The Defective Down Regulation of Fibrinolysis in Haemophilia A Can Be Restored by Increasing the TAFI Plasma Concentration

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Summary
TAFI (thrombin activatable fibrinolysis inhibitor) down regulates fibrinolysis after activation by relatively high concentrations of thrombin generated during coagulation via thrombin mediated factor XI activation and subsequent activation of the intrinsic pathway. It is this secondary burst of thrombin that is severely diminished in haemophilia A, a deficiency of coagulation factor VIII. We therefore investigated the role of TAFI in haemophilia A by measuring the clot lysis times of tissue factor induced fibrin formation and iPA mediated fibrinolysis. In haemophilia A plasma clot lysis times were normal at relatively high tissue factor concentrations but severely decreased at moderate to low tissue factor concentrations, indicating that the thrombin generation via the extrinsic pathway was insufficient to activate TAFI. Addition of factor VIII, TAFI or thrombomodulin restored the clot lysis times at low tissue factor concentrations. This confirms the hypothesis that the bleeding disorder in haemophilia A is not merely a defect in the initial clot formation but is in fact a triple defect: reduced thrombin formation via the extrinsic pathway at low tissue factor concentrations, a reduced secondary burst of thrombin generation via the intrinsic pathway and a defective down regulation of the fibrinolytic system by the intrinsic pathway.

Introduction
Haemophilia A is an X-linked hereditary bleeding disorder characterized by a deficiency or a functional defect in coagulation factor VIII that affects approximately 1-2 in 10,000 males (1). The severity of the bleeding symptoms is dependent on the residual factor VIII clotting activity. Patients with a severe form of haemophilia (factor VIII:C <1%) suffer from spontaneous joint and muscle bleedings whereas bleedings in moderate haemophilia (factor VIII:C 2-5%) are generally preceded by minor trauma (2).

Factor VIII participates in the intrinsic pathway of coagulation and is an essential cofactor in the tenase complex which catalyzes the activation of factor X by factor IXa. Coagulation is initiated by exposure of tissue factor to the blood. At high tissue factor concentrations, factor X is mainly activated by the factor VIIa-tissue factor complex, whereas at low tissue factor concentrations the activation of factor IX and the contribution of the tenase complex in the activation of factor X becomes more pronounced (3). This predicts that haemophilia A patients bleed predominantly from areas of the body which contain low tissue factor concentrations and indeed deep skeletal muscles and joints were found to have undetectable to low tissue factor expression (4). Interestingly, haemostatic plug abnormalities in haemophiliacs could not be detected directly after plug formation, but only after 2 h (5). Although this has previously been explained by the impaired fibrin formation this is unlikely in view of the small amounts of thrombin that are necessary for fibrin formation and the presence of an unaffected extrinsic pathway of coagulation in these patients. Recently a revised model of blood coagulation was proposed which provides an alternative explanation for the delay in haemostatic plug abnormalities (6). In this model initiation of coagulation and fibrinolysis are closely regulated via the activation of Thrombin Activatable Fibrinolysis Inhibitor (TAFI; EC 3.4.17.20) (7). TAFI, also referred to as procarboxypeptidase U (8) or plasma procarboxypeptidase B, (9) is a carboxypeptidase B like proenzyme that after activation inhibits fibrinolysis by removing carboxy-terminal lysine residues from fibrin that play a role in plasminogen binding and activation (10). Activation of TAFI is mediated by relatively high concentrations of thrombin that are formed via the intrinsic pathway in a factor XI dependent way (11). This secondary burst of thrombin was found to protect the clot against premature lysis and was dependent on an intact intrinsic pathway of coagulation and on TAFI (12).

