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Plasma proteolytic activity in liver transplant rejection

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Abstract In this study, we evaluated the role of proteolytic enzymes belonging to the coagulation, fibrinolytic, and plasma contact systems in the early postoperative phase after orthotopic liver transplantation (OLT). Twenty-nine patients were studied at the time of OLT and during the first 2 postoperative weeks. Blood samples were collected daily after OLT and analyzed for kallikrein-like activity (KK), functional kallikrein inhibition (KKI), plasmin-like activity (PL), and α_2-antiplasmin (AP). In addition, prekallikrein (PKK), prothrombin (PTH), antithrombin III (AT III), plasminogen (PLG), prothrombin/antithrombin III complexes (TAT), prothrombin fragment 1 + 2 (F 1 + 2), and plasmin/α_2-antiplasmin complexes (PAP) were measured. Nineteen patients experienced biopsy-verified acute rejections (AR) and ten patients had uneventful courses and served as controls. Plasma analyses showed that the contact, coagulation, and fibrinolytic systems were activated during OLT. Following OLT, continuous thrombin and plasmin generation was observed, and these effects were more pronounced in the group having an uneventful course than in patients with AR. Factors that could possibly affect plasma proteolytic activity, such as blood product usage during and after OLT and cold ischemia time of the liver graft, did not differ between the groups, nor did the routine liver function tests, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Key words Proteolytic activity, rejection, liver transplantation · Rejection, liver transplantation · Liver transplantation, proteolytic activity, rejection

Introduction

Hemostasis is the result of a fine balance between activation and inhibition of the coagulation, kallikrein-kinin, and fibrinolytic systems. During orthotopic liver transplantation (OLT), both the coagulation and fibrinolytic systems are activated [13, 23, 25], and activation of complement has also been demonstrated [27, 31]. Recently, we have found activation of the kallikrein-kinin system with liberation of bradykinin in the reperfusion phase of OLT [28]. The role of these plasma protease systems and their natural inhibitors in the development of early complications after OLT is uncertain, although many of the components of these systems are synthesized and metabolized in the liver [17, 21].

Acute rejection (AR) in the early postoperative phase following OLT occurs with a prevalence of 40%–70% and is still a major obstacle to increased graft survival in spite of improvements in immunosuppression [5, 14, 16, 33].

Proenzymes, enzymes, and inhibitors of the kallikrein-kinin, coagulation, and fibrinolytic systems are well established as central diagnostic parameters in the evaluation of critically ill patients [2]. Hepatic circulato-

Table 1 Indications for OLT and patient characteristics

Indication	*n*	F	M	Age (years)
Primary biliary cirrhosis	8	7	1	18–58
Primary sclerosing cholangitis	6	3	3	25–62
Posthepatic cirrhosis	5	3	2	10–62
Hepatocellular carcinoma	3	1	2	22–46
Autoimmune hepatitis	1	1	0	60
Laennec's cirrhosis	2	0	2	56–63
Cryptogenic cirrhosis	1	1	0	55
Metastatic cancer	1	0	1	40
Polycystic liver/kidney disease	1	1	0	57
Acute hepatic failure	1	1	0	43
	29	18	11	Mean = 46

ry changes have an immediate effect on the synthesis and metabolism of these factors [21]. Alterations in plasma activities of components of the cascade systems after OLT, including complications such as AR, can thus be expected.

The purpose of this study was to characterize and evaluate the plasma activity profiles of key factors of the coagulation, kallikrein-kinin, and fibrinolytic systems after OLT and to assess whether these activities are affected by AR.

Materials and methods

Twenty-nine patients (18 female ad 11 male; mean age 46 years, range 10–63 years) who underwent OLT at Baylor University Medical Center, Dallas, Texas, USA ($n = 9$) and at The National Hospital, Oslo, Norway ($n = 20$) from September 1991 until September 1994 were studied (Table 1). All patients gave their informed consent to the study, which followed the ethical standards of the 1964 Declaration of Helsinki. The operative procedures used at the two centers were similar.

