optical and physical characteristics of this material facilitates both en face and cross-sectional analysis, allowing the quantification of platelet and fibrin interactions with the hTF-rich substrata. In our experimental studies this PVDF surface covered with hTF, was exposed to circulating human anticoagulated blood perfused at low and moderate shear rates. Thromborel S ®, a semi-purified preparation of TF originated from human placenta was used as a source of hTF. This preparation of hTF is widely used for the determination of the prothrombin and different aspects of activity and purity have been previously characterized [15;24]. It is very likely that the TF preparation used in our studies is compatible with the currently accepted presentation of TF in circulating microvesicles [25], and presumably as the TF-rich material that may become exposed at sites of ruptured atherosclerotic plaques [4].

In a previous report Orvim et al [24] exposed the same preparation of hTF to blood directly drawn from the antecubital vein of healthy donors. Under those experimental conditions the previous authors noted a characteristic pattern of interaction with large deposits of

fibrin laying immediately on the TF interface and with platelets adhering exclusively to the upper strands of fibrin formed on the basal TF layer. These observations suggested that fibrin generated on the hTF surface preceded platelet thrombus formation. In contrast with the cited report we were unable to detect evidence of fibrin deposition in our studies using blood anticoagulated with LMWH. Platelets appeared in direct interaction with the hTF substrate. Interestingly, we were able to reproduce the characteristic deposits of fibrin at the perfused hTF interface with platelets thrombi attaching to the upper layers of fibrin in studies performed in the presence of rFVIIa (figure 2B). These observations are consistent with previous studies from our group suggesting that the beneficial effects of rFVIIa under different conditions of altered hemostasis would be mainly related to an enhanced fibrin formation at sites of vascular damage, with platelets becoming adherent on the fibrinogen/fibrin strands [21;26].

We are convinced that minimal amounts or activated FVII are generated during studies with non-anticoagulated blood and that FVIIa generated would be responsible for the initial fibrin deposits observed in earlier studies [24]. The use of LMWH in our system prevents activation of the coagulation system [19] and allowed the investigation of an intrinsic proadhesive activity of the hTF preparation towards platelets that seems not dependent of fibrinogen deposition or fibrin generation. Our immunocytochemical analysis at the hTF interface, exposed to blood anticoagulated with LMWH indicate that vWF binding would precede deposition of fibrinogen or fibrin. In fact, our observations suggest vWF would mediate the initial interaction of platelets with hTF whereas, fibrinogen would play a more relevant role in subsequent steps of platelet deposition or when fibrin is formed. These observations could be of interest in the clinical setting, since a large population of patients at risk of ischemic events receive LMWH for prophylaxis or treatment of thrombotic complications [27]. Implications of these mechanisms in the

pathophysiology of thrombosis in anticoagulated vs. non anticoagulated patients should be the object of further investigations.

The composition of thrombi formed on a damaged vascular area is highly dependent on the shear rate. Under low shear rate conditions (<400 s^{-1}) such as those taking place in venous territories, activation of coagulation factors and fibrin generation prevail over platelet mediated events [8]. Conversely, at elevated shear rate conditions developing in arterial territories (from 600 s^{-1} to 1500 s^{-1}), platelet events are predominant and fibrin formation is reduced [1;9]. In the present studies, hTF exposed on surfaces facilitated platelets interactions at low and intermediate shear rates, favoring fibrin formation when activated rFVIIa was present. These observations will go along with the classic concept on the role of vascular TF in the maintenance of correct hemostasis [5].

Under our experimental settings, rFVIIa consistently showed a tendency to enhance the procoagulant activity of TF exposed to intermediate shear rates. Elevations in F1+2 levels indicated that these effects were partially related through an increase in thrombin generation. The enhanced procoagulant activity generated on the TF substrata when rFVIIa was added to the anticoagulated blood was similar to that previously reported by our group in studies on vascular segments exposing collagen and TF [26;28]. Collagen exposed on damaged vascular surfaces is highly reactive for platelets and is known to induce maximal expression of anionic phospholipids on activated platelets. The combined presence of collagen, TF and activated phospholipids would potentiate the thrombogenic of the vascular damaged areas. Our experimental studies suggest that the thrombogenic potential of vascular damaged areas could be further enhanced in the presence or rFVIIa. Recent surveillance studies seem to indicate that administration of rFVIIa could be associated with an elevated risk of thromboembolic complications [29]. While this thrombogenic potential could be acceptable for patients with altered

hemostasis [30], there is a reasonable concern that rFVIIa could promote thromboembolic complications in damaged arterial territories when administered in patients with preserved hemostasis.

Very elevated wall shear rates (>2500 s^{-1}) are uncommon under physiological conditions, but can develop in the microvasculature or under pathological conditions at sites of arterial stenosis [31]. Since recreation of very high shear conditions is problematic with conventional parallel perfusion chambers, we decided to use the PFA-100 to test the prothrombotic potential of the association of hTF and collagen in the presence of FVIIa. For this purpose we used specially devised cartridges exposing collagen alone (COL), or collagen and hTF in their apertures [32]. Our data in this system confirm that hTF has a positive impact on the hemostatic performance as assessed by the significantly reduction in closure times with respect to values observed with cartriges containing collagen alone. Results of our studies at very high shear rate would be in agreement for a role of vascular TF promoting hemostasis [5] with possible implications on microvascular thrombosis [16] .

In contrast with results of studies at lower shear rates, presence of rFVIIa did not cause a further potentiation of hemostasis at the very elevated wall shear generated in the PFA-100. suggesting that the hemostatic action of this agent would not be evident in this particular condition. A possible rationale for these findings would be the observations by Hathcock et al [12] that have postulated important limitations for the progression of an occlusive thrombi initiated by TF. According to these authors platelets deposited on damaged vascular areas would act themselves as a physical barrier, restricting the convective and diffusive exchange of substrates and coagulation products between the blood and reactive vessel wall, thus limiting the role TF plays in thrombus growth.

