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Ⅲ．研究成果の刊行物・別刷

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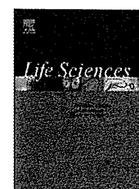

Inactivation of plasminogen activator inhibitor type 1 by activated factor XII plays a role in the enhancement of fibrinolysis by contact factors *in-vitro*

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ABSTRACT

Aims: Several activated coagulation factors have been reported to enhance fibrinolysis by inactivating plasminogen activator inhibitor type 1 (PAI-1), a serine protease inhibitor. We analyzed the interaction between PAI-1 and the three serine proteases generated during contact activation of plasma, activated factor XII (FXIIa), FXIa, and kallikrein, and evaluated their effects on fibrinolysis in-vitro.
Main methods: Effects of kaolin on euglobulin clot lysis time (ECLT) and behavior of PAI-1 in factor-depleted plasma were analyzed.
Key findings: The ECLT of pooled plasma obtained from normal volunteers (designated as 100%) was shortened to $62.1 \pm 3.1\%$ by Ca^{2+} (5 mM) and $29.9 \pm 3.1\%$ by kaolin. Activated protein C reversed the ECLT shortened by Ca^{2+}-supplementation ($86.3 \pm 17.4\%$), but did not affect the ECLT shortened by kaolin ($31.4 \pm 2.1\%$). Thus, in contrary to Ca^{2+}-supplementation, kaolin appeared to shorten the ECLT by a mechanism independent of thrombin generation. In three kinds of contact factor-depleted plasma, kaolin did not shorten ECLT only in FXII-depleted plasma. PAI-1 was cleaved to its inactive form in the Ca^{2+} as well as the kaolin-supplemented euglobulin fraction in normal plasma, the latter of which, however, was not observed in FXII-depleted plasma. Similarly, a high molecular weight complex between FXIIa and PAI-1, as well as a cleaved form of PAI-1, was observed in kaolin-supplemented normal plasma, but neither was found in kaolin-supplemented FXII-depleted plasma.
Significance: PAI-1 inactivation by FXIIa appears to be a mechanism by which contact phase coagulation factors enhance fibrinolysis independently of thrombin generation.

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Introduction

The fibrinolytic system plays an essential role in maintaining vascular patency by quickly dissolving excess amounts of accumulated fibrin clot. Tissue type plasminogen activator (tPA) is the main PA in the vasculature, and its activities both in plasma and on vascular endothelial cells (Suzuki et al. 2009) are finely tuned by plasminogen activator inhibitor type 1 (PAI-1), a member of the serine protease inhibitor superfamily (SERPINS) (Loskutoff et al. 1989). As other members of SERPINS, PAI-1 inactivates PA activity by forming a high molecular weight complex with it (Travis and Salvesen 1983). PAI-1 also forms high molecular weight complex with other serine proteases involved in the coagulation cascade, including activated contact factors (Berrettini et al. 1989), thrombin (Ehrlich et al. 1990), and Ca^{2+} bound factor Xa (FXa) (Urano et al. 1996), especially in the presence of the cofactors vitronectin (Vn) and heparin. Such complex formation naturally leads to the mutual loss of activities of both protease and PAI-1.

Euglobulin clot lysis time (ECLT) is a long-established global fibrinolytic assay basically determined by a function of plasma levels of tPA and PAI-1. It is useful to evaluate regulatory mechanisms at the initial phase of the fibrinolytic system. Supplementation of Ca^{2+} into the ECLT assay system triggers thrombin generation in the euglobulin fraction (Urano et al. 2003). The generated thrombin appeared to form a high molecular weight complex with PAI-1, which results in the loss of PAI-1 activity. Thus, thrombin generation by the coagulation cascade activation appears to trigger the expression of tPA activity and to enhance fibrinolysis through the inactivation of PAI-1. This is one possible mechanism for the coagulation-associated enhancement of fibrinolysis (Gaffney et al. 1999), which plays an essential role in maintaining vascular patency by limiting over-accumulation of fibrin clot.

The intrinsic-coagulation cascade pathway has been considered more deeply involved than the extrinsic pathway in the enhancement of fibrinolysis (Colman 1984; Kaplan and Silverberg 1987). The intrinsic pathway is triggered by activation of so-called "contact factors" by negatively charged surfaces such as kaolin, sulfatides,

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dextran sulfates, phospholipids, urate crystals, etc. The proteins prekallikrein (PK), factor XII (FXII), factor XI (FXI) and high molecular weight kininogen (HK), a non-enzymatic cofactor, are involved in this contact reaction (Yarovaya et al. 2002). The enhancement of fibrinolysis by contact factors was first reported as an increased euglobulin fibrinolytic activity of plasma by kaolin-activated FXII (Niewiarowski and Prou-Wartelle 1959). Several coherent mechanisms have been suggested by which activated FXII (FXIIa) could enhance fibrinolysis, including direct activation of plasminogen (Goldsmith et al. 1978) and/or its indirect activation through generation of kallikrein and FXIa (Mandle and Kaplan 1979). The activation of single chain urokinase by kallikrein has also been suggested as a mechanism (Ichinose et al. 1986). However, the efficacies of these activations are much lower than those of PAs in activating plasminogen and the subsequent plasmin activation of single chain urokinase.

ECLT was measured in the present study and the mechanism for kaolin-dependent enhancement of fibrinolysis was analyzed, focusing primarily on the initial phase of fibrinolysis, which might be modified by activated contact factors. FXIIa enhanced fibrinolysis by directly inactivating PAI-1, and this enhancement was thus independent of a thrombin-generation mechanism.

Materials and methods

Reagents

The following materials were purchased from the indicated companies: human thrombin (Mitsubishi Pharma Corporation, Osaka, Japan), factor-depleted plasma (FXII, XI and PK) (George King Bio-Medical, Inc., KS, USA), human factor XIIa (Enzyme Research Laboratories, IN, USA), and human tissue factor (TF) (recombiplastin) (Instrumentation Laboratory, MA, USA), human α-thrombin and prothrombin (Diagnostica Stago, Asnieres, France), pre-stained broad range molecular weight standards and polyvinylidene difluoride (PVDF) membranes and nitrocellulose membranes (BioRad Labs, Richmond, CA, USA), and enhanced chemiluminescence Western blotting detector reagents (ECL; Amersham Life Sciences, Buckinghamshire, England). Activated factor VII (FVIIa) and activated protein C (aPC) were kindly provided by Novo Nordisk A/S, Denmark, and Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan, respectively.

