Evaluation of a New Blood Coagulation Factor Xa Inhibitor, KFA-1411, as an Anticoagulant in a Cynomolgus Monkey Hemodialysis Model: Parameters Suitable for Monitoring Anticoagulant Activities

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A Cynomolgus monkey hemodialysis model was used to evaluate the efficacy of a new factor Xa (FXa) inhibitor as an anticoagulant for hemodialysis. We tested the selective FXa inhibitor KFA-1411, using doses of 0.15 mg/kg/hr, 0.3 mg/kg/hr, and 0.6 mg/kg/hr by continuous infusion in combination with a single loading-dose at the start. As a reference control, dalteparin, which is a low-molecular-weight heparin, was used at a dose of 20 IU/kg/hr plus 30 IU/kg single loading. As a monitoring method for KFA-1411, clotting time was measured in blood samples obtained from the body (systemic) and from the extracorporeal circulation (in-circuit). At KFA-1411 doses of 0.3 mg/kg/hr and 0.6 mg/kg/hr hemodialysis was maintained with essentially no change in the intra-bloodline pressure in the extracorporeal circulation. With dalteparin, the pressure increased almost to the upper limit (set at 250 mmHg). The clot-deposition score measured, after testoration of normal circulation, in the dialyzer and air-trap chamber in the extracorporeal circulation was improved in a dose–dependent manner in the KFA-1411 groups. There were no large differences between humans and Cynomolgus monkeys in the *in vitro* plasma anticoagulant activities of KFA-1411 and dalteparin. These results indicate that this selective FXa inhibitor could be used as an anticoagulant for hemodialysis. Both activated partial thromboplastin time (APTT) and prothrombin time (PT) can be used to monitor the anticoagulant activity in the extracorporeal circulation, and a drug dose that has sufficient anticoagulant efficacy for hemodialysis will prolong in-circuit-APTT about 2 times and in-circuit-PT about 3 times compared to normal.

Key words —— factor Xa inhibitor, hemodialysis, coagulation, Cynomolgus monkey, platelet, thrombin,

INTRODUCTION

Heparin has been widely used as an anticoagulant in hemodialysis, and its action in the coagulation cascade involves not only thrombin, but also the clotting factors factor Xa (FXa), FIXa, FXIa, and FXIIa.[1)] Heparin exerts its anticoagulant effect *via* activation of antithrombin III (AT III), and has, but its use leads to same problems (increased bleeding tendency, effects on platelets, and effects on lipid metabolism).[2–4)] To address these problems, low-molecular-weight heparin (LMWH), made by artificial decomposition of standard heparin, is now being used clinically.[5)] In contrast to standard heparin, LMWH has selectivity against FXa, decreased platelet effects, and reduced bleeding risk.[6,7)] The anticoagulant effect of LMWH is also mediated through AT III, and since its basic mechanism of action is the same as that of standard heparin, and the platelet problems associated with AT III-dependent anticoagulants are not completely resolved.[8)] Hence, a selective and AT III-independent FXa inhibitor would be expected to be preferable to LMWH as far as these problems are concerned. We have recently developed a new FXa-selective inhibitor, KFA-1411, (3-[*N*-(3-amidinophenyl)-*N*-[*N*-[4-[1-(1-iminoethyl)

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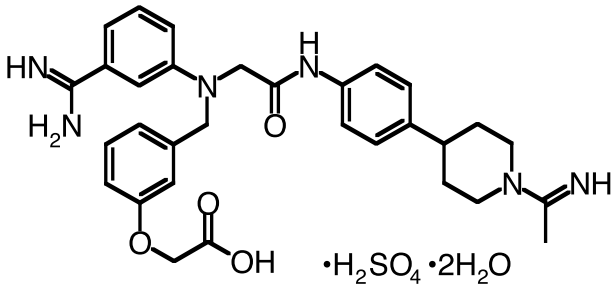

Fig. 1. Structural Formula of KFA-1411

piperidin-4-yl]phenyl]carbamoylmethyl]aminomethyl] phenoxy acetic acid monosulfonate dihydrate) (Fig. 1).

In the present study, we evaluated whether KFA-1411 can achieve anticoagulant effects in hemodialysis equivalent to those of LMWH. In general, to monitor the anticoagulant activity of standard heparin during hemodialysis, whole-blood-clotting or activated partial thromboplastin time (APTT) are measured. On the other hand, no parameter is available for monitoring LMWH except anti-FXa activity. We measured various blood parameters during use of an extracorporeal circulation, and determined the conditions necessary for KFA-1411 to be effective as the hemodialysis anticoagulant in the extracorporeal circulation. Other FXa inhibitors have been reported to be species-dependent in their inhibitory effects.[9] In the present study, to look for evidence of a species difference in KFA-1411 activity, we examinened inhibition of Russell's Viper Venom X-activated FX (RVV-X-activated FX), instead of FXa, and assayed anti-coagulant activities both *in vitro* in human plasma and in monkey plasma.

MATERIALS AND METHODS

Reagents —— KFA-1411 was synthesized in the laboratories of Kissei Pharmaceutical Co. Ltd. (Nagano, Japan). Dalteparin sodium (Fragmin injection®) a LMWH manufactured by Pharmacia K.K. (Tokyo, Japan), was used. Snake venom (RVV-X) was purchased from Enzyme Research Laboratory Inc. (IN, U.S.A.), frozen human normal blood plasma from George King Bio-Medical Inc. (KS, U.S.A.), and frozen Cynomolgus monkey blood plasma, which was sampled as 3.13% citrate blood, from SLC. (Hamamatsu, Japan). Purified human FXa was purchased from Calbiochem (CA, U.S.A.), and the synthetic substrates for FXa, S-2222® and Test-them heparin-S®, from CHROMOGENIX AB. (Mölndal, Sweden). Neoplastin Plus®, for prothrombin time (PT) measurements, and PTT reagent®, for APTT measurements, were purchased from Roche Diagnostics (Tokyo, Japan). Human AT III was purchased from SIGMA Chemical Co. (MO, U.S.A.).

