Ability of Recombinant Factor VIIa to Generate Thrombin During Inhibition of Tissue Factor in Human Subjects

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Background—In view of the central role of the tissue factor–factor VIIa pathway in the initiation of blood coagulation, novel therapeutic strategies aimed at inhibiting this catalytic complex are currently being evaluated. A limitation of this new class of anticoagulants may be the lack of an appropriate strategy to reverse the effect if a bleeding event occurs. The aim of this study was to investigate the in vivo potential of recombinant factor VIIa (rVIIa) to induce thrombin generation in healthy subjects pretreated with recombinant nematode anticoagulant protein c2, a specific inhibitor of the tissue factor–factor VIIa complex, in a double-blind randomized crossover study.

Methods and Results—Administration of nematode anticoagulant protein c2 (3.5 μg/kg) caused a prolongation of the prothrombin time from 13.7±0.6 to 16.9±1.2 seconds. The subsequent injection of rVIIa (90 μg/kg) resulted in an immediate and complete correction of the prothrombin time and a marked generation of thrombin, reflected by increased levels of prothrombin activation fragment F1+2 and thrombin-antithrombin complexes from 0.75±0.64 to 3.29±6.3 nmol/L and from 2.4±0.6 to 10.7±3.9 μg/mL, respectively. Factor X and IX activation peptides showed a 3.5-fold and a 3.8-fold increase, respectively, after the administration of rVIIa in the presence of nematode anticoagulant protein c2.

Conclusions—During treatment with an inhibitor of the tissue factor–factor VIIa complex, the infusion of rVIIa resulted in thrombin generation. Our results indicate that rVIIa may be a good candidate as an antidote for inhibitors of tissue factor. (Circulation. 2001;103:2555-2559.)

Key Words: anticoagulants • coagulation • thrombosis • drugs • inhibitors

Recent experimental and clinical studies have shown that under normal physiological conditions, the initiation of blood coagulation is catalyzed by the tissue factor–factor VIIa pathway.1,2 The tissue factor–factor VIIa complex is able to generate factor Xa via direct activation of factor X or indirectly after the activation of factor IX and subsequent formation of the tenase complex with the activated cofactor VIII. It is well established that tissue factor plays a crucial role in the many disease processes resulting from the activation of the blood coagulation response or thrombosis. For example, the systemic activation of blood coagulation in disseminated intravascular coagulation was shown to be completely dependent on tissue factor.3 In addition, the abundant presence of tissue factor in the atherosclerotic plaque has linked pathological plaque rupture to the subsequent thrombotic occlusion of a coronary artery leading to acute coronary syndromes.4,5 In view of this central role of tissue factor–factor VIIa in the activation of coagulation, novel therapeutic strategies aimed at inhibiting this catalytic complex are currently being evaluated in experimental and clinical trials.6–8 One of these inhibitors is recombinant nematode anticoagulant protein c2 (rNAPc2), a potent and selective inhibitor of the tissue factor–factor VIIa complex in the presence of factor Xa.9 In initial clinical trials, rNAPc2 has been shown to produce a dose-dependent anticoagulant effect with a systemic half-life of >50 hours after subcutaneous administration in normal volunteers.9 The potential clinical success of such inhibitors of the tissue factor–factor VIIa complex as rNAPc2 may be limited by the lack of an appropriate strategy to reverse the effect of these anticoagulants if a serious bleeding event is encountered. Recent reports from in vitro studies indicate that therapeutic doses of recombinant factor VIIa (rVIIa) may be able to induce activation of coagulation, resulting in subsequent thrombin generation possibly independent of tissue factor activity.10 The relevance of this mechanism in vivo, however, has not been confirmed thus far, although recombinant factor VIIa has been shown to be an important agent in controlling bleeding episodes in various clinical scenarios.11–13 If tissue factor–independent thrombin generation can be achieved,
then the administration of recombinant factor VIIa might be a suitable antidote to the tissue factor–factor VIIa class of anticoagulant agents. Therefore, the aim of the present double-blind, randomized, placebo-controlled multiple-period crossover study was to investigate the in vivo potential of recombinant factor VIIa to induce thrombin generation in healthy subjects pretreated with rNAPc2.

