# MEDIATORS OF LEUKOCYTE ACTIVATION PLAY A ROLE IN DISSEMINATED INTRAVASCULAR COAGULATION DURING ORTHOTOPIC LIVER TRANSPLANTATION

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**Leukocytes play an important role in the development of disseminated intravascular coagulation (DIC). In the reperfusion phase of OLT a DIC-like situation has been described and has been held responsible for the high blood loss during this phase.**

**We investigated the role of leukocytes in the pathogenesis of DIC in OLT by measuring the leukocytic mediators released upon activation (cathepsin B, elastase, TNF, neopterin) and the levels of thrombin-antithrombin III (TAT) complexes, seen as markers of prothrombin activation. Arterial blood samples were taken at 10 different time points during and after OLT. Samples were also taken of the perfusate released from the liver graft vein during the flushing procedure before the reperfusion phase. Aprotinin was given as a continuous infusion (0.2–0.4 Mill. KIU/hr) and its plasma levels were determined.**

**Significantly elevated levels of neopterin (15-fold; $P<0.01$), cathepsin B (440-fold; $P<0.01$) in the perfusate, as compared with the systemic circulation, as well as their significant increases in the early reperfusion phase suggested that they were released by the graft liver. This was paralleled by elevated levels of elastase (1.3-fold, $P<0.05$), TNF (1.5-fold, $P$=NS), and TAT complexes (1.4-fold; $P<0.1$) in the perfusate. Significant correlations could be identified between the parameters of leukocyte activation and TAT complexes, whereas no correlation was observed between any of the parameters investigated and the aprotinin levels.**

**Our results strongly indicate a release of leukocytic mediators from the graft liver during its reperfusion which seems to be related to the parallely increased prothrombin activation. No correlation could be seen between levels of aprotinin and levels of leukocytic mediators.**

Leukocytes play an important role in the development of disseminated intravascular coagulation (DIC),* as shown in sepsis and promyelocytic leukemia-associated DIC. Several mediators are released upon stimulation of different leukocytic subpopulations. TNF$\alpha$, an antitumoral cytokine produced by activated monocytes and macrophages as well as from peripheral T cells (*1*), promotes the adherence and transmigration of granulocytes and monocytes to endothelium and induces tissue factor synthesis in monocytes and endothelial cells (*2*). Furthermore, recent studies have proposed the role of TNF in DIC (*3*). In OLT, TNF has been seen to precede the clinical manifestation of liver allograft rejection (*4, 5*) and first-week TNF levels are believed to be useful predictors of long-term graft outcome (*5*).

Neopterin is a pyrazino-pyrimidine compound derived from guanosine triphosphate within the biosynthetic pathway of tetrahydrobiopterin—an important cofactor for tyrosine and tryptophan hydroxylation. It plays an important role in the biosynthesis of serotonin and catecholamines (*6*). It is released exclusively from activated monocytes and macrophages (*7*). The biological significance of neopterin is so far only poorly understood (*8*). Serum neopterin increased sig-

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* Abbreviations: DIC, disseminated intravascular coagulation; TAT, thrombin-antithrombin III.

nificantly 24 hr after TNF was administered to 6 healthy volunteers (*9*). The infusion of TNF and IFN-γ led to a significant rise of neopterin 24 hr later in 24 patients with advanced malignancies (*10*). The lysosomal cysteine proteinase cathepsin B is released from macrophages upon activation, as is the lysosomal serine proteinase elastase from polymorphonuclear granulocytes. Both are believed to be important nonspecific mediators of inflammation (*11*). Furthermore, in previous studies (*12, 13*), we have proved their occurrence in high concentrations in plasma during the reperfusion phase of OLT.

In OLT bleeding complications due to hyperfibrinolysis in the anhepatic phase (*14-16*), followed in the early reperfusion phase by signs of DIC (*17, 18*) together with decreased platelet count and platelet aggregability (*19, 20*), are still altering the patient's outcome intra- and postoperatively. Recent investigations (*14-16*) have shown that hyperfibrinolysis is most certainly due to an increased release and reduced clearance of plasminogen activators, and a continuous intraoperative infusion of aprotinin, a proteinase inhibitor (*21*), has been demonstrated to reduce signs of increased fibrinolysis as well as the bleeding tendency during OLT (*22*). However, the mechanism leading to DIC in the reperfusion phase is still lacking a pathophysiological explanation.

