HEMOSTATIC DISORDERS IN ORTHOTOPIC AND AUXILIARY LIVER TRANSPLANTATION

Clinical and experimental studies

HEMOSTASE AFWIJKINGEN BIJ ORTHOTOPE EN AUXILIAIRE LEVERTRANSPLANTATIES

Klinische en experimentele studies

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A.H.G. RINNOOY KAN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP
WOENSDAG 8 FEBRUARI 1989 OM 15.45 UUR

door

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Geboren te Schiedam

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The publication of this thesis was financially supported by Organon Teknika and The Blood bank of Rotterdam.

Aan mijn ouders

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CHAPTER 1

INTRODUCTION

INTRODUCTION

In patients with severe liver disease, hepatic dysfunction may deteriorate to such an extent that conservative medical treatments are no longer sufficient to maintain the, for life essential, liver functions. For these patients the transplantation of a donor liver represents the only hope for survival.

Livers can be transplanted in two different manners: either in the orthotopic position after removal of the diseased native liver, or in a heterotopic position as an auxiliary liver.

1.1 Orthotopic liver transplantation

Since the first orthotopic liver transplantation (OLT) in man was performed by Starzl in 1963[1], more than 2000 orthotopic liver transplantations have been performed all over the world[2,3]. During the sixties and seventies the results were rather poor with one year survival rates of about 30%[3,4]. Uncontrollable bleeding leading to large amounts of blood loss was the main cause of death in these years[3,4]. After 1980 important improvements in surgical techniques, anesthesiologic management and changes in immunosuppressive treatment have contributed to a steady increase of the survival rate. This has encouraged more centers in the world to start a liver transplantation program for liver patients for which no satisfactory medical therapy is available, and the number of transplants the world over is now almost doubling annually[5]. Although most centers now report one year survival rates of 60-70%, OLT is still associated with a high intraoperative blood loss and the need for large blood transfusions[3,6]. Even in a large center as Pittsburgh, with a long experience, the mean intraoperative use of red blood cell concentrates and fresh frozen plasma in adult first-time liver transplants in 1987 was still 23 and 12 U, respectively and transfusions of more than 50 U red blood cell concentrates are no exception (Dr. J.H. Lewis, personal communication). Intraoperative use of red blood cell concentrates of more than 30 U has been found to be associated with almost 70% mortality[7].

During the last 25 years many investigators have tried to elucidate the origin of this hemorrhagic diathesis. This has resulted in a large number of papers on the changes in hemostasis during OLT and on factors that may predispose for, or correlate with a high intraoperative blood loss. In general, it has been observed that a poor preoperative hemostatic function due to the underlying liver disease and the occurrence of specific intra-

operative deteriorations in the hemostatic system are factors that play an important role in the development of bleeding complications in many patients. However, several investigators have proposed different and sometimes contradictory explanations for their observations and the exact mechanisms underlying the intraoperative hemostatic deterioration are still not fully understood.

1.2 Auxiliary liver transplantation

The first auxiliary liver transplantation was performed by Absolon et al. in 1964[8]. The initial results of auxiliary liver transplantation were discouraging and the number of auxiliary liver transplantations performed so far is much lower than that of OLT. Until 1985, this type of liver transplantation was reported in a total of 48 patients and only two patients survived longer than one year[9]. In spite of these disappointing results the concept of auxiliary liver transplantation was never abandoned completely. Some groups have tried to clarify the reasons of failure and have further investigated new surgical techniques in experimental models. Much of this research work has been performed in The Netherlands and it has led to the development of an improved method using a partial liver graft[10,11]. After the technical feasibility of this modified technique of auxiliary partial liver transplantation (APLT) was proven in a large number of experimental studies[10-14], it was decided to perform this technique in humans also. APLT is currently used in the University Hospital of Rotterdam to treat patients with end-stage liver disease, which were rejected for OLT, or in patients with acute hepatic failure[15,16].

APLT may theoretically be associated with a reduced risk of bleeding since the surgical trauma is less severe. Furthermore, there is no anhepatic phase during the operation, which may prevent the occurrence of serious intraoperative changes in the hemostatic system. This may be an important advantage in patients with a poor preoperative condition and at too high a risk for successful OLT. However, knowledge about the effect of this type of liver transplantation on hemostasis is limited.

1.3 Objectives of the study

In this thesis studies on hemostatic changes during and after OLT and APLT are described. The studies were designed to investigate the mechanisms underlying specific hemostatic disorders that occur during both types of liver transplantation and to study the differences between APLT and OLT,

concerning the effects on the hemostasis mechanism.

The second chapter gives an extensive overview of the literature on hemostasis in liver transplantation. The different hemostatic changes and the proposed underlying mechanisms which have been described in liver transplantation are evaluated and discussed. Based on this review the working hypothesis for the studies described in the next chapters were formulated.

The aims of these studies were:

1. To compare orthotopic and auxiliary partial liver transplantation regarding the changes in the hemostatic system and intraoperative blood loss in animal studies (chapter 3).

2. To study the role of the donor liver in the origin of hemostatic deteriorations in animal studies, after connection of the graft to the recipient's blood circulation (chapter 4).

3. To develop a practical laboratory system for the intraoperative monitoring of hemostasis and anticoagulant therapy during major surgical procedures (chapter 5).

4. To study the origin and systemic effects of hyperfibrinolysis in orthotopic liver transplantation (chapter 6).

4. To make a first evaluation of the changes in hemostasis that may occur during and after auxiliary partial liver transplantation in humans (chapter 7).

5. To study the fibrinolytic system during auxiliary partial liver transplantation in humans and the mechanisms that cause hyperfibrinolysis (chapter 8).

6. To develop an in vitro model for investigations on the regulation of hemostasis proteins synthesis by culturing primary human hepatocytes (chapter 9).

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CHAPTER 2

HEMOSTASIS IN LIVER TRANSPLANTATION: A REVIEW

HEMOSTASIS IN LIVER TRANSPLANTATION: A REVIEW

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This chapter has been submitted for publication

2.1. INTRODUCTION

More than twenty five years ago, Starzl reported the first clinical application of orthotopic liver transplantation (OLT) (1). The patient, who died of blood loss due to hemostatic disturbances, had complications that would become a serious problem in subsequent patients. Although the success rate of OLT has improved markedly in recent years, and OLT is now an accepted treatment for patients with end-stage chronic liver disease (2,3), massive blood loss is still a major problem (4,5,6). In the recently published first Report of the European Liver Transplant Registry, bleeding complications were the most frequent (34%) cause of death intraoperatively and during the first postoperative week (7). Several investigators have demonstrated that large peri-operative blood loss is correlated with a high mortality (9,10). Patients undergoing OLT who received less than 30 U of red blood cells had a survival rate of 70%, whereas the survival rate decreased to about 14% in patients receiving 30 U or more (8). Disturbances in hemostasis appeared to be a major contributing factor to a high blood loss (9,11) and surviving and non-surviving cases of OLT can be partly classified by discriminant analysis of hemostasis parameters (12).

Many studies have examined the pathogenesis of the hemorrhagic diathesis in OLT and have contributed to a better understanding of the hemostatic changes that occur in OLT. Results of several studies however are contradictory. The use of different operation techniques and management procedures and variations in assay methods makes comparison of the results difficult. The relation between specific hemostatic disorders and bleeding complications is still a matter of debate.

To have a better overview of the intraoperative changes in hemostasis that have been found in clinical and experimental OLT and the underlying processes we have surveyed the literature. In view of the renewed interest in the heterotopic transplantation of an auxiliary liver graft, the effects of auxiliary liver transplantation on hemostasis will be compared with those induced by OLT.

2.2. THE HEMOSTASIS SYSTEM AND THE LIVER

Extensive research on hemostasis has led to a marked development of this field of medicine during the last decade. New proteins have been identified and more accurate assays have become available. A schematic presentation of the hemostatic system, based on present insights is given in figure 1.

The hemostatic mechanism is currently regarded as a dynamic balance between coagulation and fibrinolysis. Coagulation results in the formation of a fibrin clot and fibrinolysis leads to the resolution of fibrin clots. Under physiological conditions activities of both processes are in balance and neither thrombotic events nor bleeding disorders are seen. Either enhanced formation or delayed resolution may lead to increased fibrin deposition. Both a delayed formation and an accelerated removal may result in decreased fibrin deposition. This means that thrombosis or bleeding may be due to defects in the coagulation system or in the fibrinolytic system or both (for reviews see 13,14).

The liver plays an important role in the synthesis of coagulation and fibrinolytic proteins (15,16). In addition, the reticulo-endothelial system of the liver contributes to the clearance of hemostasis factors and their degradation products (17,18). In chronic liver disease, disturbances of these functions may lead to complex hemostatic disorders (15,19). The hemostasis disorders are often complicated by thrombocytopenia and by platelet dysfunction and by a defective platelet-vessel wall interaction (15,20). Bleeding complications in patients with severe liver disease are often found in combination with reduced levels of clotting factors (21,22), increased fibrinolytic activity (21,23) and changes in blood platelets (20,24).

Changes in coagulation and fibrinolysis are also encountered during different types of liver surgery. In an review of hemostatic problems in liver surgery, Rö (25) pointed out that dangerous defects seldom occur during most surgical procedures of the liver, but that they may become a threat during liver transplantation.

The preexisting hemostatic disorders in cirrhotic patients undergoing OLT, in combination with the specific intraoperative hemostatic changes, contribute largely to the complexity of hemostatic management of these patients.

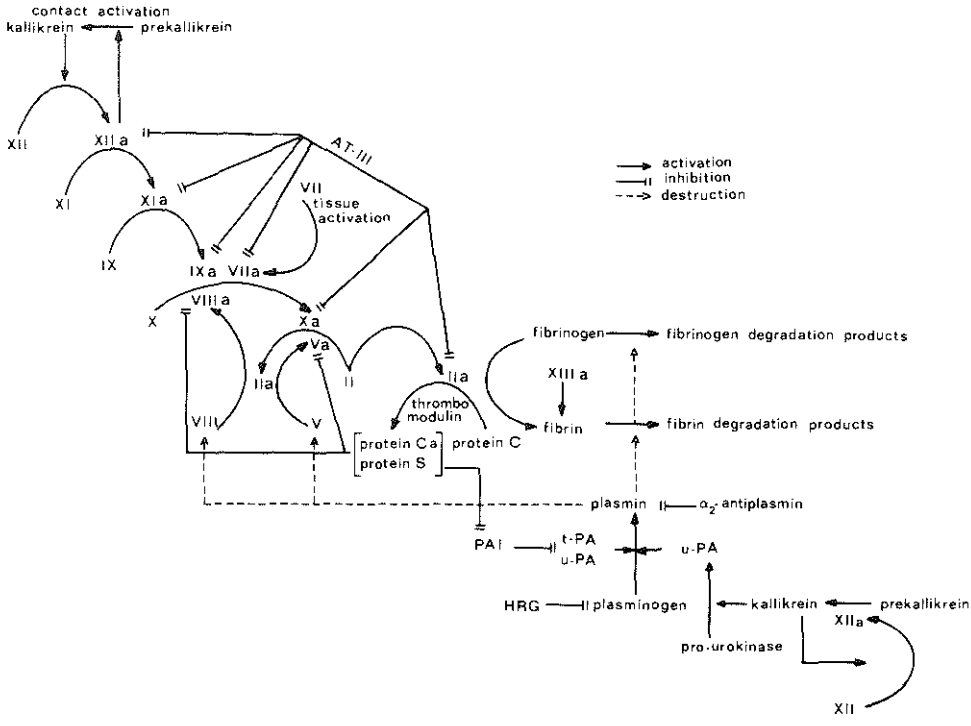

Fig. 1. Schematic presentation of the hemostatic system.

After contact activation or tissue activation each activated (a) coagulation factor converts the next coagulation factor into its enzymatic active form. Blood clots when thrombin (IIa) converts fibrinogen into fibrin. Fibrin formation is regulated by several activating and inhibiting pathways. The most important inhibitors of the coagulation system are antithrombin III (AT-III) and protein C, with its cofactor protein S.

The amount of fibrin is also regulated by the fibrinolytic system. Under physiologic conditions, fibrinolysis is initiated when plasminogen is converted into plasmin by tissue-type plasminogen activator (t-PA), released from endothelial cells, or by activation of pro-urokinase into urokinase-type plasminogen activator (u-PA) or by a still unknown proactivator. Plasmin does not only lead to the breakdown of fibrin, but, when present in high concentrations in the circulation, it may also lead to the destruction of fibrinogen and factor V and VIII. The activity of plasminogen activators is regulated by inhibitors (PAI), of which PAI-1, the rapid inhibitor of t-PA, in turn may be inhibited by protein Ca.

2.3. HEMOSTASIS IN ORTHOTOPIC LIVER TRANSPLANTATION

2.3.1 THE PREOPERATIVE CONDITION AND HEMOSTATIC PROFILE

In OLT patients with severe coagulopathy the very high operative blood loss appears sometimes to be resistent to massive infusion of plasma or plasma products (9,26). Some investigators have looked for preoperative parameters that correlate with the intraoperative use of blood products, in order to predict which patients are at risk for massive bleeding during transplantation and for a high mortality. Patient factors that are known to influence intraoperative blood loss are summarized in table 1. In adult OLT patients it was found that the liver's synthetic function, as reflected by preoperative coagulation studies, is significantly correlated to intraoperative blood loss and survival (9,11,26,27).

By relating pre-transplantation recipient parameters to intraoperative blood loss, Haagsma et al. (11) found that a 24 hr urinary sodium excretion ≤10 mmol and serum sodium ≤132 mmol/l have a 100% predictive value for blood loss over 10 l. Blood loss during the operation is also correlated with the presence of ascites or low values of antithrombin III, the main inhibitor of coagulation. These negative effects of the liver disease on hemostasis may last to the end of the recipient operation. In attempts to optimize the preoperative clotting function in patients undergoing OLT, preoperative transfusion of plasma-products and platelet concentrates (28,29), as well as plasmapheresis (28) have been used. Although these measures were reported to lead to reduction of intraoperative blood usage, they have not been examined in controlled studies. In Pittsburgh we found that the total degree of coagulation disturbances, measured by a coagulation abnormality score may predict intraoperative blood usage and survival in OLT (9). Although a comparable correlation was found by others in a limited number of pediatric OLT patients (30), the situation in pediatric OLT may be different, since no such correlation was found in a larger series of 216 children undergoing OLT (31).

In addition to these reports many authors have concluded that intra-operative changes in hemostasis and blood loss are strongly associated with the diagnosis of the liver disease (5,10,26,32). This is not surprising as the magnitude of pre-existing hemostasis disorders, the extent of collateral circulation and portal hypertension are factors that vary in severity and frequency among the different types of liver disease (15). Liver diseases in

which parenchymal cells are extensively damaged, such as postnecrotic cirrhosis, fulminant hepatitis and α_1-antitrypsin deficiency, are frequently accompanied by disorders in coagulation (9,19) and fibrinolysis (21,33). In patients with malignancy or when bile ducts are involved (primary biliary cirrhosis, sclerosing cholangitis) large areas of normal parenchymal tissue are still able to produce normal levels of coagulation factors. Collateral circulation may cause serious operative difficulties and excessive blood loss during removal of the host liver (2,10,34).

Previous abdominal surgery resulting in adhesions has been recognized as a risk factor for severe bleeding and a high mortality in adults (2,10,35,36). The performance of a routine mini-laparotomy in patients with tumors and without cirrhosis for staging purposes, however, was not found to cause serious problems by the group in Cambridge (37). In pediatric patients, previous surgery may also be associated with a greater blood loss, but the mortality rate was found to be similar to children without previous surgery (38). Apparently, in pediatric patients who have had previous abdominal surgery, mortality depends on other factors than intraoperative blood loss alone.

Table 1. **Patient factors influencing intraoperative blood loss in liver transplantation**

1. Primary diagnosis
2. Severity of the liver disease
3. Preoperative hemostatic disorders
4. Previous abdominal surgery
5. Extent of collateral circulation
6. Presence of ascites
7. Renal function

2.3.2 INTRAOPERATIVE CHANGES IN HEMOSTASIS DURING OLT

In addition to the preexisting hemostatic disorders, specific intraoperative changes in the hemostasis system have been found in OLT. Hemostatic changes that have been described in OLT can be related to three different parts of the surgical procedure: the preanhepatic period after induction of anesthesia until ligation of the patients own liver blood supply, the anhepatic period and the postanhepatic period after reperfusion of the liver graft until the end of surgery. A hemorrhagic diathesis is most often seen during the anhepatic period and in the early postanhepatic period.

2.3.2.1 The Preanhepatic period

In the preanhepatic period serious changes in coagulation and fibrinolytic activity are usually not found. (32,39,40) The hemostatic profile mainly reflects the patient's preoperative hemostatic defects, which are dependent on the etiology and severity of the liver disease (9,41). Patients with severe liver dysfunction due to extensive loss of functioning hepatocytes (i.e. postnecrotic cirrhosis) may at OLT show all the signs of hemostatic disturbances that are seen in severe liver cirrhosis (8,11,26).

Some investigators have reported signs of hypercoagulability, as measured by thrombelastography, in the preanhepatic period (42,43). This may be due to some activation of the coagulation system by surgical trauma, as can also be seen during other types of liver surgery (25,42,44). It is important to note that these observations were mainly made in patients undergoing OLT for a liver tumor (42). Most of the patients had preoperatively elevated levels of fibrinogen and platelet count, which are usually not found in patients with a cirrhotic liver. Indeed, in later reports of larger series of OLT in patients with severe hepatic dysfunction, early hypercoagulation was not found to be a frequent phenomenon (32,39). These patients suffer from hypocoaguloability as a result of the reduced synthetic function of the liver, rather than hypercoaguloablity.

Enhanced fibrinolytic activity may be found during this period in 10-20% of the cirrhotic patients (45,46), but the most important cause of blood loss in this period is the removal of the diseased liver and the transection of collaterals in the abdomen (8,10,11,34), that are often found in these patients.

2.3.2.2 **The Anhepatic Period**

More significant changes in hemostasis occur during the anhepatic period, which is often associated with increased blood loss. In several studies, signs of disseminated intravascular coagulation (DIC) (47-50), hyperfibrinolysis (32,39,44,51), or a combination of both have been found (52,53). Theoretically, DIC may contribute to bleeding complications by two different mechanisms. Firstly, consumption of coagulation factors, due to intravascular fibrin formation may lead to extremely low plasma levels of these coagulation factors. Secondly, intravascular thrombin formation during DIC may cause secondary activation of the fibrinolytic system. Both processes have been considered to be major causes of hemorrhage during the anhepatic period of OLT (41,47,48,52-54). In experimental and clinical studies a simultaneous reduction of platelet count, fibrinogen, clotting factors II, V, VII, VIII, X and antithrombin III has been observed and interpreted as signs of DIC (41,48,52-58). Lack of hepatic clearance of activated coagulation factors and lack of synthesis of coagulation factors and inhibitors during the anhepatic period have been mentioned as factors provoking DIC (41,43,54). However, in other studies no clear signs of DIC were found and the role of DIC has been disputed (32,59,60). Although some authors have reported a beneficial effect of intravenous heparin (42,57), others could not influence the abnormalities by administration of heparin (48,55). Currently, the use of heparin is generally avoided as even small amounts of heparin may lead to severe hemorrhage (54).

It is unlikely that lack of synthesis of coagulation factors really plays a role in the abrupt changes observed during the anhepatic period, since plasma half-life of most of these proteins is much longer than the duration of this period. An intraoperative reduction of plasma levels of fibrinogen and antithrombin III has also been found during other types of surgery (61,62), even in patients that were systemically heparinized (61). Hemodilution by saline infusions or unbalanced transfusion of red blood cells and plasma may be a cause of reduction of plasma levels of coagulation factors and an intraoperative decrease of hematocrit may be found in some reports on OLT (49,50). This suggests that hemodynamic changes may play a role in the intraoperative decrease of hemostasis factors, which is supported by the rapid rise in plasma levels of coagulation factors which has sometimes been observed a few hours after transplantation.

In recent reports of large series of OLT only minor changes in coagulation profiles were described during the anhepatic period (32,39,40,45,63).

These observations do not support the theory of DIC in this period Increased fibrinolysis, as measured by shortened whole blood clot lysis time, euglobulin clot lysis time (47,48,44,52) and thrombelastography (32,45) however, has been found during the anhepatic period in many studies. Several authors have speculated that increased fibrinolytic activity is an important factor in the origin of hemorrhage (45,51,57). The presence of an active fibrinolytic process has been demonstrated by a simultaneous decrease of α_2-antiplasmin, the major inhibitor of plasmin, and of plasminogen activity during the anhepatic stage (39,40,57,63). However, it is still a point of discussion whether the increased fibrinolytic activity in OLT is the result of a primary process or secondary to low grade DIC. In several studies increased fibrinolytic activity has been found without signs of DIC (39,45,51). Lewis et al (39,63) observed a disproportionate decrease in factors V and VIII:C during periods of hyperfibrinolysis, suggesting that these factors might be destroyed selectively by circulating plasmin in the process of active fibrinolysis.

Primary hyperfibrinolysis may result from an impaired balance between activators and inhibitors. The most potent plasminogen activator, tissue-type plasminogen activator (t-PA), is synthesized and released into the circulation by endothelial cells (64,65). The liver has been shown to have an important function in the rapid clearance of t-PA from the circulation (66,67), with a plasma half-life of about three to five minutes (68). In recent small studies it was found that t-PA activity reaches a peak at the end of the anhepatic period (46,69). Increased plasma levels of t-PA were associated with hyperfibrinolysis and high intraoperative blood loss (69). Lack of hepatic clearance of plasminogen activators may explain the increased fibrinolysis during the anhepatic period (49,70,71). Support for this view has been found in studies of animals undergoing hepatectomy. An increased fibrinolytic activity was found in plasma after hepatectomy (44,70,72) and after exclusion of the liver from the animal's circulation (66,71,72). Furthermore, it has been demonstrated that patients with liver cirrhosis are prone to activation of the fibrinolytic system by stimuli that cause release of t-PA (33,73). In addition, the plasma half-life of t-PA is prolonged in cirrhotic patients, probably due to a diminished hepatic clearance (74).

In general, the assessment whether increased fibrinolytic activity is due to a primary process or secondary to DIC is hampered by difficulties in differentiation by laboratory tests (75). Recently, specific enzyme immuno-

assays (ELISA's) have been developed for the separate determination of fibrinogen degradation products and fibrin degradation products in plasma (76). These assays appear to be promising tools for studies on disturbances in the equilibrium of the coagulation and fibrinolysis (76,77). Although these assays have not been used in OLT yet, they may give a better understanding of whether the observed fibrinolytic activity is of primary origin or secondary to DIC.

Independent of hemostatic disorders, the use of a venous bypass in the anhepatic period may reduce intraoperative blood loss (78). The use of a venous bypass has been propagated in order to improve cardiovascular stability and decompress the portal venous system during the anhepatic phase (79). In Pittsburgh, the use of venous bypass, without systemic heparinization, resulted in significantly less blood use during surgery when compared with patients in a previous series undergoing OLT without bypass (78). However, the methodology of this study does not exclude the possibility that other factors including improved surgical techniques, contributed to this effect. In some centers a different technique is used (80), whereas others do not use hepatic veno-venous bypass in all patients (10,40). Therefore, a comparison of different reports is difficult and further reports from randomized controlled studies have to be awaited before definite conclusions can be drawn.

2.3.2.3 The Postanhepatic Period

Reperfusion of the liver graft is associated with major deteriorations of blood coagulation and an increase of fibrinolytic activity. Abnormal bleeding is often seen in this period and many studies have been specially designed to clarify the mechanisms that are responsible for this hemorrhagic diathesis. The processes that may explain the increased bleeding tendency after graft reperfusion are summarized in table 2 and will be discussed in more detail below.

Table 2. **Processes that may effect hemostatic function after graft reperfusion**

a. Disseminated intravascular coagulation (DIC)
b. Increased fibrinolytic activity
c. Trapping of platelets
d. Release of heparin or heparin-like substances
e. Dilutional effect of preservation fluid
f. Humoral and metabolic factors
g. Graft preservation damage

a) Disseminated intravascular coagulation (DIC)

During ischemic preservation of a liver, the endothelial lining of the blood vessels may become damaged and desquamation of endothelial cells has been found in badly preserved livers (81). The injured endothelium and/or release of thromboplastic material may lead to platelet aggregation and activation of the coagulation system after graft reperfusion (54,82). DIC has been proposed by several investigators as the explanation of the observed hemostatic changes. A decrease of clotting factors (fibrinogen, factors II, VII, VII, IX and X) and of the platelet count and antithrombin III, and prolonged clotting times (activated partial thromboplastin time, prothrombin time, thrombin time) have been found after graft reperfusion in both clinical (44,52-54) and experimental situations (44,48,57,60).

In man, as in animals, the severity and duration of coagulation abnormalities seems mainly to be related to the quality of the donor liver (44,52,54). By using liver grafts after different preservations times in animals the depression of platelets and clotting factors was shown to be directly related to the amount of ischemic damage of the graft (48,59,70,83). In experiments where blood samples were taken from both the arterial inflow and venous outflow of the recirculated liver graft, an arterio-venous platelet gradient was found across the graft (70,84,85). Attempts to counteract this phenomenon by the administration of heparin have not been successful (55).

In recent large series of OLT, DIC was not found to be a major cause of excessive bleeding, since only minor changes in clotting factors, suggesting

this diagnosis, were observed (32,39,63,86). Since histopathological demonstration of fibrin depositions are the only objective and definite evidence for DIC, some investigators have performed histopathologic studies after liver transplantation. In none of these studies microthrombi could be demonstrated despite clinical suspicion of DIC in human (59,87) and animal homotransplantation (60,70,84). Improvements in organ procurement and preservation techniques seem to have reduced the intraoperative signs of DIC, which were frequently reported in the past. Therefore one may conclude that DIC is no longer an important component of the disturbed hemostatic function after graft recirculation, provided that the quality of the donor liver is good. Other processes are probably responsible for the bleeding tendency in this period.

b) Increased fibrinolytic activity

Increased fibrinolytic activity is almost invariably found after graft recirculation in both clinical and experimental studies. However, there has been discussion whether this fibrinolytic activity is of primary origin or secondary to DIC, mainly because of difficulties in differentiation by laboratory tests. In the initial studies in the sixties, activation of fibrinolysis was thought to be predominantly secondary to DIC (48,52). The significance of increased fibrinolytic activity in the pathogenesis of hemorrhages was later questioned by several investigators (53-56,60,71). In more recent publications, a marked increase in fibrinolytic activity, as demonstrated by thrombelastography (TEG), was found in 34% of the OLT patients and the enhanced fibrinolytic activity was thought to be a primary event and not secondary to DIC (45). The endothelium of the liver is an important source of plasminogen activators (88) and release of these activators from the preserved and ischemically damaged transplanted liver has been suggested as an explanation for the increased fibrinolytic activity (44,70-72). Theoretically, increased amounts of t-PA may be released from ischemically damaged endothelium. Thus far only a limited number of OLTs have been reported in which t-PA levels were measured (46,69). In these patients no increase of t-PA was seen after graft reperfusion, but larger studies have to be awaited before definite conclusions can be drawn. Contact activation of the intrinsic fibrinolytic system (89), activation of protein C (90) or activation of pro-urokinase (91) are other mechanisms that may theoretically lead to an accelerated fibrinolysis after graft reperfusion. These processes need further investigation too.

Although in most studies the increased fibrinolytic activity was found to be a transient and self-limiting process (44,92), some investigators have tried antifibrinolytic treatment. The results of these studies, unfortunately, do not seem to allow a uniform conclusion. Some authors reported the prevention of primary increased fibrinolytic activity, reduction of blood loss and operative mortality in humans and animals following treatment with epsilon-aminocaproic acid (EACA) or natural antiproteolytic agents (57,58,92). However, other investigators could not find such positive effects and in some OLT cases thromboembolic complications were observed after administration of EACA (44,48). The different effects may be due to differences in doses of EACA and differences in surgical techniques, and the clinical studies comprised only small numbers of patients.

When antifibrinolytics are given to patients with DIC, the balance between coagulation and fibrinolysis may be further pushed towards the side of increased fibrin formation. Antifibrinolytic agents should be reserved for the treatment of uncontrollable oozing where excessive primary fibrinolysis is considered likely (93). Using this policy, favorable therapeutic effects of antifibrinolytic agents in OLT have been reported by Kang et al. (45). In an open study administration of a low dose of EACA intravenously (1 g) to 20 patients who showed active fibrinolysis and generalized oozing, was found to have a positive effect on the hemostatic profile when the drug was proven effective by thrombelastography of EACA treated blood in vitro.

The use of antifibrinolytic agents in OLT, however, will remain controversial as long as it is not clear whether the increased fibrinolytic activity is a primary process or secondary to DIC. Furthermore, objective evidence for the effectiveness of antifibrinolytic therapy in reducing blood loss in OLT can only be obtained from randomized controlled studies.

c) Trapping of platelets

The systemic platelet count often decreases during and after OLT (59,94). The drop in platelet count is most dramatic after graft recirculation and thrombocytopenia may last for 3-5 days postoperatively (40,59,94). Severe thrombocytopenia may contribute to the high incidence of perioperative bleeding complications.

Since the observed thrombocytopenia is not necessarily accompanied with a decrease of clotting factors, it cannot be explained as a sign of DIC only. Experimental studies suggest a role of the transplanted liver in the origin of the thrombocytopenia (43,59,70). By simultaneous measurement of platelets in

arterial inflow and venous outflow up to 55% reduction of platelet count was found across reperfused liver grafts (70). Infusion of radiolabeled autologous platelets in four patients who underwent OLT provided evidence for sequestration of platelets in the transplanted liver (94). Histologic studies performed to elucidate the pathophysiologic mechanisms of this fall in platelet count have not provided a uniform explanation. Hutchinson et al. (59) were unable to demonstrate intravascular coagulation as a cause of reduction of platelets. They instead found evidence for the extravasation of platelets into the spaces of Disse in many of the homografts biopsied shortly after transplantation in dogs. However, others have not confirmed these observations.

Increased phagocytosis of platelets by Kupffer cells of the transplant has also been suggested to explain the thrombocytopenia after OLT (95). Other investigators found changes of platelet function to be of greater importance than changes in platelet number, giving transient prolongation of the bleeding time in dogs after OLT, while platelet counts remained in the low normal range (96). These authors suggested a process of reversible platelet aggregation in association with an activation of the reticulo-endothelial system after graft recirculation, later followed by a release of nonfunctioning platelets into the circulation (96). Although it is likely that the donor liver plays an important role in the decreased platelet count found after OLT, the underlying mechanism remains unsolved.

d) Heparin or heparin-like substances

Stremple et al. (60) have postulated that hemostatic dysfunction after graft recirculation may be caused by a release of heparin or heparin-like substances. Much evidence for the release of a heparin-like substance has been obtained from animal experiments (57,60,70). In these studies, an isolated prolongation of the partial thromboplastin time or thrombin time, that could be partially neutralized with protamine sulfate, was observed after graft recirculation. Dogs especially are highly vulnerable to release of these substances during periods of shock or acute phase reactions (97,98). Therefore, these observations do not necessarily apply to humans. An anticoagulant effect may also be produced by fibrinogen degradation products (47), and it has not always been possible to differentiate these substances from heparin.

