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Platelet activity of high-dose factor VIIa is independent of tissue factor

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Summary. High-dose recombinant factor VIIa has been successfully used as therapy for haemophiliacs with inhibitors. The mechanism by which high-dose factor VIIa supports haemostasis is the subject of some controversy. Postulating a mechanism in which activity is dependent on tissue factor at the site of injury explains the localization of activity but not the requirement for high doses. Postulating a mechanism in which factor VIIa acts on available lipid independently of tissue factor explains the requirement for high doses but not the lack of systemic procoagulant activity. We report that factor VIIa bound weakly to activated platelets ($K_d \sim 90$ nM). This factor VIIa was functionally active and could initiate thrombin generation in the presence of plasma concentrations of prothrombin, factor X, factor V, antithrombin III and tissue factor pathway inhibitor. The activity was not dependent on tissue factor. The concentration of factor VIIa required for detectable thrombin generation agreed well with the lowest concentration of factor VIIa required for efficacy in patients. High-dose factor VIIa may function on the activated platelets that form the initial haemostatic plug in haemophilic patients. These observations are in agreement with clinical trials which have shown that high-dose factor VIIa was haemostatically effective without causing systemic activation of coagulation.

Keywords: factor VII, thrombin, platelet, factor X, haemostasis.

High-dose recombinant factor VIIa (NovoSeven®) has been used very successfully in haemophiliacs with inhibitors who have uncontrollable bleeding including synovectomy (Hedner *et al*, 1988), retropharyngeal haemorrhage (Macik *et al*, 1989), joint haemorrhage (Macik *et al*, 1993), orthopaedic surgery (O'Marcaigh *et al*, 1994), intracranial haemorrhage (Schmidt *et al*, 1994) and for central line insertion (Smith & Hann, 1996). This therapy has had remarkably few side-effects, including little or no evidence of thrombotic complications (Macik *et al*, 1993; O'Marcaigh *et al*, 1994; Schmidt *et al*, 1994). The mechanism by which high-dose factor VIIa provides haemostasis is the subject of some controversy. If the mechanism was dependent on available tissue factor (Telgt *et al*, 1989), then it would be difficult to explain the requirement for doses of 60–100 μg/kg, since this gives plasma levels at least an order of magnitude above the K_d for binding to tissue factor. Furthermore, the amount of tissue factor exposed at a bleeding site should be quite limited. On the other hand, if the mechanism of high-dose factor VIIa was independent of tissue factor (Rao & Rapaport, 1990), it would be difficult to explain how enough activated factors could be generated for haemostasis without causing thrombotic complications. Previous work (Bom & Bertina, 1990; Rao & Rapaport, 1990; Telgt *et al*, 1989) has shown that factor VIIa can activate factor X independently of tissue factor, at least on synthetic phospholipid vesicles containing concentrations of phosphatidyl serine that are much higher than found physiologically.

The fact that there is not systemic activation of factors following administration of high-dose factor VIIa implies that the activity of factor VIIa is localized to the site of bleeding. Platelets are the primary site of thrombin generation and localize normally to a site of injury in haemophiliacs. We speculated that, even though platelets do not express tissue factor (Drake *et al*, 1989), factor VIIa in sufficiently high doses might have some ability to promote thrombin generation on the surface of activated platelets. We tested this hypothesis by examining the ability of factor VIIa to bind to and express activity on the surface of activated platelets. We found that factor VIIa could bind to activated platelets with an affinity similar to the affinity for binding to

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1. **Postulating a mechanism in which activity is dependent on tissue factor at the site of injury expla...**
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2. **These observations are in agreement with clinical trials which have shown that high-dose factor VII...**
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phospholipid vesicles. We have also found that at levels similar to those effective for treating haemophiliacs, factor VIIa activated factor X and promoted thrombin generation on activated platelets.

MATERIALS AND METHODS

Materials. TenStop and Spectrozyme FXa were purchased from American Diagnostica (Greenwich, Ct.). Chromozyme Th was purchased from Boehringer-Mannheim (Indianapolis, Ind.). Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN) was purchased from Multiple Peptide Systems (San Diego, Calif.) as the amide form. All other reagents were of a high commercial grade.

