Plasma From Patients Undergoing Liver Transplantation Is Resistant to Anticoagulant Activity of Soluble Thrombomodulin

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Recombinant human soluble thrombomodulin (ART-123) is an anticoagulant and anti-inflammatory agent clinically used for treatment of disseminated intravascular coagulation. Preclinical studies have shown that ART-123 reduces hepatic ischemia/reperfusion. Although ART-123 may therefore have clinical benefit in orthotopic liver transplantation, the substantial alterations in the hemostatic system may complicate its use in this setting. Here, we studied the in vitro effect of ART-123 on coagulation of patients with end-stage liver disease undergoing liver transplantation. Ten patients with end-stage liver disease undergoing liver transplantation were included in this study. Plasma samples of 10 healthy individuals were included to establish reference values. Different concentrations of ART-123 were added to plasma samples, and peak thrombin generation and clot lysis times (CLTs) were determined. In patient samples, plasma was profoundly resistant to the anticoagulant action of ART-123, as reflected by significantly higher median inhibitory concentration (IC_{50}) values of peak thrombin generation compared with controls. This might be partially explained by low levels of protein C, protein S, and elevated levels of factor VIII during transplantation. Intraoperative levels of thrombin activatable fibrinolysis inhibitor were significantly lower when compared with controls. However, ART-123–dependent prolongation of CLTs was not significantly different from healthy controls. In conclusion, this study suggests that ART-123 is unlikely to provoke bleeding in patients undergoing liver transplantation because proposed clinical dosages have a virtually absent anticoagulant effect in these patients. Clinical studies are required to confirm the safety of ART-123 and efficacy on alleviating ischemia/reperfusion injury during liver transplantation.

Liver Transplantation 25 252–259 2019 AASLD.
Received June 12, 2018; accepted July 23, 2018.

Orthotopic liver transplantation (OLT) remains the only treatment option for patients with end-stage liver failure, including cirrhosis. Worldwide organ scarcity has led to an increased utilization of suboptimal donor livers, such as livers from donation after circulatory death donors, elderly donors, and fatty livers. These extended criteria donor livers are, however, more prone to ischemia/reperfusion injury (IRI)–related complications after transplantation, including graft dysfunction, early graft loss, and posttransplant cholangiopathy.[1,2] IRI in liver transplantation refers to the deleterious biphasic phenomenon of absence of oxygen during static cold preservation of the graft and restoration of oxygen supply upon reperfusion. The underlying mechanisms of ischemia/reperfusion (I/R)–related injury to the liver are complex and multifactorial.[3,4]

Recombinant human soluble thrombomodulin (ART-123) is a novel drug composed of the active, extracellular domain of thrombomodulin (TM). TM is a transmembrane glycoprotein ubiquitously expressed

Abbreviations: APC, activated protein C; ART-123, recombinant human soluble thrombomodulin; ASH, alcoholic steatohepatitis; BMI, body mass index; CAT, calibrated automated thrombography; CLT, clot lysis time; DIC, disseminated intravascular coagulation; FVIII, factor VIII; HMGB1, high-mobility group box 1; IC_{50}, median inhibitory concentration; INR, international normalized ratio; I/R, ischemia/reperfusion; IRI, ischemia/reperfusion injury; MELD, Model for End-Stage Liver Disease; NASH, nonalcoholic steatohepatitis; OLT, orthotopic liver transplantation; POD, postoperative day; PSC, primary sclerosing cholangitis; SD, standard deviation; TAFI, thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin.

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on vascular endothelial cells. TM plays a key role in both coagulation and inflammation by binding thrombin and accelerating the activation of protein C into activated protein C (APC).[5] In addition, TM enhances the rate of activation of thrombin activatable fibrinolysis inhibitor (TAFI), a crucial regulator of clot breakdown, by more than 1000-fold.[6] Like membrane-bound TM, ART-123 binds to thrombin to inactivate coagulation via activation of protein C.[7] Interestingly, APC exhibits important cytoprotective functions, including antiapoptotic, anti-inflammatory, and barrier stabilization properties.[8] Furthermore, ART-123 inhibits high-mobility group box 1 (HMGB1) by enhancing thrombin-mediated proteolytic cleavage of HMGB1 or by a direct interaction between ART-123 and HMGB1 that neutralizes its proinflammatory effects.[9,10]

ART-123 is in clinical development for treatment of sepsis and disseminated intravascular coagulation (DIC).[11,12] ART-123 has been approved for clinical use in Japan in 2008, and safety and efficacy in patients with sepsis and DIC has been demonstrated in a global phase 2 study.[11] Currently, a phase 3 study is ongoing to examine safety and efficacy in patients with severe sepsis and coagulopathy (clinicaltrials.gov, NCT01598831). In addition, a phase 3 study on the use of ART-123 for the treatment of acute exacerbation of idiopathic pulmonary fibrosis (NCT02739165) and a phase 2 study on the use of ART-123 for the prevention of cancer treatment–related symptoms such as chemotherapy-induced peripheral neuropathy[13] in patients with postoperative stage II/III colon cancer (NCT02792842) are ongoing.