The same triple-drug immunosuppression protocol was used by both centers. Immunosuppression with cyclosporin A (Sandimmun, Sandoz, East Hanover, N.J., USA), azathioprine (Imurel, The Welcome Foundation, London, UK), and methylprednisolone (Solu-Medrol, Pharmacia & Upjohn, New Jersey, New York, USA) was used in 28 patients. One patient received tacrolimus (Prograf, Fujisawa, Japan) and methylprednisolone, and one patient was converted from triple therapy to tacrolimus and methylprednisolone.

Blood samples were collected daily, in addition to routine liver function tests, from a central venous line or by puncture of a peripheral vein, and the blood samples were divided into tubes containing trisodium citrate or ethylene diamine tetra-acetic acid (EDTA). After centrifugation at 2000 *g* for 10 min, the plasmas were removed and stored in 500-µml aliquots at –70 °C. Plastic syringes, tubes, and pipettes were used throughout to reduce the risk of contact system activation during the processing and storage of the plasmas. The plasma samples were rapidly thawed at 37 °C to limit the effects of cold activation of the contact system [3, 4, 6].

KK-like activity (KK), prekallikrein (PKK), kallikrein inhibition (KKI), prothrombin (PRTH), antithrombin III (AT III), plasminogen (PLG), plasmin-like activity (PL), and α_2-antiplasmin (AP) values in plasma were determined by functional chromogenic substrate assays, as previously described [3, 4, 6], using an automated enzyme analyzer (Cobas Bio, Hoffman La Roche, Basel, Switzerland). Precision studies of the assays gave coefficients of variation that were all less than 5% [4]. KK and PL values were expressed as units per liter (U/l). Normal values for KK (14 ± 11, range 0–54) and PL (33 ± 29, range 2–144) were obtained in plasma samples from 118 healthy control volunteers. All other enzyme activities were expressed as percentages of the values found in a normal plasma pool prepared from plasma samples from the 118 healthy donors.

Proenzyme functional inhibition index (PFI) is defined as the sum of deviations from the control values for proenzymes and functional inhibition values of the coagulation, fibrinolytic, and kallikrein-kinin systems. Increased values are counted as positive, whereas reductions compared with the normal plasma pool values are recognized as negative [2]. The following specific parameters are included in the PFI index calculation: PRTH, AT III, PLG, AP, PKK, and KKI. We have included this index as it has been proven to be useful in evaluating the generalized proteolytic activation of patients with septicemia and of severely injured patients [1].

Plasmin/α_2-antiplasmin complex (PAP), thrombin/antithrombin III complex (TAT), and prothrombin fragment F 1 + 2 (F 1 + 2) levels were determined by enzyme immunoassay techniques (Enzygnost, Behringwerke, Marburg, Germany). PAP and TAT values were expressed as micrograms per liter (µg/l) and F 1 + 2 values as nanomoles per liter (nmol/l). Normal values for PAP (median 214, range 99–368 µg/l; 2.5%–97.5% percentiles), TAT (median 1.5, range 1.0–4.1 µg/l; 2.5%–97.5% percentiles), and F 1 + 2 (median 0.7, range 0.44–1.1 nmol/l; 2.5%–97.5% percentiles) were those assigned by the kit manufacturer.

Rejection diagnosis was based on clinical, biochemical, and histopathological findings. In this study acute rejection (AR) was defined as the time at which a liver biopsy was taken either as a protocol biopsy or due to clinical suspicion of AR and in fact revealed AR [7, 9]. Treatment was then initiated with doses of 1 g of methylprednisolone within the first 2 weeks after transplantation. None of the patients in the AR group had any other significant complications at the time of AR. For each patient in the AR group, the biopsy-verified diagnosis of rejection was defined as day 0, and the time span from day –5 to 5 is given with 2-day intervals in the figures. Nineteen patients experienced biopsy-verified AR and ten patients had uneventful courses and served as controls.

