Quantitative Assessment of Fibrinogen Cross-Linking by ϵAminocaproic Acid in Patients With End-Stage Liver Disease

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Analysis of the effectiveness of antifibrinolytic therapy for liver transplant recipients is hampered by lack of quantitative assays for assessing drug effects. We adapted chemical engineering tools used in polymerization studies to quantify fibrinogen cross-linking by plasma from liver transplant patients obtained before and after ϵaminocaproic acid (EACA) therapy. A target fluorescein isothiocyanate–fibrinogen (FITC—fibrinogen) molecule was constructed; it fluoresces in a quantifiable pattern when in solution, and undergoes cross-linking in the presence of plasmin inhibitors. Cross-linking quenches the fluorescent signal, and the quenching is a quantifiable endpoint. Thus fluorescence from this reporter molecule can be used to assess functional improvement in fibrinogen cross-linking as a result of antifibrinolytic therapies, and it is sensitive to picomolar amounts of plasmin inhibitors and activators. Cross-linking of FITC-fibrinogen by patient plasma, before and after EACA therapy, was assessed using fluorescence spectrometry. Fluorescence patterns from FITC-fibrinogen indicated no significant cross-linking of the target fibrinogen as a consequence of EACA in posttreatment plasma. When the fibrinogen-FITC target was assayed without plasma in the presence of EACA at concentrations that bracket therapeutic levels (100 and 400 μg/ml), significant fluorescence quenching (target FITC-fibrinogen cross-linking) was achieved. These results suggest that fibrinogen-FITC fluorescence is sensitive enough to detect EACA activity in clinically relevant ranges, but that EACA given in usual doses is insufficient to promote fibrinogen cross-linking in patients with end-stage liver disease. (*Liver Transpl 2004;10:123–128.*)

End-stage liver disease (ESLD) is often associated with low-grade primary fibrinolysis, a complication of liver failure recognized almost a century ago.[1,2] A recent study, for example, suggests that about a third of cirrhotic patients have hyperfibrinolysis as assessed by shortened euglobulin lysis times.[3] Furthermore, worsening of primary fibrinolysis is a well-known superimposed complication of liver transplantation, characteristically most pronounced after reperfusion of the graft.[4] Antifibrinolytic therapy is used by many transplant anesthesiologists to prevent or respond to fibrinolysis during liver transplantation, though the details of antifibrinolytic therapy differ considerably from center to center. Epsilon-aminocaprioic acid (EACA) has a long history of use during liver transplantation, and its empiric use has been generally safe and well tolerated. Dosing regimens for EACA are based on observational studies that were not blinded or controlled, or in which intraoperative transfusion was used as the primary endpoint for analysis.[5,6] Furthermore, dosing regimens adapted for use during liver transplantation are based on those established for patients without liver disease, and do not account for markedly altered volume of distribution in patients with ESLD.[7]

Recognizing the lack of reliable endpoints for antifibrinolytic therapy, this study describes the first attempts to develop a new quantitative assay for studying the effectiveness of EACA therapy in patients undergoing liver transplantation. Human fibrinogen was labeled with the fluorescent marker fluorescein isothiocyanate (FITC). The labeled FITC-fibrinogen exhibits a predictable fluorescence pattern, which is quenched when the fibrinogen is cross-linked due to close proximity of the fluorescent FITC molecules.[8] On the other hand, fluorescence of the engineered target molecule increases (dequenches) with disruption of the fibrinogen cross-linking (fibrinolysis).[9] Thus the fluorescence pattern is a quantitative indicator of fibrinogen cross-linking, and is sensitive enough to detect even picomolar levels of plasminogen activators and inhibitors.[10] Here we describe preliminary results using this

Abbreviations: FITC, fluorescein isothiocyanate; EACA, ϵ- aminocaproic acid; ESLD, end-stage liver disease; ELISA, enzyme linked immunosorbent assay; HPLC, high pressure liquid chromatography; OPA, o-phtalaldehyde.

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novel assay to quantitate EACA effectiveness in patients presenting for liver transplantation.