Results of our experimental studies indirectly suggest that possible thromboembolic complications of rFVIIa may be more relevant in venous territories and less frequent on arterial ones. An important limitation of our studies being that they were performed with blood from healthy volunteers. Presence of circulating TF has been considered to be important for thrombus growth [13]. Since a determinant for the safety of rFVIIa is its specific mechanism acting on exposed, but also on potentially circulating TF, caution is required when this activated factor is used for unlabeled indications in patients that have a pre-existing condition for elevated thrombotic risk.

In summary, data from our experimental studies substantiate that hTF exposed in surfaces as it may become expressed at damaged vascular areas is an adhesive substrate for circulating platelets. Our data indirectly suggest that vWF may play a role facilitating platelet adhesion under our experimental conditions. In addition, our studies with anticoagulated blood from healthy individuals, support the concept that activated FVII enhances the procoagulant activity of the hTF surface under low and intermediate shear rates, but this action would be less relevant at very elevated shear rates.

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FIGURE LEGENDS

Figure 1: Results from perfusion studies performed at low and moderate shear rate (250 and 600 s^{-1} respectively). A) Bar diagram displays the coverage percentage of the perfused surface by platelets before (empty bars) and after addition of 5 µg rFVIIa /ml of plasma (striped bars). Panel B summarizes the values of fibrin formation on the perfused surface under the same experimental settings. Results are expressed as % of coverage of the screened surface as mean±SEM. *$p<0.05$ vs experiments in the absence of rFVIIa

Figure 2: Bright field micrographs illustrate representative interaction patterns cross-sections of Tissue Factor-coated PVDF surfaces perfused at 600 s^{-1} for 10 minutes. A) Perfusion experiments performed with blood samples from healthy donors in basal conditions. B) Perfusion experiments performed with blood samples incubated with 5 µg rFVIIa/ml of plasma. Addition of rFVIIa to the blood samples improved fibrin formation on the surface significantly. Platelet deposition showed a clear tendency to increase in the presence of FVIIa, however, results did not reach levels of statistical significance. p: platelet interaction; f: fibrin deposits. (x 400)

Figure 3: Scanning electron micrographs of the Tissue Factor-coated surfaces perfused at intermediate shear rates ($600s^{-1}$) for 10 minutes. A) Polyvinilidene difluoride membranes (PVDF) coated with human thromboplastin prior to perfusion. B) Fibrin formation (F) and platelets deposition (p) were observed in the perfused areas. C) Detail of the perfused area at high magnification.

Figure 4: Bar diagram summarizes closure times (CT) results for those experiments performed at very high shear rate (5000 s-1). Presence of TF in the apertures of the cartridges reduced CTs (* $p<0.05$ vs COL). Presence of rFVIIa did not induce a further reduction of CT. Results are expressed in seconds as mean ± SEM.

Figure 5: Immunocytochemical localization of the von Willebrand Factor involvement in thrombus formation in perfusion studies performed on TF rich surfaces at 250 s-1 for 10 minutes. A) Low magnification bright field microscopy of a surface perfused with blood depleted of platelets rendered thrombocytopenia through a filtration procedure. B) Under the same experimental settings, von Willebrand Factor labelling was observed along the TF coated surface. Panels C and D correspond to perfusion experiments performed with whole blood. Panel C shows a detail of fibrin formation and platelet deposition on the TF coated surface. Immunolocalization of Von Willebrand Factor (D) in the same field revealed labeling on the whole surface, including portions located under the platelet aggregates.

Figure 6: Immunocytochemical localization of the Fibrinogen involvement in thrombus formation in perfusion studies performed on TF rich surfaces at 250 s-1 for 10 minutes. A) Bright field microscopy of a surface perfused with blood depleted of platelets at low magnification. B) Under the same experimental settings, fibrinogen labeling was observed along the TF coated surface. Panels C and D correspond to perfusion experiments performed with whole blood. Panel C depicts a detail of fibrin formation and platelet deposition on the TF coated surface. Immunolocalization of fibrinogen (D) in the same field revealed labeling on the TF surface, but not under the platelet aggregates.

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Table 1: Thrombin generation measured as prothrombin fragments 1+2 (F1+2).

	250s^{-1} ***n=4***		***600s^{-1}*** ***n=4***	
	Pre-perfusion	***Post- perfusion***	***Pre-perfusion***	***Post- perfusion***
Baseline (BAS)	0.73±0.09	1.94±0.46*	0.73±0.09	3.80±0.49†*
5 μg/ml rFVIIa	0.94±0.23	3.73±0.66*‡	0.94±0.23	4.86±0.32*

Results are expressed in nM as mean and standard error of the mean. *$p<0.05$ vs pre-perfusion values; ‡$p<0.05$ vs BAS; †$p<0.05$ vs $250s^{-1}$.