Cynomolgus Monkey Hemodialysis Model ——

Animals: Male Cynomolgus monkeys weighing over 4 kg were purchased from Hamry Co. Ltd. (Ibaragi, Japan). Animals were chosen for this study only if their APTT was within the normal range (17.8 sec to 20.4 sec). Before the experiments, animal conditioning was carried out for 1 week. The protocol for this study was approved by the Laboratory Animal Committee of Kissei Pharmaceutical Co. Ltd. (Nagano, Japan). Animal experiments followed the ethical standards formulated in the guidelines issued by the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture, No.141, 1987: "Animal Experiments in Universities *etc*."

Preparation, Dose, and Administration of Study Drugs: KFA-1411 was dissolved in physiological saline to the appropriate concentration, then filtered through a membrane filter (0.45 μm) for sterilization. Dalteparin form ampoules (1000 IU/ml) was diluted with physiological saline. Drugs were prepared so that the amount administered was ml/kg. Drugs were administered by two methods: single loading at the start and continuous infusion during the use of the extracorporeal circulation. The KFA-1411 doses were 0.15 mg/kg loading + 0.15 mg/kg/hr, 0.3 mg/kg loading + 0.3 mg/kg/hr, and 0.6 mg/kg loading + 0.6 mg/kg/hr. The dalteparin dose was 30 IU/kg loading at the start + 20 IU/kg/hr, which corresponds to the clinical dose. For each dose, 3 tests were carried out.

Hemodialysis Study: Cynomolgus monkeys were anesthetized by administration of 25 mg/kg of pentobarbital sodium solution (Tokyo Kasei Kogyo, Tokyo, Japan) into the saphenous vein, set in the recumbent position, and then given additional anesthesia (1–5 ml of Nembutal sodium solution; Abbott Laboratories, IL, U.S.A.) during the examination. For blood access, 16G and 14G indwelling catheters (Terumo, Tokyo, Japan) were placed into the femoral artery and vein. The extracorporeal blood circulation was set up using a blood line S-5® and dialyzer MCA-0.05® for animals (Senkou-Ika Kogyo, Tokyo, Japan) attached to hemodialysis equipment

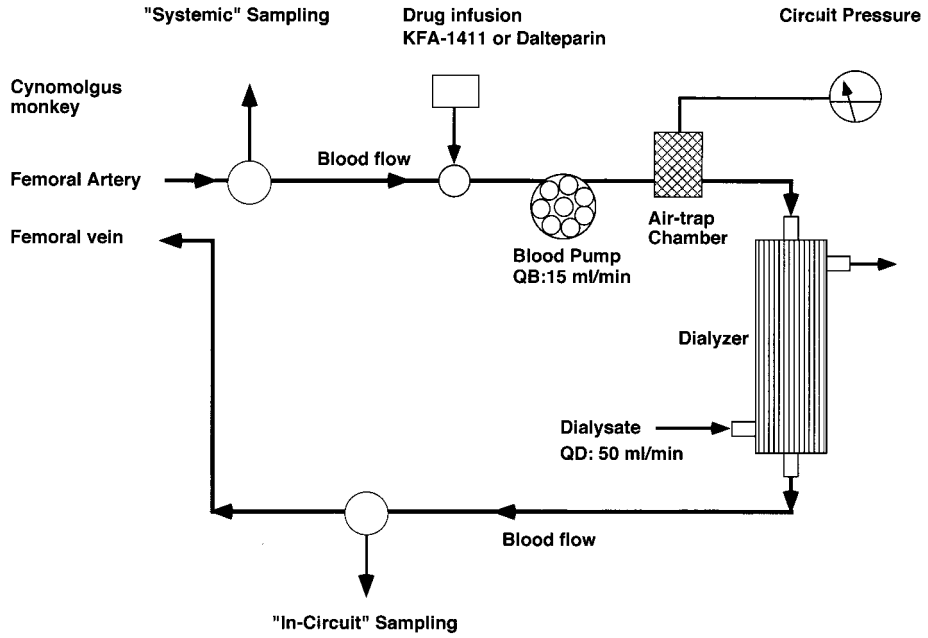

Fig. 2. The Extracorporeal Circulation Model of in Cynomolgus Monkeys

(DBB-22®; Nikkso, Tokyo, Japan), and the in-line fluid was circulated using a small peristaltic circulation pump (ATTO, Tokyo, Japan) (Fig. 2). Prior to use, the line was flushed with 200–500 ml of physiological saline, then washed and filled (about 28 ml) with about 300 ml of 1 mg/100 ml KFA-1411 or 2 IU/ml dalteparin in physiological saline as the priming solution. Immediately prior to the initiation of the extracorporeal circulation, a single drug dose was injected into the venous line *via* a blood sampling port, and simultaneously the 4-hr constant-rate maintenance infusion was begun *via* the arterial-side drug-injection port. The extracorporeal blood circulation (15 ml/min) was begun using bicarbonate-buffered dialysate (50 ml/min) (Kindary solution AF-2®; Fuso, Osaka, Japan). The line-pressure in the hemodialysis equipment was monitored from the air-trap chamber located on the arterial-side. The extracorporeal circulation was continued for 4 hr; then, the animal remained anesthetized for further 3 hr so that more blood samples could be collected.

Pressure in Extracorporeal Circuit and Extent of Clot-Deposition: Arterial line-pressure was measured at the air-trap chamber using the pressure monitor on the dialysis equipment at the initiation of the extracorporeal circulation, and at 15 min, 30 min, 1 hr, 2 hr, and 4 hr thereafter. Clot-deposition in the dialyzer and air-trap chamber was evaluated separately (after the termination of the extracorporeal circulation and washing the circuit with 500 ml of physiological saline) using the visual scoring system of Suzuki.[10)]

The dialyzer clot-deposition was graded as follows: 0, no clot-deposition (0%); 1, clot-deposition on a few fibers or scattered (less than 5%); 2, < 10%; 3, < 25%; 4, < 50%; 5, (≥ 50%), and 6, maintaining the extracorporeal circulation was difficult due to clot-deposition in the fibers.

The air-trap chamber clot-deposition was graded as follows: 0, little or none; 1, very slight; 2, slight; 3, moderate; and 4, severe.