The ten patients in the complication-free (CF) group did not experience any significant complications during the observation period of 2 weeks. The clinical course, biochemical parameters for liver function, and protocol-based liver biopsies in this group did not reveal signs of AR. The mean 1st day of rejection in the AR group was day 6 postoperatively (5.74 ± 0.32, mean ± SEM) and day 6 was thus defined as day 0 in the CF group in order to compare the groups at equivalent time points postoperatively.

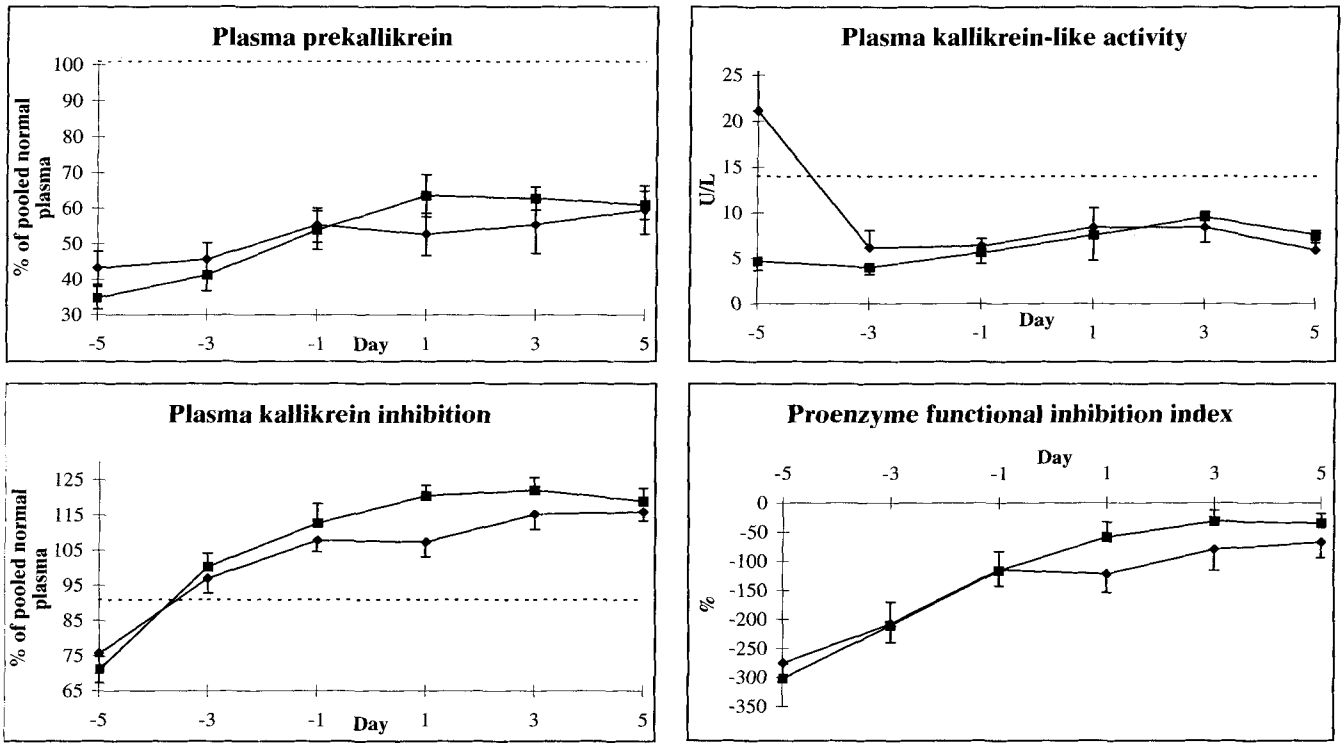

Fig. 1 *The kallikrein-kinin system and PFI index following OLT. The observation period is given as 5 days proor to and after the time of acute rejection in 2-day intervals. Values are expressed as mean + SEM (-◆- acute rejection group, -■- complication-free group, ---- mean normal value)*

The results for the same time span from day –5 to day 5 in 2-day interval as for the AR group are presented in the figures.