Proteins. Prothrombin was purified using barium citrate, DEAE-cellulose, and a copper chelate column. Factor IX was purified as described previously (McCord *et al*, 1990). Factor X was purchased from Enzyme Research Labs (South Bend, Ind.). All zymogen coagulation factors were treated with an inhibitor mixture (tosyl-lysyl chloromethyl ketone, tosyl-phenylalanine chloromethyl ketone, phenylmethyl sulphonyl fluoride, Phe-Pro-Arg chloromethyl ketone, and dansyl Glu-Gly-Arg chloromethyl ketone) for 1 h, then repurified on Q Sepharose fast flow using calcium chloride elution essentially as described previously (Yan *et al*, 1990). Factor V was purchased from Calbiochem (San Diego, Calif.). Factor VIII was repurified from Profilate (from the UNC Hospital's Pharmacy) by gel filtration on Sepharose CL-2B. Antithrombin III was prepared as described previously (Church *et al*, 1988). Factor VIIa and full-length tissue factor pathway inhibitor were the generous gift of Dr Ulla Hedner (Novo Nordisk, Gentofte, Denmark). Anti-tissue factor antibody (neutralizing antibody catalogue no. 4509) was purchased from American Diagnostica (Greenwich, Ct.). Rabbit polyclonal anti-factor X was purchased from Dako (Carpinteria, Calif.). Rabbit polyclonal anti-factor VII serum was purchased from Calbiochem (San Diego, Calif.) and the IgG fraction purified on a column of protein A Sepharose. Specificity of the antibody was determined by Western blotting with chemiluminescent detection (Amersham, Bucks., U.K.) which showed that the immunoglobulin reacted with only a single band in plasma and did not react with other plasma proteins including factor IX, factor X or prothrombin. Fluorescein-conjugated anti-rabbit immunoglobulin and protein A Sepharose were purchased from Sigma (St Louis, Mo.).

Platelet purification. Platelets were prepared as described previously using Accu-prep Lymphocyte separation medium (Accurate Chemicals, Westbury, N.Y.) followed by gel filtration on Sepharose CL-2B (Hoffman *et al*, 1992a, b). Analysis by flow cytometry did not detect any cells except platelets present in the preparation ($<1 \times 10^6$ contaminating cells).

Immunofluorescence and flow cytometry. Platelets (200×10^9/l) were activated by incubation with 2 nM thrombin for 15 min at 37°C. Activated platelets were incubated with factor VIIa in the presence or absence of 3 mM calcium for 15 min at 37°C. Samples were fixed for 30 min by addition of paraformaldehyde to a final concentration of 1%. Samples were then diluted 10-fold with Tyrode's buffer containing 1 mg/ml bovine serum albumin and incubated for 30 min. Fixed platelets were centrifuged out and incubated with rabbit polyclonal anti-factor VII antibody (1:200) for 1 h at room temperature. Platelets were diluted 10-fold with 20 mM HEPES (pH 7·4), 150 mM NaCl and incubated overnight in order to reduce non-specific binding of the primary antibody. Platelets were centrifuged out and incubated with fluorescein-conjugated anti-rabbit immunoglobulin (1:20) for 1 h at room temperature. Samples were diluted 10-fold with 20 mM HEPES (pH 7·4), 150 mM NaCl and analysed on a FACSCAN flow cytometer with amplifiers (FSC, SSC and FL1) on log settings. 5000 events were collected for each sample and the mean fluorescence intensity determined. The amount of background fluorescence was determined from a platelet sample that was incubated only with the anti-rabbit antibody with no primary antibody. Background fluorescence was subtracted from raw values to give the mean fluorescence values. In order to combine data from different experiments, mean fluorescence values were converted to relative fluorescence by dividing the mean fluorescence values by the mean fluorescence value of the sample with 100 nM factor VIIa. Platelet activation was verified by flow cytometric analysis of the expression of the platelet activation marker CD62 (phycoerythrin-conjugated anti-CD62, Becton Dickinson Immunocytometry Systems, Mountainview, Calif.).