Extending previous investigations (*12, 13*), we investigated the role of leukocytes in the pathogenesis of DIC in OLT by measuring the mediators that they release upon activation. We determined levels of cathepsin B, elastase, TNF, neopterin, and thrombin-antithrombin III (TAT) complexes before, during, and after 13 OLTs. These data are correlated for each time point of blood sampling to levels of aprotinin, which was given during and after OLT.

## MATERIALS AND METHODS

We investigated 13 patients with terminal liver disease (Table 1) who underwent their first OLT at the University Hospital Rudolf Virchow, Berlin, Germany. All 13 patients were continuously treated with aprotinin infusion starting with a dose of 200,000 KIU/hr with the induction of anesthesia, which was increased to 400,000 KIU/hr with the onset of the anhepatic phase until skin closure. Aprotinin was continued until the third postoperative day with 100,000 KIU/hr. Heparin was started at the end of the operation with 250 IU/hr and increased to 500 IU/hr 12 hr later at least, until the third postoperative day. OLT was carried out by established surgical techniques using a venovenous bypass. Packed RBC and fresh frozen plasma were substituted to compensate for intra- and postoperative blood loss, but neither platelets nor concentrates of hemostatic factors were administered. Belzer UW-CSS solution (Du Pont, Paris, France) was used during cold storage of the graft liver (median duration of the cold storage time was 11 hr).

Blood samples were taken from the arterial line, after induction of anesthesia and before the start of the operation (1), 5 min before (2) and 10 min after (3) the beginning of the anhepatic stage. Further samples were collected 5 min before reperfusion (4), as well as 5 min (5), 15 min (6), and 60 min (7) afterward. Furthermore, samples were taken 12 hr (8), 36 hr (9), and 60 hr (10) after the onset of the reperfusion phase. In addition, a sample of the perfusate released from the liver graft vein during the flushing procedure with arterial blood was taken before opening of hepatocaval anastomosis and portal blood flow. The total volume of the perfusate is approximately 500 ml and the perfusate sample was taken towards the end of the flushing procedure.

Blood samples were collected in plastic syringes prefilled with 1/10 vol of trisodium citrate.

*Assays.* TAT complexes were determined by ELISA (Behring Werke AG, Marburg, Germany). Cathepsin B was measured by its enzymatic activity against the aminopeptidase substrate Z-Phe-Arg-NMec (*11*). Elastase was estimated in complex with a1-proteinase inhibitor according to Neumann et al. (*23*).

Neopterin was estimated by RIA (Henning, Berlin, Germany) and TNF by immunoradiometric assay (Medgenix Diagnostics, Fleurus, Belgium). Plasma concentration of aprotinin were determined by ELISA according to Müller-Esterl et al. (*24*).

*Statistical analysis.* As the distribution of the parameters was found to be abnormal (*25*), the nonparametric Wilcoxon signed ranks test was used for statistical evaluations. Values of $P \leq 0.05$ were considered to be significant. Results are given as median and range.

TABLE 1. Patient data

| Diagnosis | n | Female | Male | Age Median (range) |
|---|---|---|---|---|
| Postnecrotic cirrhosis | 8 | 4 | 4 | 49 (24–65) |
| Alcohol toxic cirrhosis | 1 | 0 | 1 | 47 |
| Primary biliary cirrhosis | 2 | 2 | 0 | 49,52 |
| Primary sclerotic cholangitis | 2 | 2 | 0 | 42,48 |
| Total | 13 | 8 | 5 | 46 (24–65) |

## RESULTS

TAT complexes (Fig. 1) slowly increased during the preanhepatic and anhepatic phases and showed a more prominent rise after reperfusion, with maximal levels 15 min after reperfusion. This was followed by a steep decrease between 1 hr and 12 hr after reperfusion and a less pronounced but significant decrease between 12 hr and 60 hr. The perfusate