An objective of our study was to compare two groups of OLT patients, the only difference between them being the occurrence of AR in one of the patient groups. Patients excluded from the study due to septic symptoms experienced three or more of the following characteristics at some point during the observation period: (1) temperature above 38 °C, (2) the need for respiratory support, (3) leukocyte counts above 15×10^9/l or below 0.5×10^9/l, (4) thrombocyte counts below 100×10^9/l, and (5) a positive blood culture or an obvious septic focus. Patients with vascular thrombosis of the liver graft or biliary obstruction or leakage were also excluded.

Values for the individual groups of parameters are expressed as mean ± SEM. An independent samples *t*-test was used for comparisons between the groups. *P* values less than 0.05 were considered significant and Bonferroni's correction for multiple comparisons was included to account for chance findings.

Results

Contact activation

KK-like activity was higher in the AR group than in the CF group immediately post-transplantation (Fig. 1). The KK-like activity in the AR group decreased, however, to lower values than the mean normal value (14 U/l) by day –3, and KK-like activities remained low throughout the observation period in both groups. PKK values in both groups were very low on day –5. In the AR group, PKK started to rise but leveled off at the time of rejection and did not reach 60% of the mean normal value (100%) during the observation period (Fig. 1). In the CF group, PKK values did not exceed 70% of normal throughout the observation period. Functional KKI activities were reduced on day –5 but reached values above the mean normal value (91%) after 2 days and rose further after this time in both groups (Fig. 1).

Coagulation

PRTH values in both groups were lower than the mean normal value of 100% (38% ± 5.2% and 41% ± 4.4%, mean ± SEM in the AR and CF groups respectively) on day –5 (Fig. 2). A rise was seen in both groups preceding the time of rejection, whereafter PRTH values remained unchanged in the AR group during the next 5 days. In the CF group, PRTH values tended to rise by day 1, whereafter the increase leveled off. AT III values in both the AR and CF groups were also significantly lower (50% and 44%, respectively) than the mean normal value (106%) on day –5 (Fig. 2). A rise above normal values from 109% on day 1 to 123% and 117% on days 3 and 5, respectively, was found in the CF group,

