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Preclinical toxicity biomarkers for combination treatment with clotting factors rFXIII and rFVIIa

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Abstract

Combination treatment with the clotting factors recombinant activated factor VII (rFVIIa), serine protease, and recombinant factor XIII (rFXIII), protransglutaminase, is being explored for haemostatic therapy. We performed a single-dose toxicology study in the cynomolgus monkey, with four dose groups receiving $0.1+0.34$ mg kg^{-1} (group 1), $0.33+1.12$ mg kg^{-1} (group 2), $1.67+5.60$ mg kg^{-1} (group 3) and $5.00+16.80$ mg kg^{-1} (group 4) of a rFVIIa+rFXIII combination. In the three lower dose groups, no clinical, histopathological or blood chemistry changes were observed. In group 4, the animals died at 4 h post-dosing, with histopathology revealing a systemic coagulopathy resembling, but distinct from, disseminated intravascular coagulation. Due to the absence of toxicity warning signs, toxicity biomarkers were identified by a Western blot-based screening of approximately 20 plasma proteins known to be involved in the clotting cascade. Three of the examined proteins were specifically affected by rFVIIa+rFXIII treatment. Fibronectin and fibrinogen exhibited dose-dependent reductions from less than 10% reduction (group 2) to more than 90% reduction (group 4). These reductions were reversible, and specific. For vitronectin, a dose-dependent conversion to the 65-kDa form was found to occur in groups 3 and 4. Thus, fibrinogen, fibronectin and vitronectin represent the first biomarkers for clotting factor toxicity.

Keywords: *Cynomolgus monkey, rFXIII, rFVIIa, toxicity, biomarker, Western blot*

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Introduction

Uncontrolled haemorrhage can constitute a life-threatening situation. The situation can arise in numerous medical and surgical circumstances, including accidental trauma. In recent years, it has become clear that injection with pharmacological doses of key clotting factors is of value in treatment and prophylaxis of haemorrhagic episodes. Biopharmaceutical haemostatic treatment rests on two observations: (i) redundancy in the clotting cascade, such that single key proteins can substitute for parts of the clotting cascade in generating a blood clot; (ii) dependence of clotting cascade proteins on activated cell surfaces for binding and allosteric activation, such that the activity of injected clotting factors is not unleashed in the systemic circulation, but restricted to the site of injury. In short, it appears that the highly complex blood

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clotting cascade can efficaciously as well as safely be overridden by pharmacological doses of selected clotting proteins, leading to the 'general haemostatic agent' concept (Jurlander et al. 2001, Hedner 2002, Von Depka 2002, Hoffman & Monroe 2003, Roberts et al. 2004).

The 'general haemostatic agent' concept is currently best illustrated by factor VII (FVII). By administering pharmacological doses of activated FVII (FVIIa), the requirement for a fully functioning clotting cascade is partly bypassed, and clot formation is enhanced (Jurlander et al. 2001, Hedner 2002, Von Depka 2002, Roberts et al. 2004).

Clot formation *per se* is not sufficient for efficient haemostasis: the initial fibrin clot must be stabilized by the transglutaminase action of factor XIII (FXIIIa). FXIIIa enhances mechanical clot stability by cross-linking fibrinogen γ and α chains, and reduces fibrinolysis by cross-linking α_2-plasmin inhibitor into the fibrin mesh (Figure 1). The function of FXIII is essential for normal haemostasis, with congenital FXIII deficiencies causing haemorrhagic diathesis and, if untreated, high mortality (Muszbek et al. 1999).

Both FVIIa and FXIII can be manufactured in recombinant eukaryotic expression systems (Bishop et al. 1990, Jurlander et al. 2001). Recombinant FVIIa (rFVIIa) is an active serine protease (Jurlander et al. 2001), whereas recombinant FXIII (rFXIII) is a zymogen that is converted to the active transglutaminase (rFXIIIa) by thrombin (Bishop et al. 1990; for comprehensive review of FXIII activation, see Muszbek et al. 1999). As mentioned above, dependence of the clotting cascade on activated cell surfaces ensures that the activities of rFVIIa and rFXIII are restricted to sites of vessel wall injury (Figure 1). First, rFVIIa binds to ligands that are only found at sites of vessel wall injury (tissue factor (TF) and activated platelets) (Jurlander et al. 2001, Hedner 2002, Von Depka 2002, Roberts et al. 2004). Second, free rFVIIa has negligible proteolytic activity, but is allosterically activated at sites of injury (10^5-fold enhancement in proteolytic activity following TF binding (Eigenbrot 2002). Third, FXIII requires thrombin as well as fibrin for effective activation, thrombin and fibrin normally being restricted to sites of vessel wall injury (Muszbek et al. 1999). Fourth, even though traces of rFXIIIa could be generated in circulation by the normal 'idling' of the clotting system, the transglutaminase activity of FXIII is higher on a polymerized (fibrin) than on a soluble (fibrinogen) substrate (Muszbek et al. 1999), again restricting activity to sites of vessel wall injury. Control mechanisms such as these allow systemic treatment with these two recombinant proteins (Roberts et al. 2004, Reynolds et al. 2005, Visich et al. 2005). However, it has been reported that in cynomolgus monkeys treated with rFXIII doses exceeding expected human exposure by at least 20-fold, sudden death occurs (Ponce et al. 2005b).

Generally, in dose-escalation toxicity studies, a gradual increase in toxic effects is seen with increasing dose. In contrast, for rFXIII, it has been reported that the dose-response curve is unusually steep, with no ill effects seen in cynomolgus monkeys after a single dose of 20 mg kg^{-1}, whereas single doses of 22.5 mg kg^{-1} proved lethal (Ponce et al. 2005b). Lethal rFXIII doses triggered occlusive coagulopathy in organs such as lung, kidneys, heart and brain (Ponce et al. 2005b). Because of the steep toxicity curve of rFXIII, it is desirable to identify toxicity biomarkers that could, for example, be used to guide preclinical dose escalation trials with rFXIII and rFVIIa. In this context, we would define 'toxicity biomarkers' as proteins that

exhibit quantitative changes correlating to rFXIII+rFVIIa dose and toxicity in the cynomolgus monkey. Plasma (as opposed to biopsy or postmortem material) would be considered the most relevant biomarker source, allowing intravital and repeated assaying of the biomarker proteins. Strikingly, routine haematology and clotting parameters have been described as not informative regarding rFXIII toxicity (Ponce et al. 2005b), and to our knowledge, no biomarkers for clotting factor toxicity have been described. Therefore, in the present study, we identified biomarkers for rFXIII+rFVIIa toxicity in the preferred preclinical species, the cynomolgus monkey (Birndorf et al. 1971, Lijnen et al. 1984, Karges et al. 1994, Ponce et al. 2005a,b).

Materials and methods

Single-dose animal experiment

Eight purpose-bred cynomolgus monkeys (*Macaca fascicularis*, Bioculture Ltd, Mauritius), approximately 2–2.5 years old, were housed in bedded pens, on a 12 h light/dark cycle. Feeding was communal (SQC Mazuri Primate Diet, Special Diets Services Ltd, Witham, UK), and water was offered ad libitum in bottles. Diet was supplemented daily with mixed fruit juice drink (Booker, Wellingborough, UK) and fresh fruit, vegetables, peanuts, raisins, sunflower seeds or Bonio biscuits. Animals were provided with toys, ropes, swings and foraging materials. The animals weighed 2.7–3.7 kg (males) or 2.6–3.3 kg (females).

Eight animals were allocated into treatment groups based on social groupings. Four treatment groups, each consisting of two animals (one male and one female), received rFVIIa (NovoSeven®, 0.6 mg ml^{-1}, reconstituted in sterile water) (Jurlander et al. 2001) and rFXIII (5.0 mg ml^{-1}, reconstituted in sterile water) (Bishop et al. 1990) by slow saphenous vein injection (5 ml min^{-1}). rFVIIa was given first, followed by a saline flush of the catheter and rFXIII injection, reflecting the position of these factors at the start and end of the clotting cascade (Figure 1). The doses were: group 1, 0.1 mg kg^{-1} rFVIIa and 0.34 mg kg^{-1} rFXIII; group 2, 0.33 mg kg^{-1} rFVIIa and 1.12 mg kg^{-1} rFXIII; group 3, 1.67 mg kg^{-1} rFVIIa and 5.60 mg kg^{-1} rFXIII; and group 4, 5.00 mg kg^{-1} rFVIIa and 16.80 mg kg^{-1} rFXIII.