The first clinical OLTs were performed with heparin in the preservation fluid or administered to the donor or recipient. In this situation it was

difficult to estimate whether release of endogenous heparin (-like substance) played an important role in the diminished coagulability found after recirculation. Later it was noticed that the use of exogenous heparin promotes bleeding (54). The complete absence of clotting activity observed in the first clinical OLTs was probably partially due to use of heparin in the preservation fluid, in combination with systemic heparinization of the recipient. Heparin in the preservation fluid may become sequestered in the hepatic graft during preservation and enter the circulation after reperfusion (54,86,99). Heparin tolerance is greatly diminished in the recipient at the end of the operation and the capacity to eliminate exogenous heparin is often reduced or nearly lost. The avoidance of excess circulating heparin has been strongly recommended (54). Although systemic heparinization of the recipient is no longer performed as a standard procedure, and most centers use a preservation fluid without heparin, a "heparin effect" may still be seen after graft recirculation in some patients. This can be easily treated with protamine sulphate and suggests that release of endogenous heparin (-like substances) may play a role in the bleeding tendency in some patients (YG Kang 1988, personal communication).

e) Dilutional effect of preservation fluid

Influx of organ preservation solution from the donor liver has been suggested to dilute coagulation factors (32). This may be the main explaination for the decrease in coagulation factors if histological evidence for DIC is absent. From analysis of studies that included measurement of hematocrit it appears that a decrease of coagulation factors often parallels the decrease of hematocrit (49,50). If this dilution is not taken into account an unjustified diagnosis of DIC may be made, which may explain the overestimation of the occurrence of DIC in some early studies.

f) Humoral and metabolic factors

Metabolic acidosis, decrease in cardiovascular performance (100,101), decrease in plasma ionized calcium level (102), decrease in body temperature (103) and hyperkalemia (100,101) have been observed during OLT. Since all of these can adversely affect the hemostatic system, the role of humoral and metabolic factors in the occurrence of hemostatic disorders after graft recirculation has been emphasized. In a large clinical study, in some patients blood coagulability, as measured by TEG, improved without significant changes in plasma levels of coagulation factors occuring but coinciding with

the recovery of the above metabolic abnormalities about 30 min after graft recirculation (32). The effect of changes in plasma electrolytes and metabolic factors on hemostasis in OLT has received little attention and more investigations on this subject are warranted.

g) Preservation damage

The importance of preservation damage to the donor liver in deterioration of hemostasis has been emphasized by several investigators (47,52,55,70,71,83,92). In animal, transplantation of livers preserved for 24 hours invariably led to severe and lasting depression of platelets and clotting factors and almost all animals died of uncontrollable bleeding (48,55). Experiments with auxiliary heterotopic transplantation of livers preserved for 24 hours provided direct evidence that the systemic changes in coagulation are at least partly due to events occurring in the damaged graft and not only to loss of the clearance and synthesizing function of the damaged liver graft (70,84,85). Marked hypocoagulopathy occurred both in pigs that had received an orthotopic transplant and those with an auxiliary heterotopic transplant. The only difference between the two groups was that orthotopically transplanted animals developed in addition a very marked increase in fibrinolysis whereas the heterotopic group did not (70,84,85). Although contact activation of blood by the injured endothelium and/or release of materials with thromboplastic activity were suggested to be responsible for the arterio-venous gradients of platelets and clotting factors across the auxiliary grafts, no attempt was made to have this confirmed by histopathological examination. In contrast to the changes seen in coagulation, the diminished clearing function of the damaged graft undoubtedly contributes to the increased fibrinolytic activity. Heterotopic transplantation of 24 hrs-preserved livers had no systemic effect on fibrinolysis, whereas orthotopic transplantation caused a marked shortening of the euglobulin lysis time in pigs (70,84,85). These studies confirm that hemostatic disorders may result from reperfusion of badly preserved liver grafts. Optimal graft preservation has been shown to be of utmost importance in preventing major disturbances of the hemostatic balance (55,70,83,92). The development of better graft preservation techniques will probably further improve the success rate of OLT (3). Hopefully, better graft preservation will also contribute to a more stable hemostatic level and less bleeding complications after graft recirculation.

2.3.3 INTRAOPERATIVE MONITORING AND CORRECTION OF HEMOSTASIS DEFECTS

A difficulty in the intraoperative management of OLT has been in the monitoring of both the coagulation and fibrinolytic system. Since disorders in hemostasis during OLT often occur acutely and may become serious in a short time, close and rapid monitoring is necessary (104). The number and choice of hemostasis tests that are used intraoperatively varies somewhat from center to center (105). Some centers only perform conventional clotting assays, while others also include assays for individual clotting factors and euglobulin lysis time. However, as long as the exact mechanism underlying the hemostatic abnormalities in OLT are not understood, there are no clear indications for the use of any specific test.

Thrombelastography (TEG), a method by which both fibrin formation and fibrinolysis can be monitored, (106) has been proposed as an overall measurement of the complete hemostasis process (42,44). Some centers have included TEG or Sonoclot^{R}, a comparable instrument, in their monitoring scheme. The clinical effectiveness of TEG in guiding replacement therapy has been demonstrated by Kang et al. (32). The required volume of fluid infusion decreased by 33% after the introduction of TEG monitoring during OLT. These results can be criticized since the effect of TEG was not demonstrated in a randomized study and a historical control group was used. Improved surgical techniques and the use of venous bypass in all but two patients in the TEG-monitored group, but not in the control group, may have contributed to the differences between the two groups. A beneficial role of TEG monitoring has also been suggested by others (107), although randomized studies have not been reported. An important advantage of TEG may be the earlier detection of increased fibrinolytic activity than is possible with ordinary laboratory methods (42,108). In a renewed attempt to combat enhanced fibrinolytic activity during OLT in Pittsburgh, TEG was found to be promising in guiding antifibrinolytic treatment (45).

Differences in methods, large variations in the amounts of transfused blood products, variations in the selection of patients for OLT and small numbers of patients in some studies have made it difficult to compare the results of different monitoring strategies. Recently, the National Institute of Health started to establish a liver transplant data base by pooling information from five liver transplant centers in the U.S.A. This includes the results of 11 hemostasis tests at 10 points during and after OLT. It is hoped that

this study will provide more information on which hemostasis tests are useful to guide replacement of blood products during OLT (105).

2.4. HEMOSTASIS IN AUXILIARY LIVER TRANSPLANTATION

Auxiliary transplantation of a liver graft in a heterotopic position is an alternative method of liver transplantation, with the theoretical advantage of leaving the host liver in situ (109,110). As pointed out above, many of the bleeding problems and hemostatic disturbances in OLT are related to the anhepatic phase. In auxiliary heterotopic liver transplantation the laborious and blood consuming removal of a cirrhotic liver with the subsequent anhepatic phase and serious hemostatic disorders is avoided. Therefore, changes in hemostasis may be less prominent, but information on this subject is limited. Only a small number of auxiliary liver transplantations have been performed in humans (109) since the first report in 1964 (111). A further application of this technique was held up by the discouraging results: most patients died within one month. Recently, however, many of the technical problems of this technique seem to have been resolved by improving the surgical procedure and the transplantation of a partial graft (112,113). This improved technique of auxiliary partial liver transplantation (APLT) is now performed in Rotterdam in patients with acute fulminant hepatic failure, or with end-stage chronic liver disease who have been rejected for OLT because of poor physical condition (114,115).

Sparse information on the influence of APLT on hemostasis was obtained from the initial animal studies in the sixties and seventies (60,70,85). In most of these studies heterotopic auxiliary liver transplantation was used to examine the effect on the hemostasis system of graft damage after long-term preservation (70,85). In addition, the studies were performed during a period when heparin was generally used, both systemically in the recipient and in the preservation fluid. Only a few experimental studies (60) and some case reports in humans (42,59,99) on the changes in hemostasis during and after transplantation of better-preserved auxiliary livers grafts were published in the sixties and seventies. The results of the first 8 APLTs in Rotterdam were published recently (116,117). In these patients and in the earlier studies it appears that important changes in hemostasis are first observed after recirculation of the auxiliary graft and that these resemble the changes that are seen after graft reperfusion in OLT. Most of these hemostatic changes

have been described above. An acute drop of platelet count has been seen after recirculation, probably due to sequestration in the auxiliary graft (59). In animal studies, release of heparin-like factors from the livers was seen (60,70). A major difference with OLT seems to be the lack of markedly increased fibrinolytic activity, which can be explained by the remnant clearing function of the host liver (50,70,85,116). Even after heterotopic transplantation of an auxiliary liver in pigs with fulminant hepatic failure, no activation of fibrinolysis was detected by TEG (118). As mentioned earlier, other factors including preservation damage have hampered interpretation of some of the earlier experimental studies. Furthermore, experiences with APLT in humans are still limited. Therefore, definite conclusions cannot be drawn and new studies are necessary to investigate the influence of APLT on hemostasis and compare APLT with OLT.

Whether pre-existing hemostatic disorders are less hazardous in APLT and whether the advantage of not removing the host liver lead to increased survival in high risk patients undergoing OLT, can only be answered from further clinical experience.

2.5. CONCLUDING REMARKS

The magnitude of bleeding during orthotopic liver transplantation (OLT) appears to result from two factors: poor preoperative hemostatic capacity, complicated by specific intraoperative deteriorations, and the technical difficulties of the surgical procedure. Preoperative hemostasis parameters correlate with the underlying liver disease, intraoperative blood loss and mortality. Improvement of hemostatic function by substitution is therefore an important aspect of the preoperative management. Blood loss during the preanhepatic phase of the operation is mainly of surgical origin. In cirrhotic patients with portal hypertension, bleeding occurring during the transsection of the collateral circulation may be aggravated by a poor hemostatic function, secondary to the underlying liver disease. The hemorrhagic diathesis during the anhepatic and postanhepatic period has been explained by the occurrence of DIC, hyperfibrinolysis or a combination of these processes. Most of the evidence for the role of DIC in uncontrollable bleeding dates form studies in the sixties and seventies. Since then, improvements in surgical and anesthesiologic techniques, and more stringent intraoperative substitution therapy may have contributed to the prevention of

DIC. In recent large studies no important signs of DIC were found during the anhepatic period. Histologic studies of biopsies of the liver graft taken at different intervals after reperfusion have also never provided convincing evidence for the intravascular formation of microthrombi or platelet aggregates. Signs of increased fibrinolytic activity, however, are still frequently found during OLT. This hyperfibrinolysis is probably due to a combination of increased release and reduced hepatic clearance of t-PA, resulting in increased plasma levels of t-PA. After graft recirculation, restoration of the hemostatic balance is largely dependent on the quality of the liver graft. Optimal graft preservation has been found to be of paramount importance in the prevention of intraoperative hemostatic deterioration.

For intraoperative patient care a bedside monitoring system, such as thrombelastography, in combination with specific hemostasis tests, including the prothrombin time, activated partial thromboplastin time, thrombin time, fibrinogen, euglobulin clot lysis time and platelet count is a minimal requisite. Hemostasis tests should be performed at intervals of 0.5 - 2 hours, depending on the stage of the surgical procedure. Frequent testing is especially advised during the first two hours after graft reperfusion.

Prolonged coagulation times should be treated with fresh frozen plasma. Cryoprecipitate is indicated when fibrinogen levels are low, and platelet concentrates should be available to treat severe thrombocytopenia. In patients with marked blood loss, in whom primary hyperfibrinolysis is likely, antifibrinolytic therapy (EACA, Tranexamic acid) may be beneficial in treating life-threatening hemorrhage.

Further investigations on the etiology of hemostatic deteriorations in OLT and application of recent developed specific and sensitive assays may give better insight into the mechanisms underlying the hemostatic disorders in OLT, and provide new theoretical bases for improved treatment. To obtain objective evidence whether a new therapeutic procedure leads to a reduction of intraoperative blood loss, randomized clinical studies, using standard surgical and anesthetic methods in all patients, should be performed. Hopefully this will lead to a further reduction in usage of blood products during liver transplantation in the future.

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CHAPTER 3

A COMPARATIVE STUDY ON CHANGES IN HEMOSTASIS IN ORTHOTOPIC AND AUXILIARY LIVER TRANSPLANTATION IN PIGS.

A COMPARATIVE STUDY ON CHANGES IN HEMOSTASIS IN ORTHOTOPIC AND AUXILIARY LIVER TRANSPLANTATION IN PIGS.

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This chapter has been submitted for publication.

SUMMARY

We compared the effects of orthotopic liver transplantation (OLT) (group A, n=12) and auxiliary, heterotopic partial liver transplantation (APLT) (group B, n=11) in pigs, on blood loss and on the hemostasis system. Blood samples were taken at regular intervals during and after the operations. In both groups nine animals survived longer than 24 hrs and data from these animals were used for analysis. Intraoperative blood loss in OLT was 850.0 ± 23.1 ml and in APLT 437.5 ± 12.9 ml (mean ± SD; $p<0.01$). Routine clotting times, such as the activated partial thromboplastin time, prothrombin time and thrombin time showed no major intraoperative changes in both groups. Fibrinogen levels decreased in both groups, but no significant differences were found between the two groups. The only significant difference between group A and B was a more sustained increase in fibrinolytic activity after graft recirculation in group A. Postoperatively, restoration of fibrinogen, AT-III and α_2-antiplasmin levels was slightly faster in group B, resulting in significant higher levels at the first day postoperatively. We conclude that, in this animal model, APLT is associated with significant lower blood loss and less severe fibrinolytic activity, compared with OLT. This difference might result from the lack of an anhepatic period and the reduced surgical trauma in auxiliary, heterotopic liver transplantation.

INTRODUCTION

Although orthotopic liver transplantation (OLT) has become an accepted method to treat patients with an end-stage of chronic liver disease, it is still associated with massive intraoperative blood loss and the use of large amounts of blood products (1,2). Excessive blood loss is associated with an increased perioperative mortality and morbidity (3). Massive transfusion of blood products also contributes significantly to the total costs of liver transplantation (3).

Bleeding of surgical origin may be seriously complicated by specific hemostatic deteriorations, which occur especially during the anhepatic and post-anhepatic periods (2,4). Disseminated intravascular coagulation (DIC), primary increased fibrinolysis, or a combination of both processes has been suggested as a major cause of bleeding in orthotopic liver transplantation (4-9). Lack of hepatic clearance of activated coagulation factors or activators of the fibrinolytic system may lead to the severe disturbances of the hemostasis system in the anhepatic phase of OLT (7,8). Auxiliary, hetero-topic liver transplantation has been proposed as an alternative to hepatic replacement. The main theoretical advantage of auxiliary liver transplantation is avoidance of the technical hazards of recipient hepatectomy and the lack of an anhepatic period (10,11). A second advantage may be that the recipient is not at the outset totally dependent on the function of the homograft. These differences might attribute to less severe hemostatic disorders and the usage of smaller amounts of blood products. However, information on this technique and its effect on hemostasis is limited. Only a small number of auxiliary liver transplantations have been performed in humans in the past (10,12). The initial discouraging results have held up further application of this technique. Recently many of the technical problems seem to be resolved by improving the procedure and by using a partial graft (13,14). The first clinical applications suggest that this improved technique of auxiliary partial liver transplantation (APLT) may be performed successfully without serious changes in blood coagulation and fibrinolysis, even in patients with poor preoperative hemostatic function (15-17). However, objective information on the advantages and disadvantages of both techniques of liver transplantation can only be obtained from studies in which both techniques are evaluated under identical conditions.

Therefore, we started a comparative study on orthotopic and auxiliary partial liver transplantation in pigs. In this paper we report on the changes

in the hemostatic system. Results of differences in surgical and anesthetic management will be described elsewhere.

MATERIALS AND METHODS

Twenty-three female Yorkshire pigs were used in the experiments. The animals were randomly allocated to two groups: animals in group A (n=12) received an orthotopic liver transplant and group B (n=11) consisted of animals that underwent auxiliary partial liver transplantation. The donor and recipient were matched according to a negative reaction in the mixed lymphocyte culture test (MLC) (18).

All operations were carried out under general anesthesia and the animals were ventilated using a Siemens 900 B Servo ventilator. During the operations Ringers solution, 0.9% NaCl and Haemaccel^{R} were given for hemodynamic support as needed. Depending on the amount of bloodloss, 1-2 U donor blood was given in the period after graft recirculation.

Donor hepatectomy was performed using a conventional technique. After harvesting, the donor liver was perfused ex-vivo by portal vein cannulation with one liter Euro-Collins (4^{o}C) and grafted within 4 hrs. Details of the surgical technique relevant to this study are given below.

Orthotopic liver transplantation (OLT).

In group A, OLT was performed by standard procedure (19). During the anhepatic period blood flow from the operative area and the inferior part of the body was shunted away by a bypass from the portal and femoral vein to the jugular vein, using a heparin-coated extracorporeal circuit (20). No systemic heparin was given.

Heterotopic, auxiliary liver transplantation (APLT).

In group B an auxiliary transplantation was performed by using a partial liver graft. During bench surgery the left medial and lateral lobes of the liver were resected as described previously (13). The partial graft, consisting of 62% of the donor liver, was placed in the right subhepatic space, anastomosing the suprahepatic vena cava of the graft end-to-side to the infrahepatic vena cava of the recipient. The donor portal vein was anastomosed end-to-side to the recipient portal vein and an end-to-side anastomosis was made between the graft hepatic artery to the recipient's infrarenal aorta.

Bile flow was reconstituted by a choledochoduodenostomy. In this type of liver transplantation no shunt is necessary for the decompression of the splanchnic circulation during portal clamping. None of the animals received systemic heparin. No immunosuppressive drugs were given in both groups.

Blood loss and blood sampling.

Blood loss was quantified by measuring the amount of blood sucked away from the surgical field, and collected in Buleaux-bottles during the operation.

Intraoperatively, blood samples (20ml) were taken from an arterial line, while in the post operative period blood was collected from a central venous line or by puncturing of the jugular vein. Eighteen ml of blood was divided into two polystyrene test tubes, containing 1 ml ice-cold trisodium citrate 0.11 mol/l (9vol+1vol) and immediately placed on melting ice. Plasma was collected after centrifugation (2800 g, $4^{o}C$, 30 min), snap-frozen and stored in small aliquots at $-70^{o}C$ until used. Two ml blood was also collected into 0.045 ml 15% sol 6.75 mg EDTA.

In both groups preoperative blood samples were taken direct after induction of anaesthesia. The other blood samples were taken 5 min after anastomosis of the portal vein (recirculation of the graft), 5 min after anastomosis of the hepatic artery, 2 hr and 3 hr after transplantation and on postoperative day 1, 2, 7, 10, 14, 21, 28 and 35. In group A (OLT), one extra blood sample was taken 10 min before the end of the anhepatic phase.

Biochemical measurements.

Graft function was monitored by determination of the levels of ASAT and bilirubin by standard laboratory techniques. Of each blood sample hematocrit was measured to detect possible dilution effects.

Hemostasis studies.

Coagulation.

The activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (ThT) were measured as described previously (21). Thrombelastography (TEG) was performed in citrated plasma (22). The r-value was defined as the time interval between the start of the registration and the first deflection on the TEG recording. The k-value was defined as the time interval after the first deflection until an amplitude of 10 mm was reached. The maximum amplitude (MA) represented the maximum deflection on the TEG recording. Fibrinogen was measured according to Clauss (23) and

antithrombin-III (AT-III) activity was assayed as described by Abildgaard et al (24). The vitamin K dependent factors II, VII and X were determined by Normotest^R, according to instructions of the manufacturer (Nyegaard Diagnostica, Oslo, Norway).

Fibrinolysis.

To determine the euglobulin clot lysis time (ECLT), standard euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10 (25). Precipitates were redissolved in Tris/Tween buffer (0.1 M Tris/HCl, containing 0.1% Tween 80 (v/v) pH 7.5) and to 0.2 ml aliquots of the dissolved euglobulin fractions 0.1 ml portions of calcium thrombin solution ($CaCl_2$ 25 mmol/l and thrombin 10 NIH U/l) were added to induce clot formation. The lysis time of the clot was recorded. The disappearance of air bubbles was regarded as the endpoint of lysis. α_2-Antiplasmin (α_2-AP) activity was measured according to Friberger et al (26). Plasminogen was assayed in acidified plasma using Urokinase (Choay, Paris, France) for activation of plasminogen and S-2251 (Kabi Vitrum Haematology BV, Amsterdam, The Netherlands) as a substrate (27).

Statistical Analysis.

Statistical analysis was performed using the Wilcoxon signed rank test for paired data and the two-sample test for unpaired data. Values for $p<0.05$ were considered to be significant.

RESULTS

In both groups nine animals survived longer than 24 hr after transplantation and the data of these animals were used for analysis. In table 1 body weight and blood loss in group A (OLT) and group B (APLT) are compared. Mean intraoperative bloodloss in group A (OLT) was about twice as high as in group B (APLT) ($p<0.01$). Survival and causes of death in both groups are shown in table 2. None of the animals died of postoperative hemorrhage. The main causes of early mortality were secondary to surgery and included intestinal strangulations.

Table 1. **Body weight and intraoperative blood loss in pigs that underwent orthotopic (OLT) and auxiliary (APLT) liver transplantation**

	Group A OLT (n=9)	Group B APLT (n=9)	p-value
Bodyweight (kg)	24.9 ± 1.8	23.4 ± 2.1	NS
Blood loss (ml)	850.0 ± 23.2	437.5 ± 12.9	p<0.01
	95% confidence limits		
	(834.6 - 865.4)	(428.9 - 446.1)	

Results are mean ± SD

Table 2. **Survival and causes of death after orthotopic (OLT) and auxiliary partial (APLT) liver transplantation**

Pig No.	Group A (OLT)		Group B (APLT)	
	Survival (days)	Cause of death	Survival (days)	Cause of death
1.	7	rejection	35	sacrificed in good health
2.	17	cholangitis	8	volvulus of jejunum
3.	2	unclear	15	volvulus of jejunum
4.	94	sacrificed in good health	50	sacrificed in good health
5.	72	sacrificed, leg abscess	86	sacrificed in good health
6.	33	unclear	91	strangulated hernia
7.	25	cholangitis	159	sacrificed in good health
8.	5	volvulus of jejunum	93	sacrificed in good health
9.	38	bile leakage	23	purulent pneumonia

Biochemical measurements

Liver function and cellular damage, as characterized by bilirubin and ASAT levels are depicted in figure 1. In group B (APLT) only minimal changes in bilirubin and ASAT were found. OLT (group A) was associated with higher levels of ASAT at 3 hrs after transplantation ($p<0.05$) and a maximum was found at the first and second postoperative day. Bilirubin levels were also higher after OLT (group A) ($p<0.01$), reaching a peak at the end of the first week.

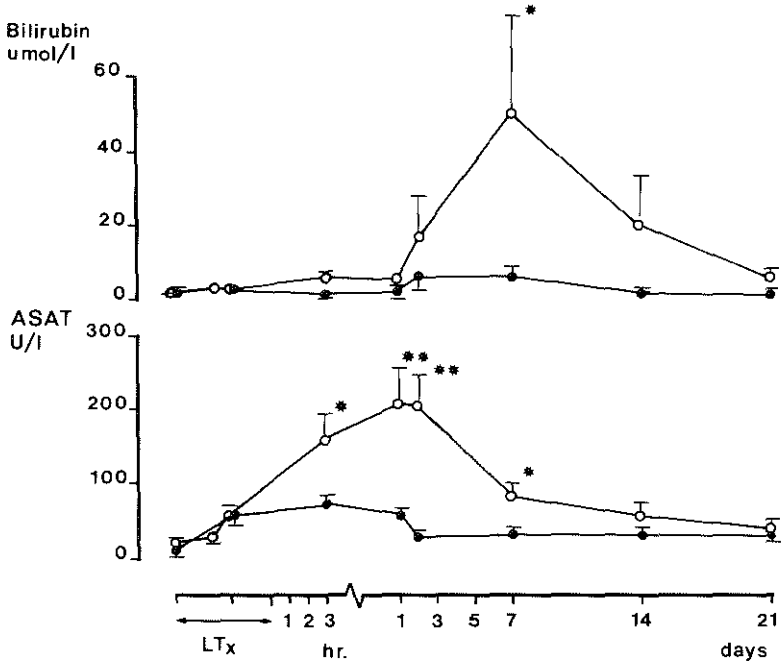

Fig. 1. Bilirubin and ASAT levels (mean±SEM) in animals that underwent OLT (group A, 0—0) and APLT (group B, ●—●). * $p<0.05$, ** $p<0.01$, comparison between group A and B.

Coagulation

Intraoperatively, none of the investigated coagulation parameters was different between the two groups. A rather stable course of APTT and PT was found in both groups (figure 2A), as was also found for ThT and NT. In fact, the only coagulation parameter which showed important changes during

the operations was fibrinogen. A more than 40% decrease of the mean fibrinogen levels was found during the operation in both groups (figure 2B). This was also reflected by a prolongation of the k-value and a decrease of the MA on the TEG-recordings (figure 2A). These changes could not be explained by hemodilution since no important changes in hematocrit were found (figure 2B). A slowly decreasing pattern was also seen for AT-III and platelet count (figure 2B).

Fibrinolysis

Important intraoperative changes were found in the investigated fibrinolytic parameters in both groups. Hyperfibrinolysis, as characterized by shortened ECLTs and reduction of α_2-AP and plasminogen concentrations, was found 5 min after graft reperfusion in both OLT and APLT (figure 3). During OLT an increased fibrinolytic activity, as measured by ECLT, was already present during the anhepatic period. In group A (OLT) a further increase of fibrinolytic activity was seen during the post- reperfusion period, resulting in significant shorter ECLTs at the time of completion of the arterial anastomosis, compared with group B (APLT) ($p<0.05$). In both groups, ECLT became normal (>180 sec) at 3 hrs after the operation. At this time plasma levels of plasminogen and α_2-AP were still reduced. Although restoration of α_2-AP levels was apparently faster in group B (APLT) this difference was not statistically different.

Long-term postoperative changes

In both groups, a recovery of the hemostatic parameters was observed during the first postoperative week. There were no differences in APTT, PT and NT. Fibrinogen, AT-III and α_2-AP levels at the first day after surgery were higher in group B (APLT) ($p<0.01$) (figure 4). Fibrinogen levels showed a peak at day 2 in group A (OLT) and at day 1 and 2 in group B (APLT) (figure 4). Platelet count reduced further during the first postoperative days, reaching a nadir at the second day in both groups. Thereafter a rapid recovery of platelet count was observed, resulting in a thrombocytosis (mean values higher than $500x10^9$/l) at the end of the first week (figure 4).

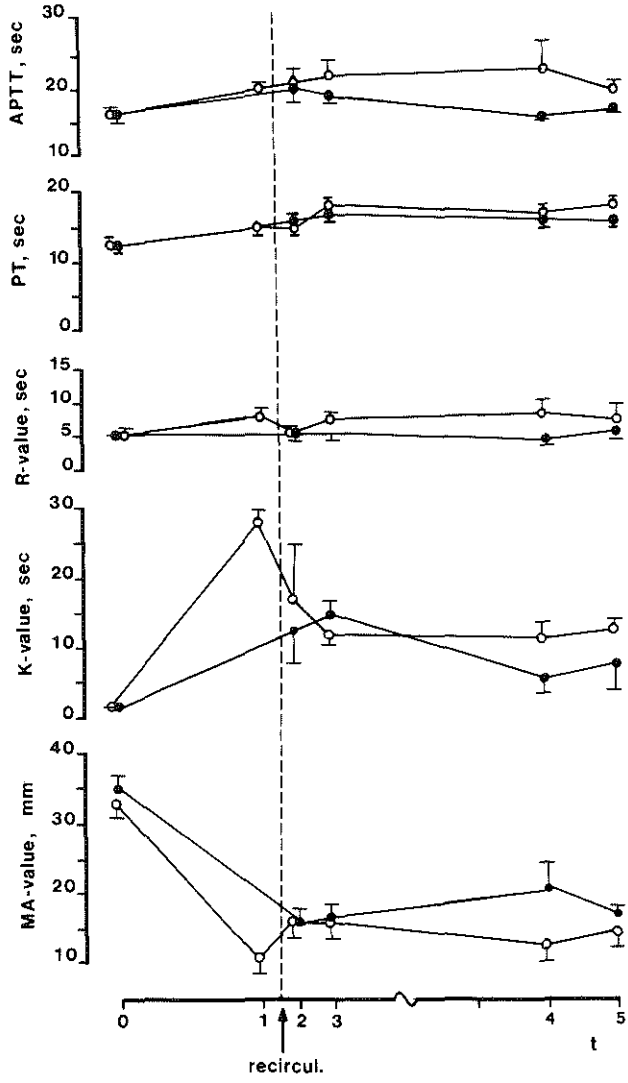

Fig. 2A. APTT, PT, r-value, k-value and maximum amplitude (MA) of thrombelastogram in animals that underwent OLT (group A, O—O) and APLT (group B, ●—●) (mean±SEM). 0 = preoperative; 1 = end anhepatic phase in OLT; 2 = 5 min after recirculation; 3 = 5 min after hepatic artery anastomosis; 4 = 2hr postoperative; 5 = 3hr postoperative.

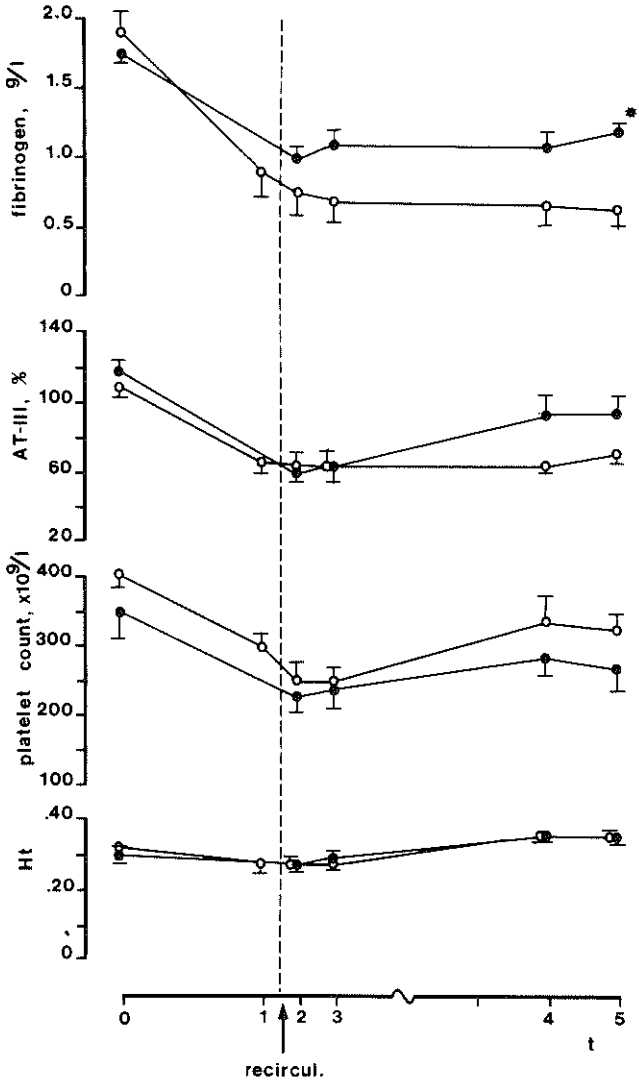

Fig. 2B. Fibrinogen, AT-III, platelet count and hematocrit (Ht) in animals that underwent OLT (group A, O--O) and APLT (group B, ●—●) (mean±SEM). * $p<0.05$, comparison between group A and B. Legends as in fig. 2A.

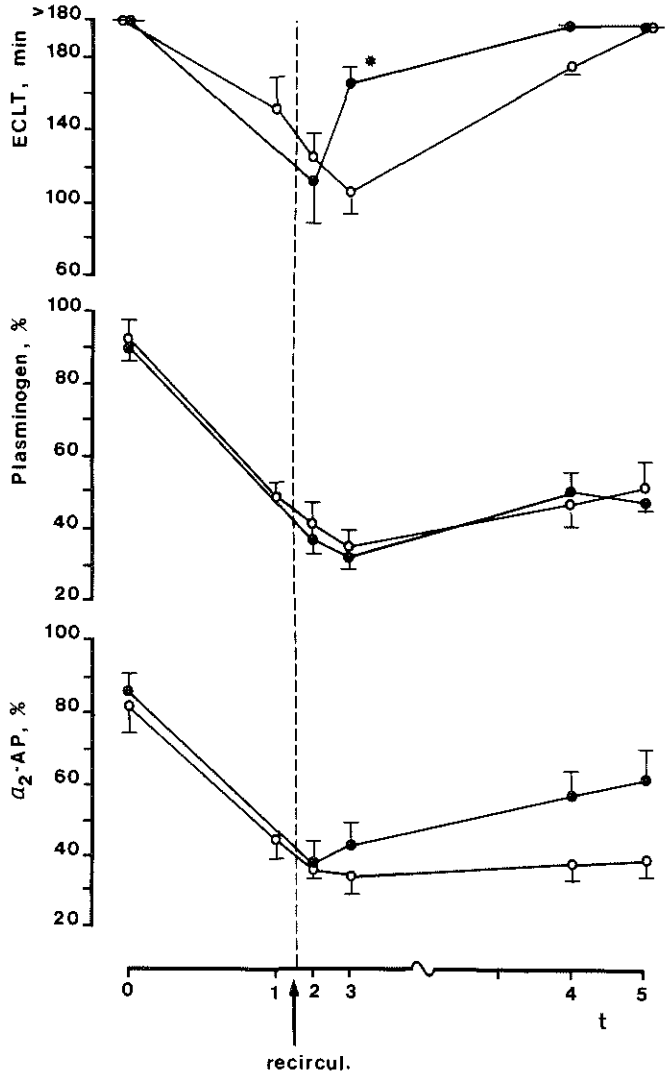

Fig. 3. ECLT, plasminogen and α_2-antiplasmin (α_2-AP) in animals that underwent OLT (group A, O—O) and APLT (group B, ●—●) (mean±SEM). * $p<0.05$, comparison between group A and B. Legends as in fig. 2A.