Blood samples

Venous blood was drawn from an antecubital vein of five healthy volunteers into silicone-coated tubes containing 1/10th volume of 3.2% sodium citrate. After centrifugation at 4 °C at 3000 ×g for 10 min, all plasma were pooled, which were then aliquoted and kept frozen at −80 °C for future investigation. All volunteers involved gave informed consent to participate in the present study, which was approved by the Institutional Review Board of Hamamatsu University School of Medicine.

ECLT assay

The ECLT assay was conducted using microtiterplates as previously reported (Urano et al. 1991). Briefly, plasma was diluted 20 times and acidified by 10 mM acetate buffer to pH 4.5, then left at 4 °C for 1 h. After centrifugation at 4 °C at 1500 ×g for 10 min, the pellet was resuspended in 0.5 ml of 0.1 M Tris buffer (pH 7.4) and used immediately as a plasma euglobulin fraction. A 150 µl aliquot of the euglobulin fraction was added to the wells of a microtiterplate containing α-thrombin at 2.5 U/ml (final concentration) in the presence of Ca^{2+} (final conc. 10 mM), kaolin (final conc. 2.5 mg/ml) or the same volume of the buffer (Urano et al. 1991). To investigate the possible influence of the extrinsic coagulation pathway, ECLT was conducted in the presence of FVIIa (final conc. 100 nM), TF (final conc. 11.3 ng/ml) and their combination. When necessary, aPC (final conc. 80 nM) was added prior to clot formation. All ECLT results were shown as a percentage of results obtained in a standard ECLT assay conducted without aPC.

Thrombin generation during ECLT

Thrombin generation from prothrombin in the euglobulin fraction was detected by Western immunoblotting. Thirty minutes after the initiation of either a standard ECLT, or a Ca^{2+}- or a kaolin-ECLT, sample buffer for SDS-PAGE was added and the euglobulin clot was thoroughly dissolved. After separation of protein bands on a 7.5% SDS-PAGE gel and transblotting onto a nitrocellulose membrane, protein bands were visualized using affinity-purified sheep anti-human prothrombin IgG (Cedarlane, Ontario, Canada), followed by the horseradish peroxidase (HRP)-conjugated $F(ab)'_2$ fragment of donkey-anti sheep IgG (Chemicon Int. Inc., Temecula, CA, USA) together with enhanced chemiluminescence Western blotting detector reagent (ECL; Amersham Life Sciences, Buckinghamshire, England).

Biotin-labeled PAI-1

Human recombinant prokaryotic PAI-1 (rpPAI-1) purified as previously reported (Urano et al. 1992) was labeled by biotin using a commercially available kit (ECL protein biotinylation module; Amersham Life Sciences, Buckinghamshire, England) as was reported before (Urano et al. 1992). At least two-thirds of the labeled material was confirmed to be active by its ability to form a high molecular weight complex with tPA.

Analysis of PAI-1 cleavage during euglobulin clot lysis

To confirm the PAI-1 cleavage in control plasma and FXII-depleted plasma, each euglobulin clot containing the minimum dose of biotin-labeled rpPAI-1 (final concentration 50 nM) was dissolved by the sample buffer for SDS-PAGE 1 h after clot formation and analyzed by SDS-PAGE. After separation of protein bands on a 7.5% SDS-PAGE gel and transblotting onto a PVDF membrane, biotin-labeled rpPAI-1 related bands were visualized using a streptavidin-HRP conjugate (Amersham Life Sciences, Buckinghamshire, England). To assess the reaction between FXIIa and PAI-1, purified FXIIa (5.1 µM) and biotin-labeled rpPAI-1 (25 nM) were incubated at 37 °C for 1 h and then subjected to the same analysis.

PAI-1 depletion from plasma

To deplete PAI-1, normal plasma was treated with increasing amounts of Sepharose 4B-conjugated anti-PAI-1 antibody for 1 h at room temperature, and the supernatant was obtained by centrifugation at 4 °C at 1500 ×g was subjected to a standard – a Ca^{2+} – and a kaolin-ECLT. A polyclonal antibody against human PAI-1 was raised in a rabbit immunized with purified rPAI-1. The IgG fraction was purified by ammonium sulphate precipitation followed by DEAE sepharose chromatography, and was coupled with CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's recommended procedure.

Analysis of two-chain high molecular weight kininogen (HKa) formation during ECLT

We investigated a possible generation of HKa, which dissociates PAI-1 and Vn binding and suppresses PAI-1 activity (Chavakis et al. 2000), during ECLT as follows: euglobulin clot prepared from either

normal plasma or PK-depleted plasma in the presence of kaolin was dissolved by adding a sample buffer for SDS-PAGE 1 h after clot formation. After separation of protein bands on a 5.0% SDS-PAGE gel and transblotting onto a nitrocellulose membrane, kininogen-related protein bands were visualized using goat anti-human kininogen antibody (R&D systems, Minneapolis, USA), (HRP)-conjugated rabbit anti-goat IgG, and enhanced chemiluminescence Western blotting detector reagent.

Statistics

Data are shown by mean ± standard deviation. Statistical significance was evaluated using Mann–Whitney's *U* test. *P* value <0.05 was considered statistically significant.

Results

Effects of Ca2^{+} and kaolin supplementation on ECLT

Clot dissolution of the euglobulin fraction of plasma was successfully monitored by a microtiterplate reader assay even in the presence of supplemented kaolin. Each ECLT was expressed as a percentage of the mean standard ECLT. ECLT was shortened to 62.1 ± 3.1% by the addition of Ca^{2+} at 5 mM to normal plasma. The ECLT was similarly shortened to 29.9 ± 3.1% by addition of kaolin (Fig. 1), and it was further shortened to 23.4 ± 1.4% by simultaneous supplementation with kaolin plus Ca^{2+}.