For biomarker analysis by Western blotting, blood samples were drawn from the femoral vein into tubes containing 1/9 vol. of 0.13 M trisodium citrate at 0 h (predose) and then at 0.25, 1, 2, 4, 6, 8, 24, 48 and 72 h after single-dose administration of the rFVIIa+rFXIII combination. The tubes were gently mixed, and centifuged at 4000*g* within 10 min. Plasma was transferred to new tubes within 5 min of centrifugation, and immediately placed at $-80^{\circ}C$ (within 10 min of centifugation).

Blood cells were analyzed using EDTA-anticoagulated blood drawn at 0 h (predose) and 24, 48 and 72 h after single-dose administration of the rFVIIa+rFXIII combination. Parameters included haemoglobin concentration, packed cell volume, mean cell volume, mean cell haemoglobin concentration, red cell distribution width, platelet crit, platelet distribution width, red blood cell count, reticulocyte count, mean cell haemoglobin, haemoglobin distribution width, platelet count, mean platelet volume, and total and differential white cell count.

Clinical chemistry was done on lithium heparin stabilized plasma, with samples drawn at 0 h (predose) and 72 h after single-dose administration of the rFVIIa+ rFXIII combination. Parameters included aspartate aminotransferase, alkaline

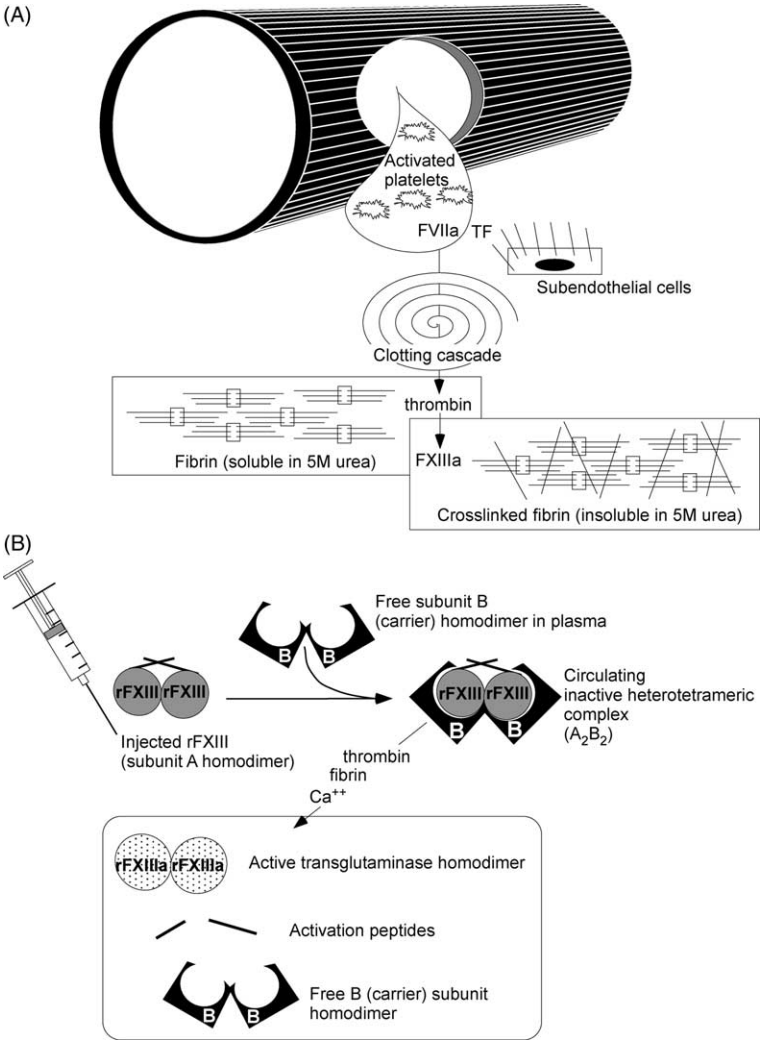

Figure 1 (Continued)

phosphatase, potassium, inorganic phosphorus, total protein, globulin, total cholesterol, urea, creatinine, alanine aminotransferase, sodium, calcium, chloride, albumin, glucose, total bilirubin and γ- glutamyl transferase.

Clotting-related parameters were determined on citrate-stabilized plasma drawn at 0 h (predose), and 6, 24, 48 and 72 h post-single dose of rFVIIa and rFXIII. They included prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer and thrombin–antithrombin (TAT) levels, determined by coagulation analyser and ELISA (Ponce et al. 2005b).

After the single-dose administration of the rFVIIa+rFXIII combination, the animals were twice daily given detailed physical examinations, and observed for signs of ill health or overt toxicity. Scheduled necropsy was performed at 72 h, after sedation with ketamine, and euthanasia by intravenous sodium pentobarbitone injection.

Group 4 animals were autopsied approximately 4 h after dosing (see Results). Histopathology was carried out on known target organs for rFXIII-induced occlusive coagulopathy (lungs, kidneys, heart, brain and eye) (Ponce et al. 2005b).

Western blot biomarker analysis

Plasma samples were thawed to room temperature, and immediately placed on ice. The plasma samples were mixed, and cleared by centrifugation at 1000*g*, 4°C for 5 min.

Cleared plasma (3.3 µl) was added to 997 µl reducing and denaturing sample buffer (SABU). The SABU was made by mixing 22.5 ml NuPage LDS sample buffer concentrate (Invitrogen, Taastrup, Denmark), 9 ml 10 × NuPage reducing agent (Invitrogen), 900 µl 0.5M EDTA, and 12.6 ml ultrapure water. The NuPage LDS sample buffer concentrate contains SDS, tracking dye and density-increasing agent, and is a proprietary form of the sample buffers usually used for fully denaturing and reducing SDS-PAGE.

Samples were reduced and heat denatured by incubating at 70°C for 15 min, and electrophoresed in precast 4–12% gradient NuPage BisTris gels (1 mm thick, Invitrogen), using MOPS-SDS buffer (Invitrogen), at 200 V for 1 h. Electrotransfer to 0.45 µm polyvinylidine fluoride (PVDF) membranes was performed in 1 × transfer buffer (Invitrogen) supplemented with 10% methanol, at 30 V for 1 h, in X-Cell modules (Invitrogen). A prestained molecular weight ladder spanning 250 to 10 kDa was included on all gels, to control the electrophoresis and transfer steps (full range rainbow marker, GE Healthcare, UK). Following transfer, the protein-side of the filters, and the prestained molecular weight ladder bands were marked with a methanol-resistant pen.

The dried PVDF filters were rehydrated in methanol and distilled water, blocked, and incubated with primary antibodies overnight at 4°C (Table I). Blocking and incubation with primary antibodies was in PBS with 0.1% Tween-20 (washing buffer),

Figure 1. Highly simplified overview of the functions of FVIIa and FXIII in blood clotting. (A) Functions of FVIIa and FXIII in the clotting cascade. Vessel wall injury brings into contact tissue factor (TF) expressed on subendothelial cells, and circulating FVIIa. Binding of FVIIa to TF augments the proteolytic activity of FVIIa 10^5-fold, thus initiating the clotting cascade. At pharmacological concentrations, TF-independent binding of FVIIa to activated platelets also initiates clotting. The clotting cascade delivers a localized burst of thrombin formation. Thrombin cleaves fibrinopeptides A and B from fibrinogen, causing fibrinogen to form a fibrin polymer. Thrombin also activates FXIII, allowing the FXIIIa transglutaminase to increase the physical stability of the fibrin clot in two ways: cross-linking of the fibrinogen α and γ chains, and incorporation of antifibrinolytic proteins (e.g. α_2-antiplasmin). For a more detailed description of the pharmacological use of rFVIIa in haemostasis, see Jurlander et al. (2001), Eigenbrot (2002), Hedner (2002), Von Depka (2002) and Roberts et al. (2004). (B) Detailed activation mechanism for FXIII. In blood, FXIII circulates as a heterotetramer consisting of two 'B' carrier subunits and two 'A' zymogen subunits. Free B carrier subunits exist in excess in plasma; thus, injected rFXIII reconstitutes the native heterotetramer (Ponce et al. 2005b). The circulating FXIII heterotetramer associates with fibrinogen and fibrin, probably mediated by the B subunits. Activation of the circulating FXIII heterotetramer to enzymatically active FXIIIa transglutaminase involves thrombin, fibrin and calcium: thrombin cleaves the N-terminal activation peptides of the 'A' subunits, a process accelerated 100-fold by the presence of fibrin. This weakens the association between the A and B subunits, allowing release of the A2 homodimer (active transglutaminase) in a process that is conditionally calcium dependent (the presence of fibrin reduces calcium dependence). Following activation of FXIII, the B carrier homodimer is released from fibrin. For a recent review of FXIII function and activation, see Muszbek et al. (1999). FVIIa, activated FVII; FXIIIa, activated FXIII; rFXIII, recombinant human FXIII; rFXIIIa, activated recombinant human FXIII.