Methods

Subjects and Study Design
The study was approved by the research and medical ethical committees of the Academic Medical Center, Amsterdam, Netherlands. Written informed consent was obtained from all subjects. The study was designed as a randomized, placebo-controlled multiple-period crossover study. Six healthy male volunteers (mean age 22 years, range 21 to 26 years) were enrolled in the study. All subjects were tested at 3 different occasions in a random order, separated by a washout period of ≥10 days. Treatment consisted of rNAPc2, a specific inhibitor of the tissue factor–factor VII complex, either in combination with recombinant factor VIIa or in combination with placebo. On a third occasion, they received recombinant factor VIIa alone (control).

Study Agents
rNAPc2 (Corvas Inc) is a small protein anticoagulant derived from the family of nematode anticoagulant proteins, which were originally isolated from hematophagous hookworm nematodes, that is currently produced as a recombinant protein under good manufacturing practices established by the FDA. rNAPc2 forms a ternary inhibitory complex with factor VIIa–tissue factor after the binding to factor Xa.10 rNAPc2 was administered in a volume of 0.3 mL at a dose of 3.5 µg/kg as a single subcutaneous injection at the start of the study. The rationale for the dose of rNAPc2 was based on previous phase-I studies in humans, in which rNAPc2 was shown to produce a dose-dependent anticoagulant effect and was considered safe and well tolerated in doses up to 5 µg/kg.8

rVIIa (NovoSeven, Novo Nordisk) was administered as an intravenous bolus injection at a dose of 90 µg/kg in 10 mL saline, 4 hours after the administration of rNAPc2. The dose rationale for rVIIa was based on the clinically effective dose in patients with bleeding disorders and previous studies with rVIIa in humans using acenocoumarol.14 In the study period in which rNAPc2 was given in combination with placebo, an intravenous injection with 0.15 mL/kg saline was administered. In the study period in which the study subjects received rVIIa alone, the injection of rVIIa was preceded (by 4 hours) by the administration of saline subcutaneously (instead of rNAPc2).

Blood Collection and Assays
Blood was drawn from the forearm by separate venipunctures into tubes containing the appropriate anticoagulants before the administration of rNAPc2, 4 hours after the administration of rNAPc2 but immediately before the administration of rVIIa/placebo, and at 0.5, 1, 1.5, 2, 3, 4, 6, and 20 hours after the administration of rVIIa or placebo.

Thrombin generation was assessed by measurement of the prothrombin activation fragment F1+2 and thrombin-antithrombin (TAT) complexes (ELISA, Behring).

Factor IX activation peptide and factor X activation peptide were assayed by a radioimmunoassay, as previously described.15,16

Plasma levels of soluble tissue factor and tissue factor pathway inhibitor were measured with commercially available ELISAs (American Diagnostics Inc).

The plasma levels of factor VIIa were determined by use of a newly developed enzyme assay for determining factor VIIa activity in human plasma according to a previously described principle.17 Briefly, solid-phase bound monoclonal antibodies raised against rVIIa enabled capture of rVIIa. In the next step, bound rVIIa was detected by secondary antibodies conjugated to horseradish peroxidase, followed by development with tetramethylbenzidine (TMB). The absorbance at 450 nm was measured.

Results

Clotting Times and Factor VIIa Levels
The administration of rNAPc2 caused a prolongation of the prothrombin time (PT), which was similar for all subjects in both study periods in which rNAPc2 was given. As shown in Figure 1A, the PT increased from 13.0 ± 0.8 seconds at baseline to 15.4 ± 1.2 seconds at 4 hours after administration of rNAPc2 (P < 0.05). The subsequent injection of rVIIa resulted in an immediate overcorrection of the PT to 9.7 ± 0.5 seconds, which was significant compared with the sustained
prolongation of the PT in the placebo controls \( (P<0.001) \). Treatment with rVIIa alone also depressed the PT from 12.0±0.8 seconds at baseline to a minimum of 8.9±0.3 seconds 30 minutes after administration of rVIIa. The difference in the shortening of the PT between the treatment groups that received rNAPc2 + rVIIa and rVIIa alone was significant \( (P<0.05) \). The effect of rVIIa administration on the PT gradually decreased after 4 hours but was still present at 6 hours after administration. The activated partial thromboplastin time (aPTT) was not significantly affected by the administration of rNAPc2 and rVIIa.

The plasma levels of factor VIIa were below the limit of detection in the period before the administration of rVIIa (Figure 1B). The plasma levels increased sharply immediately after the administration of rVIIa and reached peak values of 23.7±7.5 nmol/L at 30 minutes after administration. Thereafter, the levels gradually decreased and were below the limit of detection at 20 hours after administration. The calculated half-life of rVIIa in this experiment was \( \sim 120 \) minutes (which is slightly shorter than the reported half-life of rVIIa of 170 minutes in patients with hemophilia and acquired inhibitors to factor VIII or IX).11 As shown in Figure 1B, the administration of rNAPc2 had no effect on the plasma levels of factor VIIa.