Figure 1

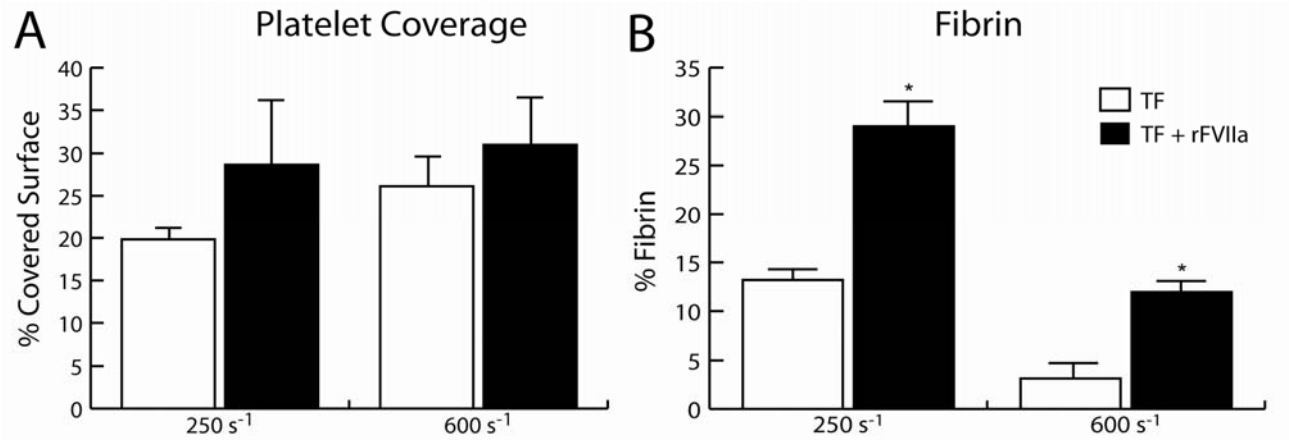

A
Platelet Coverage
% Covered Surface
40
35
30
25
20
15
10
5
0
250 s-1
600 s-1
B
Fibrin
% Fibrin
35
30
25
20
15
10
5
0
*
*
TF
TF + rFVIIa
250 s-1
600 s-1