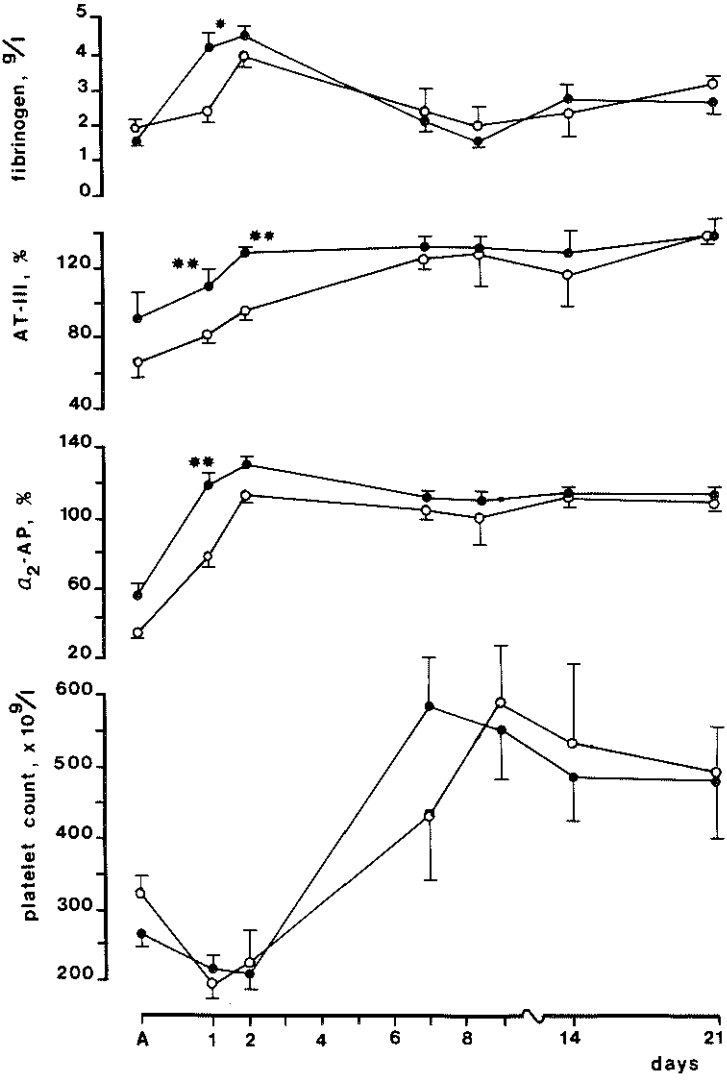

Fig. 4. Postoperative course of fibrinogen, AT-III, α_2-AP and platelet count in animals that underwent OLT (group A, O—O) and APLT (group B, ●—●) (mean±SEM). A: 2h after surgery. * $p<0.05$, ** $p<0.01$, comparison between group A and B.

DISCUSSION

The anhepatic phase in OLT is associated with serious changes in hemostasis, which may further complicate surgical bleeding and attribute to the need of massive blood transfusions (4,9). Auxiliary liver transplantation has potential benefits over OLT, as the technical hazards of the recipient hepatectomy and the subsequent anhepatic period are avoided. Therefore, auxiliary liver transplantation may theoretically be associated with less severe hemostatic deteriorations and it may be an attractive alternative for OLT. However, objective evidence on the potential advantages of auxiliary liver transplantation, regarding blood loss and disturbances of hemostasis are lacking. Although a comparison of orthotopic and auxiliary liver transplantation has been made in one earlier study, this was primarily designed to study the effect of graft preservation damage on hemostasis and insight in the specific changes due to the auxiliary transplantation procedure cannot be obtained from this study (28).

In the present study we investigated the effect on hemostasis of orthotopic and auxiliary partial liver transplantation, using techniques which are currently used in humans. Both types of liver transplantation were performed in a controlled study in healthy pigs. Hereby, the effect of preexisting differences in coagulation defects, as may be found in liver patients, were avoided. This made it possible to study the specific changes in hemostasis due to the surgical procedures only.

An important difference between OLT and APLT, found in this study, was a lower blood loss in the animals that underwent APLT. This is in accordance with our experience in clinical APLT (17). When comparing the hemostatic profile of the two groups, differences were not found in the blood coagulation system, but rather in the fibrinolytic system. In fact, both groups showed only minor changes in the routine clotting times. The only coagulation parameter that showed serious alterations was fibrinogen. A decrease of fibrinogen levels and subsequent prolongation of k-value, representing clot formation rate, and a decrease in the MA-value, representing clot stiffness, on TEG-recordings was found in both OLT and APLT. Although the intraoperative decrease of fibrinogen was apparently more severe in OLT, this difference was not statistically significant. However, an ongoing postoperative decrease of fibrinogen after OLT did result in significant lower levels compared with the APLT group at 3 hrs after the operation. Several investigators have described a reduction of fibrinogen

levels in experimental and clinical OLT (4-9,29). Mechanisms of consumption, disseminated intravascular coagulation (DIC), trapping in the graft, fibrinogenolysis and the effect of hemodilution have been suggested to explain this phenomenon (4,9,29). We did not find convincing evidence for DIC, and/or hemodilution. AT-III levels never reached extreme low values and no serious changes in any of the clotting times and hematocrit were observed. The decrease of fibrinogen is most probably caused by a local consumption around the surgical wounds.

We also found signs of hyperfibrinolysis, as characterized by a shortened ECLT and decrease of plasminogen and α_2-antiplasmin levels after graft recirculation in both groups. However, fibrinolytic activity was more severe and lasted longer in animals that underwent OLT. After recirculation of auxiliary grafts, the ECLT showed a fast normalization, and ECLT was less diturbed in this group at the time of completion of the hepatic artery anastomosis. Many investigators have stressed the role of hyperfibrinolysis in the origin of bleeding complications in OLT. Recent studies in clinical OLT suggested that increased fibrinolytic activity is predominantly of primary origin (9,29) and may result from a combination of reduced hepatic clearance and an increased release of tissue-type plasminogen activator (30). The lack of an anhepatic phase and the remnant clearing function of the host liver might explain why we observed less severe fibrinolytic activation in APLT. In agreement with this, we recently demonstrated in a clinical study that signs of hyperfibrinolysis are only found in a minority of patients undergoing APLT (16). Although the clinical relevance of hyperfibrinolysis in liver transplantation is still a point of discussion, this may be an important difference between OLT and APLT, and it may attribute to less frequent bleeding complications in APLT.

Another difference between OLT and APLT, found in this study, was an earlier restoration of some hemostasis parameters after the operation. Levels of fibrinogen, α_2-antiplasmin and AT-III were significantly higher in the APLT group at the first postoperative day. Although this study was performed in healthy animals and it cannot be deduced whether this effect is not predominantly due to synthesis by the host liver, we have observed an similar fast restoration of α_2-antiplasmin and AT-III levels in a cirrhotic patient with severe preoperative coagulation defects, who underwent APLT (16). Postoperatively, we observed a further decrease of platelet count in both groups. This is in agreement with clinical findings (5), but the mechanisms underlying this drop of the platelet count are still not clear and are

the subject of further research. The thrombocytosis, observed in both groups after the first week, can be ascribed to a reactively increased thrombopoiesis. Rebound thrombocytosis occurs because of a lag in the feedback mechanism associated with the platelet/megakaryocyte control mechanism (31). In cirrhotic patients undergoing liver transplantation a moderate to severe thrombocytopenia due to splenomegaly is usually present preoperatively. This may explain why such a thrombocytosis is less striking after successful clinical liver transplantations.

In conclusion, in comparing OLT and APLT in a controlled study, we observed a halving of intraoperative blood loss in animals that underwent APLT. No differences in the investigated coagulation parameters were found between the two groups. Although an increase in fibrinolytic activity was found during both types of liver transplantation, signs of hyperfibrinolysis were present during a longer period after graft recirculation in OLT. Postoperatively, an earlier normalization of disturbed hemostatic parameters was seen after APLT. The lack of an anhepatic period in APLT and a remnant synthesis and clearing function of the host liver in APLT may explain these differences. A controlled study in humans is necessary to investigate whether APLT is an good alternative for OLT in severely diseased liver patients.

ACKNOWLEDGEMENT

The personal of the Laboratory for Experimental Surgery is gratefully acknowledged for their help during the experiments.

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CHAPTER 4

ROLE OF THE DONOR LIVER IN THE ORIGIN OF THROMBOCYTOPENIA AND HEMOSTATIC DISORDERS IN PORCINE LIVER TRANSPLANTATION

ROLE OF THE DONOR LIVER IN THE ORIGIN OF THROMBOCYTOPENIA AND HEMOSTATIC DISORDERS IN PORCINE LIVER TRANSPLANTATION

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This chapter has been submitted for publication.

SUMMARY

We investigated the role of the donor liver in the origin of thrombocytopenia and hemostatic disorders in liver transplantation. Eighteen pigs received an orthotopic or an auxiliary, heterotopic liver graft. Liver biopsies were taken for electron microscopic studies 5-10 min after reperfusion in nine animals. Blood samples were taken from the first hepatic outflow and from the systemic circulation before and 5 min after graft recirculation. Electron microscopy did not show any evidence of microthrombi or platelet aggregation in the graft, neither after orthotopic liver transplantation nor after auxiliary liver transplantation. Most blood platelets were lying free in the sinusoids. They showed cell processes and many of them seemed to have lost their granulae. There were also signs of phagocytosis of platelets by the Kupffer cells. In the hepatic outflow, platelet count was significantly lower and fibrinolytic activity significantly higher, compared with the systemic post-reperfusion values ($p<0.01$). There were no important changes in the coagulation parameters. No significant changes were found between the effects on hemostasis of orthotopic and auxiliary graft reperfusion. This study suggests that thrombocytopenia and increased fibrinolytic activity after graft recirculation in liver transplantation are not a result of intravascular coagulation. Sequestration of platelets in the graft is probably caused by a combination of phagocytosis by Kupffer cells and a accumulation of platelets in the sinusoids. Increased fibrinolytic activity is apparently caused by primary activation in the hepatic graft.

INTRODUCTION

Orthotopic liver transplantation (OLT) is associated with serious bleeding problems, which often require the use of large amounts of blood products (1,2). Data from clinical studies suggest that bleeding of surgical origin may be enhanced by disturbances of the hemostatic system and blood loss seems to be at least to some extent dependent on intraoperative deteriorations of the hemostatic function (3,4). A dangerous period of hypocoaguloability has been recognised especially after reperfusion of the new liver (4,5), suggesting a direct effect of the reperfused donor liver on the hemostatic system.

A decrease in platelet count is one of the changes that are uniformly seen after recirculation of the donor liver, despite the infusion of platelets (3,6). Although thrombocytopenia may be a sign of disseminated intravascular coagulation (DIC), it is often seen as an isolated phenomenon and several investigators have proposed other mechanisms for platelet sequestration in the graft. Hutchison et al (6) reported morphological evidence for the extravasation of platelets into the spaces of Disse after canine OLT, but in their publication no photographs of histopathologic preparations were shown. Schalm et al. (7) suggested a reversible platelet aggregation in association with an increased activity of the reticuloendothelial system after graft reperfusion, but in this study no objective evidence by histologic studies was given. The exact mechanism underlying thrombocytopenia after OLT is therefore still not clear.

Another phenomenon that has been described after graft recirculation in OLT is an increase in fibrinolytic activity (4,8). It is still a matter of debate whether hyperfibrinolysis in OLT is a primary process or secondary to DIC. Some investigators observed increased fibrinolytic activity in combination with signs of DIC and suggested a secondary activation of fibrinolysis (5,9). Others could not find important signs of DIC (4,10) and direct histologic evidence for the presence of fibrin deposits in liver grafts after recirculation has never been found. Therefore, primary activation of the fibrinolytic system has been proposed as an important underlying mechanism of the hyperfibrinolysis after graft recirculation (10,11). However, the mechanism and origin of this fibrinolytic activation are still unclear.

In this study we investigated the role of graft reperfusion in the origin of thrombocytopenia, as well as coagulation and fibrinolytic disorders in orthotopic and auxiliary liver transplantation in pigs. In order to find morphologic evidence for microthrombi and/or platelet sequestration, electron

microscopy was performed on biopsies from the transplanted livers. Furthermore, hemostatic parameters in venous blood from the liver grafts were examined in comparison with peripheral blood samples of the general circulation.

MATERIALS AND METHODS

In all experiments female Yorkshire pigs were used, weighing 23.5 ± 3.3 kg (mean ± SD; n=18). Nine animals received an orthotopic transplant and heterotopic, auxiliary liver transplantation was performed in nine other pigs. The animals were used in a comparative study on the surgical techniques of orthotopic and heterotopic, auxiliary partial liver transplantation, the results of which will be described elsewhere. Details of the surgical procedures relevant to this study are given below.

Donor hepatectomy was performed using a conventional technique. Livers were preserved by means of an ex-vivo cold perfusion ($4^{o}C$) via the portal vein, with one liter Euro-Collins and used for transplantation within 4 hr. OLT was performed by a standard procedure (12). Heterotopic transplantation of a partial, auxiliary liver graft (APLT) was performed after resection of the left medial and lateral lobes of the donor liver during bench surgery, as described previously (13). The partial liver was inserted in the right upper abdominal cavity, anastomosing the donor infrahepatic vena cava to the host suprahepatic vena cava and the donor portal vein end-to-side to the host portal vein. The donor hepatic artery was anastomosed end-to-side to the infrarenal aorta and the infrahepatic part of the donor vena cava was ligated.

Ultrastructural and morphological studies

Wedge-like liver biopsies of about 2 gram were taken 5-10 min after graft reperfusion in 9 consecutive transplantations (6 OLTs and 3 APLTs). Pieces of liver were fixed for transmission electron microscopy either by immersion fixation in ice cold 3% glutaraldehyde or by perfusion fixation. Liver tissue fixed by immersion fixation was further processed according to standard procedures. For the perfusion fixation the liver biopt was canulated by a Braunula 0.5-G 18 into a larger vessel exposed at the periphery of the tissue block and secured tightly and perfused with buffered saline for 2 min, followed by 2% glutaraldehyde in 0.15 M sodium cacodylate buffer pH 7,4 for

5-10 min. All solutions were used at 10 ^{o}C. Postreperfusion was done with 0.05 M Tris - HCl buffer containing 7% sucrose for 6 min. The flow rate was about 5 ml/min. Vibratome sections of 150 µm were cut and postfixed in 1% OsO_4 in 0.15 M sodium cacodylate buffer for 45 min, dehydrated in a graded series of ethanol and embedded in LX 112 (Ladd Research Industries, Inc., Burlington, Vermont). Transmission electron microscopy was performed according to routine procedures (14). Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 410.

Hemostasis studies in graft blood

Collection of blood.

In order to examine the direct effect of the donor liver on hemostasis, blood samples were taken from the first venous effluent from the graft after recirculation. After completion of the portal vein anastomosis, the graft was reperfused, while reentrance of blood into the systemic circulation was prevented by leaving the vena cava anastomosis temporarily clamped off. In OLT, blood was taken from the donor infrahepatic caval vein (before suturing), using a 20 ml plastic syringe. In APLT, blood was taken from the infrahepatic vena cava before it was ligated. After graft blood was obtained in this manner, the caval venous clamp was removed, allowing complete systemic recirculation. For reference values, systemic blood samples (20 ml each) were taken from an arterial line, during the first period of the recipient operation (systemic pre-perfusion sample) and 5 min after recirculation of the donor liver (systemic post reperfusion sample). In OLT, systemic pre-perfusion samples were taken shortly before the end of the anhepatic period to avoid measuring effects already present during this period.

Eighteen ml of each blood sample was immediately transferred to two polystyrene tubes, containing 1 ml ice cold trisodium citrate 0.11 mol/l (9vol +1vol) and immediately placed on melting ice. Another 2 ml was collected into 0.045 ml 15% sol 6.75 mg EDTA. Citrated blood was centrifuged (2800 g, 4^{o}C, 30 min) and plasma was collected, snap-frozen and stored in small aliquots at -70^{o}C until used.

Assays.

Platelet count and mean platelet volume (MPV) were determined in EDTA-treated blood, using a Platelet Analyser 810 (Baker Instruments, Allentown, PA, U.S.A.). The activated partial thromboplastin time (APTT), prothrombin

time (PT) and thrombin time (ThT), were employed as described previously (15). Fibrinogen was measured according to Clauss (16) and antithrombin-III (AT-III) activity was assayed according to Abildgaard et al (17). α_2-Anti-plasmin (α_2-AP) activity and plasminogen were measured by chromogenic substrate methods (18,19). Euglobulin clot lysis time (ECLT) was determined as described previously (20), using standard euglobulin fractions of plasma, prepared at pH 5.9 with a plasma dilution of 1:10 (21). Hematocrit (Ht) was determined in EDTA-blood.

Statistical Analysis.

Statistical analysis was performed using the Wilcoxon signed rank test for paired data and the two-sample test for unpaired data. Values for $p<0.05$ were considered to be significant.

RESULTS

Morphological studies

Ultrastructural studies were performed on liver biopsies and in none of these biopsies signs of intravascular fibrin formation or platelet aggregation were found in the small sinusoids or larger vessels. In specimens fixed by perfusion fixation, both the sinusoidal cells and parenchymal cells showed a well-preserved ultrastructural morphology without signs of ischemic cell damage (figure 1). Especially the endothelial lining of the sinusoids was well-preserved. Most platelets were found to lie free in the sinusoids, independent of the fixation technique. They showed cell processes and many of them seemed to have lost part of their granulae. Also ballooned platelets, lacking microtubules and granulae were seen (figure 2). In some cases phagocytosis of whole platelets by Kupffer cells was found (figure 3). Platelets were sometimes found extravascularly in the interstitial fibrous septa, but never in the space of Disse (figure 4). There were no differences found between livers transplanted in the orthotopic position and livers transplanted as auxiliary, heterotopic grafts.

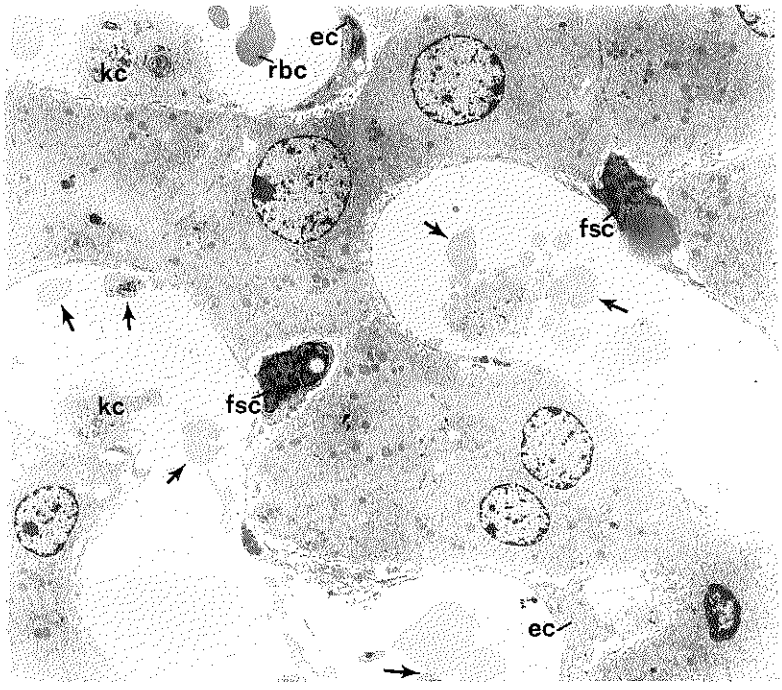

Fig. 1. Low magnification transmission electron micrograph with overview of hepatic cords and sinusoids. Some red blood cells (rbc) and numerous platelets are seen in the sinusoids (arrows). Endothelial cells (ec), fat storing cells (fsc), and Kupffer cells (kc) can also be seen. Enlargement: 2,730 x.

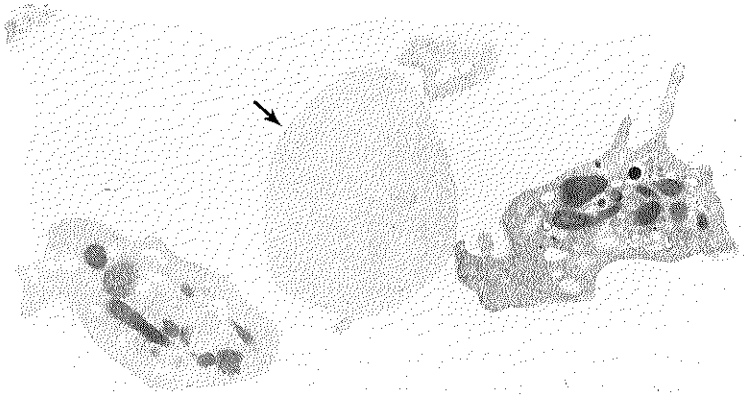

Fig. 2. Platelets in a sinusoid forming some cell processes. They lack most of their secretory granulae (arrow) and microtubules. Enlargement: 15,840 x.

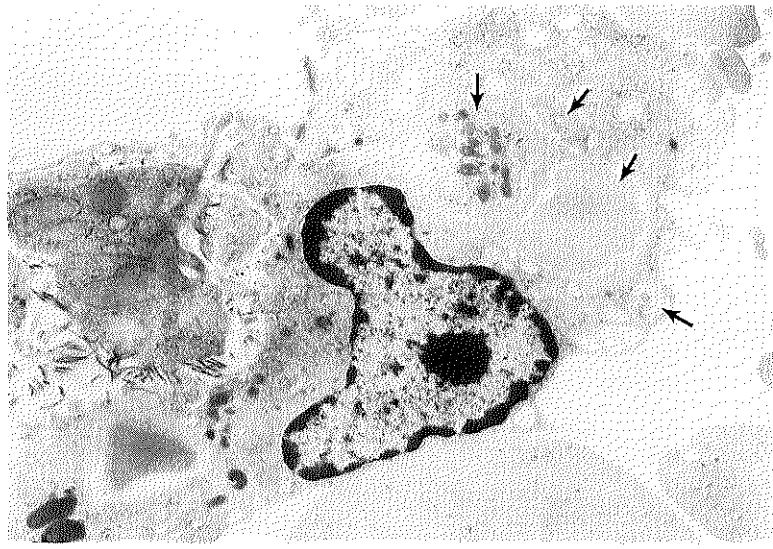

Fig. 3. Kupffer cells with internalized platelets (arrows). Enlargement: 10,340 x.

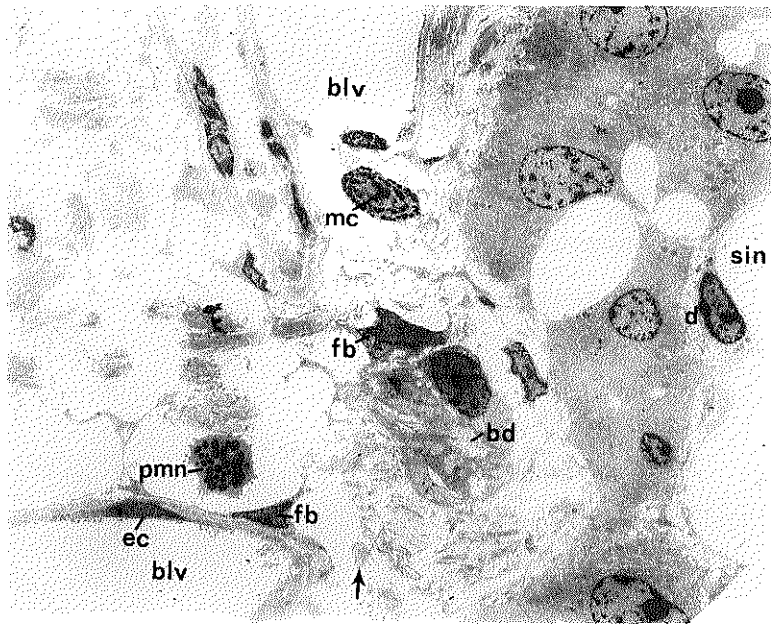

Fig. 4. Low power magnification of parenchym cells, sinusoid (sin), blood vessel (blv), space of Disse (d) and a bileduct (bd). Most of the platelets can be seen in sinusoids and blood vessels, but one is present in the fibrous septa (arrow). Also a polymorph nucleocyte (pmn), a mast cell (mc), and fibroblasts (fb), are illustrated. Enlargement: 2,130 x.

Hemostasis studies in graft blood

Comparison of platelet count and mean platelet volume in systemic pre-reperfusion samples, graft samples and systemic post-reperfusion samples is shown in figure 5. A significant drop in platelet count was found in systemic post-reperfusion blood, when compared with the preperfusion values ($p<0.01$). This systemic effect was not different in OLT and APLT and there was no difference in platelet number in graft blood of the two groups. There were no indications that the observed platelet gradient across the graft was due to a reduction of platelets of a specific size, since no differences were found in MPV of the three samples in both groups.

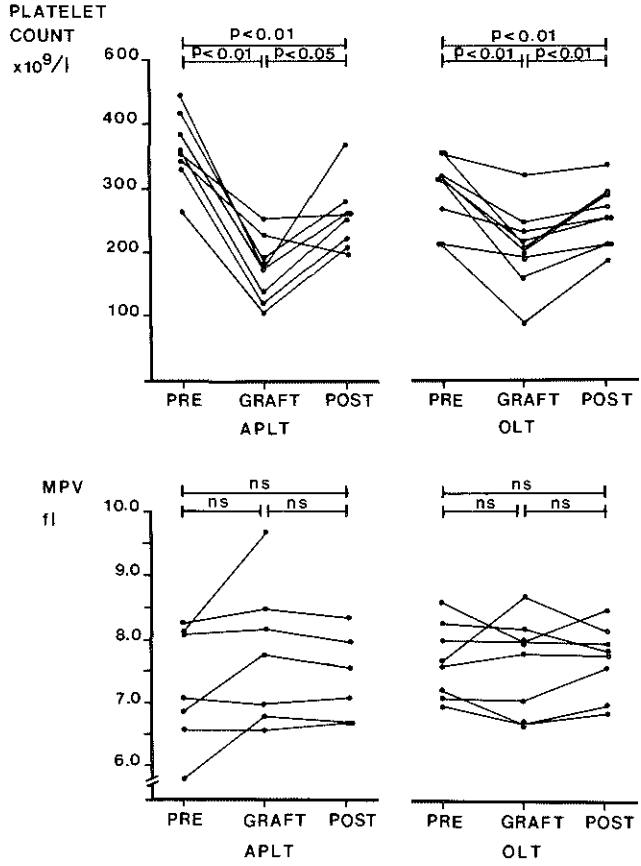

Fig. 5. Direct effect of reperfusion of orthotopic (OLT) and auxiliary (APLT) liver grafts on platelet count and mean platelet volume (MPV). PRE, systemic pre-perfusion sample; GRAFT, graft blood and POST, systemic post-reperfusion sample.

The results of the coagulation parameters and Ht values are shown in table 1A. In general, no serious changes in the coagulation system were found after graft reperfusion. None of the investigated coagulation parameters in graft blood was significantly different from the systemic reperfusion values. There were also no significant differences in hematocrit values of the three blood samples in both types of transplantation.

Table 1A. Comparison of coagulation parameters and hematocrit before and after reperfusion of orthotopic (OLT) and auxiliary partial (APLT) liver transplants (n=9 in both groups)

		PRE	GRAFT	POST
		median (range)	median (range)	median (range)
APTT (sec)	OLT	19 (15-24)	24 (19-35)	21 (17-28)
	APLT	16 (15-20)	32 (15-37)	20 (16-26)
PTT (sec)	OLT	14 (12-18)	17 (14-20)	15 (12-20)
	APLT	14 (11-16)	20 (12-25)	15 (13-24)
ThT (sec)	OLT	37 (27-60)	42 (32-55)	41 (29-60)
	APLT	47 (37-60)	35 (17-53)	35 (16-46)
Fibrinogen (g/l)	OLT	0.9 (0.5-1.6)	0.7 (0-1.1)	0.7 (0-1.5)
	APLT	1.8 (1.3-2.1)	1.1 (0-1.4)	1.2 (0.6-1.4)
AT-III (%)	OLT	70 (43-102)	55 (35-58)	50 (47-74)
	APLT	120 (77-154)	45 (22-78)	56 (42-95)
Ht	OLT	0.26 (0.22-0.33)	0.28 (0.20-0.43)	0.27 (0.21-0.30)
	APLT	0.30 (0.25-0.38)	0.35 (0.20-0.45)	0.29 (0.24-0.35)

The abbreviations used are: PRE, systemic pre-perfusion; GRAFT, graft blood; POST, systemic post-reperfusion.

The effect of graft reperfusion on fibrinolysis is shown in table 1B. Fibrinolytic activity, as measured by the ECLT, was increased in systemic blood after reperfusion, compared with the pre-perfusion values in both OLT and APLT ($p<0.05$). Shortest ECLTs were found in the graft blood samples of both orthotopic and auxiliary grafts (table 1B). In the APLT group, values of ECLT in graft blood were shorter than the systemic post-reperfusion values ($p<0.05$). Changes in α_2-antiplasmin and plasminogen were less prominent (table 1B). Only in the APLT group a reduction of α_2-antiplasmin was found in graft blood, when compared with the systemic post-reperfusion values ($p<0.05$).

Table 1B. Comparison of fibrinolytic parameters before and after reperfusion of orthotopic (OLT) and auxilary partial (APLT) liver transplants (n=9 in both groups)

		PRE median (range)	GRAFT median (range)	POST median (range)
ECLT (min)	OLT	180 (55-180)	75 (45-180)*	115 (60-180)
	APLT	180 (180-180)	40 (15-180)*	120 (25-180)
α_2-AP (%)	OLT	43 (24-72)	33 (22-65)	35 (22-61)
	APLT	88 (63-109)	25 (19-74)**	43 (10-67)
Plasminogen (%)	OLT	50 (25-65)	38 (21-50)	43 (10-67)
	APLT	94 (66-104)	29 (10-55)	37 (24-54)

ECLT, euglobulin clot lysis time; α_2-AP, α_2-antiplasmin; other abbreviations as in table 1 A. *$p<0.01$, **$p<0.05$, compaired with the corresponding POST-value.

DISCUSSION

Orthotopic liver transplantation is often associated with a drop in platelet count, which may lead to severe thrombocytopenia and may play a role in the origin of hemorrhages (3,6). This thrombocytopenia may be caused by an increased platelet consumption, hemodilution, immunologic reactions or by a sequestration of platelets in the reperfused liver graft (6,22). Since signs of DIC, characterized by a simultaneous decrease of coagulation factors and presence of fibrin degradation products are not invariable found in liver transplantation, the role of platelet sequestration in the liver graft has been subject of speculations and further research.

In the present study we observed a significant drop in platelet count in the systemic circulation after reperfusion of auxiliary and orthotopic liver grafts. Fall in platelet count was unlikely to be caused by hemodilution since no significant differences were found in hematocrit values of the corresponding blood samples. Recently, a specific accumulation of platelets in the newly grafted liver was suggested by platelet labeling studies (22). In this study we provided additional evidence that the systemic decrease of platelet count is due to a process of platelet accumulation in the liver graft, since a sharp drop of platelet count was found in the venous outflow of the graft after reperfusion.