Over the last years, evidence from animal experiments is emerging that ART-123 has important organ protective effects and that it has cytoprotective effects on the endothelium.[14,15] In a rodent model of hepatic warm ischemia, livers that were ex vivo perfused with ART-123, after 6 hours of static cold preservation, showed significantly improved bile production and decreased sinusoidal narrowing compared with controls.[16] Binding of ART-123 to HMGB1, a factor closely associated with necrotic cell damage, has been suggested as a pathophysiological mechanism, whereby ART-123 minimizes IRI. HMGB1 is used as a marker of injury in human liver and kidney transplantation, and animal studies show that inhibition of HMGB1 with a neutralizing antibody significantly decreased liver damage after I/R.[17-20] In fact, rats that were given ART-123 as an inhibitor of HMGB1 demonstrated less liver injury after partial hepatic ischemia followed by reperfusion.[21]

The hemostatic system of patients with end-stage liver disease is substantially different from healthy individuals, and these changes may further aggravate during transplantation.[22] In short, the hemostatic profile of a transplant recipient is characterized by thrombocytopenia, which appears balanced by high plasma levels of the von Willebrand factor,[23] reduced plasma levels of both procoagulant and anticoagulant proteins,[24] and reduced plasma levels of both profibrinolytic and antifibrinolytic proteins.[25] During transplantation, there is persistent elevation of the von Willebrand factor, a further decline in procoagulant and anticoagulant proteins, and a further decline in profibrinolytics and antifibrinolytics.[26-28] Moreover, we have previously demonstrated that thrombin generation in patients undergoing liver transplantation is equal or even superior to thrombin generation in healthy volunteers when tested in the presence of exogenous TM.[27] However, these previous studies were performed with a soluble form of rabbit TM. For safe application of ART-123 in transplant recipients, it is of utmost importance to investigate the anticoagulant and profibrinolytic effects of ART-123 in plasma taken during the transplant procedure. In this study, we aimed to study the in vitro effects of ART-123 on coagulation and fibrinolysis in samples taken from patients with end-stage liver disease during and in the first days after OLT.

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This study was financially supported in part by Asahi Kasei Pharma Corporation (Tokyo, Japan).

Takumi Sakai is an employee of and has received grants/contracts from Asahi Kasei Pharma Corporation. Tom Lisman has received grants/contracts from Asahi Kasei Pharma Corporation.

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DOI 10.1002/lt.25318

Patients and Methods

PATIENTS

Ten adult patients previously diagnosed with cirrhosis, who underwent OLT at the University Medical Center

Groningen, the Netherlands, between December 2015 and April 2017 were included in the study. Exclusion criteria were acute liver failure, documented hereditary thrombophilia, a recent deep vein thrombosis (<30 days), transfusion of blood products in the past 7 days, and current use of anticoagulant drugs. After transplantation, all patients received a standard immunosuppression regimen consisting of basiliximab, prednisolone, mycophenolate mofetil (CellCept), and tacrolimus (PROGRAFT). Moreover, after transplantation all patients received standard thrombosis prophylaxis with low-molecular-weight heparin (Nadoparin 2850 IE/day via a subcutaneous injection). Individual plasma samples of 10 adult healthy volunteers working at our institution were used to establish reference values. Exclusion criteria for the control group were documented hereditary thrombophilia, a documented history of a recent deep vein thrombosis (<30 days), transfusion of blood products in the past 7 days, and current usage of anticoagulant drugs. The study protocol was approved by the medical ethical committee of the University Medical Center Groningen (METc2015.206). Written informed consent was obtained from all patients in this study prior to inclusion.