Figure 2

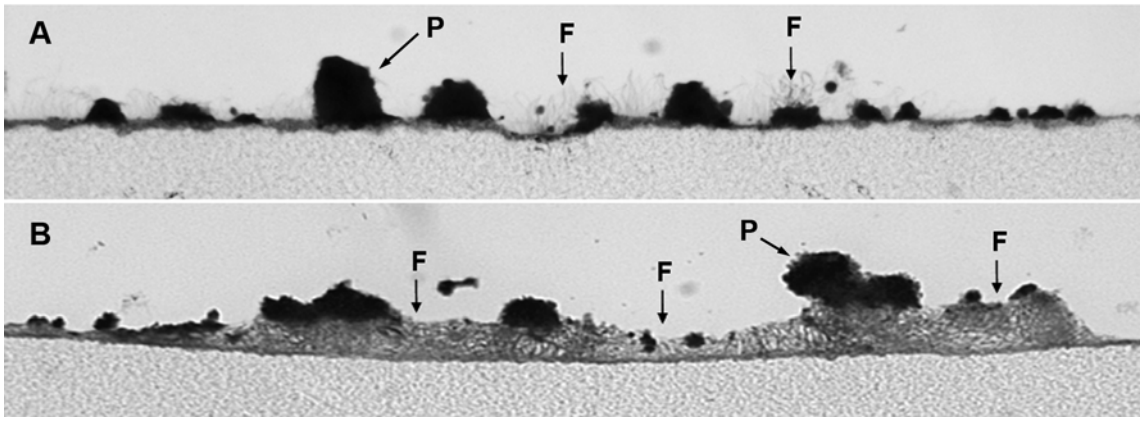


Figure 3

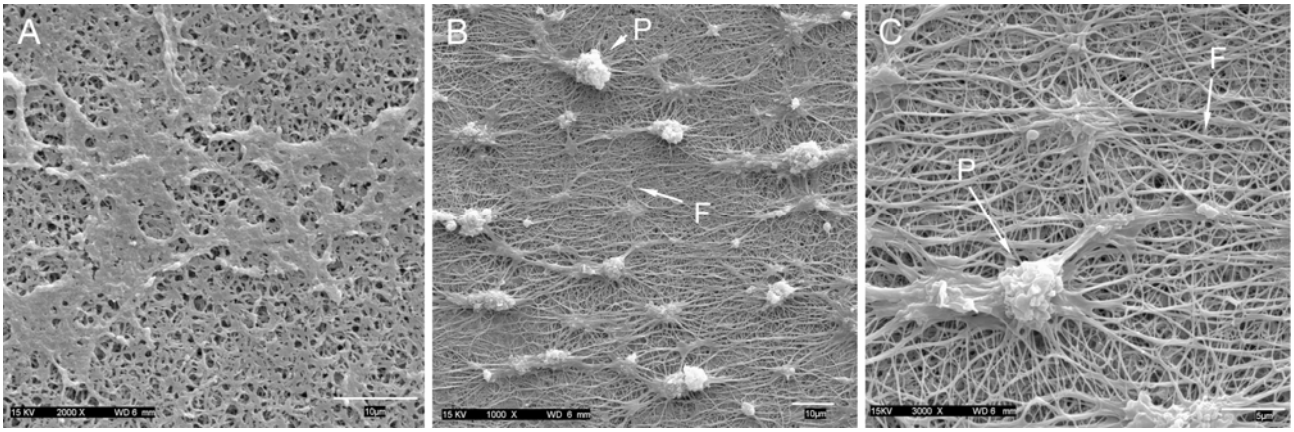


Figure 4

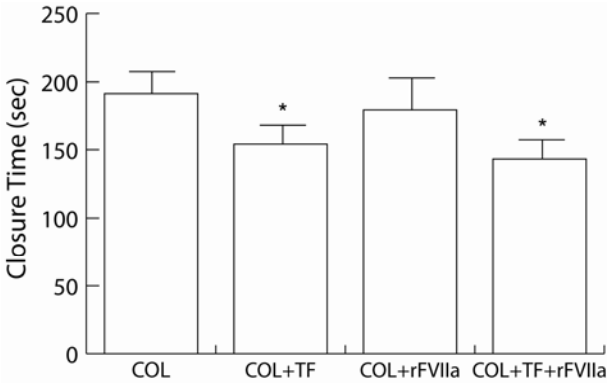

Closure Time (sec)
250
200
150
100
50
0
*
*
COL
COL+TF
COL+rFVIIa
COL+TF+rFVIIa

Figure 5

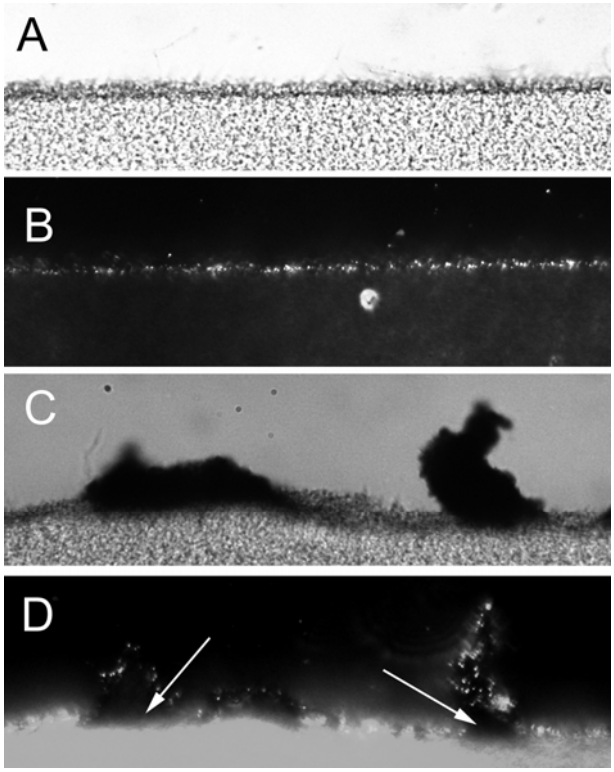
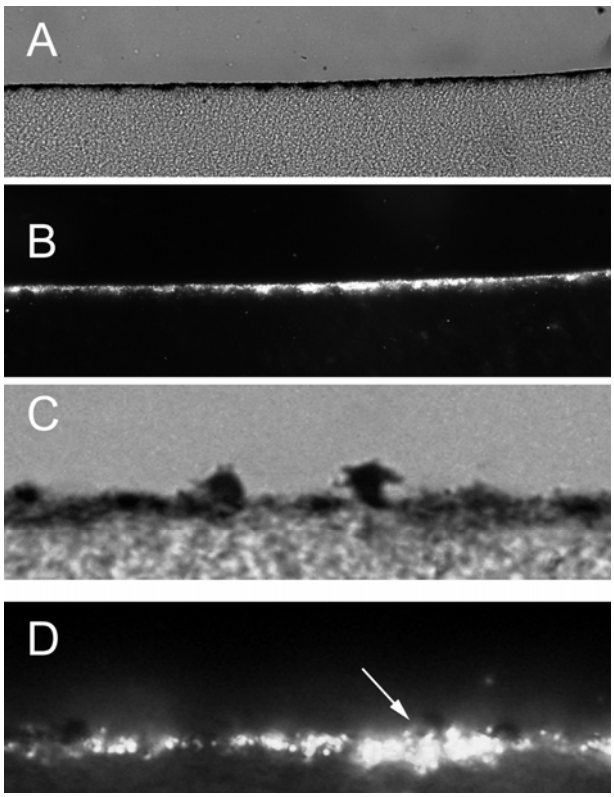


Figure 6



Recombinant FVIIa (rFVIIa) improves platelet dysfunction in patients with hemophilia: studies under flow conditions with collagen-tissue factor surfaces.

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ABSTRACT

Background: Although the bleeding tendency in hemophilia A can be explained by a defect in coagulation, a possible platelet dysfunction has not been formally investigated.

Methods: We studied collagen and TF-induced hemostasis using the blood of patients with severe hemophilia A in an in vitro set-up. Blood samples from healthy donors (n=11) or from patients (n=9) were anticoagulated with low molecular weight heparin. Perfusion studies at moderate shear rates ($600s^{-1}$), were performed using damaged vascular segments; whereas PFA-100® with specially devised cartridges of collagen alone (COL) or collagen-tissue factor (COL-TF) were used for high shear rates ($5000s^{-1}$). We also tested the ability of rFVIIa to improve hemostasis.

Results: Addition of rFVIIa (equivalent to 380 μg/Kg) to blood samples from healthy individuals induced an increase of fibrin formation (53.8±12.1% vs. 37.7±8.2%). In experiments performed with blood from hemophiliacs, fibrin deposition was almost absent (< 5%), but increased to 48.1±9.2% with rFVIIa. Experiments at high shear rate showed a prolonged closure times in samples from hemophilic patients using COL cartridges (255±22s vs.191±16s in healthy donors; $p<0.05$). Presence of TF in the apertures caused a 20% shortening in closure times in both cases. Addition of rFVIIa to hemophilic blood samples induced a further statistically significant reduction of closure times in COL-TF cartridges ($p<0.05$). This reduction was not observed in healthy individuals.

Conclusions: Under our experimental settings, hemophilic patients showed a platelet dysfunction at high shear rate. rFVIIa, in the presence of TF, contributed to a partial correction of this effect. Studies with healthy donors, indirectly suggest no additional prothrombotic profile for rFVIIa under very high shear rate conditions. Potentially, PFA-100 could be used as a system for monitoring FVIIa in hemophilic patients.

INTRODUCTION

Normal hemostasis is the result of an adequate balance of platelet function interplaying with activators and inhibitors of the coagulation system. Predominance of activating factors can lead to a prothrombotic state, whereas lack or impairment of the coagulation factors can result in severe bleeding. After vessel injury, highly reactive proteins, such as collagen and Tissue Factor, are exposed to flowing blood. The TF/FVIIa complex activates the coagulation protease cascade, which leads to fibrin deposition and platelet activation though thrombin generation [1;2]. Absences of factor VIII or factor IX (hemophilia A and B respectively) are the coagulopathies that course with the most severe bleeding episodes. Interestingly, the hemorrhagic symptoms seem to be related to alteration in coagulation mechanisms since platelet functions are thought to be normal. Recombinant factor VIIa (rFVIIa) was developed for the management and prevention of bleeding episodes in patients with FVII deficiency [3], as well as in patients with hemophilia A and B who have developed inhibitors [4;5]. Additionally, in vivo and in vitro studies have evidenced that rFVIIa improves hemostasis in several coagulopathies and thrombopathies favoring the development of an hemostatic response at sites where TF is exposed [6-13].