Only a few histopathological studies have been performed in the first hours after transplantation, in order to find morfological evidence for the mechanism of platelet accumulation in the reperfused liver graft (6,8). Groth et al. (8) and Hutchison (6) performed electron microscopy studies after canine and human OLT and described an extravasation of single, undamaged platelets into the perisinusoidal space of Disse. By performing electron microscopy on biopsies taken 5-10 min after graft recirculation we indeed found some evidence for the extravasation of platelets into the interstitial fibrous septa. But the number of platelets found extravascularly was neglectable in comparison with the number in the sinusoids. The difference between the earlier studies and our observations may be due to differences in period of time after revascularisation. We found a rather well-preserved endothelial lining of the sinusoids and no widening of the space of Disse. In conformity with other studies (6,8,23) we could not find morphologic evidence of microthrombi or platelet aggregates in the sinusoids. The problem remains as to what happens with the blood platelets during reperfusion of the liver graft. In some preparations we observed phagocytosis of whole

platelets, normal in appearance, by Kupffer cells. Comparable observations have been made by other investigators (6,24). Cossel (24) observed phagocytosis of whole platelets by Kupffer cells at a much later stage (138 days) after OLT in pigs. He proposed an increased reaction of Kupffer cells of the transplant against host thrombocytes as a possible mechanism for thrombocytopenia after liver transplantation (24). Our data suggest that this process may also contribute to the drop of platelets early after graft reperfusion. Interesting was the agranular appearance of the platelets that were lying free in the sinusoids. This might be a sign of slight platelet activation, which was just not strong enough to cause complete platelet aggregation (25). Release of granulae may not be a result of mechanical stress activation, as should occur during perfusion fixation, seen the differences in ultrastructural characteristics of platelets which are mechanically activated (26). Since blood platelets become non-functional after loosing their granulae (25), the finding of agranulair platelets may be compatible with the acquired platelet dysfunction and the process of delayed release of non-functionating platelets into the circulation after liver graft perfusion, as suggested by Schalm et al. (7). It is well known that large platelets have a higher potential of thromboxane A_2 and are more prone to activation than smaller platelets (27). We did, however, not find any difference in mean platelet volume (MPV) before and after graft reperfusion, indicating that there seems to be no preferential sequestration of larger, and hence more reactive platelets during liver graft reperfusion.

Analysis of the blood samples of the first hepatic outflow after recirculation did not reveal any serious changes of the investigated coagulation parameters, when compared with the systemic values. These observations are in agreement with the results of recent large clinical studies (3,4) and suggest that reperfusion of optimal and short-term preserved livers has no important acute effect on the coagulation system. Lack of signs of clinical relevant activation of the clotting system confirms that the observed decrease of platelet count is unlikely to be due to a process of DIC.

On the other hand, we observed a significant increase of fibrinolytic activity, as measured by the ECLT, in the blood samples of the hepatic outflow of both orthotopic and auxiliary grafts. We could not find convincing evidence for DIC by either histopathologic or hemostasis studies, therefore we think that the increased fibrinolytic activity is of primary origin. Since the ECLT measures predominantly tissue-type plasminogen activator (t-PA), it is likely that an increase of t-PA levels in the circu-

lation has caused the observed increase in fibrinolytic activity. The more so as it was recently demonstrated that systemic hyperfibrinolysis in human orthotopic (28) and auxiliary partial liver transplantation (29) is related with increased plasma levels of t-PA. The mechanisms underlying the increased release are still unclear and need further study.

In conclusion, the most prominent early effects of graft reperfusion on hemostasis, found in this study, were a drop of the platelet count and increase in fibrinolytic activity. By using electron microscopy we could not find any evidence for intravascular coagulation or platelet aggragation. Early changes in platelets may be partly due to phagocytosis by Kupffer cells. Increased fibrinolytic activity appeared to be a primary process, originating from the reperfused liver graft.

ACKNOWLEDGEMENT

The authors wish to thank Mr. W. J. Visser from the dept. of Cell biology and Genetics, Erasmus University Rotterdam, for assistance in the electron microscopy studies and the personal of the Laboratory for Experimental Surgery for their help during the experiments. Dr. P. Roholl is gratefully acknowledged for his helpful comments and suggestions regarding the electron microscopy studies.

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CHAPTER 5

MONITORING HEPARIN AND HEMOSTASIS DURING RECONSTRUCTIONS OF THE ABDOMINAL AORTA

MONITORING HEPARIN AND HEMOSTASIS DURING RECONSTRUCTIONS OF THE ABDOMINAL AORTA

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This chapter has been published before in:
Eur. J. Vasc. Surg. 1987; 1: 397-402.

SUMMARY

In spite of its unpredictable kinetics, heparin is still not generally monitored during peripheral vascular surgery. To evaluate heparin levels and neutralisation, plasma heparin concentrations were measured using a chromogenic substrate method during 20 consecutive operations on the Abdominal Aorta. This was combined with measuring activated partial thromboplastin time (APTT), thrombin time (ThT), prothrombin time (PT), antithrombin-III (AT-III) and fibrinogen concentration. Heparin concentration 5 min after administration and the elimination rate showed a wide variation. Using a standard dosage for all patients resulted in plasma levels that are potentially too low in some patients. The APTT and ThT were found to be unsuitable for an exact calculation of heparin levels. Protamine administration based on the surgeon's judgement of hemostasis was inadequate. Furthermore an intra-operative decrease of AT-III and fibrinogen was seen in eight patients. It is advisable and possible to have direct monitoring of heparin concentration during peripheral vascular surgery.

INTRODUCTION

Surgery on bloodvessels without frequent thrombotic complications has become possible by the use of intraoperative anticoagulation. Since the first described use of heparin as an anticoagulant in vascular surgery in 1940 (1), a variety of regimens have been described. Most vascular surgeons use a standard dose with an average of 5,000 to 8,000 units given intravenously (2,3), usually without monitoring its anticoagulant effect. Others use a bodyweight dependent dose of heparin i.v. (4-6). Recently we have become more aware of the complex and individually variable kinetics of heparin, which makes it impossible to predict the activity and elimination of heparin in an individual person after a standard dose is given intravenously (7-10).

Although monitoring of heparin activity during cardiopulmonary bypass is a standard procedure and some authors have emphasized the need for a close monitoring during peripheral vascular surgery also, its measurement during this type of surgery is only slowly gaining acceptance (11-15). The most widely used tests for monitoring heparin are the whole-blood clotting time (16-18) and the activated partial thromboplastin time (19). However, with these tests, heparin activity is measured only indirectly and recent evidence indicates that these methods correlate poorly with heparin levels (13,20).

With a more recently developed test, using a specific chromogenic substrate for factor Xa, heparin activity can be measured directly (21). This offers a method of more precise monitoring of heparin activity and an exact calculation of the protamine dose necessary for heparin neutralisation. As far as we know this has not been evaluated in vascular surgery before. To use this method in combination with standard clotting assays in one apparatus, one should be able to perform both kinetic assays (chromogenic substrates) and end-point assays (clotting assays). We used a spectrophotometer in which both methods can be rapidly performed. Its compactness made it possible to use it easily in the operating theatre.

This communication reports the use of a chromogenic substrate method for monitoring heparin in combination with clotting assays during 20 abdominal aortic reconstruction procedures.

PATIENTS AND METHODS

Patients

The hemostasis parameters and heparin concentration were monitored during 20 consecutive, elective abdominal aortic bifurcation prosthesis operations. The ages of the patients ranged from 39 to 72 years, with a mean of 57.8 years. The weight ranged from 56 to 84 kg, a mean of 68.7 kg. Standard operative techniques were used in all procedures. Dextran 40 (Rheomacrodex^{R}, Pharmacia Fine Chemicals, Uppsala, Sweden) was used to substitute plasma volume, at a dose of 10 ml/kg body weight (10% (w/v) dextran in 0.9% NaCl). A standard dose of ±5,000 IU heparin (Thromboliquine^{R}, Organon, Oss, The Netherlands) was given by intravenous injection, about 10 min prior to clamping of the vessels. After completion of the anastomosis and removal of the clamps, protamine was administered, the amount varied between zero and 50 mg, depending on the time passed after heparin administration and the judgement of the surgeon.

Material

A FP-910 coagulation system (Labsystems, Helsinki, Finland) is a combination of a spectrophotometer, an analyzer, an incubator with thermoblocks, a mixer and 9-channel pipettes. The instrument is designed for conventional clotting assays, chromogenic methods and enzyme immunoassays with micro or semi-micro reagent volumes. Nine samples can be measured in one cuvetteblock by means of the vertical measurement principle., range 340-620 nm. For this study the FP coagulation system and an Eppendorf centrifuge were placed in the operating theatre.

Thrombin and Actin^{R} (activated cephaloplastin reagent) were obtained from Merz Dade, Dijdingen, Switzerland; Simplastin^{R} from General Diagnostics, Morris Plane, New Jersey, U.S.A. Acetate-Veronal buffer was prepared according to Michaelis, pH7.4. Thrombin was dissolved in Michaelis buffer with final concentrations of ±3 IU/ml.

Normal plasma, necessary for the heparin assay and used as control sample for other assays was obtained from twenty apparently healthy hospital staff volunteers (male/female ration about 1). Plasma was pooled and stored in 1 ml aliquots at -70^{o}C.

Methods

Blood was collected from an arterial line into 3.2% trisodiumcitrate

(9v/1v). The first 10 ml of blood were discarded to remove traces of heparin from the line. All samples were centrifuged immediately in an Eppendorf centrifuge (3.5 min, 10,000 g, R.T.). After separation, plasma samples were kept on melting ice ($0^{o}C$) until measured. Activated partial thromboplastin time (APTT) was determined with Actin^{R} (22) (N: 22-37 s). Prothrombin time (PT) was assayed according to Quick (23), with Simplastin^{R} (N: 10-14 s). Thrombin time (ThT) was measured by adding 200 µl Thrombin (±3IU/ml) to 200 µl prewarmed plasma (24) (N: 10-15 s). Fibrinogen was assayed according to Clauss (25) (N: 1.2-4.8 g/l). Antithrombin-III (AT-III) activity was measured using an amidolytic method (26) with the synthetic peptide substrate S-2238 (Coatest^{R} antithrombin, Kabi Vitrum Haematology bv., Amsterdam, The Netherlands) and expressed in U/ml (normal plasma = 1.00 U/ml). Heparin concentration was determined by measuring anti-factor Xa activity, using the chromogenic substrate S-2222, bovine factor Xa and human AT-III concentrate (21) (Coatest^{R} heparin, Kabi Vitrum Haematology BV, Amsterdam, The Netherlands). Blood samples were taken after induction of anaesthesia, 5 min after heparinisation, and subsequently every 30 min until heparin neutralisation with protamine chloride and 5 min afterwards.

RESULTS

Hemostasis parameters

In all plasma samples the APTT, PT, ThT, fibrinogen and antithrombin-III concentrations were determined. There were no major abnormalities of the investigated parameters in the samples taken prior to heparin administration, except for an unexplained prolongation of the APTT in one case (50s).

Five minutes after heparin administration, APTT and ThT were longer than 180 s and 60 s, respectively (upper limits of measuring time), which indicated that a good heparin effect was obtained in all but one case, which will be discussed in the section on Case Reports. However the exact heparin concentration could not be measured with these assays.

AT-III and fibrinogen concentrations did not remain constant during the operation in all cases. A decrease of AT-III of more than 25% of the initial value was seen in 10 patients, which resulted in values lower than 0.50 U/ml in 8 of these patients. AT-III decrease was combined with a decline in fibrinogen of more than 25% of the initial value in 6 cases.

Heparin monitoring

The standard dose of heparin, when divided by the patient's weight, resulted in median dose of 68.5 IU/kg (range 40.0 to 86.2 IU/kg). The median heparin concentration measured 5 min after heparinisation was 0.9 IU/ml (range (0.2 to 2.8 IU/ml). The elimination of heparin from plasma for all cases is shown in Figure 1. The period between heparin administration and neutralisation by protamine varied between 47 and 132 min (median 95 min, n=15). Based on the individual surgeon's judgement of hemostasis no protamine was given in 5 cases. Heparin activity was still measurable at the end of the operation in four of these patients (range: 0.2-0.8 IU/ml) and the heparin level in only one case was below the detection limit of the assay (<0.05 IU/ml). The dose of protamine chloride, administered in the other 15 patients, varied between 20 and 40 mg, primarily based on empirical data and preference of the surgeon. No heparin was detectable after protamine administration in any of these cases.

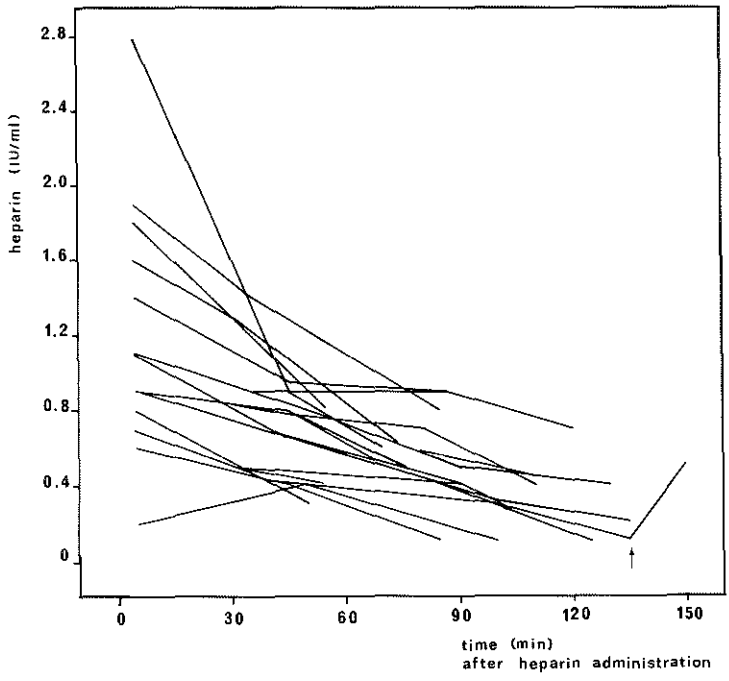

Fig. 1. Elimination curves of heparin after an intravenous bolus injection of ±5,000 IU heparin during 20 abdominal aorta reconstruction operations. Arrow indicates the extra heparin injection in case B (see text).

Case Reports

In Figure 2 data on three operations with unusual patterns of hemostasis parameters (cases B-D) are compared with a representative case with a normal course (case A).

Case A: This patient represented a normal course (Fig.2). The heparin concentration 5 min after administration was 0.9 IU/ml. During the operation the heparin level fell gradually to 0.4 IU/ml at the end, just before protamine was given. As can be seen from Figure 2, the APTT remained prolonged while heparin concentrations already decreased. This was also found for the thrombin time (data not shown). Both AT-III and fibrinogen levels remained stable at normal values during the operation.

Case B: This operation was complicated by the formation of thrombi. The initial plasma heparin concentration 5 min after a bolus injection of 4,000 IU heparin i.v. (57.1 IU/kg) was low (0.6 IU/ml) (Fig.2). Together with a fast disappearance of heparin, this resulted in a shorter period of optimal heparinisation (±45 min) than expected from the initial dosage. This may have contributed to the formation of thrombi, that were seen in both the left and right iliacal artery at the end of the procedure. A thrombectomy was performed successfully, showing two freshly formed thrombi on a pre-existing artheromatous degenerated vessel wall. At that moment the heparin concentration was 0.1 IU/ml and APTT was 45 s. Based on the clinical findings the surgeon decided to give an extra dose of 2,000 IU heparin after which the heparin level rose to 0.5 IU/ml and APTT was longer than 180 s.

Case C: A patient with low levels of both AT-III and fibrinogen. A remarkable decline of both AT-III and fibrinogen concentrations to 30% and 37% of the initial value respectively, was seen in this case, which finally resulted in values of 0.35 U.ml and 0.8 g/l respectively (Fig.2).

Case D: During surgery AT-III levels decreased and reached values below the sensitivity of the assay (0.05 U/ml) at the end of the operation (Fig.2). Fibrinogen levels, however, remained in the normal range.

Logistic data on the coagulation system

Using the FP-910 coagulation system anti-factor Xa heparin activity could be measured within 10 min after a blood sample was taken. The complete program, by which APTT, PT, ThT, fibrinogen, AT-III and heparin concentration were assayed could be performed within 30 min after blood-sampling. The intra- and intervariations of the assays are shown in Table 1. These data are comparable to other systems (e.g. coagulometer) and reflect

the applicability of this system. The costs for the total program of assays, measured per sample, together with control samples are approximately $15.00 for chemicals and $1.50 for disposable materials.

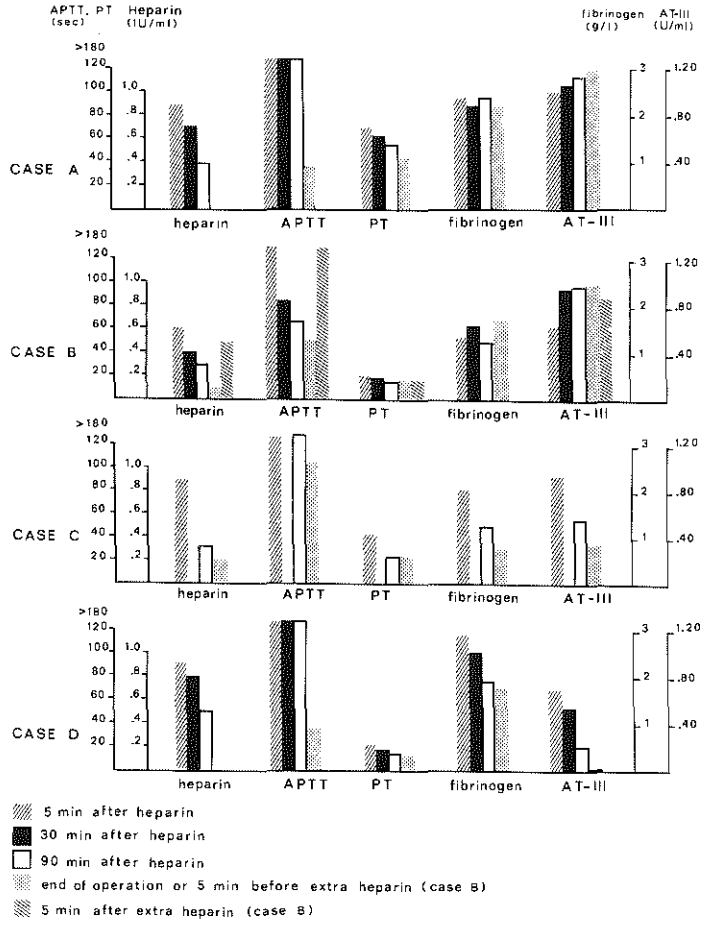

Fig. 2. Coagulation data of one patient as a representative example of a stable coagulation profile during surgery and three complicated cases. Case A: Normal pattern of hemostasis parameters and gradual disappearance of heparin; Case B: An extra dose of 2,000 IU heparin was necessary after a thrombotic complication during a period of low heparin concentration; Case C: Intraoperative decline of both AT-III and fibrinogen; Case D: Extreme intraoperative decline of AT-III.

Table 1. **Intra- and inter-assay variations on the FP-910 coagulation analyser**

	Intra-assay variations	Inter-assay variations	
Heparin	4.9%	8.9%	n=36
APTT	1.8%	5.0%	n=45
Thrombin time	3.9%	3.8%	n=45
Prothrombin time	3.3%	4.6%	n=41
Fibrinogen	7.5%	11.0%	n=36
Antithrombin III	5.0%	6.1%	n=36

DISCUSSION

Since the first described use of heparin as an anticoagulant in vascular surgery by Murray in 1940 (1), heparinisation has become standard procedure during this type of surgery (2).

Most surgeons use standard regimens for heparin administration without monitoring its activity. In recent years knowledge of the kinetics of heparin has considerably increased and we now know that heparin response after a single dose is unpredictable and the elimination rate has a wide individual variation, making it impossible to predict individual heparin effect, even when a body weight dependent dosage is used (7-9). In the few studies on monitoring of heparin in vascular surgery, usually indirect clotting assays, e.g. the activated clotting time, were used (3,11,12). However, these assays have been demonstrated to correlate poorly with heparin levels (13,20).

We report here for the first time the measurement of heparin concentration during vascular surgery with a direct chromogenic substrate method. This chromogenic substrate assay and the clotting assays used in this study were shown to have an acceptable intra- and inter-assay variation, which reflects a good reproducibility and reliability, whether performed in the laboratory or the operating theatre. We demonstrated that, with the chromogenic substrate assay, heparin concentration could be measured within 10 min

after blood sampling thus allowing it to be measured at regular intervals. By doing so it is, theoretically, possible to prevent over- or underheparinisation and to calculate the exact protamine dose.

What relevant outcomes were obtained from this study?

Firstly, by using the chromogenic substrate method we showed a wide variation in heparin response and elimination after a single intravenous dose, compatible with other studies in vascular surgery using different methods (3,11,15). These differences in elimination rate resulted in low heparin levels, faster than expected in some cases. However, in the literature there is no consensus on the minimal heparin concentration necessary to prevent thrombotic complications during vascular surgery. Based on studies of the prevention and progression of a thrombotic process in non-surgical patients a range of 0.2-0.5 IU/ml heparin was found to be effective (24,27). During surgery, however, the operative trauma may lead to activation of the clotting system, which probably requires higher heparin concentrations to prevent fibrin formation. This is also found during hemodialysis, for which it has been demonstrated that full suppression of fibrinopeptide A generation requires heparin anti-Xa levels of 0.7-1.0 IU/ml (28). It is likely that the same heparin concentration is needed during major vascular surgery, while the much higher levels (above 2.0 IU/ml) used during cardiopulmonary bypass may not be necessary (29,30). As can be seen in Figure 1 we found heparin levels to fall below the assumed effective level of 0.7-1.0 IU/ml in most cases. On the other hand, the complex kinetics of heparin were once more illustrated by the fact that, based on the time past after heparin administration and the surgeon's judgement in 4 patients no protamine was given, while in fact heparin was still measurable.

Secondly, the activated partial thromboplastin time (APTT) and the thrombin time (ThT) were found to become excessively prolonged at higher heparin levels. When heparin concentrations decreased, as measured with the chromogenic substrate assay, the APTT and ThT often remained longer than the fixed measuring time (180 s and 60 s, respectively). This demonstrates the insensitivity of these assays, which makes them unsuitable for an exact determination of heparin concentration at the levels used during vascular surgery.

Thirdly, this study showed a change in antithrombin-III and fibrinogen concentrations in patients undergoing major vascular surgery. Since the anticoagulant effect of heparin is known to be a result of its accelerating effect on inhibition of thrombin and factor Xa (and to a lesser degree factor

IXa and XIa) by AT-III (31,32), a decrease of AT-III to values lower than 0.50 IU/ml, as seen in 8 of the 20 patients may be of clinical importance. Although different explanations have been proposed to explain a decrease of AT-III during heparinisation or surgery (30.33-38), the cause of the decrease of AT-III, found in this study is still unclear.

In conclusion we can state that the individual differences in heparin kinetics, together with the changes in hemostatic parameters, found in patients undergoing major vascular surgery, warrants close monitoring in each patient. We have demonstrated that for this purpose the chromogenic substrate assay for heparin is feasible and it can be quickly performed in the operating theatre in combination with different hemostatic parameters.

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CHAPTER 6

SYSTEMIC EFFECTS OF TISSUE PLASMINOGEN ACTIVATOR ASSOCIATED FIBRINOLYSIS AND THE RELATION WITH THROMBIN GENERATION IN ORTHOTOPIC LIVER TRANSPLANTATION

SYSTEMIC EFFECTS OF TISSUE PLASMINOGEN ACTIVATOR ASSOCIATED FIBRINOLYSIS AND THE RELATION WITH THROMBIN GENERATION IN ORTHOTOPIC LIVER TRANSPLANTATION

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This chapter has been accepted for publication in:
Transplantation.

SUMMARY

Orthotopic liver transplantation is frequently associated with hyperfibrinolysis. The origin and clinical relevance of which are largely unknown. In 20 orthotopic liver transplantations we studied the occurrence and systemic effects of hyperfibrinolysis. Severe fibrinolysis was defined to be present when the euglobulin clot lysis time and the whole blood clot lysis time, as measured by thrombelastography, were shorter than 60 and 90 min respectively, at some time during the operation. Based on these criteria, seven patients had minimal fibrinolysis (group I), and thirteen patients had severe fibrinolysis (group II). In group II a gradual increase of t-PA activity was seen during the anhepatic stage, followed by an "explosive" increase immediately after graft reperfusion ($P=0.0004$, compared with group I), and a reduction of PAI activity. Plasma degradation products of fibrinogen and fibrin increased parallel to t-PA activity and levels were significantly higher at 45 min after graft reperfusion in group II compared with group I ($p<0.04$). Thrombin-antithrombin III complexes showed an identical steady increase in both groups, indicating that increased t-PA activity was not related to thrombin formation. A combination of increased endothelial release and reduced hepatic clearance may have caused the increased t-PA activity. The t-PA associated destruction of fibrinogen and fibrin after graft reperfusion is consistent with the clinical signs of severe oozing often seen in this period. These observations may have important clinical implications for the treatment of bleeding in patients undergoing orthotopic liver transplantation.

INTRODUCTION

Orthotopic liver transplantation has become an accepted and clinically useful treatment for patients with a variety of irreversible liver diseases (1). The gradual improvements of the surgical technique, anesthesiologic management and immunosuppressive therapy have contributed to an increase in the success rate and long-term survival (1,2). The surgical operation, however, is an extensive procedure, which may be frequently associated with serious bleeding, requiring massive blood transfusions (3). Maintenance of surgical hemostasis may be seriously complicated by disturbances in the hemostatic system. Previous studies have suggested an important role of hyperfibrinolysis in the origin of bleeding complications during orthotopic liver transplantation (4,5)

Recently, studies involving only a few subjects have shown that the hyperfibrinolysis during orthotopic liver transplantation may be related to increased plasma levels of tissue-type plasminogen activator (t-PA) (6,7), a key enzyme of the fibrinolytic system (8). Under normal physiological conditions t-PA activity in the circulation is low, due to its rapid inactivation by formation of complexes with PA-inhibitors (PAI), but t-PA activity may increase several fold after specific stimuli (8). Both t-PA and its major inhibitor (PAI-1) are produced and secreted by endothelial cells, whereas hepatocytes and blood platelets are additional sources of PAI-1 (8,9). Elimination of t-PA from the blood is mainly regulated by the liver with a t1/2 of 3-5 minutes (10). Increased levels of t-PA during orthotopic liver transplantation probably result from a combination of increased endothelial release and decreased hepatic clearance during the anhepatic stage (6). The mechanisms underlying the increased release of t-PA and the role of PAI in the regulation of t-PA activity during liver transplantation, however, are still unknown.

Some investigators have suggested that hyperfibrinolysis in orthotopic liver transplantation may be secondary to disseminated intravascular coagulation (DIC) (7,11,12). Differentiation between secondary and primary fibrinolytic activity, however, has been difficult, mainly due to lack of appropriate laboratory tests. Lack of specific parameters has also hampered the assessment of the role of increased fibrinolytic activity in the actual breakdown of coagulation factors and the development of a bleeding diathesis. Especially under strongly hyperfibrinolytic conditions fibrinogenolysis may also occur (13). Although increased serum levels of fibrin(ogen) degrada-

tion products have been found during liver transplantation (4,11), whether these were the result of fibrin breakdown or plasmin destruction of fibrinogen could not be determined.

In the present study we examined the origin and clinical relevance of hyperfibrinolysis in orthotopic liver transplantation. Measurement of t-PA and PAI activity in combination with the separate quantitation of plasma degradation products of fibrinogen and fibrin enabled us to study the origin of hyperfibrinolysis and its role in the development of a systemic lytic state. Thrombin-antithrombin III (TAT) complexes were measured to study the role of clotting activation and thrombin formation in the origin of hyperfibrinolysis.

MATERIALS AND METHODS

Patients

Twenty three adult patients who underwent their first liver transplantation at Presbyterian University Hospital between June 1st and 27th, 1988 were observed prospectively. In an otherwise consecutive series, three patients of whom more than two blood samples were missing due to technical errors, were omitted. The remaining 20 patients were categorized by pathological diagnosis as described previously (14). Five different diagnostic groups could be distinguished, as shown in table 1.

Table 1. Diagnosis and characteristics in 20 patients undergoing orthothopic liver transplantation

Diagnosis	No.	F	M	Age range
Postnecrotic cirrhosis	9	3	6	27 - 54
Primary biliary cirrhosis	5	5	-	27 - 60
Sclerosing cholangitis	3	1	2	29 - 41
Carcinoma	1	-	1	63
Miscellaneous*	2	1	1	22, 37
Total	20	10	10	22 - 63

*Two patients with Wilson's disease.

Orthotopic liver transplantation was performed by a standard technique, using a veno-venous bypass in all patients (15). The surgical procedure can be divided into three stages. During the preanhepatic stage (stage I) the host liver is isolated. The anhepatic stage (stage II) begins with the clamping of the vessels of the native liver and ends with the completion of the vascular anastomosis of the graft liver. The postanhepatic stage (stage III) lasts from graft reperfusion to the end of surgery. Intraoperative blood loss was compensated by the transfusion of modified whole blood (from which platelets have been removed) or packed RBC and fresh frozen plasma in an approximate ration of 1:1. In case of massive blood loss a rapid infusion system was used, by which a mixture of packed RBC, fresh frozen plasma and Plasmalyte A was infused in a ratio of 1 U: 1U: 250 ml. Platelets and cryoprecipitate were usually not given before stage III. All patients gave their informed consent for blood sampling during the operation, as part of the intraoperative patient care.

Intraoperative blood samples for hemostasis monitoring were collected from an arterial line. Blood (9 ml) was collected in 1 ml 0.13 mol/l trisodium citrate and immediately centrifuged at 2800 g for 10 min. Plasma was either directly used for testing or frozen at -70 ^{o}C. Whole blood (0.36 ml) was used for thrombelastographic monitoring within 2 min after sampling. Blood samples were taken according to the following schedule: immediately after induction of anesthesia (BASE); 30 min before removing the liver (II-30); 5 min in the anhepatic stage (II+5); 5 min before graft reperfusion (III-5); 5 min after graft reperfusion (III+5); 45 min after reperfusion (III+45); 150 min after reperfusion (III+150) and at the end of the operation (END).

Assays

Standard hemostasis tests were performed using previously described methods (16,17). Thrombelastographic monitoring of whole blood coagulation and fibrinolysis was performed using a Thromb Elastograph-D (Haemoscope Corporation, Morton Grove, Ill). The whole blood clot lysis time (WBLT) was defined as the time between the maximum amplitude and the registration of complete lysis on the thrombelastographic recording (normal >150 min) (18).

Levels of t-PA activity (normal range, 0-1 IU/ml) and PAI activity were measured using chromogenic substrate methods (Coasets tPA and PAI, Kabi Vitrum Hematology, Stockholm, Sweden). For the measurement of t-PA activity 100 ul of plasma was acidified (pH 4.0 - 4.1) with 100 ul of acetate buffer and 20 ul of 20% acetic acid, both supplied in the assay kit. T-PA

activity was determined by measuring the amidolytic activity of plasmin onto the chromogenic substrate S-2251, after incubation in the presence of plasminogen and human fibrin(ogen) fragments (19,20). The fibrinolytic activity of t-PA was expressed in International Units (IU) assessed by calibration against the international standard of t-PA from human melanoma cells (lot 83/517, National Institute for Biological Standards and Control, London, UK). PAI activity was measured by adding 40 IU/ml t-PA to an equal volume of plasma. After incubation for 10 min at room temperature samples were diluted with sterile water (1:80) and residual t-PA activity was determined as described above. PAI activity was expressed in arbitrary units (AU), defined as the amount which inhibits one IU of t-PA in 10 min (21) (normal, 0-40 AU/ml).

Two different sandwich-type enzyme linked immunosorbant assays (ELISA) (Fibrinostika, Organon Teknika, Turnhout, Belgium) were used for the quantitation of plasma levels of fibrinogen degradation products (FgDP; normal <0.5 µg/ml) and fibrin degradation products (FbDP; normal <0.5 µg/ml). In both ELISAs a monoclonal antibody, which reacts exclusively with FgDP and FbDP and not with intact fibrinogen or fibrin, is used as catching antibody. The FgDP ELISA contains a monoclonal tagging antibody which is specific for covalently bound fibrinopeptide A. Since fibrinopeptide A is split off during the activation of fibrinogen by thrombin, this ELISA tags only FgDPs which result from the plasmin-mediated destruction of fibrinogen (22). The FbDP ELISA gets its specificity for FbDP by using a monoclonal antibody which is elicited with D-dimer as immunogen (23).

TAT complexes (normal range, 1.0-4.1 µg/l) were measured by an ELISA (Behringwerke, Marburg, FRG), based on rabbit antibodies to human thrombin and antithrombin III respectively (24). Samples with TAT levels exceeding the highest standard contained in the assay kit (60 µg/l), were diluted (1:2 or 1:4) in normal pooled plasma, which had been shown to have a TAT concentration of 1.2 µg/ml.

Statistical Analysis

Statistical analysis was performed using the NPAR1WAY computer program of the Statistical Analysis System^{R} (SAS Institute Inc., Cary, NC, U.S.A.). The significance of differences within and between groups were tested using the Wilcoxon rank-sum test and two-sample test respectively. Values for $p < 0.05$ were considered to be significant.