Patients and Methods

Blood Collection

This study was approved by the University of Michigan Institutional Review Board, and informed consent in writing was obtained from all patients. All adult liver-transplant patients were suitable for study, except those with fulminant hepatic failure or history of thrombosis. After induction of general anesthesia, a radial arterial catheter was placed. A baseline (pre-EACA) blood sample was drawn from the arterial catheter (20 ml). All blood samples were collected into plastic centrifuge tubes, stored on ice until centrifugation, 1,300 g for 5 minutes, then the plasma was stored at −80°C until assay. EACA was administered, 5 g bolus followed by 1 g/hr IV infusion. At the end of this hour (during which vascular access was established, and before surgical incision), the second blood sample was obtained. Demographic data from each patient were collected at the time of study from the hospital record.

FITC-Fibrinogen Labeling

FITC (Molecular Probes, Eugene, OR), 10 mg/ml, and human fibrinogen (Enzyme Research Labs, South Bend, IN), 10 mg/ml, were incubated at 22°C in 0.1 M sodium bicarbonate (pH 9.0) for one hour with stirring. Addition of hydroxylamine (0.15 M final) stopped the reaction. Unbound FITC molecules were removed via dialysis in 0.1 sodium bicarbonate (pH 9.0) at 4°C overnight. The solution was transferred to 100,000 molecular weight cutoff (MWCO) cellulose-ester tubing (Spectrum Laboratories, Rancho Dominguez, CA) and dialyzed overnight with two buffer changes of 1.5 L each. Final product concentration was assayed at 280 nm with a Beckman DU530 Spectrophotometer and then stored at −80°C.

Fluorometry

A Perkin-Elmer LS-50B fluorometer was used for fluorescence assays. Scanning was performed at 488 nm excitation and 520 nm emission wavelengths and a slit width of 5 mm, and FITC-fibrinogen concentration of 2 mg/ml. Cuvettes were preincubated with 2 mg/ml bovine serum albumin (Sigma, St Louis, MO) for at least 30 minutes prior to use to minimize substrate adherence to cuvette walls. For each reaction, reagents were added to the cuvette in the following order: 10× buffer (final concentration: 0.1 M NaC1, 0.05 M Tris-HC1, 5 mM $CaC1_2$); water to normalize volume; FITC-fibrinogen; plasmin (0.5 mg/ml (American Diagnostica, Greenwich, CT); then EACA or plasma, depending on the assay. Thirty microliters of plasma were used per sample. Data were collected for 1,200 seconds.

Statistical Analysis

Curve slopes were calculated using computer algorithms. Comparisons of slopes generated by pre- and post-EACA plasma samples added to the target FITC-fibrinogen were done using paired *t* tests, with $P < 0.05$ considered a significant difference. Slopes were analyzed over several different times in the reaction, in order to more fully characterize the fibrinogen cross-linking potency of plasma before and after EACA administration. Data are presented as mean ± standard deviation (SD), except as noted.

HPLC Measurement of Plasma EACA Levels

High-pressure liquid chromatography (HPLC) for EACA was performed based on published protocols with some modifications.[12,13] One hundred microliters of plasma (containing tranexamic acid as an internal standard) were analyzed. Ten microliters of 10% zinc sulfate was added, followed by 100 μl of acetonitrile. After mixing and centrifugation, 50 μl of clear supernatant was transferred to an injection vial containing 300 μl borate buffer. Just prior to injection, 50 μl of OPA (20mg o-phtalaldehyde and 25 mg N-acetyl-L cysteine in 6mL 50% methanol/water) was added for derivatization. The derivatized sample was separated on a 15 × 4.6 cm Nucleosil 5, C18 column, and eluted with a mobile phase consisting of 13% acetonitrile in buffer (copper sulfate, proline, and ammonium acetate) and delivered at 2 ml/min. The OPA derivative of EACA was monitored using a fluorescence detector (Shimadzu 551) set at 252 nm (excitation) and 450 nm (emission).

ELISA Measurement of D-dimer Levels

D-dimer levels in plasma samples were quantitated by enzyme linked immunosorbent assay (ELISA) using a commercial kit according to the manufacturer's instructions (Diagnostica Stago, Asnières, France). The normal D-dimer level in this assay is <400 ng/ml.

Results

Demographic Data on Study Patients

Mean age of the patients was 46 ± 10 years. Two were women, and eight were men. Four patients had hepatitis C; four patients had hepatitis C and alcoholic cirrhosis; one patient had hepatitis B; and one patient was transplanted for primary sclerosing cholangitis. Complete blood count and coagulation profiles (mean ± SD.) are shown in Table 1. As expected, patients were thrombocytopenic, and coagulation profiles were consistent with moderate hepatic synthetic dysfunction.