ETHICS STATEMENT

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PLASMA SAMPLES

Blood samples from patients were collected during and after liver transplantation in a single-center setting at 7 predefined time points:

1. 30 minutes after induction of anesthesia.
2. 30 minutes after the start of the anhepatic phase.
3. 30 minutes after graft reperfusion.
4. At the end of surgery.
5. 24 hours after transplantation.
6. 3 days after transplantation.
7. 6 days after transplantation.

Intraoperative blood samples were taken from a dedicated nonheparinized arterial line while samples on the postoperative days (PODs) and from controls were drawn by venapuncture. Blood samples from all patients were collected in tubes containing 3.2% sodium citrate (9:1, vol/vol). Platelet-poor plasma was obtained by centrifuging blood samples at 18˚C for 10 minutes at 2000*g* and subsequently for 10 minutes at 10,000*g* within 30 minutes after blood collection. Plasma samples were stored at –80˚C until use.

ASSAYS

Peak Thrombin Generation

Peak thrombin generation capacity was assessed with calibrated automated thrombography (CAT) using platelet-poor plasma, as previously described by Hemker et al.[29] In short, the CAT method enables the quantification of thrombin generation capacity in an individual sample after induction of coagulation by using a fluorescent substrate. Fluorescence was continuously measured by a fluorometer, Fluoroskan Ascent (ThermoFisher Scientific, Helsinki, Finland). All procedures were according to the protocol suggested by Thrombinoscope BV (Maastricht, the Netherlands). Peak thrombin generation capacities were determined in the absence and presence of different concentrations of ART-123 (0, 0.03, 0.3, 3, 30, and 300 μg/mL), and median inhibitory concentration (IC_{50}) values were determined. The IC_{50} values represent the concentration of ART-123 that is required to inhibit 50% of the peak thrombin generation in vitro.

Clot Lysis Assay

Clot lysis assays were performed as described previously.[30] In short, clot formation was induced by adding a 50-μL mixture of phospholipid vesicles, tissue plasminogen activator, tissue factor, and $CaCl_2$ diluted in 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–buffered saline, to 50 μL of each sample. A clot lysis profile was generated by analyzing the optical density at 405 nm every 20 seconds in a kinetic microplate reader. The clot lysis time (CLT) was derived from this clot lysis profile and is defined as the time from maximum turbidity to clear transition. CLTs were determined in the absence or presence of different concentrations of ART-123 (0, 0.03, 0.1, 0.3, and 1 μg/mL), and IC_{50} values were determined. The IC_{50} value represents the concentration of ART-123 that is required for half-maximal prolongation of the CLT.

Protein C, Protein S, Factor VIII, and TAFI

Plasma levels of protein C, protein S, and factor VIII (FVIII) were determined on an automated coagulation analyzer (ACL 300 TOP) with reagents and protocols from the manufacturer (Werfen, Breda, the Netherlands). Plasma TAFI levels were determined by a commercially available enzyme-linked immunosorbent assay (Zymutest activatable TAFI, Nodia, Amsterdam, the Netherlands).

STATISTICAL ANALYSIS

Continuous data are presented as means with standard deviations (SDs) or medians with interquartile ranges as appropriate. Categorical variables are presented as numbers with percentages. Differences in IC_{50} values between controls and patients at multiple time points were examined using the Kruskal-Wallis test followed by the Dunn's post test. *P* values of 0.05 or less were considered statistically significant. Statistical analyses were performed with GraphPad Prism (San Diego, CA) and IBM SPSS Statistics, version 23.0 (IBM, Chicago, IL).

Results

PATIENT CHARACTERISTICS

Ten patients with cirrhosis were included in this study. The main demographic and clinical characteristics of the study population are shown in Table 1. Furthermore, 10 healthy patients, referred to as controls, were included to establish reference values for the coagulation tests.

TABLE 1. Clinical Characteristics of 10 Patients Who Underwent OLT

Characteristics	Numbers
Age, years	58.2 ± 7.6
Sex, male	4 (40)
Etiology of cirrhosis	
ASH	2 (20)
NASH	2 (20)
PSC	2 (20)
Wilson's	1 (10)
Cryptogenic	2 (20)
Other	1 (10)
Relevant pretransplant medication	
Proton-pump inhibitor	7 (70)
Beta-blocker	5 (50)
Diuretic (thiazide, loop or potassium-sparing)	7 (70)
Rifaximin or lactulose	5 (50)
Ursodeoxycholic acid	3 (30)
Ferrous fumarate	2 (20)
Insulin	2 (20)
Prednisone	1 (10)
Prophylactic antibiotics*	4 (40)
MELD	13 ± 7
INR	1.2 ± 0.1
Prothrombin time, seconds	13 ± 2
Activated partial thromboplastin time, seconds	30 ± 4
Fibrinogen, g/L	2.6 ± 1.1
Creatinine, μmol/L	75 ± 30
Platelet count, 10^9/L	123 ± 34
BMI, kg/m^2	25.9 ± 4.6
Ascites, mild	4 (40)
Encephalopathy, mild	3 (30)

NOTE: Numbers are either represented as means ± SD or n (%). One patient had cirrhosis based on ASH combined with hepatitis C.
*Prophylactic antibiotics were given to 4 patients with spontaneous bacterial peritonitis.