Table I. Summary of Western blot assays

Western blot assay description[a]	Primary antibody layer[b]	Reactivity of primary antibodies[c]	Secondary antibody layer[d]
Western blot assay 1			
Multiplex quantitation of all three fibrinogen chains, fibronectin and immunoglobulin λ light-chain	mAb 2G10, mouse IgG1 κ, catalogue no. NYB2G10 (Accurate Chemical) 1:160 000 (6.3 ng ml^{-1}) mAb T15, mouse IgG1 κ, catalogue no. NYBT15 (Accurate Chemical) 1:160 000 (6.3 ng ml^{-1}) mAb T103, mouse IgG1 κ, catalogue no. NYBT103 (Accurate Chemical) 1:160 000 (6.3 ng ml^{-1}) mAb Ab-3, mouse IgG1, FBN11, catalogue no. CP70 (Calbiochem) 1:1 500 (133 ng ml^{-})	Human fibrinogen γ (gamma 15–35 & 1–411), reduced or reduced/alkylated, ~48 kDa Human fibrinogen B β (Bß 134-461, frags X/Y/D/D-dimer), fibrin, ~54 kDa Human fibrinogen A α (A alpha 290-348/349-406 & Hi2-DSK), ~66 kDa Human fibronectin, ~220 kDa	Goat antimouse immunoglobulin, HRP conjugate, catalogue no. 7076 (Cell Signaling) Goat antihuman immunoglobulin λ light-chain, HRP conjugate, catalogue no. A 5175 (Sigma), ~25 kDa 1:100 000 (68 ng ml^{-1})
Western blot assay 2			
Characterization of the distribution between 75- and 65-kDa vitronectin forms	Sheep polyclonal antibody, purified IgG fraction, catalogue no. ab8870 (abcam) 1:5 000	Human vitronectin, ~75 and 65 kDa	Rabbit antisheep immunoglobulin, HRP conjugate, catalogue no. ab6747 (abcam) 1:5 000

[a]Western blot assay 1 allowed multiplex quantitation of five plasma proteins. Assay 2 was used for vitronectin quantitation. In all cases, fully denatured and reduced whole plasma (citrate-stabilized, platelet-poor, not fractionated) was used for SDS-PAGE. Protein bands were quantitated using chemiluminescense and an ultrasensitive CCD camera.

[b]For Western blot assay 1, the primary antibody layer consisted of a cocktail of four different monoclonal antibodies; mAb, murine monoclonal antibody. All primary antibodies were confirmed to be cross-reactive to cynomolgus antigen. Primary layer antibodies were diluted as stated in the Table, in PBS supplemented with 0.1% Tween-20 and 5% bovine serum albumin. Incubations were carried out overnight at 4°C.

[c]Reactivity is as stated by the antibody supplier. The expected molecular weights of all antigens are stated in the Table. Specificity and cross-reactivity with cynomolgus antigen was confirmed experimentally for all antibodies (see Figure 2).

[d]Secondary layer antibodies were diluted as stated in the Table, in PBS supplemented with 0.1% Tween-20 and 5% dry skim milk. Incubations were carried out for 1 h at room temperature.

supplemented with 5% bovine serum albumin. Then, membranes were washed in washing buffer, for 1 h at room temperature, with gentle shaking, and changing the wash buffer every 15 min. Finally, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in washing buffer with 5% dried skim milk, washed, and developed with ECL^{Advance} chemiluminescent substrate (GE Healthcare).

Light emission from the filters was captured with a CCD camera (LAS3000, Fujifilm). The marks made on the filters with the methanol-resistant pen appeared in white against the weak diffuse background that is invariably present on Western blot filters. This allowed simultaneous visualization of the light-emitting biomarker protein bands and molecular weight ladder bands on each image, and ensuring that the PVDF filters were exposed protein-side up. Quantitative analysis of captured Western blot images was performed with MultiGauge software (v2.3, Fujifilm).

Results

Clinical signs

Following a single dose of rFVIIa combined with rFXIII, animals in dose groups 1, 2 and 3 exhibited no clinical signs during the 3-day observation period. Also, food consumption and body weights were unaffected by the treatments. In contrast, in dose group 4, both animals became subdued and lethargic at 4 h after treatment. One animal died before compassionate euthanasia could be performed, the other animal was euthanized.

Necropsy and histopathology

Animals in dose groups 1, 2 and 3 exhibited neither macroscopic nor microscopic pathological changes, excepting slight phlebitis and cellulitis at the injection sites. In contrast, both animals in group 4 exhibited signs of a systemic coagulopathy resembling disseminated intravascular coagulation (DIC), highly similar to a previous rFXIII study (Ponce et al. 2005b). Combining gross necropsy and histopathology, the changes included diffuse tubular necrosis and glomerular thrombi (kidney), myocardial necrosis (heart), thrombi and oedema (lungs), and haemorrhages in the brain, cranial cavity and meninges (data not shown).

Haematology, clinical chemistry and clotting assays

A comprehensive panel of blood parameters was monitored during the 72-h observation period (listed in Materials and methods), but no consistent changes were observed in the dosed animals (not shown).

Because DIC was observed by histopathology in dose group 4, DIC-relevant laboratory values were scrutinized in detail (Table II). A slight reduction was seen in PT (males and females) and aPTT (females), most pronounced at 6 h post-dosing (Table II). This probably represented the well-documented ability of high plasma rFVIIa levels to interfere with these assays (Telgt et al. 1989, Rao & Rapaport 1990, Keeney et al. 2005).

Apart from those changes, animals in dose groups 1, 2 and 3 exhibited no consistent changes in DIC-relevant blood parameters (Table II). The male in dose group 4

Table II. Selected haematology and clotting values. Data are presented in the format 'male'/'female', with individual animals being blood sampled at successive time points during a 72-h observation period. In dose group 4, both animals died approximately 4 h post-dosing.

Dose group[a]	Time	Throm ($1000\ \mu l^{-1}$)	PT (s)	aPTT (s)	DDM ($ng\ ml^{-1}$)	TAT ($\mu g\ l^{-1}$)
1	Predose	450/347	13.2/13.7	27.1/30.5	1680/398	6.3/4.1
	6 h	401/334	11.0/10.9	25.8/28.0	1831/720	6.2/4.6
	24 h	450/315	12.9/13.2	27.7/28.6	1099/559	3.3/3.1
	48 h	457/276	12.5/12.5	25.9/31.1	5854/654	2.3/18.1
	72 h	469/322	12.5/13.1	27.3/23.5	1110/595	2.5/5.1
2	Predose	372/315	13.5/13.3	29.5/28.8	794/691	14.6/11.3
	6 h	352/ND	10.4/10.4	29.0/26.8	2132/1856	>60/10.3
	24 h	367/344	12.7/13.2	28.5/29.0	1624/1488	6.4/10.6
	48 h	355/335	12.4/12.7	29.2/29.5	866/1484	4.5/27.6
	72 h	363/342	12.9/13.5	22.3/22.8	1687/3162	3.4/4.8
3	Predose	330/357	13.9/13.1	30.0/34.2	529/722	3.5/3.6
	6 h	313/244	9.3/8.8	30.3/31.8	1720/1540	5.3/13.2
	24 h	314/348	11.0/11.3	31.4/32.1	1275/1423	12.2/3.9
	48 h	344/362	12.0/12.1	26.5/40.5	645/1049	>60/6
	72 h	349/415	12.6/11.6	23.1/26.1	854/8768	>60/3.5
4	Predose	302/534	13.2/13.1	25.7/29.0	821/371	7.5/2.5
	4 h	ND/223	ND/ND	ND/ND	>10000/ND	>60/ND

[a]Eight cynomolgus monkeys received a single intravenous injection with rFXIII+rFVIIa. The doses were: group 1, 0.1 mg kg^{-1} rFVIIa and 0.34 mg kg^{-1} rFXIII; group 2, 0.33 mg kg^{-1} rFVIIa and 1.12 mg kg^{-1} rFXIII; group 3, 1.67 mg kg^{-1} rFVIIa and 5.60 mg kg^{-1} rFXIII; and group 4, 5.00 mg kg^{-1} rFVIIa and 16.80 mg kg^{-1} rFXIII. The four dose groups each consisted of one male and one female.
Platelet count (throm) was determined by flow cytometry (Bayer ADVIA); prothrombin time (PT) – fibrin formation following sample activation with rabbit brain thromboplastin – was determined with an ACL Futura analyser; activated partial thromboplastin time (aPTT) was determined with an ACL Futura analyser; D-dimer (DDM) was determined by latex particle agglutination assay; thrombin–antithrombin (TAT) was determined by sandwich immunoassay. ND, not done, due to sample clotting.

exhibited increased D-dimer and TAT levels just prior to death, whereas clotting prevented analysis of the plasma sample drawn from the female.