FITC-Fibrinogen Fluorescence is Quenched by Therapeutic Concentrations of EACA

The sensitivity of the FITC-fibrinogen reporter system was assayed by adding EACA (Abbott Laboratories, Abbott Park, IL) at 100 μg/ml or 400 μg/ml to the

Table 1. Preoperative Blood Count and Coagulation Profiles of Study Patients

	Mean	SD
Hemoglobin (g/dl)	11.0	0.8
Hematocrit (%)	32.5	5.1
Platelets (/mm^3)	64,000	21,000
Prothrombin time (s)	14.3	1.7
Int'l normalized ratio	1.44	0.3
Partial thromboplastin time (s)	40.8	10.0
Fibrinogen (mg/dl)	160	47

Abbreviation: s, second. Laboratory data from study patients undergoing liver transplantation (n = 10) just prior to surgery.

Table 2. Slopes of Curves Generated by Fluorometric Analysis of FITC-Fibrinogen Signaling in the Presence of Patient Plasma Before and After EACA Treatment

	Slopes (mean ± SD)		
Times Assayed (seconds)	Pre-EACA	Post-EACA	*P* Value
300–400	1.74 ± 0.54	2.49 ± 0.65	0.72
300–500	1.21 ± 0.30	1.49 ± 0.41	0.66
300–700	1.05 ± 0.24	1.25 ± 0.31	0.30

FITC-fibrinogen cross-linking was assessed in the presence of plasma from ESLD patients collected before and after EACA (5 g bolus followed by a 1 g/hr intravenous infusion). In this assay, increased fibrinogen cross-linking is indicated by a reduction in slope (quenching). Plasma after EACA treatment did not significantly alter fibrinogen cross-linking compared to baseline plasma, as assesed by paired *t* testing.

FITC-fibrinogen target molecule in the presence of plasmin. These EACA concentrations were chosen because they bracket the manufacturer's recommended therapeutic concentration (130 μg/ml) as well as therapeutic concentration (260 μg/ml) suggested for children undergoing cardiopulmonary bypass, where an increased volume of distribution is assumed.[11] Furthermore, the assay used in this study is sensitive to 0.1 to 10 mM EACA, well below the levels achieved in plasma.[9] EACA at either 100 or 400 μg/ml significantly increased fibrinogen cross-linking as assessed by fluorescence emission slope patterns, and there was more quenching of fluorescence (more cross-linking) at the higher concentration of EACA than the lower (Fig. 1).

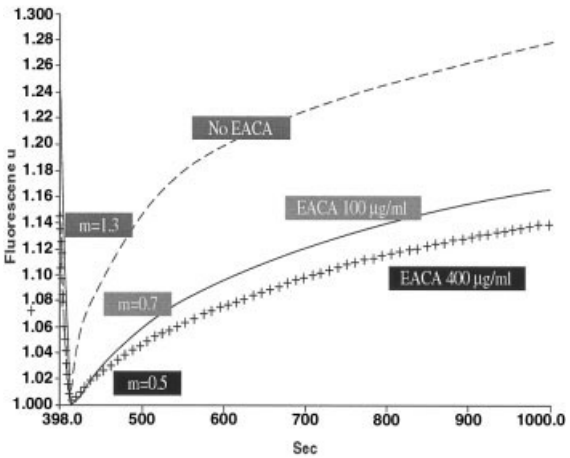

Figure 1. Fluorescence patterns of FITC-fibrinogen in the presence of plasmin and EACA. Y axis is arbitrary fluorescence units; x axis is time in seconds. EACA concentrations of 100 μg/ml and 400 μg/ml, which bracket recommended therapeutic levels, both quench the fluorescence signal. Slopes (m) were measured over the first 200 seconds of the reaction. Significant cross-linking of the target FITC-fibrinogen construct occurs in the presence of 100 or 400 μg/ml EACA, confirming sensitivity of the assay.