Activity assays. To measure thrombin generation, platelets were activated by incubation with 50 μg/ml of the thrombin receptor agonist peptide SFLLRN for 15 min at 37°C (Hung *et al*, 1992). Activated platelets (200×10^9/l) were added to a concentrated solution of calcium and proteins to give 3 mM calcium and plasma concentrations of: prothrombin (100 μg/ml), factor X (8 μg/ml), factor V (7 μg/ml), factor VIII (0·2 μg/ml), antithrombin III (150 μg/ml), and tissue factor pathway inhibitor (0·1 μg/ml). Factor IX was included in selected experiments at 4 μg/ml. Factor VIIa was mixed with the other proteins immediately prior to addition to platelets. The zymogen proteins had been preincubated with 20-fold plasma concentrations of antithrombin III and tissue factor pathway inhibitor for at least 12 h to ensure that there were no activated proteases contaminating the zymogens. At timed intervals, 10 μl samples were removed and assayed for thrombin generation by addition to 90 μl of a solution of 5 mM EDTA, 0·5 mM Chromozyme Th, and 50 μM TenStop (sufficient to block any factor Xa activity in these experiments). EDTA chelates the calcium and stops further activation of coagulation proteins. After 13 min, cleavage of the synthetic substrate Chromozyme Th was stopped by the addition of 100 μl of 50% acetic acid. The amount of thrombin was determined by measuring the absorbance at 405 nm. There was no detectable cleavage of Chromozyme Th by any of the factor VIIa concentrations used in this study.

To measure factor X activation, activated platelets (200×10^9/l) were added to a concentrated solution of calcium and proteins to give 3 mM calcium and plasma concentrations of factor X with varying concentrations of factor VIIa. Factor IX and factor VIII (free from von Willebrand factor) were included in selected experiments.

Factor VIIa was mixed with the other proteins immediately prior to addition to platelets. At timed intervals, 15 μl samples were removed and added to 35 μl of EDTA to stop further activation of coagulation proteins. After all of the samples had been taken, 50 μl of 0·2 mM Spectrozyme FXa was added. The amount of factor Xa was determined from a kinetic measurement of the absorbance at 405 nm and compared to a standard curve. Factor VIIa cleavage of Spectrozyme FXa could not be detected at any of the factor VIIa concentrations used.

Elution of platelet-bound proteins and Western blotting. Platelet-bound factor X(a) was eluted from platelets with EDTA as previously described (Hoffman *et al*, 1995). The activation state of eluted factor X was visualized by polyacrylamide gel electrophoresis and Western blotting using chemiluminescent detection (Hoffman *et al*, 1995).

RESULTS

Factor VIIa binding to platelets

We have previously shown that indirect immunofluorescence and flow cytometry can be used to characterize factor IXa binding to activated platelets (Hoffman *et al*, 1992a). In this study we have examined the binding of factor VIIa to activated and unactivated platelets. The indicated concentration of factor VIIa was incubated with resting platelets or thrombin-activated platelets for 15 min before platelets were fixed and the amount of bound factor VIIa determined by indirect immunofluorescence as described in Methods. Relative fluorescence was plotted versus the concentration of factor VIIa as shown in Fig 1. The data for activated platelets was fitted well by an equation for a single class of binding sites (equation shown in the figure legend) and gave a K_d of approximately 90 nM. This binding was dependent on both platelet activation and the presence of calcium (Fig 1). The increase in fluorescence on binding of factor VIIa was distributed across all of the activated platelets in the population rather than being due to a few cells becoming very bright. This showed that factor VIIa was binding to all of the platelets and not just to a few platelets (or other contaminating cells) that contained tissue factor.

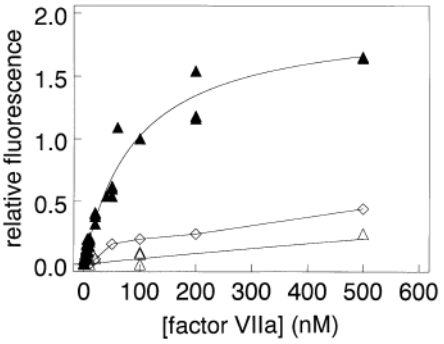

Fig 1. Binding of factor VIIa to activated platelets. Activated platelets (triangles) and unactivated platelets (open diamonds) were incubated with the indicated concentrations of factor VIIa either with 5 mM calcium (filled triangles) or without calcium (open triangles). The amount of factor VIIa associated with platelets was determined by indirect immunofluorescence as described in Methods. The curve shown was determined by fitting the data to the equation: $F = F_{max}*[VIIa]/K_d + [VIIa]$, where F is the relative fluorescence, F_{max} is the calculated maximum relative fluorescence, [VIIa] is the concentration of factor VIIa, and K_d is the binding constant for factor VIIa to activated platelets. All data points from three experiments are shown.