Effects of Ca2^{+} and kaolin on ECLT of coagulation factor-depleted plasma in the contact phase

Three kinds of contact factor-depleted plasma, i.e. FXII-, FXI-, and PK-depleted, were subjected to standard, kaolin and Ca^{2+}-ECLT, and results were compared with those obtained using normal plasma (Fig. 2). Standard ECLT values for control plasma and for FXII-, FXI-, PK-depleted plasmas were 4.59 ± 0.67 h, 18.52 ± 0.65 h, 14.44 ± 1.04 h, 20.26 ± 1.14 h, respectively. Therefore ECLT results were expressed as a percentage of the mean standard ECLT of the corresponding plasma. The kaolin-ECLT for FXII-depleted plasma (83.2 ± 2.3%) was not significantly different from the standard ECLT, whereas the Ca^{2+}-ECLT (37.0 ± 1.8%) was significantly shorter than the standard ECLT. The same tendency was seen for the FXI-depleted plasma. For the PK-depleted plasma, however, both the kaolin-ECLT (6.9 ± 1.2%) and the Ca^{2+}-ECLT (29.8 ± 1.8%) were significantly shorter than the standard ECLT. FXII appeared to be the most important among these three contact phase factors in the shortening of ECLT by kaolin, and PK appeared not to play a significant role. In all of these cases the ECLT was significantly shortened by Ca^{2+} supplementation, suggesting that a similar amount of thrombin is generated during clot lysis in the presence of Ca^{2+} in plasmas depleted of contact phase factors. This thrombin then inactivated PAI-1 and shortened the ECLT, as previously reported in normal plasma (Urano et al. 2003).

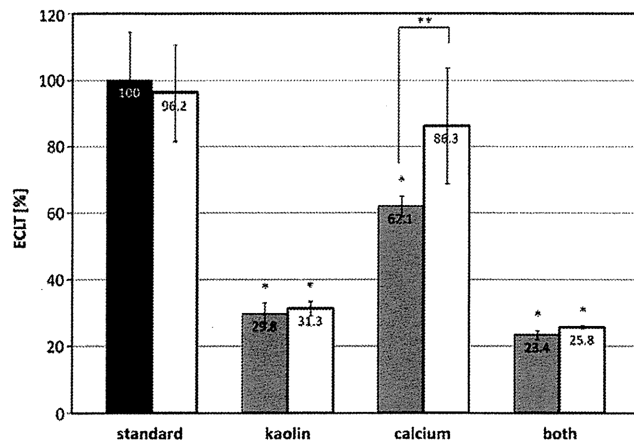

Fig. 1. Effects of aPC on regular-, kaolin- and Ca^{2+}-ECLT. Regular ECLT, kaolin-, Ca^{2+}-, and kaolin and Ca^{2+}-supplemented ECLT were measured in the absence (lt column) or presence (rt column) of 80 nM aPC. Each ECLT is expressed as a percentage of the mean standard ECLT ($n=8$–12), and is shown as mean ± standard deviation. *: Statistical difference was observed vs standard ECLT in the absence of aPC ($P<0.01$). **: Statistical difference was observed in Ca^{2+}- ECLT between assays carried out in the absence and presence of aPC ($P<0.01$).

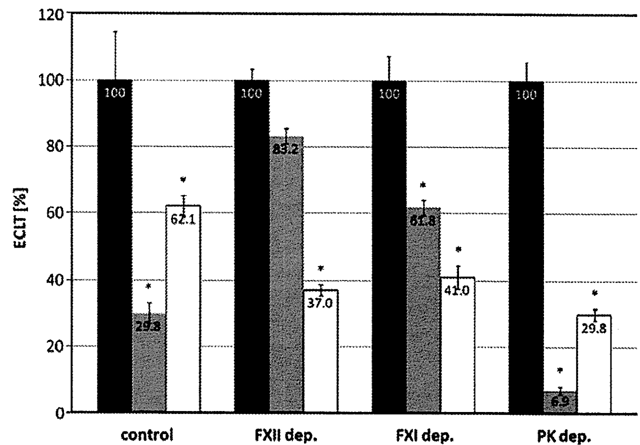

Fig. 2. Effects of supplemented Ca^{2+} and kaolin on the ECLT of plasma depleted of contact phase coagulation factors. Standard (■), kaolin- (□) or Ca^{2+}- (▩) ECLT were measured in FXII, FXI, and PK-depleted plasma. Every ECLT is expressed as a percentage of the mean of standard ECLT ($n=4$–6) and is shown as means ± standard deviation. *: Statistical difference was observed vs standard ECLT for each factor-depleted plasma ($P<0.05$).

Effects of aPC supplementation on ECLT

To evaluate possible roles of thrombin generated in the euglobulin fraction during clot lysis assay in kaolin-ECLT, the effect of aPC was analyzed. Each ECLT was expressed as a percentage of the mean standard ECLT without aPC. APC attenuated the shortening of ECLT by Ca^{2+} (86.3 ± 17.4%) (Fig. 1). In contrast, aPC did not affect the shortening of ECLT by kaolin (31.4 ± 2.1%), suggesting that thrombin generation was not required for this effect. The absence of thrombin generation during kaolin-ECLT was confirmed by Western blotting

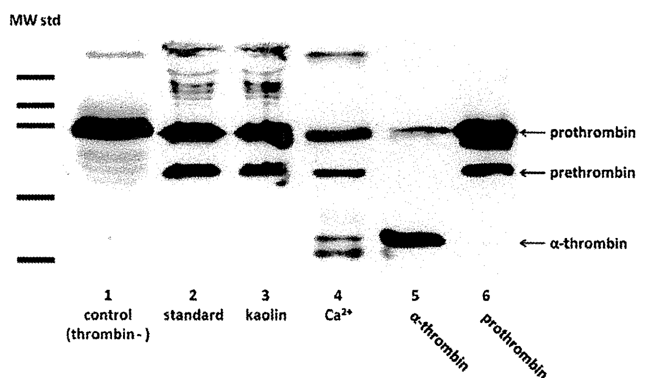

Fig. 3. Thrombin generation in the euglobulin fraction during the ECLT assay. Thrombin generation was analyzed by Western blotting employing an anti-prothrombin polyclonal antibody. Samples were obtained during the ECLT 15 min after clot formation, in the presence of kaolin or 10 mM Ca^{2+}. α-thrombin (Lane 5) and prothrombin (Lane 6) were used as positive controls. The conversion of prothrombin to thrombin was clearly demonstrated only in the presence of Ca^{2+} (Lane 4). Molecular weight standard indicates 201, 120, 100, 56 and 38 kDa, respectively.