RESULTS

In all but one patient slightly to severely increased fibrinolytic activity, as measured by shortening of the euglobulin clot lysis time (ELT; normal >120 min) or WBLT (normal >150 min), was found in at least one blood sample during the operation. Signs of hyperfibrinolysis were most frequent at the end of the anhepatic stage and early after graft reperfusion of the donor liver. Fibrinolysis was defined as minimal if the ELT was longer than 60 min or when the WBLT was longer than 90 min in all blood samples. Severe fibrinolysis was defined to be present when the ELT and WBLT were shorter than 60 and 90 min respectively, in at least one of the intraoperative blood samples. According to these criteria the patients were divided into two groups. Group I was formed by 7 patients with minimal fibrinolysis. Group II consisted of 13 patients with severe fibrinolysis. Comparison of the preoperative hemostasis parameters showed no significant differences between the two groups (table 2). Both groups included patients with different diagnoses, without an accumulation of any diagnostic group in either one of the two groups.

Mean intraoperative levels of t-PA activity and PAI activity of group I and II are depicted in figure 1. There were no significant changes in t-PA and PAI activity during the preanhepatic stage in both groups. In group I t-PA levels remained below 12 IU/ml during the rest of the operation in all patients. In group II t-PA activity increased after clamping of the vessels of the native liver and levels were significantly higher at the end of the anhepatic stage (III-5), compared with group I ($p=0.002$). During reperfusion of the graft t-PA levels increased sharply, resulting in a more than doubling at 5 min after reperfusion, compared with the values at 5 min before reperfusion ($p< 0.007$). At this time t-PA activity in group II (65.1±8.5 IU/ml, mean±SEM) was about 30 times higher than the preoperative value and more than 10 times higher than t-PA activity in group I ($p=0.0004$). Later in the postanhepatic stage a rapid disappearance of t-PA activity was seen and levels fell into the normal range (0-1 IU/ml) at the end of the operation in all but two patients. In these two patients t-PA levels were still moderately increased (3.4 and 10 IU/ml). In group II free PAI activity showed a pattern inverse that of t-PA activity and only minimal PAI activity (1.1±0.7 AU/ml) was left at the peak of fibrinolytic activity, but levels increased during the later postanhepatic period in both groups.

Mean plasma levels of FbDP and FgDP in group I and II are shown in

figure 2. Although an increase of FbDP and FgDP was seen in both groups, levels in group II were significantly higher in the postanhepatic period at 45 min after graft reperfusion when compared with group I ($p<0.04$). In group II the highest FgDP level (18.4±7.9 µg/ml) coincided with the peak in t-PA activity (II+5), whereas the maximum in FbDP (32.5±11.2 µg/ml) occurred somewhat later in the postanhepatic stage (III+45).

Table 2. Comparison of preoperative hemostasis profile in patients with minimal (group I) and severe (group II) intraoperative fibrinolysis

Variables	Reference values	Group I median (range)	Group II
Coagulation			
PT (sec)	10.8-13.0	11.3 (10.4-21.2)	12.2 (9.7-21.2)
aPTT (sec)	26-34	36.0 (28.9-51.6)	42.9 (29.1-127)
ThT (sec)	13-18	17.1 (15.3-32.9)	22.0 (14.3-47.7)
Fibrinogen (mg/dl)	150-450	285 (159-460)	140 (85-350)
Factor II (%)*	50-150	66 (32-130)	38 (15-135)
Factor VIII (%)*	50-150	185 (130-300)	120 (82-280)
TAT complex (µg/ml)	1.0-4.1	8.0 (2.0-16.0)	4.3 (1.6-60.0)
Platelets (10^9/l)	150-450	81 (56-510)	118 (39-336)
Fibrinolysis			
ELT (min)	> 120	> 120 (105->120)	60 (15->120)
WBLT (min)	> 150	> 150**	> 150**
t-PA act. (IU/ml)	0-1.0	2.4 (0-6.0)	1.4 (0-8.0)
PAI act. (IU/ml)	0-40.0	18.5 (14.0-36.0)	18.8 (3.0-37.5)
FgDP (µg/ml)	< 0.5	0.28 (0.20-3.0)	0.50 (0.20-2.6)
FbDP (µg/ml)	< 0.5	0.30 (0.26-4.5)	0.84 (0.22-6.0)

* % of pooled normal plasma
** for all patients.

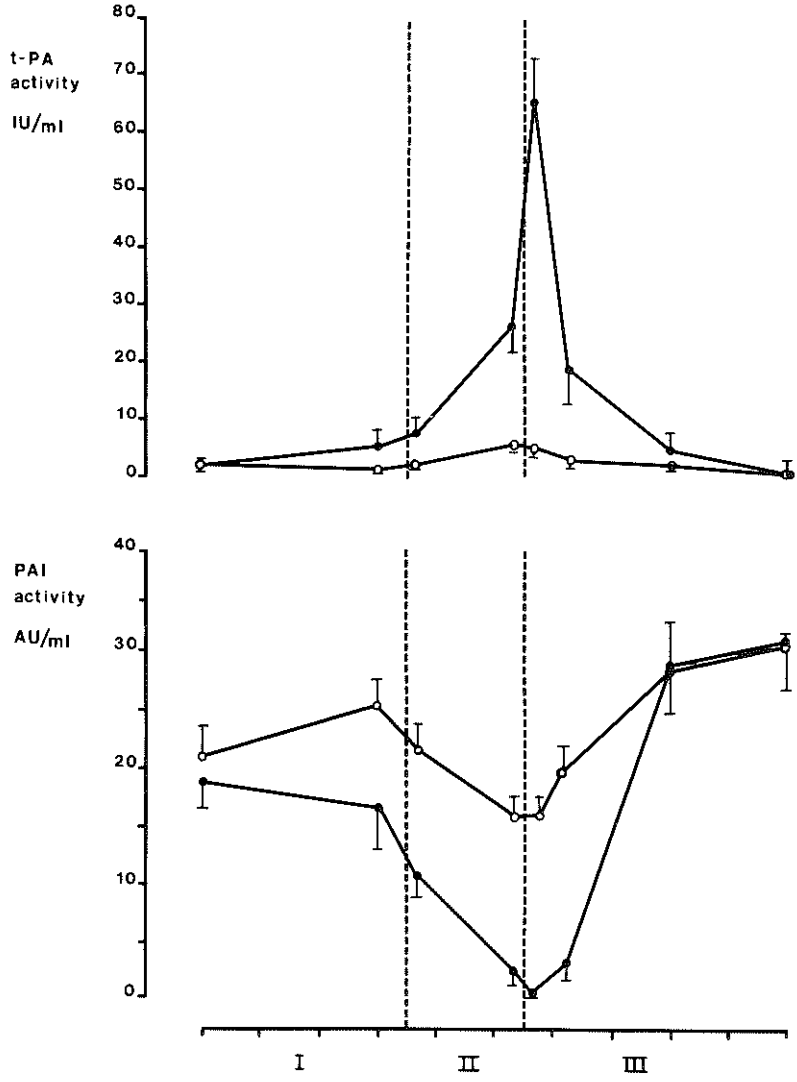

Fig. 1. Intraoperative levels of t-PA activity and PAI activity (mean±SEM) in patients with minimal (O–O, group I, n=7) and severe fibrinolysis (●—● , group II, n=13). Each tic on the abscissa indicates one hour. The area between the dotted lines represents the anhepatic stage (stage II).

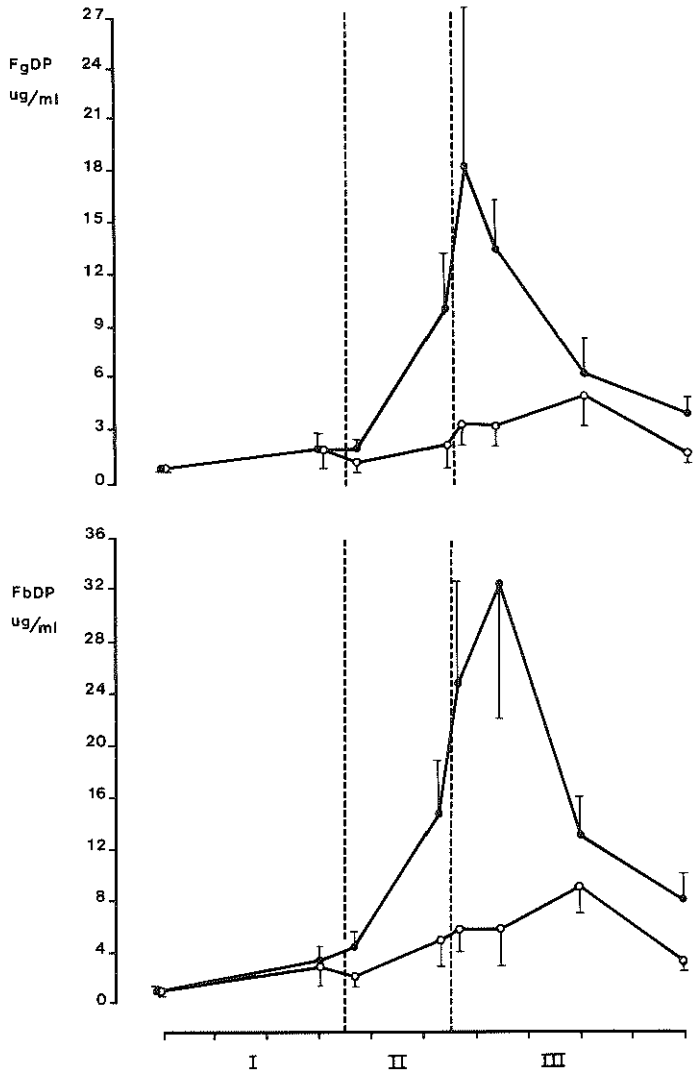

Fig. 2. Intraoperative levels (mean±SEM) of fibrinogen degradation products (FgDP) and fibrin degradation products (FbDP) in orthotopic liver transplantation. O–O = group I: patients with minimal fibrinolysis (n = 7); ●–● = group II: patients with severe fibrinolysis (n = 13). Each tic on the abscissa indicates one hour. The area between the dotted lines represents the anhepatic stage (stage II).

Mean levels of TAT complexes in group I and II are shown in figure 3. An identical increasing pattern was seen in both groups and at none of the time points were TAT levels significantly different between the two groups. Highest TAT levels were found during the postanhepatic stage and levels were still above the normal upper limit (>4.1 µg/l) at the end of the operation in all patients.

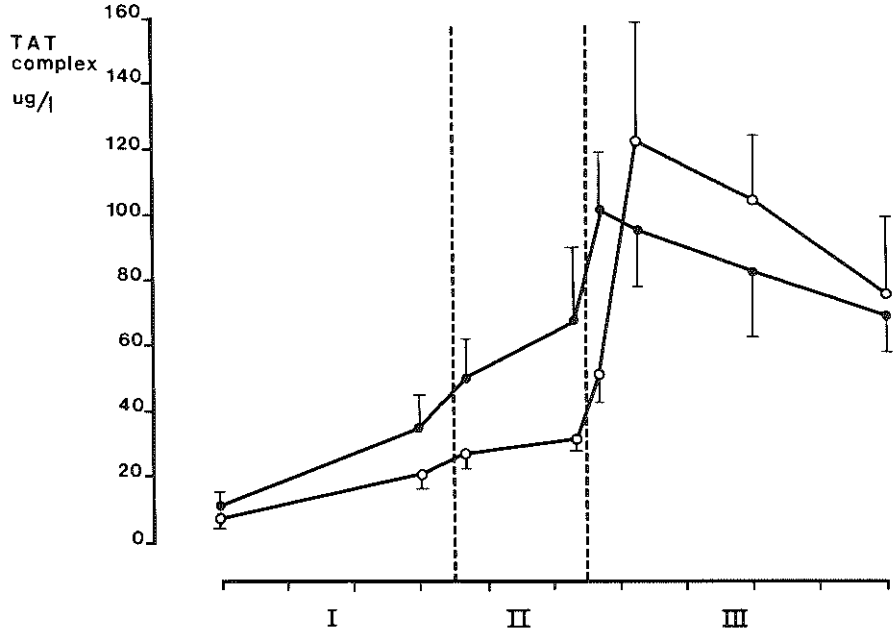

Fig. 3. Intraoperative levels (mean±SEM) of thrombin-antithrombin III (TAT) complexes in orthotopic liver transplantation. O-O = group I: patients with minimal fibrinolysis (n = 7); ●—● = group II: patients with severe fibrinolysis (n = 13). Each tic on the abscissa indicates one hour. The area between the dotted lines represents the anhepatic stage (stage II).

Comparison of changes in arterial pH, pO_2 and blood pressure in group I and II are shown in table 3. There was no evidence for a relationship between intraoperative changes in hemodynamics and the increase in t-PA activity. Periods of shock, as determined by a drop in blood pressure and pH were found among both patients with high and low t-PA activities.

The intraoperative use of blood products is shown in table 4. There were no differences in the use of cryoprecipitate and platelets between the two groups. However, intraoperative blood loss, as reflected by the total use of modified whole blood or packed RBC and fresh frozen plasma, was significantly higher in group II than in group I ($p<0.02$).

Table 3. Comparison of intraoperative hemodynamic changes in patients with low (group I) and high (group II) t-PA activity

	Group I (n=7)	Group II (n=13)
Hemodynamics at the end of stage II		
Arterial pH	7.37 (7.33-7.45)	7.40 (7.34-7.46)
Arterial pO_2 (mm Hg)	218 (113-311)	279 (122-397)
Number of patients with a hypotensive period	1/7 (14%)	3/13 (23%)
Hemodynamics early in stage III		
Arterial pH	7.31 (7.22-7.42)	7.32 (7.15-7.42)
Arterial pO_2 (mm Hg)	272 (150-486)	275 (114-531)
Number of patients with a hypotensive period	1/7 (14%)	5/13 (38%)

pH and PO_2 are expressed in median (range)

Table 4. Intraoperative blood use in patients with low (group I) and high (group II) t-PA activity.

		Group I (n=7)	Group II (n=13)
RBC* (U)	mean	5	19
	median (range)	4 (1-8)	10 (3-101)
FFP (U)	mean	1	11
	median (range)	0 (0-8)	4 (0-68)
RBC + FFP (U)	mean	6	30
	median (range)	4 (2-16)	10 (3-169)
Cryoprecipitate (U)	mean	0**	3
	median (range)	0**	0 (0-12)
Platelets (U)	mean	3	5
	median (range)	0 (0-10)	8 (0-28)

* RBC includes packed red blood cells and modified whole blood.
** for all patients.

DISCUSSION

In earlier studies we have found that increased fibrinolytic activity, as measured by the ELT or thrombelastography (TEG), occurs in about 80% of the patients undergoing orthotopic liver transplantation (18,25). Fibrinolytic activity may increase during the anhepatic stage and is most often severe early after graft recirculation. A simultaneous decrease of plasminogen and α_2-antiplasmin, the main inhibitor of plasmin, has been found during this period, which supports the view of an active fibrinolytic process (5). However, the use of ELT and TEG in these studies did not allow an exact characterization of the fibrinolytic defect, and the origin and clinical relevance of the increased fibrinolytic activity have remained largely unclear.

In this study we found an extreme increase of t-PA activity, and concomitant decrease of PAI activity during the anhepatic and early ostan-hepatic period in patients with severe fibrinolysis, as measured by the ELT and TEG. Reduction in PAI activity can be explained by the formation of complexes with t-PA. After saturation of free PAI, a further increase of t-PA will result in the increase of free t-PA activity in the circulation (9,21).

Recently, Dzik et al (6) and Palareti et al (7) have reported a similar increase of t-PA during the anhepatic stage in a limited number of patients undergoing OLT. However, these studies did not show the explosive t-PA increase, occurring directly after graft reperfusion, as seen in our patients. Since we observed a rapid normalization of t-PA activity after its maximum, this peak could have been easily missed in the other studies if no blood samples were taken within 10 minutes after reperfusion.

The intraoperative course of t-PA activity suggests that there are two different mechanisms responsible for the increase of t-PA during orthotopic liver transplantation. The initial rise of t-PA during the anhepatic stage has been explained by a combination of increased release of t-PA and reduced hepatic clearance (6). This view is supported by the lack of fibrinolytic activation during auxiliary, heterotopic liver transplantation in which the native liver is not removed (26,27). Dzik et al. (6) found that increase of t-PA during orthotopic liver transplantation may be associated with signs of shock. They suggested a mechanism of increased t-PA release due to hypotension and acidosis. We could not find any differences in blood pressure, arterial pH or pO_2 in patients with high or low t-PA levels. Probably other mechanisms than changes in the arterial circulation may also attribute to an increased release of t-PA. Patients with liver disease are known to be prone to activation of their fibrinolytic system upon specific stimuli as physical stress and exercise (28,29). The surgical stress, with the manipulations and extensive trauma to the vascular bed and abdominal circulation, therefore, might have contributed to an increased release of t-PA.

The rapid increase during graft reperfusion suggests a second mechanism which is associated with the restoration of blood flow through the donor liver. In studies with pigs we recently demonstrated that fibrinolytic activity in the hepatic venous outflow immediately after graft reperfusion is significantly higher than the fibrinolytic activity in the systemic circulation (Porte et al, 1988. unpublished data). These data, in combination with the "explosive" t-PA increase after reperfusion in this study, strongly suggest an increased release of t-PA from the reperfused donor liver. Several factors, including vasoreactive agents, venous occlusion, anoxia and thrombin have been found to stimulate t-PA release from endothelial cells in in vitro and in vivo studies (8,30,31). It can be theorized that, during graft reperfusion and the subsequent restoration of normal portal blood flow, one or more of these factors, or leakage from ischemic damaged endothelial cells, contribute to an increase of t-PA. The normalization of t-PA activity during the late postan-

hepatic stage can be explained by the restoration of the normal hepatic clearance of t-PA after the implantation of a viable donor liver. Reduction of t-PA activity might have been enhanced by an increase of PAI towards the end of the operation, which is generally seen after major surgery and which is consistent with the behavior of PAI as an acute phase reactant (32).

Some investigators have suggested that hyperfibrinolysis in orthotopic liver transplantation may be secondary to thrombin formation during DIC (7,11). For several reasons we do not believe that DIC is the main cause of t-PA increase during orthotopic liver transplantation.

First of all, in large series of patients, we previously found no evidence for a combined decrease of coagulation factors and inhibitors, as occurs during DIC (24,33). Repeatedly negative findings regarding thrombo-embolic processes in histopathologic and clinical examination support the view that DIC does not play a clinically important role in OLT (34,35).

Secondly, this study provided evidence for a thrombin independent increase in t-PA during liver transplantation. Although TAT complexes, which are formed early during clotting activation by complex formation between thrombin and antithrombin III (24), increased steadily from the beginning of the operation and were still elevated at the end of the operation, t-PA increase was clearly limited to the anhepatic and early postanhepatic stage. Additionaly, there was no difference in TAT increase in patients with high or low t-PA levels, indicating that the t-PA increase was independent of thrombin formation. The rise in TAT levels in our patients was most probably a sign of local clotting activation at the wound surface. Further studies, preferably with patients undergoing other major abdominal surgical procedures as control group, however, are necessary to establish the role of increased TAT levels in orthotopic liver transplantation.

Thirdly, secondary fibrinolysis during DIC is probably a more local process which does not result in detectable increased fibrinolytic activity in the systemic circulation. In an analysis of 346 patients with DIC, Spero et al. (36) found evidence of systemic fibrinolytic activation, as demonstrated by shortened ELT or recalcified clot lysis time, in only 10% of the patients. Francis and Seyfert (37) recently demonstrated that although t-PA antigen is elevated in patients with DIC, detectable free t-PA activity is less frequently present than in hospitalized controls. These investigators concluded that PAI levels are also increased in DIC, leading to masking of the increased endothelial secretion of t-PA. This view is supported by experiments with

cultured endothelial cells in which thrombin was shown to stimulate the release of both t-PA and PAI (30), resulting in no detectable net free t-PA activity despite increased levels of t-PA antigen. We observed a 40-50 fold systemic increase of t-PA activity and a concomitant saturation of PAI, resulting in a decrease of free PAI activity during the period of hyper-fibrinolysis. Another process than DIC, therefore, seems responsible for the increased t-PA activity in orthotopic liver transplantation. Why some patients develop a high increase of t-PA activity, whereas others do not, remains unclear. We could not find any difference in diagnosis, preoperative hemostasis profile or intraoperative hemodynamics in patients with high or low intraoperative t-PA activity levels.

Irrespective of its origin, the extremely high t-PA levels during the early postanhepatic stage may be clinically important. Especially in this period, formation of fibrin and stable hemostatic clots is necessary to prevent or stop bleeding from the vascular anastomosis and the extensive wound surface. Although the systemic increase of t-PA activity had a transient character, the clinical effect may extend over a considerably longer period, as t-PA may bind to fibrin and become incorporated in newly formed hemostatic clots, resulting in early lysis and delayed bleeding from fresh wounds (38). The role of t-PA in the development of a systemic lytic state was demonstrated in this study by the increase of plasma levels of both FbDP and FgDP in patients with high t-PA levels. This confirms earlier studies, which suggested a plasmin-mediated destruction of coagulation factors during orthotopic liver transplantation (5,33). FgDPs are known to have an anticoagulant effect by inhibiting the polymerization of fibrin monomers into crosslinked fibrin (39). The occurrence of FgDPs in the circulation, therefore, may well be attributed to a further deterioration of the hemostatic function in patients undergoing orthotopic liver transplantation, as has been suggested before by Blecher et al. (40). The peak of FbDPs in the early postanhepatic stage is consistent with the clinical picture of delayed oozing and increased blood loss in this period (18,25). We indeed found an significantly higher blood loss in patients with severe fibrinolysis (group II). However, these data should be interpreted with some reserve, as blood loss is influenced by multiple factors and larger series of patients are necessary to confirm this observation.

Since the primary increase in t-PA activity was associated with an active proteolytic destruction of fibrinogen and fibrin and a high blood loss, the use of antifibrinolytic drugs seems justified in patients with life

threatening hemorrhages where active fibrinolysis is likely. To identify these patients, it is advisable to include at least one test method for the assessment of fibrinolytic activity in the intraoperative hemostasis monitoring in orthotopic liver transplantation.

ACKNOWLEDGEMENT

This study was sponsored by the Netherlands Digestive Diseases Foundation and the A.A. Van Beek Fund. Assay kits were gifts from Behringwerke (Marburg, FRG), Kabi Vitrum Haematology (Stockholm, Sweden) and Organon Teknika (Turnhout, Belgium).

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CHAPTER 7

COAGULATION AND FIBRINOLYSIS IN THE FIRST HUMAN AUXILIARY PARTIAL LIVER TRANSPLANTATION IN ROTTERDAM

COAGULATION AND FIBRINOLYSIS IN THE FIRST HUMAN AUXILIARY PARTIAL LIVER TRANSPLANTATION IN ROTTERDAM

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This chapter has been published before in:
Fibrinolysis 1988; 2: 111 - 117.

SUMMARY

Coagulation and fibrinolysis parameters were extensively monitored during and after a case of auxiliary partial liver transplantation (APLT). Preoperative coagulation tests demonstrated a severe liver dysfunction. Routine coagulation tests were prolonged and low levels of fibrinogen, antithrombin III (AT-III), and α_2-antiplasmin (α_2-AP) were measured. Pro-urokinase (pro-Uk), total Uk-antigen and plasminogen activator inhibitor (PAI) activity were increased (6.2; 5.4 ng/ml and 12.5 IU/ml, respectively). During transplantation no major changes in hemostasis were detected by either standard coagulation tests or thrombelastography and only minimal increase of fibrinogen degradation products (0.6-1.0 μg/ml) was seen just before graft recirculation up till the end of the operation. PAI levels remained high during the operation and neither tissue-type plasminogen activator (t-PA) nor active urokinase (u-PA) were detectable. After APLT, graft function was reflected by normalization of coagulation parameters and increase of AT-III and α_2-AP to normal levels. Pro-Uk and PAI activity levels decreased after transplantation to normal values; a transient increase of t-PA activity (max. 1360 mIU/ml) was seen from the tenth to nineteenth day. It was demonstrated that a heterotopically placed liver allograft can provide synthesis of hemostasis factors and inhibitors and restore clearance which is adequate to reverse coagulopathy.

INTRODUCTION

Liver transplantation, which began as highly experimental surgery 20 years ago, has reached such a high level of success that it is now recognised as a major means of therapy for patients with end-stage liver disease (1). The most commonly performed technique is an orthotopic transplantation by which the diseased host liver is replaced by a liver allograft. Orthotopic liver transplantation (OLT) in humans is often complicated by a severe bleeding tendency which is associated with a marked (post-) operative mortality (2,3). Auxiliary liver transplantation is a different technique by which a liver graft is transplanted in a heterotopic position without removal of the diseased host liver. Theoretically, auxiliary liver transplantation would be expected to have a reduced bleeding tendency, because the surgical trauma is less and the patient's own liver retains the ability to produce clotting factors and remove procoagulant factors from the circulation. After extensive experimental studies with auxiliary liver transplantation in animals (4-6), an auxiliary partial liver transplantation (APLT) program for humans has been started in the University Hospital of Rotterdam in cooperation with the University Hospital Leiden and the Municipal Hospital of Arnhem, The Netherlands in 1986. In this paper we report the results of monitoring coagulation and fibrinolysis parameters during and after APLT in the first case. Details of the surgical procedure will be described elsewhere.

METHODS

During transplantation and in the first 3 postoperative days, blood samples were taken from an arterial line. (The first 10 ml were discarded to flush traces of heparin from the line). During the further postoperative course blood was collected by venapuncture with minimal stasis. Blood was collected into polystyrene test tubes, containing 1 ml ice-cold trisodium citrate 0.11 mol/l (9 vol + 1 vol), immediately placed on melting ice and centrifuged (2800g, 4^{o}C, 30 min) within 20 min. Plasma was collected at 4^{o}C, snap-frozen and stored in small aliquots at -70^{o}C until used. A few ml of blood were also collected in a plastic syringe and used within 3 min for thrombelastographic monitoring. Blood samples were obtained at various intervals during transplantation: directly after induction of anaesthesia, thereafter every 1.5 h; 5, 15 and 30 min after reperfusion of the liver graft

and thereafter every hour. During the first postoperative weeks blood samples were taken daily between 8:00 and 11:00, with exception on days 1 and 8 when blood samples were taken at 16:00 and 14:30, respectively. Routine coagulation tests were performed using standard techniques. Antithrombin III (AT-III) activity was measured according to Abildgaard et al (7) using Coatest^R antithrombin (Kabi Vitrum BV, Amsterdam, The Netherlands) (Normal values: 0.70-1.28 U/ml), α_2-antiplasmin (α_2-AP) was assayed according to Friberger et al (8) using Coatest^R antiplasmin (Kabi Vitrum BV). α_2-AP antigen was assayed by Rocket immunoelectrophoresis according to Laurell (9), using a 1.5% rabbit anti α_2-AP antiserum (batch 31-1085) obtained from Nordic Immunology, Tilburg, The Netherlands (N: 80-140%). The ratio of the two molecular forms of α_2-AP, the plasminogen binding (PB) and the non-plasminogen binding (NPB) form, was determined by modified crossed immunoelectrophoresis as described by Kluft & Los (10) (N: 2.1-2.8). Fibrinogen was measured according to Clauss (11) (N: 1.2-4.8 g/l). Three different sandwich-type enzyme immuno assays (EIA), based on monoclonal antibodies, were used for the separate quantitation of fibrinogen degradation products (FgDP) (12), of fibrin degradation products (FbDP) (13), and the total of FgDP and FbDP (TDP-assay) (14) (N: <0.5 µg/ml). Fibrinolytic activity of the euglobulin fraction of plasma was measured as the euglobulin clot lysis time (ECLT). Standard euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10 (15). Precipitates were redissolved in Tris/Tween buffer (0.1M Tris/HCL, containing 0.1% Tween 80 (v/v) pH 7.5) and to 0.2ml aliquots of the dissolved euglobulin fractions 0.1 ml portions of calcium-thrombin solution ($CaCl_2$ 25 mmol/l and thrombin 10 NIH U/l) were added to induce clot formation. The lysis of the clot was recorded. The disappearance of air bubbles was regarded as the endpoint of lysis. Tissue-type plasminogen activator (t-PA) activity and plasminogen activator inhibition (PAI) activity were measured by spectrophotometric assays according to Verheijen et al (16,17). Total urokinase-type plasminogen activator antigen (Uk-Ag) was assayed by EIA (18) (N: 2.1-4.9 ng/ml), active urokinase (u-PA) antigen and pro-urokinase antigen were assayed by a biological immuno assay (BIA) (19) (N; 1.7-2.5 ng/ml). The activity of whole blood coagulation and fibrinolysis, including cellular and humoral elements such as interactions of red blood cells, platelets, coagulation and fibrinolysis factors, and calcium were recorded by Thrombelastography (TEG), using whole blood (20). Blood (0.36 ml) was placed in the Thrombelastograph (Hellige Company, Freiburg, FRG) and two drops of paraffin oil were spread

over the blood surface to prevent evaporation. Recording was started 2-3 min after the blood sampling time. The TEG repeatedly measures the shear elasticity of a blood clot from the time when the first fibrin strands are formed to the completion of the clot formation, including fibrinolysis. The following variables were measured: reaction time (r-value), clot formation time (k-value), maximum amplitude (MA) and whole blood clot lysis.

RESULTS

Case Report

A 36-year-old white male, known to have chronic persistent hepatitis B since 1976, developed liver cirrhosis. He had two periods of hepatic coma due to a severe liver cirrhosis in 1985 and 1986. At those times active viral replication was no longer detectable. After having been accepted as the first patient in our auxiliary partial liver transplantation program he was admitted in coma (grade IV, encephalopathy) caused by a change in diuretic treatment for ascites. He was treated with lactulose and neomycin for encephalopathy, regained consciousness and was placed on the urgent list for transplantation. He underwent an APLT on October 23, 1986. After resection of the lateral segment of the left liver lobe, the graft was placed in the upper right abdominal cavity. The suprahepatic vena cava of the graft was anastomosed to the recipient infrahepatic vena cava, the portal vein of the graft was anastomosed end-to-side to the portal vein and the hepatic artery with an oval aorta patch was anastomosed end-to-side to the infrarenal aorta.

The patient received a fixed loading dose of blood products during the anaesthesia induction. This consisted of fresh frozen plasma (FFP) (550ml), cryoprecipitate (4x250 U factor-VIII) and platelets concentrates (16 donor units). Further supplements of blood products during the operation are shown in figure 1A, 1B and 2A. During the APLT, which lasted for almost 7h, 11 FFP, 4x250 U F-VIII cryoprecipitate and 16 donor units of platelet concentrates were given, in addition to the loading dose. The total red blood cell (RBC) supplement during the whole transplantation was 4.5 l.

The postoperative course was uncomplicated, except for a small rise of creatine and bilirubin with a maximum on the fourth postoperative day. One generalized epileptic attack occurred as a result of low plasma levels of phenobarbital on the fourth postoperative day (the patient was known to have primary grand mal epilepsy from the age of 14 years). From the first

postoperative day, the patient was treated prophylactically with a low dose unfractioned heparin i.v. (12,000 IU/24h), later followed by oral anticoagulant therapy for 3 months.

Four weeks after APLT, the patient was discharged from our hospital in good condition. During his regular visits to the out-patient clinic, he showed further improvement until the present-time, 8 month after transplantation.

Hemostasis Study

Preoperative hemostasis parameters were stable at reduced levels for months. The values just before transplantation were: prothrombin time (PT) 21 sec (N: 10-14 sec), thrombin time (THT) 21 sec (N: 10-15 sec), activated partial thromboplastin time (APTT) 51 sec (N: 22-37 sec), fibrinogen level 1.0 g/l, AT-III activity 0.28 U/ml, platelet count $31x10^9$/l (N: 140-320$x10^9$/l), α_2-AP activity 0.33 U/ml and antigen level 44%, with a PB:NPB ratio of 1.4. PAI, Uk-Ag and pro-Uk plasma levels were 12.5 IU/ml, 6.2 and 5.4 ng/ml, respectively. No t-PA activity, or active u-PA were detectable.