Several studies have investigated the mechanism of action of rFVIIa in patients with hemophilia in detail [14;15]. Recent studies suggest that, in these patients, hemostatic action of rFVIIa is the result of an increase of coagulation mechanisms and a decrease of fibrinolysis due to the activation of an inhibitor of fibrinolysis such as Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) [16]. Previous in vitro studies from our group and others consistently found that rFVIIa can exert procoagulant effects in blood from patients with severe thrombocytopenia, major glycoprotein deficiencies [11;17;18], patients with impaired coagulation system as hemophiliacs and patients treated with oral

anticoagulation [19] and cirrhotic patients [10] through an enhancement of thrombin generation.

It is well established that flow conditions can modulate hemostatic events and favoring different hemostatic responses [20]. It has been reported that high shear rate conditions prime platelet accumulation. This has been observed in vitro for platelet deposition on the subendothelium [21-23] and collagen-coated surfaces [24-26]. The effect of shear rate has also been demonstrated in human [27;28]. Most of the studies performed on procoagulant effect of rFVIIa do not take hemorrheologic mechanisms and different flow condition along the vascular system into consideration. Thus, further studies on the influence of shear rate conditions in hemostatic or prothrombotic profile of rFVIIa are required.

We wanted to investigate the implications of shear rate on platelet functions in patients with hemophilia and evaluate the contribution to hemostasis of TF. For this purpose, the present study is focused in the area of moderate shear rate found at the venous territory and the very high shear rate conditions that occur under pathologic conditions as stenosis, acute coronary syndromes [20]. Finally, we compared hemostatic/prothrombotic balance of rFVIIa at different shear rates in blood taken from patients with hemophilia and normal donors.

MATERIALS AND METHODS

Subjects and blood samples

The study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. Twenty subjects, 11 healthy donors and 9 patients with severe hemophilia A, were enrolled in the study. Blood samples were obtained by arm venipuncture from patients or donors who had not taken any drug known to affect either platelets or the coagulation system in the previous 10 days. Patients features are summarized in table I. Blood samples were anticoagulated with 20 U/ml low molecular weight heparin (Fragmin, Pharmacia & Upjohn, Stockholm, Sweden) which keeps blood anticoagulated but allows thrombin generation [29].

Moderate shear rate experiments ($600s^{-1}$)

Prior to the initiation of experiments, blood samples were incubated with either diluent or aliquots of rFVIIa (rFVIIa, NovoSeven®, Bagsvaerd, Denmark) for 1 minute. The amount of rFVIIa added was calculated to reach a concentration of 10 µg/ml at the plasma interface, which approximately corresponds to a dose of 380 µg/kg body weight if extrapolated into a 70 Kg patient. After incubation, blood samples were immediately perfused through annular chambers containing denuded arterial segments, as described by Baumgartner [30]. These damaged vessels are rich in collagen and Tissue Factor [31]. Blood was recirculated for 10 min at 37ºC using a peristaltic pump with the flow previously adjusted to give rise to a shear rate equivalent to 600 s^{-1} [10]. The hemostatic effectiveness of the blood samples was assessed using morphometric evaluation of platelet and fibrin deposition onto the subendothelium of the arterial segments [23].

Processing of vessel segments and morphometric evaluation

At the end of each perfusion, the arterial segments were rinsed with 20 ml of phosphate buffered saline, pH 7.2, sliced off from the chamber and fixed with the same buffer containing 2.5% glutaraldehyde. The fixed segments were processed histologically, as described in previous works [32].

Fibrin deposition on the subendothelium as well as platelet interactions, were morphometrically evaluated in the light microscope. Studies were conducted according to a single blind design. The technician performing the morphometric evaluation was unaware of the experimental design. Platelet interaction and fibrin deposition were analyzed using a specially devised program [33], which automatically classifies and quantifies the total percentage of the vessel surface covered by platelets (% CS) or fibrin (%F) in the same microscopic fields.

Scanning electron microscopy

For scanning electron microscopy (SEM) analysis, vascular segments perfused with blood samples from healthy donors or from hemophilic patients were fixed in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH= 7.4) at 4ºC overnight. Samples were washed in phosphate buffer and underwent a fixation process at 4 ºC in osmium tetraoxide (1%) with potassic ferricianure (0.8%) for 1 hour. After further washing, surfaces were dehydrated in graded ethanol solutions. Critical point drying techniques were applied to the samples in a CPD 7501 apparatus, and then were mounted on a holder, where they were coated with a thin layer of gold (sputtering). Samples were

observed by Scanning Electron Microscopy using a Zeiss DSM 940A microscope at 15 KV of acceleration.

Very high shear rates experiments using the Platelet Function analyzer (PFA-100).

Platelet function of healthy donors and hemophilic patients was assessed in the absence or in the presence of rFVIIa at high shear rates using the PFA-100^{TM} [34]. In this system, anticoagulated blood samples are pumped through a 150 µm aperture cut under controlled flow conditions (5000 s^{-1}). The hemostatic capacity of the blood sample is reflected by the time required for the platelet plug to occlude the aperture (closure time) [35]. Results are expressed in seconds. In this study, we used experimental cartridges coated only with collagen (COL) or with a combination of collagen and Tissue Factor (COL-TF). To prepare the latest ones we incubated collagen cartridges with 40 µl of human Thromboplastin and allowed to settle down for 2 minutes [36]. The estimated amount of TF is 0.52 ng/ cartridge. We incubated the blood either with saline solution or with rFVIIa (equivalent to a bolus of 380 µg rFVIIa/Kg of bodyweight) for 1 minute before the blood got in contact with the membrane. After the PFA-100 test finished, the membranes were sliced off from some of the cartridges and processed histologically as mentioned above. The structure of the hemostatic plugs formed on the membrane apertures were further analyzed using light microscopy on thin cross-sections.

Data analysis

All results are expressed as mean±SEM. Statistical evaluation of differences among groups of studies was performed using Student's t-test. The level of statistical significance was established at $p < 0.05$.