Identification of toxicity biomarkers

By preliminary screening (see Discussion), we identified three plasma proteins that appeared to have toxicity biomarker value. These plasma proteins were fibronectin, fibrinogen and vitronectin (Table I and Figure 2).

Fibronectin is a moderately abundant plasma protein ($\sim$300 $\mu g\ ml^{-1}$), that exists in plasma as a disulfide-linked dimer of $\sim$220 kDa subunits (Mosher 1984, Ni et al. 2003). The monoclonal antibody used in the present work reacted with a protein species of the expected $\sim$440 kDa in human as well as cynomolgus plasma, and the expected shift to $\sim$220 kDa bands was observed following reduction of the samples (Figure 2A, compare panels 1 and 2).

Fibrinogen is an abundant plasma protein (1–4 $mg\ ml^{-1}$), that circulates as a $\sim$340-kDa disulfide-linked complex of two α (66 kDa), two β (54 kDa), and two γ (48 kDa) chains (Ni et al. 2000, Gaffney 2001, Doolittle 2003). We assayed the fibrinogen chains individually, using three monoclonal antibodies (Table I and Figure 2B). The three monoclonal antibodies used in the present work reacted with protein

species of the expected apparent molecular weights in human plasma (Table 1 and Figure 2B, panels 4, 5 and 6). While the antibodies were clearly cross-reactive with cynomolgus fibrinogen chains, some intriguing species differences were observed: cynomolgus fibrinogen α chain migrated as a double band, with human fibrinogen α chain comigrating with the lower cynomolgus band (Figure 2B, panel 4). Also, human fibrinogen β and γ chains had slightly larger and smaller apparent molecular weights, respectively, than their cynomolgus counterparts (Figure 2B, panels 5 and 6). Splice variants of human α as well as γ fibrinogen chains have been described (Wolfenstein-Todel & Mosesson 1980, Fu et al. 1995), and it is possible that the cynomolgus α-doublet represents two equally prevalent isoforms. This would be different from the human situation, where >90% of α-fibrinogen consists of a single dominant isoform (Fu et al. 1995).

Vitronectin is a moderately abundant plasma protein (200–400 μg ml^{-1}), that circulates in human plasma as two species, a ~75-kDa form, and a disulfide-stabilized form nicked between Arg-379 and Ala-380, which migrates at ~65 kDa and ~10 kDa under reducing conditions (Preissner & Seiffert 1998, Koschnick et al. 2005, Reheman et al. 2005). Thus, a polyclonal antibody against vitronectin would be expected to recognize both ~65- and ~75-kDa forms, as was also the case in human plasma (Table I, Figure 2C, panel 10). In contrast, in cynomolgus plasma, only the ~75-kDa band was observed (Figure 2C, panel 10). Human, mouse, rat, rabbit and pig vitronectins share 80% amino acid homology (Preissner & Seiffert 1998), making it highly unlikely that a polyclonal antibody that reacts with the human ~65-kDa form would not react with the cynomolgus ~65-kDa form. Thus, the apparent lack of the ~65-kDa band in cynomolgus plasma indicates a species difference between man and cynomolgus monkey in plasma vitronectin processing (Figure 2C, panel 10). A weak band below the ~65-kDa vitronectin form was due to non-specific reaction from the secondary antibody (Figure 2C, compare panels 10 and 11).

Immunoglobulins are abundant plasma proteins (~10 mg ml^{-1}), consisting of two heavy chains (~75 kDa) and two light chains (~25 kDa), forming a disulfide-linked complex of ~150 kDa. The anti-λ light chain antibody used in the present work reacted with a protein species of the expected apparent molecular weight in human as well as cynomolgus plasma, with a slight preference for the human protein, against which the antibody is raised (Table I, Figure 2B, panel 7).

In summary, the antibodies used in the present work were specific, and cross-reactive between man and cynomolgus monkey (Figure 2, Table 1). Because of the clean reaction of the antibodies, and the lack of non-specific bands, we opted to use the fibronectin, fibrinogen and immunoglobulin light-chain antibodies as a cocktail, allowing multiplex identification of all five protein species in a single lane (Figure 2B, panel 8). Thus, two Western blot assays were used in the present work: a multiplex assay for fibronectin, fibrinogen α, β, and γ, and immunoglobulin λ light-chain, and a vitronectin assay (Table I).

Multiplex biomarker assay validation

Because the multiplex Western blot assay was technically the most complicated, it was characterized in more detail (Table 1, Western blot assay 1). The linear range of the assay was examined on serial dilutions of human and cynomolgus plasma (Figure 3). In both species, quantitation of fibronectin, fibrinogen β, and γ, and immunoglobulin

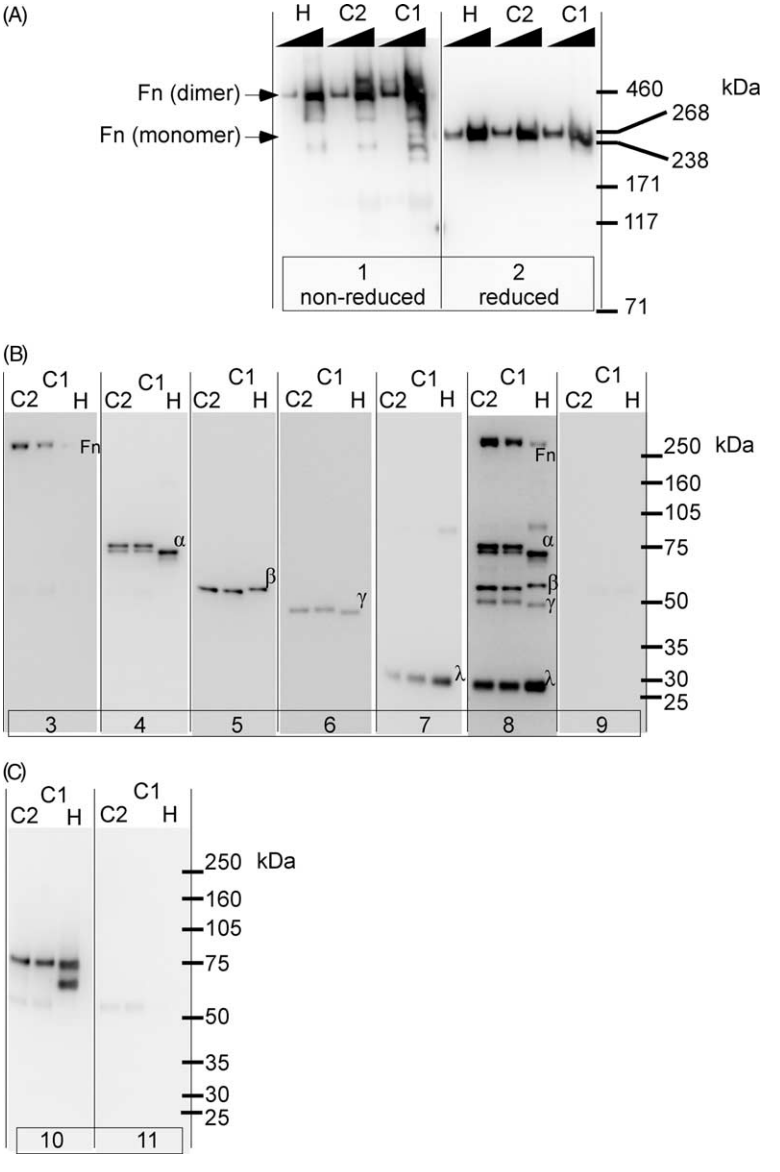

Figure 2 (Continued)

λ light-chain was linear over a 30-fold concentration range (Figure 3). Quantitation of fibrinogen α chain had a lower linear range, due to a high sensitivity of this antibody, and consequent saturation of the chemiluminescent signal at high plasma concentrations (Figure 3). The monoclonal antibodies against human fibronectin and fibrinogen α, β, and γ chains had comparable sensitivities on human and cynomolgus proteins, suggesting that these epitopes are well conserved (Figure 3, compare A and B). In contrast, the polyclonal antibody against human immunoglobulin λ light-chain had a lower sensitivity on cynomolgus immunoglobulin λ light-chain (Figure 3, compare A and B).