Intraoperatively, TEG monitoring showed no major changes in coagulation, or enhanced fibrinolytic activity (Fig. 3.). The individual hemostasis parameters measured during the operation are shown in Figures 1A and B. A prolongation of the APTT was seen after recirculation of the graft (Fig. 1A), but it had returned to the preoperative value before the end of the operation. During surgery, PAI levels were > 25 IU/ml and neither t-PA nor active u-PA were detectable. FbDP and TDP levels fluctuated between 1.4-3.6 µg/ml and 2.0-5.1 µg/ml respectively, without showing acute changes. FgDP reached detectable levels just before recirculation of the graft (0.6 µg/ml) and showed a minimal increase up to the end of the operation (1.0 µg/ml).

Postoperatively, APTT, PT and THT, as well as fibrinogen plasma concentrations stabilized at normal levels during the first days (data not shown). AT-III and α_2-AP plasma levels on the days after transplantation are shown in Figure 2A. From the first day an increase of both AT-III and α_2-AP plasma levels was seen and normal levels were reached on the third and second day respectively. The ratio of the PB:NPB forms of α_2-AP, measured on the nineteenth day was significantly higher as compared with the preoperative ratio (2.9 and 1.4, respectively) (Fig. 2B). Levels of plasminogen activators, ECLT and platelet count are shown in Figure 4. Enhanced fibrinolytic activity was demonstrated by lysis on TEG and shortened ECLT

from the tenth to the nineteenth day. On these days, t-PA activity was found to be greatly increased reaching a maximum of 1360 mIU/ml on the tenth day. Pro-Uk decreased immediately after the transplantation and reached normal values at the end of the second week. PAI remained high during the first day (12.5 IU/ml) but declined thereafter to 2.5 IU/ml on the fifth day. From the first day on FgDP were no longer detectable. FbDP levels decreased slowly, but remained detectable at a low level (1.0-2.7 μg/ml) until 25 days after transplantation.

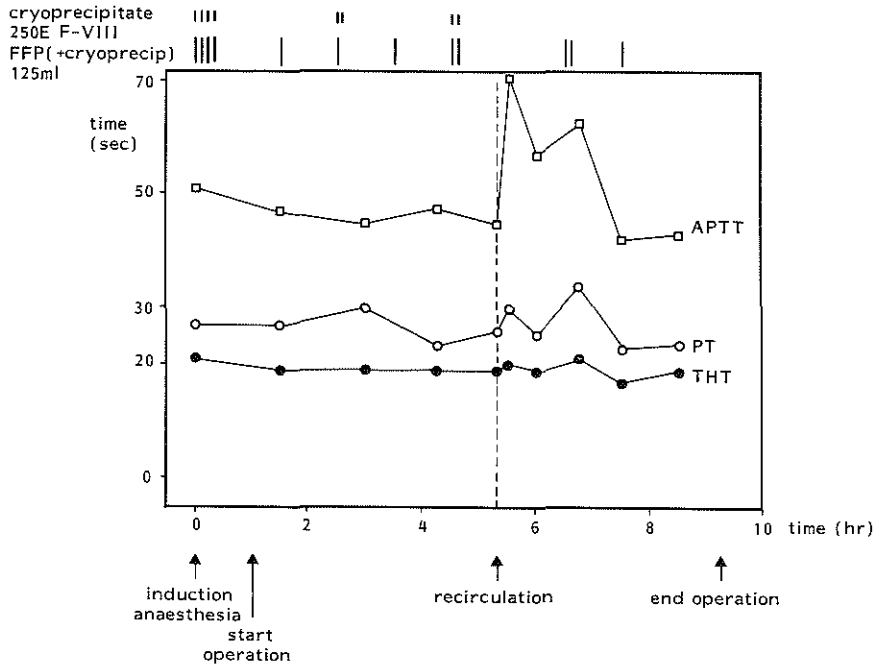

Fig. 1A. Hemostasis parameters measured during the APLT showed stable plasma levels, except for a single rise of APTT after recirculation. Supplements of blood products are shown at the indicated intervals. Activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (THT) during APLT.

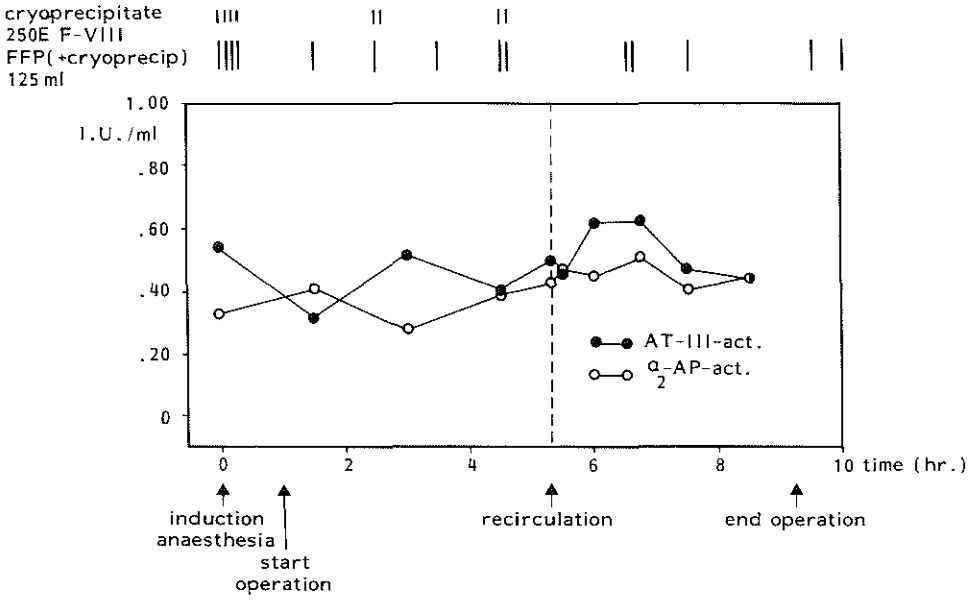

Fig. 1B. Stable hemostasis parameters were found during the APLT. Antithrombin-III activity (AT-III-act) and α_2-antiplasmin activity (α_2-AP-act).

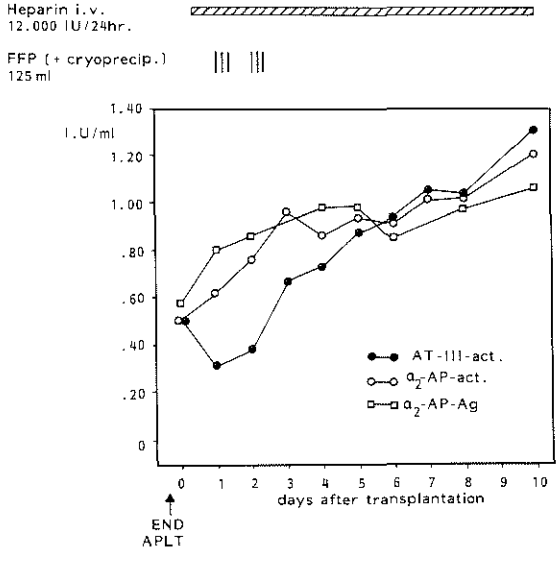

Fig. 2A. Synthesizing capacity of the graft was reflected by the normal values reached on the third postoperative day for antithrombin-III activity (AT-III-act) and second postoperative day for α_2-antiplasmin activity (α_2-AP-act) and α_2-antiplasmin antigen (α_2-AP-Ag).

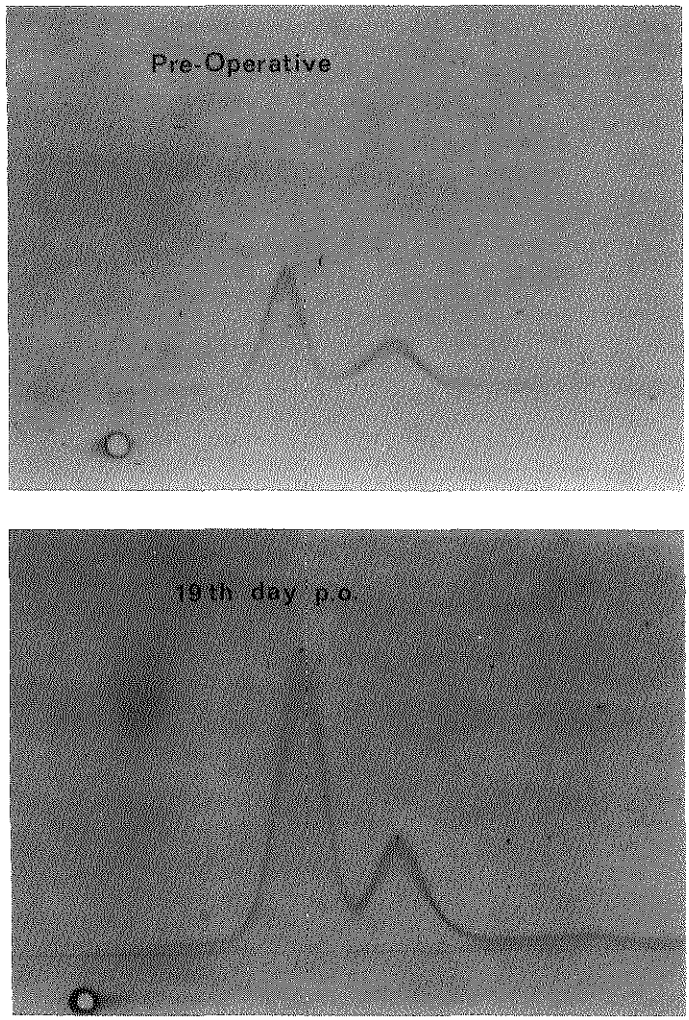

Fig. 2B. A rise of the PB form of α_2-AP (synthesized by the liver) was demonstrated by a higher ratio of the PB:NPB forms on the nineteenth day, in comparison with the preoperative sample. The PB:NPB ratios determined by crossed immunoelectrophoresis of plasma were 2.9 and 1.4 respectively (normal range: 2.1-2.8).

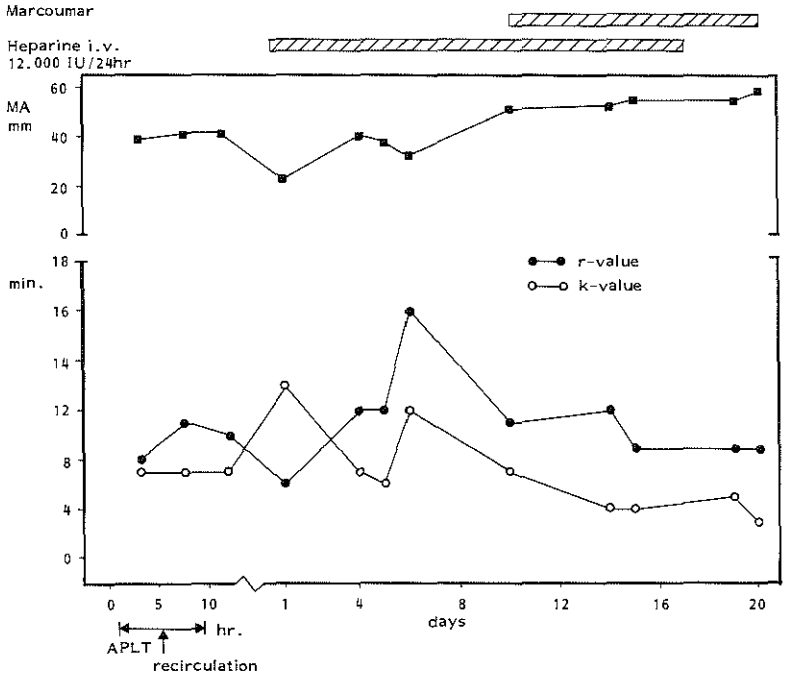

Fig. 3. Thrombelastographic results: r-value (reaction time), k-value (clot formation time) and MA (maximal amplitude) during APLT and subsequent days after transplantation.

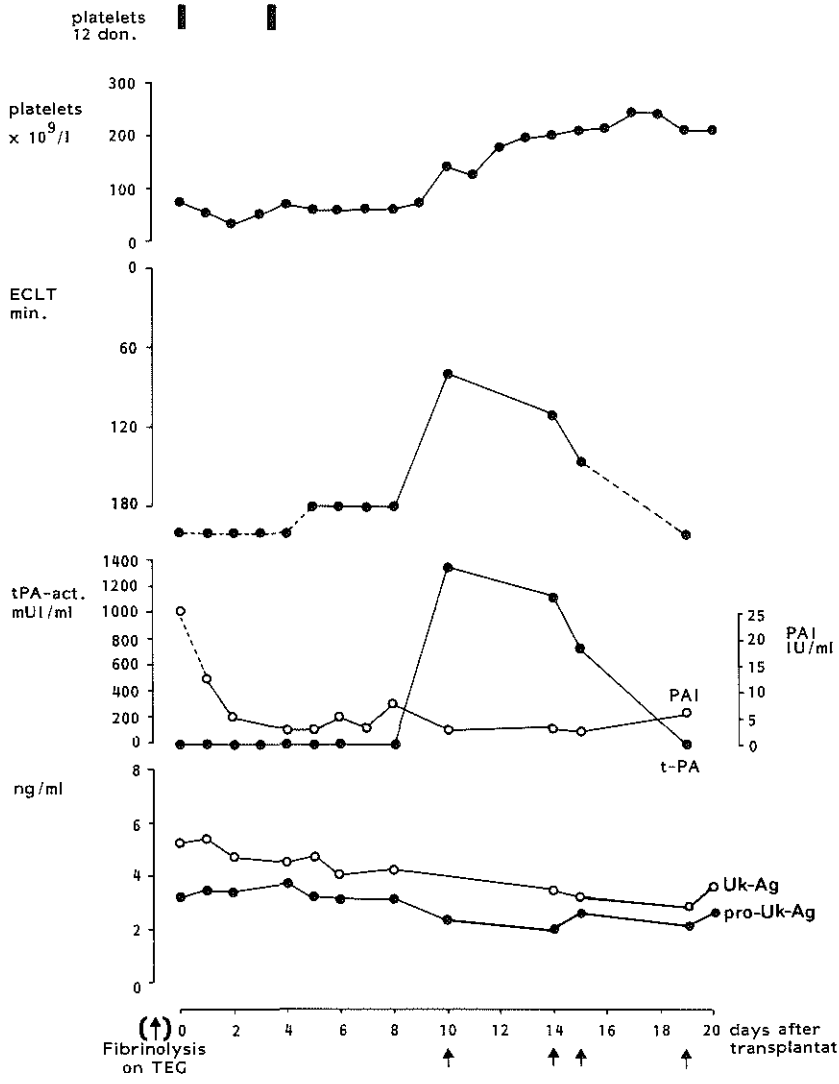

Fig. 4. Postoperatively a period of increased firbinolytic activity was found from day 10 till 19, based on an increase of t-PA actvity. Platelet count, euglobulin clot lysis time (ECLT), tissue-type plasminogen activator (t-PA) activity, plasminogen activator inhibitor (PAI) activity, pro-urkinase antigen (pro-Uk-Ag) and total urokinase antigen (Uk-Ag) plasma levels. The days on which fibrinolysis was seen on TEG are indicated by arrows.

DISCUSSION

Massive blood loss and disturbances of hemostasis are still major problems during orthotopic liver transplantation. Most changes are seen during the anhepatic phase and after reperfusion of the liver allograft (21-23). At these times a serious coagulopathy is seen, which is due to reduced function of clotting factors, including fibrinogen, and of platelets, in combination with an increase of fibrinolytic activity. Auxiliary liver transplantation has been demonstrated in experimental studies in animals to be associated with less surgical trauma. with only minor changes in hemodynamics, and with less intraoperative blood loss (24). The lack of an anhepatic phase probably contributes to the fact that the major disturbances in hemostasis seen during OLT are less extensive in APLT. In this paper we report the results of both intraoperative and postoperative monitoring of several coagulation and fibrinolysis parameters in the first patient of our APLT-program.

Preoperatively, the hemostasis profile of the patient reflected end-stage liver cirrhosis with severe dysfunction including prolongation of standard coagulation tests and low individual hemostasis factors.

Intraoperatively, only minor changes in hemostasis were detected which was probably partly due to the supplement therapy. Even after reperfusion of the liver graft no serious changes in hemostasis were seen. The lack of an anhepatic phase probably contributed to prevent deterioration of the pre-existing coagulopathy.

Postoperatively, normalization of the coagulation function tests (APTT, PT and THT) as well as fibrinogen concentration indicated that the liver allograft synthesized sufficient clotting factors to reverse the coagulopathy. The synthetic activity of the liver allograft was demonstrated by the rise of AT-III and α_2-AP levels and increase of the ratio PB:NPB forms of α_2-AP. Recently it was demonstrated that the very active PB form of α_2-AP, which is synthesized by the liver, is converted in the circulation into the less active NPB form (25). Enhanced synthesis of α_2-AP therefore results in a temporary increase of the PB:NPB ratio. The early rise of α_2-AP, i.e. before AT-III, is compatible with the acute phase reacting properties described for this protein (25).

Our observations on PAI and pro-Uk deserve special comment. The increased levels of PAI and pro-Uk before transplantation are consistent with the high levels found in patients with severe liver dysfunction by other investigators (26,19). Although it has been suggested that this is due

to a decreased clearance by the diseased liver, the mechanism of the increase in these factors is still unclear. In our patient, levels of PAI remained high during the first day after transplantation, which is also found in other types of surgery (27), and is ascribed to its acute phase reactant properties. The decrease of pro-Uk levels immediately after APLT, strongly suggests a role of the liver in the clearance of pro-Uk from the blood.

Although pro-Uk further declined to normal levels during the postoperative period, a transient rise of the other plasminogen activator, t-PA, was seen from the tenth to nineteenth day. A mild degree of activation of fibrinolysis after an auxiliary liver transplantation has also been described by Howland et al (28). However, this occurred during the first 3 days after transplantationand the cause of this phenomenon also remains unclear.

Since our findings are based on only one patient it may be premature to draw general conclusions on the influence of APLT on hemostasis. We believe. however, that this case shows that a heterotopically placed partial liver allograft can synthesize hemostasis factors sufficiently well to reverse the coagulopathy. Whether hemostasis will remain as stable during and after APLT in our next patients remains to be seen. Apart from this direct patient care, extensive monitoring provides the opportunity to study the role of the liver in the synthesis and clearance of several coagulation and fibrinolysis factors.

ACKNOWLEDGEMENT

The authors wish to thank the Hellige Company of Germany for the loan of equipment.

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CHAPTER 8

FIBRINOLYSIS DETECTED BY THROMBELASTOGRAPHY IN HETEROTOPIC, AUXILIARY LIVER TRANSPLANTATION: EFFECT OF TISSUE-TYPE PLASMINOGEN ACTIVATOR.

FIBRINOLYSIS DETECTED BY THROMBELASTOGRAPHY IN HETEROTOPIC, AUXILIARY LIVER TRANSPLANTATION: EFFECT OF TISSUE-TYPE PLASMINOGEN ACTIVATOR.

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This chapter has been published before in:
Fibrinolysis 1988; 2, supp 3: 67 - 73.

SUMMARY

Orthotopic liver transplantation (OLT) is associated with haemostatic disturbances and a severe bleeding diathesis. Fibrinolytic activity may be increased, especially during the anhepatic phase and after graft-recirculation and this has been mentioned as a possible causative factor in the occurrence of uncontrollable bleeding. However, most studies were based on global assays and could not clarify the origin of the increased fibrinolysis. Recently, a programme of auxiliary liver transplantation (APLT) was started in Rotterdam. APLT is a surgically less traumatic procedure in which no anhepatic phase occurs. We examined fibrinolytic activity in the first 8 cases of APLT by thrombelastography (TEG), and also by measuring plasma levels of tissue-type plasminogen activator activity (t-PA-act) and antigen (t-PA-Ag) and its inhibition (PAI). Intraoperatively, in only two of the eight APLTs, a period of enhanced fibrinolytic activity was observed on TEG-recordings. This could be explained by an increase of t-PA-act (max. 8840 mIU/ml and 3760 mIU/ml) and t-PA-Ag (≥ 60 ng/ml). Both patients had signs of increased bleeding during these periods. Postoperatively, in patients with a good graft function PAI levels decreased to normal values, whereas persistently elevated PAI levels (≥ 25 IU/ml) were found in cases with primary non-functioning liver grafts.

INTRODUCTION

Orthotopic liver transplantation (OLT) is associated with massive blood-loss[1], which is correlated with a high perioperative mortality[2]. In the first report of the European Liver Transplant Registry, haemorrhage was the main cause of death (34%) during the first 7 postoperative days[3]. Although the number of transplants the world over is doubling annually, the etiology of this bleeding diathesis is still not fully elucidated and can only be partially explained by the surgical trauma and changes that occur in the haemostatic mechanism.

Increase in fibrinolytic activity has been reported in liver transplantation and it has been mentioned as a possible causative factor in the occurrence of uncontrollable bleeding[4,5]. However, since most studies were based on global assays for determination of fibrinolytic activity, they could not clarify the origin of the increased fibrinolytic activity. From recent insights in fibrinolysis, we know that increased fibrinolysis may be caused by an increase in plasma levels of tissue-type plasminogen activator (t-PA), which itself was found to be associated with a haemorrhagic diathesis[6]. Specific assays for the determination of both t-PA and plasminogen activator inhibition (PAI) in plasma have been developed and we are now able to investigate the role of these factors in the origin of increased fibrinolytic activity in liver transplantation.

A program of auxiliary partial liver transplantation (APLT) in patients with severe liver disease was started in Rotterdam, The Netherlands, in 1986. Heterotopic transplantation of an auxiliary liver-graft is a surgically less traumatic procedure than OLT and it is not attended with an anhepatic phase[7]. Since clearance and synthesis of plasmatic factors of coagulation and fibrinolysis may still be possible at a low level throughout the whole operation, changes in haemostasis may be less prominent[8].

The aim of this study was to examine whether changes in the fibrinolytic system, as found by thrombelastographic monitoring during OLT[9], do also occur during APLT and if so, whether these can be explained by changes in plasma levels of t-PA.

PATIENTS AND METHODS

Patients

Between October 1986 and November 1987, 8 APLTs were performed in 7 patients, one of whom required retransplantation during this period. In four patients the indication was an end-stage of chronic liver disease and the diagnosis acute hepatic failure (AHF) was made in the other cases (including one retransplantation). Heterotopic transplantation of an auxiliary partial liver graft was performed essentially as described before in animal studies[10]. Technical details of the procedure and clinical course will be described elsewhere.

Fibrinolysis study

Blood samples were drawn from an arterial line during the operation and in the first postoperative days and by venapuncture in the later postoperative period. Blood (9 ml) was collected in 1 ml ice-cold trisodium citrate 0.11 mol/l, immediately placed on melting ice and centrifuged (2800 g, 4°C, 30 min) within 20 min. Plasma was collected at 4°C, snap-frozen and stored in small aliquots at -70°C until used. Blood samples were taken at various intervals during transplantation: directly after induction of anaesthesia, thereafter every 1.5 hr; 5 min before recirculation, 15, 30 and 60 min after recirculation of the liver-graft and every hour thereafter. Postoperative bloodsamples were taken daily between 8:00 and 10:00 hrs.

The thrombelastographic (TEG) registration of whole blood coagulation and fibrinolysis was performed by using a Thromb-Elastograph-D (Hellige Company, Freiburg, FRG). The whole blood clot lysis index was calculated as the ratio of the maximum amplitude (MA) of the TEG-recording and the amplitude 60 min after MA (A_{60}), (A_{60}/MA x 100%). Fibrinolytic activity was defined as a clot lysis index ≤ 80%[9].

Tissue-type plasminogen activator activity (t-PA-act) and plasminogen activator inhibition (PAI) were assayed according to Verheijen et al[11,12] (reference values: 0-200 mIU/ml and 0-25 IU/ml, respectively). Tissue-type plasminogen activator antigen (t-PA-Ag) was measured by using an enzyme immuno assay (Biopool IMULYSE^{TM} 5-t-PA)[13] (reference values: 5-10 ng/ml)[13]. In this assay, both free t-PA and t-PA in the inactive complex form with PAI, are measured.

RESULTS

Preoperative period

Preoperative TEG was performed in 5 cases (APLT 3,4,5,7 and 8), none of which demonstrated fibrinolytic activity (figure 1). No t-PA-act was detectable in the preoperative plasma sample in 6 cases, while t-PA-act (1200 mIU/ml) was increased in one patient with AHF. Median preoperative t-PA-Ag level was 33 ng/ml (range 18-55 ng/ml) and PAI was 14 IU/ml (range 1.2->25 IU/ml).

Intraoperative period

Intraoperatively, two different patterns of TEG recordings could be distinguished, demonstrating either an absence of significant fibrinolytic activity (pattern A) or a periodical increase of fibrinolytic activity (pattern B).

Pattern A, without any signs of fibrinolysis (clot lysis index >80%), was observed in 6 cases of APLT (figure 1). During these transplantations PAI levels increased and remained elevated (≥25 IU/ml), and t-PA-act was either undetectable or very low (≤80 mIU/ml). Levels of t-PA-Ag showed no major changes during these operations. In figure 2 the data of two representative patients from this group are depicted.

Pattern B, with increased fibrinolytic activity (clot lysis index ≤80%), was found in two patients. The period of increased fibrinolytic activity occurred in the period before graft-recirculation in one patient (APLT 5, clot lysis index 56% and 23%) and 1 hr after recirculation in the second patient (APLT 7, clot lysis index 0%) (figure 1). High levels of t-PA-act (max 8840 mIU/ml and 3760 mIU/ml) were measured in these periods (figure 3). Increased t-PA-act coincided with a fast rise of t-PA-Ag and a concurrent reduction of PAI. The increase in t-PA-act lasted for 3 to 3.5 hrs and was self-limiting. During this period, both patients suffered high bloodloss due to generalized oozing from the surgical field.

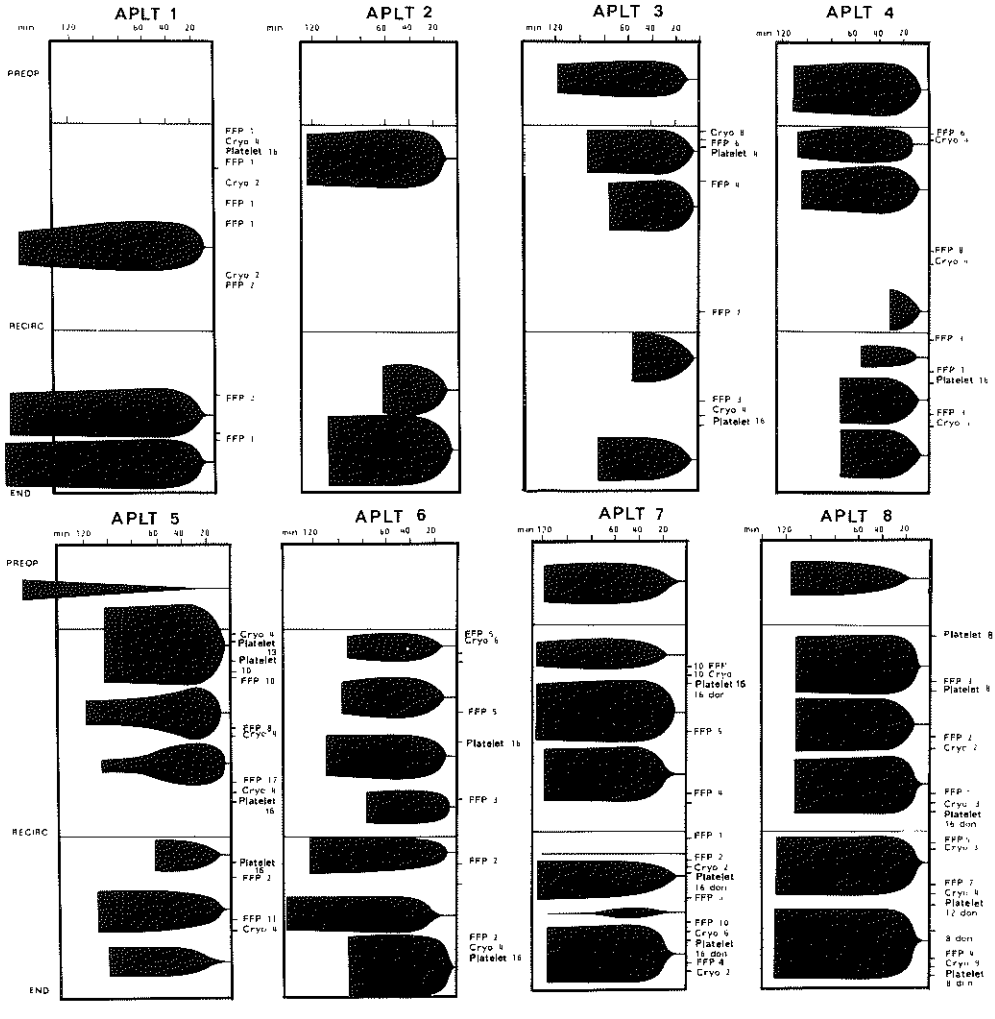

Fig. 1. Thrombelastographic recordings in eight cases of auxiliary liver transplantation (APLT). Increased fibrinolytic activity was demonstrated before graft recirculation in APLT 5 and after recirculation in APLT 7 (pattern B, see text). No fibrinolytic activity was found in the remaining cases (pattern A, see text).

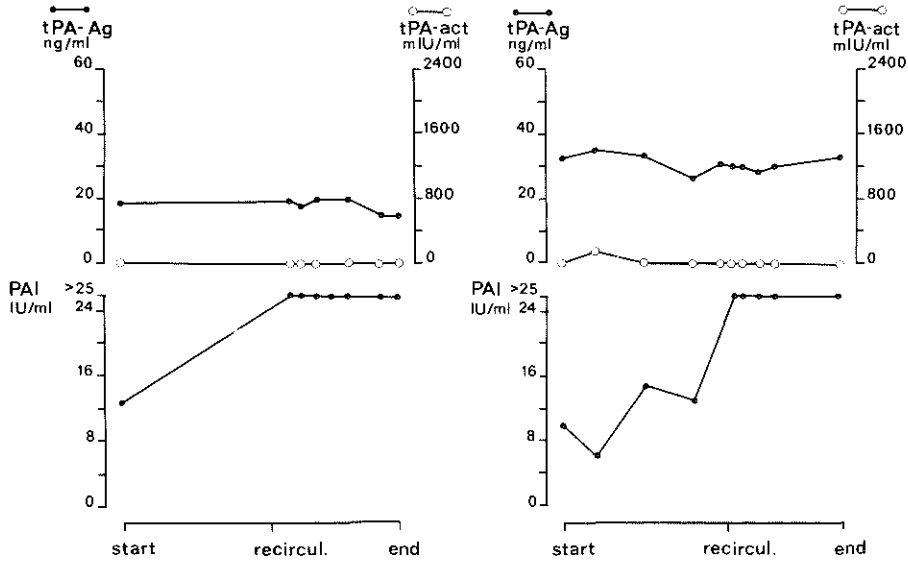

Fig. 2. Intraoperative plasma levels of t-PA-act, t-PA-Ag and PAI in two representative cases of auxiliary liver transplantation, without signs of fibrinolysis (APLT 1 and 6). t-PA-act was either undetectable or very low, while PAI levels increased and remained high during the operation.

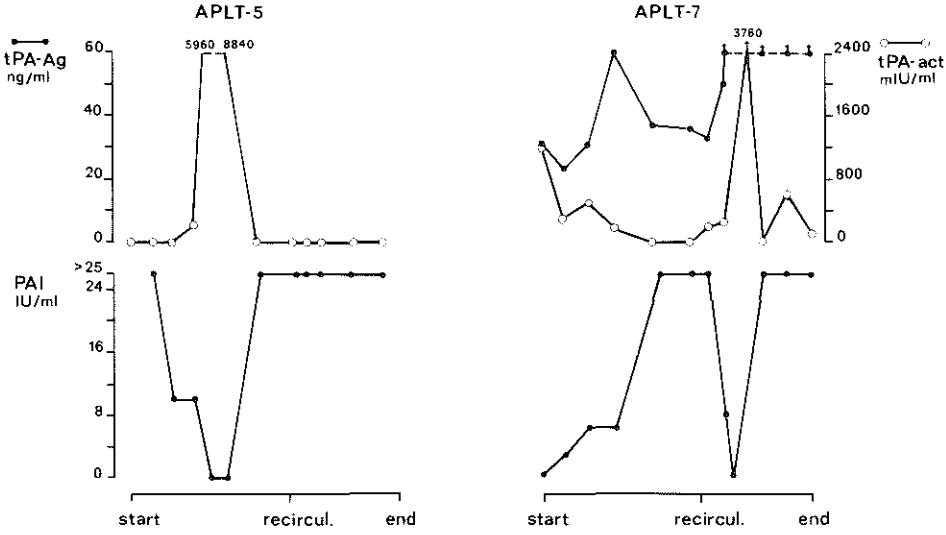

Fig. 3. Intraoperative plasma levels of t-PA-act, t-PA-Ag and PAI in two cases of auxiliary liver transplantation (APLT 5 and 7) with increased fibrinolysis on thrombelastographic recordings. t-PA-Ag levels in APLT 5 were always ≥ 60 ng/ml.