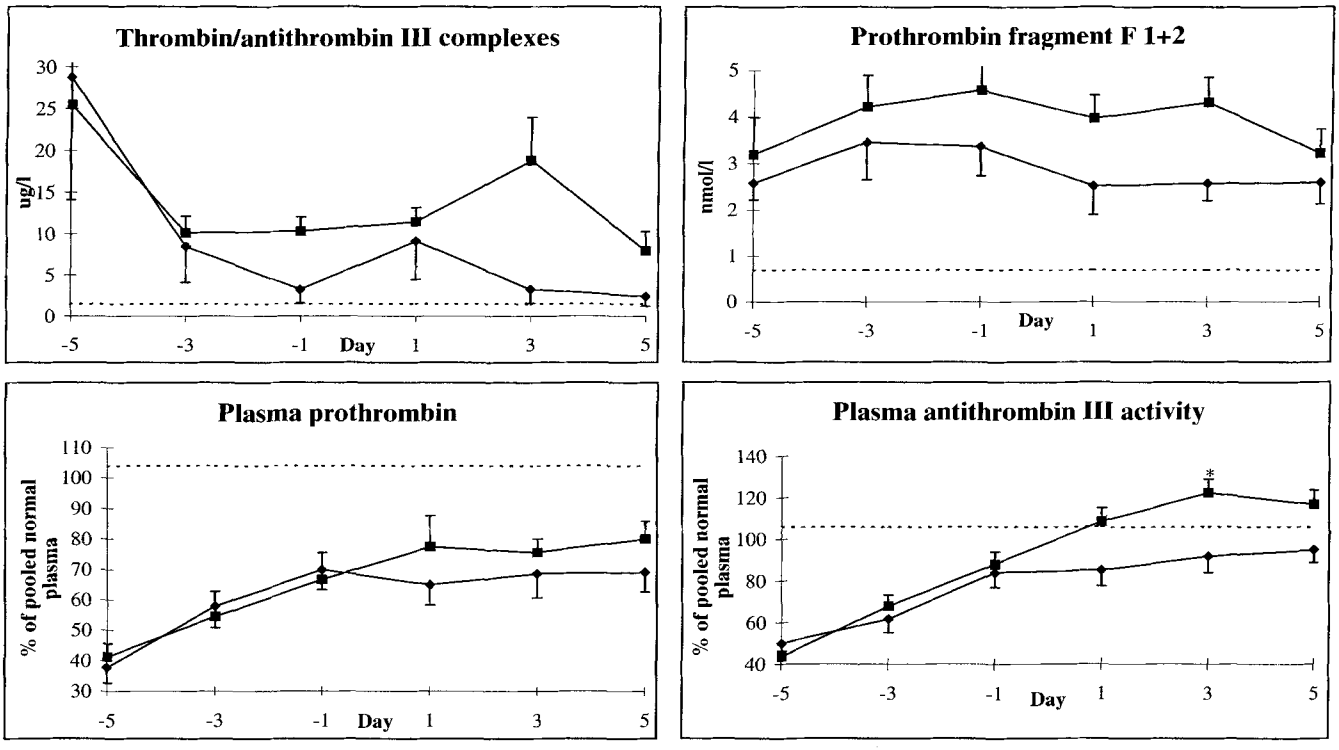

Fig. 2 The coagulation system following OLT. The observation period is given as 5 days prior to and after the time of acute rejection in 2-day intervals. Values are expressed as mean + SEM (-◆- acute rejection group, -■- complication-free group, ---- mean normal value) * $P \leq 0.05$

while AT III values in the AR group were significantly lower than those in the CF group on day 3 (92%, $P < 0.05$). TAT complexes were quite elevated on day -5 in both groups (19-fold in the AR group and 17-fold in the CF group) compared to the reference value of 1.5 µg/l found in healthy normals, but they fell markedly during the next days (Fig. 2). After day -3, TAT values were lower in the AR group than in the CF group for the rest of the observation period, though the values for both groups were above normal throughout the entire observation period. F 1 + 2 values were much higher in both patient groups (0.7 nmol/l) than in healthy controls throughout the whole period (Fig. 2). The values for the CF group were well above those of the AR group, and the difference was most pronounced on days 1 and 3.

Fibrinolytic activity

PLG values on day -5 were significantly lower in both the AR and CF groups (49% and 37%, respectively) than those in the healthy controls (100%) and did not reach normal values at any time during the observation period (Fig. 3). PLG values rose gradually in both groups until day -1; they tended to rise further in the CF group, while in the AR group a slight, insignificant reduction was seen on day 1 and values remained lower until day 5. Plasmin-like activity (PL) was highest in the AR group on day -5, but on days -3 and -1 these enzyme activities were found to be within the normal range in both groups (Fig. 3). From days 1 to 5, the PL values rose in both groups, but the highest values were in the CF group, with the most pronounced difference on day 1 (57 U/l vs 28 U/l, respectively). AP values were significantly lower than normal on day -5 in both groups but rose to normal values in both groups by day 0 (Fig. 3). PAP complexes were significantly higher than normal values in both groups throughout the study period (Fig. 3). The values peaked on day -1 in the AR group and then started to fall, while in the CF group they continued to rise until day 3, when they were significantly higher than in the AR group (1006 µg/l and 2092 µg/l, respectively, $P = 0.005$).

PFI index

The PFI index revealed very low values in both groups on day -5 (Fig. 1). In the CF group, the values rose and approached the normal value (0) while in the AR group the values tended to fall from day -1 to day 1, whereafter a gradual increase was found.