To examine the effect of protein transfer efficacy on quantitation results, the same plasma sample was loaded in all wells of the 17-well precast gels, and CVs were calculated for all five protein species detected by the multiplex Western blot assay (Table I). Such intra-assay CVs were 6–9% for fibrinogen α, β, and γ and immunoglobulin λ light-chain, and up to 20% for fibronectin. The larger CV for fibronectin was expected, because larger protein species would exhibit more variable transfer to the PVDF membranes. The low CVs for fibrinogen and immunoglobulin λ chains were also expected, because for these small proteins, transfer would be expected to be efficient and reproducible from the 1-mm thin PAGE gels utilized in this study.

In short, the multiplex biomarker assay (Table 1, Western blot assay 1) was applicable to quantitative analysis on human and cynomolgus plasma. A fixed dilution of 1:300 plasma was selected for further work, to minimize non-specific bands, and remain in the linear range. Similarly, it was confirmed that the vitronectin assay (Table 1, Western blot assay 2) was applicable to quantitative analysis on human and cynomolgus plasma (Figure 2, and not shown).

Toxicity biomarker changes following rFXIII+rFVIIa treatment

When plasma from rFXIII+rFVIIa-treated animals was examined with the multiplex assay (Table 1, Western assay 1), a reduction in fibronectin and fibrinogen α, β, and γ chains was observed at 0.25 and 1 h post-dosing (Figure 4).

To quantitate the changes, we divided protein levels at 0.25 h post-dosing, with protein levels in the same animal before dosing, to obtain 0.25 h/predose ratios (Figure 4B). For all biomarker bands (fibronectin and fibrinogen α, β, and γ chains), there was no significant change in the lowest dose group (Figure 4B). However, in dose groups 2 and 3, we observed a dose-dependent reduction in biomarker bands (Figure 4B). The dose dependency in dose groups 2 and 3 was most apparent in males, with females displaying an almost complete reduction in biomarker proteins

Figure 2. Confirmation of the specificity and species cross-reactivity of antibodies on normal plasma (in the absence of rFXIII or rFVIIa treatment). All antibodies are listed in Table I. In all cases, SDS-PAGE and Western blotting was performed on whole (non-fractionated) human and cynomolgus plasma. H, human plasma pool; C1 and C2, two cynomolgus plasma pools; Fn, fibronectin; α, fibrinogen α chain; β, fibrinogen β chain; γ, fibrinogen γ chain; λ, immunoglobulin λ light chain. (A) Detailed examination of the reactivity of monoclonal antibody (mAb) Ab-3 (human fibronectin). To examine more stringently antibody specificity against this large antigen (220 kDa), SDS-PAGE was carried out in 3–8% gradient gels (Invitrogen), and electrophoresis was performed under non-reducing as well as reducing conditions. Plasma was assayed at two dilutions, 1:30 and 1:200 (corresponding to 0.15 and 0.02 μl plasma per well). (B) Formulation of a cocktail of five antibodies for multiplex Western analysis. Fully reduced and denatured plasma was electrophoresed on 4–12% gradient SDS-PAGE gels as described in Materials and methods. A 1:300 dilution of plasma was used (corresponding to 0.017 μl plasma per well). In panels 3–6, the following mAbs were used together with goat antimouse HRP-conjugated secondary antibody: Panel 3, mAb Ab-3 (human fibronectin), panel 4, mAb T103 (human fibrinogen α), panel 5, mAb T15 (human fibrinogen β), panel 6, mAb 2G10 (human fibrinogen γ). Panel 7, HRP-conjugated goat antihuman immunoglobulin λ light-chain. Panel 8, a cocktail of the antibodies used individually in panels 3–6, as described in Table I. Panel 9, goat antimouse HRP-conjugated secondary antibody alone. (C) Reactivity of sheep antihuman vitronectin polyclonal antibody. Fully reduced and denatured plasma was electrophoresed on 4–12% gradient SDS-PAGE gels as described in Materials and methods. A 1:300 dilution of plasma was used (corresponding to 0.017 μl plasma per well). Panel 10, sheep antihuman vitronectin polyclonal antibody together with HRP-conjugated rabbit antisheep secondary antibody. Panel 11, HRP-conjugated rabbit antisheep secondary antibody alone.

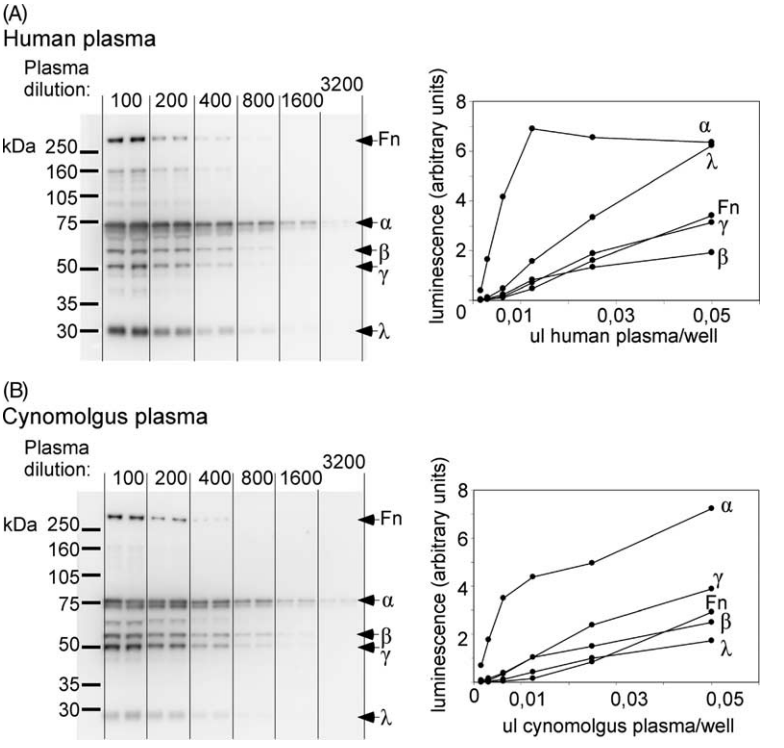

Figure 3. Linear range of the multiplex Western blot assay on normal plasma (in the absence of rFXIII or rFVIIa treatment). Two-fold dilutions of human and cynomolgus plasma, spanning 1:100 through 1:3200, were analysed by fully reducing and denaturing SDS-PAGE and multiplex Western blot (Western blot assay 1, Table I). The dilutions corresponded to 50, 25, 13, 6, 3 and 2 nl plasma per gel well. The following five protein bands were quantitated by chemiluminescence: Fn, fibronectin; α, β, γ, fibrinogen α, β, γ chains; λ, immunoglobulin λ light chain. The extra bands appearing at high plasma concentrations would be expected to represent primarily protein oxidation and aggregation during electrophoresis, possibly with contribution from minority fibrinogen isoforms (Wolfenstein-Todel & Mosesson 1980, Fu et al. 1995). (A) Human plasma. The linearity of the multiplex Western assay was estimated by r^2 values across the whole plasma dilution range (Fn, β, γ and λ), or using only the 1:400, 1:800, 1:1600 and 1:3200 data points (α). The r^2 values were: Fn, 0.94; α, 0.97; β, 0.97; γ, 0.99; λ, 1.00. (B) Cynomolgus plasma. The linearity of the multiplex Western assay was estimated by r^2 values across the whole plasma dilution range for all five bands (Fn, α, β, γ and λ). The r^2 values were: Fn, 0.95; α, 0.97; β, 0.97; γ, 0.98; λ, 0.99.

between dose levels 1 and 2 (Figure 4B). However, even in females, a dose dependency in dose groups 2 and 3 could be observed, best seen with the fibrinogen α antibody (Figure 4B), which was the most sensitive of the antibodies used in the multiplex assay (Figure 3).