(Fig. 3). When both Ca^{2+} and kaolin were added, aPC only partially but significantly attenuated the shortening of the ECLT.

Assessment of the role of PAI-1 in kaolin-dependent shortening of the ECLT

Two distinct facts, that ECLT is essentially regulated by the balance between tPA and PAI-1 (Urano et al. 1991) and that the inactivation of PAI-1 by generated thrombin is responsible for the shortening of ECLT by Ca^{2+} (Urano et al. 2003), prompted us to analyze the possible role of PAI-1 in the kaolin-dependent shortening of the ECLT. We employed biotin-labeled PAI-1, which was supplemented into the euglobulin fraction before forming a euglobulin clot. Thirty minutes after initiation of the ECLT assay, biotin-labeled recombinant PAI-1 was cleaved to lower weight molecular forms in the normal euglobulin fraction in the presence of Ca^{2+} (Fig. 4A), as reported previously (Urano et al. 2003). PAI-1 was similarly cleaved to a smaller molecular weight form in the presence of kaolin, and this was more pronounced in the presence of kaolin together with Ca^{2+}. The synchronized shortening of ECLT and cleavage of PAI-1 caused by kaolin suggest that PAI-1 cleavage into an inactive lower molecular weight form by proteases activated by kaolin plays a role in ECLT shortening.

In the euglobulin fraction prepared from FXII-depleted plasma, however, biotin-labeled PAI-1 was cleaved to lower weight molecular forms only in the presence of Ca^{2+} (Fig. 4B), but it was not cleaved in the presence of kaolin. These results suggest that FXIIa activated by kaolin is the protease responsible for both the shortening of ECLT and the PAI-1 cleavage. Cleavage of PAI-1 was observed when it was incubated with purified FXIIa for 1 h, whereas a high molecular weight complex was not clearly observed under these conditions (Fig. 5).

Further confirmation of the relevance of PAI-1 cleavage in kaolin-dependent shortening of the ECLT

To investigate the role of PAI-1 on shortening of the ECLT, PAI-1 depleted plasma was prepared by treating normal plasma with an anti-PAI-1 neutralizing antibody and was subjected to ECLT assay (Fig. 6). Not only standard ECLT, but kaolin- as well as Ca^{2+}-ECLT were shortened in a dose-dependent manner by addition of the anti-PAI-1 antibody, and the differences between the standard and other ECLTs were lessened. However, definite differences remained between the standard and the Ca^{2+}-ECLT, even after treatment by the highest concentration of antibody. These results suggest the following: First, all of these ECLT are under the control of PAI-1. Second, the Ca^{2+}-dependent shortening of the ECLT depends largely on the existence of PAI-1, thus thrombin-dependent inactivation of PAI-1 is essential for this shortening. Third, though FXIIa-dependent inactivation of PAI-1 plays a role in kaolin-dependent shortening of the ECLT, other mechanisms independent of PAI-1 are also involved.

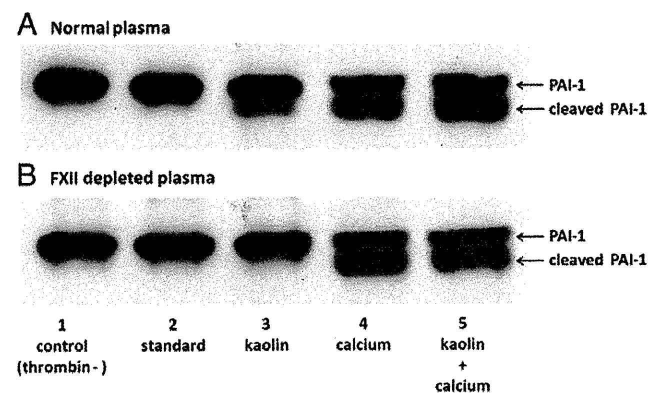

Fig. 4. Modification of biotin-labeled PAI-1 in the euglobulin fraction during the ECLT. The proteolytic modification of biotin-labeled PAI-1 was analyzed during the ECLT. The euglobulin fraction was supplemented with biotin-labeled PAI-1, and samples were obtained during the ECLT 30 min after clot formation, in the absence and the presence of kaolin, Ca^{2+} or both. The euglobulin fraction supplemented with biotin-labeled PAI-1 without thrombin treatment was employed as a positive control (Lane 1). In the normal plasma, lower molecular weight forms of biotin-labeled PAI-1 were observed in the presence of both Ca^{2+} and kaolin (A. Lanes 3 and 4). In the FXII-depleted plasma, however, biotin-labeled PAI-1 was cleaved to lower weight forms only in the presence of Ca^{2+} (B. Lanes 3 and 4).

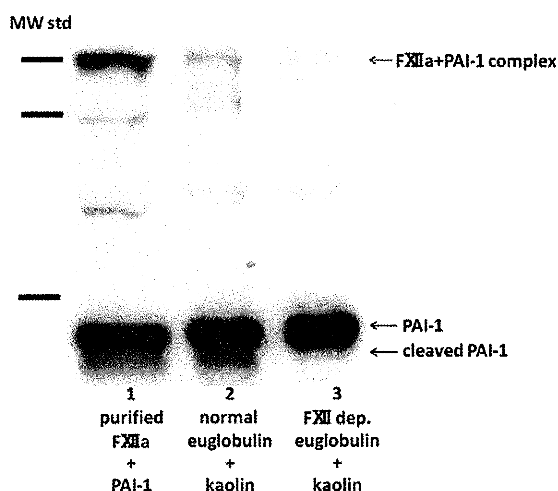

Fig. 5. The interaction between FXIIa and biotin-labeled PAI-1 in the euglobulin fraction during the ECLT. Using purified FXIIa, the interaction between biotin-labeled PAI-1 and FXIIa was analyzed by Western blotting. After 1 h incubation of purified FXIIa and biotin-labeled PAI-1, both the high molecular weight complex and the lower molecular weight cleaved form of PAI-1 were observed (Lane 1). In samples obtained during the kaolin-ECLT assay at 1 h after clot formation, both the cleaved form of biotin-PAI-1 and a small amount of the FXIIa/biotin-labeled PAI-1 complex were observed in the euglobulin prepared from control plasma (lane 2), but not in that from FXII-depleted plasma (lane 3). Molecular weight standard indicates 56, 38 and 30 kDa, respectively.