Usual Doses of EACA Do Not Result in Enhanced Fibrinogen Cross-Linking by Plasma In Vitro

Analysis of fluorescence patterns revealed that maximum quenching (indicating maximum fibrinogen cross-linking) occurred early in the reaction after addition of plasma to the FITC-fibrinogen target in the presence of plasmin. The fluorescence quenching data are presented in Table 2, showing the calculated slopes

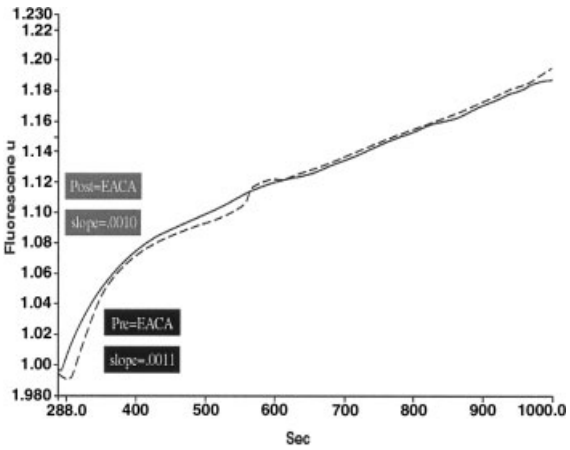

Figure 2. Fluorescence pattern of FITC-fibrinogen generated in the presence of plasma before EACA treatment, and after EACA. The two signals are virtually indistinguishable, indicating no change in target fibrinogen cross-linking after treatment. Slope (m) was calculated over the first 200 seconds of the reaction.

integrated over various times in the reaction. A representative assay from a single patient's samples is depicted in Figure 2, illustrating the time-course of a typical in vitro fluorescence quenching reaction. In Figure 2, the pre-EACA plasma and post-EACA plasma of a single patient were not different from each other. Not only was there no significant increase in fibrinogen cross-linking induced by plasma after EACA therapy on average, but also in some patients the post-treatment plasma actually caused dequenching of the baseline signal.

EACA, 5 g Bolus then 1 g/hr Infusion, Achieves "Therapeutic" Plasma Concentration in Patients With ESLD, Despite Increased Volume of Distribution

To determine whether subtherapeutic drug levels contributed to the lack of potency in fibrinogen cross-linking, EACA plasma levels were measured. All pre-EACA (baseline) plasma samples contained no EACA by HPLC. The range of levels in posttreatment plasma was 207 to 501 ug/ml with a mean of 346 ± 36 ug/ml (SE). These levels approximate those considered therapeutic in other surgical settings.

D-Dimer Levels Before and After EACA

D-dimer levels varied widely at baseline, but did not change significantly in post-EACA plasma samples. Mean D-dimer levels were 1,554 ± 524 (SE) at baseline, and were 2,253 ± 664 ng/ml after EACA ($P = 0.21$).

Discussion

The study represents a first step in developing quantitative methodologies for studying antifibrinolytic drugs, with a long-term goal of using this methodology to refine antifibrinolytic therapy for patients with end-stage liver disease undergoing liver transplantation. Details about the aberrations in pharmacokinetics and pharmacodynamics of drugs used in the intraoperative care of cirrhotic patients are remarkably limited, partly because these patients often have disordered renal and cardiac function and are inherently difficult to study.[14] EACA is not an exception to this information void, despite its potential importance in liver transplantation.

Though many transplant anesthesiologists feel that EACA (or the related lysine analog antifibrinolytic, tranexamic acid) plays an important role in preventing blood loss during liver transplantation, EACA has not been formally studied with objective, quantitative endpoints of its effectiveness in this setting.[15] Furthermore, studies on antifibrinolytic drugs during liver transplantation have been difficult to interpret because of methodological limitations (historic controls, small numbers of patients, wide patient variability in bleeding risk, varied experience of the transplant teams) as reviewed in Kufner.[16] The most influential study supporting the use of EACA during transplantation was not controlled, but it suggested that EACA 1 g should be given as needed with thrombelastographic evidence of fibrinolysis.[5] Twenty patients were treated in this study. A subsequent retrospective analysis of 36 patients (15 received EACA) showed no benefit to using EACA in preventing blood loss during liver transplantation.[17] A later description of clinical experience (without data presented) suggested that doses as low as 250 mg (instead of 1 g) could be used in response to thrombelastographic evidence of fibrinolysis.[18] Only one blinded, prospective analysis of EACA has been reported in the liver transplant literature. In this report, 42 liver transplant patients received EACA (6 mg/hr), and 40 did not. EACA reduced transfusion requirements but not significantly.[6] Taken together, EACA has been formally studied in very few liver transplant patients, and the published endpoints of analysis in these studies are difficult to interpret. Furthermore, all studies to date have used fixed dosing regimens, though the degree of fibrinolysis during transplantation is widely variable and likely to require widely varying dosages of antifibrinolytic therapy. Development of methods as presented in this study, combined with rigor in study design as well as larger studies, may be useful to readdress the value of antifibrinolytic therapies in liver transplant recipients.