In contrast to the changes observed in the biomarker proteins, immunoglobulin λ light-chain was not affected by treatment (Figure 4). This showed that the reduction in fibronectin and fibrinogen α, β, and γ chains was specific, and not a general toxicity effect of high rFXIII+rFVIIa doses, affecting all plasma proteins.

Finally, it should be mentioned that consistently in all animals, a 100-kDa band was observed following rFXIII+rFVIIa treatment (Figure 4B). This band likely represented a dimer of the fibrinogen γ chain, generated by the transglutaminase activity of rFXIII (see Discussion).

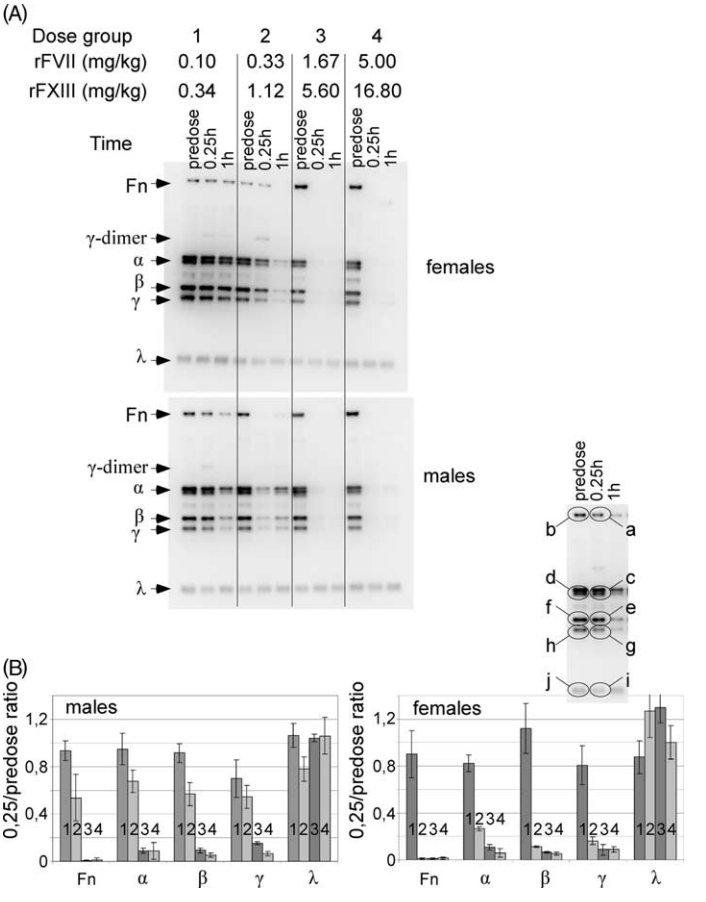

Figure 4. rFXIII+rFVIIa combination treatment causes a dose-dependent and specific reduction in plasma fibrinogen and fibronectin levels. Eight cynomolgus monkeys (four males and four females) received a single intravenous injection with the indicated doses of rFXIII+rFVIIa. The doses were: group 1, 0.1 mg kg^{-1} rFVIIa and 0.34 mg kg^{-1} rFXIII; group 2, 0.33 mg kg^{-1} rFVIIa and 1.12 mg kg^{-1} rFXIII; group 3, 1.67 mg kg^{-1} rFVIIa and 5.60 mg kg^{-1} rFXIII; and group 4, 5.00 mg kg^{-1} rFVIIa and 16.80 mg kg^{-1} rFXIII. Plasma drawn predose and 0.25 h and 1 h post-dosing was analysed by multiplex Western blot (Table I, Western blot assay 1). (A) Representative multiplex Western images, showing the five protein bands quantitated by the assay. Fn, fibronectin; α, β, γ, fibrinogen α, β, γ chains; λ, immunoglobulin λ light chain. The immunoglobulin λ light chain served as an internal control, to demonstrate the specificity of the biomarker changes. The γ-dimer band at ~100 kDa was consistently observed following treatment, using slightly longer exposures. This band represents covalently linked fibrinogen γ chains, reflecting increased plasma transglutaminase activity. (B) Quantitative analysis of multiplex Western data. The effect of rFXIII+rFVIIa treatment was determined as the ratio between 0.25 h and predose plasma levels. An example of 0.25/predose ratio calculation is provided in the image insert: Fn ratio = a/b; α ratio = c/d; β ratio = e/f; γ ratio = g/h; λ ratio = i/j. The λ ratio was used to demonstrate the specificity of the fibrinogen and fibronectin reductions. In the column diagrams, the 0.25h/predose ratios were plotted for each of the five protein bands. Columns are labelled 1–4, corresponding to rFXIII+rFVIIa dose groups. Error bars, one SD determined from 4-plicate determinations (four SDS-PAGE gels and Western assays on a single plasma sample).

In contrast to fibronectin and fibrinogen, where straightforward reductions in plasma levels were seen (Figure 4), a more complex picture was seen for plasma vitronectin in rFXIII+rFVIIa-treated animals (Figure 5). Before treatment, and in low-dose groups 1 and 2, plasma vitronectin invariably migrated as a single ~75-kDa

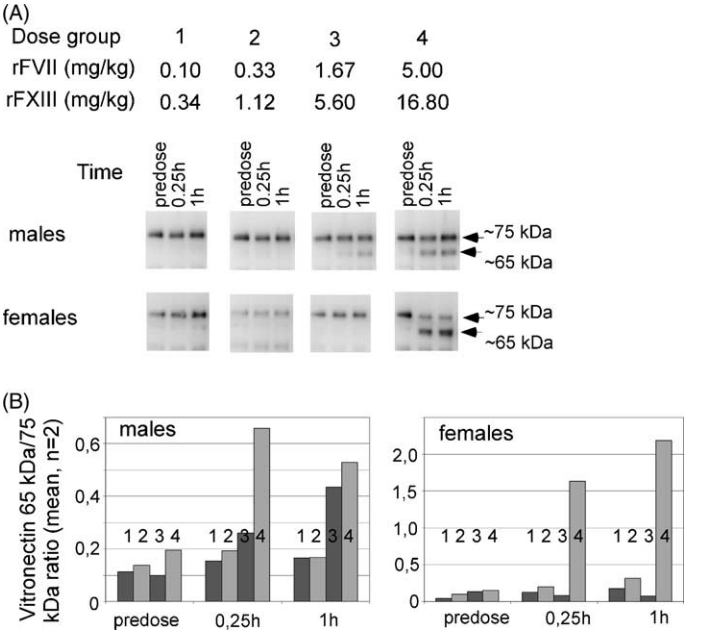

Figure 5. Near-lethal doses of rFXIII+rFVIIa cause the appearance of the 65-kDa vitronectin form, without affecting total plasma vitronectin levels. Eight cynomolgus monkeys (four males and four females) received a single intravenous injection with the indicated doses of rFXIII+rFVIIa. The dose groups are given in the legend to Figure 4. Plasma vitronectin levels were analysed by Western blot (Table I, Western blot assay 2). (A) Representative Western images, showing the ~75-kDa vitronectin band that is dominant in cynomolgus monkey plasma, and the ~65-kDa vitronectin band that was induced by rFXIII+rFVIIa treatment. (B) Quantitative analysis of Western data. At each time point, the effect of rFXIII+rFVIIa treatment was determined as the ratio between ~65-kDa and ~75-kDa bands. Values are the mean of duplicate determinations (two SDS-PAGE gels and Western assays on a single plasma sample). Columns are labelled 1–4, corresponding to rFXIII+rFVIIa dose groups.

band (Figure 5). However, in high-dose groups 3 and 4, there was a reduction in the ~75-kDa band, with a simultaneous appearance of a ~65-kDa band (Figure 5), akin to the band pattern seen in human plasma (Figure 2C, panel 10). Total levels of plasma vitronectin, estimated by the sum of the ~75-kDa and ~65-kDa bands, was not affected by rFXIII+rFVIIa treatment (Figure 5).