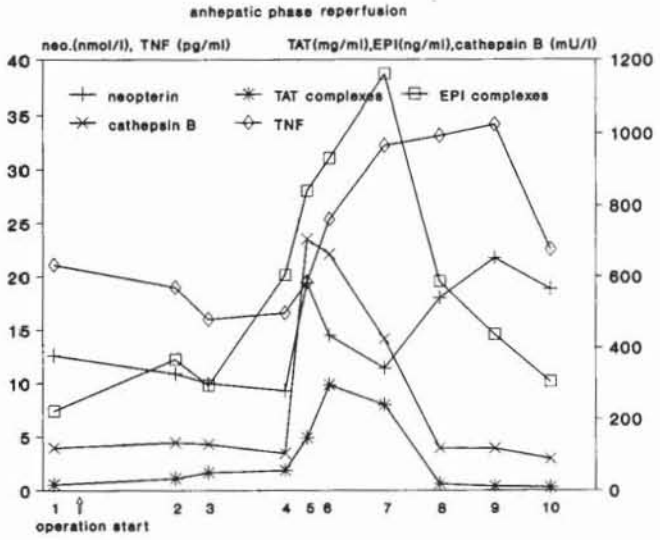


FIGURE 1. Median levels of TAT complexes, TNF, neopterin, cathepsin B, and elastase inhibitor (EPI) complexes before, during, and up to 3 days after OLT (for time points, see *Materials and Methods*). Significant changes between 2 time points are given as *P*-values. TAT complexes: $P_{(1/2)}=0.002$, $P_{(2/3)}=0.012$, $P_{(3/4)}=0.04$, $P_{(4/5)}=0.045$, $P_{(5/6)}=0.009$, $P_{(7/8)}=0.002$, $P_{(9/10)}=0.002$; TNF: $P_{(4/6)}=0.02$, $P_{(4/7)}=0.002$, $P_{(9/10)}=0.003$; neopterin: $P_{(4/5)}=0.002$, $P_{(5/6)}=0.04$, $P_{(6/7)}=0.003$, $P_{(8/9)}=0.03$; cathepsin B: $P_{(4/5)}=0.002$, $P_{(6/7)}=0.02$, $P_{(7/8)}=0.002$; EPI complexes: $P_{(3/4)}=0.03$, $P_{(4/5)}=0.006$, $P_{(6/7)}=0.01$, $P_{(7/8)}=0.005$, $P_{(9/10)}=0.003$.

TABLE 2. Comparison of parameters in perfusate and systemic circulation

| Parameter | Perfusate | Systemic circulation (4) | *P* (P/4) |
|---|---|---|---|
| | Median (Range) | Median (Range) | |
| TNF | 24 (2.5–1,020) | 16.6 (6.8–52.4) | NS |
| Neopterin | 79 (80–1,008) | 9.3 (5.8–26.4) | 0.0022 |
| Cathespin B | 45,513 (976–524,265) | 103.5 (55.7–204.4) | 0.0022 |
| Elastase | 761 (399–1,396) | 604 (276–1,205) | 0.0499 |
| TAT complexes | 77 (32–1,300) | 56 (17–381) | 0.0995 |

levels of TAT complexes were tendentiously higher than in corresponding samples taken 5 min before reperfusion (Table 2).

Levels of TNF (Fig. 1) remained unchanged during preanhepatic and anhepatic phases. With revascularization of the graft liver, a highly significant and sustained increase was seen, with maximal values 36 hr after reperfusion followed by a highly significant decrease. The higher TNF concentration in the perfusate than in the systemic circulation 5 min before reperfusion was not significant (Table 2).