RESULTS

Studies with flowing blood at moderate shear rate experiments (600 s^{-1})

Exposure of blood samples from healthy donors to damaged vascular surfaces under conditions of moderate shear rate induced 26.1±4.2 % coverage of the screened surface by platelets and 37.5±8.1 % by fibrin. When we perfused blood samples from patients with severe Hemophilia fibrin formation was almost absent (less than 5%), though platelet deposition barely changed in comparison to experiments performed with blood samples from healthy donors (23.4±5.6 %). These results are summarized in figure 1.

In vitro addition of rFVIIa (equivalent to a bolus of 380 µg rFVIIa/Kg) to blood samples from healthy donors did not modify platelet deposition (22.3±4.2 %) significantly. However, it induced a remarkable increase of fibrin formation (53.8±12.1 % vs. 37.7±8.2 % in basal conditions). In experiments performed with blood samples from hemophilic patients, addition of rFVIIa resulted in 20.9±2.8% coverage of the screened surface by platelets and 48.1±9.2% by fibrin (figure 1). Scanning electron microscopy pictures in figure 2 illustrate the most remarkable features observed in cross-sections of the perfused vascular segments are shown.

Studies with flowing blood at very high shear rate (5000s^{-1})

Hemophilic patients showed a statistically significant prolongation of closure time vs. control donors with COL (255±22 s. vs. 191±16 s.; $p<0.05$) and COL-TF (216±23 s. vs. 154±14 s.; $p<0.05$) cartridges suggesting a functional impairment of primary hemostasis in these patients. In both cases, healthy donors and hemophilic patients, the presence of TF on the cartridge induced a 20% reduction of closure times (Figure 3).

In the presence of TF in the membranes, addition of rFVIIa to blood samples reduced closure times in both groups, but differences only reached levels of statistical

significance ($p<0.05$) in the group of patients with hemophilia, thus overcoming the initial platelet dysfunction. In these patients, the addition of rFVIIa enhance the recruitment of platelets interacting with COL-TF cartridges (176±28 s. vs. 216±23 s.; $p<0.05$). These results are summarized in figure 3.

Microscopic analysis of the plugs formed in the apertures showed that in hemophilic patients occlusive thrombi formed in the presence of TF were more compact and had higher occlusive capacity than the ones formed in the lone presence of COL. In the presence of TF, addition of FVIIa led to the formation of more effective platelet plugs as inferred by a shortening in the closure times. The observation of the plug revealed the possible presence of microenvironments, where the flow stress would be decreased and fibrin formation could be favored (see figure 4).

DISCUSSION

Hemophilia is the hemostatic disorder that courses with most frequent and severe bleeding episodes. However, pathophysiologic mechanism underlying the profuse bleeding in hemophilia is not fully understood since bleeding occurs in a condition in which primary hemostasis is basically normal. In the present study we explore hemostatic primary performance at intermediate and very high shear rate in blood samples from healthy donors and patients with severe hemophilia A in the presence of an anticoagulant that enables thrombin generation (LMWH) [29]. We found an alteration of primary hemostasis in patients with hemophilia at very high shear rate ($5000s^{-1}$). This impairment of platelet function, not manifest at intermediate shear rate ($600s^{-1}$), was partially corrected by TF and further corrected by rFVIIa in the presence of TF.

Exposure of TF at sites of vascular damage [37;38]contributes to the initiation of hemostasis by priming thrombin generation in the vicinity of a growing platelet aggregate. This initial hemostatic plug must be consolidated by the progressive local generation of factor Xa and thrombin. Thrombin is important both, for fibrin generation and for further platelet activation leading to the primary arrest of bleeding [33;39]. Exogenously added rFVIIa seems to increase the rate of thrombin generation on thrombin-activated platelet surfaces. This mechanism has been suggested to promote a thrombin burst necessary to make the plug resistant to premature lysis [8]. Since thrombin is so important for hemostasis, in our experimental settings blood samples were always anticoagulated with LMWH, which maintains blood anticoagulated but allows thrombin generation when blood is exposed to thrombogenic surfaces containing collagen and tissue factor [29].

Our experiments at moderate shear rates ($600s^{-1}$) with circulating blood samples from hemophilic patients revealed levels of platelet interactions with the damaged vessels that were essentially similar to those observed in studies with blood from healthy donors. Fibrin formation was practically absent in perfusion studies with blood samples from hemophilic patients but, presence of fibrin was observed when rFVIIa was added in experiments with blood from hemophilic patients. The restoration of fibrin formation observed after the addition of rFVIIa could explain the beneficial effect of this activated factor in hemophilia, as suggested by earlier studies from our group [19]. Other studies have reported that hemophiliacs have a defective inhibition of fibrinolysis produced by low levels of Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) [40] suggesting that rFVIIa does also enhance antifibrinolytic mechanisms in these patients [41]. It is very likely that the inhibition of fibrinolysis found by Lisman and coworkers could explain the long term effects of rFVIIa in hemophilia facilitating the consolidation of initial hemostatic plugs and preventing re-bleeding of original vascular injuries.