Postoperative period

Postoperatively, PAI levels also showed two patterns. Either a decrease to normal values or a persistent high level of PAI was found. A decrease of PAI to normal values below 25 IU/ml was found in four patients with a good graft function (figure 4). Persistent elevated levels of PAI (>25 IU/ml) were measured in three cases that were complicated by primary non-function of the graft. t-PA-act was almost always undetectable, in spite of the relative high levels of t-PA-Ag in these patients (figure 5). A postoperative period with enhanced fibrinolysis on TEG was found in two patients at the end of the first and second week and this was also correlated with increased levels of t-PA-act and t-PA-Ag[14].

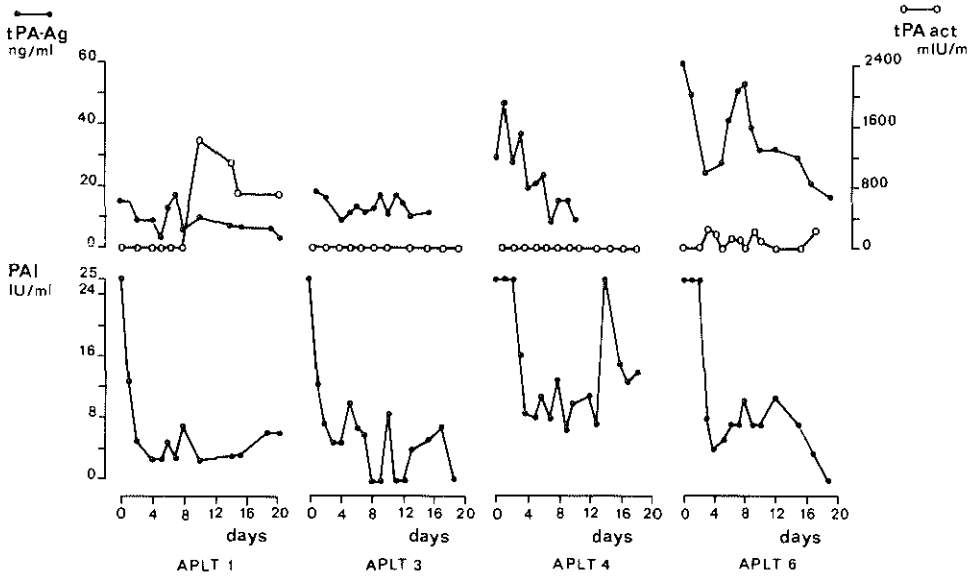

Fig. 4. Postoperative plasma levels of t-PA-act, t-PA-Ag and PAI in four patients with a good function of their auxiliary liver graft.

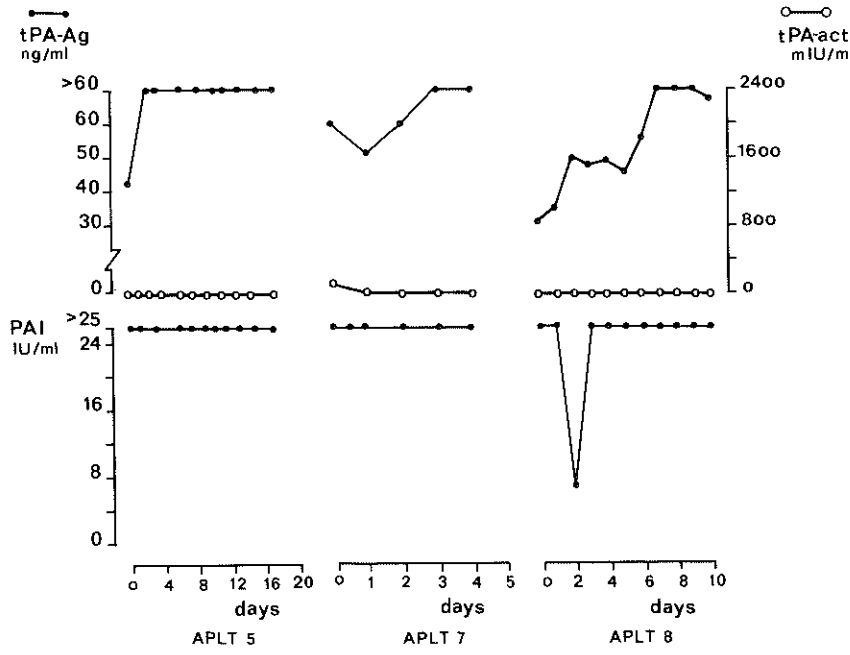

Fig. 5. Postoperatively, persistent high levels of PAI in three cases of auxiliary liver transplantation (APLT) with primary non-functioning liver grafts.

DISCUSSION

Increase in fibrinolytic activity was already observed during the first human liver transplantations in the sixties[15], and has been reported by several investigators later[4,5]. Haemostatic disorders were found to be most significant during the anhepatic phase and to deteriorate upon graft-recirculation. However, until now all studies were based on global assays for the overall detection of fibrinolytic activity, such as the euglobulin clot lysis time and TEG and the origin of this increased fibrinolysis is still unclear. In recent years, knowledge of the fibrinolytic system has greatly increased and we now know that high plasma levels of t-PA may cause enhanced fibrinolytic activity, associated with a bleeding tendency[6]. Since t-PA is produced by endothelial cells and cleared from the circulation by the liver[16], it may be hypothesized that fibrinolysis during OLT is caused by a diminished clearance of t-PA during the anhepatic phase or release from ischaemically damaged graft-endothelial cells after recirculation. However, plasma levels of

these factors during liver transplantation have not been reported. Furthermore no data are available on the occurrence of increased fibrinolytic activity during APLT, a technique of liver transplantation in which no anhepatic phase occurs[10].

In this study we observed increased fibrinolytic activity during the operation in only two of the eight cases of APLT. This increased activity, detected by TEG, could be explained by a sharp increase of t-PA-act. During the other 6 operations moderate amounts of t-PA-Ag were detectable in plasma, but this did not result in measurable levels of t-PA-act since plasma levels of PAI were high in these cases. It can be concluded that most t-PA was present in the inactive complex form with PAI. From figure 3 it may be concluded that the increased t-PA-act in APLT 7 was caused by a sharp increase of t-PA-Ag, to such high levels that the excess PAI could be overcome. Since in APLT no anhepatic phase occurs, the increase in fibrinolytic activity, observed in these operations is unlikely to be caused by a diminished clearance of plasminogen activators but is probably due to an increased release of t-PA into the circulation.

In the postoperative period we found that in patients with a good graft function, PAI levels decreased to normal values, while persistent high levels were found in cases with a primary non-functioning liver graft. These high levels of PAI may be due to disminished hepatic clearance during graft failure or may be an expression of general deterioration of the patient's condition.

In summary we can state that increased fibrinolysis was found by TEG in only two of the eight cases of APLT. Enhanced fibrinolytic activity can be explained by an increase of plasma levels of active, free t-PA. Although the increase of t-PA has a transient character, the clinical effect may extend over a considerably longer period, since t-PA may bind to fibrin and become incorporated in newly formed haemostatic clots, resulting in an early lysis and insufficient haemostatic function.
Larger series are necessary to correlate plasma levels of t-PA with intraoperative bloodloss.

ACKNOWLEDGEMENTS

The Hellige Company (Freiburg, FRG) is gratefully acknowledged for the loan of their equipment.

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CHAPTER 9

SECRETION OF PROTEIN C AND PLASMINOGEN BY HUMAN HEPATOCYTES IN PRIMARY CULTURE: APPLICATION OF MODIFIED, SENSITIVE ENZYME IMMUNO-ASSAYS.

SECRETION OF PROTEIN C AND PLASMINOGEN BY HUMAN HEPATOCYTES IN PRIMARY CULTURE: APPLICATION OF MODIFIED, SENSITIVE ENZYME IMMUNO-ASSAYS.

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This chapter has been submitted for publication.

SUMMARY

Primary cultures of human hepatocytes were analyzed for their ability to secrete the plasma hemostasis proteins protein C and plasminogen. Human hepatocytes were isolated from five left liver lobes by a two step perfusion technique with collagenase. Cells were cultured as a monolayer and culture medium was refreshed every 24 h. For quantification of human plasminogen we developed a specific enzyme immuno-assay (ELISA). Another specific ELISA was used for the determination of human protein C. Protein C and plasminogen concentrations in 24 hrs-culture medium increased for 5 days and rapidly declined thereafter. Addition of triiodothyronine resulted in a significant reduction of protein C (18 to 21%, $p<0.02$) and of plasminogen secretion (26 to 34%, $p<0.01$). Low doses of dexamethasone stimulated plasminogen secretion. Addition of insulin resulted in a significant reduction of protein C (20%, $p<0.02$) and plasminogen secretion (17%, $p<0.05$). It was demonstrated for the first time that normal human hepatocytes in primary culture secrete protein C and plasminogen. These data suggest that human hepatocytes in primary culture may be an interesting and useful model to investigate the molecular processes underlying regulation of biosynthesis and secretion of human coagulation proteins.

INTRODUCTION

Protein C is a vitamin K dependent protein which possesses anticoagulant and profibrinolytic activity upon activation by thrombin and the endothelial cofactor thrombomodulin (1,2). In clinical studies, low plasma levels of protein C were found in patients with severe liver disease (3,4). Recently, a child with a homozygous protein C deficiency had a complete restoration of protein C activity after orthotopic liver transplantation (5). These observations strongly suggest the liver as its site of synthesis. Although Wion et al. (6) have reported the detection of protein C antigen in perfused human whole liver and liver cells suspensions, contradictory results were obtained from studies using the established human liver cell line Hep G_2. Fair and Marlar (7) reported the synthesis of protein C antigen by cultured Hep G_2 cells, but Morito et al. (8) were unable to detect protein C antigen in culture media of Hep G_2 cells.

Plasminogen is a plasma glycoprotein which can be converted into the serine protease, plasmin, upon proteolytic modification. Although the site of plasminogen synthesis has been controversial for a long time, it was recently demonstrated that plasminogen is mainly synthesized by the liver (9,10). Examination of genetic types of plasminogen from a donor and a recipient before and after orthotopic liver transplantation have demonstrated that the liver is the principal site of synthesis of human plasminogen (9). Furthermore, primary cultures of rat hepatocytes and human hepatocellular carcinoma cell lines were found to synthesize and secrete plasminogen (10-12).

Recently, it was demonstrated that normal human hepatocytes can be isolated from pieces of human liver tissue and that these cells retain their morfology and several specific liver cell functions, when maintained as monolayers in cell cultures (13,14). Although primary cultures of human hepatocytes have been found to synthesize and secrete some plasma proteins (14,15), thus far no studies have been reported on the production of hemostasis proteins. Since the amount of hemostasis proteins, secreted by liver cells in cultures is expected to be low, highly sensitive immuno-assays are necessary for quantification of the trace amounts of proteins in the culture medium.

In this study we used two enzyme immuno-assays (ELISA) for the determination of human protein C and plasminogen, to examine whether these hemostasis proteins are secreted by primary cultures of human hepatocytes.

In addition, we investigated the effect on protein C and plasminogen secretion of some hormones that are generally used in cell culture and that may have stimulating effects on hepatocyte function and survival (15).

MATERIALS AND METHODS

Isolation of Human Hepatocytes

Human hepatocytes were isolated from the left lobes of livers which were taken during human donor nephrectomy in one case, and from four livers that were obtained through the Auxiliary Partial Liver Transplantation Program carried out at the University Hospital Dijkzigt in Rotterdam, The Netherlands. Permission was given by the Medical Ethical Committee to use the remaining not transplanted part of the donor liver for scientific research. The livers were taken from five physically healthy organ donors (4 females and 1 male, aged from 6 to 42 years), who died after brain hemorrhages or severe traumatic brain injury and in one case carotid artery occlusion. During resection of the left lobe, the liver was perfused by portal vein cannulation with Euro-Collins ($4^{o}C$). Isolation of human hepatocytes was essentially as described before (16,17). After resection, the left liver lobe was transported to the perfusion site within 45 min in a cold buffer ($4^{o}C$) containing 10 mM HEPES, pH 7.4, 142 mM NaCl, 16.7 mM KCl and 0.5 mM EGTA. Perfusion with 3 liter of this buffer at a rate of 40 ml/min per catheter was started after insertion of four polyethylene catheters (18 gauge) in the vascular orifices that could be identified at the dissection surface. After the pre-perfusion the liver was perfused successively with 500 ml of a HEPES buffer, pH 7.6, containing 5mM $CaCl_2$ without recirculation, and with 200 ml of this buffer containing 0.05% and 0.1% collagenase, respectively, with recirculation for 20 min each. Liver tissue was dissociated in a Hanks buffer containing 2% bovine serum albumin, cells were filtered through a 250 µm filter, centrifuged (75 x g for 5 min) and washed three times in a cold culture medium ($4^{o}C$) to remove damaged and non-parenchymal cells. Viabilities were examined by trypan blue exclusion test, using 0.11% trypan blue.

Human Hepatocyte Cultures

The hepatocytes were seeded on 6-well cluster plates (35 mm diameter) (Costar, Cambridge, MA, U.S.A.) at a density of 1.25×10^5 cells/cm^2 and

were maintained in Williams E medium (Flow Laboratories, Irvine, Scotland, UK) supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml kanamycin (WE/10%FCS), and 135 nM insulin and 50 nM dexamethasone at 37 ^{o}C in a 5% CO_2 / 95% air atmosphere. The medium (1 ml per 10 cm^2) was renewed 10-16 h after seeding to remove unattached non viable cells and every 24 h thereafter. The cells were examined daily by light-microscopy. Following this procedure the percentage of parenchymal cells after attachment of the cells and during 72 hours in culture was found to be 96% or more (18). At the indicated intervals medium was removed, centrifuged (10000 x g, 1 min) and the supernatants were collected and stored at -20 ^{o}C. After washing three times using cold Hanks' buffer (4^{o}C), cells were harvested by scrapping the wells and stored at -20^{o}C for total protein determination.

Additional experiments were performed with cells cultured for 40 - 88 h, to assess the effect of hormones on the secretion of protein C and plasminogen. In these experiments hepatocytes were initially cultured in WE/10%FCS, supplemented with dexamethasone and insulin, for 16 h. During this period the cells were able to attach to the culture dishes and form monolayers. Culture medium was removed and replaced by hormone-free medium for the following 24 h. After 40 h, cells were refreshed with WE/10%FCS to which one of the following hormones was added: dexamethasone (Dex) (in concentrations of 0.05 µM; 0.2 µM; 1 µM or 10 µM), triiodothyronine (T_3) (concentrations: 1 µM or 10 µM), insulin (Ins; 135 nM), or a combination of 10 µM T_3 and 50 nM Dex.

Determination of proteins

Total protein was measured according to Lowry et al (19) with bovine serum albumin as standard.

In order to quantify trace amounts of human protein C and plasminogen in the presence of an excess of fetal calf serum, we used a commercially available ELISA for protein C and we developed an ELISA for the specific quantification of human plasminogen.

Enzyme immuno-assay for human protein C

The ELISA for human protein C was based on two polyclonal anti-human antibodies (Boehringer Mannheim, Mannheim, FRG) (20). For quantification of the secreted amount of protein C antigen, culture medium was diluted 1:1

in the dilution buffer, supplied in the assay-kit. A calibration curve was made from pooled normal plasma diluted in a mixture of the same dilution buffer and Williams E medium (1:1). The amount of protein C antigen secreted in the culture media was expressed in U/mg cell protein/24 h (1 U = 1% normal plasma, containing approximately 30 ng/ml).

Enzyme immuno-assay for human plasminogen

The ELISA, we developed for quantification of human plasminogen, is based on the double antibody sandwich principle and can be performed on microtitre plates.

Assay procedure.

Microtitre plates (Nünc Immunoplate, Nünc, Roskilde, Denmark) were coated with 200 µl rabbit polyclonal anti-human plasminogen (IgG conc. 11.7 g/l, Dakopatts Ltd, Glostrüp, Denmark) dissolved in a phosphate buffered saline (PBS) coating buffer (2.5 mM $NaH_2PO_4.H_2O$, 7.5 mM $Na_2HPO_4.2H_2O$, 145 mM NaCl, pH 7.2) and left covered with plastic film overnight at 4 ^oC. The plates were washed three times with a washing buffer [PBS buffer, pH 7.2, with 0.1% (v/v) Tween 20 (Merck, Darmstadt, FRG) and 1 mM Na_2-EDTA (Merck)]. Pooled citrated plasma, prediluted ($1:10^4$ - $1:16x10^4$) in a dilution buffer [PBS buffer, pH 7.2, with 0.1% (v/v) Tween 20, 1mM Na_2-EDTA and 3% (w/v) PEG 6000 (Merck)] served as standard and for controls we used pooled EDTA-plasma, diluted 1:40,000 and 1:20,000 in the dilution buffer. Test samples were diluted with dilution buffer and 100 µl was added to each well. The plates were incubated at room temperature for 2 h and shaken continuously. After washing, 100 µl specific peroxidase-conjugated rabbit anti-human plasminogen antibody (IgG conc. 1.3 g/l, Dakopatts Ltd) diluted 1:2000 in dilution buffer was added to each well and incubated at room temperature for 1 h under continuous shaking. After washing, enzyme color reaction was started by adding 100 µl of freshly prepared color reagent, orthophenylediamine (0.67mg/ml) in citric acid phosphate buffer (34.7 mM citric acid, pH 5.0 containing 66.7 mM Na_2HPO_4) to each well. After incubation for 15 min at room temperature, the reaction was stopped by adding 150 µl 1M H_2SO_4 to each well. The absorbance was measured after 15 min at 492 nm with a Titertek Twinreader (Flow Laboratories, Virginia, USA). The standard curve was plotted on log-log paper with log A 492 nm as ordinate and log concentration of standards as abscissa. Concentration of the test samples were read by means of interpolation of this standard curve.

Statistical analysis

Statistical analysis was performed using Wilcoxon's signed rank test. Differences were assumed to be statistically significant when p-values were less than 0.05.

RESULTS

Isolation and culture of human hepatocytes

Hepatocytes were isolated from 5 left liver lobes. Cell yield varied from 537 to 1670 $x10^6$ hepatocytes with a viability of 70% - 81% (table 1). In culture, the initially spherical hepatocytes attached themselves to the culture plastic within 6 h, aggregated and formed monolayers of cells with a characteristic polygonal shape during the first 40 h. During the first 5-6 days, hepatocytes were morfologically in a stable condition and resembled cells in vivo (figure 1).

Table 1. **Cell yield and viability of five human hepatocyte isolations**

Isolation	Cell Yield	Viability (%)
1	537×10^6	71
2	1280×10^6	70
3	1290×10^6	81
4	1151×10^6	77
5	1670×10^6	73

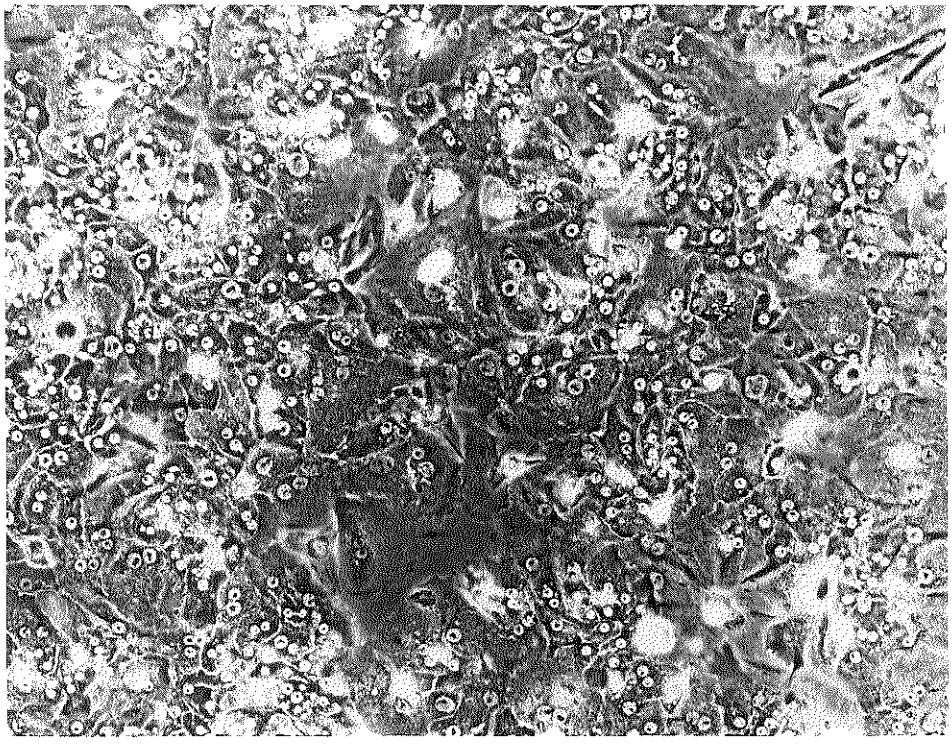

Fig 1. Phase contrast micrograph of human hepatocytes cultured for 72 h in primary monolayers (original magnification x 100).

Secretion of protein C by human hepatocytes

The ELISA used for protein C was found to be highly specific for human protein C and did not cross-react with the FCS, as no protein C antigen was detected in control experiments with unconditioned Williams E medium, supplemented with 10% FCS. In experiments from two separate cell isolations, concentrations of protein C in the culture medium was measured during a period of 10 days. After 4-5 days, protein C concentration in culture medium reached a maximum of about 0.5% of the concentration in normal plasma and rapidly declined thereafter (figure 2).

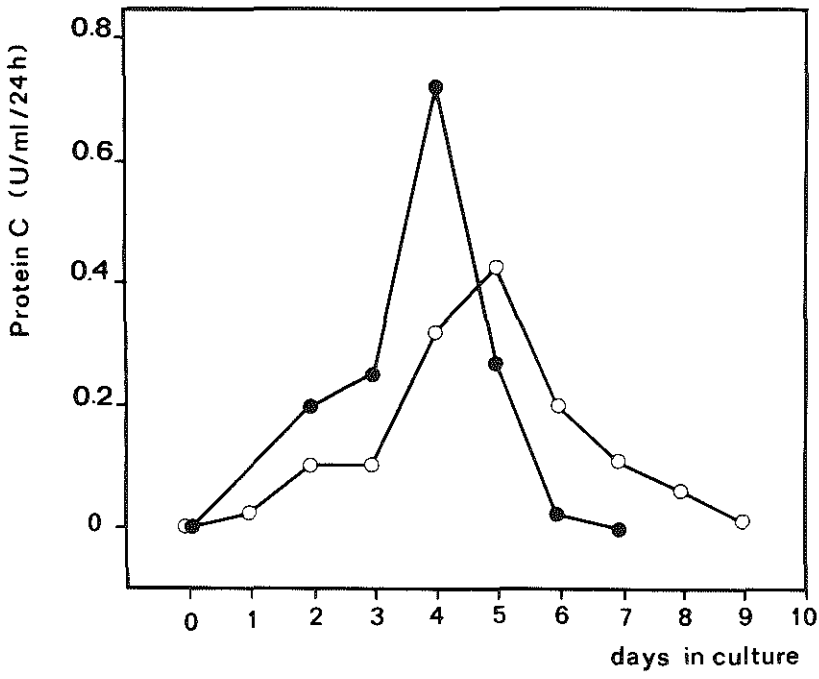

Fig 2. Time course of protein C secretion by primary cultures of human hepatocytes, obtained after isolation from the left lobes of two different livers. Cells were cultured in Williams E medium, supplemented with 135 nM insulin and 50 nM dexamethasone. The values are expressed in units per milliliter of medium per day (1 U = 1% normal plasma).

Determination of human plasminogen and secretion by human hepatocytes

Human Plasminogen ELISA.

The standard curve of plasminogen ELISA procedure is shown in figure 3. Using the ELISA it was possible to quantify plasminogen concentrations from about 6.25×10^{-4} to 1×10^{-2}% of normal pool plasma (conc. 1.5 µmol/l). The intra-assay coefficient of variation for plasma plasminogen measured within the ranges of about 40 pM to 80 pM was 5.3% (n=40), and the inter-assay variation (n=21) over the same range was 15%. The specificity of the assay was checked by determination of the recovery of purified plasminogen (more than 98%), and the negligible interference of other plasma proteins. This included apoprotein (a), which is a lipoprotein (a)-specific antigen that is structurally closely related to plasminogen (21).

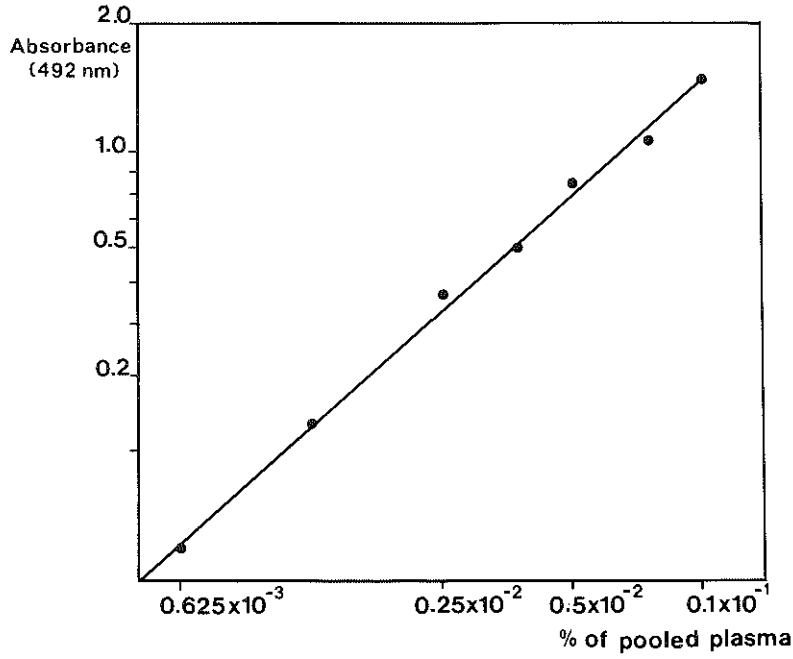

Fig 3. Standard dilution curve of the ELISA for human plasminogen, obtained by dilution of pooled normal citrate plasma (plasminogen conc. 1.5 μM) (logarithmic scales).

Plasminogen in cell cultures.

When unconditioned Williams E medium, supplemented with 10% FCS was analyzed with the plasminogen ELISA, very low absorbances were measured, indicating some cross-reactivity with FCS. Therefore all results were corrected, using WE/10%FCS as the blank. In experiments from two separate cell isolations, plasminogen secretion was measured during a culture period of 10 days. Concentration of plasminogen in culture medium increased for 5 days, reaching a maximum value of about 0.5% of its plasma concentration, and rapidly declined thereafter (figure 4).

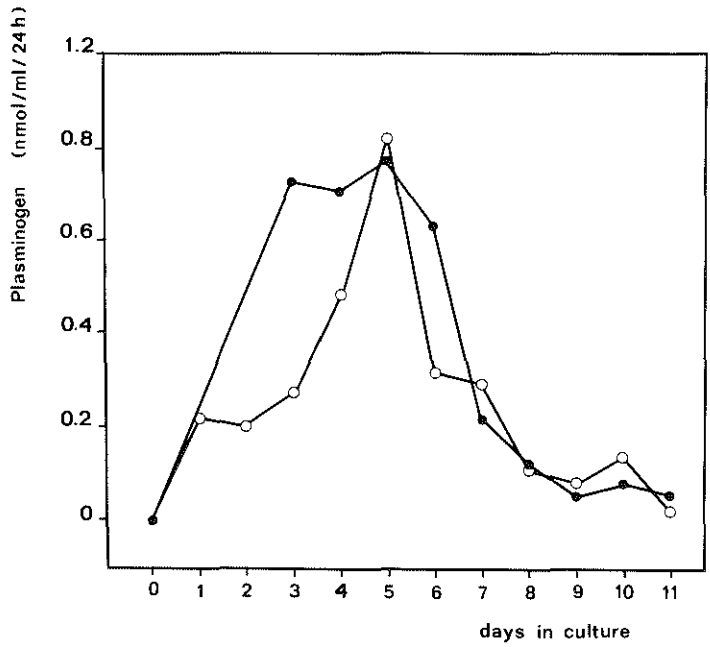

Fig 4. Time course of plasminogen secretion by primary cultures of human hepatocytes, obtained after isolation from the left lobes of two different livers. Cells were cultured in Williams E medium, supplemented with 135 nM insulin and 50 nM dexamethasone. The values are expressed in nmoles per milliliter of medium per day.

Effect of hormones on secretion of protein C and plasminogen by human hepatocytes

Hormonal incubations were performed during a time interval of 40-88 h, using hepatocytes obtained from three different cell isolations. The secretion of protein C and plasminogen, in control experiments with hormone-free medium, during this period is shown in table 2. The amount of secreted protein varied somewhat between the experiments, using cells from different livers. The effect of the hormonal incubations on the secretion of protein C and plasminogen, expressed in percentage of the corresponding control experiments is shown in table 3. Addition of 0.05 and 0.2 μM Dex to the culture medium resulted in an increase of plasminogen secretion by 28%

($p<0.02$) and 25% ($p<0.05$), respectively, but no significant effect on protein C secretion was found. Higher amounts of Dex did not have any significant effect on plasminogen secretion. After incubating the cells with 1 μM or 10 μM T_3, a significant reduction of both protein C and plasminogen secretion was seen (table 3). If 0.05 μM Dex and 10 μM T_3 were added to the culture medium simultaneously, protein C and plasminogen secretion were not significantly different from the control experiments. When the cells were grown in medium with 0.135 μM insulin, protein C and plasminogen secretion decreased by 20% ($p<0.02$) and 17% ($p<0.01$), respectively.

Table 2. Secretion of protein C antigen and plasminogen antigen by human hepatocytes in hormone-free medium, cultured for 40-88h

Experiment	Protein C (U/mg cell protein/24 h)	Plasminogen (nmol/mg cell protein/24h)
Control 1	1.32±0.44	NA*
Control 2	1.99±0.17	4.33±0.29
Control 3	1.04±0.12	2.56±0.20

Results are means ± SEM of 6-8 fold cultures, with duplicate determinations of protein C and plasminogen antigen. *NA = not assayed.

Table 3. The effect of hormonal incubations on protein C and plasminogen secretion in human hepatocytes, cultured for 40-88h

Hormone treatment	Effect on Protein C % of controls[a]		Effect on Plasminogen % of controls[a]	
Dexamethasone (Dex)				
0.05 μM	- 8%	NS[b]	+ 28%	p<0.02
0.2 μM	+ 2%	NS	+ 25%	p<0.05
1 μM	+ 8%	NS	+ 10%	NS
10 μM	+ 9%	NS	+ 10%	NS
Triiodothyronine (T_3)				
1 μM	- 22%	p<0.02	- 26%	p<0.01
10 μM	- 18%	p<0.02	- 34%	p<0.01
10 μM T_3 + 0.05 μM Dex	- 9%	NS	- 2%	NS
0.135 μM Insulin	- 20%	p<0.02	- 17%	p<0.05

Results are means of 2-3 isolations with 2-4 fold incubations and duplicate determination of protein C and plasminogen.
[a]See table 2, [b]NS = not significant .

DISCUSSION

Almost all proteins of the hemostasis system have been found to be synthesized by the liver (22). Indications for the synthesis of hemostasis proteins by the liver were partly based on clinical data, documenting reduced plasma levels of the proteins in patients with severe liver disease. Furthermore, the human hepatoma cell line, Hep G_2, was found to have the capacity to synthesize several components of the hemostatic system (11,12,23). This cell line has been used to study intracellular processes and regulation of synthesis of some of these proteins (24). However, cell lines originating from liver tumors may physiologically not be fully comparable with normal human liver cells. Theoretically, cultures of normal human hepatocytes represent the ideal in vitro model for studies of specific liver

cell functions in a well-defined environment (15). Recently, it was demonstrated that human hepatocytes can be isolated successfully from pieces of normal liver tissue by means of a perfusion technique with collagenase (13,14). Isolated hepatocytes could be maintained in cell culture for several days, during which they demonstrated several specific liver cell functions (15). Although human hepatocytes in primary culture were found to produce albumin (14,25) and some other plasma proteins (15), reports on the production of hemostasis proteins are limited to a study on plasminogen activator inhibitor (16). Primary cultures of human hepatocytes may be a physiologically good model to investigate synthesis of these proteins. However, the use of normal liver tissues for scientific research is limited, especially for ethical reasons. A rather unique situation resulted from the auxiliary liver transplantation, in which only a part of the donor liver is transplanted (26). The resected part (usually the left lobe) is not used for transplantation and is very suitable for isolation of liver cells.