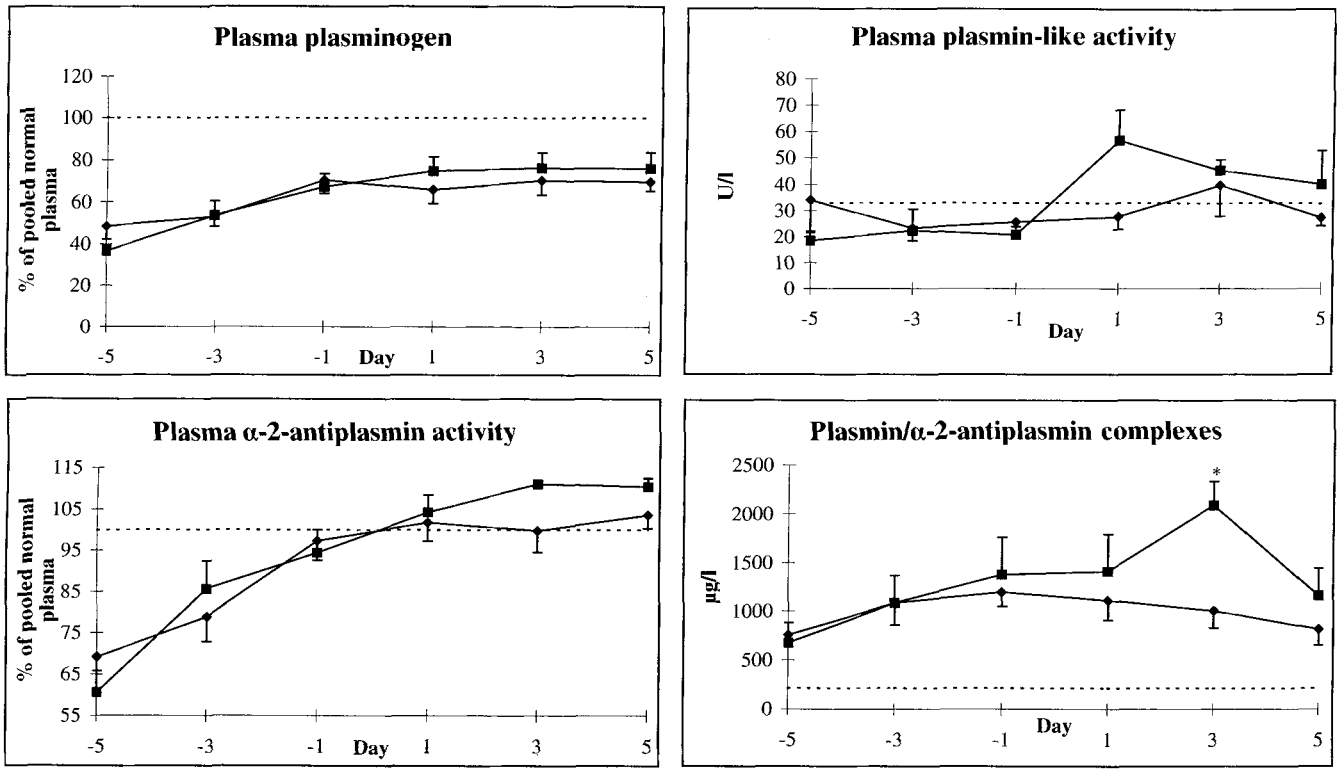

Fig. 3 The fibrinolytic system following OLT. The observation period is given as 5 days prior to and after the time of acute rejection in 2-day intervals. Values are expressed as mean + SEM (-◆- acute rejection group, -■- complication-free group, ---- mean normal value) $*P \leq 0.05$

Blood usage, bilirubin, transaminases, and cold ischemia time

The use of packed red blood cells (PRBC), fresh-frozen plasma (FFP), and platelets was recorded during and after OLT. No differences with respect to the use of blood products were found between the two groups (Table 2). The routine liver function tests – bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) – were recorded on day 6 postoperatively and did not differ between the groups (Table 2). The cold ischemia time of the liver grafts was equal in the two groups (Table 2).