The concentration of neopterin (Fig. 1) showed no changes during preanhepatic and anhepatic phases and increased significantly with reperfusion of the graft, with maximal values 5 min after revascularization of the graft followed by a significant decrease with minimum 1 hr after reperfusion and a second increase with maximal values 36 hr after reperfusion. Median levels measured in the perfusate reached more than 15-fold higher concentrations than in the systemic circulation before reperfusion, a difference that was highly significant (Table 2). Cathepsin B (Fig. 1) increased highly significantly with reperfusion reaching maximal levels 5 min after reperfusion followed by a significant decrease 10 min later reaching preoperative levels after 12 hr. Levels in the perfusate were excessively (440-fold) higher than in corresponding samples from the systemic circulation (Table 2).

Concentration of elastase proteinase inhibitor complexes (Fig. 1) decreased significantly with the start of the anhepatic phase and increased highly significantly with reperfusion until maximal values were reached 60 min after the start of reperfusion, followed by a continuous decrease. Levels in the perfusate were significantly higher (1.3-fold) than in the systemic circulation 5 min before reperfusion (Table 2).

Significantly increasing levels of aprotinin (Fig. 2) were measurable throughout the whole OLT, with maximal values in the early and late reperfusion phase. No difference was observable in the perfusate and the corresponding systemic circulation.

The plasma levels of the markers of leukocyte activation and prothrombin activation were correlated for each time point of plasma sampling (*1–10*) and for the perfusate.

Significant correlations became apparent among TNF, neopterin, elastase, and cathepsin B, especially when confined to reperfusion parameters (Fig. 3). Furthermore, a significant correlation was observed between TAT complexes and the leukocytic mediators. Only cathepsin B and TAT complexes did not correlate during and after OLT. Aprotinin levels did not correlate with most of the parameters investigated. However, there was a correlation between neopterin and aprotinin that was tendentiously significant 5 min after reperfusion ($P$=0.098, $r$=0.5) and highly significant 36 hr

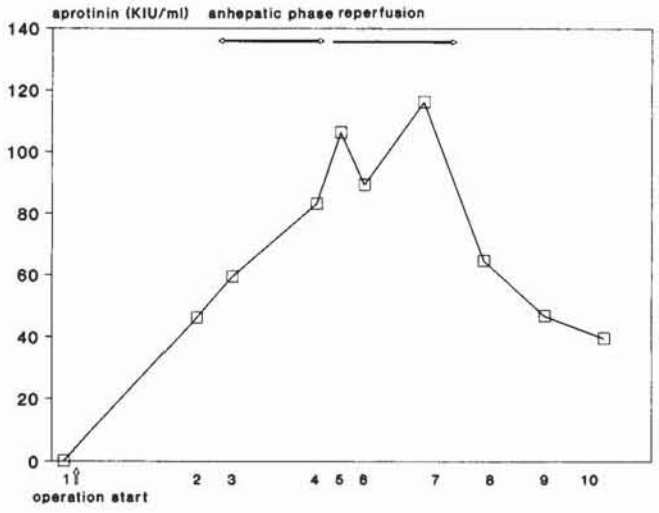


FIGURE 2. Median levels of aprotinin before, during, and up to 3 days after OLT. Significant changes are given as $P$-values: $P_{(1/2)}$ =0.006, $P_{(2/3)}$ =0.03, $P_{(3/4)}$=0.004, $P_{(7/8)}$=0.002, $P_{(8/9)}$=0.04.