Rheological conditions play a critical role in the maintenance of adequate hemostasis and in pathological events. Bleeding symptoms often initiate at microvascular areas subjected to very high shear rates. These conditions are difficult to reproduce with regular perfusion techniques. To test primary hemostasis in hemophilia we adapted the PFA-100 to these investigations using specially devised cartridges containing collagen alone or collagen-TF. The PFA-100 has proven to be an useful *in vitro* bleeding time device for the detection of bleeding diathesis [42;43]. The shear rate obtained at the level of the aperture in the PFA-100™ cartridges reaches values equivalent to 5000 s^{-1}, similar to those found in microvascular areas. An interesting finding of our studies in the PFA-100 was that closure times were significantly prolonged in the group of patients with hemophilia, suggesting that these patients may have a disorder of primary hemostasis only evidenced at

high shear rates. Earlier studies in the middle 80´s had already reported a prolongation of Simplate bleeding times in some hemophilic patients [44-46], though little attention was paid to these observations. Recent studies using the PFA-100 but in blood samples anticoagulated with CPD, also reported prolonged closure times in hemophilic samples [47]. It is likely that the platelet dysfunction observed in patients could be related to a dysfunctional role of the vWF/FVIII complex by the quantitative alteration of FVIII. Addition of rFVIIa to blood samples from healthy donors did not modify closure times significantly, whereas in experiments performed with blood from hemophilic patients the presence of rFVIIa circumvented a pre-existent platelet adhesion defect. Under such shear rate conditions, activated coagulation factors are cleared away downstream, and fibrin generation is almost negligible [48]. rFVIIa has been suggested to increase the rate of thrombin generation on the surface of activated platelets [14], favoring the activation of neighboring platelets. Microenvironments of diminished clearance could enhance the rate of thrombin formation. Microscopic analysis of the plugs formed in the apertures of the cartridges, confirmed that addition of rFVIIa to blood samples from hemophilic patients enhanced recruitment of platelet interacting with the COL-TF-coated apertures with little impact on fibrin.

According to the information provided by our studies, the short term favorable effects of rFVIIa in hemophilia may well be the result of fibrin generation in vascular areas subjected to intermediate shear (where coagulation events prevail) and enhance platelet thrombus growth (platelet events) in damaged areas of microvasculature subjected to more elevated shear rates where long-term fibrinolytic mechanisms [49] may have a limited effect.

Our data provide indirect mechanistic information on the possible side effects associated to rFVIIa. The fact that rFVIIa caused an increase in fibrin formation in studies with blood form normal donors, but never resulted in further shortening of closure time in closure

times in PFA-100 may indicate that thrombotic complications related to rFVIIa could be more frequent in vascular areas with moderate shear (venous territory), and more infrequently bound to occlusive/ischemic thrombi in vascular areas subjected to elevated shear (arterial territory or stenotic injuries). Our experimental results raise interesting concepts not only in our understanding physiologic hemostasis, but also on the mechanism underlying of hemostatic action of rFVIIa and its potential role to generate thrombotic complications. Finally, our studies indirectly suggest that the PFA-100 could be used for monitoring the hemostatic action of rFVIIa in patients with hemophilia. This potential use should be explored in further studies.

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Table I: Features of the patients studied

Age	29,67±3,42
FVIII	<1%
PLT	185,67±23,36
%Htc	38,41±2,1
HIV (N/Y)	4/5
HCV (N/Y)	0/9

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Figure legends

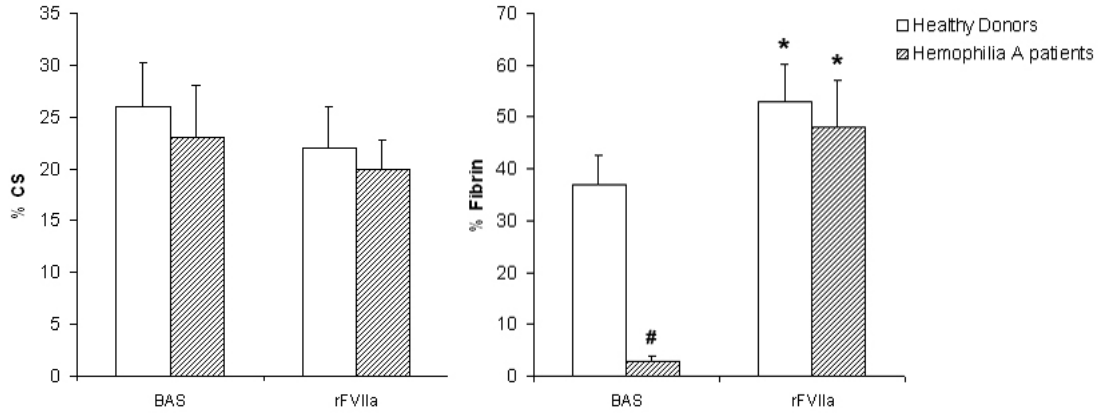
Figure 1: Results from perfusion studies with blood from hemophilic patients or healthy donors at moderate shear rate (600 s^{-1}). Bar diagram in panel A displays the coverage percentage of the perfused surface by platelets before and after addition of 10 µg rFVIIa /ml of plasma. Panel B summarizes fibrin formation before and after addition of rFVIIa. Empty bars correspond to healthy individuals (n=11) results and striped bars correspond to results obtained with blood samples from patients with severe hemophilia A (n=9). Results are expressed as % of coverage of the screened surface as mean±SEM. # $p<0.05$ vs healthy donors; *<0.05 vs basal conditions.

Figure 2: Scanning electron microscopy pictures illustrate representative patterns of interaction of hemophilic blood samples with denuded vascular segments. **A)** In basal conditions, fibrin formation was barely observable. **B**) Addition of rFVIIa to blood from hemophilic patients increased fibrin formation.

Figure 3: Very high shear rate (5000 s^{-1}) results. Bar diagram summarizes closure times (CT) for all experimental settings. Empty bars correspond to experiments performed with blood samples from healthy donors, and striped bars to experiments performed with blood from patients with severe hemophilia A. Hemophilic patients showed CTs significantly longer than healthy donors (# $p<0.05$). Presence of TF in the apertures of the cartridges reduced CTs in healthy donors and in hemophilic patients. Presence of rFVIIa only reduced CT in the presence of TF in those experiments performed with blood samples from hemophilic patients (***** $p<0.05$ vs col-TF cartridges). Results are expressed in seconds as mean±SEM.

Figure 4: Microsections of the plugs formed in the apertures of the PFA-100 cartridges. All pictures in this figure correspond to experiments performed with blood samples from hemophilic patients. Plugs formed in the presence of TF (B) were more consistent than the ones formed on collagen alone (A). Addition of rFVIIa (C) induced the formation of thrombi more efficient, as deducible by the reduction of CT.