Patients with end-stage liver disease have both an effective increase in total blood volume and an increased volume of distribution for highly water-soluble drugs.[19] The increase in volume of distribution has been best characterized for neuromuscular blockers and was first recognized for d-tubocurarine.[20] For example, patients with end-stage liver disease have an estimated 50% increase in the volume of distribution for pancuronium.[21]

Though the pharmacokinetics of EACA in end-stage liver disease patients have not been directly addressed, investigations in other surgical populations may be informative for liver transplant recipients. Recently EACA pharmacokinetics have been studied and modeled extensively in patients undergoing cardiopulmonary bypass. These patients also develop fibrinolysis during surgery, though the molecular mechanisms underlying fibrinolysis during cardiopulmonary bypass and during liver transplantation are likely to be different. Nonetheless, cardiopulmonary bypass patients,

like those undergoing liver transplantation, have increased volume of distribution for EACA such that these studies may be somewhat informative for cirrhotic patients. Based on measurement of EACA levels and pharmacokinetic models, Butterworth found that a loading dose of 70 mg/kg followed by an infusion of 30 mg/kg/hr would maintain EACA levels at approximately theoretical therapeutic concentration in patients undergoing cardiopulmonary bypass.[22] This recommended loading dose is approximately the same used in our study, but the recommended infusion rate is about twice that used in our study. Our results indicate that approximately the same levels of drug are achieved in patients with ESLD as in those undergoing cardiopulmonary bypass.

Nonetheless, the new assay used in this study suggested that EACA at these plasma levels has no functional antifibrinolytic effect *on average* in patients with ESLD. When data from individual patients are analyzed, however, some patients showed increased fibrinogen cross-linking in response to EACA, though most did not. Similarly, clinicians who use thrombelastography to monitor EACA therapy can detect wide variance in the qualitative response to therapy. Our pooled data suggest that this EACA dose (and plasma levels) may not be sufficient for a functional effect on plasminogen and plasmin to effect fibrinogen cross-linking, and more drug may be required in patients with ESLD. Furthermore, the data suggest that the risk of inducing too much procoagulant effect is unlikely using EACA in patients with ESLD. One theoretical issue raised by the results is the possibility that functional inhibitors of plasmin are present in the plasma of patients with end-stage liver disease. These issues require further study in order to optimize intraoperative antifibrinolytic therapy.

D-dimer levels are used to monitor EACA therapy in some surgical settings (for example, cardiac surgery), and the absence of an expected rise in D-dimers is considered an indication that fibrinolysis has been treated.[23] Degradation of cross-linked fibrin causes release of products that are measured in D-dimer assays. These assays are used clinically to quantify of the amount of active fibrin formation and lysis.[24] In this study, D-dimer levels suggested no antifibrinolytic activity by the infused EACA, supporting the fluorescence assay. Although surgical incision was not made before both sets of blood were collected, large bore catheters including percutaneous venovenous bypass lines were inserted during the hour of EACA therapy. In some patients these procedures may have contributed to an increase in D-dimer levels over the hour of EACA infusion. On average, D-dimer levels increased during the EACA infusion, but not significantly so, a pattern similar to that obtained fluorometrically in FITC-fibrinogen cross-linking assay. Surprisingly, D-dimer levels have not been reported as outcome monitors for EACA therapy during liver transplantation, and thus the correlation between the FITC-fibrinogen assay and D-dimer levels will also require further study.

In conclusion, progress in modeling pharmacokinetics and pharmacodynamics of EACA, as acknowledged in published reports, is hindered by a lack of functional studies to assess antifibrinolytic effectiveness.[11,12] The approach taken here can be used to augment our understanding of the optimal regimens for EACA not only in liver transplant patients, but also in other patient populations that may benefit from EACA therapy.

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