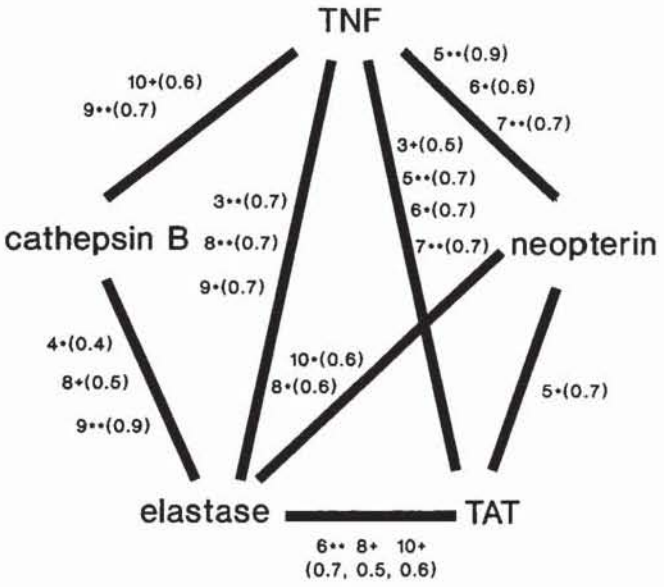


FIGURE 3. Correlation among levels of cathepsin B, TNF, elastase, neopterin, and TAT complexes and the systemic circulation during 13 OLT. Time point of blood sampling, significant level (+$P$<0.1, *$P$<0.05, **$P$<0.01), and correlation coefficient (in parentheses) are given.

($P=0.002$, $r=0.8$) and tendentiously 60 hr ($P=0.062$, $r=0.9$) after the onset of reperfusion.

With regard to the perfusate levels, a significant correlation was observable between TNF levels and neopterin ($P=0.03$, $r=0.6$) as well as between TAT complexes and elastase ($P=0.002$, $r=0.8$).

## DISCUSSION

Fifteen-fold elevated neopterin levels and 440-fold elevated cathepsin B levels in the perfusate together with a 100% increase of systemic neopterin levels and 700% increase of systemic cathepsin B with graft reperfusion suggest a neopterin and cathepsin B release when blood passes the partially damaged vascular bed of the graft liver.

This was paralleled by slightly, but significantly, elevated levels of elastase and tendentiously elevated levels of the marker of prothrombin activation, TAT complexes, in the perfusate. In the perfusate, TAT complexes and elastase correlated significantly, suggesting an elastase-mediated increased prothrombin activation in the graft liver and an independent increase of neopterin and cathepsin B.

In the systemic circulation, cathepsin B, elastase, TNF, and neopterin increased significantly, with starting reperfusion parallel to a significant increase in TAT complexes. In addition, with reperfusion of the graft liver there were correlations between the parameters of leukocyte activation as well as TAT complexes. Only cathepsin B did not correlate with neopterin and TAT complexes. These results are an argument for a strong relationship between leukocyte activation and increased thrombin generation with revascularization of the liver graft so that the DIC-like state causing increased bleeding tendency in the reperfusion phase (*15*, *17*, *18*) may in part be initiated by leukocyte activation. The observed decrease in platelet count and platelet aggregability in the reperfusion phase (*20*) may also be initiated by leukocytic mediators. As correlations between leukocytic mediators as well as TAT complexes are weak in the graft liver's perfusate and strong in the systemic circulation (Fig. 3), the vascular bed of the patient's systemic circulation seems to be more important in initiating the release of leukocytic mediators than the graft liver's vessel wall.

Levels of TNF and neopterin increase in parallel with reperfusion of the graft. However, perfusate levels of neopterin are much higher than in the systemic circulation—a difference that is not seen with TNF. The high neopterin levels do not, therefore, seem to be induced primarily by TNF (*9*), but by a direct activation of monocytes. However, a TNF-mediated activation of the monocytes may secondarily contribute to the high levels of neopterin.

Recently, it has been demonstrated that elevated TNF levels up to 100 pg/ml at the end of OLT were preceding graft rejection within 10 days after OLT (*5*). In our investigated group of 13 patients, no graft rejection occurred postoperatively and TNF levels higher than 100 pg/ml were measurable at the end of the operation in 2 patients only.

A highly significant correlation was seen between aprotinin levels and neopterin 36 hr and 60 hr after reperfusion, whereas none of the other parameters seem influenced by aprotinin. This may be explained by a retarded host reaction against aprotinin as bovine protein.

In conclusion, our results strongly indicate a release of leukocytic mediators out of the graft liver during its reperfusion which seems to be connected to the parallely increased prothrombin activation. Aprotinin did not completely control the release of leukocytic mediators. For ethical reasons, there was no control group of patients without aprotinin treatment during OLT, so that nothing can be said about the potential effect of aprotinin.

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