Possible contribution of HKa in the shortening of the ECLT by kaolin

It has been reported that HKa interferes with the binding of PAI-1 to vitronectin and that it suppresses both stability and activity of PAI-1 (Chavakis et al. 2002). To study the possible involvement of such mechanisms in shortening of the ECLT by supplemented kaolin, we analyzed the generation of HKa during kaolin-ECLT. The euglobulin fractions prepared from both control and PK-depleted plasma were

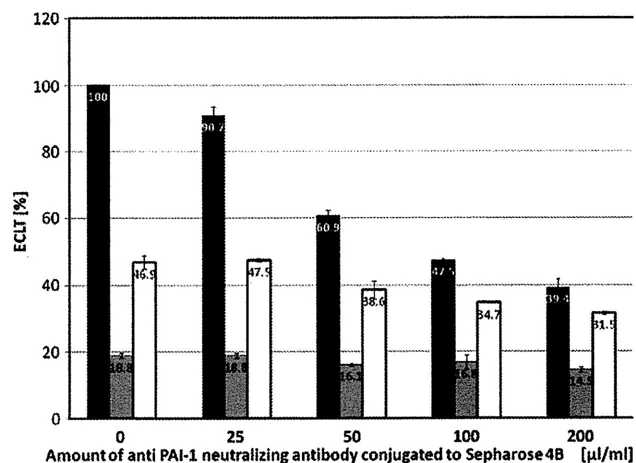

Fig. 6. The effect of PAI-1 depletion on the shortening of the ECLT. The euglobulin fraction was pretreated with increasing amounts of anti-PAI-1 neutralizing antibody conjugated to Sepharose 4B (0, 25, 50, 100, and 200 μl/ml), and standard- (■), kaolin- (□)or Ca^{2+}- (▒) ECLT were measured. Every ECLT is expressed as a percentage of the mean standard ECLT ($n = 2$) and is shown as mean ± standard deviation.

incubated with either kaolin or the corresponding volume of distilled water at 37 °C for 30 min, and the mixture was subjected to SDS-PAGE followed by Western blotting using anti-human kininogen (Fig. 7). In the euglobulin fraction of control plasma incubated with distilled water, only the high molecular weight kininogen band (approximately 120 kDa) was recognized. When the incubation was carried out with kaolin, lower molecular weight bands were detected, suggesting the release of bradykinin. In the case of PK-depleted plasma, only a small fraction of lower molecular weight bands were detected even after incubation with kaolin, suggesting that PK is largely responsible for the generation of HKa. Kaolin shortened the ECLT of PK-depleted plasma, making it similar to the ECLT obtained for normal plasma. This attenuated generation of HKa in PK-depleted plasma suggests that HKa generation is not a significant factor in shortening of the ECLT by kaolin.

Effects of TF and FVIIa on the ECLT

To evaluate the possible involvement of the extrinsic coagulation factors in enhancing fibrinolysis, the effects of TF/FVIIa on the ECLT were analyzed. We added either TF alone, FVIIa alone, or a combination of these factors to the euglobulin fraction and measured the clot lysis times (Fig. 8). The ECLT was shortened by TF ($59.4 \pm 7.4\%$), and was further shortened by supplementation with FVIIa ($50.3 \pm 3.8\%$). Addition of only FVIIa did not shorten the ECLT ($107.5 \pm 4.8\%$). Shortening of the ECLT by TF alone or in combination with FVIIa was reversed by aPC ($90.9 \pm 7.2\%$, $82.3 \pm 3.4\%$, respectively), suggesting that shortening of the ECLT by either TF alone or TF/FVIIa was dependent on thrombin generation, and that neutralization of PAI-1 by TF/FVIIa complex was not a significant factor in this process.

Discussion

Contact factors activated by kaolin were found to enhance fibrinolysis by a mechanism that did not involve thrombin generation. Inactivation of PAI-1 by activated contact factors, notably FXIIa, appeared to be at least partially involved in this enhancement.

ECLT, a classical global fibrinolytic assay, is determined by the balance between tPA and PAI-1; it is thus an appropriate assay to analyze mechanisms modifying the initial phase of fibrinolysis. Physiological concentrations of Ca^{2+} drastically shortened the ECLT and this effect was attenuated by aPC in a dose-dependent manner. Inactivation of PAI-1 by thrombin generated in the euglobulin fraction

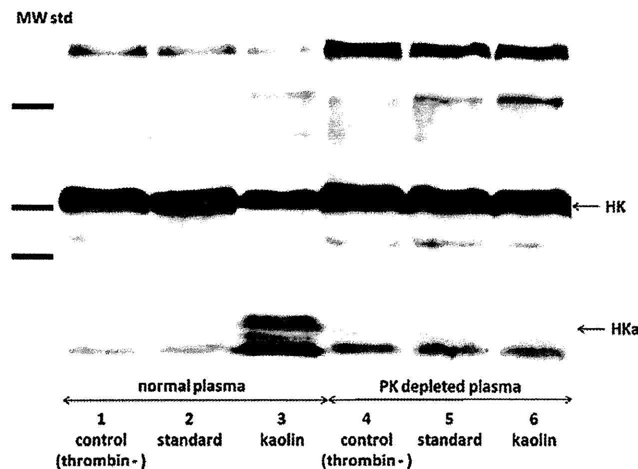

Fig. 7. Generation of HKa in the euglobulin fraction during the ECLT. Cleavage of HK was analyzed by Western blotting. Samples were obtained during ECLT 15 min after clot formation of either normal or PK-depleted plasma, either in the presence or absence of kaolin. In the normal plasma HKa was clearly observed only in the presence of kaolin (Lane 3), while it was much less apparent in the PK-depleted plasma (Lanes 5 and 6). Molecular weight standard indicates 201, 120, and 100 kDa, respectively.