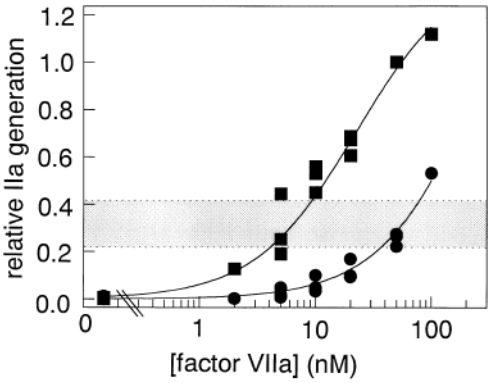

Fig 2. Activity of factor VIIa on activated platelets. As described in Methods, activated platelets were incubated with plasma concentrations of prothrombin, factor X, factor VIII, factor V, antithrombin III, tissue factor pathway inhibitor, and the indicated concentration of factor VIIa either with factor IX (squares) or without factor IX (circles). Thrombin generation was measured as described in Methods. For each of the three experiments shown, thrombin generation was normalized so that the amount of thrombin made in the presence of factor IX and 50 nM factor VIIa was defined as 1. The shaded region indicates the amount of thrombin made on platelets from these individuals when the model system was initiated with 0·2 nM factor VIIa (plasma concentration) in the presence of tissue factor (Sumner *et al*, 1996).

Thrombin generation on activated platelets

We next examined the functional activity of platelet-bound factor VIIa. Platelets were activated with the thrombin agonist peptide SFLLRN so that no proteases were added. Activated platelets were incubated with plasma concentrations of prothrombin, factor X, factor V, factor VIII, antithrombin III and tissue factor pathway inhibitor. For some treatments, factor IX was added at its plasma concentration. To initiate thrombin generation, factor VIIa was added to the proteins and activated platelets. Thrombin generation was measured as described in Methods. As previously described (Sumner *et al*, 1996), there was considerable variability in thrombin generation among different donors. The rates of thrombin generation in each experiment were normalized to the amount of thrombin made in the well containing factor IX with 50 nM factor VIIa. For each donor, treatments with and without factor VIIa

were run simultaneously so that the relative rates were directly comparable. In the absence of added factor VIIa there was no thrombin generation on the activated platelets over the time course examined. As shown in Fig 2, increasing concentrations of factor VIIa gave increasing rates of thrombin generation on the activated platelets. When factor IX was present, 2 nM factor VIIa gave enhanced thrombin generation. In the absence of factor IX more factor VIIa was required to catalyse thrombin generation and less thrombin was produced on the activated platelets. To ensure that this activity was not due to trace contamination by tissue factor we included an antibody to tissue factor that potently inhibits tissue factor activity by blocking formation of the factor VIIa/tissue factor complex (Hoffman *et al*, 1994). Addition of this antibody did not decrease thrombin generation. In addition, we examined platelets for tissue factor using indirect immunofluorescence and flow cytometry. In agreement with a previous immunohistochemical study (Drake *et al*, 1989), we did not observe any measurable tissue factor antigen on platelets.

Factor Xa generation on activated platelets

We speculated that thrombin generation was the result of direct activation of factor X by platelet-bound factor VIIa. Platelets were activated with SFLLRN and incubated with plasma concentrations of factor X with and without 50 nM factor VIIa. Platelets were centrifuged through sucrose to separate bound and unbound proteins and factor X(a) was eluted from platelets. As shown in Fig 3, factor Xa was generated within 30 min on the platelet surface in the presence but not the absence of factor VIIa. As we have observed previously, factor Xa remained bound to the platelets and was not released into the supernatant (Hoffman *et al*, 1995). We examined the time course of