POSTTRANSPLANT OUTCOME

Six-month and 1-year graft and patient survival rates were 100% (10 out of 10). Delayed graft function, primary nonfunction, and thrombosis were not observed in this study.

INHIBITION OF THROMBIN GENERATION

Thrombin generation tests were performed in platelet-poor plasma wherein thrombin formation was initiated by tissue factor. The IC_{50} values were determined for peak thrombin generation by fitting ART-123 concentration versus peak thrombin curves by 1 phase exponential decay curves. In a number of samples, particularly in samples taken during surgery, the inhibitory effect of ART-123 on peak thrombin generation was too low to obtain curve fits using 1 phase exponential decay. In these samples, we therefore estimated IC_{50} values by using linear regression. Figure 1A shows peak thrombin IC_{50} values for the various time

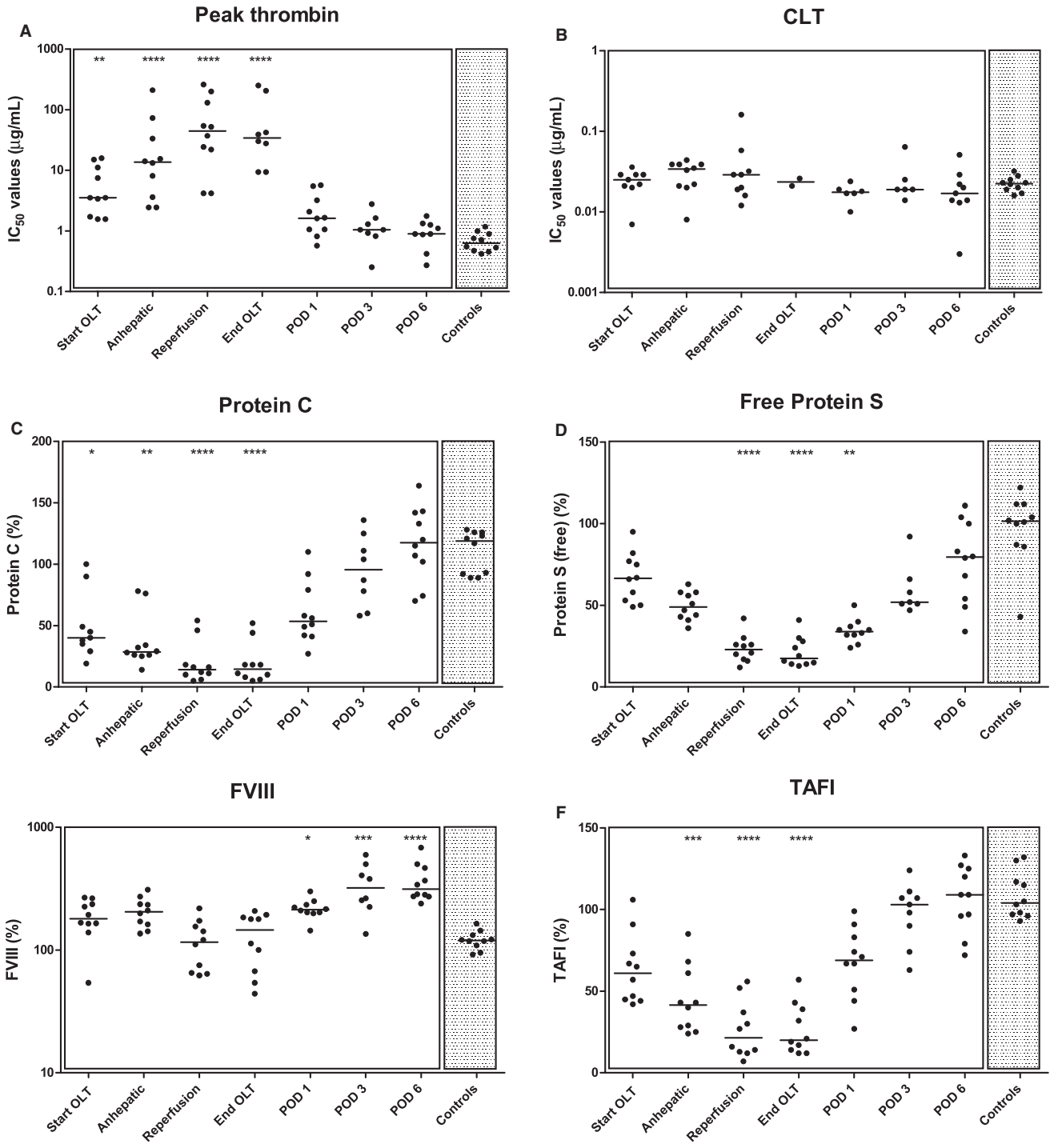

FIG. 1. Overview of analyses in platelet-poor plasma samples of 10 patients and 10 healthy controls. Samples were collected at the start of OLT, during the anhepatic phase, after reperfusion, and at the end of the procedure. Moreover, samples were collected during POD 1 as well as PODs 3 and 6. IC_{50} values were determined by adding different concentrations of ART-123 (A and B). Plasma levels of protein C, free protein S, FVIII, and TAFI were determined in all plasma samples and are depicted as a percentage of normal pooled plasma samples (set at 100%) (C-F). All values during different time points in patient plasma samples were compared with values in healthy controls. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Horizontal lines indicate medians.

points examined. Compared with peak thrombin IC_{50} values in plasma of healthy controls, IC_{50} values in patients were significantly increased at the start of surgery, indicating resistance to the anticoagulant action of ART-123. Resistance against ART-123 anticoagulant activity increased during surgery. Compared with peak thrombin IC_{50} values in healthy controls, values were significantly higher during the anhepatic phase, the reperfusion phase, and at the end of surgery. From POD 1 onward, ART-123 sensitivity normalized and was comparable with controls at day 6.

INHIBITION OF CLOT LYSIS