The defect in the intrinsic pathway in haemophilia A would predict a diminished TAFI activation resulting in a rapid lysis of the clot. Indeed Broze et al. have shown that the clot lysis time is decreased in factor VIII, IX, X and XI deficient plasma and could be restored by addition of the deficient factor (13). Also addition of thrombomodulin (TM), an endothelial cell receptor that stimulates the thrombin mediated TAFI activation more than 1000 fold, was shown to correct the clot.
lysis times in haemophilia plasma (13, 14). This suggests that the clot lysis times could be restored in haemophilia A by restoring the activation of TAFI. However, TM also stimulates the activation of the anticoagulant protein C pathway, thereby inhibiting thrombin formation, an undesired side effect in haemophilia A (15, 16). Recently we have shown that at low concentrations, TM predominantly stimulates the activation of TAFI whereas at 5 nM or higher the activation of protein C becomes more pronounced and subsequently TAFI activation is down-regulated by activated protein C (16). Another way of increasing the activation of TAFI would be by increasing the TAFI plasma concentration, since the plasma concentration of TAFI (4-15 μg/ml) is well below the Km for thrombin mediated TAFI activation (14, 15, 17). Addition of purified TAFI to normal plasma resulted in an increased activation of TAFI and a dose dependent down regulation of fibrinolysis (17).

In this study we characterize the activation of TAFI in severe haemophilia A and compare the effects of addition of TAFI and TM on the clot lysis in individual patients with severe haemophilia A and patients with high anti-factor VIII antibody titres.

Results

Tissue factor induced clot lysis in normal- and haemophilia A plasma. Activation of TAFI in normal- and pooled haemophilia A plasma was determined in a clot lysis assay by measuring the turbidity of a tissue factor-induced (dilution of Innovin) clot over time. Clot lysis times were determined in normal plasma (panel A) and pooled haemophilia A plasma (panel B). Coagulation was initiated by different tissue factor concentrations (1*10^5 – 1*10^7 dilution of Innovin) and tPA (30 U/ml) was used to initiate fibrinolysis. Clot lysis times were determined in the absence (□) and presence of an antibody against factor XI (■), 50 μg/ml CPI (○) or 1 U/ml factor VIII (●). Each point represents the mean of two independent experiments.
Innovin), where feedback via the intrinsic pathway is essential. Accordingly the clot lysis times in pooled haemophilia A plasma were normal at high tissue factor concentrations but decreased to those in the presence of CPI at low tissue factor concentration (Fig. 1b). Reconstitution of the haemophilia A plasma with plasma concentrations of factor VIII (1 U/ml) restored the clot lysis time at low tissue factor concentrations.

**Normalization of clot lysis times in haemophilia A plasma by factor VIII and TAFI.** The effect of different concentrations of factor VIII on the clot lysis time in haemophilia A plasma was tested. Addition of 0.01 U/ml factor VIII to pooled haemophilia A plasma was already sufficient to restore the clot lysis time to the same level as observed in normal plasma (Fig. 2). Increasing the factor VIII level to 5 U/ml did not result in a further prolongation of the clot lysis time.

Previously we found a correlation between the clot lysis time and the TAFI concentration in normal plasmas (17). Addition of purified TAFI to pooled haemophilia A plasma resulted also in a dose dependent increase of the clot lysis times (Fig. 3). Increasing the plasma TAFI concentration two fold to 30 μg/ml resulted in a 3 fold increase of the clot lysis time in haemophilia A plasma whereas a 10 fold increase of the TAFI plasma concentration (addition of 150 μg/ml TAFI) resulted in a 7 fold increase of the clot lysis time which could be completely inhibited by the TAFIa inhibitor CPI.

**Clot lysis in individual haemophilia A patients.** The clot lysis times were determined in plasmas of 56 individual patients with severe haemophilia A. Clot lysis times in the haemophilia A patients (median, 21.2 min) were similar to the clot lysis times in the presence of CPI (18.8 min), indicating that only limited TAFI activation had occurred (Fig. 4). Addition of factor VIII (1 U/ml) induced a prolongation of the clot lysis times (39.9 min). Doubling the TAFI concentration resulted in a 2 fold increase of the clot lysis times (43.5 min), whereas addition of TM (1 nM) resulted in a 3.7 fold increase of the clot lysis time (83.9 min). The tissue factor (1*10^6 dilution of Innovin) induced clotting times were determined (data not shown). A median value of 12.4 min was obtained and addition of 1 U/ml factor VIII resulted in a shortening of the mean clotting time to 6.3 min in the haemophilia A patients. The clotting times were not significantly different after addition of TAFI, TM or CPI (11.9, 13.8 and 11.3 min, respectively).