Measurement of Blood Parameters: During the use of the extracorporeal circulation, blood samples were collected from 2 blood access-points: from bloodline immediately after passage through the dialyzer (which reflected the "in-circuit" conditions) and immediately after exit from the body (which reflected "systemic" conditions) (Fig. 2). Blood samples were also collected from blood access-points in the femoral artery and vein prior to the initiation of the extracorporeal circulation, and from the femoral vein after its termination. For hematologic studies, part of the blood sample (0.3 ml) was mixed with EDTA, and the platelet count was measured prior to the initiation of the extracorporeal circulation, and at 30 min, 1 hr, 2 hr, and 4 hr thereafter using an automated hematologic analyzer (K-4500®; Sysmex, Kobe, Japan). For coagulation studies, plasma was obtained by centrifuging blood in 3.13% citrate (4°C, 3000 rpm, 10 min) using blood

samples collected prior to the initiation of the extracorporeal circulation and at 15 min, 30 min, 1 hr, 2 hr, and 4 hr thereafter. APTT and PT in the collected plasma were measured using a clotting-meter KC4A® (Baxter, Tokyo, Japan). The concentration of anti-FXa substance in plasma, which reflects the concentration of KFA-1411, was measured using part of each sample for a clotting-time assay (after storage at –80°C). The concentration of anti-FXa substance in plasma samples was calculated from the inhibition of human FXa activity with the aid of a KFA-1411 FXa inhibition calibration curve and the modified method of Hara.[11)]

The reaction mixture was incubated at the room temperature for 10 min on a 96-well plate continaing, 2.5 μl of plasma sample or reference plasma, 0.027 units purified human FXa, 0.2 mM S-2222, 20 mM NaCl, and 40 mM tris(hydroxymethyl) aminomethane/HCl buffer (pH 8.4) (Tris/HCl buffer). The reaction was stopped by the addition of the 50 μl of 60% acetic acid, and the absorbance at 405 nm was measured using a plate reader (Spectra MAX 250®; Molecular Devices, CA, U.S.A.).

The concentration of dalteparin was assayed using a commercial heparin-assay kit (Test-them heparin-S®) using a reference curve for dalteparin.

***In Vitro* Measurements for Species Differences** ——

Measurement of Inhibitory Activity Against RVV-X-Activated FX: Endogenous FX in 40 μl of human or Cynomolgus monkey plasma was activated using 2.5 μg/ml of RVV-X, then allowed to react in a tube containing the test drug, 0.2 mM S-2222, 50 mM $CaCl_2$, 50 mM NaCl, and 100 mM Tris/HCl buffer (pH 8.4) for 15 min (human plasma) or 25 min (Cynomolgus monkey plasma). The reaction was stopped by the addition of 100 μl of 60% acetic acid, and the supernatant from the reaction mixture was isolated by centrifugation. The absorption of the product at 405 nm was then measured. The reference activity was that observed when vehicle alone was added to the reaction mixture, and the concentration of the drug that inhibited 50% of the activity of the reference (IC_{50}) was calculated from the concentration-inhibition curve.

Measurement of Anticoagulant Activity: PT and APTT were measured in normal human and Cynomolgus monkey plasmas using a clotting-meter. For APTT measurement, 75 times the final concentration of drug was prepared in physiological saline, and presented in an assay cuvette with plasma and PTT reagent added. After pre-incubation for 60 sec, the measurement started with the addition of 25 mM $CaCl_2$. For PT measurement, 75 times the final concentration of drug was prepared in physiological saline, the presented in an assay cuvette with plasma, and the measurement started with the addition of PT reagent. The anticoagulant activities of the test drugs (CT_2) were expressed as the concentration necessary to double the clotting time of the reference (in which only vehicle was added), as calculated from the concentration-clotting time prolongation curve.

Statistical Analysis —— The results are expressed as mean ± S.E. If an intra-group comparison by means of an analysis of variance of quantitative data over time showed a significant difference at 5% level, the relevant *t*-test was conducted (based on the pre-circulation value as the reference). In the case of inter-group comparisons of temporal and non-temporal quantitative data, if the analysis of variance was significant at 5% level, Dunnett's multiple-comparison test was conducted using the dalteparin group as the reference. Qualitative data was tested using the non-parametric Dunnett's multivariate method, with the dalteparin group as the reference. Results were considered significant if $p < 0.05$.

RESULTS

Cynomolgus Monkey Hemodialysis Model

Effect on Pressure in Extracorporeal Circuit: A basic requirement of hemodialysis is that blood flow be maintained without clotting in the blood line and/or the dialyzer, and generally the line-pressure is used to monitor the clotting status. In the present animal model of hemodialysis, use of the extracorporeal circulation without anticoagulants resulted in clotting in the circuit after about 20 min. As a consequence, the line-pressure reached the upper limit of 250 mmHg, and blood flow in the circuit could no longer be maintained (data not shown). Figure 3 shows the line-pressure changes observed in this study. All three doses of KFA-1411 permitted maintenance of the circulation for 4 hr, and at 0.3 mg/kg/hr and 0.6 mg/kg/hr there was no increase in the line-pressure. A line-pressure increase was seen in the 0.15 mg/kg KFA-1411 group, but did not reach significance compared with the value at the start. These results indicate that KFA-1411 at 0.3 mg/kg/hr or greater was sufficient to maintain blood flow in the present model. In the dalteparin group, maintenance

of the circulation was possible, but there was a significant increase in line-pressure peaking at 1 hr after the start ($p < 0.01$). Thus, a dose of dalteparin the same as that in clinical use in humans showed a low efficacy as an anticoagulant under this conditions.

Effect on Clot-Deposition in Dialyzer and Air-Trap Chamber: The presence of clot-deposits in the circuit or in the dialyzer after the completion of hemodialysis is an indication of insufficient circuit anticoagulation, and is not clinically desirable. In this study, the extent of clot-deposition was expressed by means of a numerical scoring system to en able us to evaluate the two anticoagulants (Table 1). The extent of clot-deposition in the dialyzer decreased in the KFA-1411 groups in a dose–dependent manner. Clot-deposition in the air-trap chamber also decreased in a dose–dependent manner in the KFA-1411 groups. Clot-deposition in the dialyzer in the dalteparin group was similar to that seen with KFA-1411 at 0.3 mg/kg/hr. On the other hand, all the animals in the dalteparin group showed the maximum clot-deposition score of "4" in the air-trap chamber, with large clot-mass formation. This value was significantly different from that obtained for the 0.6 mg/kg/hr KFA-1411 group ($p < 0.05$). These results also indicated that dalteparin 20 IU/kg/hr was ineffective as an anticoagulant in this animal model. KFA-1411 at doses of 0.3 mg/kg/hr and 0.6 mg/kg/hr tended to show greater efficacy with respect to clot-deposition than the dalteparin group.