Discussion

In this study, we found activation of the coagulation, fibrinolytic, and kallikrein-kinin systems following OLT in the patients experiencing AR as well as in the CF group. Markedly elevated values of both thrombin and plasmin in complex AT III and AP, respectively, were found. In addition, very high values of F 1 + 2, a specific marker of thrombin generation, was observed. We found that the plasma cascade system activation was more pronounced in patients with uneventful courses following OLT. Proenzyme values were generally low, probably due to consumption, as a result of activation, and decreased synthesis. Inhibitor values reached the normal range 2–6 days after OLT, and values for functional kallikrein inhibition rose well above normal values, giving evidence of rapid restoration of synthesis of these proteins in the liver graft. The inhibitor levels tended to be higher in the CF group, possibly correlating with the recovery of liver function. Based on our findings, it seems that an ongoing, increased activation of the plasma cascade systems is part of a regular phenomenon following OLT and that reduced proteolytic activity can be associated with AR. Other factors that may have an impact upon the plasma factors monitored, such as blood product usage during and after OLT, cold ischemia time of the donor livers, and liver graft function, as reflected by ALT and AST, were evaluated (Table 2). However, no differences between the two groups with respect to these parameters were found. Bilirubin and ALT values tended to be higher but did not reach statistical significance in the AR group on day 6, the mean day of acute rejection.

Activation of plasma protease systems, including the complement, kallikrein-kinin, coagulation, and fibrino-

Table 2 Blood usage, liver function tests, and cold ischemia time in OLT (*PRBC* packed red blood cells, *FFP* fresh-frozen plasma, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase)

Variable	PRBC[a] (Units)	Platelets[a] (Units)	FFP[a] (Units)	PRBC (Units)	Platelets (Units)	FFP (Units)	ALT[b] U/l	AST[b] U/l	Bilirubin[b] μmol/l	Cold ischemia time (hours)
Rejection group:										
Mean	9	6	7	2	4	2	182	112	156	9
SEM	1.3	1.1	1.8	0.4	3.6	1.8	30.4	21	25.3	0.7
Complication-free group:										
Mean	15	6	14	1	0	0	67	88	59	11
SEM	7.7	2.6	5.2	0.6	0	0	10.6	19.6	10.9	1.5

[a] Blood product usage during operation. Only blood products given before or during the 11-day observation period are recorded
[b] ALT, AST, and bilirubin values were recorded on day 6 postoperatively in both groups
No statistically significant differences between the groups were found for any variable

lytic systems, is known to occur during OLT and is attributed to both the underlying liver disease and to the surgical trauma [11, 13, 15, 22–24, 27, 28, 31]. Investigations on blood coagulation before and during OLT have mainly been focused on the derangements caused by the transplantation procedure in order to reduce bleeding and blood product requirements [19]. Intraoperative impairments in hemostasis leading to excessive blood usage have been related to higher rates of postoperative complications and have been found to influence graft and patient survival [18, 20]. However, the degree of activation of these systems during the early postoperative phase of OLT in relation to the occurrence of acute complications such as AR and outcome has, to our knowledge, not been extensively investigated, although the production of most coagulation factors takes place in the liver [13]. Furthermore, the important role of the liver in hemostasis has been illustrated by the correction of inherited coagulation defects by OLT [10, 19]. Previous studies have also indicated that parameters of the coagulation system are of importance as predictive indicators of kidney transplant rejection [29].