Reversibility of toxicity biomarker changes

As mentioned above, in dose groups 2 and 3, we observed a 50–90% reduction in the levels of plasma fibrinogen and fibronectin within 15 min of dosing (Figure 4). Strikingly, this was not associated with any clinical changes during a 72-h observation period, and histopathology at 72 h post-dosing was also unremarkable in dose groups 2 and 3. To explore this further, we examined later time points post-dosing for animals in dose groups 3 and 4. We found that in dose group 2, a nadir in fibrinogen and fibronectin levels was reached around 2–4 h post-dosing, with return to predose levels by 24 h (Figure 6). In dose group 3, the nadir was of a longer duration, and beginning recovery of plasma fibrinogen and fibronectin was not apparent until 24 h post-dosing (Figure 6). As mentioned above, no systematic changes in haematology and clotting

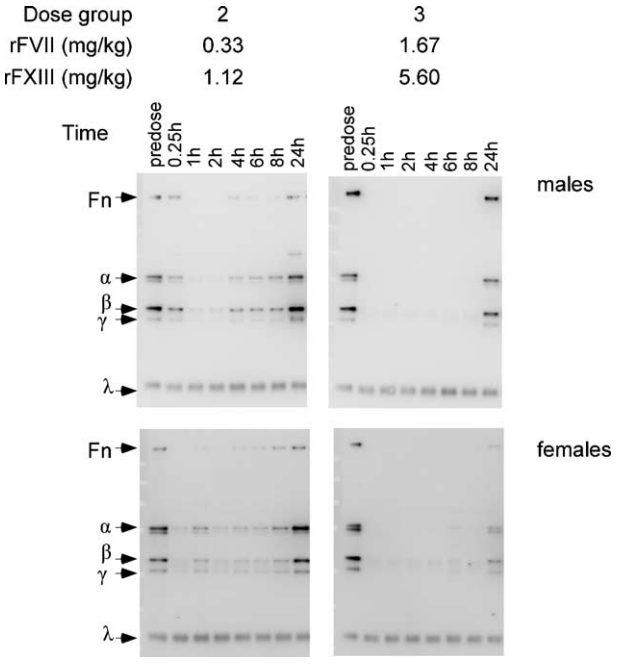

Figure 6. The reduction in plasma fibrinogen and fibronectin caused by intermediate doses of rFXIII+rFVIIa is reversible. For dose groups 2 and 3, representing animals that exhibited reductions in blood fibrinogen without any clinical signs during a 72-h observation period, the reversibility of the biomarker changes was examined. Plasma drawn predose and at the indicated times post-dosing was analysed by multiplex Western blot (Table I, Western blot assay 1). Representative multiplex Western images show the five protein bands quantitated by the assay: Fn, fibronectin; α, β, γ, fibrinogen α, β, γ chains; λ, immunoglobulin λ light chain.

paramaters were observed in animals exhibiting these profound changes in fibrinogen and fibronectin levels.

In short, as expected from the unremarkable clinical and histopathological findings at 72 h, survival from sublethal doses of rFXIII+rFVIIa was associated with recovery of blood fibrinogen and fibronectin levels during a 24-h period (Figure 6).

Importantly, as mentioned above, plasma levels of vitronectin and immunoglobulin light-chain λ levels were not affected at any dose or time point (Figure 6). Thus, despite the severity and duration of the disturbances caused by rFXIII+rFVIIa treatment, these were quite specific for a subset of plasma proteins (Figures 4 and 6, and not shown).

Discussion

While the safety of rFXIII monotherapy has been documented in preclinical as well as clinical trials (Bishop et al. 1990, Dodds et al. 2005, Ponce et al. 2005a,b, Reynolds et al. 2005, Visich et al. 2005), combination treatment with rFXIII and rFVIIa has not yet been examined in preclinical models. Here, we performed a single-dose intravenous study with a combination of rFXIII and rFVIIa in the cynomolgus monkey. The doses were chosen based on published monotherapy studies, to span from expected human exposure to just below the maximal tolerated dose for each

compound (Roberts et al. 2004, Ponce et al. 2005a,b, Reynolds et al. 2005, Visich et al. 2005).

At dose levels 1, 2 and 3, no ill effects were observed during the 3-day observation period. In contrast, at dose level 4, both animals died approximately 4 h post-dosing. In previous monotherapy single-dose studies in the cynomolgus monkey, rFXIII has been reported to be tolerated at up to 20 mg kg^{-1} (Ponce et al. 2005b). Thus, the deaths in dose group 4 (5.00 mg kg^{-} rFVIIa and 16.80 mg kg^{-1} rFXIII) may to be due to a combination of rFXIII and rFVII toxicities. Alternatively, the deaths in dose group 4 could be due to technical differences and biological variation between our study and previous studies (Ponce et al. 2005b). In any case, the steep toxicity response described for rFXIII monotherapy (Ponce et al. 2005b) appeared to be present also with rFXIII+rFVIIa combination therapy.

Previously, a syndrome resembling DIC has been described following rFXIII toxicity in the cynomolgus monkey (Ponce et al. 2005b). The histopathological findings described for lethal doses of rFXIII were similar to the histopathological changes found at dose level 4 in this study. Thus, as expected, the histopathological picture did not appear to differ between rFXIII monotherapy and rFXIII+rFVIIa combination therapy, and showed in both cases DIC-like pathology in decedent monkeys.

To identify toxicity biomarkers, we screened a number of plasma proteins known from the literature to be substrates for the transglutaminase activity of FXIII, or to be involved in blood clotting, inspired by recent publications describing the preclinical development of rFXIII for monotherapy use (Ponce et al. 2005b). Our screening procedure consisted of two phases. First, because the cynomolgus monkey is the preferred species for toxicology studies with human clotting factors (Birndorf et al. 1971, Lijnen et al. 1984, Karges et al. 1994, Ponce et al. 2005a,b), we screened commercially available antibodies against human clotting-cascade proteins for cross-reactivity to cynomolgus proteins. Second, using antibodies identified in the first phase, we examined the effect of rFXIII+rFVIIa treatment on the given clotting cascade protein. Western blotting was the preferred method for two important reasons: first, Western blotting represents a gold standard for protein detection, in terms of specificity and sensitivity; second, coagulation cascade proteins as a matter of course physically interact in the plasma matrix. However, because proteins are fully denatured, reduced and separated by size in the SDS-PAGE step, Western blotting assays are generally accepted not to exhibit matrix effects (Rand et al. 1996, Dempfle et al. 2000). Finally, we used platelet-poor plasma, drawn and stored as described in Materials and methods, because we assumed that using whole (non-fractionated) plasma would minimize the risk of introducing sample preparation artefacts, and increase the throughput and practical use of biomarker assays. Using the screening strategy described above, we screened approximately 25 'anti-human' antibodies (not shown), and found approximately 20 antibodies that were cross-reactive to cynomolgus monkey proteins (not shown), of which three antibodies had biomarker assay value. The three antibodies and corresponding clotting cascade plasma proteins that appeared to have toxicity biomarker value were fibronectin, fibrinogen and vitronectin (Table I, Figures 4–6).

In our opinion, the 3/20 success rate confirms that antibody-based screening of selected, relevant proteins is a highly productive way of biomarker identification, so much more so because with an antibody-based strategy, the transition from initial biomarker identification to subsequent biomarker assay development is very simple

and rapid (Table 1). However, because Western blotting is slow compared with, for example, enzyme-linked immunosorbent assay (ELISA), we sought to increase the throughput of Western blotting by a multiplex approach. The fibronectin, fibrinogen and immunoglobulin λ antibodies were in this case most amenable to multiplexing (Figures 2 and 3, Table I). The optimized multiplex Western assay was linear over a 30-fold protein concentration range (Figure 3), and allowed quantitative analysis using just 17 μl plasma per SDS-PAGE gel well, in a total volume of 5 μl sample buffer.