**Thrombin Generation**

There was a nonsignificant trend toward lower levels of circulating markers for thrombin generation (F1 \( \alpha \) +2, TAT complexes) after injection of rNAPc2 (Figure 2). Administration of rVIIa in subjects pretreated with rNAPc2 resulted in a marked increase in thrombin generation, as reflected by a sharp rise in plasma levels of both F1 \( \alpha \) +2 and TAT complexes, which reached a maximum 30 to 60 minutes after the administration of rVIIa. Both F1 \( \alpha \) +2 and TAT complexes showed significant 3.6-fold and 4.5-fold increases with peak levels of 3.2±1.5 mmol/L and 10.5±1.6 \( \mu \)g/L, respectively, in response to rVIIa administration (Figure 2). The injection of rVIIa alone resulted in an additional increase in thrombin generation of 25% to 40% over that observed in subjects receiving both rNAPc2 and rVIIa, with F1 \( \alpha \) +2 and TAT complex peak levels of 4.0±0.3 nmol/L and 14±1.3 \( \mu \)g/L, respectively \( (P<0.05) \). Therefore, these results suggest that rNAPc2 significantly blunted thrombin generation induced by rVIIa.

**Factor IX and X Activation**

As shown in Figure 3, the plasma levels of factor X activation peptide after the administration of rNAPc2 decreased gradually during the observation period, but this change was not statistically significant. The observed thrombin generation after the administration of rVIIa appeared to be mediated by the activation of factors IX and X, as evidenced by the significant increase in activation peptides derived from these zymogens. Pretreatment with rNAPc2 resulted in lower rVIIa-induced peak levels of factor X activation peptide and a trend toward lower rVIIa-induced peak levels of factor IX activation peptide compared with injection of rVIIa alone. Factor IX activation peptide increased from 221±41 to 839±157 pmol/L after the administration of rVIIa and pretreatment with rNAPc2 compared with a maximal level of 977±65 pmol/L achieved after the administration of rVIIa alone \( (P=0.07) \). Levels of factor X activation peptide rose from 87±23 to 302±61 pmol/L after injection of rVIIa preceded by rNAPc2 and to 387±12 pmol/L after the injection of rVIIa alone \( (P<0.05) \). Plasma levels of soluble tissue factor and tissue factor pathway inhibitor did not change during the experiment in any experimental group.

**Plasma Levels of rNAPc2**

Maximum plasma concentrations of rNAPc2 of 60.6±3.7 ng/mL for the rVIIa group and 68.5±4.0 ng/mL for the placebo group occurred at 6.7±0.6 and 8.5±3.1 hours after the administration of rNAPc2, respectively (Figure 4). The area under the plasma concentration-time curve (AUC 0 to 24) was comparable for each group; 1172±72 ng · h\(^{-1} \) · mL\(^{-1} \) for the rVIIa group versus 1298±100 ng · h\(^{-1} \) · mL\(^{-1} \) for the placebo group. Estimation of the apparent terminal half-life (t1/2) was 72.6±9.4 hours for the rVIIa group and 78.2±5.0 hours for the placebo group.

**Discussion**

The initiation of the blood coagulation response to vascular injury or inflammation is mediated by the exposure of tissue
factor to factor VIIa that circulates at low levels in the blood. The resulting factor VIIa–tissue factor complex catalyzes the proteolytic activation of zymogen factor X to the active protease factor Xa, which is then incorporated into the prothrombinase complex on appropriate membrane surface with factor Va. The prothrombinase complex catalyzes the formation of thrombin through the activation of circulating prothrombin. The tissue factor–factor VIIa–factor Xa complex can also indirectly contribute to factor Xa generation through the activation of zymogen factor IX to factor IXa, which when bound to factor VIIa on an appropriate membrane surface forms the tenase complex, which generates additional factor Xa. We previously confirmed this pivotal role of tissue factor in the initiation of factor Xa– and factor IXa–mediated thrombin generation in a chimpanzee model. On the basis of the notion of the essential role of the tissue factor–factor VIIa complex in the initiation of coagulation, recombinant factor VIIa was developed and was indeed shown to be a potent prohemostatic agent in patients with severe coagulation disorders. Recent in vitro studies, however, have questioned the tissue factor–dependency of coagulation activation, showing generation of thrombin in the presence of relatively high concentrations of factor VIIa and in the absence of tissue factor–bearing cells. On the basis of these experiments, it was hypothesized that factor VIIa might be able to contribute to thrombin generation by a tissue factor–independent mechanism, although it was unclear whether this mechanism might play a role in vivo.