Our studies have revealed the following variations in levels of some components of the kallikrein-kinin, coagulation, and fibrinolytic systems, which lead us to believe that activation of these systems occurs not only during OLT but also during the period following OLT:

1. The kallikrein-kinin system: Activation of the kallikrein-kinin system during OLT was demonstrated in an earlier study by our group [28]. In the present study, we found increased KK activity in combination with a low PKK level and decreased KKI activity in the AR group on day –5, suggesting activation of this system after OLT. In the CF group, with an uneventful postoperative course, the PKK, KK, and KKI values were all below normal on day –5. Thus, there seems to be a different level of activation of the kallikrein-kinin system immediately after OLT in the two groups. Inhibitory values rose to above normal on day –3 in both groups and continued to rise, possibly due to acute phase synthesis of complement factor 1 esterase inhibitor (C1-INH), a major plasma inhibitor of plasma kallikrein [8]. PKK and KK values remained well below normal values in both groups throughout the observation period. The PKK and KKI values tended to be slightly lower in the AR group from the day of rejection; this could have been caused by further contact activation or by a fall in the production of PKK and kallikrein inhibitors in this group.

2. The coagulation system: Following OLT, a hypercoagulable state persisted, which is in accordance with the findings of others [30, 32]. This activation of the coagulation system was reflected in highly elevated values of TAT complex and F 1 + 2 in combination with low PRTH values during the entire observation period. Interestingly, this activation was more pronounced in the patients without complications. The AR group and the CF group seemed to diverge at the time of AR, with thrombin generation continuing in the CF group but decreasing in the patients developing AR. AT III values reached the normal range in the CF group by day 1 and were significantly higher than in the AR group on day 3. This suggests that normal, or even enhanced, synthesis of AT III was occurring in the CF group despite the evidence from the TAT complexes and F 1 + 2 data of enhanced thrombin generation.

3. The fibrinolytic system: Evidence of ongoing fibrinolytic activity after OLT was found in both groups, reflected in very low PLG values in combination with markedly elevated PAP complex values during the whole observation period. As with the kallikrein-kinin and coagulation systems, the fibrinolytic system also responded differently in the two groups. On day 0 and throughout the rest of the observation period, all parameters measured continued to increase in the group of patients with an uneventful course, while in the AR group a decreasing pattern for these was found. A sig-

nificant difference was found for PAP complexes on day 3.

The raised levels of TAT and PAP complexes in both patient groups may indicate impaired function of the reticuloendothelial system (RES), which removes enzyme-inhibitor complexes from the circulation [26]. However, it would be expected that KK activities, which reflect KK-α_2-macroglobulin complexes [4], would be elevated in both patient groups and this was not observed (Fig. 1). Also, evidence for ongoing coagulation was detected not only in high values for TAT complexes but also in high values for F 1 + 2, which is a specific indicator of prothrombin activation. One would have expected the RES to be functioning better in the CF group than in the AR group following AR and to yield lower values for TAT and PAP complex levels. In fact, much higher values for these complexes were found in the CF group (Figs. 2, 3).

Despite the observations that proenzyme levels (PKK, PRTH, PLG) failed to reach normal levels in either of the patient groups, the rapid normalization of inhibitory function for all of the systems indicates that the synthetic function of these inhibitors by the grafted liver is quickly restored. The role of these inhibitors is to ensure that no active proteases circulate in the blood and to limit their activities to local targets. The deviations from normal in the other parameters measured were probably due to varying levels of synthesis and activation in the various systems at different time points. This hypothesis is sustained by the PFI index, which indicates differences from day –1 between the two groups (Fig. 1).

The fact that methylprednisolone was administered at the time of AR in the AR group could partly be responsible for the different courses in the two groups from this time point, as it could affect all the plasma cascade systems investigated. However, to our knowledge, the exact effect of methylprednisolone on these systems has not yet been demonstrated in vivo.

Historically, serine proteases such as thrombin, urokinase, and plasmin were thought to function solely to maintain hemostasis. Recent studies have shown, however, that these enzymes stimulate growth of a variety of cells and are involved in tissue repair and wound healing [12, 25]. Although the less pronounced increases in thrombin and plasmin generation observed in the AR group (particularly 3 days after the onset of rejection) could be a consequence of the rejection process, it is possible that activation of the plasma defense systems has protective, rather than deleterious, effects on the transplanted liver, as is currently thought.

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