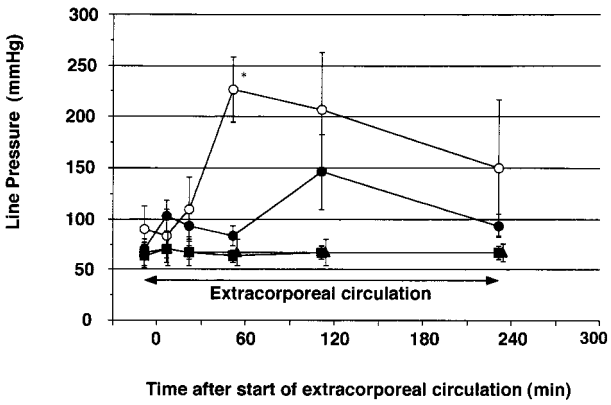

Fig. 3. Effects of KFA-1411 and Dalteparin on Circuit Line-Pressure in Cynomolgus Monkey Hemodialysis Model

The circuit line-pressure on the arterial side of the extracorporeal circulation was measured using the pressure monitor of the dialysis equipment (see Fig. 2). In the present study, the upper limit for circuit line-pressure was 250 mmHg. Use of the extracorporeal circulation without anticoagulants resulted in occlusion of the circuit by clot within about 20 min. Symbols are as follows: KFA-1411 0.15 mg/kg/hr + 0.15 mg/kg loading. (●), 0.30 mg/kg/hr + 0.30 mg/kg loading. (▲), 0.60 mg/kg/hr + 0.60 mg/kg loading. (○), Dalteparin 20 IU/kg/hr + 30 IU/kg loading. (■). Data show the mean ± S.E. from three animals. *$p < 0.01$ significantly different from at 0 min.

Effects on In-Circuit and Systemic Coagulation: The ideal anticoagulant for hemodialysis should have anticoagulant effects in the circuit, but no systemic effects. To investigate the relationship between in-circuit coagulation conditions and systemic conditions, blood samples were simultaneously collected from the appropriate sampling points.

APTT: The average APTT prior to the initiation of the extracorporeal circulation was about 18 sec. In the KFA-1411-treated animals, the in-circuit APTT became prolonged in a dose–dependent manner immediately after the start, and it plateaued at about 1.7 times the value obtained prior to the start in the 0.15 mg/kg/hr group, at about 2 times in the 0.3 mg/kg/hr group, and reached a plateau at about 2.7 times in the 0.6 mg/kg/hr group (Fig. 4A). These data indicate that the dose of KFA-1411 needed to prolong the in-circuit APTT to 2 times the normal value was around 0.3 mg/kg/hr, and this should provide sufficient anticoagulation for the extracorporeal circulation. Because a loading dose of KFA-1411 was used at the initiation of the extracorporeal circulation in this study, a transient prolongation of the

Table 1. Effects of KFA-1411 and Dalteparin on Clot-Deposition in Dialyzer and Air-Trap Chamber in Cynomolgus Monkey Hemodialysis Model

		Dialyzer	Air-trap chamber
KFA-1411	0.15 mg/kg/hr + 0.15 mg/kg loading	3.3 ± 0.33	3.3 ± 0.33
KFA-1411	0.30 mg/kg/hr + 0.30 mg/kg loading	2.3 ± 0.33	1.7 ± 0.33
KFA-1411	0.60 mg/kg/hr + 0.60 mg/kg loading	1.3 ± 0.88	0.3 ± 0.33*
Dalteparin	20 IU/kg/hr + 30 IU/kg loading	2.7 ± 1.45	4.0 ± 0.00

Each value shows the mean ± S.E. of the clot-deposition scores in 3 animals. In the dialyzer: 0, no clotting; 1, < 5%; 2, 5–9%; 3, 10–24%; 4, 25–49%; 5, Over 50%; 6, occlusion due to clot-deposition in the fibers. In the air-trap chamber: 0, little or none; 1, very slight; 2, slight; 3, moderate; 4, severe. *: $p < 0.05$ significantly different from dalteparin group (Kruskal-Wallis test, followed by Dunnett-type multiple-comparison test).

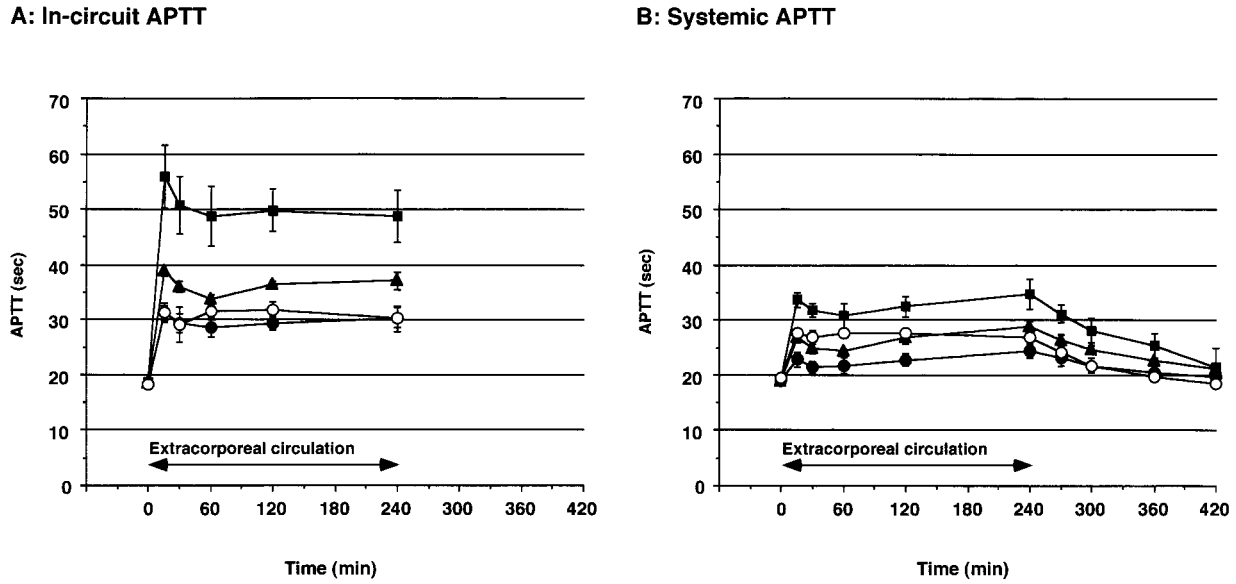

Fig. 4. Effects of KFA-1411 and Dalteparin on In-Circuit and Systemic APTT in Cynomolgus Monkey Hemodialysis Model