Using the optimized multiplex Western assay (Table 1), we investigated the effect of rFXIII + rFVIIa treatment in depth. In dose group 1, minimal changes were observed in plasma fibronectin and fibrinogen, regardless of sex. In dose groups 2, 3 and 4, we observed a gradual reduction in the plasma levels of fibronectin and fibrinogen, with females exhibiting stronger reductions than males (Figure 4). As expected, virtually identical results were obtained with monoclonal antibodies to fibrinogen α, β and γ chains, suggesting that rFXIII + rFVIIa treatment affected the mature disulfide-linked fibrinogen complex, as opposed to free fibrinogen chains. In dose groups 3 and 4, plasma fibronectin and fibrinogen was reduced by approximately 90% at 1 h post-dosing, but returned to normal levels within 24 h in males, with slower recovery in females (Figure 6). This tallied with the absence of clinical signs in dose groups 2 and 3. Importantly, rFXIII + rFVIIa had no effect on the levels of a number of other plasma proteins, with immunoglobulin λ light chain being used as control here (Figures 4 and 6). In short, plasma fibrinogen and fibronectin exhibited the required features for toxicity biomarkers: (i) minimal changes at the high end of the expected human dose (Figure 4, dose group 1), (ii) dose-dependent changes following exposure to suprapharmacological rFXIII + rFVIIa levels (Figure 4), (iii) reversibility in surviving animals (Figure 6), (iv) specificity, with changes restricted to biologically relevant proteins (Figures 4 and 6, and see below). Perhaps the main shortcoming of fibronectin and fibrinogen is the poor dose response at high rFXIII + rFVIIa levels (Figure 4), but vitronectin was serendipitously found to cover the near-lethal levels of rFXIII + rFVIIa exposure (Figure 5). Thus, fibrinogen, fibronectin and vitronectin represent the first preclinical toxicity biomarkers for general haemostatic treatment ever described. While this study reports the results from a single animal experiment, these biomarkers have been confirmed in our lab using material from independent animal studies (not shown).

The process of blood sampling can in itself activate coagulation proteins (despite chelating agents), and such sampling artefacts might become more pronounced for blood containing supraphysiological levels of clotting factors. For example, a recent study described sampling/storage artefacts in citrate stabilized plasma drawn from rFXIII-treated cynomolgus monkeys (Ponce et al. 2005a). In this regard, we observed similar reductions in fibrinogen and fibronectin in rFXIII-treated animals regardless of whether plasma was stabilized with citrate or EDTA (not shown). Also, our observation of sex differences strongly argues against sampling artefacts producing the biomarker changes (Figures 4–6). Nevertheless, to examine directly whether biomarker changes occurred post-sampling (i.e. represented sampling artefacts), we spiked citrate-stabilized plasma with either rFXIII or thrombin-activated rFXIIIa, and exposed the spiked samples to freeze/thawing followed by successive incubations at 0°C and 37°C for 30 min. Despite the spiked samples containing rFXIII levels similar to those obtained in treated animals in dose groups 3 and 4, fibrinogen and

fibronectin levels did not change at any point in the spiked samples (not shown). Thus, sampling/storage artefacts cannot be responsible for the reductions in fibrinogen and fibronectin we observed in dosed animals (Figures 4 and 6). On the other hand, it is entirely credible that the severe reduction in fibrinogen and fibronectin represents a biological effect of the rFXIII+rFVIIa combination in the live animal: histopathology shows that a DIC-like condition occurs following overdosing with rFXIII+rFVIIa, and acute reduction in blood fibrinogen can be observed during DIC in species as diverse as rodents and man (Levi & ten Cate 1999, Toh & Dennis 2003, Kaspereit et al. 2004).

Fibrinogen, fibronectin and vitronectin all participate in blood clotting, and all three proteins have been described as substrates for the transglutaminase activity of FXIIIa (Mosher 1984, Preissner & Seiffert 1998, Muszbek et al. 1999, Ni et al. 2000, 2003, Koschnick et al. 2005, Reheman et al. 2005). However, fibrinogen and fibronectin are heavily incorporated in blood clots, and incorporation is strongly stimulated by FXIIIa (Bale et al. 1985, Okada et al. 1985). In contrast, although vitronectin is a physiologically important component of blood clots (Koschnick et al. 2005, Reheman et al. 2005), incorporation into clots occurs at lower levels, and is not dependent on FXIIIa (Preissner & Seiffert 1998, Podor et al. 2002). Thus, the observed plasma 'fingerprint' of reduced fibrinogen/fibronectin (Figure 4), and largely unchanged vitronectin (Figure 5), is fully consistent with substrate depletion during a blood clotting reaction, especially one driven by unphysiologically high levels of FXIII. This inferred clotting reaction could hypothetically yield two products (Gaffney 2001): (i) insoluble fibrin/fibronectin clots, and (ii) soluble cross-linked complexes of fibrinogen and fibronectin. Previously, in animals dosed only with rFXIII, soluble, FXIII cross-linked fibrinogen-containing complexes have been observed in plasma, with frank thrombi appearing in tissues only at doses associated with mortality (Ponce et al. 2005b). Here, in rFXIII+rFVIIa-dosed animals, we observed in all dose groups the treatment-dependent formation of a ~100-kDa band, which most likely represented fibrinogen γ chains covalently cross-linked by FXIII (Figure 4, and not shown), and thrombi by histopathology in animals only at dose level 4. Thus, our findings support and extend to rFXIII+rFVIIa combination treatment the previously suggested toxicity mechanism for rFXIII monotherapy (Ponce et al. 2005b). In the previously proposed rFXIII monotherapy toxicity model (Ponce et al. 2005b), suprapharmacological levels of rFXIII exceeding the capacity of the B-carrier subunit (Figure 1) lead to increased plasma transglutaminase activity, formation of cross-linked protein complexes, and ultimately a systemic coagulopathy resembling DIC (Ponce et al. 2005b). In further support of the validity of the rFXIII monotherapy model also for rFXIII+rFVIIa combination treatment, we have found that plasma fibrinogen and fibronectin levels are similarly affected by rFXIII+rFVIIa (Figure 4) as by rFXIII alone (not shown). On the other hand, preliminary results suggest that the conversion of the ~75-kDa vitronectin form to the ~65-kDa form that occurs with rFXIII+rFVIIa treatment (Figure 5) is not seen with rFXIII treatment alone (not shown). This opens the possibility that while the toxicological mechanisms of mono- and combination therapies are largely identical, there are unique features contributed by the rFVIIa component. Interestingly, plasminogen-mediated proteolysis of vitronectin has been shown to cause loss of plasminogen activator inhibitor 1 (PAI-1) binding, which would be expected to impact the thrombotic effect of vitronectin (Chain et al. 1991, Kost et al. 1992). It was originally noted that while the

systemic coagulopathy induced by rFXIII alone histopathologically resembles DIC, the pathogenesis of rFXIII factor toxicity differs from that of DIC (Ponce et al. 2005b). DIC can arise from different aetiologies, but is generally characterized by a thrombin- and plasmin-generating, procoagulant state in plasma, that can be clinically graded from mild to severe (Levi & ten Cate 1999, Toh & Dennis 2003). In contrast, with rFXIII monotherapy, a procoagulant state was not induced in the plasma of animals receiving doses only fractionally lower that lethal, neither did clinical symptoms occur (Ponce et al. 2005b). Yet, in animals receiving lethal rFXIII doses, haematological as well as histopathological changes consistent with DIC were seen (Ponce et al. 2005b). In the current study, the hallmark change following rFXIII+rFVIIa treatment was severely reduced fibrinogen (Figure 4). The high level of fibrinogen reduction after rFXIII+rFVIIa treatment resembles the picture in severe DIC (Levi & ten Cate 1999, Toh & Dennis 2003), or during fibrinolytic treatment with ancrod (Dempfle et al. 2000). However, in contrast to what is expected in DIC or fibrinolytic treatment, platelet counts, TAT levels and D-dimer levels did not exhibit consistent changes in dose groups 1, 2 and 3 (Table II). A slight reduction in PT was observed, while the aPTT was unaffected (Table II). Changes in PT and aPTT were not described for rFXIII monotherapy, but are well known to occur after rFVIIa dosing (Telgt et al. 1989, Rao & Rapaport 1990, Keeney et al. 2005). Thus, the present study confirms in the setting of rFXIII+rFVIIa combination therapy that while the systemic coagulopathy caused by coagulation factor toxicity histopathologically resembles DIC, the pathogenesis is quite different (Ponce et al. 2005b).

In summary, this study provided the first description of biomarkers for the toxicity of rFXIII+rFVIIa combination treatment. The biomarkers are in full agreement with the proposed mechanism of rFVIIa/rFXII toxicity, supporting their validity. The biomarkers were specifically developed for preclinical toxicology testing. Thus, their applicability to the clinical situation, where patients may have severely perturbed haematological parameters at the time of treatment, remains to be determined. Also, for clinical applications, methods are required that are simpler to perform than Western blotting, such as ELISA.

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