**TAFI levels in haemophilia A.** In haemophilia A patients the mean TAFI level was decreased compared to the 40 normal individuals that comprise the normal plasma pool (82.0% vs. 97.7% (17); p<0.0044 (t-test)) or (82.0% vs. 98.1%; p<0.0029) when women were omitted from the control group. Because TAFI levels were found to increase with age (18) and the haemophilia A patients were on average younger than the control group (13.6 vs. 36.1 years), TAFI levels were determined in a group of young children (n = 11; mean age 7.5 years). In this group of children TAFI levels were also decreased compared to the control group and were not significantly different from the haemophilia A patients (81.9% vs. 82.0%).

Since TAFI levels were found to determine the clot lysis times in normal healthy individuals (17) the clot lysis time or the TAFI level might give an insight in the severity of the bleeding complications of...
the haemophilia A patients. TAFI levels correlated with the clot lysis times in haemophilia A patients in the presence of factor VIII ($r = 0.63; \ p < 0.0001$) but not in the absence of factor VIII (data not shown). However no correlation between the TAFI levels and the severity of the bleeding complications in haemophilia A patients (as characterized by age of first bleeding, frequency of bleeding in first year or the total amount of prophylaxis per year) could be observed.

**Addition of TM or TAFI to haemophilia A patients with factor VIII inhibitors.** In approximately 25% of the patients with severe haemophilia A inhibitory antibodies directed against factor VIII develop after multiple transfusions with factor VIII concentrate (19). To determine if addition of TAFI or TM also increased the clot lysis times in patients who have developed inhibitory antibodies against factor VIII, clot lysis times were determined in four patients with high factor VIII antibody titres (three congenital inherited haemophilia A patients: 188, 133 and 114 BU/ml and one acquired haemophilia A patient: 181 BU/ml). As anticipated, addition of factor VIII had no effect on the clot lysis times. In contrast, addition of both TAFI or TM increased the clot lysis times, similarly to that observed in the haemophilia A patients without an inhibitor (Fig. 5).

**Discussion**

Here we report that the down regulation of fibrinolysis is severely impaired in haemophilia A patients due to the absence of TAFI activation at low tissue factor concentrations. The bleeding complications in haemophilia A patients have always been attributed to defective clot formation despite the fact that bleeding occurs hours or days after injury (after the clot had been formed). This can not be explained by a defective clot formation alone and suggests that additional mechanisms are involved in the manifestation of bleeding in haemophilia A patients. Recently, a revised model of blood coagulation provided a plausible explanation for the defect in haemophilia A. (6) In this model coagulation is initiated via the extrinsic system by the exposure of tissue factor resulting in the generation of sufficient amounts of thrombin to induce fibrin formation. Thrombin formation continues inside the fibrin clot via the intrinsic pathway by the activation of factor XI by thrombin (11). Although only small amounts of activated factor XI are formed, a secondary burst of thrombin formation is provided by the intrinsic pathway due to the continued activation of factor XI by thrombin and the amplification power of both the tenase and the prothrombinase complex. These high concentrations of thrombin are needed for the activation of TAFI and results in a down regulation of fibrinolysis (12). Thrombin thus not only forms the fibrin clot but also protects the fibrin clot against lysis. This model implies that haemophilia A patients have a defective thrombin formation via the extrinsic pathway at low tissue factor concentrations, a reduced secondary burst of thrombin formation via the intrinsic pathway and a defective TAFI mediated down regulation of the fibrinolytic system. We studied the regulation of tissue factor induced coagulation and fibrinolysis in haemophilia A patients using a clot lysis assay that measures the efficiency of the coagulation system to generate thrombin to down regulate fibrinolysis. We found a defective TAFI dependent downregulation of fibrinolysis when low tissue factor concentrations were used to initiate coagulation, whereas at high tissue factor concentrations a normal TAFI dependent clot lysis time was obtained. This indicates that in haemophilia A plasma at low tissue factor concentrations not enough thrombin is generated via both the extrinsic and intrinsic pathway to activate TAFI whereas at high tissue factor concentrations the extrinsic pathway generates sufficient thrombin to activate TAFI independent of the intrinsic system. In previous studies we found in normal individuals a correlation between the TAFI levels and the clot lysis time in the presence of an intact coagulation system (17). An abnormal clot lysis time could therefore also be explained by a decreased TAFI level. The TAFI levels in the haemophilia A patients were found to be lower compared to a control group of normal individuals, but the TAFI level was not significantly different compared to a group of young children. TAFI levels are known to increase with age (18, 20). In agreement with our previous study where correlation between the TAFI level and clot lysis was lost upon disruption of the intrinsic pathway, (17) the TAFI level and the clot lysis time did not show a significant correlation in the haemophilia A patients. No correlation was found between TAFI levels and bleeding complications (age of first bleeding, frequency of bleeding in the first year or the total amount of factor VIII used for prophylactic treatment per year).