In this study we used lobes from 5 livers for cell isolation. The cell yield was rather constant with a good viability of the cells. The ELISA used to detect protein C was found to be highly specific for human protein C as no cross-reactivity occurred with unconditioned Williams E medium containing 10% FCS in control experiments. Therefore, hepatocytes could be maintained on medium supplemented with FCS throughout the experiment, which is found to be of importance for long term culture of human hepatocytes (27,28). For quantification of plasminogen in the culture medium we developed a specific ELISA for human plasminogen. The range for plasminogen detected by this assay, was sufficiently broad to measure accurately plasminogen in culture medium. Secretion of protein C and plasminogen by human hepatocytes was found to increase for 4-5 days and rapidly declined thereafter. Other investigators have found a comparable pattern for the secretion of albumin by human hepatocytes in primary culture (14,25).

Experiments with hormonal incubations were performed between 40 and 88 h. In this period the cells were morfologically in a stable and good condition and had an optimal secretion of protein C and plasminogen. It is known that hormones either alone or in a mixture may strongly influence synthesis of certain plasma proteins (15). We examined the effect on protein C and plasminogen secretion of three hormones that are generally used in cell culture and that may have a stimulating effect on the hepatocytes. In none of the used concentrations an effect of Dex was found on protein C secretion, but plasminogen secretion was stimulated in the presence of low

amounts of Dex. A similar stimulating effect has been observed for other proteins (29,30), and this may result from the beneficial effect of this hormone on cell metabolism and various functions in hepatocyte cultures (15). Lack of effect on protein C secretion may be explained from differences in the in vivo synthesis rate of the two proteins. From the plasma half lives and plasma concentrations it can be calculated that in vivo plasminogen synthesis is about twice the synthesis of protein C. An in vitro stimulating influence of Dex may have a greater effect on the protein with the highest rate of synthesis, which is plasminogen in this case. Noteworthy was the lack of effect by higher amounts of Dex, which suggests a dose dependent mechanism. Triiodothyronine seemed to decrease the secretion of both protein C and plasminogen. This is probably due to a non specific effect on cell metabolism, since T_3 at concentrations used in this study may cause a depletion of the ATP cell content (31). Another explanation may be that general protein turnover and breakdown is stimulated by T3, as was recently found in cultured rat hepatocytes (32). When cells were grown in the presence of both Dex and T_3 the effect of both hormones seemed to counteract each other, resulting in a protein C and plasminogen secretion not different from control experiments. Maekudo et al. (33) found that human hepatocytes in primary culture retain the ability to bind and respond to insulin. Although insulin stimulates albumin synthesis in rat hepatocytes (34,35), we observed a slightly negative effect of insulin on both protein C and plasminogen secretion, for which we do not have a good explanation.

In conclusion we found for the first time that human hepatocytes in primary culture secrete protein C and plasminogen into their medium. Furthermore, it seems that human hepatocytes in primary culture maintain reactivity to some hormones, probably indicating that receptors at the cell membrane are preserved during the isolation procedure. Thus, human hepatocytes in primary culture seem to provide an interesting and useful model to investigate the molecular events underlying the regulation of biosynthesis and secretion of human coagulation proteins.

ACKNOWLEDGEMENT

This study forms part of the projects, performed within the Human Liver cell Foundation, in cooperation with the Auxiliary Liver Transplantation Program, of the University Hospital Dijkzigt in Rotterdam. All parties involved are thanked for their assistance. Thanks are also indebted to Cand. pharm. Ms. Agnete Ingild, Dakopatts Ltd, for labelling rabbit anti-human plasminogen.

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CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

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9.1 Rationale of the study

Orthotopic liver transplantation (OLT) is now generally accepted as a realistic treatment for patients with end-stage liver disease[1,2]. However, the surgical procedure is technically demanding and sometimes the poor clinical condition of patients is considered to be too a high a risk for a successful OLT. In some centers OLT is currently not considered in patients with fulminant hepatic failure. For these patients auxiliary partial liver transplantation may be a reasonable alternative.

In spite of the improved results of OLT, the surgical procedure is still associated with large amounts of blood loss[3,4], which is correlated with an increased perioperative mortality[4,5]. Part of this blood loss is related to the magnitude of the surgical trauma[6]. However, a poor preoperative hemostatic function and specific intraoperative deteriorations of the hemostatic system may contribute[7,8]. Although there are theoretical grounds to assume that blood loss and the deteriorating effect on hemostasis will be less severe in APLT, there is no objective clinical or experimental evidence to sustain this view. A large number of papers have been published on the hemostatic disorders in liver transplantation, but the mechanisms that are responsible for the hemorrhagic diathesis in liver transplantation are still poorly understood. The role of the fibrinolytic system has been subject of many speculations. An increased fibrinolytic activity is found in may patients undergoing OLT[9-11], but the underlying mechanisms of this activation and its clinical relevance are not clear. Interpretation and comparison of the results of different groups is difficult due to differences in surgical and anesthesiologic techniques and in laboratory methods. Interpretation of hemostasis tests has also often been difficult, and many of the assay methods used in the earlier studies are now known to be nonspecific or insensitive. During the past decade, knowledge about the hemostatic system has increased greatly. Many new proteins and activating or inhibiting pathways of the hemostatic system have been recentely discovered (for reviews see 12). The introduction of specific chromogenic substrate methods and sensitive enzyme immuno-assays have provided tools to study these new aspects of the hemostatic system.

Some of these new assays have been used in the studies described in this thesis. The aim of the investigations was to study the effect of APLT on the hemostatic system and the mechanisms that cause some specific

hemostatic changes during both OLT and APLT. We also evaluated the possibility of using resected parts of human donor livers used for APLT for in vitro studies. From this human liver tissue, hepatocytes were isolated and maintained in primary culture to study the secretion of plasma hemostasis proteins.

9.2 Hemostasis in orthotopic liver transplantation

An important problem in OLT is the poor preoperative hemostatic function which is found in many patients with severe hepatic dysfunction[7,8]. This sometimes warrants attempts to improve the hemostatic function by the substitution of blood and blood-products preoperatively or during the first part of the surgical procedure. Partly for this reason some centers now tend to select patients for liver transplantation at an earlier stage of their liver disease, when they have less severe secondary coagulopathy.

Specific changes occur in the hemostatic mechanism intraoperatively. These changes may further complicate bleeding of traumatic origin, and may necessitate the transfusion of large amounts of blood. The observed intraoperative deterioration of hemostasis has been interpreted as a sign of disseminated intravascular coagulation (DIC)[13-15], hyperfibrinolysis[16,17] or a combination of both processes[18,19]. Although DIC may have played a role in the earlier experiments and clinical studies, some investigators dispute its clinical relevance and its role in the increased bleeding tendency during OLT[16,20]. The occurrence of DIC is largely dependent on the quality of the donor liver[21,22]. Improvements in graft preservation techniques, surgical and anesthetic procedures and a more stringent substitution therapy have undoubtedly contributed to the decreased occurrence of hemostatic changes suggestive of DIC. The concept that DIC does not play a clinically important role in OLT is supported by the failure to find evidence for thrombo-embolic processes in histological or clinical examination[23-25]. Using electron microscopy, performed on liver biopsies taken 5-10 min after graft reperfusion, we were also unable to find microthrombi or signs of platelet aggregation (chapter 4). Some authors, however, have described an intraoperative decrease of clotting factors and inhibitors like antithrombin III in their patients undergoing OLT and interpreted these as signs of DIC[18,26]. However, we have also seen a decrease of antithrombin III and fibrinogen levels

in heparinized patients undergoing major vascular surgery (chapter 5). This strongly suggests that other, less specific processes, such as hemodilution, may play a role in the changes in coagulation during liver transplantation as well.

Despite all the improvements in surgical and anesthetic management during recent years, intraoperative signs of hyperfibrinolysis are still found in a majority of the OLT patients[10,11,20]. Although the origin of this increased fibrinolytic activity is still controversial, there is growing evidence for a primary mechanism. By comparing fibrinolytic activities, as measured by the euglobulin clot lysis time, during both OLT and APLT we observed a more sustained increase in fibrinolytic activity during OLT (chapter 3). We also found that reperfusion of the donor liver is associated with an increase of fibrinolytic activity. In blood samples taken from the first hepatic outflow, fibrinolytic activity was significantly increased compared with the systemic circulation (chapter 4). In a study in 20 OLTs we found an increase of tissue-type plasminogen activator (t-PA) activity during the anhepatic stage, followed by an "explosive" 30-fold increase immediately after graft reperfusion (chapter 6). Recently, Dzik et al.[11] have reported a similar increase of t-PA during the anhepatic stage in a limited number of patients undergoing OLT. However, this study did not show the explosive t-PA increase, occurring directly after graft reperfusion, as seen in our patients. Since we observed a rapid return to normal of t-PA activity after the maximum levels, this peak could easily have been missed if blood samples were not taken within 10 minutes after reperfusion. In both studies, however, patients with high levels of t-PA suffered from extensive intra-operative blood loss. We also found significantly higher plasma levels of degradation products of fibrinogen and fibrin in patients with high t-PA levels. We found a similar steady increase of thrombin-antithrombin III complexes in patients with severe and minimal fibrinolysis, indicating that increased t-PA activity was not related to thrombin formation (chapter 6).

The intraoperative course of t-PA activity in patients undergoing OLT, in combination with the results of the experimental studies (chapter 4), strongly suggest that hyperfibrinolysis during OLT is caused by two different mechanisms. In the first place hyperfibrinolysis may occur during the anhepatic stage due to a combination of an increased endothelial release of t-PA and a reduced hepatic clearance. Secondly, reperfusion of the donor liver may have a stimulatory effect on the fibrinolytic system. Our findings suggest that release of t-PA from the intrahepatic endothelium may cause a

temporary increase in fibrinolytic activity after graft reperfusion. This process might depend on the quality of the graft and the magnitude of the ischemic damage. Since the primary increase in t-PA activity was associated with an active proteolytic destruction of fibrinogen and fibrin and a high blood loss, the use of antifibrinolytic drugs may be justified in patients with life threatening hemorrhages when there is evidence for active fibrinolysis. To identify these patients, it is advisable to include at least one test method for the assessment of fibrinolytic activity in the intraoperative hemostasis monitoring in orthotopic liver transplantation.

Another phenomenon that is often encountered in patients undergoing OLT and that may play a role in the hemorrhagic diathesis, is a decrease in platelet count[23,27]. Despite transfusion of platelet concentrates, severe thrombocytopenia may occur during the first postoperative days[27,28]. In blood samples taken from the venous outflow of the reperfused liver graft, we found significantly lower platelet counts, compared with the systemic circulation (chapter 4). This is in agreement with the findings of other investigators and strongly suggests sequestration of blood platelet in the liver graft[25,29]. Since we could not find any histologic evidence for intravascular coagulation or platelet aggregation in the livers, other processes than DIC are probably responsible for this phenomenon. In electron microscopy studies we found signs of a phagocytosis of platelets by Kupffer cells while other platelets were lying free in the sinusoids (chapter 4). It is of interest that we could not confirm the earlier observations of Hutchison et al.[23], who described an extravasation of platelets into the space of Disse.

In general, it seems that bleeding problems in OLT are largely dependent on the preoperative condition and hemostatic status of patients undergoing OLT. Intraoperatively, primary hyperfibrinolysis may play an important role in the further deterioration of the hemostatic function. Rapid restoration of the hemostatic balance after recirculation seems to be largely dependent of the quality of the liver graft.

9.3 Hemostasis in auxiliary partial liver transplantation

Auxiliary partial liver transplantation (APLT) is theoretically an attractive alternative to OLT. The traumatizing and blood consuming surgical removal of the host liver can be avoided and there is no period in which the hepatic function is completely absent[30,31]. APLT might have less disturbing effects

on the hemostatic system and the procedure might be associated with a reduced need of blood transfusion. In patients with acute hepatic failure it might provide temporarily life support during a period in which the own liver has a change to recover to normal functional capacity.

We indeed found less blood loss during APLT, compared with OLT, in a controlled study in healthy pigs (chapter 3). The main difference between the effects of APLT and OLT on hemostasis was a less sustained increase in fibrinolytic activity during APLT (chapter 3). This supports the theory that increased hyperfibrinolysis in OLT is at least partly due to reduced hepatic clearance of plasminogen activators during the anhepatic stage. After recirculation, we found a similar decrease in platelet count and an increase in fibrinolytic activity in the venous outflow of auxiliary and orthotopic liver allografts. Remnant clearing function of the native liver might have contributed to a rapid normalization of fibrinolytic activity in the systemic circulation during APLT. Although signs of hyperfibrinolysis have been reported in about 80% of the patients undergoing OLT[16], we have seen a period of increased fibrinolytic activity in only 2 of the 8 patients undergoing APLT (chapter 7). The signs of hyperfibrinolysis, as measured by thrombelastography, were found before graft recirculation in one patient and after recirculation in another. Both patients had high levels of t-PA activity and suffered from increased intraoperative blood loss, which gives further support for the role of t-PA in hyperfibrinolysis and uncontrollable bleeding in liver transplantation.

Based on the present studies it seems that APLT has, in general, a less disturbing effect on hemostasis than OLT. Larger series of patients are necessary to evaluate whether APLT is indeed associated with a lower bleeding risk and whether it is a realistic alternative for patients at high risk for OLT.

In the currently used technique for auxiliary liver transplantation, only a part of the liver is grafted[31,32]. Since the resected part is not used for transplantation, this provides interesting material for scientific research. In the last study presented in this thesis, the resected parts of the donor livers used for APLT were used for liver cell isolation (chapter 8). It was demonstrated that the isolated hepatocytes can be kept in primary culture and are capable of secreting some plasma hemostasis proteins. This in vitro model seems to be an interesting method to study the regulation of the biosynthesis of hemostasis proteins by "normal" human hepatocytes. The unique

situation in which one part of a liver is used for transplantation, whereas the other part can be used for scientific research might also provide an interesting model to study other aspects of liver transplantation, such as graft preservation damage, primary non-function and hepatic regeneration. More studies are necessary to explore the scientific possibilities provided by this combination of clinical liver transplantation and in vitro experiments.

9.4 Conclusions

1. By comparing orthotopic and auxiliary partial liver transplantation it was demonstrated that, in healthy animals, APLT may be associated with a 50% reduction in intraoperative blood loss and a less pronounced activation of the fibrinolytic system. These findings support the theoretically different effects of both types of liver transplantation on hemostasis.

2. We showed that the donor liver may play a role in the origin of thrombocytopenia and hyperfibrinolysis during liver transplantation. Graft reperfusion was associated with a sequestration of blood platelet in the sinusoids and phagocytosis by Kupffer cells of the donor liver. Blood samples taken directly from the hepatic vein showed an activation of the fibrinolytic system.

3. Using a compact coagulation analyzer, heparin levels and some hemostatic parameters could be measured in the operation room at 20 min intervals. Rapid determination of heparin levels provided new insights in the heparin concentrations and the elimination of heparin during major vascular surgery.

4. In 20 OLTs the occurrence and systemic effects of hyperfibrinolysis were studied. An 30-fold increase of tissue-type plasminogen activator (t-PA) activity was seen immediately after graft reperfusion in 13 of the 20 patients. High levels of t-PA activity were associated with the destruction of fibrinogen and fibrin, which is consistent with the clinical signs of severe oozing often seen in this period of the operation.

5. In the first patient which underwent APLT, coagulation and fibrinolytic parameters were studied extensively. It was concluded that in a severely

diseased patient with an end-stage of liver cirrhosis, APLT may be performed without serious changes in the hemostatic system and without serious intraoperative blood loss. Synthetic function of the auxiliary liver was reflected in the normalization of hemostasis parameters during the first postoperative days.

6. The fibrinolytic system was studied during and after eight consecutive cases of APLT in man. Intraoperative signs of fibrinolytic activation were found in only two of the eight cases and they were associated with increased levels of t-PA activity in the circulation. Postoperatively, persistent high levels of plasminogen activator inhibitor activity were found in patients with a primary non-functioning liver graft.

7. An in vitro model was developed to study the regulation of synthesis of hemostasis proteins by human hepatocytes. Human hepatocytes were successfully isolated from the resected parts of donor livers, used for APLT. Maintained in primary cell culture, the cells were able to secrete protein C and plasminogen. There was evidence for some reactivity of the cells to triiodothyronine, dexamethasone and insulin.

We think that the results of these experiments have provided more insight in the mechanisms underlying the hemostatic disorders in OLT and APLT. Larger series of patients are necessary to study the exact role of specific hemostatic disorders in the occurrence of severe intraoperative bleeding. This may further increase our understanding of the origin of the hemorrhagic diathesis in liver transplantation. Hopefully, this will provide the basis for improvements in therapeutical management and will lead to a further reduction of the usage of blood products in liver transplantation.

9.5 References

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SUMMARY

In this thesis clinical and experimental studies on hemostatic disorders in orthotopic (OLT) and auxiliary partial liver transplantation (APLT) are described. The studies were designed to investigate the effect of APLT on the hemostasis system and to study the differences between OLT and APLT, regarding intraoperative blood loss and changes in hemostasis. In addition, the underlying mechanism of specific hemostatic disorders, which occur during both types of liver transplantation was studied.

In the introduction to this thesis in chapter 1, general information on the evolution of OLT and the development of an improved surgical technique for APLT is given. The theoretical differences between those two types of liver transplantation, regarding the effect on hemostasis is also briefly discussed. A review of the literature on hemostasis in liver transplantation is given in chapter 2. Based on this review, the working hypothesis for our own experimental and clinical studies were formulated. The results of these studies are reported in chapter 3, 4, 5, 6, 7, 8 and 9.

In the first study, described in chapter 3, the effects of OLT and APLT on the hemostasis mechanism were compared in an controlled study in pigs. Intraoperative blood loss in APLT was about half the blood loss in OLT. There were no differences in the coagulation parameters during OLT and APLT. However, in OLT there was an increase in fibrinolytic activity during the anhepatic stage, and after graft recirculation a longer period of increased fibrinolytic activity was observed, compared with APLT. Differences in surgical trauma and lack of an anhepatic stage in APLT may explain these observations. In the study described in chapter 4, the effect of graft reperfusion on the hemostatic mechanism and the role of the donor liver in the origin of hemostatic disorders was studied in more detail. This study was performed in pigs that underwent either OLT or APLT. A significantly lower platelet count and increased fibrinolytic activity were found in venous blood from the graft, compared with blood from the systemic circulation. Electron microscopy performed on liver biopsies did not show evidence for intravascular coagulation or platelet aggregation. However, there were signs of phagocytosis of platelets by Kupffer cells of the graft. Although most platelets were lying free in the sinusoids, some of them had an agranulair

appearance. Since there was no evidence found for intravascular coagulation, either by hemostasis tests or histopathologic examination, the increased fibrinolytic activity after graft reperfusion was due to primary activation.

In chapter 5, a laboratory system for the monitoring of hemostasis and heparin levels during major vascular surgery was tested. It was possible to perform a general hemostasis study, including heparin concentration, in a short period of time during major vascular surgery. It was demonstrated that the kinetic and elimination rate of heparin have a wide individual variation, which may lead to subtherapeutic heparin levels in some patients.

In chapter 6 the origin and systemic effects of hyperfibrinolysis in OLT were studied. Hyperfibrinolysis, as measured by the euglobulin clot lysis time and thrombelastography, was associated with an high increase of tissue-type plasminogen activator (t-PA) activity and plasma fibrinogen and fibrin degradation products. Highest t-PA activity was found immediately after graft reperfusion, which is consistent with the clinical signs of severe oozing at this stage of the operation.

The results of studies on hemostatic changes in APLT in man are described in chapter 7 and 8. In agreement with the animal studies (chapter 3), it was found that APLT in patients with severe liver disease, may be associated with only minor changes in hemostasis. Especially signs of hyperfibrinolysis may occur less frequently and the increase in fibrinolytic activity may be less severe, compared with OLT. Increased fibrinolytic activity, as measured by thrombelastography, was found in only 2 of 8 consecutive cases of APLT. In these two patients it was demonstrated that the increased fibrinolytic activity was caused by an enhanced t-PA activity.

In chapter 9, the isolation and culturing of human hepatocytes, as an model for studying the regulation of the synthesis of hemostasis proteins by human hepatocytes is described. In these experiments liver tissue from donor livers that were used for APLT, was used. It was found that human hepatocytes in primary cultures are capable to secrete plasmatic hemostasis proteins (i.e. protein C and plasminogen) into their culture medium. Using the resected part of a donor liver for in vitro studies provides a unique situation in which one liver is used both for transplantation and scientific research.

Further clinical studies in both orthotopic and auxiliary partial liver transplantation are necessary to evaluate the role of the described hemostatic deteriorations in the origin of severe intraoperative bleeding.

SAMENVATTING

In dit proefschrift worden klinische en experimentele studies naar hemostase-afwijkingen in orthotope (OLT) en auxiliaire partiele levertransplantaties (APLT) beschreven. De studies waren opgezet om het effect van APLT op het hemostase-systeem te bestuderen en om de verschillen tussen OLT en APLT ten aanzien van intraoperatief bloedverlies en veranderingen in de hemostase te onderzoeken. Tevens werden de onderliggende mechanismen van specifieke hemostase-afwijkingen, welke voorkomen tijdens beide typen van levertransplantatie, bestudeerd.

Algemene informatie over de evolutie van OLT en de ontwikkeling van een verbeterde chirurgische techniek voor APLT wordt gegeven in de inleiding van dit proefschrift in hoofdstuk 1. In het kort wordt ook ingegaan op de theoretische verschillen tussen deze twee typen levertransplantatie, ten aanzien van het effect op de hemostase. Een overzicht van de literatuur over de hemostase bij levertransplantaties wordt gegeven in hoofdstuk 2. Aan de hand van dit overzicht werden de werkhypotheses voor onze eigen experimentele en klinische studies geformuleerd. De resulaten van deze studies worden vermeld in de hoofdstukkken 3, 4, 5, 6, 7, 8 en 9. In het eerste onderzoek, beschreven in hoofdstuk 3, worden de effecten van OLT en APLT op het hemostasemechanisme vergeleken, in een gecontroleerde studie bij varkens. Het intraoperatief bloedverlies tijdens APLT was ongeveer de helft van dat tijdens OLT. Er waren geen verschillen in de stollingsparameters tijdens OLT en APLT. Tijdens OLT was er echter een toename in de fibrinolytische activiteit in de anhepatische fase, en na recirculatie van het transplantaat werd een verhoogde fibrinolytische activiteit waargenomen gedurende een langere periode dan bij APLT. Deze bevindingen kunnen verklaard worden door verschillen in het chirurgisch trauma en het ontbreken van een anhepatische fase in APLT. In het onderzoek beschreven in hoofdstuk 4 wordt het effect van reperfusie van het transplantaat op het hemostasemechanisme, en de rol van de donorlever in het ontstaan van hemostase afwijkingen meer in detail bestudeerd. Dit onderzoek werd uitgevoerd bij varkens die een OLT of een APLT ondergingen. Veneus bloed rechtstreeks afkomstig uit het transplantaat vertoonde een significant lager aantal bloedplaatjes en een verhoogde fibrinolytische activiteit, vergeleken

met bloed in de systemische circulatie. Electronenmicroscopisch onderzoek van leverbiopten, toonde geen aanwijzingen voor intravasale stolling of bloedplaatjesaggregatie. Er waren wel tekenen van phagocytose van bloedplaatjes door de Kupffercellen van het transplantaat. Hoewel de meeste bloedplaatjes vrij in de sinusoiden lagen, vertoonden sommige een agranulair aspect. Daar zowel hemostasetesten als histologisch onderzoek geen aanwijzingen gaven voor intravasale stolling, was er sprake van een primaire activatie van de fibrinolyse na reperfusie van het transplantaat.

Hoofdstuk 5 beschrijft een laboratorium-systeem dat werd getest voor de monitoring van de hemostase en heparineconcentraties. Het bleek mogelijk om tijdens grote vaatoperaties een algemeen hemostase-onderzoek, inclusief heparine bepaling, uit te voeren in een kort tijdsbestek. Tevens werd aangetoond dat de kinetiek en verdwijningssnelheid van heparine een brede individuele variatie vertonen, die kunnnen leiden tot subtherapeutische heparinespiegels in sommige patienten.

In hoofdstuk 6 worden de oorzaak en de systemische effecten van hyperfibrinolyse tijdens OLT bestudeerd. Hyperfibrinolyse, gemeten met de euglobulinestolsel-lysistijd en thrombelastografie, bleek geassocieerd met een sterke toename van de weefsel-type plasminogeen activator (t-PA) en plasma fibrinogeen- en fibrine-afbraakprodukten. De hoogste t-PA activiteit werd gevonden direct na reperfusie van het transplantaat, wat in overeenstemming is met de klinische tekenen van ernstig diffuus bloedverlies ("oozing") gedurende deze periode van de operatie.

In de hoofdstukken 7 en 8 werden de resultaten beschreven van onderzoeken naar hemostaseveranderingen tijdens APLT bij de mens. In overeenstemming met de dierexperimenten (hoofdstuk 3), bleek dat APLT bij patienten met ernstige leverziekten gepaard kan gaan met slechts minimale veranderingen in de hemostase. Met name komen tekenen van hyperfibrinolyse minder frequent voor en de toename in fibrinolytische activiteit kan minder ernstig zijn, vergeleken met OLT. Tijdens slechts 2 van de 8 APLTs werd met behulp van thrombelastography een verhoogde fibrinolytische activiteit gemeten. Bij deze twee patienten werd aangetoond dat de verhoogde fibrinolytische activiteit veroorzaakt wordt door een toegenomen activiteit van t-PA. Synthesefunctie van het transplantaat werd aangetoond door de normalisatie van hemostaseparameters gedurende de eerste dagen na de operatie.

In hoofdstuk 9 wordt de isolatie en het kweken van humane hepatocyten, als model voor de bestudering van de regulatie van de synthese van hemo-

stase-eiwitten door humane hepatocyten, beschreven. In deze experimenten werd gebruik gemaakt van leverweefsel afkomstig van de donorlevers, welke gebruikt werden voor APLT. Het bleek dat humane hepatocyten in primaire kweek in staat zijn om hemostase-eiwitten (t.w. protein C en plasminogeen) uit te scheiden in hun kweekmedium. Het gebruik van het verwijderde stuk van een donorlever voor in vitro onderzoek levert een unieke situatie op, waarbij een lever zowel gebruikt wordt voor transplantatie als voor wetenschappelijk onderzoek.

Verder klinisch onderzoek, bij zowel orthotope als auxiliaire levertransplantaties is noodzakelijk om de klinische relevantie van de beschreven verstoringen in de hemostase ten aanzien van het intraoperatieve bloedverlies vast te stellen.

ACKNOWLEDGEMENTS

This thesis was prepared in the department of Internal Medicine II of the Erasmus University Rotterdam and University Hospital Rotterdam-Dijkzigt. Much of the work was performed in cooperation with other departments and institutes. I have worked on this thesis with great pleasure and I am very grateful to everybody who contributed to it. To some I would like to express my special thanks.

Dr. E.A.R. Knot, Eddy, I am very grateful to you for initiating and supervising the experiments and for the opportunity to work on this thesis before completing my medical degree.

Prof. J.H.P. Wilson, thank you for your willingness to be my promotor and for the freedom you gave me to work on this thesis in your department.

Drs. F.W.G. Leebeek, Frank, you were of great support during the clinical studies. Your constructive criticism and comments were very helpful and encouraging. I always enjoy your sense of humour and hope that our personal friendship will last for many years.

Ms. M.P.M. de Maat, Moniek, your assistance during the experiments was invaluable. I am grateful for the many laboratory techniques you taught me.

Prof. Dr. O.T. Terpstra, Onno, thank you for the opportunity to join in the experimental studies at the Laboratory for Experimental Surgery and for your enthusiasm during all the studies. You and Prof. Dr. J. Abels and Prof. Dr. H.G. van Eijk are warmly acknowledged for your readiness to be members of the doctorate committee.

Dr. J. Stibbe, I am very grateful to you for introducing me to the field of hemostasis during the period I worked in the Department of Hematology. I have learned a lot from your extensive knowledge on the hemostasis.

Ms. M.J. Gomes, Maria, many thanks for correcting my English. Your support and interest in my work were of great help.

I wish to thank Dr. J.W. van der Berg and the personal of the Laboratory of Internal Medicine II for providing me with laboratory facilities and especially Ms. Gardie Voortman for her help in the experiments with the hepatocytes cultures.

Dr. T.H.N. Groenland and his colleagues from the department of Anesthesiology are acknowledged for their assistance in the blood sampling

during some of the studies.

Special thanks are extended to Prof. J.H. Lewis from the Department of Medicine, University of Pittsburgh and the Central Blood Bank for her hospitality and support during the study on orthotopic liver transplantation.

Dr. F.A. Bontempo, Frank, the many pleasant discussions I had with you were very instructive. Your enthusiasm and support ensured an unforgettable stay in Pittsburgh.

Dr. H.M.G. Princen, Hans, thank you for your help in the isolation and culture of human hepatocytes. Your assistance and that of several others from the Gaubius Institute TNO is much appreciated.

I am indebted to Dr. J. Jespersen and Mrs. M. Toft Nielsen from the Department of Clinical Chemistry, Ribe County Hospital, Esbjerg. They provided a plasminogen assay and performed the plasminogen determinations in the hepatocytes cultures.

I also wish to thank Mr. E.C.C. Colly, Mr. E. Ridderhof, Mr. Spruyt and Mr. Schalkwijk from the Laboratory for Experimental Surgery, who helped me during the animal studies.

Ms. E. Blauw and Ms. C. de Ruiter from the Institute for Experimental Gerontology TNO and Mr. W.J. Visser from the Department of Cell biology and Genetics assisted in the electron microscopy study.

During the period that I worked on this thesis, financial support to visit congresses and institutes was received from "De Stichting De Drie Lichten", The Dutch Liver Patient Society, The Rotterdam Liver Research Foundation, The A.A. van Beek Fund and the Netherlands Digestive Diseases Foundation (Nederlandse Lever Darm Stichting).

Finally I want to thank my parents for a carefree youth and the opportunity they have given me to study medicine. Without their confidence and support I would never have been able to work on this thesis. I am also much indebted to my brothers Dirk Willem and Erwin, for their support in many fields and for their friendship.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren in 1962 te Schiedam. Hij groeide op in Hellevoetsluis en behaalde zijn eindexamen V.W.O. aan de Chr. Scholengemeenschap "Blaise Pascal" te Spijkenisse in 1981. In hetzelfde jaar werd de geneeskunde studie aangevangen aan de Erasmus Universiteit te Rotterdam. Als onderdeel van het candidaats examen deed hij, o.l.v. Dr. J. Stibbe gedurende een half jaar onderzoek op de afdeling Hematologie van de Erasmus Universiteit (hoofd: Prof. Dr. J. Abels) naar hemostase afwijkingen tijdens open-hart chirurgie en streptokinase behandeling van het acute myocard infarct. Na het behalen van het candidaats examen in 1984, werkte hij twee jaar in het studenten-team van de Afdeling Inwendige Geneeskunde III van het Academisch Ziekenhuis Dijkzigt te Rotterdam. Hierna was hij gedurende een jaar als student-assistent werkzaam o.l.v. Dr. E.A.R. Knot op de Afdeling Inwendige Geneeskunde II (hoofd: Prof. J.H.P. Wilson). Tijdens deze periode werd de eerste aanzet gegeven voor de experimenten beschreven in dit proefschrift. Na het behalen van het doctoraal examen in 1986, werd voltijds gewerkt aan dit onderzoek. In de periode juni tot oktober 1988 was hij werkzaam op het Coagulation Laboratory (hoofd: Prof. J.H. Lewis) van het Presbyterian University Hospital te Pittsburgh, P.A., U.S.A., waar onderzoek werd gedaan naar de fibrinolyse tijdens orthotope levertransplantaties (hoofd levertransplantatie groep: Prof. T.E. Starzl).