A: Change in in-circuit APTT. Blood samples for in-circuit values were collected from the bloodline immediately after passage through the dialyzer. B: Change in systemic APTT. Blood samples for systemic value were collected from the bloodline immediately after exit from the body during, and from the femoral vein after use of the extracorporeal blood circulation. APTT in the collected plasma was measured using a clotting meter, as described in Materials and Methods. Symbols are as follows: KFA-1411 0.15 mg/kg/hr + 0.15 mg/kg loading. (●), 0.30 mg/kg/hr + 0.30 mg/kg loading. (▲), 0.60 mg/kg/hr + 0.60 mg/kg loading. (■), Dalteparin 20 IU/kg/hr + 30 IU/kg loading. (○). Data are the mean ± S.E. from three animals.

in-circuit APTT was seen in the 0.3 mg/kg/hr and 0.6 mg/kg/hr groups. In dalteparin-treated animals, the APTT was prolonged to about 1.7 times the value obtained prior to the start.

The systemic APTT become prolonged after the initiation of the extracorporeal circulation in the KFA-1411-treated animals. It was maintained at about 1.2 times the start value in the 0.15 mg/kg/hr group, at about 1.5 times in the 0.3 mg/kg/hr group, and at 1.7 times in the 0.6 mg/kg/hr group (Fig. 4B). The prolongation of systemic APTT in dalteparin-treated animals was to 1.4 times the start value.

PT: Because *in vitro* measurements of clotting time showed that PT is more sensitive than APTT to the anti-coagulant effect of KFA-1411, PT was also monitored in this study. The in-circuit PT in the KFA-1411-treated animals became prolonged in a dose–dependent manner immediately after the initiation of the extracorporeal circulation, and plateaued at about 2 times, about 3 times, and about 4.2 times the start value in the 0.15 mg/kg/hr, 0.3 mg/kg/hr, and 0.6 mg/kg/hr groups, respectively (Fig. 5A). In the systemic circulation, the prolongation of the PT seen in the KFA-1411 groups was only 2.4 times the start value even at the highest dose of KFA-1411 used (0.6 mg/kg/hr) (Fig. 5B). These data also indicated that the PT prolongation caused by KFA-1411 is greater in magnitude than the APTT prolongation, and that PT is suitable for use as a monitor of anti-coagulant activity during hemodialysis, like APTT.

In the dalteparin-treated animals, there was essentially no prolongation of the in-circuit or systemic PT compared to the start values (Fig. 5B). This may have been due to the inclusion of the heparin inhibitor, polybrene, in the commercial PT reagent (to allow investigation of the coagulation profile of the patient even though the blood sample contains heparin).[12,13]

Comparison of In-Circuit and Systemic Coagulation: Comparison between the prolongations of in-circuit and systemic clotting times in the KFA-1411 groups indicated that both the in-circuit APTT and PT were greater by about 1.5 fold than the systemic values. In contrast, in the dalteparin group there was no difference in the extent of APTT prolongation between in-circuit and systemic values (Figs. 4 and 5).

After termination of the extracorporeal circulation, the systemic APTT decreased with time in all groups, to reach the pre-circulation value 3 hr later in the KFA-1411 group (Fig. 4B). The systemic PT also recovered with time, and even at the maximal dose of KFA-1411, 0.6 mg/kg/hr, the value was only 1.4 times the pre-circulation value at 3 hr after termination of the circulation (Fig. 5B).

Effect on Platelet Counts: Since heparin is

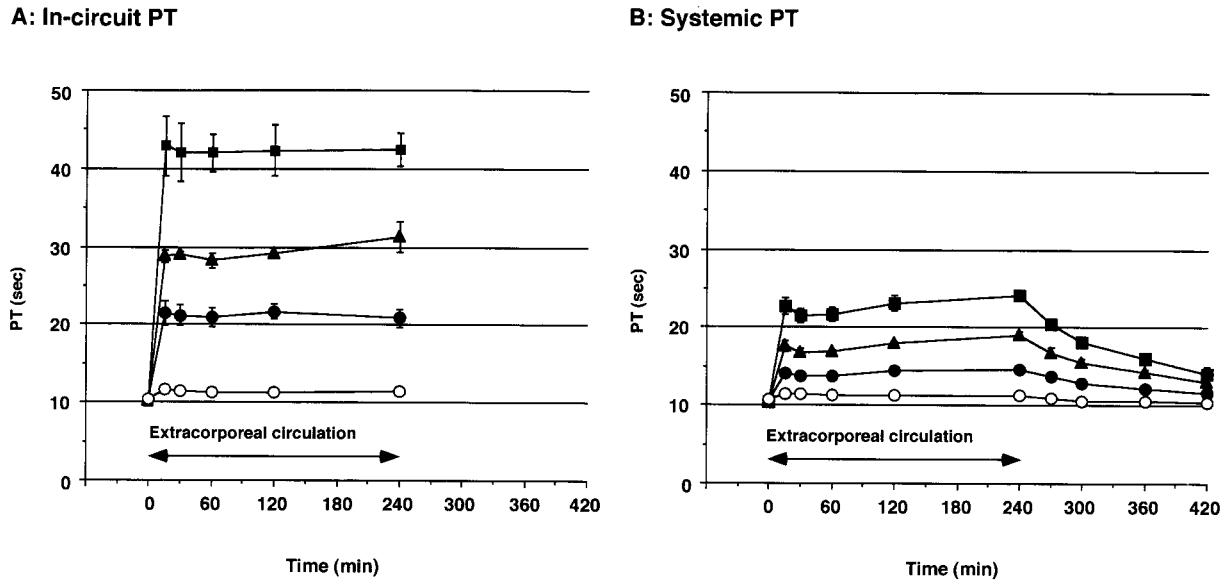

Fig. 5. Effects of KFA-1411 and Dalteparin on In-Circuit and Systemic PT in Cynomolgus Monkey Hemodialysis Model