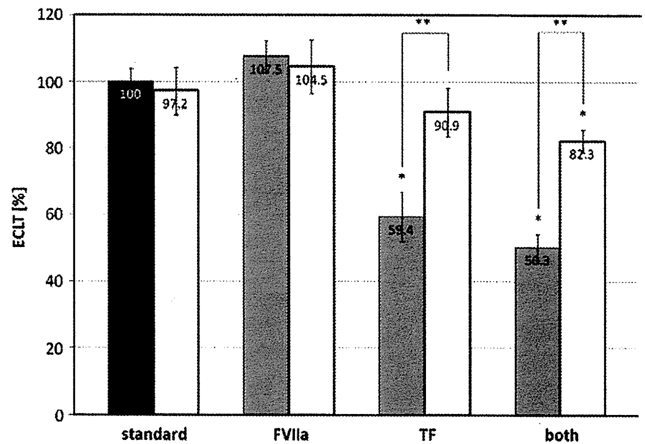

Fig. 8. Effects of FVIIa and TF on ECLT both in the presence and absence of aPC. Regular ECLT, FVIIa-, TF-, and FVIIa/TF- supplemented ECLT were measured in the absence (lt column) and the presence (rt column) of 80 nM aPC. Each ECLT is expressed as a percentage of the mean standard ECLT ($n=8$) and is shown as mean ± standard deviation. *: Statistically significantly different vs standard ECLT in the absence of aPC ($P<0.01$). **: Statistical difference was observed in TF- and FVIIa/TF- ECLT in the absence vs presence of aPC ($P<0.01$).

appeared to be the primary mechanism. We proposed that this is one mechanism of coagulation-associated enhancement of fibrinolysis.

Kaolin was found to shorten the ECLT. Such marked enhancement of fibrinolytic activity after exposure of plasma to negatively charged surfaces is known as intrinsic fibrinolysis, in which activation of contact phase factors is essential (Klüft et al. 1987). Kaolin was more effective than Ca^{2+} in shortening the ECLT. An important difference between kaolin-dependent and Ca^{2+}-dependent shortening of the ECLT is that the former is not dependent on thrombin generation. Absence of thrombin generation in the kaolin-supplemented euglobulin fraction was confirmed by Western blotting. Furthermore, the facts that aPC did not affect the ECLT shortening by kaolin whereas it quenched that by Ca^{2+} suggest that the thrombin generation is not involved in the former process.

Berrettini et al. (1989) reported that each of the contact system enzymes formed high molecular weight complexes with PAI-1 that resulted in reciprocal inhibition of PAI-1 and the proteases. They also speculated that the neutralization of PAI-1 activity by FXIa may represent a relevant mechanism for the enhancement of clot lysis during contact activation of plasma. In the present study, we found that supplemented biotin-PAI-1 formed high molecular weight complexes and was cleaved into smaller fragments by limited proteolysis during kaolin-ECLT, but not during a standard ECLT.

To identify the factor(s) responsible for ECLT shortening and inactivation of PAI-1, we employed plasma depleted of FXII, FXI and PK to analyze the effect of kaolin on their ECLT. The ECLT shortening by kaolin was more pronounced for FXII-depleted plasma than for FXI-depleted plasma. Surprisingly, kaolin shortened the ECLT of PK-depleted plasma more significantly than control plasma. These results suggest that FXII was more important than PK in the kaolin-enhanced shortening of the ECLT.

FXIIa was reported to inhibit PAI-1 activity by forming a high molecular weight complex and by proteolytic degradation of PAI-1 (Berrettini et al. 1989). We observed degradation products of biotin-PAI-1 as well as a small amount of FXIIa-PAI-1 complex in the kaolin-supplemented euglobulin fraction of control plasma and in a reconstituted system of purified factors. These products were not recognized in the euglobulin fraction of normal plasma in the absence of kaolin or in the kaolin-supplemented euglobulin fraction of FXII-depleted plasma. Thus FXIIa appeared to be a candidate to inactivate PAI-1 and to shorten the ECLT in the presence of kaolin.

Contact factors may suppress PAI-1 activity by another mechanism involving dissociation of the complex formed by VN and PAI-1. PAI-1 binds

to VN in both plasma and matrices, thereby stabilizing the conformation and preserving the function of active PAI-1 (Lawrence et al. 1997). HK, especially HKa generated mainly by kallikrein-dependent limited proteolysis, was reported to compete the binding of PAI-1 to VN (Nishikawa et al. 1992) and to suppress the stabilizing effect of VN. To address the possibility that this mechanism is involved in the kaolin-dependent ECLT shortening, we looked for a possible cleavage of HK to form HKa during kaolin-ECLT. HKa was generated in the euglobulin fraction of control plasma only in the presence of kaolin. Importantly, much less HKa was generated in kaolin-supplemented euglobulin of PK-depleted plasma, although the magnitude of the ECLT shortening by kaolin was larger than for control plasma. These findings suggest that HKa-dependent suppression of PAI-1 activity is not significant in kaolin-dependent shortening of the ECLT.

To confirm how deeply the tPA-PAI-1 system is involved in ECLT, and how deeply PAI-1 inactivation is involved in the shortening of ECLT either by thrombin generated in Ca^{2+}-ECLT or by FXIIa in kaolin-ECLT, PAI-1 was depleted from plasma using an anti-PAI-1 neutralizing antibody. This antibody dose-dependently shortened not only the standard ECLT, but also both the Ca^{2+}- and the kaolin-ECLT, suggesting that PAI-1 regulated the ECLT in all three conditions. Furthermore, the difference between standard-, Ca^{2+}-, and kaolin-ECLT became smaller, suggesting that inactivation of PAI-1 by either thrombin or FXIIa plays a less important role at lower levels of active PAI-1. These results suggest again that PAI-1 is an important regulator of fibrinolytic system initiation and that inactivation of PAI-1 by activated coagulation factors is important in enhancing fibrinolysis. The amount of cleaved PAI-1 products in the presence of kaolin, however, was lower than that produced with Ca^{2+} supplementation, even though the ECLT was shortened more dramatically with kaolin supplementation. This suggests that additional mechanisms to enhance fibrinolysis may be involved in kaolin-dependent enhancement of the ECLT. The activation of single chain urokinase by activated contact factors may contribute to this effect.