Our present experiments in human subjects demonstrate that during inhibition of the tissue factor–factor VIIa complex with rNAPc2, the administration of factor VIIa is still able to induce thrombin generation. Whereas the shortening of the PT and aPTT may be interpreted as in vitro artifacts, the significant increase in thrombin activation markers F1 and TAT complexes provides convincing evidence for rVIIa-induced thrombin generation. In fact, the factor VIIa-induced 4- to 5-fold elevation in markers for thrombin generation is blocked only ~25% by pretreatment with rNAPc2. We can provide a number of possible explanations for these observations. First, the dose of rNAPc2 may not have been sufficient to completely block the amount of tissue factor–factor VIIa complex after the administration of recombinant factor VIIa. This is supported by the fact that administration of a dose of 5 µg/kg in a previous study led to an increase in the extent of PT elevation compared with what was observed with the 3.5-µg/kg dose used in this study. An alternative explanation might be provided by the hypothesis that factor VIIa is able to overcome the inhibitory effect of factor VII on thrombin generation, thereby displacing factor VII bound to tissue factor, resulting in additional thrombin generation. This mechanism may also explain why the plasma level of recombinant factor VIIa required for adequate hemostatic efficiency far exceeds the $K_d$ for binding of factor VIIa to tissue factor. Another explanation may be provided by recent studies in a cell-based model of the coagulation system, demonstrating the ability of high concentrations of factor VIIa to restore platelet surface–localized and prothrombinase-mediated thrombin generation. Last, and partly in accordance with the previous explanation, it has been shown in a model of thrombus formation on a collagen-coated glass slide (ie, in the absence of tissue factor–bearing cells) that tissue factor might still play a role in the blood clot formation.

Interestingly, our present observations indicate that factor VIIa–induced activation of factors IX and X is sustained.
much longer than the effect on thrombin generation. Because differences in plasma elimination time of these molecular markers cannot explain this discrepancy,\cite{20,25} the most likely explanation for this phenomenon is the inhibition of thrombin generation by physiological anticoagulant pathways, such as the protein C pathway.\cite{26} The ability of factor VIIa to produce thrombin generation during inhibition of tissue factor activity is attractive in view of the upcoming introduction of pharmacological tissue factor inhibitors. Because of the pivotal role of tissue factor in the initiation of blood coagulation and the pathogenesis of thrombosis, it is quite understandable that a novel generation of anticoagulant agents like nAPc2 or recombinant tissue factor pathway inhibitor is being developed to interfere with this pathway.\cite{1,4–6,27} The observation that administration of factor VIIa is able to induce transient thrombin generation during the partial inhibition of tissue factor–factor VIIa indicates that rVIIa may be an effective antidote in this situation, which is particularly useful in agents with prolonged elimination half-lives.

The safety of the administration of factor VIIa requires some reflection. We did not observe any clinical effect of the administration of factor VIIa, but our study was limited to a small number of relatively young, healthy volunteers. Although there are some case reports on thrombotic complications probably related to the administration of factor VIIa,\cite{28,29} infusion of factor VIIa is generally considered to be safe in patients with coagulation disorders. Previous studies have shown that factor VIIa at doses up to 320 μg could be safely administered to subjects receiving warfarin.\cite{14} In addition, in a recently completed trial in 32 patients without any coagulation abnormality undergoing abdominal prostatectomy, the use of factor VIIa (for reduction of perioperative blood loss) was not associated with any thrombotic adverse event during the 10-day study period (P.W.F., manuscript in preparation). Hence, although the use of factor VIIa appears to be relatively safe, the available information is still limited, and the potential occurrence of thrombotic complications (for the potential, in elderly subjects with atherosclerotic disease or organ failure) cannot be fully excluded at this stage.

In conclusion, we demonstrate the ability of factor VIIa to generate thrombin during inhibition of the tissue factor–factor VIIa complex in vivo. Recombinant factor VIIa may therefore be a suitable antidote if anti–tissue factor pathway–directed anticoagulant treatment is complicated by bleeding.

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References


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