A: Change in in-circuit PT. Blood samples for in-circuit values were collected from the bloodline immediately after passage through the dialyzer. B: Change in systemic PT. Blood samples for systemic values were collected from the bloodline immediately after exit from the body during, and from the femoral vein after use of the extracorporeal blood circulation. PT in the collected plasma was measured using a clotting meter, as described in Materials and Methods. Symbols are as follows: KFA-1411 0.15 mg/kg/hr + 0.15 mg/kg loading. (●), 0.30 mg/kg/hr + 0.30 mg/kg loading. (▲), 0.60 mg/kg/hr + 0.60 mg/kg loading. (■), Dalteparin 20 IU/kg/hr + 30 IU/kg loading. (○). Data are the mean ± S.E. from three animals.

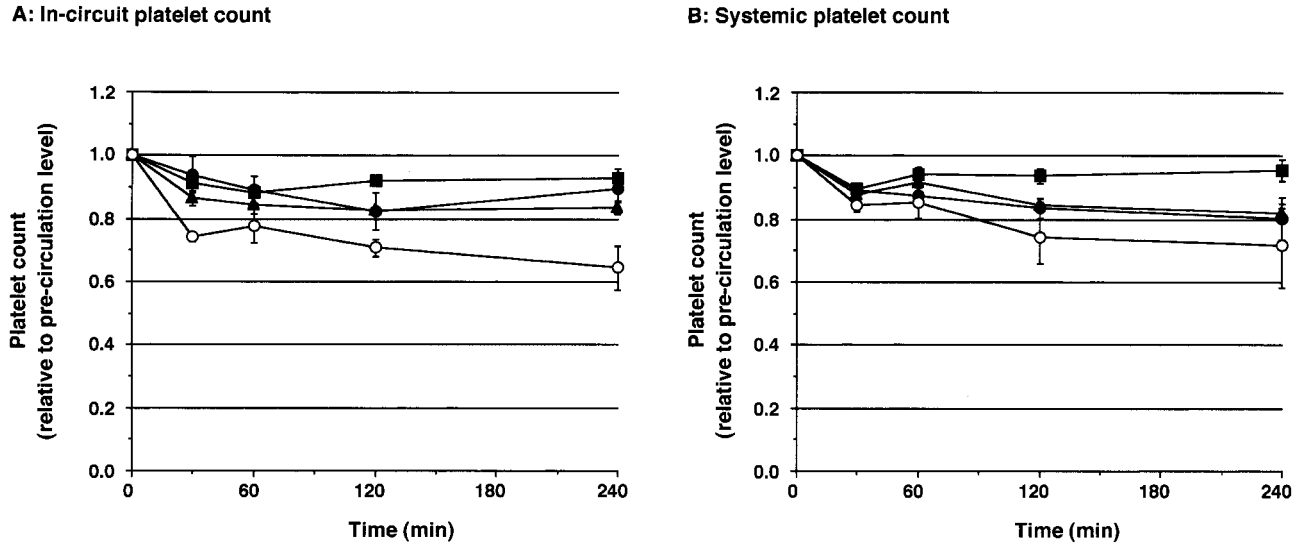

Fig. 6. Effects of KFA-1411 and Dalteparin on Platelet Counts in Cynomolgus Monkey Hemodialysis Model

A: Change in in-circuit platelet count. Blood samples for in-circuit values were collected from bloodline immediately after passage through the dialyzer. B: Change in systemic platelet count. Blood samples for systemic values were collected from bloodline immediately after exit from the body. Platelet counts were obtained measured using an automated hematology analyzer, as described in Materials and Methods. Symbols are as follows: KFA-1411 0.15 mg/kg/hr + 0.15 mg/kg loading. (●), 0.30 mg/kg/hr + 0.30 mg/kg loading. (▲), 0.60 mg/kg/hr + 0.60 mg/kg loading. (■), Dalteparin 20 IU/kg/hr + 30 IU/kg loading. (○). Data are the mean ± S.E. from three animals.

known to activate platelets during treatment,[3] the effect of KFA-1411 on platelets was investigated in this study. The average platelet count prior to the initiation of the extracorporeal circulation was about 37×10^4 /ml. The in-circuit and systemic changes in platelet count are presented in Fig. 6. In the KFA-1411 groups, both counts had decreased by about 10% within 30 min of the start. Thereafter, the counts were maintained at a relatively constant value. For the KFA-1411 groups at all doses, there were no dif-

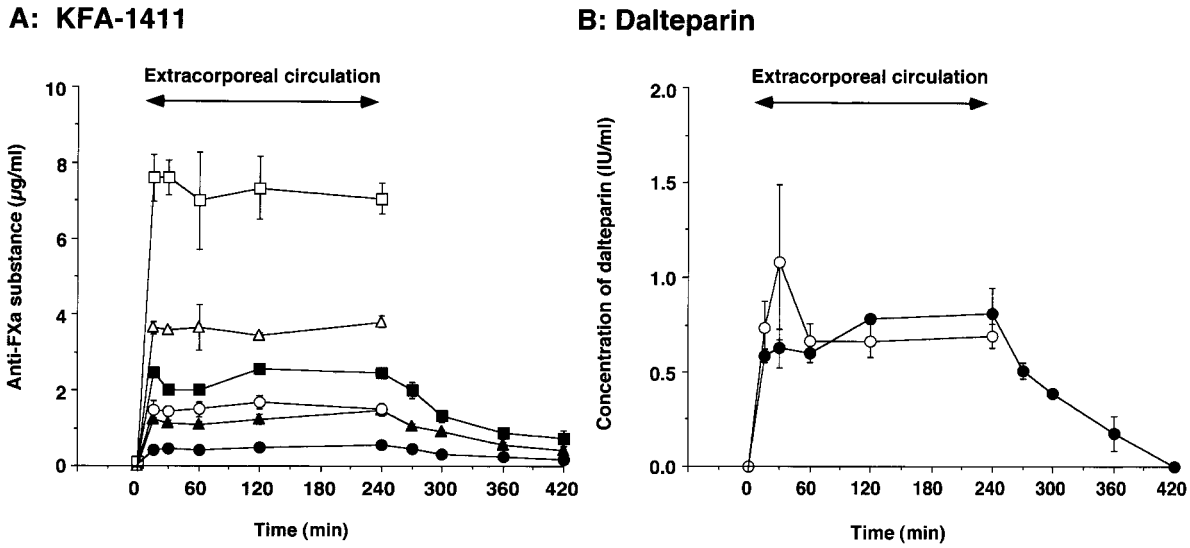

Fig. 7. Concentrations of Anti-FXa Substance (KFA-1411) and Dalteparin in Plasma in Cynomolgus Monkey Hemodialysis Model