To evaluate the possible effects of extrinsic coagulation pathway initiation factors on the ECLT, we analyzed the effects of TF, FVIIa and TF/FVIIa on ECLT assays. The ECLT was shortened by both TF and TF/FVIIa, and this effect was attenuated by aPC, suggesting that the shortening was dependent on thrombin generation. TF and TF/FVIIa seem to have generated a small amount of thrombin even in the absence of Ca^{2+}, resulting in the enhancement of fibrinolysis by PAI-1 inactivation. Thus the enhancement of fibrinolysis by contact factors, which is independent of thrombin generation, appeared to be an additional unique pathway for coagulation-dependent enhancement of fibrinolysis.

Recently, FXIIa is reported to suppress fibrinolysis by enhancing the activation of both thrombin activatable fibrinolysis inhibitor (TAFI) and FXIII as a result of the enhanced generation of thrombin (Nielsen et al. 2006). Since both the removal of C-terminal lysine by activated TAFI of partially degraded fibrin to which plasminogen binds and the cross linkage of a2-antiplasmin to fibrin by activated factor XIIIa are physiologically important inhibitory pathways of fibrinolysis, the inhibition of fibrinolysis by FXIIa through these mechanisms may have either physiological- or pathological-meanings (Rijken and Lijnen 2009). However, these results were obtained in the tPA-induced plasma clot lysis assay using thromboelastography, in which large amounts of tPA is supplemented to overwhelm PAI-1 activity in plasma. Thus profibrinolytic ability of FXIIa which appears through PAI-1 inactivation as is shown in the present study, seems difficult to emerge and rather masked in such an assay method. FXIIa, therefore, may play both inhibitory- and enhancing-roles in fibrinolysis under different conditions *in-vivo*.

Conclusion

The inactivation of PAI-1 by FXIIa was found to be responsible at least in part for the enhancement of fibrinolysis by activated contact factors *in-vitro*.

Acknowledgments

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A case of intraocular yolk sac tumor in a child and its pathogenesis

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While yolk sac tumor is one of the most common malignant germ cell tumors occurring in young children, it is rarely found in extragonadal sites. We report a case of intraocular yolk sac tumor in a 4-year-old boy. The diagnosis was confirmed by histologic examination and by the rapid normalization of serum α-fetoprotein level following enucleation. We propose that yolk sac cells can potentially migrate into the eye at 22 days of embryonic life during neural tube formation, when the head and tail of the neuropore open contemporaneously and communicate with the amniotic cavity.

Yolk sac tumor, if not treated aggressively, has a poor prognosis.[1] Most frequently occurring in the testis of young boys,[2] they have also been reported in the liver, mediastinum, the third ventricle, and pineal gland.[3] There are several reports of yolk sac tumor in the orbit.[4-8] Yolk sac tumors are characteristically accompanied by an elevation of the serum α-fetoprotein level.

Case Report

A 4-year-old boy visited a nearby hospital in July 2007 complaining of decreased vision in the right eye; optic neuritis was diagnosed. Steroid pulse therapy was used but proved ineffective. He was seen at another hospital, where Coats disease was diagnosed. During this period, his visual acuity worsened.

He presented to our clinic 5 months later. Upon examination, his visual acuity was no light perception in the right eye and 20/20 in the left eye. A yellowish white mass with retinal detachment was found in the right eye. Computerized tomography scan showed a large tumor in the right eye with no evidence of calcification (Figure 1). Serous tumor markers were examined and an excessively high level of serum α-fetoprotein, 4,700 ng/mL (normal, 0-10 ng/mL) was detected. Other markers, such as neuron-specific enolase, ferritin, and carcinoembryonic antigen were found at normal levels. Whole body computerized tomography scan revealed no evidence of neuroblastoma or other malignant tumors. Retinoblastoma was considered as a possible diagnosis, and the patient was treated by 2 cycles of chemotherapy consisting of carboplatin, vincristine, cyclophosphamide, and pirarubicine. After treatment, the tumor size was significantly reduced, and serum α-fetoprotein decreased to 102 ng/mL on February 27. In light of these results, the tumor in the eye was considered to be an α-fetoprotein-producing malignant tumor. The right eye was assessed to have no prospect of light perception and it was enucleated (Figure 2). After the enucleation, the patient's serum α-fetoprotein level decreased to normal within 2 weeks. Histologic examination of the eye showed a posterior segment tumor with tumor cells infiltrating mainly the retina and the choroid. Some tumor cells were also found scattered in the optic disk. The tumor itself had lumina lined with flat-to-cuboidal epithelial cells, and liver-like cells were present (Figure 3). α-Fetoprotein was clearly identified by immunohistochemistry (Figure 4). From these findings, we concluded that the diagnosis was most consistent with yolk sac tumor in the eye. One and a half years after the histologic diagnosis, his general condition has been good; serum α-fetoprotein is 0 ng/mL, and no other tumors have been found.

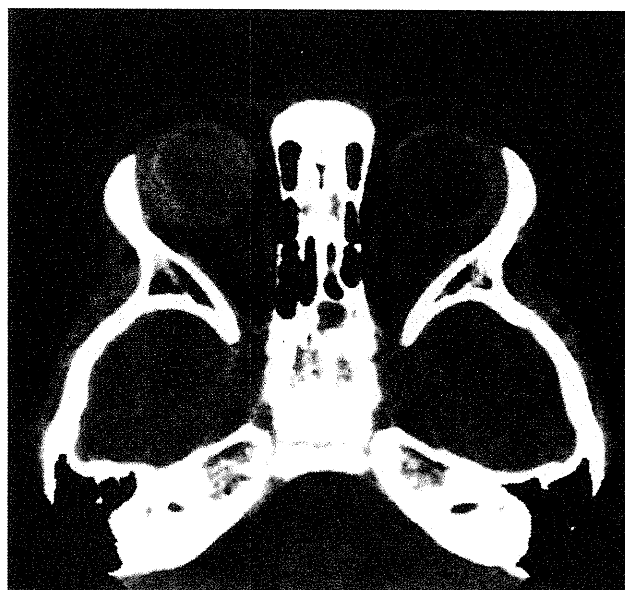

FIG 1. CT scan before chemotherapy showed a large tumor in the right eye with no evidence of calcification.