ART-123 accelerates thrombin-mediated activation of TAFI and thereby substantially prolongs plasma CLTs. CLTs were determined by a plasma-based clot lysis assay after the addition of different concentrations of ART-123. We subsequently determined IC_{50} values (Fig. 1B). In general, ART-123 prolonged CLTs to a similar extent in patients and controls. In a number of samples, no clot lysis occurred within the 3 hours of the assay even in the absence of ART-123 (at the end of surgery and POD 1). In these cases, no IC_{50} values could be calculated. In samples taken during OLT and during the PODs, the antifibrinolytic activity of ART-123 was comparable to controls.

PROTEIN C LEVELS

Protein C levels were determined in all plasma samples (Fig. 1C). At the start of OLT, protein C levels were significantly decreased compared with healthy controls. During OLT, protein C levels decreased further and were significantly decreased at all time points compared with levels in heathy controls; during the anhepatic phase, after reperfusion, and at the end of surgery, respectively. From POD 1 onward, protein C levels normalized with values comparable to controls on day 6.

PROTEIN S LEVELS

Protein S levels were determined in all plasma samples (Fig. 1D). At the start of surgery, median protein S levels were lower compared with controls, but this difference did not reach statistical significance. Protein S levels decreased even further during OLT. Protein S levels were significantly lower during reperfusion and at the end of surgery compared with levels in healthy controls. At POD 1, protein S levels were still decreased compared with controls, but levels normalized from then onward.

FVIII LEVELS

FVIII levels were determined in all plasma samples (Fig. 1E). During the start of surgery and during the anhepatic phase, median FVIII levels were elevated compared with levels in healthy controls, yet the differences did not reach significance. Over the course of surgery, FVIII levels normalized. During PODs 1, 3, and 6, FVIII levels were significantly higher compared with levels in healthy controls.

TAFI LEVELS

TAFI levels were determined in all plasma samples (Fig. 1F). At the start of surgery, median TAFI levels were lower compared with controls, yet this difference did not reach statistical significance. TAFI levels decreased even further during OLT. TAFI levels were significantly decreased during the anhepatic phase, during reperfusion, and at the end of surgery compared with levels in healthy controls. From POD 1 onward, TAFI levels normalized with values comparable to controls on day 3.