Blood samples for in-circuit values and systemic values were collected from the blood-access point in the circuit during use of the extracorporeal circulation, while those for systemic values were collected from the femoral artery and vein prior to the initiation of extracorporeal circulation, and from the femoral vein after its termination. The concentration of anti-FXa substance was measured and calculated from the inhibition of human FXa activity in the plasma samples using the FXa inhibitory calibration curve for KFA-1411. The concentration of dalteparin, was measured using a commercial heparin assay kit (Test-them heparin-S®) using a reference curve for dalteparin as described in Materials and Methods. A: Changes in anti-factor FXa substance concentration in-circuit and systemically. Symbols are as follows: KFA-1411 0.15 mg/kg/hr + 0.15 mg/kg loading. (●: systemic values, ○: in-circuit values), 0.30 mg/kg/hr + 0.30 mg/kg loading. (▲: systemic values, △: in-circuit values), 0.60 mg/kg/hr + 0.60 mg/kg loading. (■: systemic values, □: in-circuit values). B: Changes in dalteparin concentration in-circuit and systemically. Dalteparin 20 IU/kg/hr + 30 IU/kg loading. (●: systemic values , ○: in-circuit values). Data are mean ± S.E. from three animals.

ferences between the in-circuit and systemic platelet counts. This indicates that even in the maximum dose of KFA-1411 used in this study, no apparent loss of platelets from the circulation occurred from 30 min after the start to completion. In the dalteparin group, both the in-circuit and systemic platelet counts had decreased by about 20% at 30 min after the start, and thereafter they continued to decrease with time. In addition, in the dalteparin group the in-circuit count was less than the systemic count at all time points, although statistical significant was not tested. This indicates a loss of platelets during passages through the dialyzer and in-circuit blood line.

Anti-FXa Substance in Blood during Extracorporeal Circulation: To make a preliminary examination of the pharmacokinetics of KFA-1411 for the purposes of hemodialysis, the in-circuit and systemic drug concentrations were measured. The blood concentration of KFA-1411 was estimated as anti-FXa substance (μg/ml equivalent to KFA-1411) (Fig. 7A), while dalteparin was expressed in International Units (IU/ml) (Fig. 7B). In the KFA-1411 groups, the in-circuit and systemic levels in the blood began to increase at the start, and then remained at a fixed level through out, the effects being dose–dependent. The in-circuit concentration of anti-FXa substance was about 3 times higher than the systemic concentration at each dose of KFA-1411. This difference is in accord with the difference in the values obtained for the prolongation of clotting time. In contrast, in the dalteparin group although the dalteparin concentration was maintained at a fixed level after the start, there was no significant difference between the in-circuit and systemic concentrations. After the completion of dialysis, the systemic concentrations of KFA-1411 and dalteparin decreased with time.

Evaluation of Species Differences between Humans and Cynomolgus Monkeys *in Vitro*

FXa-Inhibitory Effects of KFA-1411 and Dalteparin: The species differences in the effects of KFA-1411 on FXa activity were measured *in vitro* using human and Cynomolgus monkey plasma (Table 2). The Ki value for the effect of KFA-1411 against purified human FXa had already been calculated as 1.73 nM, and the inhibition was of the competitive type (data not shown). RVV-X-activated FX (see Materials and Methods) was used in this study. There was no effect of KFA-1411 on other enzymes related to this assay system, including FVIIa/tissue factor and FIXa, at the low concentrations that nevertheless had the inhibitory actions on

Table 2. Effects of KFA-1411 and Dalteparin on RVV-X-Activated FX and Coagulation in Plasma of Humans and Cynomolgus Monkeys

	KFA-1411		Dalteparin	
Inhibition of RVV-X-activated FX	(CI_{50} μM)	(ratio)	(IC_{50} IU/ml)	(ratio)
human	0.051	(1)	0.475	(1)
Cynomolgus monkey	0.080	(1.57)	0.391	(0.82)
APTT prolongation	(CT_2 μM)	(ratio)	(CT_2 IU/ml)	(ratio)
human	0.83	(1)	0.44	(1)
Cynomolgus monkey	1.53	(1.84)	0.81	(1.84)
PT prolongation	(CT_2 μM)	(ratio)	(CT_2 IU/ml)	(ratio)
human	0.38	(1)	> 10	(—)
Cynomolgus monkey	0.48	(1.26)	> 10	(—)

Inhibitory effects of KFA-1411 and dalteparin on RVV-X-activated FX activities in plasma of humans and Cynomolgus monkeys. The FX present in plasma was activated using snake venom RVV-X, and activity was measured, using a chromogenic assay, at 405 nm. The reference activity was observed by adding the vehicle alone to the reaction mixture. RVV-X-activated FX inhibition: IC_{50} values are given for the inhibitory effects of KFA-1411 and dalteparin on RVV-X-activated FX. Effects of KFA-1411 and dalteparin on coagulation of human plasma and Cynomolgus monkey plasma (APTT and PT prolongation): CT_2 indicates the drug concentration needed to double the clotting time in the vehicle experiment (calculated from the concentration-clotting time prolongation curve). Each value was obtained from 3–7 assays. The ratio given in parenthesis for each parameter indicates the activity of each drug relative to the activity in humans.

FXa (data not shown). The KFA-1411-mediated inhibitory activity against RVV-X-activated FX (IC_{50}) was about 1.6 times more potent in humans than in Cynomolgus monkeys. The corresponding value for dalteparin was 0.82 times.