Reconstitution of haemophilia A plasma with factor VIII resulted in a significant correlation between the clot lysis time and TAFI level as was also observed in the plasmas of normal individuals. This indicates that the activation of TAFI and the down regulation of fibrinolysis proceeds normally in haemophilia A plasma provided factor VIII is present.

The defective clot lysis time in haemophilia A was also corrected by increasing the TAFI level. Previous studies had shown that the TAFI concentration in plasma is well below the Km of the activation reaction of TAFI by thrombin. Increasing the TAFI level two fold resulted in a normalization of the clot lysis time and the clot lysis times continued to prolong at increasing TAFI concentrations. This indicates that some thrombin is generated via the intrinsic pathway at low tissue factor concentrations that is capable of activating TAFI which becomes more pronounced when the TAFI level is increased. This is in contrast to the effect of increasing the factor VIII level. At >1% factor VIII a normal clot lysis time is observed and higher levels of factor VIII do not result in a further prolongation of the lysis time suggesting that the
thrombin formation already proceeds at its maximal rate at about 0.01 U/ml of factor VIII.

Thrombomodulin was shown before to correct the impaired clot lysis in plasma of patients with haemophilia A (13). Thrombomodulin increases the efficiency of thrombin to activate TAFI many fold making trace amounts of thrombin more effective in the activation of TAFI (14). Thrombomodulin also stimulates the activation of protein C which acts not only as an anticoagulant but also as an antifibrinolytic effect because it reduces the generation of thrombin (15). Recently we showed that at low concentrations thrombomodulin predominantly stimulates the activation of TAFI by thrombin and thus has an antifibrinolytic effect whereas at high concentrations of thrombomodulin protein C is activated and the antifibrinolytic effect becomes more pronounced (16). The shift from antifibrinolytic to profibrinolytic depending on the concentration of thrombomodulin hampers an eventual clinical use of thrombomodulin. Recently, critical residues have been identified in both thrombin and thrombomodulin that selectively stimulate TAFI or protein C activation (21, 22). Such variants might be useful tools for selectively increasing the rate of TAFI activation in haemophiliacs.

TAFI dependent down regulation of fibrinolysis is severely impaired in haemophilia A patients with an inhibitor against factor VIII. In these patients the clot lysis could not be corrected by reconstitution with factor VIII. However addition of TAFI or thrombomodulin normalized the clot lysis test indicating that trace amounts of thrombin generated by the extrinsic pathway could be used effectively in these patients in the down regulation of fibrinolysis.

These data suggest that antifibrinolytic therapy would contribute to reduce the bleeding complications in haemophilia A. The concept of an antifibrinolytic therapy in haemophilia A is not new and has been advocated for 30 years. Systemic fibrinolysis inhibitors, such as tranexamic acid, a lysine analog that competes with the binding of tPA and plasminogen to fibrin were successfully used during tooth extraction and are indicated for 30 years. Systemic fibrinolysis inhibitors, such as tranexamic acid, a lysine analog that competes with the binding of tPA and plasminogen to fibrin were successfully used during tooth extraction and are indicated for 30 years.

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