Discussion

This study shows that plasma of patients undergoing OLT is extremely resistant to the anticoagulant activity of ART-123. The resistance of ART-123, as shown by elevated peak thrombin IC_{50} values, was significantly higher in the plasma of patients undergoing liver transplantation at the start of surgery, during the anhepatic phase, after reperfusion, and at the end of surgery compared with healthy controls. These results are in line with our previously published data showing resistance to rabbit-derived TM in similar samples.[27] We expanded our previous studies by now testing a compound that is in clinical development, and we assessed the effects of multiple doses in order to obtain IC_{50} values.

For effective clinical treatment of DIC, the plasma concentration of ART-123 is less than 2 μg/mL, although it has been suggested that, based on in vivo studies with nonhuman primates, plasma levels of ART-123 above 10 μg/mL might increase the risk of bleeding in humans.[31] Clinical treatment levels

of ART-123 to ameliorate IRI during liver transplantation have yet to be established, but a dose similar to that used in the treatment of DIC appears plausible based on the proposed mode of action. At such plasma concentrations, clinical application of ART-123 during liver transplantation is unlikely to provoke a clinically significant anticoagulant effect as peak thrombin IC_{50} values are far above the expected plasma concentrations of the drug in vivo. With respect to bleeding, this dose may therefore be much safer in the transplant recipient than in an individual with adequate liver function. The resistance of plasma to the anticoagulant action of ART-123 during liver transplantation is partly explained by the decreased levels of both protein C and protein S. Consequently, a bleeding risk may be present in those transplant recipients who have preserved liver function and (near) normal levels of protein C and S, for example patients with metabolic disorders. Conversely, a bleeding risk may be absent at even higher doses of ART-123 in patients with exceptionally low plasma levels of protein C and S, for example, patients with acute liver failure.[32]

If ART-123 was tested clinically, it might be relevant to avoid the use of prothrombin complex concentrates during OLT because these concentrates contain appreciable levels of protein C and S, which would increase the anticoagulant potency of ART-123 and potentially contribute to (paradoxically) increased perioperative bleeding. During the postoperative period, the normalization of protein C and protein S levels allowed for a more effective ART-123–mediated inhibition of thrombin generation. A mild anticoagulant effect of ART-123 in the postoperative period may be beneficial to avoid early hepatic artery thrombosis or venous thrombotic events.[33]

FVIII is mainly synthesized by hepatic sinusoidal endothelial cells and is typically elevated in chronic or acute liver failure. Although in this study we were unable to detect a significant increase in levels of FVIII at the start of surgery compared with levels in controls, elevated FVIII levels might also contribute to ART-123 resistance during surgery as was previously demonstrated by us in experiments with rabbit TM.[27] In our study, FVIII levels further normalized during surgery and levels were significantly increased compared with levels in healthy controls during the PODs, which may be related to persistent elevations in the platelet adhesive protein von Willebrand factor,[26] which is a carrier protein for FVIII in circulation.

We demonstrated that, in general, ART-123 prolonged CLTs, but not in samples in which no clot lysis occurred within the 3 hours of the assay (at the end of surgery and POD 1). The lack of fibrinolysis at these time points has been well described as the postoperative fibrinolytic shutdown.[28,34,35] Moreover, we have shown that intraoperative TAFI plasma levels were decreased compared with levels in controls. At POD 1, TAFI levels were already increasing and normalized at POD 3 and 6. Interestingly, despite very low intraoperative TAFI levels, ART-123–mediated inhibition of fibrinolysis is still intact as evidenced by the clot lysis assay, which is in line with our previously published results using rabbit TM.[28] Thus, ART-123 is expected to exert a balanced antifibrinolytic effect during liver transplantation, which is beneficial in preventing excessive blood loss as evidenced by the clinical efficacy of antifibrinolytic agents such as the serine protease inhibitor aprotinin.[36]

In conclusion, the thrombin generation capacity in plasma of patients undergoing liver transplantation is remarkably resistant to the anticoagulant action of ART-123. During liver transplantation, ART-123 might therefore be safely tested as an agent to ameliorate IRI via its anti-inflammatory effects as no bleeding diathesis is expected to be induced by ART-123 plasma levels of 1 μg/mL or even higher. Clinical studies are needed to confirm the safety profile assessed here in vitro, to determine a dosage profile for patients with end-stage liver disease, and to assess the efficacy in reducing IRI.

Acknowledgments: We are very appreciative of all volunteers who donated blood to establish reference values for this experiment. We also want to thank the students of the liver team of the University Medical Center Groningen who helped to collect samples during the liver transplantations.

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