Figure 1


% CS
35
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rFVIIa
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70
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BAS
rFVIIa
Healthy Donors
Hemophilia A patients

Figure 2

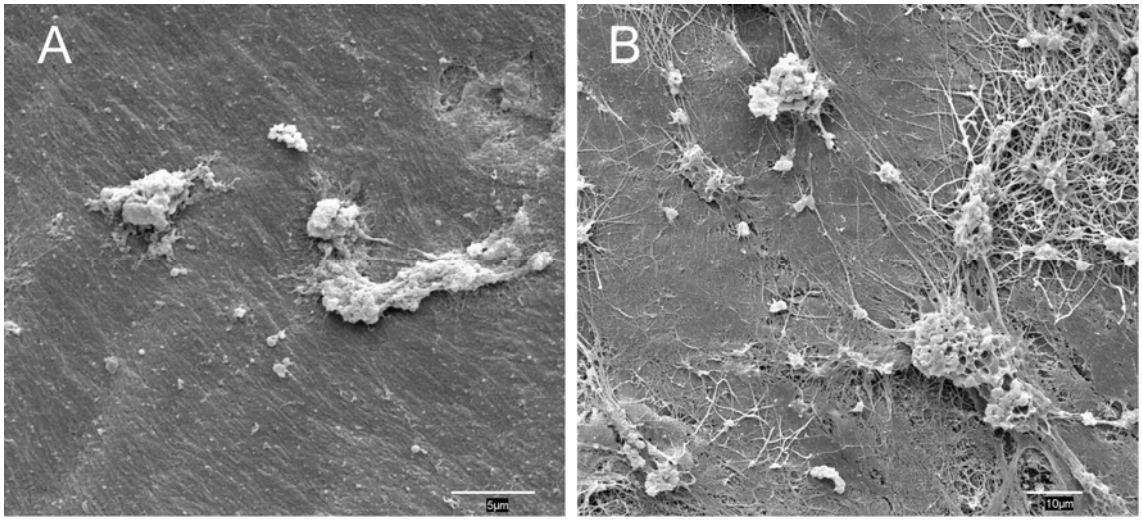


Figure 3

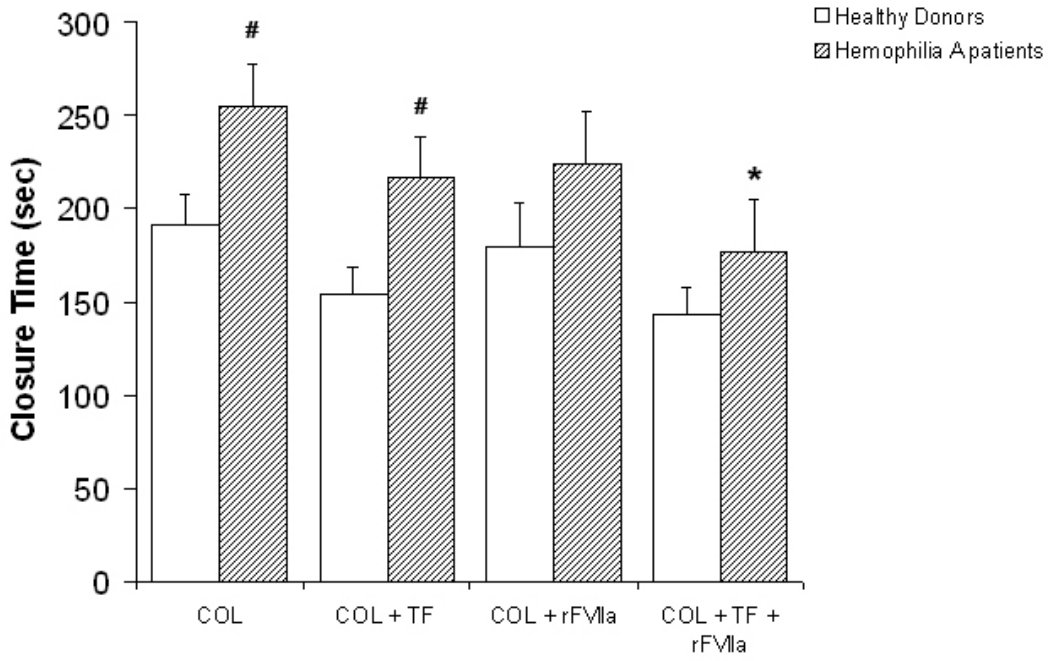
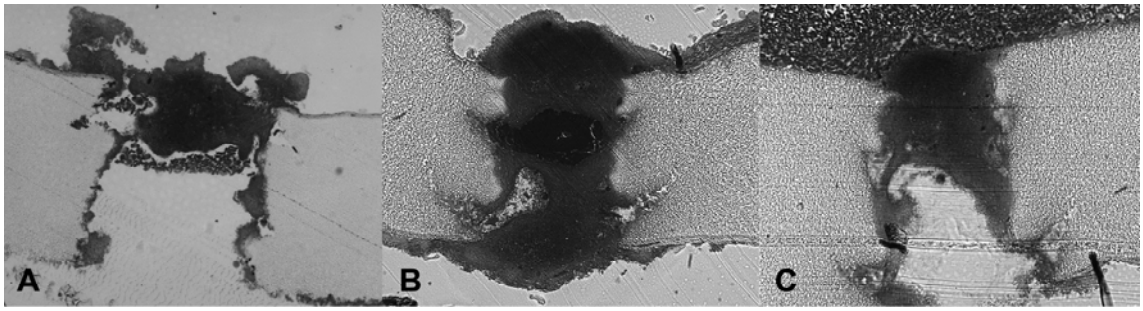

Healthy Donors
Hemophilia A patients
Closure Time (sec)
300
250
200
150
100
50
0
#
#
*
COL
COL + TF
COL + rFVIIa
COL + TF + rFVIIa

Figure 4



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