Discussion

In this extremely rare case, the diagnosis was confirmed by the histologic examination and the rapid normalization of

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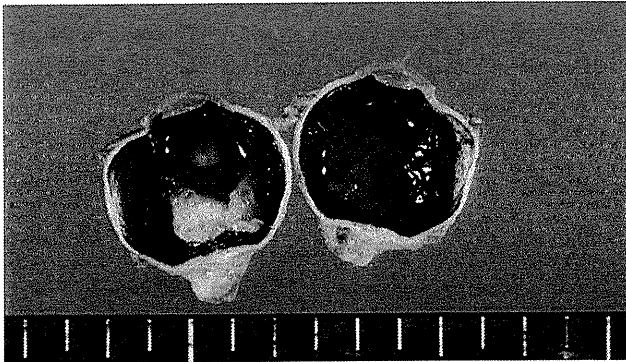
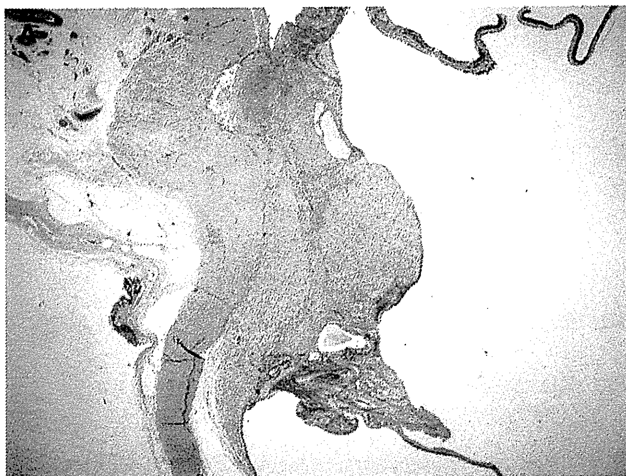


FIG 2. Bisected enucleated eye showing posterior segment tumor.



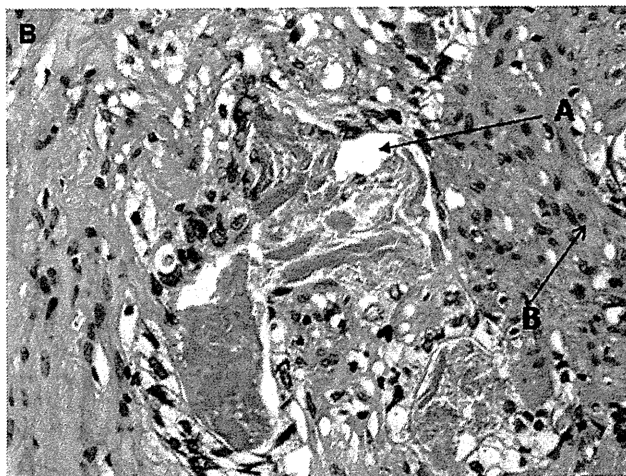


FIG 3. A, Tumor mass at the posterior area of the eyeball (hematoxylin-eosin, ×40). Tumor cells were found infiltrating mainly in the retina and the choroid. Some tumor cells were also found scattered in the optic disk. B, Lumina lined with flat-to-cuboidal epithelial cells (arrow, A) were seen; liver-resembling cells (arrow, B) were present (hematoxylin-eosin, ×400).

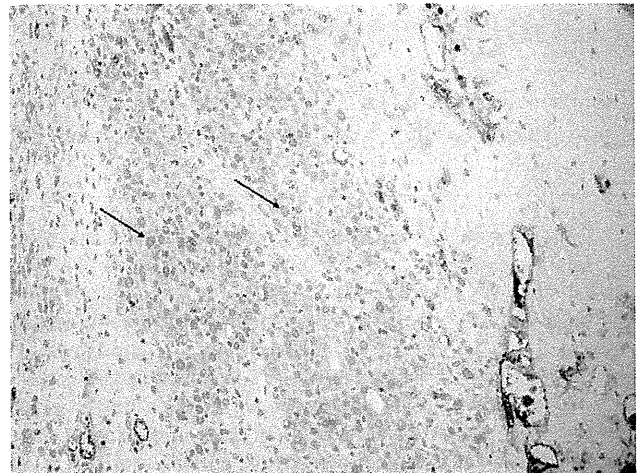


FIG 4. Histochemical stain for α-fetoprotein was positive (arrows) in the tumor after chemotherapy (×200).

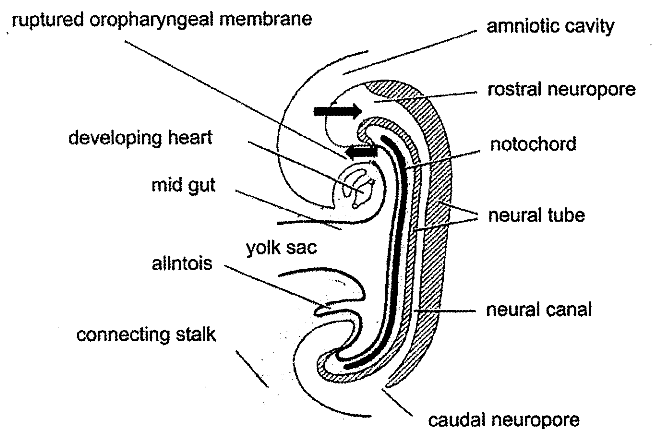


FIG 5. Schematic showing how germ cells can migrate incorrectly from the amniotic cavity to the rostral neuropore.

serum α-fetoprotein level. The characteristics of intraocular yolk sac tumor included rapid progression of the tumor size, yellowish colored tumor, no calcification within the tumor, liver-like cells in histology, and a high level of serum α-fetoprotein.[6]

There have been several reports of the tumor in the orbit.[4-8] Ours appears to be the second reported case of intraocular yolk sac tumor.[9] These reports show proptosis is a main initial finding, along with elevated serum α-fetoprotein levels. One patient, who died 10 months after proptosis was discovered, developed massive intracranial extension and metastasis to the calvarium and lung despite therapy.[5] Other patients of orbital yolk sac tumor treated with chemotherapy, radiotherapy, and surgery have survived.[4,5]

In our case rapid growth of the yolk sac tumor suggested malignancy, and retinoblastoma seemed to be the most likely diagnosis. Aggressive treatment for a malignant tumor at the first stage may have contributed to the good prognosis.