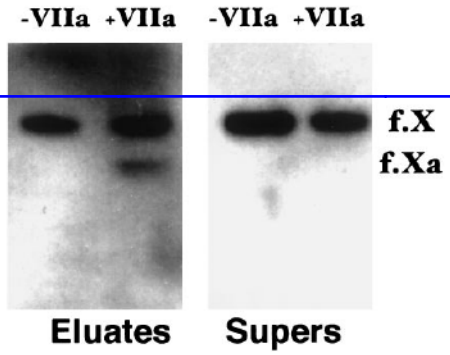

Fig 3. Factor X activation on platelets in the presence of factor VIIa. Platelets were activated with SFLLRN. Plasma concentrations of factor X with or without 50 nM factor VIIa were added and incubated for 30 min. Platelets were centrifuged through sucrose to separate bound and free factor X. Bound factor X was eluted from the platelets and both bound (Eluates) and free (Supers) factor X were visualized as described in Methods. The locations of factor X and factor Xa were established from standards run under similar conditions.

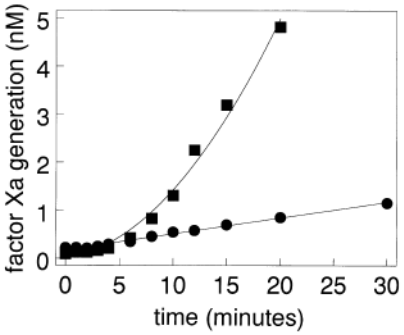

Fig 4. Time course of factor X activation on activated platelets. Platelets were activated with SFLLRN. Factor VIIa (50 nM) and plasma concentrations of factor X with (■) or without (●) plasma concentrations of factor IX and factor VIII were added. Timed samples were removed and assayed as described in Methods. The fitted lines were derived from either a linear regression (factor X alone) or a second-order polynomial (with factor IX/factor VIII).

activation of factor X under these conditions, as shown in Fig 4. Factor Xa generation was linear for 30 min. When we used varied concentrations of factor VIIa from 2 to 50 nM, there was a direct correlation between the amount of factor VIIa added and the amount of factor Xa generated (data not shown). When plasma concentrations of factor IX and factor VIII (free from von Willebrand factor) were included in the reaction mixture, factor Xa generation was no longer linear with time (Fig 4). The data could be fitted by a second-order polynomial which would be consistent with factor VIIa activating factor IX on the platelet surface. Platelet surface factor IXa in complex with factor VIIIa would then rapidly activate factor X. So, although it was not easy to quantitatively compare reactions with and without factor IX and factor VIII, it was clear that significantly more factor Xa was generated in the presence of factor IX and VIII than in their absence.

DISCUSSION

Recombinant factor VIIa has proved very effective as a therapy for bleeding in haemophilia patients who have inhibitors, but very high doses (60–100 μg/kg) are required (Hedner *et al*, 1993). The observation that factor VIIa can act in the absence of tissue factor on the surface of an activated platelet may explain the requirement for high doses if the data from the current *in vitro* studies can be extrapolated to the *in vivo* condition. This study shows that factor VIIa binds to platelets with a K_d of about 100 nM. This value is similar to the affinity of factor IXa for phospholipid vesicles (Jones *et al*, 1985) and low-affinity factor IXa binding to platelets (Hoffman *et al*, 1992a) but is tighter than previously observed for factor VIIa binding to phospholipid vesicles (Bom & Bertina, 1990).

Hedner *et al* (1993), using a modified coagulation assay described by Lindley *et al* (1994), showed that individuals

1. **Anchor Name: Factor Xa generation on activated platelets** *[Agency UK becky.stirk@hansonzandi.co.uk]*

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given 120 μg/kg recombinant factor VIIa had peak plasma levels of 1·8 μg/ml (36 nM) which dropped to 0·4 μg/ml (8 nM) after 3 h. These authors, based on studies in haemophilic dogs and humans, suggested that a plasma factor VIIa level of 0·24 μg/ml (4·8 nM) is the minimum effective level of factor VIIa (Hedner *et al*, 1993). These levels are consistent with our data showing that, in the absence of factor IX, thrombin generation on activated platelets was observed at factor VIIa concentrations >5 nM. At the concentrations of factor VIIa required for therapeutic efficacy, thrombin generation on activated platelets approached the levels attained when the model system was initiated with tissue factor and plasma concentrations of factor VIIa (0·2 nM) (Hoffman *et al*, 1995; Sumner *et al*, 1996).