Anticoagulant Effects of KFA-1411 and Dalteparin: The anticoagulant activities of KFA-1411 and dalteparin were assessed by measuring APTT and PT (Table 2). KFA-1411 and dalteparin were each 1.8 times more potent on APTT in humans than in Cynomolgus monkeys. The CT_2 value for the effects on PT, which was only available for KFA-1411, as described above, showed that this drug was 1.3 times more potent in humans than in Cynomolgus monkeys. On the basis of these data, it was considered that both KFA-1411 and dalteparin showed little species difference between humans and Cynomolgus monkeys in anticoagulant activities; there was no great differences in anti-FXa activity between these two species for either drug.

DISCUSSION

In the present study, we estimated whether a newly developed selective FXa inhibitor has an efficacy as an anticoagulant for hemodialysis that is similar to that of AT III-dependent anticoagulants, and whether it can provide conditions required for the maintenance of an extracorporeal circulation. The results of our extracorporeal-pressure and clot-deposition analysis indicated that the new selective FXa inhibitor, KFA-1411, could indeed be used as an anticoagulant for hemodialysis. Although there was slight in-circuit clot-deposition (in the dialyzer and the air-trap chamber), the doses of 0.3 mg/kg/hr and 0.6 mg/kg/hr KFA-1411 showed high enough efficacies in the Cynomolgus monkey hemodialysis model. On the other hand, with dalteparin at 20 IU/kg/hr extracorporeal pursuer increased substantially and the maximum score of 4 was obtained for clot-deposition in the air-trap chamber in all animals. Therefore, our general evaluation of the present dose of dalteparin seemed to suggest that it is unsatisfactory for hemodialysis. The dose of dalteparin used in this study was the same as the clinical dose, with which clot-deposition is essentially not observed in human use. The observation made in this study, therefore, indicates that the present conditions use for the extracorporeal circulation were more likely to induce blood coagulation than the clinical conditions used for hemodialysis in humans. One possible reason for this difference may be a lower quality (precision) of materials (*e.g.*) dialysis tubing and dialyzer intended for animals use versus those used in humans. An other possible reason is a species difference between humans and the Cynomolgus monkey. However, a species difference in respect of FXa inhibition was excluded by the present findings. The other factors located in the coagulation cascade were not examined in this study. It has been reported that other previously described FXa inhibitors exhibit

species differences in FXa-inhibitory activity,[9] and that selection of the animal species is important when attempting to estimate clinical effects in humans on the basis of animal studies. In the present study, we used RVV-X-activated FX instead of purified FXa for the *in vitro* measurements. The KFA-1411 results in humans and Cynomolgus monkeys indicated no large species differences. On the basis of these results, we consider that experimental results obtained in Cynomolgus monkeys can be used to estimate the clinical efficacy of KFA-1411 in humans.

Under conditions in which the clinical dose of dalteparin showed an insufficient anticoagulant effect, KFA-1411, at a dose of 0.3 mg/kg/hr or 0.6 mg/kg/hr, did show sufficient efficacy. It is therefore to be expected that KFA-1411, at doses of 0.3 mg/kg/hr + 0.3 mg/kg loading and 0.6 mg/kg/hr + 0.6 mg/kg loading (*i.e.*, the doses used in the present study) will have sufficient anticoagulant efficacy for the purposes of hemodialysis.

APTT, which indicates activities in the intrinsic pathway, reflects blood activation through contact with a foreign body. At the KFA-1411 dose of 0.3 mg/kg/hr, which in this study was considered sufficient to permit maintenance of dialysis, APTT was prolonged by about 2 fold in-circuit and by about 1.5 fold systemically (compared to the values obtained prior to the initiation of the extracorporeal circulation). In addition to APTT, PT (an index of the extrinsic pathway) could also be used as a monitor. On the basis of the coagulation monitoring carried out in this study, a dose of KFA-1411 has sufficient anticoagulant efficacy for hemodialysis if the in-circuit APTT and PT are maintained at about 2 times and about 3 times the normal value, respectively. In the present study, this was achieved with 0.3 mg/kg/hr KFA-1411.

From the standpoint of bleeding risk during and after hemodialysis, an anticoagulant drug should ideally have anticoagulant effects in the circuit, but no systemic effects (and/or almost immediately disappear from the systemic circulation). In the present study, even though KFA-1411 affected the systemic APTT and PT to a small extent, no abnormal bleeding was observed at any site at the completion of the experiment (data not shown). At the maximum KFA-1411 dose of 0.6 mg/kg/hr in this study, the systemic PT was prolonged about 2 times compared to the start value. In our preliminary studies in rats, KFA-1411 at a dose that caused PT prolongation to 2 times the normal PT had no significant effect on the bleeding time (data not shown). From these observations, the doses of KFA-1411 used in this study are not likely to increase the risk of systemic hemorrhage during hemodialysis.

Since the systemic KFA-1411 concentration in blood was lower than the in-circuit concentration during hemodialysis, the systemic blood anticoagulation effect of KFA-1411 should be less than its in-circuit anticoagulation effect. This indicates that KFA-1411 is a slightly better anticoagulant for hemodialysis, than dalteparin in respect of its effect on systemic coagulation. However, the mechanism underlying this phenomenon is unknown.

In the KFA-1411 groups, the platelet count had decreased slightly compared to the start values at 30 min after the start. However, at all doses there was no difference in the platelet count before versus after passage through the dialyzer (after the initial decrease). This indicates that platelet loss only occurred at an early stage in hemodialysis independent of the concentration of KFA-1411. Heparin is known to induce heparin-induced thrombocytopenia (HIT) in some patients, who experience platelet activation due to the in exposure to heparin.[3] The present results indicate that, unlike heparin, KFA-1411 would not activate platelets during hemodialysis.

To judge from out results in respect of enzyme-inhibition and anticoagulation *in vitro*, anticoagulant activities of KFA-1411 should be comparable between the human and the Cynomolgus monkey. However, since the pharmacokinetics of KFA-1411 are as yet unclear, both in humans and in Cynomolgus monkeys, the results of this study allow us only to suspect that KFA-1411 can be used as an anticoagulant for hemodialysis in humans.

The Cynomolgus monkeys used in this study were healthy, with normal renal function. On the other hand, patients who require hemodialysis have insufficient renal functions, and so the information on pharmacokinetics and pharmakodynamics are very important for a newly developed drug. From the standpoint of potential application to patients requiring hemodialysis, further studies will be needed using animal models of renal insufficiency.

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