If high-dose factor VIIa acted *in vivo* on a wide variety of lipid-containing surfaces, it might be expected that patients receiving high-dose factor VIIa would experience disseminated intravascular coagulation or thrombotic complications. In fact, such complications are surprisingly rare. The lack of thrombotic complications implies that high-dose factor VIIa is not leading to widespread (systemic) thrombin generation, but rather is acting locally to provide thrombin generation at a site of injury. This observation is consistent with several studies that found no evidence of systemic activation of coagulation following administration of high doses of factor VIIa. In a rabbit stasis model of thrombosis there was no change in platelet count or plasma fibrinogen levels up to 3 h after administration of high-dose factor VIIa (Diness *et al*, 1992). Also, a study of two haemophilic patients given five treatments with high-dose factor VIIa showed normal levels of antithrombin III and tissue factor pathway inhibitor with minimal decrease in fibrinogen levels or plasminogen activity (Schmidt *et al*, 1994). In a study of 15 haemophilic patients given high-dose factor VIIa, D-dimer, fibrinogen, platelet count, antithrombin III, thrombin–antithrombin III complexes, and α-2-antiplasmin were monitored. There was again no evidence of widespread activation of coagulation (Macik *et al*, 1993).

When present at very high concentrations, factor VIIa may be saturating available tissue factor exposed at a site of injury (Rao *et al*, 1996) as well as tissue factor in the extravascular spaces. This tissue factor bound factor VIIa is almost certainly responsible for the basal levels of factor X activation seen in studies of uninjured chimpanzees (ten Cate *et al*, 1993). However, as pointed out previously by Rao & Rapaport (1990), saturation of local tissue factor at the site of an injury would not require a peak factor VIIa level 3–4 times the baseline zymogen factor VII level (as is required therapeutically). Formation of a primary platelet plug is normal in haemophilic patients, even haemophilic patients with inhibitors. If factor VIIa could act locally on the surface of activated platelets at the site of an injury, locally generated thrombin could act to cleave fibrinogen and consolidate the initial platelet plug. This study showed that factor VIIa bound to activated platelets could catalyse thrombin generation on activated platelets in the absence of factor IX. This activity requires high concentrations of factor VIIa (at least 5 nM), consistent with the concentrations of factor VIIa required for effective therapy in haemophiliacs with inhibitors. Platelets are the primary surface for thrombin generation in physiological coagulation and have a number of control mechanisms, both positive and negative, that regulate thrombin generation. If exogenous factor VIIa were acting on the surface of activated platelets, it would be constrained to activity only at a site of injury and might also be subject to some of the normal control mechanisms that prevent thrombosis. Therefore our data are consistent with the clinical observation that high-dose factor VIIa is both effective and safe in haemophilic patients with inhibitors.

This study showed that high-dose factor VIIa is significantly more effective in promoting both factor Xa and thrombin generation in the presence of factor IX than in its absence. This is presumably because factor IX activated by factor VIIa on the platelet surface can act with its cofactor factor VIIIa to enhance factor Xa generation and thereby thrombin generation on the platelet surface. This may explain the efficacy of high-dose factor VIIa in treating thrombocytopenia (Kristensen *et al*, 1996). At the dose of factor VIIa used in thrombocytopenic patients, significantly more thrombin was generated in the model system than when the system was initiated with tissue factor and plasma concentrations of factor VIIa (0·2 nM). In the presence of high-dose factor VIIa and normal levels of other coagulation factors, a given number of platelets has a greater thrombin-generating capacity than in the absence of high-dose factor VIIa. This may allow for formation of a consolidated haemostatic plug in thrombocytopenic patients even when only small numbers of platelets accumulate at the site of injury.

It is possible that factor VIIa is acting at the site of injury on cells other than activated platelets or tissue factor containing cells. Any cell that expresses phosphatidyl serine might support factor VIIa activity (Bom & Bertina, 1990; Rao & Rapaport, 1990; Telgt *et al*, 1989). Injured and apoptotic cells might therefore provide a surface for factor VIIa generation of factor Xa. Factor VIIa activity on these cells might not be subject to the same regulatory mechanisms as platelets. This study implies that care should be taken in administering high-dose factor VIIa in cases where there is significant cell damage such as sepsis or extensive tissue necrosis.

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